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Development of Mediated Enzymatic Fuel Cells for Operation in Blood

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

School of Chemistry

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Abstract

The process of enzymatic catalysis of glucose oxidation releases electrons which, when harnessed to flow through a circuit, may be utilised to power devices. This principle has driven research towards the goal of providing implantable medical devices powered by the fuel and oxidant present in-vivo, namely glucose and oxygen respectively. Challenges exist in the form of efficiently converting chemical energy into electrical energy, communicating the chemical process to an electrode, achieving both these aims under physiological conditions over a sufficient period of time, and, in so doing, creating enough electrical energy to power a device. This thesis builds on, and draws from, over three decades of development in the field of biomolecular electronics. Enzyme electrode assembly methodologies are developed, compared, and analysed in order to improve catalytic current density and stability for glucose oxidation. The inclusion of carbon nanotubes and implementation of alternate crosslinking strategies are investigated, and a novel method of enzyme electrode preparation is established. An enzyme electrode prepared with the addition of multi-walled carbon nanotubes, and co-immobilisation of glucose oxidase with a redox polymer-bound osmium mediator, crosslinked using glutaraldehyde vapour and subsequently reduced with sodium borohydride, is demonstrated to achieve 4.7 mA cm$^{-2}$ glucose oxidation current density in phosphate buffer solution (pH 7.4, 37 °C, 150 mM NaCl) in the presence of 100 mM glucose, and retain almost 80% of that current over 24 hours, measured at an applied potential of 0.35 V (Chapter 2). Glucose oxidising and oxygen reducing enzymes are co-immobilised with redox polymer-bound osmium mediators in films upon electrodes, with the addition of multi-walled carbon nanotubes and crosslinked with glutaraldehyde vapours, and compared under pseudo-physiological conditions (Chapters 3-4). Building upon these results fully enzymatic glucose-oxidising, oxygen-reducing, fuel cells are assembled and their operation compared in pseudo-physiological buffer. A selected fuel cell is also tested for operation in artificial
plasma, containing interferents present under physiological conditions. The maximum power density observed for the selected fuel cell based on glucose dehydrogenase and *Myceliophthora thermophila* laccase enzyme electrodes as anode and cathode, respectively, decreases from 110 μW cm\(^{-2}\) in buffer to 60 μW cm\(^{-2}\) on testing in artificial plasma. This EFC provides the highest power density output reported to date for a fully enzymatic glucose-oxidising, oxygen-reducing fuel cell operating in artificial plasma.
Acknowledgements

I would like to express my gratitude to all who assisted me through my many years of study.

To my supervisor, Prof. Dónal Leech, for his wisdom and expertise, and his willingness to share it, and the patience that required.

To my friends in the office, for putting up with me.

To my friends outside the office, for reminding me that life existed outside the office.

To my parents especially, thank you for your support and for granting me a life of opportunity.

“to strive,
to seek,
to find,
and not to yield.”

–Alfred, Lord Tennyson.
Declaration of work ownership

I certify that this Thesis is all my own work and that I have not obtained a degree at NUI Galway or elsewhere on the basis of any of this work.

Domhnall Mac Aodha
Abbreviations

ΓOs  Osmium surface coverage
T1/2  Time until 50% of current remains
4-amp  4-aminomethyl pyridine
AspGDH  Glucose dehydrogenase sourced from *Aspergillus* sp.
AuNP  Gold nano-particles
Bpy  Bipyridine
BOd  Bilirubin oxidase
CDH  Cellulose dehydrogenase
CNT  Carbon nanotube(s)
CtCDH  *Corynascus thermophilus* Cellobiose dehydrogenase
DET  Direct electron transport
Dm  Dimethyl
Dmo  Dimethoxy
EFC  Enzymatic fuel cell
FAD  Flavin adenine dinucleotide
FADGDH  Glucose dehydrogenase with FAD cofactor
FWHM  Full-width-half-maximum
GA  Glutaraldehyde
GA(R)  Glutaraldehyde with sodium borohydride reduction
GDH  Glucose dehydrogenase
GeGDH  *Glomerella cingulata* GDH
GOx  Glucose oxidase
MBCO  Multi-blue-copper oxidase(s)
MET  Mediated electron transport
MFC  Microbial fuel cell
MtL  
*Myceliophthora thermophila* laccase

MWCNT  
Multiwalled carbon nanotube(s)

*MvBOD*  
*Myrothecium verrucaria* bilirubin oxidase

NAD  
Nicotinamide adenine dinucleotide

NHE  
Normal hydrogen electrode

Os(bpy)PVI  
\[\text{Os}(2,2'-\text{bipyridine})_2(\text{poly-vinylimidazole})_{10}\text{Cl}]^+\]

Os(bpy)-4AMP  
\[\text{Os}(2,2'-\text{bipyridine})_2(4\text{-aminomethyl pyridine})\text{Cl}]^+\]

Os(dmbpy)PVI  
\[\text{Os}(4,4'-\text{dimethyl-2,2'-bipyridine})_2(\text{PVI})_{10}\text{Cl}]^+\]

Os(dmobpy)PVI  
\[\text{Os}(4,4'-\text{dimethoxy-2,2'-bipyridine})_2(\text{PVI})_{10}\text{Cl}]^+\]

PBS  
Phosphate buffer solution

PDB  
Protein databank

PEGDGE  
poly(ethylene glycol) diglycidyl ether

PEM  
Proton exchange membrane

PQQ  
Pyrolo-quinoline quinone

PVI  
poly-vinylimidazole

rCtCDH  
Recombinant CtCDH

rGeGDH  
Recombinant *Glomerella cingulata* GDH

ScL  
*Streptomyces coelicolor* Laccase

SHE  
Standard hydrogen electrode
Chapter 1: Introduction

1.1 Introduction

As miniaturised medical devices\textsuperscript{1} become more sophisticated, they require small, lightweight power sources that are able to sustain operation over long periods of time. This is especially the case for biomedical devices proposed to be deployed for use in the human body. Miniaturisation can be limited by the power source requirement. The current lithium and alkaline electrolyte based battery technology used in pacemakers, for example, is dependent on use of components that are corrosive or highly toxic to humans, necessitating isolation of the battery with a membrane or casing from the surrounding environment, and from each electrode in order to avoid failure\textsuperscript{2}, with a result that the power supply can comprise over half of the device volume\textsuperscript{2}. Although such batteries can operate continuously for \textasciitilde 10 years, replacement necessitates surgery and its associated risks. Rechargeable batteries were developed in the late 1950’s, yet were deemed to be flawed due to their short life-span and the bad medical practice of placing the responsibility of recharging the batteries in the hands of the patient\textsuperscript{2}. The use of fuel cells which derive their fuel and oxidant from the \textit{in-vivo} environment would, theoretically, allow continued power generation throughout the host’s life span. Fuel cells could accomplish this by oxidising glucose and reducing oxygen present in the body, forming naturally occurring and benign metabolites. During this process electricity can be generated, with the possibility of harnessing this to power an implanted device. The redox reactions in such a fuel cell are however kinetically slow on traditional electrode materials and under physiological conditions\textsuperscript{3}. Enzymatic fuel cells (EFCs) use enzymes as a catalyst at fuel cell electrode(s) to improve electron transfer kinetics, and the specificity of the catalysis. The specificity of such catalysts can permit removal of the membrane and casings to provide a route for miniaturisation\textsuperscript{1,3}.
This thesis presents steps in the development of an enzymatic fuel cell with a view to production of a demonstration system capable of operating an implantable medical wi-fi transceiver. These steps are presented in the three publications, with myself as first co-author for each, as separate chapters of the thesis. Chapter 2, presenting the first publication, includes results demonstrating a proof of principle for the methodology used for enzyme electrode assembly and preparation. This is followed by a chapter (Chapter 3) which, using the enzyme electrode assembly methodology previously examined, details the screening of enzyme electrodes prepared from a range of glucose oxidising enzymes co-immobilised with two redox polymers as electron transfer mediators, in order to determine and assess their suitability for anodes in a glucose/oxygen enzymatic fuel cell operating under physiological conditions. In the subsequent chapter (Chapter 4), screening is performed, using the previously established enzyme electrode preparation methodology, of a range of oxygen reducing enzymes co-immobilised with a redox polymer mediator, in order to determine and assess their suitability for cathodes in a glucose/oxygen enzymatic fuel cell operating under physiological conditions. Based on the results of the anode and cathode screening, results are then presented on testing of assembled membrane-less glucose/oxygen enzymatic fuel cells in phosphate buffer solution mimicking physiological conditions and in artificial plasma.

The following sections of this chapter are intended as an introduction to selected theoretical, experimental and analytical aspects not otherwise present in Chapters 2-4 (and their respective publications), whilst providing a general overview of the literature pertaining to enzymatic fuel cells.
1.2 Fuel cells

A fuel cell converts electrochemical energy into electricity through the oxidation of a fuel at the anode, and the reduction of an oxidant at the cathode. The power \( P_{\text{CELL}} \) produced by a fuel cell, measured in Watts, is defined as the product of the current produced by the cell \( I_{\text{CELL}} \) and the potential difference of the cell \( V_{\text{CELL}} \) as measured in Volts (Equations 1.1 and 1.2). The current is the rate of transfer of electrons and is measured in Amperes, while the reversible potential difference of the cell is equal to the potential (E) difference between the anode and the cathode, minus irreversible losses in the voltage (\( \eta \)) due to kinetic limitations of the electron transfer process at the electrode interfaces, ohmic resistances and concentration gradients.

\[
V_{\text{CELL}} = (E^{\theta}_{\text{cathode}} - E^{\theta}_{\text{anode}}) - \eta \quad \text{Equation 1.1}
\]

\[
P_{\text{CELL}} = V_{\text{CELL}} \times I_{\text{CELL}} \quad \text{Equation 1.2}
\]

A fuel cell differs from a battery in that a battery possesses all the components necessary for electricity generation within a self-contained unit, whereas a fuel cell is an open system and does not contain energy, merely the means to convert chemical energy (in the form of fuel and oxidant) to electricity\(^{[4,5]}\). Both fuel and oxidant are supplied to the device, which would, ideally, continue to convert chemical energy into electricity for as long as fuel and oxidant were supplied. An example of such a fuel cell is the hydrogen-oxygen proton-exchange membrane (PEM, a proton conducting membrane impermeable to electrons or gases) fuel cell as illustrated in Figure 1. Equations 1.3 and 1.4 are the anodic and cathodic half reactions taking place at the respective electrodes for this fuel cell.
Figure 1 Simplified schematic of a hydrogen-oxygen PEM fuel cell (see text for details).

\[
\text{Anode: } H_2 \rightarrow 2 \text{ e}^- + 2 H^+ \quad \text{Equation 1.3}
\]

\[
\text{Cathode: } \frac{1}{2} O_2 + 2 \text{ e}^- + 2 H^+ \rightarrow H_2O \quad \text{Equation 1.4}
\]

Hydrogen gas, when in contact with the anode, is catalytically converted into protons and electrons via a catalyst, such as platinum\[^6\]. The protons pass through the PEM towards the cathode, whilst electrons are forced through the external circuit, creating a current. A flow of oxygen over the cathode is catalytically reduced by a catalyst such as platinum and combined with the protons to form water. The standard potential difference (\(\Delta E^0\)) between the anode and cathode of a \(H_2 || O_2\) fuel cell is 1.23 V\[^4\].

Although platinum is a highly effective catalyst for such a fuel cell, it is expensive and is specific to neither hydrogen oxidation nor oxygen reduction. Non-specificity of catalysts in a fuel cell necessitates the use of membranes to separate the fuel and oxidant, thus preventing short-circuiting. Furthermore, platinum is prone to deactivation through surface poisoning\[^7,8\].
1.2.1 Biofuel cells

Biofuel cells operate upon the same principle as all fuel cells, whereby a fuel is catalytically oxidised at the anode and an oxidant is reduced at the cathode thereby converting chemical energy into electricity. However, the catalysts used are sourced from living entities. Biofuel cells are categorised by the bio-catalyst selection: microbial and enzymatic. Microbial fuel cells (MFCs) involve entire microbial organisms that are capable of catalytically converting a fuel (and more recently oxidant) to electrical power. Enzymatic fuel cells rely upon enzymes extracted from biological sources for the chemical energy conversion process. The enzymes are usually immobilised at an electrode surface, to increase local concentration of catalyst and to permit simple separation of anode catalyst from contact with the cathode (and vice versa). Thus, while a PEM and microbial fuel cells usually require a membrane separator, enzyme electrodes can be specific (or as specific as the enzyme) to a substrate and therefore do not necessitate use of a membrane. Therefore, for miniaturised biofuel cells with potential application to powering in-vivo devices, EFCs are advantageous due to the lack of a membrane, thus allowing miniaturisation. Fuel cells often operate under harsh conditions, as efficiency is of greater concern than biocompatibility. By replacing the metal catalysts of a fuel cell with suitable enzymes, an EFC is capable of converting biochemical energy into electricity under bio-compatible conditions, a principle first demonstrated by Yahiro et al. in the 1960’s. Such research has revealed the possibility of green, non-polluting, and sustainable energy conversion processes that could replace the hazardous and corrosive batteries in current use in many applications including miniaturised electronic devices, self-powered sensors and portable electronics. By utilising the components of bodily fluids such as blood as a fuel source, implanted fuel cells could generate electrical power capable of operating implanted systems such as insulin
pumps, prosthetic elements, nerve-stimulators, or implantable biosensors[1].

The most commonly described enzymatic fuel cell prototype in the literature consists of a glucose-oxidising anode and an O$_2$-reducing cathode although other systems using other fuels such as fructose or methanol, and using peroxide as oxidant have also been reported[3,7]. As this thesis is focused solely on research leading to fuel cells based on a glucose oxidising anode and an oxygen reducing cathode, consideration of these other systems will not be undertaken.

For all the promise they offer, EFCs have shortcomings. Low power densities and a short usefully-operational lifetime of the biocatalyst are significant hurdles to prototype development and operation. For a glucose/O$_2$ biofuel cell to be considered a realistic possibility it is imperative that it operate effectively under physiological conditions (i.e. pH 7.4, 37 °C, 5 mM glucose, 150 mM NaCl). Currently, the implanted device that requires the greatest battery power is the heart pacemaker in which the lithium battery is located below the skin between the shoulder and the neck and is connected to electrodes in the heart muscle[11]. At present, the lifetime of a 1 W cm$^{-3}$ battery with a typical operating regime of 1 μW exceeds 10 years[12]. EFCs have a lifetime measured in days. It will be some time, if ever, and after a great deal of further development, before an EFC can compete with the level of stability of lithium batteries. However, it is worth noting that the power supply can be remote. It is possible that short term EFCs that are easily and safely replaceable could be used, whereby the electrodes are printed onto a disposable patch that connects to implanted miniature electrical contacts. This negates the requirement for repeated surgery and sidesteps the issue of EFC stability.

### 1.3 Enzymatic catalysts

Enzyme biocatalysts are relatively cheaper than metal catalysts such as platinum; however that is only one of several significant
advantages. Enzymes possess high substrate specificity, efficient conversion of substrates\cite{1,3,7} and, in terms of the enzymes used in this thesis, operating temperature ranges and operating pH conditions suitable for physiological conditions. As a result, there has been extensive research into enzymatic catalysis for fuel cells, with genetic engineering utilised as a means of increasing efficiency of an enzyme, or to widen the substrate specificity to a larger group of fuels\cite{13–17}.

1.3.1 Enzymes for use as biocatalysts in anodes

As the focus of the studies contained within this thesis is upon biofuel cells with \textit{in-vivo} application, glucose was targeted as the substrate that any potential enzyme must oxidise. The following sections present an introduction to the glucose-oxidising enzymes used in the thesis.

1.3.1.1 Glucose oxidase

![Figure 2](image.png)

\textbf{Figure 2} Crystal structure of the \textit{Aspergillus niger} glucose oxidase. A single unit of the dimer is shown. (PDB ID: 3QVP, Kommoju \textit{et al.}\cite{18}).
Glucose oxidase (GOx) is a dimeric glycoprotein consisting of two equal subunits with a molecular mass of 80 kDa each. It requires flavin adenine dinucleotide (FAD) as cofactor. FAD functions as the initial electron acceptor and is reduced to FADH$_2$ which in turn is then re-oxidised by the final electron acceptor, oxygen, as presented in Equations 1.5 and 1.6.

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

\[
\text{GOx(FADH}_2) + \text{O}_2 \rightarrow \text{GOx(FAD)} + \text{H}_2\text{O}_2 \quad \text{Equation 1.6}
\]

The replacement of oxygen as the natural co-substrate by a redox complex as mediator, which competes with O$_2$ as the final electron acceptor, allows shuttling of the electron from the enzyme active site, ~1.5 nm within the protein, to an electrode (see section 1.4) and generation of current at lower overpotentials compared to systems based on reduction of oxygen or oxidation of peroxide. Significant problems may arise, however, for development of in-vivo biocatalytic anodes based on GOx, as oxygen, present in the fuel cell electrolyte, competes with the redox complex. In addition, GOx catalyses the oxidation of glucose to δ-gluconolactone, producing hydrogen peroxide when oxygen is the electron acceptor. Hydrogen peroxide is a highly toxic product and can destroy bioactive material\cite{19}. As oxygen is the oxidant of choice in the EFC, oxygen depletion may also arise in the electrolyte by reaction at the GOx-based anode, leading to poor biocatalytic cathode performance. It may, therefore, be advantageous to focus on enzymes which oxidise fuels yet do not produce hydrogen peroxide nor consume oxygen, such as dehydrogenases, for application to glucose/oxygen EFCs.

Nonetheless, due to its stability, substrate selectivity, high electron turnover rate and commercial availability\cite{7}, GOx is used for comparing electrode assembly methodologies in Chapter 2. GOx was also used in Chapter 3 for comparison to other enzymes as bioanodes, with all anodes prepared in an identical manner, and
again in Chapter 4, when assessing EFCs performance in pseudo-physiological buffer.

GOx is used in ~6 billion glucose sensor strips each year\[^{20}\]. Initially, such sensors used oxidation of hydrogen peroxide, at ~0.6 V against Ag/AgCl\[^{21,22}\] to detect glucose levels. Such high potentials caused interference through the oxidation of other species present in blood serum, such as ascorbic and uric acid. With the incorporation of mediators, however, a lowering of the potential was made possible, thus avoiding such interference. Cass et al.\[^{23}\] first demonstrated this by using ferrocene derivatives as mediators, co-immobilised with GOx on a pyrolytic graphite electrode, allowing glucose detection sensors that operate close to the redox potential of the ferrocenes, ~0.35 V vs Ag/AgCl. The influence of ferrocene derivative structure on GOx mediation in aqueous solution was comprehensively reported on by Forrow et al.\[^{24}\]. By crosslinking GOx with dimethylferrocene-modified poly(ethyleneimine) polymer on glassy carbon electrodes Meredith et al.\[^{25}\] succeeded in providing ~2 mA cm\(^{-2}\) glucose oxidation current density at 0.13 V vs Ag/AgCl in pH 7.4 buffer containing 100 mM glucose. Ferrocene is, however, unstable in its oxidised form, ferricenium, in aqueous solution and is not readily soluble in its reduced form, resulting in difficulties with electrode fabrication\[^{26}\].

The use of a range of tris(4,4′-substituted-2,2′-bipyridine) complexes of iron, ruthenium, and osmium as mediators of GOx was reported on by Zakeerudin et al.\[^{27}\], with such complexes displaying a wider range of redox potentials as compared to ferrocene derivatives. These complexes may thus be more suited to EFC applications. GOx has been the enzyme of choice for much of the development of glucose oxidising electrodes\[^{26,28–32}\], including the development of redox polymer bound mediator based electrodes, discussed in section 1.4.1.
1.3.1.2 Dehydrogenases

The use of dehydrogenases such as glucose dehydrogenase (GDH) and cellobiose dehydrogenase (CDH) is gaining increased attention as catalysts for oxidation of glucose in biosensing and biocatalytic fuel cell applications. Cellobiose dehydrogenase[33] is typically a monomeric protein consisting of a flavin-containing catalytic dehydrogenase domain which is loosely connected to a smaller heme b containing cytochrome domain. The use of this enzyme in enzymatic anodes has been focused on harnessing direct electron transfer to the electrode via its cytochrome domain[33], though mediated electron transfer (see section 1.4) can be achieved via the FAD cofactor[34]. In Chapter 3, a Corynascus thermophilus cellobiose dehydrogenase (CtCDH), among other enzymes, was selected for comparison as a glucose-oxidising anode, with two osmium based redox-polymers as mediators. The catalytically active dehydrogenase domain of the CtCDH was recombinantly produced in the bacterial expression system E. coli resulting in a truncated protein with a molecular mass of only 60 kDa, compared to 85 kDa for the untruncated protein[35]. To further attempt to increase glucose turnover by the recombinant enzyme, an amino acid in the catalytic centre (cysteine 62) was exchanged to a tyrosine. To facilitate protein purification a c-terminal His6-tag was also added. Purification resulted in a homogenous protein preparation of recombinant CtCDH (rCtCDH) with activity for glucose of 19 U mg⁻¹. The GDHs can utilise either nicotinamide adenine dinucleotide (NAD)[36], pyrrolo-quinoline quinone (PQQ)[37,38], or an FAD[39] as cofactor for oxidation of glucose. The NAD cofactor is not directly bound to the enzyme but its presence is necessary because it acts as a carrier of two electrons and one proton, and importantly it activates the biocatalytic function of the enzyme. However, the NADH co-factor itself is not useful as a redox mediator because of the high overpotential for the NADH/NAD⁺ redox process[40] and the instability of NAD⁺. The PQQ co-factor has a thermodynamic
redox potential of ~0.115 V vs. Ag/AgCl at pH 7\textsuperscript{[41]}. Use of the PQQ-dependent GDH in biocatalytic fuel cells may prove limited, however, because of its relative instability, when compared to GOx\textsuperscript{[42]}. GDH may also possess, as does GOx, an FAD cofactor. FAD is typically bound within the enzyme but can be removed. FAD undergoes a two-electron, two-proton redox reaction. At pH 7, the redox potential of the free flavin is −0.23V relative to the standard hydrogen electrode (SHE)\textsuperscript{[39]}. Therefore, three GDHs, among other enzymes, were selected for comparison as a glucose-oxidising anode, with two osmium based redox-polymers as mediators in Chapter 3.

These are: a recombinant FAD-dependent glucose dehydrogenase from *Glomerella cingulata* (rGcGDH) that has been expressed in *Pichia pastoris*; a second FAD-dependent glucose dehydrogenase expressed in an *Aspergillus sp.*, of interest due to its high turnover rate, commercial availability and oxygen independence; and finally a commercially available pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQGDH) that has an optimum pH of 7.0, and an optimum temperature of 37°C.

### 1.3.2 Enzymes for use as biocatalysts in cathodes

In order to provide a membrane-less fully enzymatic fuel cell operating under physiological conditions, coupling of the glucose-oxidising enzyme electrode anodes to oxygen-reducing enzyme electrode cathodes is required. Biocatalytic processes occurring at the cathode offer selectivity advantages over the use of platinum, which may eliminate the need for anodic and cathodic compartmentalisation.

The use of “blue” multicopper oxygenases, such as laccase and bilirubin oxidase has received much attention of late because of their ability to bio-catalytically reduce oxygen to water at relatively high reduction potentials and under mild conditions\textsuperscript{[14,17,43–48]}. 

11
1.3.2.1 Laccases

The immobilisation of laccase with a redox polymer mediator to provide for oxygen reduction was first reported on by Trudeau et al.[49] using a di-epoxide crosslinked osmium redox polymer hydrogel film containing laccase on carbon electrodes. Laccases are classed as polyphenol oxidases, and tend to consist of monomers which contain 4 coppers in the active site, designated as types 1 to 3[44]. Substrate oxidation occurs at a type 1 single “blue” copper site, at which point the catalytically produced electrons are shuttled to the oxygen reduction site, ~1.5 nm away, consisting of a trinuclear cluster of a type 2 copper site and a coupled pair of type 3 copper sites[44].

Figure 3 Crystal structure of *Trametes versicolor* laccase (PDB ID: 1GYC, Piontek et al.[50]).

Laccases are obtained from various sources, ranging from plant and fungal to, more recently, bacterial sources. The redox potential of the type 1 (T1) copper site determines the potential at which reduction is driven[44]. Fungal laccases possess T1 sites of either mid-range (~ + 0.47 V to + 0.71 vs. NHE) or high range (~ + 0.78 V vs. NHE) potentials[44,51]. Most of the high range potential fungal laccases are
inhibited by hydroxyl ions, with maximal activity occurring around pH 4 to pH 5. This limits their use as biocathodes, due to low activity, in an EFC intended to operate under physiological conditions[32]. As the focus of this thesis is on the development of fuel cells for deployment under physiological conditions, enzymes were chosen that operate under physiological pH conditions: a laccase sourced from the *Streptomyces coelicolor* bacterium; and a laccase sourced from the *Myceliophthora thermophila* fungus. *Streptomyces coelicolor* laccase[46,47] (ScL) is a recently reported on bacterial laccase. It is reported to form trimeric assemblies, with each monomer consisting of two domains containing the 4 copper active sites found in monomeric fungal laccases[47]. It has a monomeric molecular mass of 32 kDa, displays a redox potential of the T1 site of ~0.43 V vs NHE[17], and is active up to 90°C. The pH optimum is substrate dependent, but typically around pH 8. The *Myceliophthora thermophila* laccase (MtL) is a monomeric glycoprotein with a molecular mass of 85 kDa, a T1 substrate-oxidising copper site redox potential of 0.48 V vs NHE, and activity up to 60°C. The pH optimum is substrate dependent, but typically ranges from pH 6-8[14,45].
1.3.2.2 Bilirubin oxidase

Figure 4 Crystal structure of *Myrothecium verrucaria* bilirubin oxidase (PDB ID: 2XLL, Cracknell *et al.*[52]).

The natural substrate of bilirubin oxidase (BOd) is bilirubin, which it converts to biliverdin, with concomitant reduction of oxygen to water in a four-electron reduction process. As with laccase, substrate oxidation occurs at a T1 copper site, while oxygen reduction occurs at a tri-nuclear T2/T3 copper site. The *Myrothecium verrucaria* bilirubin oxidase (*Mv*BOd), selected for examination as a catalyst in this thesis, is a monomeric glycoprotein with a molecular mass of 60 kDa, a T1 site redox potential of 0.67 V vs NHE and a broad pH activity region (3-7) for electron donors such as K4[Fe(CN)6] or electrodes[48]. The use of BOd as an oxygen reducing catalyst in fuel cell cathodes, has been widely reported on. Tsujimura *et al.* first reported on a *Mv*BOd-based oxygen cathode using solution-phase 2,2'-azino-bis(3-ethylbenothiazoline-6-sulphonic acid) (ABTS) mediated reduction of oxygen at carbon felt electrodes in phosphate buffer, pH 7.0[53]. Co-immobilisation of both mediator and BOd, and optimisation of mediator selection for the enzyme, led to current densities of 17 mA cm⁻² at + 0.25 V vs.
Ag/AgCl for mediated oxygen reduction, by electrostatically entrapping \( Mv\text{BOd} \) with \([W(CN)\text{4}]^{3-}/4^-\) within poly(L-lysine) at carbon felt electrodes rotated at 4000 rpm\(^{[54]}\). The \( Mv\text{BOd} \) immobilised in poly(L-lysine) layers at carbon electrodes, containing a high density of crystal edges, is reported\(^{[55]}\) to produce, via direct electron transfer (DET, see section 1.4) between the enzyme and electrode, steady-state oxygen reduction current densities of 0.85 mA/cm\(^2\) at potentials of \(-0.2\) V vs. Ag/AgCl in oxygen saturated phosphate buffer at pH 7.0 with rotation at 1400 rpm. DET between \( Mv\text{BOd} \) and electrodes has been used in DET fuel cells. In pH 7.4 phosphate buffer solution (PBS), with 5 mM glucose, Coman \textit{et al.}\(^{[56]}\) achieved an OCV of 0.62 V and a power density of 3 \( \mu \)W cm\(^{-2}\), with the cathode producing 55 \( \mu \)A cm\(^{-2}\), while Wang \textit{et al.}\(^{[57]}\) achieved an OCV of 0.66 V and a power density of 3.2 \( \mu \)W cm\(^{-2}\) with the cathode also producing \(-55\) \( \mu \)A cm\(^{-2}\) in the presence of 100 mM glucose containing PBS. Kim \textit{et al.}\(^{[58]}\) co-immobilised a 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 fibers to yield biocatalytic oxygen cathodes that provide current densities of 0.7 mA cm\(^{-2}\) at a potential of +0.3 V vs. Ag/AgCl in non-stirred phosphate buffered saline at 37 °C\(^{[58]}\). When rotated at 4000 rpm, a current density of 6.25 mA cm\(^{-2}\) at potentials of +0.3 V vs. Ag/AgCl was obtained in oxygenated phosphate buffered saline at 37 °C\(^{[59]}\).

### 1.4 Electron transfer between enzymes and electrodes

A primary concern in the selection of biocatalyst intended for use in a biofuel cell is the efficiency of the electron transfer between the biocatalyst and the electrode. The route by which electron transfer occurs between the biocatalyst and the electrode in biofuel cells has two variations: direct electron transfer (DET); and mediated electron transfer (MET)\(^{[60]}\). During DET, electrons are transferred directly from the enzyme active site to the electrode. For DET to
occur the enzyme needs to be ~20 Ångstroms\textsuperscript{[61]} or less from the electrode surface. This limits the number of layers of enzyme that can potentially adhere to the electrode and remain electrically connected. The orientation of the enzyme upon the electrode surface must also be considered to permit electron transfer between the active site and electrode. Therefore DET between the active site and the electrode is often quite difficult to achieve\textsuperscript{[20,62]}, and current densities are often low when compared to those observed using MET\textsuperscript{[7,60]}. The example of GOx catalysis demonstrates this. In order to function as a biocatalyst, GOx requires a cofactor, flavin adenine dinucleotide (FAD). In the GOx-catalysed redox reaction, FAD functions as the initial electron acceptor and is reduced to FADH\textsubscript{2}. Then FADH\textsubscript{2} is oxidised by the final electron acceptor, oxygen, the natural acceptor, or, an artificial acceptor (mediator) which replaces oxygen in the electron transfer mechanism. As the protein shell is designed to protect the redox-active centre and impart selectivity, direct electron transfer on a traditional planar electrode surface for wild-type \textit{Aspergillus niger} GOx does not occur \textsuperscript{[62–64]}. Mediators can however access the redox-active centre of GOx and shuttle electrons to electrodes efficiently, whilst being re-oxidised at lower detection potentials than the peroxide product of oxygen reduction\textsuperscript{[20]}. Therefore it may prove necessary and beneficial to use MET, by introduction of an electron transfer mediator to shuttle electrons between the electrode and the enzyme, over DET. Mediators, for example, can be used to address multiple layers of enzymes immobilised on the electrode, as orientation and proximity of the enzymes are not as crucial a factor as for DET. For redox mediation to proceed with an effective electron exchange rate, the thermodynamic redox potentials of the enzyme and the mediator should be appropriately matched\textsuperscript{[3,60,65]} in order to produce a driving force for current production, whilst not contributing overly to decreasing a fuel cell voltage output. The rate of electron transfer varies exponentially with reaction free energy, as described by Marcus theory\textsuperscript{[66]}, up until other limitations, such as diffusion and
mass transport, come into effect. Thus, a redox potential difference of approximately 50 mV between a mediator and GOx has been proposed\[31\], as a compromise between driving force, current generation and cell voltages in enzymatic fuel cell electrodes. A more recent report\[67\] examining the effect of selection of redox polymer on the kinetics of mediated *Trametes versicolor* laccase oxygen reduction at electrodes, determined an optimum compromise between driving force and current generation to occur at a redox potential difference of 160 mV.

The mediator designed and/or selected for use in enzymatic fuel cells should be stable in both reduced and oxidised states to permit continuous participation in the electro-catalytic cycle. The demonstrated stability of osmium polypyridyl redox complexes in their Os(II) and Os(III) oxidation states\[68\], has led to implementation of osmium-based redox polymers as mediators for biofuel cell applications. The redox potentials of the Os(II/III) transition in osmium polypyridyl-based redox polymers can also be tuned through chemical modification of the ligands complexed to the osmium metal centre \[68,69\], again leading to extended adoption of these systems as mediators of enzyme reactions. Developed by Forster and Vos\[68\] in 1990, the polyvinyl-imidazole (PVI)-bound osmium bipyridine series of redox polymers, of structural motif presented in Figure 5, have been used extensively in research, and development of commercial continuous glucose monitoring systems.
Figure 5 General structure of the PVI bound osmium bipyridine mediators. \([\text{Os}(2,2^\prime\text{-bipyridine})_2(\text{poly-vinylimidazole})_{10}\text{Cl}]^+\)
(Os(bpy)PVI): X=H, \([\text{Os}(4,4^\prime\text{-dimethoxy-2,2^\prime\text{-bipyridine})_2(\text{poly-vinylimidazole})_{10}\text{Cl}]^+\) (Os(dmobpy)PVI): X=O-CH₃, \([\text{Os}(4,4^\prime\text{-dimethyl-2,2^\prime\text{-bipyridine})_2(\text{poly-vinylimidazole})_{10}\text{Cl}]^+\) (Os(dmbpy)PVI): X=CH₃.

1.4.1 Enzyme electrodes based on redox polymer mediators

Electrode modification has been a subject of interest and research for over 30 years\([70–72]\), due to its potential application in areas such as biosensors and energy conversion. The modification of electrodes with redox polymer-bound mediators allows control over the electroactive nature of an electrode through the deliberate immobilisation of compounds and enzymes onto the electrode surface, unrestricted by concerns for enzyme orientation or proximity. The presence of a redox polymer-bound mediator can prevent oxidation and/or reduction of other species as overpotential is lessened, an important consideration for biosensors though possibly not for fuel cells.
Furthermore, the presence of a crosslinked redox polymer imparts greater structural stability to the film on the electrode. Preparation of films may be achieved by drop casting a solution of the required electro-active materials upon the electrode surface, often with a crosslinker included to improve stability of the film\[^{28,29,73,74}\]. Crosslinked redox polymers create a matrix into which electro-active biomaterials can then be entrapped or bound\[^{28,29}\], immobilising the enzyme as well as the redox-polymer-bound mediator. Redox polymer modified electrodes can be assembled without covalent cross-linking by simple adsorption of the species at the electrode surface. However, in the absence of any covalent bonds holding the matrix together the components are more rapidly leached from the film\[^{75}\]. Cross-linking of redox polymer and enzymes can be achieved using homo-bifunctional crosslinkers, resulting in formation of an enzyme-containing redox hydrogel on the electrode surface. For example, Gregg \textit{et al.}\[^{76}\] developed an approach to entrap enzymes and mediators at electrodes using epoxide cross-linkers to form a three dimensional biocatalytic redox matrix in a hydrogel. Electron conduction in these redox hydrogels is proposed to occur\[^{77,78}\] by self-exchange of electrons or vacancies between rapidly reduced and oxidised redox functions. Electron transfer thus takes place by collisions between the reduced and oxidised forms of the osmium pendant moieties tethered to the polymer backbone, as illustrated in Figure 6. As such, backbone mobility can effect electron transfer within a film. Subsequent to immersion in the test solution, the films may swell, aiding mass transport and even electrical connectivity to the electrode surface\[^{79,80}\] due to greater backbone mobility.
Figure 6 Collisions between labile pendant osmium moieties on the polymer backbone allows for the diffusion of electrons through the redox hydrogels that “wire” the reaction centres to the electrode. Electron exchange takes place when the reduced and oxidised species reach a certain distance $d$, as described by Dahms and Ruff[81,82].

Crosslinkers may introduce a degree of rigidity to the hydrogel that can affect electron transfer within the film[30], along with prolonging the hydrogel film structural integrity[30]. In order to improve the stability of biocatalytic electrodes, attachment of the film to pre-functionalised surfaces can be used instead of physisorption[83]. This functionalisation may be achieved through the use of diazonium salt chemistry for derivatising conducting surfaces with functional groups which are amenable to further coupling reactions[83].
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 using the agents in Figure 7. Ohara et al.\cite{29} examined the use of Os(bpy)PVI, co-immobilised with GOx on electrodes using a di-epoxide, poly(ethylene glycol) diglycidyl ether (PEGDGE), for sensing glucose levels. They determined that the resulting hydrogel were both permeable to glucose and allowed the diffusion of electrons. De Lumley-Woodyear et al.\cite{30} compared PEGDGE crosslinking to that using suberic acid bis(N-hydroxysuccinimide ester, dimethyl suberimidate or glutaraldehyde solution for the co-immobilisation of enzyme and redox-polymer bound mediators. All the crosslinked films, with the exception of those using glutaraldehyde, adhered well to the carbon electrodes and retained approximately 90% of their electroactive centres when soaked and stirred in the phosphate buffer.
buffer at room temperature for 48 hours. It was found that glutaraldehyde had a considerable negative impact on the resulting current density as compared to the current densities observed for the electrodes prepared using the other crosslinkers and tested in identical conditions. It should be noted, however, that the use of glutaraldehyde vapours instead of solutions as a method to crosslink the films was not reported on. The use of glutaraldehyde vapours as a fixative and crosslinking agent is widespread in biochemistry\cite{84}, and occasionally in the field of electrochemistry\cite{85,86}. Chapter 2 presents, for the first time, a comparison of the use of glutaraldehyde vapours to crosslink, and immobilise, films of redox polymer and GOx on electrode surfaces, to provide enzyme electrodes for oxidation of glucose under physiological conditions.

### 1.4.2 Nanoparticles as additives in enzyme electrodes

The addition of nanoparticulate matter to films of enzymes 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 in enzyme electrode development. For example, inclusion of carbon nanotubes (CNTs) to films of enzymes on electrodes has been reported to promote direct electron transfer between enzyme and electrode\cite{87,88} and results in increases to both current and stability of the enzyme electrodes for applications as biosensors\cite{36,89–91} and, more recently, as electrodes for EFCs\cite{92–95}. Incorporation of single walled CNTs and glucose oxidase (GOx) in a film on electrodes with an osmium redox polymer results in an increase to both the current signal for oxidation of the redox polymer and for oxidation of glucose\cite{91}. The same study also reported an increase in stability of enzyme activity, in which a redox polymer/enzyme film prepared with single walled carbon nanotubes was found to retain 67% of its initial glucose oxidation current over
24 h compared to a value of 55% attributed to a similar electrode prepared without single walled carbon nanotubes. Addition of gold nanoparticles (AuNP) have also been shown to improve electrical contact between enzyme and electrode, facilitating DET current generation\cite{96–98}. For example, a current density of 5.2 mA cm$^{-2}$ at 4000 rpm has been reported for oxygen reduction\cite{98} using a BOD/AuNP modified electrode, with an improvement in stability of catalytic currents also observed upon inclusion of AuNP. This BOD/AuNP electrode was connected to a fructose dehydrogenase anode to form a fully enzymatic fuel cell, with carbon paper used as the electrode substrate. This fuel cell produced a maximum current density of 0.87 mW cm$^{-2}$ in a stirred solution, operating at 0.3 V.

1.5 Towards the in vivo fuel cell

From Yahiro et al.\cite{10}, when the first enzyme based glucose oxidising/oxygen reducing fuel cell was reported in 1964, onward to today, much time, money, and effort has been invested by researchers into developing the concept of a membraneless enzymatic biofuel cell capable of operating under in-vivo conditions\cite{99–101}. Such a membraneless system requires catalysts specific to the fuel and oxidant to be immobilised at the electrode surface. The first reported example of an anode for glucose oxidation based on immobilised enzymes in a fuel cell is for a graphite electrode modified by adsorption of glucose dehydrogenase from Bacillus megaterium, immobilised on porous glass using glutaraldehyde, and NADH\cite{102}. A membrane-based fuel cell was formed with a platinum gauze electrode, and produced a current density of 0.2 mA cm$^{-2}$ at a cell voltage of ~0.8 V, with the anode rotated at 740 rpm. The first paper describing a membrane-less glucose/O$_2$ biofuel cell\cite{103} 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, as shown in Figure 8.

Figure 8: Schematic configuration of a biofuel cell employing glucose and O2 as a fuel and an oxidiser, respectively, and PQQ-FAD GOx and cytochrome c/cytochrome c oxidase-functionalised electrodes as biocatalytic anode and cathode, respectively\textsuperscript{[103]}.

Advances reported on in a series of publications\textsuperscript{[28,30,104,105]} led to 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\textsuperscript{[106]}. This fuel cell produced 0.6 μW (137 μW cm\textsuperscript{−2}) at 0.4 V cell voltage, in 37 °C chloride-free pH 5.0 citrate buffer containing 15 mM glucose. Mano et al.\textsuperscript{[107]} describe a similar carbon fibre fuel cell operating under pseudo-physiological, pH 7.4 chloride containing conditions, though at 15 mM glucose, that produced 1.9 μW (equating to 50 nW mm\textsuperscript{−1} of fibre length) at 0.52 V cell voltage. The selection of BOd as oxygen reducing enzyme permitted operation under a neutral pH condition. Gao et al.\textsuperscript{[94]}
developed an analogue of this fuel cell, but used electrodes composed of nanotube wires, and produced a power density of 740 µW cm$^{-2}$ at 0.57 V cell voltage, under the same conditions. Despite all the advances in miniaturisation, little has been reported on testing of such systems in blood or even artificial plasma. Coman et al.\textsuperscript{[56]} report on the use of BOd and cellobiose dehydrogenase enzyme electrodes to form an EFC that relies on direct electron transfer for glucose oxidation and oxygen reduction. The EFC achieved 3 µW cm$^{-2}$ in both PBS and human serum, though an additional peak was observed in serum, attributable to the presence of oxidisable interferents. Recently a mediated EFC has been tested for use in both PBS and artificial plasma\textsuperscript{[108]}. This EFC, consisting of a GOx/Os(dmbpy)PVI anode and a $Mv$BOd/Os(bpy)PVI cathode, each co-immobilised onto a gold electrode, produces a power density of 8 µW cm$^{-2}$ in PBS and only 2 µW cm$^{-2}$ in artificial plasma. Southcott and Katz\textsuperscript{[109]} examined the use of an EFC, with human blood flowing over buckypaper electrodes, to power a pacemaker, based on use of a charge pump and DC–DC converter interface circuit to boost the cell voltage. The combined device was tested for a period of 5 hours, during which it continued to power the pacemaker. Osmium redox complexes have also been as mediators in enzymatic fuel cell electrodes for testing in serum. For example, Ó Conghaile et al.\textsuperscript{[38]} assembled an EFC based on an oxygen-reducing cathode using ScL co-immobilised with the redox complex $[\text{Os}(2,2^{'-}\text{bipyridine})_2(4\text{-aminomethylpyridine})\text{Cl}]^+$ and a glucose-oxidising anode using PQQGDH co-immobilised with $[\text{Os}(4,4^{'-}\text{dimethoxy 2,2^{'-}\text{bipyridine}})_2(4\text{-aminomethylpyridine})\text{Cl}]^+$, each with MWCNT added, crosslinked using the methodology detailed in Chapters 2, 3, and 4 of this thesis. When tested in pseudo-physiological PBS (pH 7.4, 150 mM NaCl, 37°C) containing 5 mM glucose, a power density of 66 µW cm$^{-2}$ at 0.25 V cell voltage was achieved. However, these fuel cells when tested in human serum produced only 37 µW cm$^{-2}$ at 0.26 V cell voltage.
1.6 Electroanalytical techniques

The electroanalytical method of cyclic voltammetry is a core research tool utilised in this thesis in order to determine characteristic properties of films, such as assessing surface coverage of mediator detectable upon the electrode surface, and current-voltage characteristics of fuel cell electrodes. A brief description of the basic principles of this technique is therefore presented in the following sections.

1.6.1 Cyclic voltammetry

Cyclic voltammetry is the fundamental analytical technique available to the electrochemist studying redox systems. In cyclic voltammetry an initial potential is applied across the electrochemical cell, and is ramped linearly across a predetermined range to the switching potential at which point the process is reversed and the potential is ramped back, usually to the initial potential, at the same rate. During this, the current ($I$) at the working electrode is monitored as a function of applied potential ($E$) between the working electrode and a reference electrode resulting in an I-E curve called the cyclic voltammogram (CV).
During such an experiment, a standard three electrode setup is normally used. This consists of a working electrode, which is the electrode at which the reaction of interest occurs, a counter electrode, to facilitate a connection to the electrolyte so that a current can be measured through the working electrode, and a reference electrode. The potential of the working electrode is measured with respect to the reference electrode. Typical working electrode materials include graphite, glassy carbon, platinum and gold. An Ag/AgCl (in chloride solution) system is used as the reference electrode as it is simple to construct, inexpensive, and provides a stable potential. A platinum mesh is usually used as the counter electrode, to ensure that the area of, and therefore current through, the counter electrode does not limit the current at the working electrode. Use of unstirred electrolyte solution, usually phosphate buffer saline in this thesis, during the recording of the CV, simplifies interpretation of the experimental results\textsuperscript{110}.

An electrochemically reversible reaction is characterised by rate of the heterogeneous electron transfer reaction being fast enough to ensure that the relative concentrations of the reduced and oxidised
species at the electrode surface are in thermodynamic equilibrium (i.e. the Nernst equation is obeyed)\cite{110}. The sweep to more positive potentials, at a planar disk-shaped stationary working electrode produces an oxidation peak if the applied potential passes through the standard redox potential ($E^0$) of a redox couple, within the potential window applied. When the scan direction is reversed back through the $E^0$, a reduction peak current, due to conversion of electrolysis product (oxidised product) back to reduced species is obtained. The important parameters in a CV include the cathodic ($E_{pc}$) and anodic ($E_{pa}$) peak potentials and cathodic ($I_{p,\text{red}}$) and anodic ($I_{p,\text{ox}}$) peak currents, determined as shown in Figure 10.

![Figure 10](image.png)

**Figure 10** Reversible cyclic voltammogram where $E_{pa}$ and $E_{pc}$ represent the anodic and cathodic peak potentials, respectively, while $I_{p,\text{ox}}$ and $I_{p,\text{red}}$ represent the oxidation and reduction peak currents, respectively.

In a reversible system, the equilibrium ratio for a redox reaction is determined by the approximate Nernst equation (1.7), where $E$ is the electrode potential relative to the $E^0$, the formal electrode potential.

$$E = E^0 - \frac{0.059}{n} \log \left( \frac{[\text{red}]}{[\text{ox}]} \right)$$

Equation 1.7
Under these conditions, the separation of potential between the current peaks is:

\[ \Delta E = E_{p_a} - E_{p_c} = \frac{59}{n} \text{ mV} \quad \text{Equation 1.8} \]

For an electrochemically reversible process the peak currents scale proportionally to the square root of scan rate \( (\nu) \) and the ratio of peak currents is equal to one. The peak current for an electrochemically reversible reduction or oxidation is given by the Randles-Sevčík equation 1.9

\[ i_p = 0.4463.n.F \sqrt{\frac{nF}{R.T}.A.D^{1/2}.C.V^{1/2}} \quad \text{Equation 1.9} \]

where \( i_p \) is the peak current (in A), \( n \) is the number of electrons being transferred, \( A \) is the electrode area (cm\(^2\)), \( D \) is the diffusion coefficient (cm\(^2\) s\(^{-1}\)) of the oxidised or reduced species, \( C \) is the concentration (mol cm\(^{-3}\)) of the oxidised or reduced species and, \( \nu \) is the scan rate (V s\(^{-1}\)).

If the rate of electron transfer is slow relative to the scan rate the CV is said to show irreversible or quasi-reversible behaviour\([110]\). For an irreversible system electron transfer is the rate determining step and the Nernstian equilibrium is not maintained i.e. the peak to peak separation is greater than \( \frac{59}{n} \) mV and increases with increasing scan rate\([110]\). If homogeneous chemical reactions precede or follow on from the oxidation or reduction of a species at the electrode, the shape of the CV alters. The observed changes give significant information about the kinetics of the coupled reaction\([110]\). The Randles-Sevčík equation may be used to model the CV for multilayer modified electrodes if the sweep rate is rapid enough to ensure semi-infinite diffusion conditions prevail. This, therefore, allows estimation of a diffusion co-efficient for charge transport through such films. However, as the film thickness may be difficult to
determine, making the concentration of electroactive species within the film, C, also difficult to estimate, a parameter of $D^{1/2}C$ is usually reported instead$^{[111]}$. If slow scan rates are used to record a CV for surface confined redox species, such as monolayer or multilayer modified electrodes, a depletion layer extends all the way into the solution and finite diffusion prevails$^{[110]}$. In this case, the surface coverage of redox active sites confined to electrodes (Γ, moles cm$^{-2}$) can be determined from equation 1.10 by using the faradaic charge (Q) passed during complete electrolysis of the film, as determined by quantifying the area under the curve (either anodic or cathodic) on a CV,

$$\Gamma = \frac{Q}{nFA} \quad \text{Equation 1.10}$$

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

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

$$FWHM = \frac{3.53RT}{nF} = \frac{90.6}{n} \text{mV} \quad \text{Equation 1.11}$$

$$E_{pa} = E_{pc} \quad \text{Equation 1.12}$$

where FWHM is the full width at half maximum of the anodic or cathodic wave$^{[110]}$.

The half-life ($t_{1/2}$) of catalytic currents was occasionally used as a comparative measurement of stability. This was determined by dividing ln 2 (0.69314718) by the negative slope of the natural log of current density versus time.
1.7 Thesis proposition

The aim of this thesis is to investigate strategies to improve biocatalytic electrodes for use in an enzymatic biofuel cell, detailed in the subsequent 3 chapters, which have all been published in the scientific literature. The purpose of this research was to investigate the assembly of mediated enzyme electrodes capable of operating under in-vivo conditions, and of producing improved current densities and stability than would otherwise be exhibited using previously established film assembly methodologies. Development was performed by focussing on benchmarking the performance of films prepared using novel methodologies and/or enzymes against those prepared with more established enzymes and methodologies. The effect of each component, such as addition of MWCNT and alteration of crosslinker type, is analysed against analogous mediated enzymatic electrodes prepared using established methodologies and components as benchmarks. After establishing a methodology that produced high catalytic current densities and stability for glucose oxidation, and screening such methodologies using selected oxygen-reducing enzymes, the performance of fully enzymatic fuel cells was assessed using selected combinations of anode and cathode. As is shown in Chapter 4, although one anode might prove capable of producing greater glucose oxidation current densities than another, it may not necessarily prove to contribute to a greater or equal power density when forming part of an assembled EFC. Finally, operation of an EFC was compared in both PBS and artificial plasma solution containing 5 mM glucose. This is of interest as plasma may contain interferents which could affect the behaviour of the mediated enzymatic electrodes.

Chapter 2 demonstrates the proof-of-principle of a novel mediated enzyme electrode film production methodology. The Chapter compares approaches for co-immobilisation of Os(bpy)PVI and GOx within crosslinked films on glassy carbon electrodes. Electrodes prepared by crosslinking using glutaraldehyde vapour, with and
without a NaBH$_4$ reduction, provide higher glucose oxidation current than those prepared using a well-established diepoxide method. Addition of MWCNT to the film deposition solutions produces electrodes providing an enhanced glucose oxidation current density, over those films prepared without MWCNT, with current density up to 5 mA cm$^{-2}$ at 0.35 V vs Ag/AgCl in 100 mM glucose solutions, whilst improving the operational stability of the current signal. These MWCNT, glutaraldehyde vapour crosslinked, films on electrodes, reduced by NaBH$_4$, retain 77% of initial catalytic current over 24 hours of continuous amperometric testing in a 37°C, 50 mM phosphate buffer solution containing 150 mM NaCl and 100 mM glucose. Potential application of this approach to implantable enzymatic biofuel cells is demonstrated by production of glucose oxidation currents, under pseudo-physiological conditions, using films prepared with redox polymers possessing lower redox potentials than the Os(bpy)PVI initially selected.

Chapter 3 progresses the research by reporting on a demonstration of the applicability of the methodology established in Chapter 2 to screening for glucose-oxidising enzyme electrodes based on a range of enzymes. The sugar-oxidising enzymes: glucose oxidase; a range of glucose dehydrogenases (GDH) and cellobiose dehydrogenases (CDH) were co-immobilised, in the presence of MWCNTs, with osmium redox polymers on graphite electrodes. Under pseudo-physiological conditions of 5 mM glucose, 150 mM NaCl, 37°C, glucose oxidation current densities above 800 µA cm$^{-2}$ are obtained using films containing an Os(dmbpy)PVI redox polymer, and either glucose oxidase or FAD-dependant GDH. Current produced by glucose-oxidising half-cells, and stability of the current, is compared in 100 mM glucose, with films containing CDHs proving most stable.

Chapter 4 initially reports on using the same co-immobilisation methodology, described in Chapters 2 and 3, to select from a range of blue multiple copper oxygenases, active under physiological pH conditions, as components in enzyme electrode for oxygen-reducing biocathodes in an EFC operating under pseudo-physiological
conditions. The Chapter also presents a comparison of the response of assembled enzymatic fuel cells utilising the bioanodes and biocathodes developed in both the second and third Chapters in buffer and artificial plasma. It should be noted that the maximum concentration of dissolved oxygen measured (~0.065 mM dissolved O₂) within the artificial plasma produced for the research presented in this thesis did not approach that reported on in the literature (~0.134 mM dissolved O₂)[112]. The estimate, derived from Henry’s law constants for oxygen, does not consider the effect of antioxidants in artificial plasma. In addition, when left to equilibrate under ambient conditions, the dissolved O₂ concentration in the artificial plasma solutions decreased to less than 50 μM, in agreement with published reports on venous O₂ concentrations[113]. This should be taken into consideration if comparisons to reports of power output from assembled EFC, operating in buffer solutions, are undertaken, as attempted in Chapter 4.

Finally, Chapter 5 summarises the contribution made towards the scientific body of knowledge by this thesis, and outlines other research undertaken during the course of the PhD, and some opinion on the direction such research may take in the future.

As part of my PhD activities entailed synthesis of a range of redox polymers, and distribution of such systems to a diversity of collaborating partners, I have been honoured to be included as a co-author on some publications emanating from their research using my redox polymers. On some other papers, co-authorship was granted due to the use of methodologies, or expertise, that I developed during my PhD. Although these results are not included in this thesis, an appendix is included providing a complete list of my co-authored publications, as well as the oral and poster presentations I have made over the course of my PhD studies.
1.8 References


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Chapter 2

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**Co-author contributions:**
I synthesised the PVI-bound osmium redox systems, and performed the laboratory work, the analysis, and wrote the first draft of the publication.

Maria Ferrer contributed to the publication through her knowledge and experience of MWCNT, which inspired the investigation that led to this publication.

Peter Ó Conghaile contributed to the publication through his involvement in the synthesis of the osmium complex precursor to the PVI-bound osmium redox systems.

Paul Kavanagh contributed advice and guidance during the laboratory work.

Dónal Leech, as the project supervisor, contributed through guidance and advice throughout and wrote the final draft of the publication.
Crosslinked redox polymer enzyme electrodes containing carbon nanotubes for high and stable glucose oxidation current

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Abstract
Co-immobilisation approaches for preparation of glucose-oxidising films of [Os(2,2’-bipyridine)2(poly-vinylimidazole)10Cl] and glucose oxidase on glassy carbon electrodes are compared. Electrodes prepared by crosslinking using glutaraldehyde vapour, without and with a NaBH 4 reduction, provide higher glucose oxidation current than those prepared using a well-established diepoxide method. Addition of multi walled carbon nanotubes to the film deposition solutions produces an enhanced glucose oxidation current density of 5 mA cm −2 at 0.35 V vs Ag/AgCl, whilst improving the operational stability of current signal. Carbon nanotube, glutaraldehyde vapour crosslinked, films on electrodes, reduced by NaBH 4, retain 77% of initial catalytic current over 24 hours of continuous amperometric testing in a 37°C, 50 mM phosphate buffer solution containing 150 mM NaCl and 100 mM glucose. Potential application of this approach to implantable enzymatic biofuel cells is demonstrated by production of glucose oxidation currents, in pseudo-physiological conditions, using mediating films with lower redox potentials.
Introduction

Enzymatic biofuel cells (EFCs) propose replacement of metal catalysts in proton-exchange membrane fuel cells by enzymes to enhance conversion of chemical energy to electrical energy. EFCs may extend substrate (fuel) choice and remove the requirement for fuel and oxidant separation by membranes and casings, thus opening opportunities for production of micropower sources, either for devices operating at mild conditions or for implantable units\textsuperscript{1–4}. Over the past two decades advancements have been made through co-immobilisation of enzyme within films of synthetic redox polymer hydrogels on solid electrodes to provide for mediated electron transfer between redox enzyme active site and the electrode. In particular, redox polymer hydrogels containing osmium pendant moieties attached to polymers such as poly(N-vinylimidazole), poly(4-vinylpyridine) have been widely used\textsuperscript{5–11}. Films of hydrogels form a three-dimensional source of electrically connected catalyst on electrodes, compared to monolayer-based films, enabled through successive electron transfer cycling between the Os\textsuperscript{2+/3+} redox centres (self-exchange). Moreover, as they are permeable to water-soluble substrates and products, the observed current densities for substrate electrolysis by enzymes in such films are amongst the highest reported\textsuperscript{11,12}. Despite advances in harnessing the catalytic process to provide for enzyme-based biosensors and fuel cells, the low level of both power output and long-term stability has hampered the development of continuous-use enzyme electrodes. Methods for integrating components of enzyme electrodes that maximise enzyme loading while maintaining, effectively, the electrical contact between biocatalyst and electrode, with improved enzyme stabilisation are widely investigated\textsuperscript{12–14}. For example, use of conductive nano-objects shows great promise to achieving this. The presence of carbon nanotubes (CNTs) in enzyme electrodes has been reported to promote direct electron transfer between enzyme and electrode\textsuperscript{15,16}, and resulted in notable increases of both current and stability in electron transfer mediated systems, reported mostly for applications related to biosensors\textsuperscript{17–20} and more recently, to EFCs\textsuperscript{21–24}. An increase in current for both oxidation of the osmium redox complexes
and for glucose oxidation by incorporation of single walled CNTs with glucose oxidase (GOx) in a film containing an osmium redox polymer has been reported\textsuperscript{20}. The same study also reported an increase in stability of enzyme activity, in which a redox polymer/enzyme film prepared with single walled carbon nanotubes was found to retain 67\% of its initial glucose oxidation current over 24 hr compared to a value of 55\% attributed to a similar electrode prepared without single walled carbon nanotubes.

A crucial aspect to enhancing EFC operational stability is selection of immobilisation strategy of enzyme and polymer-bound mediator. Different methods have been proposed to effectively trap the biocatalyst without affecting its activity, such as the covalent grafting of enzymes on derivatised electrode surfaces\textsuperscript{25}, the use of crosslinkers to bond the biocatalyst with the redox polymer chains\textsuperscript{8}, or employing layer-by-layer techniques\textsuperscript{26,27}. Although most studies on enzymes co-immobilised within redox polymer hydrogels have been based on the use of a diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether (PEGDGE), to promote film stability the use of glutaraldehyde (GA) solutions to covalently bond enzyme and redox polymer has also been investigated\textsuperscript{28–30}. The use of GA vapours for enzyme immobilisation for biochemical applications has been widely reported\textsuperscript{31}, and recently has been applied as a crosslinking agent for electrodes\textsuperscript{32,33}, and when used in redox polymer/enzyme films has shown an increase in oxidation current and enzyme stability over non-crosslinked films\textsuperscript{33}. The use of borohydride to reduce unstable Schiff bases, formed during GA crosslinking, to amines is also well established\textsuperscript{34,35}. However, use of GA vapours followed by a borohydride reduction has not as yet been reported on, to our knowledge, for preparation of stabilised redox hydrogel-based enzyme electrodes, with air-drying of the crosslinked films the most widespread reported procedure\textsuperscript{33,36,37}.

Here we report on an investigation of methods to improve glucose oxidation current density and stability for films prepared by deposition and crosslinking of the redox polymer [Os(2,2'-bipyridine)\textsubscript{4}(poly(N-vinylimidazole)\textsubscript{10}Cl)\textsuperscript{+} (OsPVI) with GOx on glassy carbon electrodes.
These enzyme electrodes are capable of providing current for glucose oxidation in phosphate buffer, pH 7.4 above applied potentials of 0.23 V vs Ag/AgCl\textsuperscript{8-10,14}. The performance of enzyme electrodes prepared using GA vapour as a crosslinking agent, with and without the addition of multi walled carbon nanotubes (MWCNT), is compared against films prepared using the PEGDGE crosslinker, with subsequent application of the method providing the best compromise of current density and stability to preparation of enzyme electrodes containing redox polymers displaying potentials shifted more negatively, more appropriate for use as low potential glucose-oxidising anodes in EFCs.

**Experimental Details**

Synthesis of the redox polymers was achieved by adapting literature procedures\textsuperscript{38,39}. PEGDGE (average Mn ~ 526), GOx from *Aspergillus niger* (182,000 U/mg), multi walled carbon nanotubes, glutaraldehyde solution (25%) and all other chemicals were obtained from Sigma–Aldrich (Dublin, Ireland) unless otherwise indicated. Glassy carbon electrodes (3 mm diameter, IJ Cambria), polished with 0.05 µm alumina powder slurry (Buehler), were modified by drop-coating a mixture of aqueous solutions of redox polymer (6 µL of an 8 mg mL\textsuperscript{-1} solution) and GOx (4.8 µL of 10 mg mL\textsuperscript{-1} solution). For the MWCNT systems, 74 mg mL\textsuperscript{-1} of MWCNT was selected for incorporation into the redox polymer solution, based on previous results for preparation of MWCNT scaffolds\textsuperscript{40}. PEGDGE crosslinking was achieved by addition of freshly prepared aqueous solution of 1.9 µL of a 15 mg mL\textsuperscript{-1} PEGDGE to the dropcoat solution, with curing of the deposited films at room temperature for 24 hours. GA crosslinking was by exposure of the film on electrodes, previously dried for 23.5 hours, to glutaraldehyde vapours in a sealed headspace for 30 minutes. In some cases the GA crosslinked electrodes were subsequently immersed in 1 M NaBH\textsubscript{4} for ~5 seconds and then rinsed in MilliQ water. Testing commenced within 20 minutes of the completion of electrode preparation.

Electrochemical experiments were performed using a CH Instruments 1030 multichannel potentiostat in a three electrode cell at 37°C containing pH 7.4 phosphate buffer saline (PBS, 50 mM buffer, 150 mM
NaCl) purged with nitrogen, using the modified glassy carbon as working electrode, a 3 M KCl Ag/AgCl reference electrode (CH Instruments) and a platinum mesh counter electrode (Goodfellow).

**Results and Discussion**

Previous reports using redox polymers and GOx co-immobilised on electrodes for oxidation of glucose have mostly focused on optimisation of component ratios for film preparation based on maximising the glucose oxidation current signal, for application to glucose sensing or current density production in anodes of an EFC. We have undertaken a comparative study of the effect of film composition on both the glucose oxidation current density and the stability of resulting signals, in order to select an optimum mix of components for preparation of redox-polymer based glucose oxidising enzyme electrodes for application to sustainable power generation in an EFC. To test for the effect of di-epoxide PEGDGE crosslinker content on current density and stability a series of enzyme electrodes were compared using a fixed GOx to OsPVI mass ratio of 1:1.3 whilst varying the amount of PEGDGE added. All PEGDGE curing times were 24 hours, in accordance with published data that determined 24 hours as optimum compromise for stability and enzymatic activity. The observed glucose oxidation current densities, estimated from initial slow scan cyclic voltammograms, and after subsequent 20 hours storage at 4°C, in the presence of 100 mM glucose indicate maximum current density and stability for addition of 23% w/w PEGDGE to the films (not shown). Bell-shaped curves, similar to that observed previously, of increasing and then decreasing catalytic current relative to increased loading of PEGDGE are obtained. While lower ranges of PEGDGE crosslinker result in low currents due to poorer retention of enzyme and redox polymer film on the electrode surface, higher ranges of PEGDGE crosslinker also result in low currents because extensive crosslinking restricts charge transport through the films or interferes with access of substrate into the film.

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Figure 1 Normalised glucose oxidation currents from 5 mV s$^{-1}$ initial (blue diamond) and after 75 minutes (red square) CVs in 50 mM pH 7.4 phosphate buffer solution containing 150 mM NaCl and 100 mM glucose. Films prepared with fixed PEGDGE 23% w/w and a range of GOx and OsPVI % w/w. Subsequently, the effect of the ratio of OsPVI:GOx in the film deposition was examined, maintaining a 23% w/w PEGDGE as crosslinker, in order to determine the optimum combination to deliver high, and stable, glucose oxidation currents. The best compromise between current and stability is observed for the 38.5 % w/w GOx 38.5 % w/w OsPVI deposition solutions from a comparison, shown in Figure 1, of initial catalytic current and catalytic current recorded after 75 min, following continuous CV cycling, normalised to the highest initial current. This film retained 89% of its current after the 75 min test period. Films containing a higher loading of GOx (44 % w/w), whilst increasing the glucose oxidation current, resulted in a loss of relative stability of the current signal over the same time period, retaining only 58 % of initial signal. Films based on the higher loading of GOx have previously been reported to yield the highest glucose oxidation currents for a similar system$^{41}$, with however stability of signal not taken into consideration. Films prepared using this compromise composition (designated henceforth as PEGDGE electrodes) yield CVs, recorded at 1 mV s$^{-1}$ in the
absence of glucose substrate, that display a formal redox potential of 0.22 V vs Ag/AgCl, similar to that reported on previously\textsuperscript{6,30,41}, and a full width half maximum (FWHM) for the Os\textsuperscript{2+/3+} redox transition of 0.11 V, close to the ideal response of 0.09 V for a surface-confined one-electron redox system\textsuperscript{43}. The observed difference in potential between oxidation and reduction peaks (ΔE\textsubscript{p}) of 30 mV is less than the 59 mV separation expected for a diffusion-controlled process, but nonetheless deviates slightly from the ideal value (0 mV) expected for a surface-confined redox transition, presumably because of interactions within the films\textsuperscript{44}. At slow scan rates (<20 mV s\textsuperscript{-1}) the Os\textsuperscript{2+/3+} peak currents scale linearly with scan rate, with integration of the area under the peak providing an estimate of the quantity of redox active osmium on the surface of the electrode (Γ\textsubscript{Os}) of 7.5×10\textsuperscript{-8} moles cm\textsuperscript{-2}. At higher scan rates (>20 mV s\textsuperscript{-1}) peak currents scale with the square root of the scan rate indicative of a changeover from finite-diffusion (surface confined) to semi-infinite diffusion (incomplete electrolysis within the film on the timescale of the experiment) control. Under these conditions a D\textsuperscript{1/2}C value of 1.2×10\textsuperscript{-8} mole cm\textsuperscript{-2} s\textsuperscript{-1/2} can be estimated from the slope of the plot of peak current versus square root of the scan rate using the Randles-Ševčík equation\textsuperscript{43}, with such values comparable to previously published results for films of redox polymers\textsuperscript{45} and of redox polymers and enzymes\textsuperscript{41,46}. In the presence of 100 mM glucose substrate at an applied potential of 0.35 V vs Ag/AgCl, the enzyme electrodes produce an initial current density of 0.8 mA cm\textsuperscript{-2} that drops to approximately 0.2 mA cm\textsuperscript{-2}, 27% of the initial current density, after 12 hrs of constant potential amperometry, and to only 6% of the initial current density after 24 hours, suggesting that alternate immobilisation approaches should be considered.

**Glutaraldehyde crosslinked systems**

Glutaraldehyde, either in solution\textsuperscript{28–30} or as a vapour\textsuperscript{33,36,37}, has been widely used as a crosslinking agent for preparation of biocatalytic films on surfaces. It is reported that the use of glutaraldehyde solution for preparation of GOx/redox polymer films on carbon electrodes produces significantly lower glucose oxidation current, at a glucose concentration
of 48 mM, than other homo-bifunctional crosslinkers. More recently, GA vapours have been used to crosslink enzymes and, often, mediators in the preparation of films on surfaces with a wide range of GA exposure times reported. Evaluation of the performance of GA vapours as a crosslinking agent in comparison to the PEGDGE crosslinker is therefore merited. Films of GOx and OsPVI, 1:1 mass ratios, were exposed to GA vapours over a range of times, resulting in GA electrodes with an approximately threefold higher initial catalytic current for glucose oxidation for films prepared using a GA vapour exposure time of 30 minutes, compared to the PEGDGE crosslinked systems, and higher than those prepared using longer GA vapour exposure times. However, after 3 hours of continuous cycling in 100 mM glucose-containing electrolyte, the films retained only 28% of initial catalytic current, performing worse than the PEGDGE electrodes. Longer GA exposure times delivered improved stability of catalytic current for glucose oxidation, resulting in retention of between 40% and 50% glucose oxidation current after 3 hours, though with significantly lower initial currents than the film exposed to GA for 30 minutes.

The use of borohydride, to reduce the unstable Schiff bases, formed upon reaction of the aldehyde with primary amines, therefore stabilising systems crosslinked using GA solutions, has been reported. Introduction of borohydride reduction following the GA crosslinking reaction was therefore examined (termed GA(R) electrodes) to establish whether resulting films provide for improved stability, whilst retaining the high glucose oxidation currents observed using GA vapours as a crosslinking methodology. For example, GA electrodes display similar osmium surface coverages and D1/2C to that for the PEGDGE electrodes, yet produce almost 3 times the initial catalytic current in the same 100 mM glucose stirred phosphate buffer solution at an applied potential of 0.35 V. However, after 24 hours amperometry the initial current density diminishes by 98%. When GA crosslinked films are subsequently reduced using NaBH₄, high initial catalytic current density for glucose oxidation, 2.4 mA cm⁻² (similar to that of the GA electrode), is observed.
with however only marginally improved stability, with 94% decrease in initial current after 24 hours continuous amperometry at 0.35 V, Table 1.

**Carbon nanotube-based catalytic films**

Addition of CNT to biocatalytic films has been reported to result in improvement to observed biocatalytic currents and stability of such films on electrodes, for biosensor and biofuel cell applications. A comparison of the effect of addition of commercial MWCNT to the drop-coating solutions, prior to crosslinking, on the glucose oxidation current magnitude and stability was therefore undertaken. The glucose oxidation current density increases for all cases (PEGDGE, GA and GA(R) electrodes) upon inclusion of MWCNT in the drop-coating solution for film formation (Table 1). The large error observed for response of the MWCNT-containing films in some instances is likely due to differences in deposited amounts of MWCNT stemming from difficulties with obtaining reproducible dispersion within the solution. An example of the increase in glucose oxidation current densities can be observed in the MWCNT-containing films prepared by crosslinking GOx and OsPVI using GA(R) which yield an initial glucose oxidation current density of 4.7 mA cm\(^{-2}\) in PBS at 0.35 V vs Ag/AgCl constant-potential amperometry, compared to a current density of 2.4 mA cm\(^{-2}\) for analogous GA(R) electrodes without MWCNT addition. Such currents, of 4.7 mA cm\(^{-2}\), are higher than those previously reported for glucose oxidation using redox polymer/enzyme films with incorporation of carbon nanotubes.
Higher glucose oxidation current densities have only recently been obtained using macroporous nanofibre-based scaffolds on microelectrodes\(^{23,50}\).

An estimate of the stability of the response for glucose oxidation can be achieved by recording the amperometric response, whilst gently stirring (150 rpm) the bulk solution, to avoid localised substrate depletion. In order to compare stabilities of the response from the different films we plot the natural logarithm of the current as a function of time over 24 hrs, thus assuming simple first-order decay in

**Table 1** Response of films prepared using different crosslinking methodologies. Initial and 24 hr glucose oxidation current density values (median of two measurements, error estimated as range) obtained from amperometry at an applied potential of 0.35 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 100 mM glucose.

<table>
<thead>
<tr>
<th>Methodology</th>
<th>( \Gamma_{os} ) (nmoles cm(^{-2}))</th>
<th>Initial glucose current density (mA cm(^{-2}))</th>
<th>% glucose current remaining at 24 hrs</th>
<th>Calculated ( t_{1/2} ) (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEGDGE</td>
<td>75±1</td>
<td>0.80±0.04</td>
<td>11±3</td>
<td>6.5±1</td>
</tr>
<tr>
<td>PEGDGE</td>
<td>180±30</td>
<td>4.9±1.5</td>
<td>30±9</td>
<td>20±0.2</td>
</tr>
<tr>
<td>MWCNT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>64±13</td>
<td>2.4±0.2</td>
<td>2±1</td>
<td>4±0.6</td>
</tr>
<tr>
<td>GA</td>
<td>69±3</td>
<td>6.3±0.8</td>
<td>13±5</td>
<td>7.5±1</td>
</tr>
<tr>
<td>MWCNT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA(R)</td>
<td>65±18</td>
<td>2.4±0.6</td>
<td>6±2</td>
<td>3.4±0.1*</td>
</tr>
<tr>
<td>GA(R)</td>
<td>130±50</td>
<td>4.7±0.8</td>
<td>77±3</td>
<td>174±17</td>
</tr>
<tr>
<td>MWCNT</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*calculated from 12 to 24 hr decay
signal, and extract a half-life of the film response from the slopes of these plots (Figure 2). Surprisingly, assumption of such a simple model provides for linear plots for all but one of the approaches: the GA(R) electrodes in the absence of MWCNT, with the reported half-life for these films extracted only from the linear portion of the plot over the 12-24 hr time period.

**Figure 2** Decrease in glucose oxidation current (ln) over time for films prepared with (filled) and without (open) MWCNT and crosslinked using PEGDGE (red triangles), GA (blue circles) or GA(R) (black squares) methodology. Data points sampled from 0.35 V vs Ag/AgCl amperometry in 50 mM pH 7.4 phosphate buffer solution at 37°C containing 150 mM NaCl and 100 mM glucose, stirred at 150 rpm.

From results in Figure 2 and Table 1, addition of MWCNT to the films results in a dramatic increase in the estimated half-life of the glucose oxidation current signal, for all of the crosslinking methodologies investigated. Remarkably, films prepared using addition of MWCNT based on GA(R) electrodes deliver a calculated half-life of 174 hrs under operating conditions in the gently stirred solution, though it should be highlighted that this result was extrapolated from data obtained over a comparatively short time period of 24 hrs. This half-
life is approximately 10-fold longer than the half-life estimated for MWCNT-containing films using either PEGDGE or GA electrodes, under the same conditions, and nearly 40-times longer than the half-life estimated for films that do not include MWCNT. In the absence of glucose substrate, estimation of initial $\Gamma_{Os}$ for the films on electrodes highlights that addition of MWCNT to the films results in an increase in amount of Os addressed, compared to films prepared that do not include the MWCNT. Figure 3 presents an example of the increase in oxidation and reduction currents of the osmium redox polymer, due to addition of MWCNT into the GA(R) crosslinked films. Interestingly, the GA electrodes do not show such an increase in $\Gamma_{Os}$ upon inclusion of MWCNT, yet show an increase in glucose oxidation current density. These films, however, display the least improvement in signal stability upon inclusion of MWCNT, perhaps because of the reported instability of the bonds formed using the GA crosslinking approach.

**Figure 3** 1 mV s$^{-1}$ CVs of OsPVI/GOx electrodes crosslinked by GA(R) without (black) and with MWCNT (red dash) in 50 mM pH 7.4 phosphate buffer solution at 37°C containing 150 mM NaCl and in the absence of glucose.
Such results suggest that the increased stability observed for GA(R) electrodes with MWCNT may not be due solely to either inclusion of MWCNT or crosslinking methodology, but perhaps a combination of both. An increase in stability of enzyme electrode signals has also been attributed previously to inclusion of MWCNT \(^{17-20,51}\). For example, a redox polymer/enzyme film prepared with single walled carbon nanotubes was found to retain 67% of its initial glucose oxidation current over 24 hr compared to a value 55% attributed to a similar electrode prepared without single walled carbon nanotubes \(^{20}\). In addition, CNTs incorporated into films for use in EFCs resulted in a six fold increase in power density, but also more than a threefold increase in stability, as compared to films without CNT \(^{51}\). Our results, with over 77% of the initial, substantial, glucose oxidation current retained after 24 hr, for the GA(R) electrodes with MWCNT, show promise as a method to deliver EFCs that can operate in physiological conditions with increased power and stability.

**Redox polymers for fuel cell anodes**

For provision of anodes for enzymatic fuel cells that display both high glucose oxidation current density and improved stability of such currents, implementation of the film preparation methodology developed to films of redox polymers capable of glucose oxidation at lower redox potentials than the OsPVI system is of interest. This would open up the possibility of power production at higher cell voltages, for fuel cells operating using glucose oxidation coupled to oxygen reduction \(^{1,2,13}\). A lowering of the Os\(^{2+/3+}\) redox potential for PVI-co-ordinated redox complexes can be achieved by insertion of electron-donating substituents to the 4,4'-position of the 2,2'-bipyridine ligands of the complexes \(^{8,10,14}\). When such an approach is undertaken, films of redox polymers on carbon electrodes in PBS displaying redox potentials of 0.12 V and −0.04 V vs Ag/AgCl are obtained using a methyl- or methoxy-substituent, respectively, compared to the 0.23 V vs Ag/AgCl for the unsubstituted 2,2'-bipyridine polymer-bound complex, similar to results reported on previously \(^{52,53}\). Preliminary tests using anodes prepared by addition of
MWCNT to films of these redox polymers, GOx, and the GA(R) crosslinking methodology on carbon electrodes were undertaken. In order to compare the capacity of such anodes to operate under physiological conditions, slow scan CVs, displayed in Figure 4, were recorded in the presence of 5 mM glucose in 50 mM phosphate buffer solution with 150 mM NaCl at 37°C in the absence of convection. From such CVs, steady-state glucose oxidation current densities of 1 mA cm⁻² at potentials above 0.23 V are observed in the presence of 5 mM glucose for films prepared using OsPVI (2,2'-bipyridine complex), compared to current densities of 3.4 mA cm⁻² in the presence of 100 mM glucose, under the same conditions. The current density is the same for films prepared either on glassy carbon or graphite electrodes of the same diameter, suggesting that the signal is independent of the underlying roughness factor of the electrodes. Films prepared using [Os(4,4'-dimethyl-2,2'-bipyridine)₂(poly-vinylimidazole)₁₀Cl] redox polymer on graphite deliver steady-state glucose oxidation current densities of 0.7 mA cm⁻² above potentials of 0.10 V vs Ag/AgCl, whilst analogous films prepared using the [Os(4,4'-dimethoxy-2,2'-bipyridine)₂(poly-vinylimidazole)₁₀Cl] redox polymer deliver steady-state glucose oxidation current densities of 0.4 mA cm⁻² above potentials of 0 V vs Ag/AgCl, Figure 4. The decrease in glucose oxidation current density as a function of redox potential may be as a result of the lower thermodynamic driving force for current generation and/or differences in the properties (swelling, charge transport etc.) for the films, as suggested by others. Whilst direct comparison is rendered difficult, because of different film preparations, test conditions, and parameters, the response of these MWCNT-containing electrodes, crosslinked by the GA(R) methodology, compares favourably in terms of glucose oxidation current density with existing reports on macroelectrodes, including those that used CNTs. For example, a glucose oxidation current density of 0.5 mA cm⁻² in 100 mM glucose solutions is reported for films of the [Os(4,4'-dimethoxy-2,2'-bipyridine)₂(poly-vinylimidazole)₁₀Cl] redox polymer crosslinked with PEGDGE on graphite in the absence of MWCNT, providing a similar current to that recorded for the GA(R) electrode with
MWCNT for the same redox polymer in just 5 mM glucose. In terms of stability, films of redox polymer and enzyme crosslinked on a microfiber electrode, rotated at over 2000 rpm, retained 82% of initial catalytic current over an 8 hour period\textsuperscript{23}, as compared to the 77% retained by the GA(R) electrode with MWCNT over a 24 hour period in a gently stirred solution. Such a performance indicates that the GA(R) electrode with MWCNT shows promise for application to glucose/oxygen EFCs.

**Figure 4** CVs at 1 mV s\textsuperscript{-1} recorded in 50 mM pH 7.4 phosphate buffer solution at 37°C containing 5 mM glucose, 150 mM NaCl for GA(R) electrodes with MWCNT based on OsPVI on glassy carbon (black dot), graphite (pink solid), [Os(4,4’-dimethyl-2,2’-bipyridine)\textsubscript{2}(poly-vinylimidazole)\textsubscript{10}Cl] on graphite (red dash dot), and [Os(4,4’-dimethoxy-\textsubscript{2,2’-bipyridine})\textsubscript{2}(poly-vinylimidazole)\textsubscript{10}Cl] on graphite (blue dash).

**Conclusions**

The inclusion of MWCNT into an enzyme/redox polymer film crosslinked with GA vapours and reduced by NaBH\textsubscript{4} was shown to offer improved stability of glucose oxidation current over other approaches investigated, retaining 77% of the initial current over 24 hours of continuous amperometric testing in stirred 37°C PBS. This was achieved
while still allowing high current densities (1 mA cm$^{-2}$ in 5 mM glucose),
greater than those previously reported$^{3,18,56}$ with osmium redox
polymer/enzyme films incorporating CNTs on macroelectrodes. To our
knowledge, the above glucose oxidation current densities are the highest
reported in 5 mM glucose.

It was further demonstrated that the GA(R) crosslinking
methodology used in this study can be applied to lower potential redox
polymers more suited as fuel cell anodes, and work is currently
underway in this area with the aim of achieving glucose/oxygen EFCs
with improved stability and power output.

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Chapter 3

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

Peter Ó Conghaile contributed to the publication through his involvement in the synthesis of the osmium complex precursor to the PVI-bound osmium redox systems.

Brenda Egan contributed to the publication by assisting in some aspects of the laboratory work.

Paul Kavanagh contributed advice and guidance during the laboratory work.

Cristoph Sygmund and Roland Ludwig contributed through the development, and supply, of their glucose oxidising enzymes.

Dónal Leech, as the project supervisor, contributed through guidance and advice throughout and wrote the final draft of the publication.
Comparison of glucose oxidation by crosslinked redox polymer enzyme electrodes containing carbon nanotubes and a range of glucose oxidising enzymes

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Abstract

The sugar oxidising enzymes glucose oxidase, glucose dehydrogenases (GDH) and cellobiose dehydrogenases (CDH) were co-immobilised, in the presence of multiwalled carbon nanotubes, with osmium redox polymers. Under pseudo-physiological conditions of 5 mM glucose, 150 mM NaCl, 37 °C, glucose oxidation current densities above 800 µA cm⁻² are obtained from films containing an [Os(4,4'-dimethyl-2,2'-bipyridine)₂(poly-vinylimidazole)₁₀Cl]⁺ redox polymer, redox potential 0.1 V vs Ag/AgCl, and either glucose oxidase or FAD-dependant GDH. Current produced by, and stability of, glucose-oxidising half-cells is compared in 100 mM glucose, with films containing CDHs proving most stable. Such results show promise for development of glucose-oxidising enzymatic fuel cells.
1. Introduction

Coupling of enzymatic oxidation of glucose to enzymatic reduction of oxygen in an enzymatic fuel cell (EFC) can generate electrical power. Use of enzymes as catalysts, instead of non-specific transition metals, allows each electrode to be designed to be specific towards fuel or oxidant, e.g. glucose oxidised at the anode and oxygen reduced at the cathode. This allows for a membraneless system, conducive to miniaturisation [1-3]. Development of enzymatic fuel cells for commercial exploitation for powering, for example, low power medical devices or portable electronics is, however, contingent upon improvements in current generation, power production, and the stability of the devices. One potential application area involves deployment in an implantable, or semi-implantable format, of a miniaturised EFC fuelled by oxidation of glucose within the blood stream (present at a level between 4 and 8 mM) [4], with oxygen reduction at the cathode. However, operation in the presence of oxygen can affect the current output of a sugar oxidising/oxygen reducing membraneless fuel cell based on mediated glucose oxidase catalysis at the anode. Oxygen can compete with the mediator as an electron acceptor, reducing electron flow to the electrode while also depleting substrate available to the cathode. In such a case hydrogen peroxide would be produced, which is a strong oxidant, and will negatively impact the stability of the glucose oxidation currents.

Mediators allow the shuttling of electrons from the enzyme active site to the electrode, regardless of orientation or proximity of the enzyme active site to the electrode surface [1,2]. Enzyme performance has been shown to differ with different mediators [5-7] while the redox potential of the mediator will affect the characteristics of the fuel cell as well as the current produced [8]. Co-immobilisation of mediator and enzyme can be achieved by crosslinking of films deposited on electrodes [8,9]. Improved current density is possible by incorporation of micro and nano-structured conductive particles into the film [10-13].

We recently reported on optimisation of the relative amounts of an osmium redox polymer and glucose oxidase (GOx) in films co-immobilised on carbon electrodes in the presence of multiwall carbon
nanotubes (MWCNT) and bifunctional crosslinkers to provide high and stable glucose oxidation current production [8]. The resulting films produced glucose oxidation currents of 1 mA cm$^{-2}$ in 5 mM glucose, and retained over 70% of initial glucose oxidation current during 24 hours continuous operation. Although the optimisation was performed with an [Os(2,2'-bipyridine)$_2$(poly-vinylimidazole)$_{10}$Cl]$^+$ (Os(bpy)PVI) redox polymer, $E^\circ$ of 0.24 V vs Ag/AgCl for the Os$^{2+/3+}$ transition the potential of this redox polymer is too positive for use as an anode in an EFC. Films of GOx, MWCNT and redox polymers [Os(4,4'-dimethyl-2,2'-bipyridine)$_2$(poly-vinylimidazole)$_{10}$Cl]$^+$ (Os(dmbpy)PVI, $E^\circ$ = 0.1 V) and [Os(4,4'-dimethoxy-2,2'-bipyridine)$_2$(poly-vinylimidazole)$_{10}$Cl]$^+$ (Os(dmobpy)PVI, $E^\circ$ = −0.02 V) produce glucose oxidation current densities of 0.7 mA cm$^{-2}$ and 0.4 mA cm$^{-2}$ in 50 mM quiescent PBS and 5 mM glucose.

Here we present a comparison of the glucose oxidation response of films prepared using the previously optimised relative amounts of components, whilst varying the sugar-oxidising enzyme catalyst, in an attempt to provide for even greater current production at the anode, for application to EFCs. We focus the comparison on use of the two redox polymers, Os(dmbpy)PVI and Os(dmobpy)PVI.

1.1 Enzymes

A range of glucose oxidising enzymes were selected for comparison. Glucose oxidase [14] (from Aspergillus niger) is a dimeric enzyme consisting of 2 equal subunits with a molecular mass of 80 kDa each. Its specific activity towards glucose is approximately 180 U mg$^{-1}$ and GOx has been used as the benchmark sugar oxidising enzyme as it is readily available and returns high glucose oxidation currents. It uses FAD as cofactor which is reduced to FADH$_2$ that is then re-oxidised by the final electron acceptor which is naturally oxygen, or as in this case the redox polymer mediator. As oxygen competition with the mediator can affect current output in an EFC, comparison to anodes based on dehydrogenases (glucose and cellobiose), that do not donate electrons to oxygen, are of interest.
Cellobiose dehydrogenase [15] is typically a monomeric protein consisting of a flavin-containing catalytic dehydrogenase domain which is loosely connected to a smaller heme $b$ containing cytochrome domain. *Corynascus thermophilus* cellobiose dehydrogenase (*CtCDH*) is an example of an alkaline CDH and has a molecular weight of 85 kDa with activity for glucose of 18 U mg$^{-1}$. The use of this enzyme in enzymatic anodes has been focused to date on direct electron transfer to the electrode via its cytochrome domain [15]. Mediated electron transfer can be achieved with the FAD cofactor, as is the case with GOx [16]. In the present report, the catalytically active dehydrogenase domain of *CtCDH* was recombinantly produced in the bacterial expression system *E. coli* resulting in a truncated protein with a molecular mass of only 60 kDa. To further attempt to increase the glucose turnover of the recombinant enzyme an amino acid in the catalytic centre (cysteine 310) was exchanged to a tyrosine. To facilitate protein purification a c-terminal His$_6$-tag was also added. Purification resulted in a homogenous protein preparation of recombinant *CtCDH* (*rCtCDH*) with activity for glucose of 19 U mg$^{-1}$.

A recently characterised glucose dehydrogenase (GDH) [17] was also selected for comparison. The GDH in this study is a FAD-dependent glucose dehydrogenase from *Glomerella cingulata* that has been expressed in *Pichia pastoris*. The recombinant *Glomerella cingulata* GDH (*rGcGDH*) has a molecular mass, determined by SDS-PAGE, of around 100 kDa and a specific activity of 836 U mg$^{-1}$ determined with glucose at pH 5.5.

A second FAD-dependent glucose dehydrogenase expressed in an *Aspergillus* sp. with a molecular weight of approximately 97 kDa and specific activity of 900 U mg$^{-1}$ was selected for the comparison. This *Aspergillus* sp. GDH (AspGDH) [18] is of interest due to its high turnover rate, commercial availability and oxygen independence.

Finally a commercially available pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQGDH) with a molecular mass of 94 kDa was also selected for comparison. It has an optimum pH of 7.0, an optimum
temperature of 37°C and is reported as being less stable than GOx [19]. The PQQGDH preparation has a specific activity of 500 U mg⁻¹.

2. Experimental Details

Synthesis of the redox polymers was achieved by adapting literature procedures [20,21]. Multiwall carbon nanotubes, glutaraldehyde solution (25%) and all other chemicals were obtained from Sigma–Aldrich (Dublin, Ireland). Glucose oxidase type VII from Aspergillus niger (GOx, EC 1.1.3.4.) was purchased from Sigma–Aldrich (Dublin, Ireland, product G2133). CtCDH (EC 1.1.99.18) was produced according to Harreither et al. [22]. The flavodehydrogenase domain of Corynascus thermophilus CDH (rCtCDH) was heterologously expressed in E. coli. rGcGDH (1.1.99.10) was recombinantly produced in the methylotrophic yeast Pichia pastoris and purified according to Sygmund et al. [23]. AspGDH (1.1.99.10) was purchased from Sekisui (Cambridge, USA; product GLDE-70-1192). A pyrroloquinoline quinone (PQQ) dependent glucose dehydrogenase (PQQGDH, EC 1.1.99.17) was purchased from Sorachim (Lausanne, Switzerland; product GLD-321).

Electrodes were prepared from graphite rods, with a diameter of 3 mm (Graphite Store, USA) cut to a length of 8 cm and shrouded in heat shrink tubing creating a working surface area of 0.0707 cm² which was polished on fine grit paper prior to use. Film deposition was achieved by pipetting 9.6 µL of a 46.25 mg mL⁻¹ dispersion of acid treated MWCNT (20 mg mL⁻¹ in HNO₃, refluxed for 6 hours at 150°C), 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 electrode, and allowing to cure for 24 hours. The films were exposed, by placement in a sealed container for 30 mins, to glutaraldehyde vapours in the container headspace and subsequently removed and briefly submerged in a 100 mM NaBH₄ solution and rinsed in MilliQ water, prior to testing. All tests were conducted, unless otherwise stated, in a 50 mM phosphate buffer solution, pH 7.4, containing 150 mM NaCl (PBS), at 37°C using a CH Instruments (IJCambria) multi-channel potentiostat.
3. Results and Discussion

3.1 Voltammetric characterisation of redox polymer/enzyme films

The redox polymers, Os(dmbpy)PVI and Os(dmobpy)PVI, are selected for screening of mediation of glucose oxidation by co-immobilised enzymes in films on carbon electrodes, because of their lower redox potentials compared to that of Os(bpy)PVI systems, for eventual application to glucose-oxidising enzymatic biofuel cell anodes. The screening process consists of preparation, by drop-coating, of films of redox polymer and enzyme in the presence of MWCNT, shown to contribute to increased current densities [8,12,24], on graphite electrodes, and evaluation of response in parallel in the same electrolyte using a multi-channel potentiostat. The relative mass of redox polymer, enzyme and MWCNT is selected to match that determined to be optimal for glucose oxidation current density and stability by films of Os(bpy)PVI and GOx, previously [8]. Crosslinking of the components is achieved by incubation of the dried films in a chamber containing glutaraldehyde, with subsequent reduction, in a NaBH₄ solution, of the unstable imine and enamines formed [8,25,26]. The initial screening involved recording of slow-scan cyclic voltammetry (CV) of the films in the absence of glucose substrate, for comparison of the redox response of the mediating osmium polymer within the films, and then in the presence of 5 mM and 100 mM glucose, to evaluate the bioelectrocatalytic current for glucose oxidation. Conditions are chosen to simulate in-vivo (or ex-vivo [27]) situations using quiescent 37°C, 50 mM phosphate buffer, pH 7.4, containing 150 mM NaCl.

In general, in the absence of substrate, the difference in peak-to-peak potentials for the Os²⁺/³⁺ transition within films of Os(dmbpy)PVI and Os(dmobpy)PVI is less than 59 mV, and the peak current scales directly with scan rate, up to scan rates of approximately 20 mV s⁻¹, indicative of a response for surface confined redox species [28]. The estimated redox
potential of the Os$^{2+/3+}$ transition, from the midpoint between the oxidation and reduction peak potentials, is $0.100 \text{ V} \pm 0.005 \text{ V}$ and $-0.014 \text{ V} \pm 0.005 \text{ V}$ vs. Ag/AgCl for Os(dmbpy)PVI and Os(dmobpy)PVI based films, respectively, matching those values observed for such systems in previous publications [17,29,30]. Integration of the area under the CV peak, at slow scan rates such as those depicted in Figure 1, provides an estimate of the coverage of osmium sites, $\Gamma_{\text{Os}}$, on the electrode on the timescale of this experiment. The average $\Gamma_{\text{Os}}$ for all of the systems studied, based on co-immobilisation with the Os(dmbpy)PVI redox polymer, is $120 \pm 40$ nmoles cm$^{-2}$, similar to the coverages observed previously for Os(bpy)PVI co-immobilised with GOx and MWCNT on carbon electrodes [8]. The electrodes prepared with enzymes co-immobilised with Os(dmobpy)PVI were observed to have a surface coverage of only $70 \pm 10$ nmoles cm$^{-2}$, almost half that of the Os(dmbpy)PVI based electrodes. Whether this is due to a slower rate of swelling and/or charge transport, and thus difficulties in addressing the redox sites on the timescale of the experiment, or other factors, is as yet not clear.

**Figure 1** CVs, at $1 \text{ mV s}^{-1}$ in quiescent $50 \text{ mM}$ phosphate buffer solution, $150 \text{ mM}$ NaCl at $37^\circ \text{C}$, of films of PQQGDH co-immobilised with Os(dmbpy)PVI (black) in the absence (▬) and presence of $5 \text{ mM}$ glucose (−−−) and with Os(dmobpy)PVI (red) in the absence (・・・) and presence of $5 \text{ mM}$ glucose (−•).
Upon addition of glucose to the electrochemical cell, the observed glucose oxidation currents of 690 µA cm\(^{-2}\) and 350 µA cm\(^{-2}\), obtained from 1 mV s\(^{-1}\) CVs in 5 mM glucose solutions, for films of GOx co-immobilised with Os(dmbpy)PVI and Os(dmobpy)PVI, respectively, are comparable to those previously reported \[8\] under the same conditions. As an example of the voltammetric response of the films, slow scan CVs of films of Os(dmbpy)PVI and of Os(dmobpy)PVI co-immobilised with PQQGDH in absence and presence of 5 mM glucose are shown, Figure 1. The CVs performed in the presence of glucose achieve a steady level of glucose oxidation current for all sugar-oxidising enzymes, as expected in an EC’ catalytic process \[28\], by 0.25 V for electrodes containing Os(dmbpy)PVI and 0.1 V for electrodes containing Os(dmobpy)PVI. Replacement of enzyme does not affect the redox potential for oxidation of the redox polymer, nor the potentials at which steady levels of glucose oxidation currents can be achieved. This is evident by comparison of the CVs based on glucose oxidation (in the presence of 5 mM and 100 mM glucose) by films containing AspGDH and PQQGDH co-immobilised with the same Os(dmbpy)PVI redox polymer, Figure 2. Also evident is the variation in catalytic glucose oxidation current density as a function of selection of sugar-oxidising enzyme.

Further screening of the performance of the films on electrodes as a function of redox polymer and enzyme selection was undertaken by monitoring the glucose oxidation current using amperometry, with an applied potential of 0.25 V for films containing Os(dmbpy)PVI and 0.1 V for films containing Os(dmobpy)PVI.

### 3.2 Amperometric screening of redox polymer/ enzyme films for glucose oxidation

The amperometric response for films containing enzymes co-immobilised with redox polymer display glucose oxidation current densities that are similar to the current observed at the same potential in slow scan CVs. In general a slight increase in current density is obtained, probably because of the decision to introduce convection using 150 rpm stirring of the solution for amperometry, in an effort to avoid local
depletion of glucose substrate contributing to decreasing current over time. For example, a current density of 440 µA cm$^{-2}$ can be estimated at 0.25 V from the CV in Figure 1 for oxidation of 5 mM glucose by films of Os(dmbpy)PVI co-immobilised with PQQGDH, whereas a current density of 470 µA cm$^{-2}$ is obtained at the same applied potential during amperometry.

Figure 2 CVs at 1 mV s$^{-1}$ in quiescent 50 mM phosphate buffer solution, 150 mM NaCl at 37°C, of films of Os(dmbpy)PVI co-immobilised with AspGDH in 5 mM glucose (▬) and in 100 mM glucose (・・・) and with PQQGDH in 5 mM glucose (▬) and in 100 mM glucose (・・・).
Figure 3 Glucose oxidation current densities extracted from steady-state amperometry at an applied potential of 0.25 V, as a function of glucose concentration, for films prepared using Os(dmbpy)PVI co-immobilised with GOx (■), AspGDH (♦), rGcGDH (▼), PQQ-GDH (◄), CtCDH (●), or rCtCDH (▲) in pH 7.4 PBS at 37°C, solution stirred at 150 rpm.

The glucose oxidation currents were observed to increase as a function of glucose concentration for all systems studied, Figure 3 and 4, using amperometry. Apparent Michaelis constants, $K_{M^{app}}$, and maximum saturation currents, $I_{max}$, can be estimated from curve-fitting of the results with a simple Michaelis-Menten model, with results of this fitting presented in Table 1. Both CtCDH and rCtCDH have high $K_{M^{app}}$ for glucose, in excess of 100 mM glucose for the Os(dmbpy)PVI electrodes for example. These are slightly higher than the $K_{M^{app}}$ estimated from solution phase analysis, 90-mM for CtCDH and 65 mM for rCtCDH using cytochrome c as co-substrate. The three glucose dehydrogenase enzymes examined displayed lower $K_{M^{app}}$ in comparison to the CDHs, between 13-17 mM. The PQQGDH $K_{M^{app}}$ of 4.8 mM [31], estimated in solution is lower than the 17 mM in films of Os(dmbpy)PVI on electrodes. The $rGcGDH$ displayed a similar $K_{M^{app}}$, 13 mM, to its solution phase $K_{M^{app}}$ of
17 mM when 2,6-dichlorophenolindophenol is used as co-substrate at pH 7.5, whilst the AspGDH \( K_{M}^{\text{app}} \) of 17 mM, is somewhat lower than the 50 mM observed in solution [18]. Both \( K_{M}^{\text{app}} \) are however comparable to published values observed [17] for films of Os(dmbpy)PVI on graphite electrodes, but without MWCNT, under similar conditions.

**Figure 4** Glucose oxidation current densities extracted from steady-state amperometry at an applied potential of 0.1 V, as a function of glucose concentration, for films prepared using Os(dmobpy)PVI and GOx (■), PQQ-GDH (▲), and CtCDH (●) in pH 7.4 PBS at 37°C, solution stirred at 150 rpm.

**Table 1** \( K_{M}^{\text{app}} \) and \( I_{\text{max}} \) for glucose estimated from amperometric response (Fig 3 and 4) for films of redox polymer, enzyme and MWCNT on graphite electrodes in glucose solutions.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( K_{M} ) mM</th>
<th>( I_{\text{max}} ) mA cm(^{-2} )</th>
<th>( I_{\text{max}} ) mA U(^{-1} ) cm(^{-2} )</th>
<th>( K_{M} ) mM</th>
<th>( I_{\text{max}} ) mA cm(^{-2} )</th>
<th>( I_{\text{max}} ) mA U(^{-1} ) cm(^{-2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOx</td>
<td>6</td>
<td>2.2</td>
<td>0.3</td>
<td>8</td>
<td>0.7</td>
<td>0.08</td>
</tr>
<tr>
<td>rGcGDH</td>
<td>13</td>
<td>1.9</td>
<td>0.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AspGDH</td>
<td>17</td>
<td>3.8</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PQQGDH</td>
<td>17</td>
<td>1.9</td>
<td>0.8</td>
<td>30</td>
<td>1.4</td>
<td>0.06</td>
</tr>
<tr>
<td>CtCDH</td>
<td>125</td>
<td>4.9</td>
<td>5.6</td>
<td>69</td>
<td>1.6</td>
<td>1.9</td>
</tr>
<tr>
<td>rCtCDH</td>
<td>103</td>
<td>2.6</td>
<td>2.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
When co-immobilised with Os(dmobpy)PVI the estimated $K_{M^{\text{app}}}$ for GOx of 8 mM is similar to the 6mM $K_{M^{\text{app}}}$ observed when co-immobilised with Os(dmbpy)PVI. It is interesting to note, however, that the $K_{M^{\text{app}}}$ for PQQGDH co-immobilised with Os(dmobpy)PVI is 30 mM, increased in comparison to that for Os(dmbpy)PVI films, whilst the $K_{M^{\text{app}}}$ for CtCDH co-immobilised with Os(dmobpy)PVI is 69 mM, representing a decrease in comparison to that for Os(dmbpy)PVI films.

As $I_{\max}$ indicates the maximum current that can be extracted from these systems, films prepared using CtCDH will deliver higher glucose oxidation current densities than those prepared using PQQGDH for both redox polymer systems studied, under saturated glucose conditions. However, the relative performance of each redox polymer/enzyme combination is compared for a glucose concentration of 5 mM, to better mimic physiological glucose levels, with eventual application to in-vivo or ex-vivo glucose-oxidising anodes as a goal. Under these screening conditions films prepared using Os(dmbpy)PVI and either GOx or AspGDH display the highest current densities, 0.9 mA cm$^{-2}$ and 0.8 mA cm$^{-2}$, respectively. Unsurprisingly, given the high $K_{M^{\text{app}}}$ values, films prepared using the CDHs produce the lowest current densities in 5 mM glucose, ~0.2 mA cm$^{-2}$. For films with enzyme co-immobilised with Os(dmobpy)PVI the highest glucose oxidation current densities, though lower than those observed with Os(dmbpy)PVI, are also obtained for films prepared with GOx, producing ~300 µA cm$^{-2}$, compared to those prepared using PQQGDH, which produced ~200 µA cm$^{-2}$, or CtCDH which produced ~100 µA cm$^{-2}$.

To compare response under conditions where maximum glucose oxidation current density is required, for application, for example, to powering portable electronic devices, a glucose concentration of 100 mM was selected. Under these conditions, films prepared using Os(dmbpy)PVI and AspGDH produced the highest current density of 3.2 mA cm$^{-2}$, whilst films of GOx or CtCDH produced approximately 2 mA cm$^{-2}$, with, however, films of the CtCDH not yet approaching the predicted $I_{\max}$ current density of 4.9 mA cm$^{-2}$. For films prepared using Os(dmobpy)PVI, tested in 100 mM glucose, the highest glucose oxidation
current densities were observed when the redox polymer was co-immobilised with either PQQGDH or CtCDH, both producing approximately 1 mA cm$^{-2}$, though, unlike films prepared with PQQGDH, the CtCDH system had yet to achieve substrate saturation.

As an equal mass of each enzyme was used in the screening presented here, (in order to emulate the previously established methodology based on co-immobilisation of Os(bpy)PVI and GOx) the current densities normalised to the initial solution phase glucose activity of the enzyme mass added in the film preparation procedure may be of interest. Table 1 presents the results when the normalised currents are fitted, as previously, to a Michaelis-Menten model. The activity normalised current density produces similar glucose calibration curves for all three glucose dehydrogenases, Figure 5, when co-immobilised with Os(dmbpy)PVI, suggesting that the difference in glucose oxidation current density observed between them is likely due to the differing catalytic activity per mole of enzyme. GOx, per unit activity, achieved a higher activity normalised glucose oxidation current density compared to the GDHs whilst the CDHs produced increasing activity normalised glucose oxidation current throughout the entire range of glucose concentrations examined. A similar trend is observed for enzymes co-immobilised with the Os(dmobpy)PVI films on electrodes. A recent study [17] compared glucose oxidation currents for films of AspGDH and rGcGDH, each co-immobilised with Os(dmbpy)PVI on graphite electrodes, with equal specific activity for glucose of the enzymes added to the coating solutions, instead of equal mass, and no added MWCNT. These films, in a flow-injection format, produced only ~0.5 mA cm$^{-2}$ in the presence of 50 mM glucose, compared to current densities of 3.1 mA cm$^{-2}$ and 1.5 mA cm$^{-2}$ for films of Os(dmbpy)PVI co-immobilised with AspGDH and rGcGDH with added MWCNT in the presence of 50 mM glucose, in the present study. It is interesting to note, however, that the glucose oxidation current density, when activity normalised, become similar for the enzyme electrodes in both studies (~0.06 mA U$^{-1}$ cm$^{-2}$). MWCNT addition thus provides a scaffold to permit greater mass loading of enzyme, with no difference in activity of the immobilised enzymes in
the presence or absence of MWCNTs. Further research, comparing enzyme electrodes prepared under the same conditions, as a function of MWCNT, and enzyme quantity in terms of moles, activity, and mass added to deposition solutions is warranted.

**Figure 5** Glucose oxidation current densities normalised to the activity of enzyme added to the deposition solutions, extracted from steady-state amperometry at an applied potential of 0.25 V, as a function of glucose concentration from electrodes coated with Os(dmbpy)PVI and GOx (■), AspGDH (♦), rGcGDH (▼), PQQ-GDH (◄), CtCDH (●), and rCtCDH (▲) in pH 7.4 PBS at 37°C, solution stirred at 150 rpm.

3.3 Stability of glucose oxidation currents

The operational stability is a key parameter required for further development of continuous-use enzyme electrodes for application to biosensing and biocatalytic fuel cells. Operational stability testing was performed using amperometry in 50 mM pH 7.4 phosphate buffer solution, containing 150 mM NaCl and 100 mM glucose, maintained at 37°C and stirred at 150 rpm. A cell volume of 1 L assures that less than 1% of the glucose is oxidised over the 24 hour period. The percentage of remaining current for each electrode after 24 hours of continuous
amperometry at 0.25 V or 0.1 V for electrodes coated with an enzyme and Os(dmbpy)PVI or Os(dmobpy)PVI was used as a measure of operational stability.

Films prepared using GOx retain >60% of initial current after the 24 hr testing period although the highest current retention is observed for films prepared with CDHs and the Os(dmbpy)PVI redox polymer, with CtCDH films retaining 70% and rCtCDH retaining 64% of initial current after the 24 hr testing period, as expected based on the reported high stability of films of CDHs on electrodes [15,32]. Films prepared using the PQQGDH deliver, as expected [19], the poorest operational stability, retaining <35% of glucose oxidation current after the 24 hr testing period for films of both redox polymers. Interestingly films prepared using AspGDH and Os(dmobpy)PVI retain >60 % of initial glucose oxidation current, yet retain <50 % when co-immobilised with Os(dmbpy)PVI.

Although comparison between these results and those reported on by others is hampered by differences in film preparation and testing procedures, it should be noted that films of AspGDH crosslinked using a diepoxide with Os(dmbpy)PVI on a graphite electrode, without added MWCNT, displayed <40% glucose oxidation current after 25 hrs [17], highlighting the improved stability of the present system.

4. Conclusions

Co-immobilisation of a range of glucose-oxidising enzymes with redox polymers and MWCNT on graphite electrodes results in catalytic current for the oxidation of glucose in pseudo-physiological conditions. The presence of MWCNTs, compared to reports on films prepared without added MWCNT, results in a greater amount of redox enzyme on the surface [17], and an improved operational stability of the glucose oxidation response [8]. Use of the redox polymers Os(dmbpy)PVI and Os(dmobpy)PVI result in glucose oxidation at applied potentials more negative than that using an Os(bpy)PVI redox polymer.

When the response in solution of 5 mM glucose for films prepared by co-immobilisation of a fixed mass of enzyme are compared, films
containing GOx or AspGDH display the highest current densities, and films prepared using the CDHs produce the lowest current densities. Whilst the same enzymes also display highest current densities in 100 mM glucose solutions, it is noteworthy that films prepared using CtCDH do not yet approach the predicted $I_{\text{max}}$ current density of 4.9 mA cm$^{-2}$.

The glucose oxidation current density normalised to reflect the activity of the enzymes added to the coating solutions, are however similar for all GDH enzyme electrodes, indicating no difference in activity of the immobilised enzymes in the presence or absence or MWCNTs.

Such studies provide promising results in relation to the development of glucose-oxidising enzymatic fuel cells.

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Chapter 4

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Co-author contributions:
I synthesised the PVI-bound osmium redox systems, and performed the laboratory work, the analysis, and wrote the first draft of the publication. Peter Ó Conghaile contributed to the publication through his involvement in the synthesis of the osmium complex precursor to the PVI-bound osmium redox systems. Brenda Egan contributed to the publication by assisting in some aspects of the laboratory work. Paul Kavanagh contributed advice and guidance during the laboratory work. Dónal Leech, as the project supervisor, contributed through guidance and advice throughout and wrote the final draft of the publication.
Membrane-less Glucose/Oxygen Enzymatic Fuel Cells Using Redox Hydrogel Films Containing Carbon Nanotubes

Domhnall MacAodha, Peter Ó Conghaile, Brenda Egan, Paul Kavanagh and Dónal Leech*

(Dedicated to Adam Heller on the occasion of his 80th birthday)

Abstract

Co-immobilisation of three separate multiple blue copper oxidases, a Myceliophthora thermophila laccase, Streptomyces coelicolor laccase and a Myrothecium verrucaria bilirubin oxidase, with an \([\text{Os}(2,2'-\text{bipyridine})_2(\text{poly-vinylimidazole})_{10}\text{Cl}]^{+/2+}\) redox polymer in the presence of multi-walled carbon nanotubes on graphite electrodes results in enzyme electrodes producing current densities above 0.5 mA cm\(^{-2}\) for oxygen reduction at an applied potential of 0 V vs Ag/AgCl. Fully enzymatic membrane-less fuel cells are assembled using the oxygen-reducing enzyme electrodes connected to glucose-oxidising anodes based on co-immobilisation of glucose oxidase or an FAD-dependent glucose dehydrogenase with an \([\text{Os}(4,4'\text{-dimethoxy-2,2'-bipyridine})_2(\text{poly-vinylimidazole})_{10}\text{Cl}]^{+/2+}\) redox polymer in the presence of MWCNTs on graphite electrodes. These fuel cells can produce power densities of up to 145 \(\mu\text{W cm}^{-2}\) when operated in pH 7.4 phosphate buffer solution at 37 °C containing 150 mM NaCl, 5 mM glucose, and 0.12 mM O2. The fuel cells based on Myceliophthora thermophila laccase enzyme electrodes produce the highest power density when combined with glucose oxidase-based anodes. Though the maximum power density of a fuel cell of glucose dehydrogenase and Myceliophthora thermophila laccase enzyme electrodes decreases from 110 \(\mu\text{W cm}^{-2}\) in buffer to 60 \(\mu\text{W cm}^{-2}\) when tested in artificial plasma, it provides the highest power output reported to date for a fully enzymatic glucose oxidising, oxygen-reducing, fuel cell in artificial plasma.
Introduction

A fully enzymatic fuel cell (EFC) is capable of generating electrical power through enzyme-catalysed oxidation of fuel coupled to enzyme-catalysed reduction of oxidant. By using enzymes as catalysts, rather than non-specific transition metals, each electrode can be designed to be specific towards fuel or oxidant. This, if the catalysts are immobilised at the electrodes, opens up the possibility for a membrane-less, and thus miniaturised, system for powering electronic devices[1-4]. Much interest in recent years has focused on EFCs that may provide power using fuel and oxidant available in-vivo, in body fluids such as the bloodstream, e.g. glucose oxidised at the anode and oxygen reduced at the cathode.

Addition of redox mediators to enzyme-based electrodes improves shuttling of electrons between enzyme active site and electrode, making electron transfer independent of orientation or proximity of enzyme active site to electrode surface, in comparison to that for direct electron transfer mechanisms between enzyme and electrode[1,2]. Enzyme electrode current output depends on selection of mediator with appropriate structure (enzyme affinity) and redox potential (driving force) [5-7]. The selection of mediator will also affect the magnitude of an EFC voltage and its power output[8].

Co-immobilisation of mediator and enzyme, for provision of enzyme electrodes for application to membrane-less EFCs, can be achieved by cross-linking enzyme and mediator using reactive bifunctional reagents such as di-epoxides or di-aldehydes, to provide biocatalytic films on electrodes[9,10]. Improved current densities for such films is possible by incorporation of micro and nano-structured conductive particles into the film[8,11-14]. This has been demonstrated, for example, by achievement of a 6.4-fold increase in current density for glucose oxidation by films of redox polymer, glucose oxidase (GOx) and carboxylated carbon nanotubes coated onto carbon fibre electrodes, compared to the current in the absence of carbon nanotube[15].

Output from mediated EFCs, fuelled by oxidation of glucose within the blood stream (present at a level between 5 and 8 mM)[16] coupled to reduction of oxygen, may be limited by a number of factors.
For example, operation in the presence of oxygen can affect current output of glucose oxidase-based electrodes in a membrane-less fuel cell, as oxygen competes with the mediator as an electron acceptor, reducing electron flow to the electrode whilst also depleting oxygen availability to the cathode\(^2\). In addition the current and power output of EFCs can be compromised by instability of anode or cathode current signals as a result of film instability and/or enzyme instability in physiological solutions\(^17,18\).

We have reported on production of high and stable currents for glucose oxidation by films prepared by co-immobilisation on carbon electrodes of osmium-based redox polymers, GOx and multiwall carbon nanotubes (MWCNT) using glutaraldehyde (GA) vapours, with NaBH\(_4\) reduction of imine bonds formed upon crosslinking\(^8\). For example films of GOx, MWCNT and [Os(4,4'-dimethyl-2,2'-bipyridine)\(_2\)(poly-vinylimidazole)\(_{10}\)Cl\(^{+}\)/\(^{+}\) (Os-(dmbpy)PVI, E\(^{\theta}\) 0.1 V vs Ag/AgCl) or [Os(4,4'-dimethoxy-2,2'-bipyridine)\(_2\)(poly-vinylimidazole)\(_{10}\)Cl\(^{+}\)/\(^{+}\) (Os(dmobpy)PVI, E\(^{\theta}\) 0.02 V vs Ag/AgCl) produce glucose oxidation current densities of \~0.7 mA cm\(^{-2}\) and \~0.4 mA cm\(^{-2}\), respectively, in 50 mM quiescent phosphate buffered saline (PBS) solutions containing 5 mM glucose.

Co-immobilisation of the same mass of a range of glucose-oxidising enzymes with these redox polymers and MWCNT on graphite electrodes results in maximum current output for glucose oxidation, under pseudo-physiological conditions, from films prepared using either GOx or an FAD-dependent glucose dehydrogenase (GDH) isolated from Aspergillus\(^9\).

In order to provide a membrane-less fully EFC operating under physiological conditions, coupling of the glucose-oxidising enzyme electrode anodes to oxygen-reducing enzyme electrode cathodes is required. We report here on responses of enzyme electrodes using a range of oxygen-reducing multiple blue copper oxygenases (MBCO)\(^19,20\), co-immobilised with the redox polymer [Os(2,2'-bipyridine)\(_2\)(poly-vinylimidazole)\(_{10}\)Cl\(^{+}\)/\(^{+}\) (Os(bpy)PVI, E\(^{\theta}\) 0.24 V vs Ag/AgCl) and MWCNTs. Three enzymes were selected: the fungal laccase sourced from
*Myceliophthora thermophila* (*Mt*L)\(^{[21,22]}\), a bacterial laccase sourced from *Streptomyces coelicolor* (*Sc*L)\(^{[23-26]}\) and the *Myrothecium verrucaria* bilirubin oxidase (*Mv*BOd)\(^{[1-3, 19,27-30]}\). These MBCOs are selected as they have been shown to catalyse the reduction of oxygen to water at neutral pH, without producing hydrogen peroxide\(^{[21-32]}\). The *Mt*L is a monomeric glycoprotein with a molecular mass of 85 kDa, a type 1 (T1), substrate-oxidising copper site redox potential of 0.48 V vs NHE, and it is active up to 60°C. The pH optimum is substrate dependent, but typically around pH 7\(^{[21,22]}\). The *Sc*L is reported to form trimeric assemblies, with each monomer consisting of two domains containing the 4 copper active sites found in monomeric fungal laccases\(^{[25]}\). It has a reported monomeric molecular mass of 32 kDa, displays a redox potential of the T1 site of ~0.43 V vs NHE\(^{[26]}\), and is active up to 90°C. The pH optimum is substrate dependent, but typically around pH 8. The *Mv*BOd is a monomeric glycoprotein with a molecular mass of 60 kDa, has a T1 site redox potential of 0.67 V vs NHE and a broad pH activity region (3–7) for electron donors such as K\(_4\)[Fe(CN)\(_6\)] or electrodes.

Subsequent to evaluation of oxygen reduction by films of redox polymer, MBCO and MWCNT under pseudo-physiological conditions, we report on assembly of a series of oxygen-reducing, enzyme electrodes, as cathodes with glucose oxidising, enzyme electrodes, as anodes, for testing under pseudo-physiological conditions in buffer, and in an artificial plasma solution.

**Results and Discussion**

**Enzyme electrodes for reduction of oxygen**

A marked increase in catalytic current density, at substrate saturation levels, due to the presence of MWCNT has been previously observed for glucose oxidation by crosslinked films of enzymes and redox polymers on electrodes\(^{[8]}\). When MWCNTs are included in enzyme electrodes, prepared by crosslinking Os(bpy)PVI and *Mt*L with glutaraldehyde vapours, as reported on previously for the glucose-oxidising enzyme electrodes\(^{[8,9]}\) the oxygen reduction current density at saturation
increases from 0.3 mA cm\(^{-2}\) to 0.65 mA cm\(^{-2}\) (not shown). This increase in current density may be due to an increased loading of enzyme enabled by addition of MWCNT, as postulated elsewhere\(^{[8,9]}\). In any event, MWCNTs are included in the enzyme electrode preparation for all subsequent evaluation, to increase the projected current density for oxygen reduction.

A comparison of current density at an applied potential of 0 V vs Ag/AgCl for enzyme electrodes prepared with the same redox polymer, and same added mass for each of the three MBCOs as a function of oxygen concentration was then undertaken. The current densities achievable with enzyme electrodes containing ScL, producing 1.5 mA cm\(^{-2}\) at O\(_2\) saturation levels (0.22 mM), are approximately double that observed for enzyme electrodes prepared using either MvBOd (0.83 mA cm\(^{-2}\)) or MtL (0.66 mA cm\(^{-2}\)) as oxygen reduction biocatalysts. For oxygen concentrations at which fuel cell testing is performed (0.12 mM, vide infra) films prepared with ScL produce a current density of 1.1 mA cm\(^{-2}\), whereas films of either MvBOd or MtL produce 0.6 mA cm\(^{-2}\). The differences in current densities, determined using amperometry at 0 V, are confirmed by differences in steady-state oxygen reduction currents in slow scan cyclic voltammograms (not shown). The observation of a higher current density for ScL is perhaps not surprising given the lower molar mass of the protein compared to that of the other MBCOs tested. However, the enzyme electrodes containing co-immobilised MtL reach substrate saturation by 0.1 mM O\(_2\), and thus yield a significantly lower \(K_m^{\text{app}}\) for oxygen than either the ScL or the MvBOd enzyme electrodes (Table 1), with \(K_m^{\text{app}}\) values estimated using a Hanes-Woolf plot, and subsequently verified through non-linear fitting. The current density estimated by constant potential amperometry at 0 V vs Ag/AgCl, compares well to a current density of 1.5 mA cm\(^{-2}\) reported for oxygen reduction under saturation conditions for films of Os(bpy)PVI co-immobilised with ScL on rotating (900 rpm) glassy carbon electrodes, tested at pH 7, 40 °C\(^{[26]}\). In addition, Kim et al.\(^{[33]}\) report an oxygen reduction current density of 0.7 mA cm\(^{-2}\) in air saturated PBS for films of an osmium redox polymer and MvBOd crosslinked on 7 µm diameter
carbon fibre electrodes, comparable to the current density recorded in Figure 1 for MvBOd-containing films.

**Figure 1** Current density response, using constant potential amperometry at 0 V vs Ag/AgCl, as a function of oxygen concentration for enzyme electrodes containing the same mass of ScL (□), MtL (■), and MvBOd (○) in 50 mM phosphate buffer solution, pH 7.4, 150 mM NaCl, at 37°C. n≥3.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_{m}^{\text{app}}$ (mM)</th>
<th>$I_{\text{max}}$ (mA cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ScL</td>
<td>0.24 ± 0.04</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>MvBOd</td>
<td>0.19 ± 0.05</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>MtL</td>
<td>0.08 ± 0.02</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

Table 1 Michaelis-Menten constants and maximum currents for oxygen reduction by enzyme electrodes (n=3) prepared from crosslinking films of MBCO enzyme, Os(bpy)PVI, and MWCNT on graphite electrodes.
**Enzymatic fuel cells**

Assembly of each enzyme electrode in a membrane-less fuel cell under conditions mimicking that of a physiological environment was implemented for each of the MBCO-based oxygen-reducing enzyme electrodes. Enzyme electrodes chosen as anodes are based on films of an Os(dmbpy)PVI redox polymer co-deposited with enzyme and MWCNT and crosslinked, after drying, by exposure to glutaraldehyde vapours with subsequent reduction using NaBH₄[^8]. Enzymes selected for use in a glucose-oxidising anode are GOx and an FAD-dependant glucose dehydrogenase (GDH) as we previously reported[^1, 9] that these produce glucose oxidation current densities >0.8 mA cm⁻² in 5 mM glucose solution.

The assembled EFCs based on oxygen reduction by cathodes containing *Mt*L provided higher maximum power outputs than those assembled using cathodes containing *Sc*L or *Mv*BOd, Figure 2. For example, for fuel cells prepared using GOx in the anode enzyme electrode, a maximum power density of 145 µW cm⁻² was achieved when *Mt*L was used in the cathode enzyme electrode, compared to 105 µW cm⁻² or 70 µW cm⁻² produced for cathode enzyme electrodes using *Sc*L or *Mv*BOd, respectively. The EFCs based on using GOx as the enzyme in the anode provided higher maximum power density compared to their counterparts using GDH. For example, the GDH-based anodes produce maximum power densities approximately 25% lower than that produced by the GOx-based anode analogues when coupled to either *Mt*L or *Sc*L-based cathodes in an EFC.
**Figure 2** Power curves recorded for fully enzymatic membrane-less fuel cells in phosphate buffer solution, pH 7.4, 150 mM NaCl, 5 mM glucose, 0.12 mM O₂ at 37°C, where enzymes for anode/cathode are: GOx/MtL (■), GOx/ScL (×), GOx/MvBOd (□), GDH/MtL (O), GDH/ScL (—), and GDH/MvBOd (▲) and redox polymers for anode/cathode are Os(dmbpy)PVI and Os(bpy)PVI.

It is curious that the ScL-based enzyme electrodes produce higher oxygen reduction current densities than those based on MtL or MvBOd under 0 V vs Ag/AgCl constant potential amperometry, Figure 1, whilst maximum power density output is observed in the assembled EFCs by coupling enzyme electrodes based on MtL in the cathode to anodes, over those using either the ScL or MvBOd-based enzyme electrodes, Figure 2. Current at a constant applied potential may not therefore be applicable as a predictor for relative EFC power output.

The power density values observed, for the simple membrane-less EFC configuration based on combining macroscopic (3 mm diameter) enzyme electrodes compare well with those in other reports on fully enzymatic fuel cells operating under pseudo-physiological conditions. For example, an EFC assembled in a membrane-less configuration based on GOx and MvBOd co-immobilised with osmium redox polymers on 7 μm diameter, 2 cm long, carbon fibres, as anode and cathode,
respectively, produced a power density approaching 50 µW cm⁻² in quiescent solutions of phosphate buffer at 37.5°C containing 0.14 M NaCl and 15 mM glucose, compared to the 70 µW cm⁻² for the GOx/MvBOd EFC in Figure 2, under similar electrolyte conditions, but with only 5 mM glucose[33]. It should however be noted that the maximum power densities we observe are at lower cell voltages, between 0.18 to 0.26 V, compared to that reported by Kim et al.[33], because of the difference in redox potentials of the osmium redox polymers selected. Comparison to results of other fully-enzymatic fuel cells is difficult due not only to considerable differences in testing conditions, such as glucose concentration and pH, but also to the effect of the electrode preparation methodologies. For example, an EFC assembled using GOx and a fungal laccase, co-immobilised with osmium redox polymers on 7 µm diameter, 2 cm long, carbon fibres, as anode and cathode produced 350 µW cm⁻² power density in 15 mM glucose, pH 5 buffered solutions[34]. Replacement of GOx sourced from Aspergillus niger with a novel GOx sourced from Penicillium pinophilum resulted in maximum power density of 280 µW cm⁻² under the same conditions, except 5 mM glucose used as fuel instead of 15 mM[35]. Fully enzymatic fuel cells based on films of GOx or a fungal laccase on 3 mm diameter glassy carbon electrodes, but adopting a ferrocene-based redox polymer, instead of an osmium-based redox polymer as an anode electrocatalyst, provide maximum power density of 56 µW cm⁻² in stirred solutions containing 60 mM glucose, pH 5.5 at 37°C[36].
Use of GDH in an enzyme electrode operating as a glucose-oxidising anode is of interest due to its catalytic capabilities being, unlike GOx, unaffected by the presence of oxygen\textsuperscript{[37]} while use of \textit{M}t\textit{L} in an enzyme electrode operating as an oxygen-reducing cathode is of interest due to its low $K_{m}^{app}$ relative to ScL and \textit{M}v\textit{BOd} enzyme electrodes. The peak power density observed at 0.23 V for the GDH/\textit{M}t\textit{L} EFC of 110 ± 6 µW cm\textsuperscript{-2} (n=3) is amongst the highest reported to date, under the conditions used. It should be noted that a substantial power density of 36 µW cm\textsuperscript{-2} is still produced at a cell voltage of 0.40 V in this fully enzymatic fuel cell under the same conditions.

The power curve recorded for the GDH/\textit{M}t\textit{L} EFC, Figure 2 was compared to a power curve computed using the anodic, for the GOx enzyme electrode, and cathodic, for the \textit{M}v\textit{BOd} enzyme electrode, sections of the slow scan CV of each enzyme electrode in the fuel cell electrolyte, Figure 3, in an attempt to evaluate the factors limiting power
output. The enzyme electrodes produce similar steady-state current densities of ~0.7 mA cm\(^{-2}\) at potentials more oxidising than 0.1 V vs Ag/AgCl for glucose oxidation, and more reducing than 0.2 V vs Ag/AgCl for oxygen reduction, respectively. The polarisation curves, in Figure 4, for enzyme electrodes, and of the assembled fuel cell indicate that current density of the cathode limits the current density produced in that assembled fuel cell. The cathode is producing oxygen reduction current density at a substrate concentration approximately three times the \(K_{m,app}\) for oxygen, whilst the anode is operating in 5 mM glucose, approximately one-third the \(K_{m,app}\) for glucose\(^9\). The glucose concentration present in human blood ranges, in a healthy person, from 5-8 mM while the concentration of non-haemoglobin-bound O\(_2\) dissolved within the blood stream, both venous and arterial, is approximately 0.13 mM\(^{38}\). Thus implementation of the EFC in whole blood would result in a power output limited by the cathode.

The use of artificial plasma allows reproduction of physiological conditions present in human blood without the associated hazards of using whole blood samples. Operation of an EFC, prepared by co-immobilising on graphite electrodes of GDH, Os(dmbpy)PVI, and MWCNT for a glucose-oxidising anode and \(M_\text{tL, Os(bpy)PVI, and MWCNT}\) for an oxygen-reducing cathode, produces maximum power density of 60 \(\mu\text{W}\) cm\(^{-2}\) in artificial plasma, Figure 5, approximately half that observed for the same EFC operating in PBS. The current produced by enzyme electrodes has been shown to differ between operation in PBS and operation in blood\(^{39,40}\). The artificial plasma solutions adopted here contain anti-oxidants and enzyme inhibiting\(^{41-43}\) compounds present in human blood, though not the haemoglobin nor, therefore, haemoglobin bound O\(_2\). In addition the oxygen concentration in the artificial plasma was determined to be lower than that achieved in PBS, with a concentration maintained at ~0.065 mM using oxygen bubbling through the solution. These factors likely contribute to the lower power output observed for the EFC in artificial plasma compared to that in buffer. The solubility of O\(_2\) within plasma is stated in medical literature\(^{38}\) as 0.003 mL of O\(_2\) per mmHg per dL at 37 °C, with desirable systolic blood
pressure being ~100 mmHg. This equates to ~0.134 mM O₂, exceeding the concentration we measure within the artificial plasma. The estimate, derived from Henry’s law constants for oxygen, does not consider the effect of anti-oxidants in artificial plasma. When left to equilibrate under ambient conditions, the O₂ concentration in our artificial plasma solutions decreased to less than 50 µM. Cinquin et al.[45] report a venous O₂ concentration of 45 µM.

Figure 4 Polarisation curves recorded in phosphate buffer solution, pH 7.4, 150 mM NaCl, at 37°C containing 5 mM glucose and 0.12 mM O₂ for the GDH-based (dashed) and MtL-based enzyme electrodes (dotted), vs an Ag/AgCl reference electrode and for the enzyme electrodes assembled as a fuel cell (solid).

Previous reports[42] on an EFC using cellobiose dehydrogenase and bilirubin oxidase enzyme electrodes that relied upon direct electron transfer between enzyme active site and electrode observe no significant change in power output between PBS and human serum, but observe instead an additional maximum, at low cell voltage, in the power curves due to the presence of oxidisable interferents in the serum. A recent study[46], however, of EFC power output using GOx co-immobilised with...
Os(dmbpy)PVI on gold electrodes as an anode and MvBOd co-immobilised with Os(bpy)PVI as a cathode reports a significant decrease in maximum power density in artificial plasma, 2 µW cm⁻², compared to that observed in PBS, 8 µW cm⁻².

**Figure 5** Power curve recorded in artificial plasma at 37°C, ~0.065 mM O₂ for the GDH/MtL EFC.

**Conclusions**

Co-immobilisation of MBCO oxygen-reducing enzymes with an osmium-based redox polymer at graphite electrodes, in the presence of MWCNTs, proved suitable for production of enzyme electrodes capable of delivering current densities above 0.5 mA cm⁻² for oxygen reduction at an applied potential of 0 V vs Ag/AgCl, with ScL enzyme electrodes exhibiting the highest Iₘₐₓ and MtL enzyme electrodes the lowest Kₘₐₚₚ. When operated in an EFC, combined with glucose-oxidising anodes, in 50 mM PBS (at 37°C, 150 mM NaCl, 5 mM glucose, ~0.12 mM O₂), the MtL enzyme electrode EFC combinations contribute the highest power density, producing 145 µW cm⁻² when combined with GOx-based anodes and 110 µW cm⁻² when combined with GDH-based anodes. The maximum power density of the GDH/MtL EFC is only 60 µW cm⁻² when tested in artificial plasma, but is, to our knowledge the highest power
output reported for a fully enzymatic glucose oxidising, oxygen-reducing, fuel cell in artificial plasma to date.

**Experimental Section**

Synthesis of the redox polymers was achieved by adapting literature procedures[47,48]. Multiwall carbon nanotubes, glutaraldehyde solution (25%) and all other chemicals were obtained from Sigma–Aldrich (Dublin, Ireland). Glucose oxidase type VII from *Aspergillus niger* (GOx, EC 1.1.3.4.) was purchased from Sigma–Aldrich (Dublin, Ireland, product G2133). AspGDH (1.1.99.10) was purchased from Sekisui (Cambridge, USA; product GLDE-70-1192). Purified preparations of both *Myceliophthora thermophila* laccase and *Streptomyces coelicolor* laccase were provided by Novozymes A/S (Denmark).

Partly purified preparations of *Myrothecium verrucaria* Bilirubin oxidase were provided by Amano Enzyme Inc. (Nagoya, Japan). The enzyme was additionally purified to homogeneity as described in Guo et al., 1991[49]. All MBCO enzyme activities determined with syringaldazine at ~37°C.

Electrodes were prepared from graphite rods, with a diameter of 3 mm (Graphite Store, USA) shrouded in heat shrink tubing and polished on 1200 grit SiC paper prior to use. Film deposition was achieved by pipetting 9.6 µL of a 46.25 mg mL⁻¹ dispersion of acid treated MWCNT (20 mg mL⁻¹ in HNO₃, refluxed for 6 hours at 150°C), 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 electrode, and allowing to cure for 24 hours. The films were exposed, by placement in a sealed container for 30 mins, to glutaraldehyde vapours in the container headspace and subsequently removed and briefly submerged in a 1 M NaBH₄ solution and rinsed in MilliQ water, prior to testing.

All tests were conducted, unless otherwise stated in a 50 mM phosphate buffer solution, pH 7.4, containing 150 mM NaCl (PBS), at 37°C using a CH Instruments (IJCambria) 1030A multi-channel potentiostat. Platinum mesh and an Ag/AgCl (3 M NaCl) were used as counter and
reference electrode, respectively, for all voltammetry. Oxygen saturation was estimated, using a dissolved oxygen electrode and meter (EUTech Instruments), to occur at ~0.22 mM O₂, achieved by bubbling oxygen into the solution. During fuel cell testing oxygen was blown over the surface of the solution, resulting in a solution concentration, monitored using a dissolved oxygen electrode, of ~0.12 mM O₂. The artificial plasma contained uric acid (68.5 mg L⁻¹), ascorbic acid (9.5 mg L⁻¹), fructose (36 mg L⁻¹), lactose (5.5 mg L⁻¹), urea (267 mg L⁻¹), glucose (916.5 mg L⁻¹), cysteine (18 mg L⁻¹), sodium chloride (6.75 g L⁻¹), sodium bicarbonate (2.138 g L⁻¹), calcium sulphate (23.8 mg L⁻¹), magnesium sulphate (104.5 mg L⁻¹) and bovine serum albumin (7 g L⁻¹).

**Acknowledgements**

We thank Novozymes A/S for the preparations of Myceliophthora thermophila laccase and Streptomyces coelicolor laccase, and Amano Enzyme for Amano 3 preparation of Myrothecium verrucaria bilirubin oxidase. The contribution of Urszula Salaj-Kosla and Edmond Magner, in providing the artificial plasma recipe, is gratefully acknowledged. This work was supported by funding from “3D-nanobiodevice” (FP7 NMP4-SL-2009-229255), an ERA-Chemistry award through the Irish Research Council, and a Charles Parsons Energy Research Award by Science Foundation Ireland.

**Keywords:** enzymatic fuel cell, glucose oxidation, enzyme electrode, oxygen reduction, plasma


5.1 Conclusions

This thesis focused upon the investigation of strategies to improve biocatalytic electrodes for use in an enzymatic fuel cell, by investigating alternate strategies for the assembly of mediated enzyme electrodes capable of operating under in-vivo conditions, with the aim of producing improved current densities and stability over other assembly methodologies. Chapter 2 demonstrated the proof of principle that a methodology consisting of addition of MWCNT, and crosslinking using glutaraldehyde vapour, with NaBH₄ reduction, provides higher glucose oxidation current than those prepared using other methods, such as use of a diepoxide for crosslinking. Enzyme electrodes assembled in this manner, with GOx and Os(bpy)PVI co-immobilised within the electrode film, produce a glucose oxidation current density of 5 mA cm⁻² at 0.35 V vs Ag/AgCl, whilst improving the operational stability of current signal, with almost 80% of initial catalytic current over 24 hours of continuous amperometric testing in a 37°C, 50 mM phosphate buffer solution containing 150 mM NaCl and 100 mM glucose. Potential application of this approach to implantable enzymatic fuel cells was demonstrated by production of glucose oxidation currents, in pseudo-physiological conditions, using osmium polymer mediating films that display lower redox potentials. Chapter 3, demonstrated the applicability of the methodology established in Chapter 2 to a screening process to select the best combination of glucose-oxidising enzyme and osmium redox polymer for bioanodes to be used in an EFC. Under pseudo-physiological conditions of 5 mM glucose, 150 mM NaCl, 37 °C, best glucose oxidation current densities, above 800 µA cm⁻², are obtained from films containing an Os(dmbpy)PVI redox polymer, and either glucose oxidase or FAD-dependant GDH. Current produced by, and stability of, glucose-oxidising half-cells was compared in 100 mM glucose, with films containing CDHs proving most stable. Chapter 4, reported on a screening process for biocathodes to be used in an EFC, and also presented on the performance
of enzymatic fuel cells utilising combinations of bioanodes and biocathodes developed in both the second and third Chapters. The results presented in Chapter 4 also demonstrate performance a selected EFC in artificial plasma. Although the maximum power density observed for the fuel cell of glucose dehydrogenase and *Myceliophthora thermophila* laccase enzyme electrodes as anode and cathode, respectively, decreases from 110 µW cm\(^{-2}\) in buffer to 60 µW cm\(^{-2}\) on testing in artificial plasma, this EFC provides the highest power density output reported to date for a fully enzymatic glucose-oxidising, oxygen-reducing fuel cell operating in artificial plasma.

As a further test of the proof-of-concept for EFC devices developed during the research conducted for the thesis, selected methodologies developed were incorporated into tests performed by other members of the Biomolecular Electronics Research Laboratory and collaborating partners with the goal of powering a prototype implantable biosensor device. 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. An anode, prepared using a deglycosylated pyranose dehydrogenase (dgPDH) sugar oxidising enzyme co-immobilised with Os(dmbpy)PVI and MWCNT and crosslinked with GA vapours, and subsequently reduced by NaBH\(_4\), was paired with BOd on AuNP substrate as biocathode\([1–3]\). Power densities of up to 325 µW cm\(^{-2}\) were achieved, in PBS, which contained 5 mM glucose, 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 cathode is the limiting factor in the fuel cells, so 10 biocathodes were stacked against a single bioanode in the testing procedure. A signal was received thus demonstrating that the device was powered and functional.

There is still a need for further development prior to implementation of this prototype technology. For example, testing and development was undertaken using macro-scale, i.e. 3 mm diameter, electrodes. Any *in-vivo* applications would necessitate use of micro-scale electrodes\([4–7]\). One possible avenue for miniaturisation is for the electrode itself to be
constructed from the CNT components that, hitherto, formed a film on the graphite electrode surface. A possible mechanism to achieve this is the fabrication of micro-channelled CNT scaffolds. The next section details some of the preliminary tests undertaken to investigate such a route for enzyme electrode preparation.

5.2 Three dimensional micro-channelled CNT scaffolds

In order to maximise the extent of miniaturisation of implantable enzymatic fuel cells a strategy was devised, in collaboration with M. Ferrer of the Consejo Superior de Investigaciones Científicas of Madrid (CSIC), to produce micro-channelled electrodes constructed entirely from the components of the biocatalytic films usually deposited on graphite or glassy carbon electrodes. Such electrodes may efficiently utilise the large surface area allowing for high current due to greater integration and contact of the biocatalytic components and the fuel or oxidant within a given two-, and three-, dimensional area. The platform for this research was a previously published approach based on preparation by ice segregation induced self-assembly (ISISA) of the three dimensional micro-channelled CNT scaffolds as electrodes\(^\text{[8–14]}\). ISISA is a technique that is simple to deploy, consisting of harnessing unidirectional freezing of any diluted aqueous suspension, and even emulsions, by means of controlled immersion of the suspension into a liquid N\(_2\) bath, thereby obtaining a cryo-gel. The cryo-gel is formed by the propagation of ice formation. As the ice forms the matter is segregated to the external boundaries of the ice, as the growth of ice continues the matter is compacted, forming the channel walls. The resulting frozen suspension is freeze-dried in order to sublime the water leaving only empty channels surrounded by the matter, resulting in a micro-channelled structure. ISISA scaffolds using dispersed suspension of CNT and chitosan have been used, for example, as supports for microbial anodes\(^\text{[15]}\). Preliminary research directed at formation of scaffolds, as enzyme electrodes for use in EFCs, using ISISA of MWCNT suspensions dispersed using either chitosan, for subsequent coupling of
enzyme and redox complexes, or MWCNT suspensions dispersed using redox polymers.

Scanning electron microscopy (SEM) is used to confirm formation of three dimensional micro-channelled CNT scaffolds. For example, the SEM images in Figure 1, of a scaffold formed using ISISA of a suspension of 74 mg mL$^{-1}$ MWCNT and 8 mg mL$^{-1}$ Os(bpy)PVI in 1 mL syringe, after freeze-drying. The images display both the presence of micro-channels and the carbon nanotube composition of the channel walls. Occasional globules of insufficiently dispersed MWCNT clumps are also visible (image in bottom left of Figure 1).

**Figure 1** SEM images of increasing magnification of a scaffold formed using ISISA of a suspension of 74 mg mL$^{-1}$ MWCNT and 8 mg mL$^{-1}$ Os(bpy)PVI in 1 mL syringe, after freeze-drying.
An ISISA MWCNT scaffold secured by shrink-wrapping around a glass tube to Teflon tubing and electrically connected using carbon fibre tape, configured for flow-through operation.

A flow-through system of a scaffold formed using ISISA of a suspension of MWCNT and Os(bpy)PVI and crosslinked with GA vapours was created by shrink-wrapping the scaffold within a glass tube and connection to Teflon tubing, with electrical connection using carbon fibre tape, Figure 2. A cyclic voltammogram of the scaffold with PBS containing GOx flowing through produces a peak current of 0.15 mA for the oxidation of the Os(II) at the appropriate redox potential (+0.25 V vs Ag/AgCl), demonstrating retention of the redox polymer within the scaffold, and electrical connection of the redox polymer to the scaffold electrode. When 100 mM glucose is included with the PBS/GOx solutions, a sigmoidal-shaped CV, indicative of catalytic oxidation of glucose, of 1.2 mA is observed, demonstrating that the approach is feasible. However, in order to create an enzyme electrode for application to a membrane-less EFC, it is necessary to immobilise the enzyme within the scaffold.

Two methods of immobilisation of GOx within the scaffold were investigated, and the results described in the next sections. One method explored was inclusion of the enzyme into the MWCNT suspension along with Os(bpy)PVI during the ISISA process, with subsequent crosslinking after freeze drying, as before (Section 5.2.1). A second method explored was electrochemical activation (ECA) of a glutaraldehyde crosslinked...
scaffold prepared by ISISA of an MWCNT suspension in chitosan. The ECA step is reported to produce aldehyde functional groups within the chitosan structure, that are thus available to react with amino functional groups of enzymes and redox complexes, such as an \([\text{Os}(2,2'\text{-bipyridine})_2(4\text{-aminomethyl pyridine})\text{Cl}^+] (\text{Os(bpy)-4AMP})\) complex (Section 5.2.2).

### 5.2.1 Integration of components during scaffold assembly

Integration of components may be achieved using a method where 10 mg mL\(^{-1}\) GOx is included into the MWCNT and Os(bpy)PVI suspension to undergo ISISA. After freeze drying, the scaffold is exposed to GA vapours for crosslinking. However, scaffolds prepared using GA exposures of 30 mins and 1 hour lost their structural integrity within a short period of time, breaking apart and leaching material within the flow through system. Longer GA exposures were therefore investigated in order to increase the stability of the scaffolds. Slow scan (1 mV s\(^{-1}\)) CV of scaffolds exposed to GA vapours for 2 hours and 4 hours, in the absence and then presence of 100 mM glucose are shown in Figure 3. The CVs of the scaffolds in the absence of glucose show signals for the Os(II/III) redox process at the appropriate redox potential (+0.22 V vs Ag/AgCl), demonstrating retention of the redox polymer within the scaffold, and electrical connection of the redox polymer to the scaffold electrode. When glucose is added, increased oxidation currents, with a sigmoidal-shaped CV for the scaffold crosslinked for 2 hours, indicative of catalytic oxidation of glucose, are observed. The higher uncompensated resistance evident from the CVs recorded after a crosslinking duration of 4 hours, than that observed for the scaffold prepared using 2 hours crosslinking, indicated that such a long crosslinking time may not prove optimal. The electrode exposed to GA for 2 hours displays a more sigmoidal shape, while producing double the catalytic glucose oxidation current at 0.5 V compared to the electrode exposed to GA for 4 hours.
Figure 3 Slow scan CV (1 mV s⁻¹) of scaffolds prepared with Os(bpy)PVI/MWCNT/GOx and exposed to GA vapours for 2 hrs (red) and 4 hrs (black), in 50 mM PBS, pH 7.4, 150mM NaCl, in the absence (broken lines) and presence (solid lines) of 100 mM glucose at room temperature.

In order to evaluate current generation under an applied, or imposed, voltage, as would be the case in an operational EFC, amperometry was performed upon the scaffold prepared by 2 hr exposure to GA vapours. A potential of 0.35 V vs Ag/AgCl was applied in a flow of 50 mM PBS, pH 7.4, 150mM NaCl, 100 mM glucose at 37°C, to mimic physiological conditions, that resulted in a current of ~0.7 mA, as shown in Figure 4. This current can be normalised to the projected two-dimensional area of the scaffold face to yield an estimate current density of 1.05 mA cm⁻². However, such current densities are possible to achieve by simple addition of MWCNT to enzyme electrode films, operational in 5 mM glucose, instead of 100 mM, as reported on in Chapters 2 and 3. The low
current densities obtained using the scaffolds may arise if the scaffold
channels are clogged or material is lost due to collapsed channel walls.

![Graph](image)

**Figure 4** Amperometry, recorded at an applied potential of 0.35 V vs Ag/AgCl, of an ISISA electrode, prepared from a MWCNT and Os(bpy)PVI suspension, exposed to GA vapours for 2 hours. Performed in a 50 mM PBS (150 mM NaCl, at 37°C, 100 mM glucose) flow through system.

### 5.2.2 Electrochemical activation of chitosan

A second method explored is based on previous reports\cite{16-20} on the ECA of chitosan, achieved by applying an anodic voltage in the presence of NaCl to generate strong oxidant (hypochlorite) that can partially oxidise chitosan to generate aldehyde groups (proposed scheme shown in Figure 5), to functionalise GA crosslinked ISISA scaffolds prepared from a suspension of MWCNT in 1% w/v chitosan. Subsequent reaction of the aldehydes within the scaffold with amino groups on GOx and on an osmium complex, Os(bpy)-4AMP\cite{21-23}, may provide scaffolds for mediated oxidation of glucose.
Figure 5 Proposed reaction scheme for generation of aldehydes through action of hypochlorite on chitosan within a GA crosslinked scaffold.\textsuperscript{[16-20]}
Attempts to implement this strategy used GA crosslinking of MWCNT-chitosan after the ISISA process, followed by immersion in a 50 mM PBS, pH 7.4, 150 mM NaCl solution and application of a potential of 0.9 V to the scaffold. Subsequent to this, GOx (10 mg ml\(^{-1}\)) and Os(bpy)-4AMP (0.2mM) solutions are passed through the activated scaffold for 1 hr. Slow scan (1 mV s\(^{-1}\)) CVs for scaffolds prepared using a 10 minute ECA step display an Os(II) oxidation peak current of ~16\(\mu\)A in the absence of glucose, and a sigmoidal shaped catalytic current of ~25 \(\mu\)A in the presence of 5 mM glucose, and ~300 \(\mu\)A in the presence of 100 mM glucose. Control electrodes prepared without an ECA step display an Os(II) oxidation peak current of ~6\(\mu\)A in the absence of glucose substrate, but did not produce a catalytic current in the presence of 100 mM glucose, indicative of poor retention of redox complex and GOx under these conditions. The results indicate that ECA does result in improved retention of mediator and enzyme over those scaffolds that did not undergo the ECA procedure. However, as with the integrated components method (Section 5.2.1), the currents were lower than expected, considering the quantity of materials used in the fabrication of the electrodes as compared to the results in Chapter 2.

A problem of instability of the scaffold channels when subjected to a flow of buffer, as indicated by visible evidence of leaching of CNT material in the outlet channel from the scaffolds, is a likely fault with both fabrication methods explored. Creating larger, more stable, channels may well result in a high-current producing electrode. Future studies should be directed toward the optimisation of the crosslinking procedure, in an effort to stabilise the channels of the scaffolds. Research into use of the scaffolds continues, through collaboration between NUI Galway and Consejo Superior de Investigaciones Científicas of Madrid.

5.3 Proposed research directions for the future

The osmium bound poly(N-vinylimidazole) redox polymers, used throughout this thesis, are synthesised based on a target osmium to polymer monomeric unit ratio of 1:10, as established by Ohara et al.\(^{[21]}\) as
optimum for efficient charge transport through Os(bpy)PVI films containing GOx, although this conclusion is based on an assumption of complete reaction. Control of redox complex loading within these systems has been shown to be important in optimisation of, for example, oxygen reduction current by films of osmium-based redox polymers co-immobilised with a Trametes versicolor laccase on carbon electrodes\cite{22-24}. The synthesis of these redox polymers is however, difficult to control, leading to osmium loading ratios which vary between batches. Furthermore, the bulk free radical polymerisation method employed to synthesise the PVI results in batch-to-batch variations in enzyme electrode responses, due to differing molecular weights, although alternate synthetic strategies have been proposed to allow greater control\cite{25}. Osmium loading of redox polymer can be controlled by employing a synthetic route described by Pöller et al.\cite{26} in which Os is coordinated by a ligand with functional group distal to the metal binding site\cite{27}, that can then be attached to a pre-synthesised polymer via epoxide ring opening reaction. A recent report employed this strategy to bond osmium complexes containing a 4-AMP ligand to a range of epoxy-functionalised polymers\cite{27}, offering greater versatility in crosslinking strategies to promote film stability, along with the greater control over osmium loading of the redox polymers. As with the redox polymer bound osmium mediators, the redox potential may be adjusted through the substitution of electron donating or withdrawing groups to the metal ligands. For example, enzyme electrodes prepared using [Os(dmobpy)$_2$(4AMP)Cl]$^+$, [Os(dmbpy)$_2$(4AMP)Cl]$^+$ and Os(bpy)-4AMP co-immobilised with polyallylamine and GOx have been demonstrated\cite{28} to provide glucose oxidation current densities of 30, 70 and 120 $\mu$A cm$^{-2}$ respectively under pseudo-physiological conditions, though the low stability of signals is an issue. Recently, the electrode assembly methodology detailed in Chapters 2-4 of this thesis was employed using a range of enzymes, and the osmium complexes containing a 4AMP ligand as mediators\cite{29}. For example, enzyme electrodes based on the co-immobilisation of ScL, Os(bpy)4-AMP and MWCNT, crosslinked with GA vapours and subsequently reduced in sodium borohydride, produced
oxygen-reduction current density as high as 0.8 mA cm\(^{-2}\) in physiological buffer solution. The fabrication and characterisation of a membrane-less glucose-O\(_2\) enzymatic fuel cell was undertaken by combination of the ScL-based enzyme electrode for oxygen reduction as a cathode, with glucose-oxidising anodes, based on glucose dehydrogenase enzymes and 4AMP based osmium redox complexes. Combination of the PQQGDH-based enzyme electrode as anode with the ScL-based enzyme electrodes as cathode in a membraneless EFC provided a power density of 66 \(\mu\)W cm\(^{-2}\) in 5 mM glucose, 150 mM NaCl, phosphate buffer solution at 37 \(^\circ\)C, and 37 \(\mu\)W cm\(^{-2}\) in human serum. Such power densities are approaching those of PVI based EFCs, and provide a more controlled process for preparation of mediated enzyme electrodes. Further optimisation of crosslinking methodology and conditions may improve these systems.

Another approach that could prove beneficial is to investigate the use of enzyme cascades\(^{[30-32]}\), whereby more than one enzyme is used in an enzymatic electrode film, to increase coulombic efficiency of EFCs. Whereas one enzyme would oxidise, for example, glucose, a second enzyme could oxidise the resulting metabolite of the first enzyme. Such enzyme cascades exist in nature and result in the complete oxidation of fuels to highly reduced compounds, e.g. carbon dioxide produced from the citric acid cycle\(^{[30]}\). Utilisation of multiple-enzyme cascades for full or partial oxidation of fuels, such as ethanol\(^{[33,34]}\), has been demonstrated to improve power output in biofuel cell assemblies. Enzyme electrodes prepared using a combination of cellobiose dehydrogenase and pyranose dehydrogenase co-immobilised with SWCNT and an osmium redox polymer on pyrolytic graphite electrode have been used to increase oxidation currents of glucose-oxidising anodes, extracting up to six electrons from one molecule of glucose\(^{[32]}\), providing a promising route to increased power density EFCs.
5.4 References


Appendix

Publications:


Oral Presentations:
14th International Conference on Electroanalysis in Slovenia (2012)
Consortium presentation at Novozymes A/S in Copenhagen (2011)
Consortium presentation at Lund University in Sweden (2010)
Consortium presentation at Malmö University in Sweden (2010)
Consortium presentation at Consejo Superior de Investigaciones Científicas in Madrid (2010)

Poster Presentations:
Queens University, Belfast 62nd Irish Universities Chemistry Research Colloquium 2010
Dublin City University Conference on Analytical Sciences in Ireland Colloquium 2011
National University of Ireland, Galway Environmental Change Institute Research day 2010, 2011 & 2012
National University of Ireland, Galway Energy Night 2011 & 2012