Bioelectrochemical films on electrodes for application to biofuel cells

Rengaraj, Saravanan

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Bioelectrochemical films on electrodes for application to biofuel cells

Saravanan Rengaraj, M.Sc

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School of Chemistry,
National University of Ireland, Galway.

Head of School: Prof. Paul V. Murphy
Supervisor: Dr. Dónal Leech
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• Exchange research visitor to the Department of Chemistry at University of Connecticut (USA) Prof. James F Rusling laboratory to work on surface chemistry of an electrode to improve the stability of enzymatic fuel cells.
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Abstract

This thesis focuses on studies of both enzymatic (EFC) and microbial (MFC) fuel cells. In the enzymatic fuel cell studies, interactions between enzymes, osmium based complexes and redox polymers are characterized electrochemically. These enzymes and redox polymer films are immobilized by either physisorption of covalent anchoring at electrodes and their response investigated for operation as enzymatic fuel cells with a view to enhancing the power output and stability of such assemblies. Microbial fuel cell studies deal with the electrochemical characterization of model organism, Geobacter sulfurreducens and Rhodoferax ferrireducens, biofilms on electrodes that are induced to grow under fixed applied potential.

Chapter 1 introduces the enzymes, osmium based complexes and redox polymers, immobilization strategies for EFC operation and microbial electrocatalysis of electroactive bacteria.

Chapter 2 uses cyclic voltammetry to extract parameters for solution phase mediators, and their interaction with glucose oxidase, to permit screening of redox mediators for their suitability for glucose oxidation by glucose oxidase in anodes of an EFC. Osmium complexes with polypyridyl ligands exhibiting a wide range of redox potentials from -0.24 V to 0.43 V vs. Ag/AgCl were examined as mediators, and CV was used to estimate pseudo-first and second order rate constants for mediation of glucose oxidase oxidation of glucose. The rate constants increased with increase indifference between the redox potential of glucose oxidase and the mediators. Although osmium complexes with $E'_0$ above 0.2 V vs. Ag/AgCl showed high rate constants for reaction with glucose oxidase, complexes with lower rate constants and redox potentials, such as Os[(4,4′-dimethoxy-2,2′-bipyridine)(4-aminoethyl pyridine)Cl]PF$_6$ with $E'_0$ = +0.025 V vs. Ag/AgCl were selected for further study in anodes of an EFC. This was to provide a compromise between current density and anode potential. Operation of a solution phase, Nafion separated, EFC using a laccase-based cathode, revealed that selection of appropriate mediators can decrease anode overpotential.

Chapter 3 investigates an immobilization strategy for enzymes in redox hydrogels as carbon electrodes, as immobilization can eliminate the need for membrane separators. Glassy carbon and graphite electrodes were modified with films of enzyme and osmium redox polymer, cross linked with poly(ethylene glycol)diglycidyl ether, and used for elaboration of a glucose/O$_2$ enzymatic fuel cell (EFC). The redox polymers [Os(4,4′-dimethoxy-2,2′-
(polyvinylimidazole)$_{10}$]$_{\text{Cl}}$]$_{\text{+}}$ and [Os(4,4$^\prime$-dichloro-2,2$^\prime$-bipyridine)$_2$(polyvinylimidazole)$_{10}$]$_{\text{Cl}}$]$_{\text{+}}$ were selected to facilitate transfer of electrons from the glucose oxidase active site to the T1 Cu site of *Trametes hirsuta* laccase and *Myrothecium verrucaria* bilirubin oxidase. Using graphite resulted in an increased redox polymer loading, and as a consequence increased current densities, leading to a maximum power output of 43 $\mu$W cm$^{-2}$ at 0.25 V under physiological conditions for assembled EFCs. Improved stabilization of biofilms was achieved through covalent anchoring of enzyme and redox polymer on graphite electrodes, derivatized via electrochemical reduction of the diazonium cation generated *in situ* from p-phenylenediamine, using the di-epoxide cross linker. 

In order to investigate means to improve current and power densities, chapter 4 focused on Layer-by-layer (LBL) assembly of alternate osmium redox polymer and enzymes at graphite electrode to attempt to increase film component loading, and thus current densities. The same redox polymers used in chapter 3 were selected for anode and cathode. A bilayer assembly (two layers each) of redox polymer and enzymes, glucose oxidase for anode and *Trametes hirsuta* laccase for cathode, produced an EFC with a maximum power density of 103 $\mu$W cm$^{-2}$ at pH 5.5 and 40 $\mu$W cm$^{-2}$ at pH 7.4, with power limited by the acidophilic laccase at pH 7.4.

In chapter 5, an alternate immobilization strategy, based on anchoring of a poly (L-Lysine) layer on oxidized pyrolytic graphite electrodes to provide support for cross linking of redox polymer and enzyme, was investigated for application to membrane-less EFC assembly. The modified PG electrodes showed improved catalytic currents for both glucose oxidation and oxygen reduction, using redox polymers and glucose oxidase or bilirubin oxidase, compared to PG electrodes with physisorbed films of cross linked enzyme and redox polymer. Operational stability for glucose oxidation with [Os(4,4$^\prime$-dimethoxy-2,2$^\prime$-bipyridine)$_2$(polyvinylimidazole)$_{10}$]$_{\text{Cl}}$]$_{\text{+}}$(Med$_1$)/glucose oxidase films and [Os(2,2$^\prime$-bipyridine)$_2$(polyvinylimidazole)$_{10}$]$_{\text{Cl}}$]$_{\text{+}}$(Med$_3$)/bilirubin oxidase films for oxygen reduction at physiological buffer conditions and 37 °C were evaluated independently for modified and control PG electrodes. Enzyme electrodes were cured for 24 h and 48h, in order to check this influence over catalytic current and stability. Assembled EFCs produced a maximum power density of 96 $\mu$W cm$^{-2}$ at 0.33 V under these conditions, with operational stability studies revealing that the limiting factor for power loss being the decrease in anode redox polymer signal. 

There are only a few bacteria reported to be capable of transferring electrons directly to electrodes while oxidizing substrate. Chapter 6 focused on the use of cyclic voltammetry
and chronoamperometry to probe the response of *Geobacter sulfurreducens* and *Rhodoferax ferrireducens* to applied potentials. Growth of biofilms over carbon electrodes at fixed applied potentials yielded a bioelectrocatalytic response to acetate oxidation. Electrodes were held at different applied potentials to induce *Geobacter sulfurreducens* or *Rhodoferax ferrireducens* biofilm growth from culture media that did not contain a natural electron acceptor for the bacteria. This study is the first such study on the *Rhodoferax ferrireducens* electroactive bacteria. The difference in cyclic voltammograms and electron micrographs recorded for biofilms of the two bacteria highlighted differences in their rates of growth under applied potentials.

Chapter 7 summarizes the main research findings and possible future research directions are presented.
1. Introduction

1.1 Introduction

A biofuel cell is an electrochemical system capable of converting chemical energy to electrical energy using biochemical pathways (Bullen et al., 2006, Katz et al., 2003a, Shukla et al., 2004). Conversion of biochemical energy is achieved by oxidation which generates protons and electrons, coupled with reduction, which uses these electrons and protons at the cathode. Electrons are forced though an external circuit while cations diffuse through the membrane that separates anode and cathode, thereby maintaining the charge balance and completing the electrical circuit. Traditional chemical fuel cells such as polymer-electrolyte, direct methanol and solid-oxide rely on reformed fossil fuels and operate with expensive metal catalysts (Larminie and Dicks, 2003). In contrast, biofuel cells are driven by biofuels or biocatalysts (either enzymes or whole cell microorganisms). Biofuel cells that use biocatalysts are advantageous over chemical fuel cells, as they operate in mild conditions and do not require expensive metal catalysts such as platinum.

Biofuel cells are classified based on the biocatalyst used for the reaction. Fuel cells that use a specified enzyme are known as enzymatic fuel cells (EFCs) while systems that use whole microorganisms are known as microbial fuel cells (MFCs). Fig. 1.1 presents a simplified scheme representing the mechanisms of EFC and MFC. In EFCs, an enzyme can be substrate specific, while in MFC the metabolic pathways can be diverse and MFCs can thus use a wide range of substrates. Due to the specificity of the enzyme reaction, some EFCs do not require a membrane separation, particularly when the enzymes are immobilized over electrodes, whereas MFCs require membrane separation (except in the case of benthic MFC) as the reactions are not substrate specific. Enzymes have relatively short life time, which is an innate characteristic even in natural environments and contributes to power loss (Kim et al., 2006). In contrast, MFC microorganisms are able to regenerate and a power increase associated with microbial growth may be observed.
Figure 1.1 Operational principles of a) Enzymatic Fuel Cell, which uses an enzyme reaction at anode and cathode (from Kavanagh and Leech, 2011) and b) Microbial Fuel Cell, where microorganisms catalyse the anode reaction in combination with a non-biological cathode (metal catalyst).

1.2 Enzymatic Fuel Cells (EFCs)

The first enzyme based EFC was reported in 1964 using glucose oxidase as a catalyst at the anode and glucose as the fuel (Yahiro et al., 1964). Research advances in the field of EFCs have shown exciting results, but still the performance of EFCs in terms of power density and operational lifetime are far beyond that of chemical fuel cells (Aston and Turner, 1984, Palmore and Whitesides, 1994). Rather than considering EFCs as a large-scale power source, research has focused on implantable devices, biosensors, microchips, microfluidics, and power for portable medical devices (Katz and Willner, 2003b, Barton et al., 2004, Heller, 2004). Enzyme immobilization and material chemistry, especially electrodes with nanostructures, can provide a platform for these applications.

Achieving electrical connection between redox enzymes (wired) and electrodes is one of the most important approaches developed in bioelectrochemistry. Redox enzymes generally lack direct electron transfer (DET) between the active redox centers and electrode. Dimensions of redox proteins are in a range of 70-200 Å and the redox centers are sufficiently buried within the protein layers, thereby preventing electrical communication to the electrode (Heller, 1992) (Fig. 1.2a). However, DET has been observed with certain proteins and enzymes such as cytochrome c, laccases, hydrogenases and peroxidases (Varfolomeev et al., 1996, Ghindilis et al., 1997, Schuhmann, 2002, Freire et al., 2003). For example, laccase catalyzed DET for oxygen reduction was proposed to occur as a distance
of only 20 Å is observed between the copper-based enzyme active site and the electrode (Yaropolov et al., 1981). In order to overcome DET issues, electron shuttles (mediators) are introduced. Mediators transport electrons from the enzyme active site to the electrode: a process termed mediated electron transfer (MET) (Lewis, 1966, Govil and Saran, 1982, Palmore and Whitesides, 1994, Katz et al., 2003b) (Fig.1.2b). Several reports have categorized EFCs by either direct or mediated electron transfer. However, with MET the mediator enhances electron transfer from the active site of the enzyme to the electrode, and when compared to DET, a higher current density for EFC is usually found. MET can be used at both anode and cathode to form a full EFC.

![Figure 1.2](image1.png)

**Figure 1.2** a) Schematic diagram depicting electrocatalytic oxidation of glucose using glucose oxidase (denoted as Enz), where the electrons generated in the flavin active site, buried within the protein layers, prevents direct electron transfer (DET) to electrode. b) Mediated electron transfer (MET) to the electrode using a mediator (denoted as Med).

![Figure 1.3](image2.png)

**Fig.1.3** illustrates the schematic configuration of the membrane-less glucose/O₂ EFC based on a mediated glucose oxidase oxidation of glucose transferring electrons to cathode where mediated laccase reduction of oxygen consumes the transferred electrons. Transport of protons through the electrolyte helps electrons to flow through the external circuit. The electrical power density, P, of the EFC is given by I×ΔE, where I is the current density passing through the external circuit and ΔE is the voltage across a resistor load. A maximum voltage from the cell is possible by maximizing the difference between the thermodynamic redox potentials of the mediators incorporated within the electrode system. Mediators should therefore be designed or selected to possess redox potentials close to those of the redox center of the enzymes.
Enzymes used in electrocatalysis

A wide range of enzymes have been used in EFCs for the generation of electrical power. These enzymes belong to the oxidoreductase family, and are capable of catalyzing oxidation reaction at anode and reduction reactions at the cathode. For anodes, these enzymes are categorized into three types according to the redox cofactor by which electrical communications are made to the electrode (Heller, 1992). The first group consists of pyrrolo-quinoline quinone (PQQ) dependent enzyme dehydrogenases (e.g., glucose dehydrogenase, glycerol dehydrogenase). These enzymes have the coenzyme PQQ bound to the enzyme with a thermodynamic redox potential of $E^\circ = -0.06$ V vs. Ag/AgCl at pH 7 (Willner et al., 1998). Glucose dehydrogenase has high catalytic efficiency and is insensitive to oxygen, as the co-factor is bound to the enzyme (Yuhashi et al., 2005, Ye et al., 1993, Ikeda and Kano, 2003, Tsujimura et al., 2003). The second class of enzyme utilise a nicotinamide adenine dinucleotide (NADH/NAD$^+$) or nicotinamide adenine dinucleotide phosphate (NADPH/NADP$^+$) cofactor, e.g. glucose dehydrogenase and alcohol dehydrogenase (AlcDH). This co-factor is loosely bound to the enzyme, and acts as carrier for two electrons and a proton. The thermodynamic redox potential of (NADH/NAD$^+$) is $E^\circ = -0.5$ V vs. Ag/AgCl at pH 7. High overpotential for oxidation of the NADH co-factor and its poor electrochemical reversibility limits its usage in EFC and biosensor application, however. The third class of enzyme has a flavin adenine dinucleotide (FAD) co-factor buried in the protein.
layer, making it difficult for electron transfer to the electrode. Glucose oxidase (GOx) from *Aspergillus niger* is the most commonly used enzyme that belongs to this group. The redox potential of FAD/FADH$_2$ active site is $\sim E^\prime = -0.36$ V vs. Ag/AgCl at pH 7, making it useful for the anode of an EFC if DET can be achieved (Willner et al., 1996).

For biological cathodes, a class of multi-copper oxidases that are capable of four-electron reduction of O$_2$ to water have been used (Solomon et al., 1996). Laccases (for example those isolated from *Trametes versicolor* or *Melanocarpus albomyces*) and bilirubin oxidase (BOD) (from *Myrothecium verrucaria* for example) have a high activity towards biological reduction of oxygen directly to water. Laccase and BOD have four Cu$^{+/-}2+$/Cu$^{+/-}1+$ in their active site. Laccase has a copper site, designated as T1, oriented towards the protein surface (about 8 Å) that accepts electrons from the electrode, or the natural phenolic substrate. Electrons are then transferred to a trinuclear copper T2/T3 cluster (distance about 12-13 Å) where molecular oxygen, through a mechanism that is as yet poorly elucidated, is reduced to water (Katz and Willner, 2003b). The redox potential of the T1 Cu site of laccase from *Trametes hirsuta*, ThLacc, used in this thesis is approximately $E^\prime = +0.57$ V vs. Ag/AgCl (Shleev et al., 2005). Laccases generally function under slightly acidic conditions, as one of the centers (T2 center) is inactive at natural pH and inhibited by chloride ions (Xu, 1997). BOD reduces molecular oxygen in a similar mechanism to laccase, but is more active at neutral pH (Mano et al., 2002). The T1 Cu site of the multicopper oxygenases of BOD have a redox potential of $\sim E^\prime = +0.4$ V vs. Ag/AgCl for MvBOD (Xu et al., 1996). DET for oxygen reduction was reported for laccase adsorbed on carbon electrodes (Shleev et al., 2005) and for BOD on spectrographic graphite (SPG) (Ramírez et al., 2008). Fig.1.4 shows a proposed mechanism of DET to BOD on graphite electrode. BOD has gained in popularity as it can work at physiological conditions for oxygen reduction at the cathode.

**Figure 1.4** Schematic presentation of proposed mechanisms of direct electron transfer of bilirubin oxidase (BOD) from electrodes to BOD connected via T1 site (Ramírez, et al. 2008).
1.2.2 Mediated electrochemistry (enzyme – mediator interaction)

Mediators assist in the rapid transfer of electrons between the electrode and enzyme. In a simplified, ping-pong, oxidative process, the enzyme reacts with substrate and is then re-oxidized by the mediator, that can the diffuse to the electrode to be oxidized at the electrode surface. The mediator cycles the electrons continuously between the enzyme and the electrode, as it is repeatedly oxidized and reduced, generating a catalytic current, while the enzyme reacts with the substrate to give its product.

An electron transfer mediator should have (1) an appropriate redox potential that in turn enables the electrode to be polarized at a potential that does not give rise to interfering electrochemical reactions, (2) chemical stability in both oxidized and reduced forms, and (3) a high value rate constant for the electron transfer reaction with the enzyme (e.g., GOx). In order to select the appropriate mediator homogeneous solution phase studies of the enzyme and mediator electron transfer reaction kinetics offers insight into electron transfer mechanisms. Solution phase kinetics has focused on utilizing a wide range of mediators such as ferrocenes and its derivatives (D’Costa et al., 1986). For example, Forrow et al. investigated the influence of the structures of ferrocene derivatives towards GOx mediation (Forrow et al., 2002). Zakeeruddin et al. synthesized a range of tris-(4,4'-substituted-2,2'-bipyridine) complexes of the group VII metals, iron(II), ruthenium(II) and osmium(II) (Zakeeruddin et al., 1992). These complexes were characterized electrochemically with respect to their ability to act as electron transfer mediators for redox enzymes, in particular GOx. For oxygen reduction, Trudeau et al. used laccase, mediated with the osmium-based complexes, Os(bpy)$_2$Cl$_2$(bpy = 2,2'-bipyridine) and [Os(bpy)$_2$(N-methylimidazole)Cl]$^+$ to investigate homogeneous mediation kinetics (Trudeau et al., 1997). More recently Rochefort et al. investigated solution phase laccase-mediator interactions for several organic and transition metal-based mediators (Rochefort et al., 2004). Mediators were characterized electrochemically in homogenous solution phase to determine their capacity to act as an electron transfer mediator, with respect to reaction rate constant and the difference between the redox potentials of the enzyme and mediator. The introduction to, and discussions presented in, Chapter 2 provide more detailed information about enzyme mediator kinetics in solution phase, with a particular emphasis on use of osmium complexes. In addition to kinetics, solution phase EFCs have been developed and operated with the appropriate mediator to demonstrate overpotential effects on the EFC performance. However, in order to operate biosensors and membrane-less EFCs for implantable or semi-implantable devices, all
components should be immobilized on the electrode surface. Immobilization techniques can prevent the components from leaching, thus preventing decrease in signal and cross-reactions, and lead to the longer term operation of these devices.

1.2.3 Enzyme and mediator immobilization

1.2.3.1 Enzyme redox hydrogels

Immobilization techniques for the development of EFCs and biosensors include: (a) physical adsorption of mediators and enzymes on to an electrode surface, or (b) entrapment/attachment in polymer matrices (e.g. redox hydrogels). A method that co-immobilizes enzyme and mediator in a redox hydrogel (a design to prevent leaching of redox species) was used by Heller and co-workers (Mano et al., 2002, Heller, 2004, 2006). Enzymes in these redox hydrogels are electrically connected by mediation through the redox polymer network, with electron conduction controlled by collisional electron transfer (electron hopping) between the reduced and oxidized transition metal-based redox centers tethered to the polymer backbone.

The polymer backbones mostly used in redox hydrogels include polyvinylimidazole (PVI), polyallylamine (PAA) and polyvinylpyridine (PVP), with osmium complexes as the redox centers (Gregg and Heller, 1991). Fig. 1.5 shows the reaction scheme for formation of an osmium redox polymer by co-ordination of a [Os(2,2’-bipyridine)Cl]⁺ complex to a PVI backbone. The CV in the figure shows ~ +0.2 V positive shift in the [Os(2,2’-bipyridine)Cl]⁺ complex Os(II)/(III) redox potential when the chlorine ligand is replaced with PVI. Redox potentials of osmium complexes can be further manipulated by selection and chemical modification of the ligands (Kavanagh et al., 2009) and by changing the metal, so they can be tailored to a specific enzyme reaction (Heller, 2006, Kim et al., 2003).
Figure 1.5a) The chemical structure of the osmium redox polymer, formed by co-ordination of a [Os(2,2'-bipyridine)$_2$Cl]$^+$ complex (X=H) to a polyvinylimidazole backbone. b) CVs show the positive shift in the potential of the complex (black) when [Os(2,2'-bipyridine)$_2$Cl]$^+$ is co-ordinated with PVI (red).

The rate of electron transport through these redox hydrogels can be improved by including flexible chemical spacers that connect the redox-active centers to the PVI backbone, through improving collisional electron transfer (Heller, 2006, Mao et al., 2003). An example by Mao et al. using a redox polymer PVP-[Os($N,N'$-dialkylated-2,2'biimidazole)$_3$]$^{2+/3+}$ containing 13 atom spacer arms attached to the backbone of the redox.
center, showed improved charge transport as a redox hydrogel, over the redox polymer with no tethers (Mao et al., 2003). Similarly Mano et al. synthesized a redox polymer (4,4'-dimethyl-2,2'-bipyridine)(4-aminomethyl-4'-methyl-2,2'-bipyridine) complex of Os^{2+/3+} that has a high apparent diffusion coefficient ($D_{app}$) for charge transport through the film via collisional electron hopping (Mano et al., 2006).

Generally redox hydrogel electrodes are prepared by crosslinking enzyme and redox polymers using a poly(ethylene glycol)bisglycidylether (PEG) crosslinker on the electrode surface. PEG is an efficient crosslinker as it is soluble in aqueous solutions, and reacts readily with amines and the nitrogen containing heterocyclics of redox polymers at ambient temperature (Fig. 1.6).
\[ ip = 0.4463 \, nFAC(nFvD/RT)^{1/2} \] (1.1)

Where \( ip \) is the peak current, \( n \) is the number of electrons being transferred, \( A \) is the electrode area (cm\(^2\)), \( D \) is the diffusion coefficient (cm\(^2\)s\(^{-1}\)), \( C \) is the concentration (mol cm\(^{-3}\)) and, \( v \) is the scan rate (Vs\(^{-1}\)), \( R \) is the gas constant, \( T \) is the temperature, \( F \) is the Faraday constant. The transport of electrons through the redox polymer is measured by their apparent electron diffusion coefficients (\( D \)). In general, the \( D \) in redox hydrogels varies between \( 10^{-12} \) to \( 10^{-6} \) cm\(^2\) s\(^{-1}\) (Mano et al., 2006).

Several researchers have focused on GOx modified redox hydrogels. Kenausis and co-workers (Kenausis et al., 1996) incorporated GOx in films of [Os(4,4’-dimethoxy-2,2’-bipyridine)\(_2\)Cl]\(^+\) complexes in a PVP, and reported a redox potential of \( E'_o = +0.035 \) V vs. Ag/AgCl that downshifted to \( E'_o = −0.07 \) V vs. Ag/AgCl, by replacing PVP with PVI (Fig.1.7). Current densities of 110 \( \mu \)A cm\(^{-2}\) for glucose oxidation in the presence of 32mM glucose have been reported using films of this polymer crosslinked with GOx on carbon electrodes (Chen et al., 2001). Kim et al. have synthesised a redox polymer with 4,4’-diamino-2,2’ bipyridine (\( E'_o = −0.185 \) V vs. Ag/AgCl) replacing 4,4’-dimethoxy-2,2’ bipyridine, yielding a current density of 150 \( \mu \)A cm\(^{-2}\) at 15 mM glucose under physiological conditions when co-immobilised in films on electrodes with GOx (Kim et al., 2003). Mao et al. have compared films with GOx and two types of redox polymers (for 15 mM glucose oxidation using a vitreous carbon rotating disk electrode) that differ in terms of current density (Mao et al., 2003). A PVP-[Os(N,N'- dialkylated-2,2’-biimidazole)\(_3\)]\(^{2+/3+}\) (\( E'_o = −0.195 \) V vs. Ag/AgCl) polymer, with a 13 atom long flexible tether, showed a higher diffusion coefficient that yielded a current density of 1.15 mA cm\(^{-2}\) for glucose oxidation when compared to 0.15 mA cm\(^{-2}\) for a PVI-[Os(4,4’-diamino-2,2’-bipyridine)\(_2\)Cl]\(^{3+/2+}\) polymer.
Redox hydrogel modified electrodes in an EFC suffer in terms of long term stability (Calabrese-Barton et al., 2004). Loss of enzyme activity and leaching of components from the redox hydrogels have been reported (Binyamin and Heller, 1999, Boland et al., 2009). A recently developed method for improving stability consists of anchoring the hydrogel to the electrode surface via covalently attached tether groups (Boland et al., 2009, Lehr et al., 2010). Boland et al. compared the current densities and stabilities of pre-treated and bare graphite and gold electrodes (Boland et al., 2009). The pre-treated graphite electrodes were functionalised to yield amine functional groups by electrochemical reduction of a diazonium salt formed in situ from 1,4-phenylenediamine. An osmium-based redox polymer was then cross-linked over the bare and pre-treated electrodes, with GOx on the anode and BOD on the cathode. For both electrodes, retention of activity was vastly improved by pre-treatment. It was postulated that amine groups on the pre-treated surfaces covalently anchored the hydrogel through the cross-linking reaction, resulting in improved stability.

Similarly, Pellissier et al. grafted a GOx layer on a GC electrode modified using surface derivatization, through coupling with peripheral amine groups of the GOx (Pellissier et al., 2008). This enzyme layer was used as an anchoring base onto which a cross-linked enzyme layer was subsequently deposited. The authors demonstrated that these modified

**Figure 1.7** Structure of [Os (4,4′-dimethoxy-2,2′-bipyridine)₂([PVI][Cl])]⁺
electrodes retained much of their activity after 6 weeks, while control electrodes prepared by
depositing the crosslinker and GOx directly onto the GC had lost all activity within only 1
week. Surface modification and covalent anchorage of the redox species to the electrode
surface could therefore improve the stability of electrodes in assembled EFCs, a topic that is
addressed in chapter 3 and 5 of this thesis.

1.2.4 Layer by layer assembly

The layer by layer (LBL) technique can be used to assemble enzymes and mediators
alternatively, using electrostatic deposition, over an electrode. Ultrathin films formed by this
method can provide spatial distribution of redox polymers and enzymes, enhancing the
electron transfer rate within the 3D supramolecular architectures (Calvo et al., 2001). An
alternate LBL electrostatic assembly technique has been shown to produce multilayer thin
films with molecular level thickness (Decher, 1997). The advantage of the LBL self-assembly
over redox hydrogeldrop-coat deposition is that variables such as thickness, enzyme loading,
mediator concentration and the charge of the topmost layer can be controlled (Calvo et al.,
2010).

Hodak et al. used LBL self-assembled enzyme (GOx) and ferrocene redox polymer
layers to develop amperometric enzyme electrodes (Hodak et al., 1997). In this integrated
supramolecular enzyme film, a sequence of electron hopping events in the redox polymer is
followed by electron transfer between suitably positioned redox centers and the
FADH₂ prosthetic group of the enzyme (Willner, et al., 1996). Since then extensive research
on LBL assembly of alternate redox polymer and enzyme layers has been explored to form
various biosensors. Several publications on LBL assembly showed that catalytic response
was proportional to the number of self-assembled layers formed at gold electrodes (Calvo et
al., 2010, Deng et al., 2008). Enzyme electrocatalysis, such as oxidation of glucose to
glucono-lactone by GOx and reduction of oxygen to water by laccase, can be achieved at
electrodes modified with films formed from alternate enzyme and redox polymer multilayers.
Recently LBL assembled biocathodes of Trametes trogii laccase and an osmium complex
bound to poly(allylamine) (PAH-Os) at a gold electrode was used for oxygen reduction,
showing evidence useful for operation of an oxygen cathode in an EFC under stagnant
conditions (Calvo, et al., 2008). Deng et al. assembled an EFC using self-assembled
multilayers with gold nano particles and enzymes at macroporous gold electrodes with the
NADH cofactor in solution for the GDH catalysed oxidation of glucose. The EFC based on
LbL films on macroporous gold resulted in 14 times higher power densities compared to the
EFC assembled on the planar gold surfaces (Deng et al., 2008). For the most part, LBL assembly to date has focused on assembly at thiol pre-treated gold electrodes. One advantage of using graphite electrodes instead of gold is that the need for thiol modification of gold to adsorb the cationic osmium redox polymer layer is eliminated. Also, graphite electrodes are intrinsically negatively charge, so cationic redox polymers can be adsorbed directly (Zheng et al., 2004). An approach for formation of EFCs using LbL assembly of layers on graphite electrodes is presented in chapter 4. Fig. 1.8 shows a model of a glucose/O$_2$ EFC scheme using LBL redox polymer / enzyme at graphite electrodes for glucose oxidation and oxygen reduction, for such an approach.

![Figure 1.8 Schematic representation model of glucose/O$_2$ EFC utilizing Os-redox polymer-enzyme (GOx or Laccase) LBL films at graphite electrodes for mediated biocatalytic oxidation of glucose and reduction of O$_2$ to generate electrical power.](image)

**1.2.5 Assembled EFC**

The glucose/O$_2$ EFC has received most research attention due to the relatively high concentration of glucose in blood (5 – 8 mM), leading to potential application of EFCs for *in vivo* power production for low energy (~10 µW) demanding biomedical devices (Heller, 2004). Although an EFC can theoretically meet the power demands of some biomedical
devices, stability of power output of EFCs remains an issue. Leaching of enzyme and/or mediator from the electrode surface can occur, leading to short term instability of bioelectrocatalytic films (Gregg and Heller, 1991a, Gregg and Heller, 1991b, Boland et al., 2009a). A realistic goal may be the development of miniature semi-implantable glucose/O\textsubscript{2} systems to provide power for the lifetime of an implanted glucose sensor (typically <1 week) and be discarded after their first and only use, thereby eliminating the need for longer term stability (Chen et al., 2001, Kim et al., 2003).

Katz et al. reported the first single chamber EFC consisting of two immiscible electrolytes separated by a liquid–liquid interface, allowing DET to take place (Katz et al., 1999). It consisted of reconstituted apo-GOx coupled to a (PQQ) relay conjugated to thiol modified gold anode and a cross-linked cytochrome c oxidase (COx) assembled on a gold cathode. The maximum power density reported for this EFC was 5 µW cm\textsuperscript{-2}. Rapid development on EFCs has been achieved in the past decade driven by the increased demand for reliable power supplies for miniaturized medical devices. Electrical contacting of the redox enzymes with osmium polypyridine or polyvinylimidazole hydrogels was extensively studied (Gregg and Heller, 1991). Thermodynamic redox potentials of osmium complexes can be tuned using ligands electrically wired with different enzymes. In the case of the glucose/O\textsubscript{2} EFC, the anode should operate at the lowest possible redox potential, for example close to that of the FAD redox site of glucose oxidase should this be the choice for anode catalyst, whereas the biocatalytic cathode should operate at the most positive potential. Tuning of redox potentials of the redox polymer hydrogels is possible, as described previously. A glucose/O\textsubscript{2} EFC was formed using GOx mediated by [Os(4,4′-diamino-2,2′-bipyridine)\textsubscript{2}poly(N-vinylimidazole)Cl], E\textsuperscript{o′} = −0.11 V versus Ag/AgCl, and laccase mediated by its incorporation in [Os(1,10-phenanthroline)\textsubscript{2}poly(N-vinylimidazole)], E\textsuperscript{o′} =+0.49 V versus Ag/AgCl as anode and cathode, respectively. Maximum power output of 16 µW cm\textsuperscript{-2} at physiological conditions was achieved (Barrière et al., 2006) and the cell produced 0.6 V, which is the potential difference between the GOx mediator and laccase redox sites. An alternate glucose oxidase (GOx) electrically wired enzyme–hydrogel anode used [Os(N,N′-alkylated-2,2′ –bi-imidazole)\textsubscript{3}]\textsuperscript{2+} redox active moiety tethered to the polymer backbone via one of its alkylated biimidazole ligands and a 13 atom long flexible tether to improve charge transport diffusion. The polymer exhibits a redox potential of E\textsuperscript{o′} = −0.19 V vs.Ag/AgCl (Mao et al., 2003) and combined with a cathode electrocatalyst of bilirubin oxidase (BOD) from Trachyderma tsunodae and a copolymer of polyacrylamide and poly (N-vinylimidazole)
complexed with $[\text{Os} \ (4,4'\text{-dichloro}-2,2'\text{-bipyridine})_2\text{Cl}]^{+2+} \ (E^\circ' = +0.34 \text{ V vs. Ag/AgCl})$ a power density of 430 µW cm$^{-2}$ at 0.52V was produced. This EFC lost its power density at a rate of $\sim 6\%$ per day when operated at 37 °C in a physiological buffer conditions for a week (Mano et al., 2002). **Fig. 1.9** shows the operating potentials of the assembled EFC.

Mano and Heller reported a glucose/O$_2$ EFC with another redox polymer$[\text{Os}(4,4'-\text{dimethoxy}-2,2'-\text{bipyridine})_2(\text{PVI})\text{Cl}]^{+2+}(E^\circ' = −0.07 \text{ V vs. Ag/AgCl})$ and GOx, combined with PAA-PVI-$[\text{Os}(4,4'-\text{dichloro}-2,2'-\text{bipyridine})_2\text{Cl}]^{+2+}$/BOD (*Fig.1.10*) (from *Myrothecium verrucaria*) to yield a maximum power density of 244 µW cm$^{-2}$ at 0.36V when operated under physiological conditions at 37 °C. When operated at 23 °C it produced 98 µW cm$^{-2}$ at 0.36V. The maximum power density dropped by 45% during continuous operation. From these observations it is evident that the anode influences the power output (Mano and Heller, 2003).

An EFC operating in low glucose concentration of 5 mM produced a power density of 280 µW cm$^{-2}$ when GOx from *Aspergillus niger* was replaced by GOx from *Pencillium pinophilum* (Mano, 2008). The anode consisted of 7 µm diameter, 2 cm long carbon fibers cross-linked with GOx (*P. Pinophilum*) and PVP-$[\text{Os}(N,N'-\text{dialkylated}-2,2'-\text{biimidazole})_3]^{2+/3+}$. The cathode was of cross-linked laccase and PVP-$[\text{Os}(4,4'\text{-dimethyl}-2,2'-\text{bipyridine})_2(4'\text{-aminomethyl}-4'-\text{methyl}-2,2'-\text{bipyridine})]^{2+/3+}(E^\circ' = +0.55 \text{ V/AgAgCl})$. A maximum power density of 98 µW cm$^{-2}$ was produced when the same EFC operated with the GOx from *Aspergillus niger*. 

![Scheme of operating potentials of enzymes and redox polymer](image)
Figure 1.10 The chemical structure of $[\text{Os}(4,4'\text{-dichloro-2,2'\text{-bipyridine})_2\text{Cl}]^{+2+}$ complexed to a copolymer of PVI and polyacrylamide.

Other than GOx, EFCs operating with alternate enzymes in a modified electrode is possible. Tsujimura et al. substituted GOx for pyrroloquinoline quinone-dependent glucose dehydrogenase (GDH) as an anode biocatalyst (Tsujimura et al., 2002). Wiring GDH and bilirubin oxidase in the Os$^{2+/3+}$ polypyridine hydrogels, a glucose/O$_2$ EFC with a power output of 58 $\mu$W cm$^{-2}$ was constructed. Similarly cellobiose dehydrogenases (CDH) can be used as it catalyses the electrochemical oxidation of various substrates such as glucose, lactose and cellobiose. Glucose/O$_2$ EFC using CDH and Os$^{2+/3+}$ polypyridine hydrogels as anode and using platinum black cathode provide a power density of 157 $\mu$W cm$^{-2}$ (Tasca et al., 2008).

1.2.6 EFC optimization

EFCs with different enzymes, redox polymers and electrode materials discussed above have been shown to yield reasonable current and power densities. At this stage EFC optimization is required for improvement in the long-term stability under continuous operation. Improvement of performance requires optimization of all fuel cell components such as biocatalysts, mediators, enzymatic electrode assemblies and fuel cell design. Mostly, improvements in enzyme stability are the focus. It has been shown that the properties of enzymes for fuel cell application can be improved by purification (Gao et al., 2002) or deglycosylation (Courjean et al., 2009) in the case of GOx. Enzyme engineering is a powerful strategy that can be used to target applications. Site-directed mutagenesis can be used to verify the structure-activity relationship of the protein and directed evolution can be used to identify, by screening, different mutants (Wong and Schwaneberg, 2003). In an example, pyranose 2-oxidase was subjected to semi-rational design and mutants with
improved kinetics towards glucose and different mediators were identified (Spadiut et al., 2009). Another example reported for EFC devices uses engineered PQQ-GDH for improved stability (Yuhashi et al., 2004). A novel, thermostable GDH consisting of three subunits (FAD-containing, heme c containing and a chaperone-like subunit) was reported. This protein was found to exhibit DET and the performance of the subunit and the GDH complex was evaluated in anEFC (Okuda-Shimazaki et al., 2008) and a fuel-cell type biosensor (Kakehi et al., 2006). Application of these advanced engineering tools can improve the stability of the biocatalyst and can extend the knowledge to discover novel enzymes that address the stability issues.

1.3 Microbial Fuel Cells (MFCs)

Electroactive bacteria (EAB) transfer electrons to an electrode while converting biochemical energy to ATP. Electrons flow through a series of electron-carrier proteins in which the electrons are ultimately transferred to a terminal electron acceptor. This reaction is exploited in the microbial fuel cell (MFC) to generate a considerable amount of power from organic waste as a fuel (Debabov, 2008, Rabaey and Verstraete, 2005). In a MFC bacteria oxidize organic substances and transfer electrons to the anode, where they flow to the cathode combining with protons and a chemical catholyte such as oxygen to form water. A MFC device is capable of having the substrate oxidized, as it is then replenished either continuously or intermittently for the continuous generation of electrons at anode - effectively a bio-battery. Bacterial respiration involves a soluble compound (eg. oxygen, nitrate, and sulphate) as an electron acceptor, however certain bacteria respire solid electron acceptors (eg. metal oxides and metal oxide electrodes) in order to obtain energy.

1.3.1 Bacterial potential for electricity generation

Bacteria gain energy by transferring electrons from a reduced substrate at a low potential such as glucose to an electron acceptor with a high potential such as oxygen. An overview of such a redox reaction is given in Fig. 1.11 (Madigan et al., 2000). If bacteria derive reducing equivalents from glucose in the form of NADH, and subsequently shuttle electrons from NADH to oxygen the potential difference is ~1.2 V \[\Delta E = (+0.840V)−(−0.32V)\].
Figure 1.11 Table of common redox reactions, and their formal reduction potentials. Scheme shows losses to cell voltage during electron transfer in a MFC. 1. Losses owing to bacterial electron transfer. 2. Losses owing to electrolyte resistance. 3. Losses at the anode. 4. Losses at the MFC resistance and membrane resistance losses. 5. Losses at the cathode. 6. Losses owing to electron acceptor reduction (Rabaey et al., 2005).

Energy gained from electrochemical reactions can be calculated based on power output. The power depends on both the voltage and current and is linked to the fuel cell resistance by Ohm’s law $V = I \times R$, in which $R$ represents the resistance (Ohm) (Niessen et al., 2004). Maximum open circuit voltage (OCV) of 0.8 V can be observed when there is no
current flowing through the external circuit in a MFC (Madigan et al., 2000). In a closed circuit, the voltage decreases significantly as a result of overpotentials. Overpotentials are potential losses due to electron transfer resistance and internal resistance Fig. 1.11. Three kinds of overpotentials can be defined in electrolyses: activation overpotentials, ohmic losses and concentration polarization (Larminie and Dicks, 2003). This overpotential mainly depends on current density flowing through the electrode, electrochemical properties of the electrode and the presence of mediating compounds and the operational temperature.

1.3.2 Bacterial electron transfer and electrochemical characterization

Bacterial attachment to the anode electrode surface is essential for the efficient electron transfer in a MFC. Bacteria in the anode biofilm that transfer electrons to the anode are carrying out a respiratory metabolism. Fig. 1.12a shows the bacterial metabolism and the electron transfer system (ETS).

![Figure 1.12](image)

**Figure 1.12** a) Substrate oxidation - electrons are transferred from the electron donor (to intracellular reducing power (NADH) and associated ATP production. Electrons are transferred to outermembrane proteins, b) Electron transfer system to the electrode which acts as final electron acceptor.

NADH donates electrons for oxidation and the electrons are handled by various membrane bound proteins as shown in Fig.1.12b. In anaerobic conditions, bacteria reduce the terminal electron acceptor (eg, iron-respiring bacteria reduce Fe$^{3+}$ to Fe$^{2+}$). In a MFC the anode acts as terminal electron acceptor and its potential is determined by the rate of respiration (Torres et al., 2008). Therefore the anode potential is the analog to the
concentration of a soluble electron acceptor in a conventional respiration process. Bacteria respire/transfer electrons only when the anode accepts the electron. Hence in an electrochemical cell (three electrode electrochemical cell or H-cell) electrodes can be held at certain applied potentials that mimic the natural electron acceptor. The system developed for the growth of anodic biofilms under controlled applied potential conditions is known as bio-electrochemical system (BES) and such electrochemical cell set-up mimics the anode MFC. Fig.1.13 shows the electrochemical cell setup that can be used, reported in chapter 6, to grow bacteria by applying a potential to a working electrode (anode) using a potentiostat. Catalytic current generated by bacteria are continuously monitored using chronoamperometry and cyclic voltammetry. Each electrode can be held at a different applied potential in the same electrochemical cell using a multichannel device.

Figure 1.13. Electrochemical cell setup. (Electrochemical cell mimics the anode MFC)
Researchers have proposed three distinct extracellular electron transfer (EET) mechanisms for electron transfer to solid electrodes, as depicted in Fig.1.14. The first EET mechanism proposes the presence of a soluble electron shuttle, a mediator compound that is produced by bacteria that carries electrons. These shuttles diffuse in and out through the bacterial cell trapping electrons which then finally reduce the terminal electron acceptor. Electron shuttles are oxidized and reduced continuously. Bacteria are known to produce electron shuttle compounds such as melanin, phenazines, flavins, and quinones (Hernandez et al., 2004, Von Canstein et al., 2008). A second mechanism is the direct electron transfer from bacteria to electron acceptor. Presence of outer-membrane (OM) cytochromes can permit interaction directly with the electrode surface (Beliaev et al., 2001, Magnuson et al., 2001). The third mechanism is the formation of an extracellular biofilm matrix, conductive for electron transfers from the bacteria to electrode. A recent example is the proposal that cellular pili act as conductive nanowires (Reguera et al., 2005).

In microbial fuel cells and electrolysis cells, collectively called MXCs, the anode potential can be varied thus altering the thermodynamic potentials available for EAB. EAB include members of diverse phyla such as such as Alpha-, Beta-, Gamma-, and Deltaproteobacteria, Firmicutes, Acidobacteria (Logan, 2009). Mostly they are
Gramnegative, anaerobic and use Fe(III) as an electron acceptor. Substrate utilization capabilities of these bacteria are limited to acetate, but some members can utilize a wide range of substrates such as propionate, butyrate, lactate and glucose (Lovley et al., 1993, Holmes et al., 2004a, Debabov, 2008). Researchers have used various applied potentials to investigate the EET mechanism of EAB within both single and mixed cultures. In order for the reaction to be thermodynamically favorable, the anode should be held at apotential more positive than the oxidation potential of the substrate, acetate (−0.48 V vs. Ag/AgCl), thermodynamically favoring electron transfer while oxidizing acetate. Several studies have analyzed the growth of EAB at different applied potentials. Busalmen et al. showed two different electrochemical responses with Geobacter sulfurreducens films grown under either 0.1 or 0.6 V vs. Ag/AgCl–KCl, suggesting two different respiratory processes: one at low, and the other at higher, anode potentials (Busalmen et al., 2008, Busalmen et al., 2010). This bacterium is used as a model for EAB, as it has been shown to perform well in biofilms on electrodes for MFCs, providing amongst the highest currents densities for acetate oxidation in such systems. In the genome of the Geobacter sulfurreducens there are 111 different cytochromes coded, and more than 30 are located in the outer sphere of the cell (Dumas et al., 2008b). Attenuated Total Reflection-Surface Enhanced Infrared Absorption Spectroscopy (ATR-SEIRAS) revealed that the c-type cytochromes that are present in outermost location are involved in the electron transfer process (Busalmen et al., 2008, Busalmen et al., 2010). It has been proposed that the bacterial electron transfer pathway is triggered based on the potential of the electron acceptor (Busalmen et al., 2008).

EAB have not evolved to produce electricity, but conditioning can alter their respiratory pathway in response to various anode potentials. Electrodes mimic the presence of natural electron acceptors if poised at appropriate potentials, thus triggering a specific response. According to Busalmen et al. branching of the external electron transfer chain in Geobacter sp. allows the recognition of at least three different electron transfer pathways with increasing potential (Busalmen et al., 2010). The reduction of fumarate (−0.18 V Ag/AgCl–KCl sat.) by the fumarate reductase (FrdCAB) (Butler et al., 2006), the reduction of iron and metal oxides (−0.10 to +0.10 V Ag/AgCl–KCl sat.) through the pathway composed by PpcA, OmcB and probably OmcS (PpcA- Periplasmic c-type cytochrome, Omc - outer membrane c-type cytochromes) and finally the reduction of high potential acceptors (≥ 0.6 V Ag/AgCl–KCl sat.) including vanadates (Ortiz-Bernad et al., 2004). Interestingly, vanadate is the only known exocellular electron acceptor reduced by Geobacter sp at high potential (Anderson et al., 2003).
Dumas et al. individually polarized *Geobacter sulfurreducens* biofilm electrodes at different potential ranges between −0.2 V to +0.2 V vs. Ag/AgCl in the same reactors. At +0.2 V vs. Ag/AgCl they observed maximum current densities around 2.4 A m$^{-2}$ on stainless steel and 8 A m$^{-2}$ using *Geobacter sulfurreducens* grown on graphite electrode (Dumas et al., 2008a & b). Other than *Geobacter sulfurreducens*, *Shewanella oneidensis* was studied for its whole-cell voltammetry and showed that electrochemical activity was hypothetically linked to outer membrane c-type cytochromes (OM c-Cyts) (Kim et al., 2002). The redox wave has been lately confirmed as signals of OM c-Cyts OmcA/MtrC (Meitl et al., 2009), a mutant of *S. oneidensis* lacking OmcA/MtrC, did not display this redox pair. On the other hand, *S. oneidensis* is also known to secrete flavins for EET (Marsili et al., 2008). Table 1.1 shows that many different bacteria are suitable for reduction at the anode and the generation of electrical power (Logan, 2009).

Apart from pure culture, mixed-cultures were also subjected to different applied potentials to investigate the response. Finkelstein et al. grew EAB communities at different anode potentials (−0.058 to 0.618 V vs. Ag/AgCl) in a benthic microbial fuel cell (MFC) (Finkelstein et al., 2006). Their results suggested that EAB communities regulate their respiratory pathways to maximize energy efficiency and minimize extracellular potential losses. Wang et al. have obtained faster acclimation of EAB in MFCs using a constant high potential (+0.2 V vs. Ag/AgCl), resulting in a faster MFC start-up (Wang et al., 2009).

High current density, high power, and fast start-up in a MFC may require setting the anode potential to grow a biofilm most capable of achieving these traits. There is no optimal method to set anode potential using a potentiostat for bacterial growth at the anode. Theoretically, bacteria gain more energy by reducing terminal acceptors at a more positive potential. This is possible only when the bacteria have metabolic pathways capable of capturing the available energy. No one set potential will always yield the best results, suggesting that the outcome of a set potential experiment is dependent on culture conditions, electrode materials, and inoculums. Bio-electrochemical investigations of both pure and mixed cultures, over a wide range of potentials, are needed to better understand how to set and evaluate optimal anode potentials for improving MFC performance. Electrochemical tests, community analyses, and further study of the response of both pure and mixed cultures at applied potentials and different resistances will improve our understanding of the behavior of microbial communities in various redox environments and different types of MFCs. Chapter 6 describes the influence of different applied potential on *Rhodoferax ferrireducens* and *Geobacter sulfurreducens* biofilms developed at graphite electrodes. Electrochemical behaviour of
Rhodoferax ferrireducens is as yet unreported, though it has been utilized in an MFC for electricity production (Chaudhuri and Lovley, 2003). In chapter 6, an attempt has been made to compare the electrochemical characteristics of biofilms of both these bacteria induced to grow at different applied potentials.

Table 1.1 Electroactive bacteria

<table>
<thead>
<tr>
<th>Year</th>
<th>Microorganism</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td><em>Shewanella putrefaciens</em> IR-1 (Kim et al., 1999)</td>
<td>Direct proof of electrical current generation in an MFC by a dissimilatory metal-reducing bacterium (Gammaproteobacteria)</td>
</tr>
<tr>
<td>2002</td>
<td><em>Desulfuromonas acetoxidans</em> (Bond et al., 2002)</td>
<td>Identified in a sediment MFC community and shown to produce power (Deltaproteobacteria)</td>
</tr>
<tr>
<td>2003</td>
<td><em>Geobacter metallireducens</em> (Bond et al., 2002)</td>
<td>Shown to generate electricity in a poised potential system (Deltaproteobacteria)</td>
</tr>
<tr>
<td>2003</td>
<td><em>Geobacter sulfurreducens</em> (Bond et al., 2003)</td>
<td>Generated current without poised electrode (Deltaproteobacteria)</td>
</tr>
<tr>
<td>2004</td>
<td><em>Rhodoferax ferrireducens</em> (Chaudhuri et al., 2003)</td>
<td>Used glucose (Betaproteobacteria)</td>
</tr>
<tr>
<td>2004</td>
<td><em>Pseudomonas aeruginosa</em> (Rabaey et al., 2004)</td>
<td>Produced low amounts of power through mediators such as pyocyanin (Gammaproteobacteria)</td>
</tr>
<tr>
<td>2005</td>
<td><em>Geopsychrobacter electrodiphilus</em> (Holmes et al., 2004)</td>
<td>Psychrotolerant (Deltaproteobacteria)</td>
</tr>
<tr>
<td>2006</td>
<td><em>Shewanella oneidensis</em> DSP10 (Ringelstein et al., 2006)</td>
<td>Achieved a high power density (2 W per m² or 500 W per m³) by pumping cells grown in a flask into a small (1.2 mL) MFC (Gammaproteobacteria)</td>
</tr>
<tr>
<td>2006</td>
<td><em>S. oneidensis</em> MR-1 (Bretschger et al., 2007)</td>
<td>Various mutants identified that increase current or lose the ability for current generation (Gammaproteobacteria)</td>
</tr>
<tr>
<td>2008</td>
<td><em>Rhodopseudomonas palustris</em> DX-1 (Xing et al., 2008)</td>
<td>Produced high power densities of 2.72 W per m² compared with an acclimated waste-water inoculum (1.74 W per m²) (Alphaproteobacteria)</td>
</tr>
<tr>
<td>2008</td>
<td><em>Desulfovibrio desulfuricans</em> (Zhao et al., 2008)</td>
<td>Reduced sulphate when growing on lactate; resazurin in the medium was not thought to be a factor in power production (Deltaproteobacteria)</td>
</tr>
<tr>
<td>2008</td>
<td><em>Acidiphilium</em> sp. (Borole et al., 2008)</td>
<td>Current at low pH and in the presence of oxygen in a poised potential system (Alphaproteobacteria)</td>
</tr>
<tr>
<td>2008</td>
<td><em>Klebsiella pneumoniae</em> L17 (Zhang et al., 2008)</td>
<td>The first time this species produced current without a mediator (Gammaproteobacteria)</td>
</tr>
<tr>
<td>2008</td>
<td><em>Thermincola</em> sp. strain JR (Wrighton et al., 2008)</td>
<td>Phylum Firmicutes</td>
</tr>
<tr>
<td>2008</td>
<td><em>Pichia anomala</em> (Prasad et al., 2007)</td>
<td>Current generation by a yeast (kingdom Fungi).</td>
</tr>
</tbody>
</table>
1.4 Electroanalytical techniques

In EFC and MXCs, electrochemists use various voltammetric techniques to characterize electrochemical reactions at an electrode surface. Selected techniques may be cyclic voltammetry (CV), differential pulse voltammetry (DPV), and chronoamperometry (Christensen and Hammett, 1994, Bard and Faulkner, 2001). Electroanalysis is the application of electrochemistry to solve analytical problems. Electrode processes in electroanalysis involve the charge transfer across the electrode/solution, electrode/polymer and electrode/bacterial biofilm interface (heterogeneous electron transfer), either in equilibrium at the interface, or under partial or total kinetic control.

1.4.1 Voltammetric and amperometric techniques

Cyclic voltammetry measures current as a function of a cyclic applied potential. In this technique, the potential is ramped linearly at rates of $0.01 – 10^5 \text{V s}^{-1}$, with reversal of the ramp after a given time (potential) and the resulting current ($I$) is monitored as a function of applied potential ($E$) to give the I-E curve called the cyclic voltammogram (Christensen and Hammett, 1994, Bard and Faulkner, 2001). A typical electrochemical set up utilized for performing cyclic voltammetric experiments would consist of a standard three electrode system. The electrodes employed are the working electrode (WE), that is, the electrode at which the reaction of interest occurs, a counter electrode (CE), to facilitate a connection to the electrolyte so that a current can be measured at the working electrode, and a reference electrode (RE), where the potential of the working electrode is measured with respect to the reference electrode. Typical working electrodes include carbon (glassy and graphite) and gold, while Ag/AgCl usually acts as reference electrode. A platinum wire is normally used as the counter electrode. A redox system can be characterized from the potentials of the peaks on the cyclic voltammogram and from changes caused by variations in scan rate. Fig.1.17 depicts an example of a voltammetric response for the Os(II)/(III) oxidation and reduction processes of immobilized $[\text{Os(bpy)}_2\text{(PVI)}_{10}\text{Cl}]^{2+/+}$. The potential is swept from $-0.2 \text{ V}$ to $0.6 \text{ V}$ and back again to reveal a typical reversible redox waveform for a surface-immobilised system.
Figure 1.17 Cyclic voltammogram of immobilized $[\text{Os(bpy)}_2\text{(PVI)}_{16}\text{Cl}]^{2+/+}$ in aqueous phosphate buffer, 0.05M pH 7.4 on a glassy carbon electrode at a scan rate of 5 mV/s vs. Ag/AgCl. $E_{pa}$, $i_{pa}$, $E_{pc}$ and $i_{pc}$ represent the anodic and cathodic peak potentials and currents respectively. FWHM is the full width half maximum of peak current and $\Gamma$ corresponds to the surface coverage of redox active sites.

A reversible reaction is one where the kinetics of the electrode reaction is faster than the mass transport. The forward sweep produces a current flow resulting in an oxidation peak as the applied potential passes through the standard redox potential ($E^\circ$) and then the current begins to diminish. When the scan is reversed we simply move back through the equilibrium, past the $E^\circ$, gradually converting the electrolysis product (oxidized product) back to the reduced species. This potential window contains only a single electrochemical reactant. The important parameters in a cyclic voltammogram include the cathodic ($E_{pc}$) and anodic ($E_{pa}$) peak potentials and cathodic ($i_{pc}$) and anodic ($i_{pa}$) peak currents. The peak current for a reversible system displaying semi-infinite diffusion is given by the Randles-Sevcik equation (1.1). This equation describes the relationship between peak current and scan rate for a reversible redox species at standard temperature and pressure.
Cyclic voltammetry can be used in the determination of many kinetic parameters of a redox system. If homogeneous chemical reactions accompany the oxidation or reduction of a species at the electrode, the shape of the voltammogram changes. The observed changes give significant information about the kinetics of the coupled reaction. By applying slow scan rates to surface confined redox species a depletion layer extends all the way into the solution and finite diffusion becomes important. Changes in cyclic voltammograms are observed for immobilized redox active materials when compared to solution phase (semi-infinite diffusion) reactants since the redox active material does not have to diffuse to or from the electrode surface. In such cases, the surface coverage of redox active sites confined to electrodes ($\Gamma$) can be determined by using the faradaic charge ($Q$) passed during complete electrolysis of the thin film.

$$\Gamma = \frac{Q}{nFA} \quad (1.2)$$

Where $n$ is the number of electrons, $F$ is Faraday’s constant and $A$ is the area of the electrode. For an ideal Nernstian reaction where there is no lateral interaction between neighbouring redox centers a CV of surface confined species will display a full width at half maximum (FWHM) of the anodic or cathodic wave as:

$$FWHM = \frac{3.53RT}{nF} = \frac{90.6}{n} mV \quad (1.3)$$

Amperometry involves the measurement of current at fixed applied potentials as the function of time. The working electrode is held at a fixed potential at which the electron transfer reaction to the electrode is thermodynamically favorable. In the case of enzyme redox polymer electrodes, a potential positive to the redox potential of the osmium(II) based polymer will favor electron transfer to the electrode. Similarly, in microbial catalyzed electrodes, a potential is selected positively uphill of the substrate oxidation potential, thermodynamically favoring the bacterial electron transfer to the electrodes. In such conditions, the electrode acts as electron acceptor for bacterial respiration. Current produced during this reaction depends upon the applied potential.
1.5 Thesis proposition

This thesis investigates both enzymatic and bacterial electron transfer mechanisms with a view to providing bio-electrochemical systems for energy generation. Chapter 2 describes methodologies developed for screening for possible redox mediators, mostly osmium complexes with mixed ligands exhibiting a wide range of redox potentials, for glucose oxidase by estimation of solution phase kinetics of the enzyme mediator interaction. Chapter 3 deals with development of immobilization strategies for production of films used in operation of enzymatic fuel cells. This chapter particularly focuses on surface-pretreatment to form a platform amenable to anchoring enzyme-redox polymer hydrogels. Chapter 4 focuses on a layer-by-layer technique for novel and alternate assembly of osmium redox polymer and enzymes, based on electrostatic interaction over graphite electrodes, to form enzymatic fuel cells. Chapter 5 investigates an electrochemical approach to form redox hydrogels over pyrolytic graphite electrodes with a view to improving the catalytic current densities, to subsequently form improved enzymatic fuel cells. In research complementary to that undertaken on enzymes, chapter 6 uses voltammetry to probe the electron transfer properties of 
*Geobacter sulfurreducens* and 
*Rhodoferax ferrireduces* films formed over carbon electrodes held at different applied potentials. Finally, chapter 7 summarizes the advances made during the course of this research and proposes some directions for future work.
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2. Electrochemical characterization of redox mediators for glucose oxidase

2.1 Introduction

Oxidoreductases that can be coupled to electrode reactions using redox mediators have been studied extensively (Ghica and Brett, 2005, Razola et al., 2000). Enzyme mediated bioelectrocatalysis systems are used in EFCs and biosensors (e.g., glucose sensors) to convert chemical reactions to electrical signals. In an EFC oxidation/reduction reactions take place with the enzyme and mediator bound to the electrode in a solution phase with applications in generating power that may suit power implantable or miniaturized electronic devices in mild operating conditions (Barrière et al., 2006, Kavanagh et al., 2009, Barton et al., 2004). Mostly the construction of EFCs focuses on glucose oxidation coupled to oxygen reduction, and these are intended to serve as implantable devices in the human body utilizing blood glucose and oxygen for their operation. Due to the fact that most redox enzymes do not directly exchange electrons with electrodes, a wide range of chemical compounds have been described for use as mediators, allowing enzymes to transfer electrons to electrodes (Fultz and Durst, 1982, Johnson et al., 1983). Mediated electron transfer in EFCs can be achieved for glucose oxidation (Mano et al., 2003, Barton et al., 2001) and oxygen reduction (Palmore et al., 1999, Trudeau et al., 1997) using GOx and laccase respectively as biocatalysts. Optimization of an EFC with appropriate mediator, can determine the overall cell potential, and is thus crucial. At both the anode and the cathode tailoring the mediator potential to the potential closest to that of the enzyme active site will maximize cell potential. However, this could lead to low catalytic currents due to lower driving force for electron transfer.

Effects of redox potential of a mediator on enzyme–mediator electron transfer rates have been reported previously (Zakeerruddin et al., 1992, Takagi et al., 1998). Glucose oxidase (GOx) was used as a model enzyme to study the electron transfer between enzymes and redox mediators. Evaluation of GOx – mediator interaction allows the selection of optimum mediators for EFC. Kinetic parameters, such as the pseudo first-order ($k_f$) and second order rate constant ($k_{med}$) for enzyme-mediator reactions, have been evaluated in order to measure the rate of electron transfer between the enzyme and mediators (Nicholson and Shain, 1964). These studies were performed in homogenous solution phase at low mediator concentration with a fixed concentration of enzyme and a high substrate
concentration, permitting extraction of a pseudo-first order reaction rate constant. By varying the enzyme concentration one can then estimate the second order rate constant.

Two main factors are considered for selecting a mediator. Firstly, the redox potential of the mediator should be thermodynamically favorable with regards to the enzyme redox potential. Secondly, the mediator-enzyme electron transfer rate should be high. Ferrocene and its derivatives have been characterized extensively with GOx (Cass et al., 1984, Noci et al., 2008). Ferrocene in its oxidized form, ferricinium, undergoes slow hydrolysis (Yeh and Kuwana, 1976) and it is not very soluble in its reduced form in aqueous solvents. Thus ferrocene is not an ideal mediator and can cause problems in mediated electrocatalysis. Zakeruddin et al. (1992) and Fraser et al. (1992) presented a range of 4,4'-substituted-2,2'-bipyridine complexes of osmium which showed high second order rate constant values for reaction with GOx and low redox potentials. Synthesis of such low redox potential osmium complexes can be used to form glucose biosensors and EFCs. It is possible to tune osmium complexes to a desired redox potential by varying the ligands (Zakeruddin et al., 1992, Fraser et al., 1992). These complexes usually comprise of polypyridyl ligands, such as 2,2'-bipyridine (bpy), 4,4'-dichloro-2,2'-bipyridine (dClbpy) or 4,4'-dimethoxy-2,2'-bipyridine (dmobpy), bonded to metal ions through coordinate bonding. Fig. 2.1 shows the chemical structure of the osmium complex cis-[Os(bpy)2Cl2] for example. For ligands, the presence of an electron withdrawing group results in an increase of the redox potential of the metal in the complex, while the opposite occurs with electron donating substituents. In this chapter a range of osmium polypyridyl complexes have been characterised electrochemically for their ability to act as electron transfer mediators for redox enzymes, in particular GOx. In addition to osmium complexes, a number of commercially available mediators - TMPD (N,N,N',N'-tetramethyl p-phenylenediamine) and ferrocene dicarboxylic acid - were studied to validate the approach and compare the rate constants to those observed for the novel osmium-based complexes.

Mediator solubility is an issue in studying homogeneous aqueous solution phase kinetics. Solubility of osmium complexes vary according to substitutions on the coordinating polypyridyl ligand. In general, solubility decreases with increasing electron-donating ligand substituents (Lever, 1990). These problems contribute to the failure to observe reversible electrochemical properties, precipitation of molecules that change the molar concentrations, and low catalytic currents. Dissolving the mediators in more suitable solvents could resolve solubility issues provided they do not affect the mediator and enzyme properties.
In this chapter, voltammetry of osmium complexes of a wide range of redox potentials was recorded to evaluate their capacity to mediate electron transfer from GOx. Mediators were characterized electrochemically in homogenous solution phase. Evaluation of first ($k_f$) and second order rate constants ($k_{med}$) for GOX mediated oxidation of glucose was used to classify suitable mediators, taking account of their redox potential. This evaluated parameter was exploited to select the mediators that provide the best compromise of current generation and cell voltage for operation of a glucose/O$_2$ solution phase EFC.

2.2 Experimental Section

2.2.1 Enzyme Activity

GOx from *Aspergillus niger* was purchased from Sigma Aldrich and its activity was estimated as reported previously (Bergmeyer 1974, Worthington enzyme manual 1993) using a UV spectrophotometer (Agilent 8453). Absorbance was monitored at 460 nm, where the increase in absorbance is a result of the oxidation of dianisidine through a peroxidase coupled system, in phosphate buffer pH 6. GOx 10 mg /ml stock (1500 U/ml) was used for enzyme activity and bioelectrocatalysis. The activity of the laccase (*Trametes versicolor*, VTT Finland) enzyme was calculated using the spectrophotometric ABTS assay (Leech and Daigle, 1998) by monitoring the oxidation of 5 mM ABTS in 50 mM acetate buffer pH 4.5 at 420 nm.

2.2.2 Enzyme Concentration

The active concentration of a monomer of GOx is expressed in terms of catalytically active FAD (Sigma-Aldrich), determined using absorbance at 450 nm (Weibel and Bright, 1971, Swoboda and Massey, 1965). Calibration curves were established with standard
solutions of FAD in potassium acetate buffer solution. A glucose solution was added to the solution mix to observe the reduced form of FAD, and the absorbance obtained from the stock solution of GOx (10mg/ml) correlated with the calibration curve of FAD to determine the active concentration [GOx active].

2.2.3 Synthesis of osmium complexes

Chemicals used for synthesis were purchased from Sigma except for 2,2'-biimidazole which was purchased from Matrix Scientific and 4,4'-dichloro-2,2'-bipyridine purchased from CMS chemicals Ltd.

Ligand abbreviations

The following abbreviations for ligands are used: dmobpy = 4,4'-dimethoxy-2,2'-bipyridine; bpy = 2,2'-bipyridine; dmbpy = 4,4'-dimethyl-2,2'-bipyridine; dclbpy = 4,4'-dichloro-2,2'-bipyridine; phen = 1,10-phenanthroline; 4-aminomethyl pyridine (4-AMP); 4-(2-aminoethyl) pyridine (4-AEP); (Aminopropyl)-imidazole (API).

\[ \text{4-aminoethyl pyridine} \quad \text{(Aminopropyl)-imidazole} \]

\[ \begin{array}{c}
\text{N} \\
\text{H}_2 \\
\end{array} \]

\[ \begin{array}{c}
\text{N} \\
\text{H}_2 \\
\end{array} \]

4-aminomethyl pyridine

\[ \begin{array}{c}
\text{N} \\
\text{H}_2 \\
\end{array} \]

2.2.4 General synthesis of cis-Os(N-N)\(_2\)Cl\(_2\) complexes

Typical synthesis, conducted in our laboratory by other members of the team, of the osmium complexes of general formula \(\text{cis-Os(N-N)\(_2\)}\text{Cl\(_2\)}\) was carried out according to published procedures (Kober et al., 1988). Generally, \((\text{NH}_4)\text{OsCl}_6\) and a slight excess molar equivalent of the ligand \(\text{(N-N)}\) were dissolved in ethylene glycol and held at reflux for 45
minutes. After cooling to room temperature, an excess amount of aqueous sodium dithionite was added to reduce Os(III) to Os(II). The mixture was stirred in ice for 45 minutes before filtering. The precipitate was then washed with water followed by large volumes of diethyl ether. The resulting complex was dried under vacuum. Yields in excess of 85% were consistently obtained. Complexes were characterized using CV.

2.2.5 Synthesis of cis-[Os(N-N)2(L)Cl].PF6 polypyridyl complexes

Typical synthesis of complexes of general formula cis-[Os(N-N)2(L)Cl].PF6 were carried out according to published procedures (Kober et al., 1988), in our laboratory by other members of the team. Generally, cis- Os(N-N)2Cl2 and a slight excess molar equivalent of the ligand (L) were dissolved in ethylene glycol and held at reflux for 45 minutes. After cooling to room temperature, excess amount of aqueous ammonium hexafluorophosphate was added to form the insoluble PF6 adduct of the complex.

2.2.6 Electrochemical methods

The rate of reaction in solution between enzyme and mediator was studied in a conventional three-electrode cell with a working volume of 0.4 cm³ using a CH Instruments potentiostat (USA). Working, reference, and counter electrodes were glassy carbon (GC), Ag/AgCl (All potentials are quoted vs.Ag/AgCl electrode throughout the thesis unless otherwise stated) and a platinum wire respectively. The geometric surface area of the GC was calculated as 0.07 cm². The glassy carbon electrode was polished with different grade of alumina and cleaned by sonicating the electrode with distilled water. Cyclic voltammetry (CV) was performed with deoxygenated potassium phosphate buffer (PPB) solution (0.1M pH 7.4) using scan rates of 50 to 2 mV s⁻¹. Mediators were dissolved in PPB solutions to make a final concentration of 0.2 mM in 0.1 M glucose. Mediators that were insoluble in PPB solution were solubilized in organic solvent (DMSO) followed by addition of buffer to yield 5% DMSO in buffer as solvent. Catalytic current was measured with increasing [GOx active] (2 -12 µM). The change in the catalytic current was compared with the diffusion current obtained in the absence of enzyme at different scan rates, and a rate constant was obtained according to Nicholson and Shain method (Nicholson and Shain, 1964). In this method a pseudo-first order rate constant was estimated from the slope of a plot of a kinetic parameter \(k_f/a\) as function of \(1/v\), where \(k_f\) is the rate constant, \(a = nFv/ RT\) where \(n\) is the number of moles of electrons, \(F\) the Faraday constant, \(v\) scan rate (Vs⁻¹), \(R\) is the universal gas constant and \(T\) is temperature). The second order rate constant of the homogeneous reaction for the
mediation scheme can be estimated by $k_{\text{med}} = k_d/[\text{GOx active}]$.

2.2.7 Solution phase EFC

A room-temperature, 20 ± 1°C, two chambered solution phase fuel cell with a volume of 10 ml each for anode and cathode (glassy carbon electrode) separated by a Nafion 117 membrane was used for evaluation of fuel cell response. The Nafion membrane was pretreated consecutively by boiling with H$_2$SO$_4$ (0.1 M) and H$_2$O$_2$ (5%), distilled water (DW) and stored in DW prior to use. The anolyte was purged with nitrogen and catholyte with oxygen for a few minutes before startup of fuel cell testing. Polarization for both fuel cells was studied with externally connected resistors (10MΩ – 1 kΩ) and the drop in the voltage was monitored using a digital multimeter under non-stirred conditions.

2.3 Results and discussion

2.3.1 Characterization of redox mediators

For an EFC assembly it is important to select the best compromise for mediator, based on a consideration of both thermodynamics and kinetics. Estimation of the rate constant for the enzyme-mediator interaction can provide data on enzyme-mediator kinetics. In addition, electrochemical characterization of mediator, based on its redox potential, allows screening of mediators for provision of EFC assembly with high cell voltages. GOx was selected as the anodic enzyme to be investigated in a glucose/O$_2$ EFC because of its commercial availability, and its well-characterized propensity for glucose oxidation. Mediators selected for characterization are listed in Table 2.1, with their redox potentials. Mostly osmium complexes of formula $\text{cis-Os(N-N)Cl}_2$, synthesized previously in our laboratory, with redox potentials in the range ($E^{\circ} = -0.24$ to $+0.43$V vs. Ag/AgCl) were evaluated. The formal potentials ($E^{\circ}$) of the mediators tabulated are the mid values obtained between oxidation and reduction peaks in cyclic voltammograms.
Table 2.1 Redox potentials of mediators vs. Ag/AgCl estimated from CVs recorded at 5 mV/s deoxygenated potassium phosphate buffer (0.1M, pH 7.4), mediators 0.2Mm

<table>
<thead>
<tr>
<th>Mediators</th>
<th>E° (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Os (dmo bpy)₂ Cl₂]</td>
<td>-0.24</td>
</tr>
<tr>
<td>[Os (dm bpy)₂ Cl₂]</td>
<td>-0.12</td>
</tr>
<tr>
<td>[Os (bpy)₂ Cl₂]</td>
<td>-0.02</td>
</tr>
<tr>
<td>[Os (phen)₂ Cl₂]</td>
<td>0</td>
</tr>
<tr>
<td>[Os (dmobpy)₂ (4AEP) Cl] PF₆</td>
<td>0.025</td>
</tr>
<tr>
<td>[Os (dmobpy)₂ (4AMP) Cl] PF₆</td>
<td>0.060</td>
</tr>
<tr>
<td>TMPD</td>
<td>0.065</td>
</tr>
<tr>
<td>[Os (dmbpy)₂ (4AMP) Cl] PF₆</td>
<td>0.19</td>
</tr>
<tr>
<td>[Os [(bpy)₂ (API) Cl] PF₆</td>
<td>0.2</td>
</tr>
<tr>
<td>[Os (bpy)₂ (4AMP) Cl] PF₆</td>
<td>0.24</td>
</tr>
<tr>
<td>[Os (bpy)₂ (4AEP) Cl] PF₆</td>
<td>0.26</td>
</tr>
<tr>
<td>[Os (dcl bpy)₂ (4 AMP) Cl] PF₆</td>
<td>0.43</td>
</tr>
<tr>
<td>Ferrocene dicarboxylicacid</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Figure 2.2 Structure of cis-[Os(N-N)₂ (L)Cl].PF₆
Suitable ligands could be co-ordinatively bound to the starting cis-Os(N-N)2Cl2 complexes, by ligand substitution of one of the chlorines, general formula denoted cis-[Os(N-N)2(L)Cl].PF6 (Fig. 2.2), to yield complexes that possess additional functional groups for immobilization, such as amines. Substitution of Cl with pyridyl ligands shifted the redox potential more positive: Os (bpy)2Cl2 and [Os (bpy)2(4AEP)Cl]PF6 showed redox potentials of $E^{o'} = -0.02$ and $+0.26$ V vs. Ag/AgCl, respectively. The redox potentials of osmium complexes can also be manipulated by substitution of electron withdrawing or electron donating groups in the 4 and 4’ positions of the bipyridine ligand, that shifts the redox potential positively or negatively creating a library of complexes, as listed in table 2.1. As an example, 2,2’-bipyridine bonded to an osmium metal center possesses a redox potential of $E^{o'} = -0.02$ V vs. Ag/AgCl, yet the presence of an electron donating group such as methoxy at the 4 and 4’ positions of the bipyridine leads to a clear difference in redox potential of $-0.22$ V for the osmium complex, as observed by others (Boland et al., 2008). These complexes could be used as mediators for a variety of different enzymes. For example, GOx possesses a redox active site $E^{o'} = -0.35$ V vs. Ag/AgCl (Gregg and Heller, 1991a, Gregg and Heller, 1991b) and thermodynamically, all of these mediators listed in the table are predicted to act as electron-transfer mediators for glucose oxidation (Nicholson and Shain, 1964).

For determination of the electron transfer rate constant between GOx and mediator, two criteria have to be fulfilled: (i) the electrode reaction of the mediator must be fast compared to the rate of reaction between the mediator and the enzyme, and (ii) saturated glucose concentration is required to ensure a pool of reduced enzyme. Oxidation of glucose by GOx results in the generation of electrons by oxidizing the reduced GOx/FADH2 (Cardosi and Turner, 1987). Then GOx/FADH2 is oxidized by the mediator where the mediator is then oxidized at electrode transferring electrons continuously. A general scheme of mediated (osmium complex) electron transfer mechanism with GOx is given as

$$\text{Glucose} + \text{GOx}_{\text{ox}}(\text{FAD}) \xrightarrow{\text{GOx}_{\text{red}}(\text{FADH}_2)} \text{gluconic acid} + \text{GOx}_{\text{red}}(\text{FADH}_2) \quad (2.1)$$

$$\text{GOx}_{\text{red}}(\text{FADH}_2) + 2 \text{Os (III)} \xrightarrow{\text{GOx}_{\text{ox}}(\text{FAD}) + 2 \text{Os (II)} + 2 H^+} \quad (2.2)$$

$$2 \text{Os (II)} \xrightarrow{2 \text{Os (III)} + 2 e^-} \quad (2.3)$$
2.3.2 Evaluation of first order rate constants

The homogeneous electron transfer rate constant corresponding to the reaction between a mediator and an enzyme in solution can be determined by cyclic voltammetry according to Nicholson and Shain approach (Nicholson and Shain, 1964). The pseudo-first order reaction applies only in presence of high substrate concentration (that is not a limiting factor) with a known concentration of mediator. When the reaction between the reduced enzyme and the oxidized mediator is fast on the timescale of the experiment, the oxidation catalytic current reaches a plateau, the reduction disappears completely and the cyclic voltammogram acquires a sigmoidal shape. When both glucose and GOx were added together to a solution of an osmium complex, catalytic current sigmoidal waveforms were observed for most of the osmium complexes. Catalytic current was observed only when the fixed concentrations of mediators, GOx and glucose were present in the electrochemical cell. No catalytic current was seen in the absence of enzyme. Anaerobic conditions in the electrochemical cell were ensured by purging with nitrogen, to ensure no competition from oxygen and that the mediators act as final electron acceptors.

For the evaluation of the rate constant, \( k_f \), the diffusion current \( (i_d) \) of the mediator in the absence of enzyme is estimated from the CV, followed by addition of enzyme (GOx) to yield a current for mediated glucose oxidation, designated as \( i_k \). Fig. 2.2 shows the CVs of solutions of 0.2 mM mediator and 0.1 M glucose, in absence and presence of 12 µM GOx. Continuous regeneration of the oxidized or reduced state of the mediator by the enzyme takes place in the presence of an electron donor. The faster the enzymatic oxidation or reduction rate is, the larger the current increase will be. This catalytic oxidation or reduction current \( (i_k) \) is also measured and the ratio of diffusion current over catalytic current is calculated for each scan rate. The ratio of \( i_k/i_d \) can then be equated to the kinetic parameter \( (k_f/a)^{1/2} \) where \( a = nFv/RT \) (where \( n \) is the number of moles of electrons, \( F = \) Faraday constant, \( v = \) scan rate, \( R = \) gas constant, \( T = \) absolute temperature). By plotting \( k_f/a \) against inverse scan rate and evaluating the linear first order slope as shown in Fig. 2.3 we can derive the pseudo first order rate constant \( k_f \).
Figure 2.2 Cyclic voltammograms of Black [Os (bpy)$_2$ Cl$_2$], Orange [Os (phen)$_2$ Cl$_2$], Gray [Os (dmo bpy)$_2$ (4AMP) Cl]PF$_6$, Blue [Os (bpy)$_2$(API) Cl]PF$_6$, pink [Os (dm bpy)$_2$ (4AMP) Cl]PF$_6$, Red [Os[(bpy)$_2$(4AEP)Cl]PF$_6$, Dark green [Os (dcl bpy)$_2$ (4 AMP) Cl] PF$_6$ at 5mV/s in deoxygenated potassium phosphate buffer (0.1M, pH 7.4), glucose (0.1 M), mediators 0.2mM and a) in absence and b) in presence of GOx- 12 µM (30 U/ml),
Figure 2.3 Plot of kinetic parameter $k_f/a$ against inverse scan rate, $v^{-1}$ for Os (bpy)$_2$ Cl$_2$ with GOx 12 µM, 0.2 mM mediator in presence of 0.1M glucose.

Fig.2.4 demonstrates the analysis of $k_f$ using a cyclic voltammogram for Os(phen)$_2$Cl$_2$ shown as an example. A baseline was drawn that represents the background current that was subtracted to obtain $i_k$ and $i_d$ values (Yokoyama and Kayanuma, 1998) as follows:

- CVs were recorded using the CH instrument software. Lines drawn from the initial rise in the current (from −0.2 V to −0.1 V), were considered to represent the background signal.

- Redox potentials were obtained from the midpoint between the oxidation and reduction peak current potentials of the CV for the mediator in the absence of enzyme.

- The peak catalytic ($i_k$) and diffusion ($i_d$) current was measured at point b subtracting the background current at a.

- For every scan rate a baseline was drawn that was then subtracted to obtain $i_k$ and $i_d$ values.
Figure 2.4 Cyclic voltammograms of \([\text{Os (phen)}_2 \text{Cl}_2]\) showing the baseline drawn for the, a) diffusion current \((i_d)\) and b) catalytic current \((i_k)\). Point a is subtracted from the peak current b for \(i_k\) and \(i_d\) values.

2.3.3 Evaluation of second order rate constants.

Evaluation of second order rate constants for these osmium complexes was dependent on estimation of the active concentration of GOx involved in the mediation of glucose oxidation. To determine the active concentration of GOx, estimation of FAD concentration was used since it is the redox cofactor involved in glucose oxidation (Weibel and Bright, 1971, Swoboda and Massey, 1965). The calibration curve of FAD concentration was linear with correlation coefficient \((R^2 = 0.98)\) Fig.2.5. This was then correlated with the absorbance solution of GOx used in the mediated electrocatalysis studies to yield an estimate of active concentration of GOx of 12 \(\mu\)M. A second order homogenous rate constant can be obtained by dividing first order rate constant by the active concentration of GOx involved in the mediation: \(k_{med} = k_f[\text{active GOx}]\)
Figure 2.5 Calibration curve of FAD. Assay was conducted in potassium acetate (0.01M pH 5.5) and 0.01M glucose. Absorbance of solution containing 2 to 10 µM FAD measured in a standard 1 cm cuvette at 450 nm.

2.3.4 Effect of enzyme concentration on catalytic currents

The change in the catalytic current was measured by varying the amounts of GOx, keeping the concentrations of mediator and glucose constant, at a scan rate of 5 mV/s. Fig. 2.6 shows the CVs of different mediators (0.2 mM) obtained for GOx concentrations in the range of 2-12 µM. Though 2 µM GOx was sufficient to yield an increase in oxidation current, steady state catalytic currents were achieved at 12 µM GOx for these mediators. Hence, the GOx concentration value of 12 µM was chosen for all the measurements.

In the absence of the enzyme, mediators tested in cyclic voltammetry (scan rates between 0.05 – 0.002 V/s) showed reversible electrochemical properties. Currents recorded at different scan rates were plotted versus \( v^{1/2} \) and the Randles-Sevcik equation used to estimate a mediator diffusion coefficient \( (D) \) from the slope of the plots, which was in the range of \( 10^{-6} \) cm\(^2\)s\(^{-1}\) for all the mediators listed in the Table 2.4, ensuring fast mediator diffusion through the buffer solution at 0.1 M glucose.
\[ R^2 = 0.92 \]

**Potential (V)**

**Current (µA)**
c)  

**Figure 2.6** Cyclic voltammograms for a) [Os (dmobpy)$_2$ (4AEP) Cl] PF$_6$, b) [Os (phen)$_2$ Cl$_2$], c) TMPD obtained at 5mV/s in deoxygenated potassium phosphate buffer (0.1M pH 7.4) glucose (0.1M), mediators (0.2mM) with GOx concentrations - 2, 4, 8, 12 µM. (Insert shows the current vs. GOx concentration). (gray- 0 µM, brown – 2 µM, blue – 4 µM, black – 8 µM, saffron – 12 µM).

### 2.3.5 Issues with the methodology to estimate rate constants for mediated electron transfer

In general, solubility decreases with increasing electron-donating groups in osmium complexes (Lever, 1990). Low mediator solubility can affect the methodology adopted for estimation of the rate constants for mediated enzyme electron transfer kinetics. This may be alleviated by use of alternate solvents to dissolve the compounds for investigation of the homogeneous chemical reactions. Here, 5% of dimethyl sulfoxide (DMSO) was used to attempt to resolve the mediator solubility problems. Mediators were first dissolved in DMSO and then buffer and substrate were added to achieve a final DMSO level of with 5%, before analysis in electrochemical cells. To establish if the DMSO had any effect on the enzyme, GOx activity was measured as a function of %DMSO using the GOx spectrophotometric assay. As seen from the data in **Table 2.2**, the solvent, at these levels, did not overly affect the enzyme activity, when compared to the control (no DMSO). In fact a slightly enhanced enzyme activity was observed for DMSO levels up to 3%. Use of 5% DMSO to dissolve the
mediators was therefore introduced, as it did not affect GOx activity. Enhanced enzyme activity for enzymes dissolved in low levels of DMSO was reported previously (Di and Kerns, 2006, Wiggers et al., 2007).

**Table 2.2** GOx activity at different levels of DMSO

<table>
<thead>
<tr>
<th>DMSO added (µl)</th>
<th>Volume in percentage (%)</th>
<th>Enzyme activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>645</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>669</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>716</td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>637</td>
</tr>
</tbody>
</table>

(GOx activity was estimated using a UV spectrophotometer as described in section 2.2.1 in phosphate buffer (pH 6) containing different levels of DMSO added to the GOx vial)

In order to establish the influence of DMSO on mediated electrocatalysis, solvent studies were conducted in electrochemical cells using the mediator TMPD as an example. **Table 2.3** shows a comparison of the rate constants for glucose oxidation by GOx mediated by TMPD, obtained with TMPD dissolved with and without 5% DMSO in the buffer. From the observation it seems that the DMSO did not affect the electrocatalysis. **Fig. 2.8** shows an example of the CVs of the TMPD solution with or without 5% DMSO, in the presence and absence of GOx, demonstrating that the presence of this level of DMSO has no significant effect on the CVs.
**Table 2.3** Kinetics for TMPD with no DMSO and with DMSO (5%)

<table>
<thead>
<tr>
<th>Mediator</th>
<th>$E^\text{a'}$ (V)</th>
<th>$k_f$ (s$^{-1}$)</th>
<th>$k_{med}$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMPD (no DMSO)</td>
<td>0.065</td>
<td>0.15</td>
<td>1.3 $\times 10^4$</td>
</tr>
<tr>
<td>TMPD (5% DMSO)</td>
<td>0.065</td>
<td>0.21</td>
<td>1.8 $\times 10^4$</td>
</tr>
</tbody>
</table>

Scan rate 0.05 – 0.002 V/s in PPB buffer at pH 7.4 containing 0.1 M glucose with 12µM GOx for solution phase kinetics at pH 20 ± 1 °C. Redox potential of the mediators in table is given in reference with Ag/AgCl.

**Figure 2.8** Cyclic voltammograms obtained at 5mV/s in deoxygenated potassium phosphate buffer (0.1M pH 7.4) glucose (0.1M), TMPD (0.2mM) with and without GOx (12 µM). (Black line – no DMSO, Blue line – with 5% DMSO).
A decrease in the height of the steady-state catalytic current for glucose oxidation by GOX mediated by different mediators is apparent upon repetitive cycling of the potentials, particularly at slow scan rates, as shown in the example using [Os(bpy)$_2$(APt)Cl]PF$_6$ in Fig. 2.9. Substrate depletion cannot account for catalytic decay, as excess glucose of 0.1 M is present in the solution ensuring that the substrate was not depleted. For example, Flexer et al. have shown that in a similar experiment, using complex [Os(bpy)$_2$ClPyCOOH]$^+$, GOx at 2.4µM, experienced a catalytic current decay in chronoamperometry experiments; with the glucose concentration decrease in a cell from 50 mM decreased to 49.92 mM in 15 min (Flexer et al., 2008). They have shown that the enzyme inactivation at higher glucose concentration was the reason for catalytic current decay. In order to avoid too much variation in the data due to time-dependent decay in the catalytic current, CVs are recorded immediately after addition of enzyme. Slow scan rate CVs were recorded first, after the addition of the enzyme, before moving on to recording CVs at faster scan rates. Anaerobic conditions ensured that oxygen is not an electron scavenger and also thus limiting the production of H$_2$O$_2$, which can inhibits the enzyme activity.

Figure 2.9 Cyclic voltammograms of [Os(bpy)$_2$(APt)Cl]PF$_6$ obtained at 5mV/s in deoxygenated potassium phosphate buffer (0.1M pH 7.4) glucose (0.1 M), mediator 0.2mM) with GOx concentrations 12 µM showing the current decay in time during the kinetic parameter analysis. (Insert shows the current decay with respect to time taken at 0, 600 and 1200 seconds respectively).
Table 2.4 Parameters extracted from CV of mediators. Values of rate constants presented in the table are taken using a GOx concentration of 12 µM. Conditions as in Fig 2.6.

<table>
<thead>
<tr>
<th>Mediators</th>
<th>E°' (V)</th>
<th>D (\times 10^{-6} \text{cm}^2 \text{s}^{-1})</th>
<th>k_f (/s)</th>
<th>k_{med} (M(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Os (dmo bpy)_2 Cl]_2</td>
<td>-0.24</td>
<td>4.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>[Os (dm bpy)_2 Cl]_2</td>
<td>-0.12</td>
<td>4.4</td>
<td>0.081</td>
<td>6.6 \times 10^3</td>
</tr>
<tr>
<td>[Os (bpy)_2 Cl]_2</td>
<td>-0.02</td>
<td>1.7</td>
<td>0.035</td>
<td>3 \times 10^3</td>
</tr>
<tr>
<td>[Os (phen)_2 Cl]_2</td>
<td>0</td>
<td>1.7</td>
<td>0.89</td>
<td>7.4 \times 10^4</td>
</tr>
<tr>
<td>[Os (dmobpy)_2 (4AEP) Cl]_PF_6</td>
<td>0.025</td>
<td>2.7</td>
<td>1.4</td>
<td>1.2 \times 10^5</td>
</tr>
<tr>
<td>[Os (dmobpy)_2 (4AMP) Cl]_PF_6</td>
<td>0.060</td>
<td>1.1</td>
<td>1.12</td>
<td>9.3 \times 10^4</td>
</tr>
<tr>
<td>TMPD</td>
<td>0.065</td>
<td>3.4</td>
<td>0.21</td>
<td>1.8 \times 10^4</td>
</tr>
<tr>
<td>[Os (dmbpy)_2 (4AMP) Cl]_PF_6</td>
<td>0.19</td>
<td>1.8</td>
<td>2.64</td>
<td>2.2 \times 10^5</td>
</tr>
<tr>
<td>[Os ([bpy]_2 (API) Cl]_PF6</td>
<td>0.2</td>
<td>1.7</td>
<td>2.41</td>
<td>2 \times 10^5</td>
</tr>
<tr>
<td>[Os (bpy)_2 (4AMP) Cl]_PF_6</td>
<td>0.24</td>
<td>6.9</td>
<td>2.23</td>
<td>1.9 \times 10^5</td>
</tr>
<tr>
<td>[Os (bpy)_2 (4AEP) Cl]_PF_6</td>
<td>0.26</td>
<td>1.1</td>
<td>28.58</td>
<td>2.4 \times 10^6</td>
</tr>
<tr>
<td>[Os (del bpy)_2 (4 AMP) Cl]_PF_6</td>
<td>0.43</td>
<td>1.1</td>
<td>12.73</td>
<td>1.1 \times 10^6</td>
</tr>
<tr>
<td>Ferrocene dicarboxylicacid</td>
<td>0.43</td>
<td>6.2</td>
<td>0.077</td>
<td>6.4 \times 10^3</td>
</tr>
</tbody>
</table>

2.3.6 Enzyme-mediator kinetics

Generally, k_{med} values decrease as the mediator redox potential (E°') shifts to more negative potentials, and the reaction becomes less thermodynamically favourable. In the case of osmium complexes with low potentials: [Os(dmbpy)_2Cl]_2 and
[Os(bpy)$_2$Cl$_2$], low catalytic currents resulted even at relatively high enzyme concentrations. No detectable catalytic current was observed using [Os(dmbpy)$_2$Cl$_2$] as a mediator. This complex displayed a redox potential $E^\circ' = -0.24$ V vs. Ag/AgCl, close to the redox potential of GOx. Nakabayashi et al. have reported low second order rate constants of $7.86 \times 10^3$ M$^{-1}$ s$^{-1}$ for GOx mediated oxidation of glucose by the osmium complex [Os(dmbpy)$_2$(1-methylimidazole)Cl] with redox potential $E^\circ' = 0.002$ V vs. Ag/AgCl (Nakabayashi et al., 2001) which is also close to that of the GOx active site $E^\circ' = -0.35$ V vs. Ag/AgCl (Gregg and Heller, 1991a, Gregg and Heller, 1991b). When the selected mediator displays increasingly more positive redox potentials ($E^\circ'$) the reaction becomes more thermodynamically favourable and therefore an increase in $k_{med}$ values were generally observed. Flexer et al. reported a rate constant of $2.1 \times 10^5$ M$^{-1}$ s$^{-1}$ for [Os(bpy)$_2$Cl(pyridine carboxylic acid)]$^+$ with a redox potential of $E^\circ' = 0.23$ V vs. Ag/AgCl (Flexer et al., 2008). Similarly, in the present work [Os(bpy)$_2$(4AMP)Cl] PF$_6$ that displayed a redox potential of 0.24 V vs. Ag/AgCl showed a rate constant of $1.9 \times 10^5$ M$^{-1}$ s$^{-1}$. Nakabayashi et al. have shown that their osmium complexes exhibited $k_{med}$ values of $10^6$ M$^{-1}$ s$^{-1}$ for those complexes that display redox potentials around $E^\circ' = 0.2$ to 0.4 V vs. Ag/AgCl (Nakabayashi et al., 2003). Our osmium complexes, particularly [Os(bpy)$_2$(4AEP)Cl]PF$_6$ ($E^\circ' = +0.26$ V vs. Ag/AgCl) and [Os(dcl bpy)$_2$(4AMP)Cl]PF$_6$ ($E^\circ' = +0.43$ V vs. Ag/AgCl) showed second order rate constants in the order of $10^6$ M$^{-1}$ s$^{-1}$.

From the overall observations, it is not always the case that an increase in driving force leads to a relatively higher rate constant for electron transfer mediation. The affinity of the interaction between the mediator and GOx may also be important for efficient mediation (Hayashi and Nakamura, 1981). For example, ferrocenedicarboxylic acid showed a relatively low $k_{med}$ value compared to that obtained for the [Os(dclbp)_2(4AMP)Cl] PF$_6$ complex, even though both displayed the same redox potential. In a similar trend, a second order rate constant of $2.84 \times 10^4$ M$^{-1}$ s$^{-1}$ was reported for glucose oxidation by GOX mediated by ferrocene monocarboxylic acid with a redox potential of $E^\circ' = 0.3$ V vs. Ag/AgCl (Nakabayashi et al., 2001).

Estimation of redox potentials and enzyme mediation kinetics allows screening of mediators for glucose oxidation by GOx that may then be applied in a glucose/O$_2$ EFC. In order to investigate overpotential effects on EFC performance, solution phase EFCs were assembled using mediators selected based on redox potential and kinetics.
2.3.7 Solution phase EFC

Fig.2.10 shows a schematic of the solution phase EFC assembly used in this approach. The fuel is enzymatically oxidized at the anode, producing protons and electrons. At the cathode, the oxidant (oxygen) reacts with electrons and protons, generating water. Anode and cathode are separated by a proton exchange membrane that is permeable to protons. Mediated solution phase EFCs were assembled in order to compare the competing influences of mediator redox potential and current generation capabilities in the production of power and cell voltages. To this effect two of the mediators that showed promise for application as GOx mediators, as they generated glucose oxidation current, yet displayed redox potentials that are not too positive were selected from the library: \([\text{Os (dmbpy)}_2(4\text{AEP})\text{Cl}]\text{PF}_6\) and TMPD. These were coupled to similar cathodes, via a Nafion membrane.

![Figure 2.10 Schematic model of PEM EFC.](image)

Fig.2.11 shows a photograph of the setup used in these preliminary solution phase tests of EFC, with the EFC connected to a digital multimeter across a resistance load. Two individual fuel cell trials were performed using \([\text{Os (dmbpy)}_2(4\text{AEP})\text{Cl}]\text{PF}_6\) with redox potential \(E^0 = +0.025\ \text{V vs. Ag/AgCl}\) (FC 1) and TMPD with redox potential \(E^0 = +0.065\ \text{V vs. Ag/AgCl}\) (FC 2) as a mediator of glucose oxidation by GOx in the anolyte. A catholyte mediator of \([\text{Os (dclbpy)}_2(4\text{AMP})\text{Cl}]\text{PF}_6\) with redox potential \(E^0 = +0.43\ \text{V vs. Ag/AgCl}\) close to the fungal Trametes versicolor laccase redox potential \(E^0 = +0.585\ \text{V vs. Ag/AgCl}\) (Stankovitch et al., 1978, Solomon et al., 1996), with laccase in solution, was the same for both types of EFC. An electron transfer rate constant for oxygen reduction (pseudo-first order
rate constant) by laccase (5 U/ml) of $3.6 \text{ s}^{-1}$ was estimated, using the same approach as that adopted for the GOx measurements, for the selected catholyte mediator.

**Figure 2.11** Photograph of assembled mediated solution phase EFC connected to digital multimeter across a resistance load.

The assembled solution phase EFC was operated at room temperature $20 \pm 1^\circ \text{C}$ with each chamber containing 10 ml of electrolyte solution. A mediator concentration in each electrolyte of 0.2 mM was selected for the investigations, with GOx 12 µM and 0.1 M glucose in potassium phosphate buffer (pH 7.4) as anolyte and laccase from *Trametes versicolor* (230 U/ml) in sodium citrate buffer pH 4.5 containing dissolved oxygen as catholyte. **Fig. 2.12** shows slow scan rate CVs of the two half-cells in the fuel cells in the presence (a) and absence (b) of enzymes. The cathodic current densities were almost similar for both types of fuel cell, displaying catalytic current densities of 6.5 µA cm$^{-2}$ for oxygen reduction for FC 2, and slightly higher current densities of 7.5 µA cm$^{-2}$ for FC 1. Glucose oxidation current densities of 20 µA cm$^{-2}$ and 29 µA cm$^{-2}$ were obtained for the anodes in FC 1 and FC 2, respectively. The estimated rate constants for glucose oxidation for the selected mediators, given in table 2.1 are $1.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for [Os (dmobpy)$_2$(4AEP)Cl]PF$_6$ and $1.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for TMPD. Though the complex [Os (dmobpy)$_2$(4AEP)Cl]PF$_6$ showed higher second order rate constants, catalytic currents are higher for TMPD. This is due to the larger signals of the TMPD in absence of GOx when compared to the [Os (dmobpy)$_2$(4AEP)Cl]PF$_6$ as shown in the **Fig. 2.12(b)**. Hence it is recommended that the comparisons of absolute catalytic currents are more useful than the second order rate constants in an EFC.

a)
Figure 2.12 CVs of anode with of [Os (dmobpy)$_2$(4AEP)Cl)]PF$_6$FC 1(black line) and TMPDFC 2 (blue line) and cathode [Os [(dcl bpy)$_2$(4AMP)Cl)]PF$_6$ (same for both fuel cell). a) Presence and b) absence of enzymes.
Figure 2.13 Performance of the FC 1 a) showing the overall cell potential (circle) and the current density vs power density (triangle) during polarization b) stability of the FC 1 (Triangle – 0h, square – 1 h, and circle – 24h)
Figure 2.14  Performance of the FC 2 a) showing the overall cell potential (circle) and the current density vs power density (triangle) during polarization b) stability of the FC 2 (Triangle – 0h, square – 1 h, and circle – 24h)
When anode and cathode were assembled in the fuel cells open circuit voltages (OCV) of 0.6 V for FC 1 and 0.55 V for FC 2 were observed, with the decrease in OCV from FC 1 to FC 2 reflecting the more positive redox potential (higher overpotential for glucose oxidation) for TMPD compared to the selected osmium complex. Polarization studies, Fig. 2.13a (FC 1) and 2.14a (FC 2), were performed by switching resistance loads between anode and cathode, starting at high resistance and decreasing gradually the load. Such studies yield a maximum power density of 2.1 µWcm⁻² at 0.3 V, and a current density of 7 µAcm⁻² at maximum power density for FC 1. This compares to a power density of 1.8 µWcm⁻² at 0.35 V, with current density of 5 µAcm⁻² at maximum power density for FC 2.

![Figure 2.15 Cell behaviors during polarization for FC 2.](image)

The polarization studies Fig. 2.14 and the CVs Fig. 2.12a show that the cathode limits the current (mass transport, referred to as concentration polarization) in a fuel cell. For example in Fig. 2.14 the anode potential remained constant as a function of current densities, for current densities below 7.5 µA cm⁻², while the cathode potential dropped for current densities over 5 µA cm⁻². It is evident from the cell behavior studies that the cathode has been limited by oxygen mass transfer. Cathode is limiting in both, with a slightly higher cathodic current density for FC 1 compared to FC 2. This prevented us to compare the performances of FC 1 and 2 apart from OCV. The overpotential loss between the GOx and [Os(dmobpy)₂(4AEP)Cl]PF₆ is ~ 0.385 V for FC 1 and with GOx and TMPD is ~ 0.425 V for FC 2. At cathode, overpotential loss between the lacccase and [Os([(dclbp)₂(4AMP)Cl])PF₆]
is 0.13 V for FC 1 and 2 respectively, as both the FC uses the same cathode mediator. In order to maximize the cell voltage, it is important to minimize the overpotential at both the anode and the cathode, with a focus on minimizing anode overpotential as this contributes most to cell overall voltages in the EFC. The stability of the EFCs was monitored for 24h. Power density decreased by 52 % and 55 % for FC 1 (Fig. 2.13b) and FC 2 (Fig. 2.14b) respectively after 1h. At 24 h both EFCs had almost lost their power densities. It is assumed that the oxygen mass transport limitation at cathode could have contributed for the power loss in EFCs shown here.

Generally the measured OCV for these EFCs is higher than the estimated difference in redox potential of cathode and anode mediators, which is around 0.4 V for both fuel cell types. Such a difference in OCV could be attributed to the direct electron transfer (DET) occurring from the electrode to laccase, postulated to be responsible for the high cathode potential at low current densities in individual cell behavior studies during polarization (shown as an example for FC 2) Fig. 2.15 (Mano et al., 2003).

The power output is comparatively less when compared to previous reports on assembled EFC with enzyme and mediator immobilized over carbon electrodes (Barrière et al., 2006, Kavanagh et al., 2009). The power output of the solution phase EFC compares favorably to a power density of 5 µWcm⁻² for the glucose / O₂ EFC assembled by Katz et al. which consisted of GOx and cytochrome c / cytochrome oxidase on gold electrodes (Katz et al., 1999). Liu et al. assembled a glucose/O₂ EFC by loading glucose oxidase or fungal laccase within a porous carbon matrix. A power density of 2 µWcm⁻² at pH 7.0 was produced (Liu et al., 2005).

Several factors influence the power output of a fuel cell. For example, Kim et al. reported a fivefold loss in power density of their miniaturized EFC operated at 23 °C when compared to EFCs operated at 37°C (Kim et al., 2003). It is thus expected that our solution phase EFC operated at 20 ± 1°C could be improved upon by operation at higher temperatures. In addition to the temperature factor, non-stirring conditions of the homogenous reactions in a solution phase EFC, and the low solubility of oxygen in solutions obviously result in mass transfer limitations.

2.4 Conclusions

Redox mediators were electrochemically characterized using cyclic voltammetry and their propensity for interaction with GOx in the mediated oxidation of glucose studied. Thermodynamically, kinetics of redox mediators increased as the difference between the
redox potential of GOX and mediators increased. The [Os(bpy)$_2$(4AEP)Cl] PF$_6$ mediator showed efficient electron transfer with a high rate constant, although at high driving force (redox potential). As it is important, for selection of mediators for application to EFC electrodes, to achieve a compromise between current generating capability and cell voltage output, mediators with redox potentials closer to that of the FAD/FADH$_2$ couple in the active site of GOX redox should be selected for EFC anodes. In that case, the [Os(dmobpy)$_2$(4AEP)Cl] PF$_6$ mediator, which displayed Os(II)/(III) redox potentials $\sim E^0 = +0.025$ V vs. Ag/AgCl, was selected for the anode compartment of an EFC. Possible electron transfer mediators for glucose cannot be evaluated by the redox potential alone; but reactivity of the mediator and GOX is also considered to be an important factor for mediated catalysis. Solution phase EFC was assembled to demonstrate the influence over difference in redox potential of the mediators and kinetic parameters on EFC output. In conclusion, mediator kinetics with a wide range of redox potentials allowed for the selection of an appropriate mediator for construction of prototype fuel cell. Limiting factors for OCV, cell voltages, power and current densities, and stability were identified during EFC operation. For implantable EFCs it is preferable to have all the components immobilized at the electrodes surfaces. Immobilization of enzyme and mediator over electrode surfaces for the operation of membrane-less EFC will be shown in the following chapters.
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3. Redox polymer and enzyme co-immobilization on carbon electrodes to provide membrane-less glucose/O\textsubscript{2} enzymatic fuel cells

3.1 Introduction

An enzymatic fuel cell uses enzymes, in place of traditional metal catalysts such as platinum, to convert chemical energy to electrical energy. Because of enzyme diversity, fuels such as hydrogen, alcohols and sugars, with oxygen as oxidant, can be employed in research-based EFC prototypes (Palmore et al., 1998, Palmore et al., 1999, Heller, 2004, Barrière et al., 2006). Of these, the glucose/O\textsubscript{2} EFC has received most attention due to the relatively high concentration of glucose in blood (5 – 8 mM), leading to potential application of EFCs to *in vivo* power production for low energy (~10 µW) demanding biomedical devices (Heller, 2004). In chapter 2 osmium complexes with general formula cis-[Os(N-N)\textsubscript{2}Cl\textsubscript{2}] (where N-N = polypyridyl ligands) were evaluated on the basis of their rate of reaction in solution between enzyme and electrode for application towards EFC. Appropriate osmium complexes were selected for anode and cathode to form solution phase EFC with a membrane separator to study its performance. However, immobilization of EFC catalytic components at the electrode surface allows miniaturization, and has led to development of µm dimension EFCs (Mano et al., 2003). In immobilized EFCs leaching of enzyme and/or mediator from the electrode surface can occur, leading to short term instability of bioelectrocatalytic films (Gregg and Heller, 1991a, Gregg and Heller, 1991b, Boland et al., 2009a). Although an EFC can theoretically meet the power demands of some biomedical devices, stability of power output of EFCs remains an issue. For example, an implanted EFC in a rat, using enzymes and mediators contained in solution, generated intermittent power for 10 days (Cinquin et al., 2010). In that report EFC enzymes and mediators were encased within membranes to prevent reactant crossover and catalyst leakage. A realistic goal may be the development of miniature semi-implantable glucose/O\textsubscript{2} systems to provide power for the lifetime of an implanted glucose sensor (typically <1 week) and be discarded after their first and only use, thereby eliminating the need for longer term stability (Chen et al., 2001, Kim et al., 2003).

Redox hydrogels can be formed on carbon electrodes by crosslinking enzyme and osmium redox polymers using a using a diepoxide crosslinker that swells when hydrated. Electron conduction is controlled by collisional electron transfer (electron hopping mechanism) between the reduced and oxidized transition metal based redox centres tethered to the polymer backbone. In order to assist immobilization, osmium complexes were complexed, via ligand substitution of one of the chlorines, to polymeric backbones of PVI.
Redox potentials of osmium based metallopolymers are largely governed by the nature of their coordinating ligands. In general, the greater the binding energy of the ligands in the complex, the more positive the redox potential exhibited. For example, in chapter 2, redox potentials of the [Os(dmobpy)\(_2\)Cl\(_2\)] which is \(-0.24\) V vs. Ag/AgCl shifts positively by around 0.2 V when a single chlorine ligand is substituted with PVI. This osmium redox polymer potential is close to the redox potential of the FAD/FADH\(_2\) active site in glucose oxidase (GOx) and may prove useful as an anode in an EFC based on glucose oxidation (Kim et al., 2003).

In recent years, progress in development of prototype EFCs capable of meeting power and operational lifetime requirements for such applications has been made (Heller, 2004, Mano et al., 2003, Minteer et al., 2007), although comparison of power output and performance between reports is rendered difficult due to different electrode materials, modifications and approaches used. The Leech group EFC research initially focused on developing oxygen-reducing cathodes based on co-adsorption of multi-copper oxygenases and redox polymer mediators on glassy carbon and graphite disk electrodes, enabling comparison of half-cell performance (Barrière et al., 2004 & 2006, Kavanagh et al., 2008 & 2009, Jenkins et al., 2009). Combining cathodes with glucose oxidizing GOx anodes yielded EFCs producing power densities of 16 \(\mu\)Wcm\(^{-2}\) (Barrière et al., 2006) and 17 \(\mu\)Wcm\(^{-2}\) (Kavanagh et al., 2009) under pseudo-physiological conditions using a *Trametes versicolor* or *Melanocarpus albomyces* laccase cathode, respectively.

In the present investigation, we compare *Myrothecium verrucaria* bilirubin oxidase (MvBOD) and *Trametes hirsuta* laccase (ThLacc), co-immobilized with a common [Os(4,4\(^{\prime}\)-dichloro-2,2\(^{\prime}\)-bipyridine)\(_2\)(polyvinylimidazole)\(_{10}\)Cl\(_2\)]\(^+\) redox polymer (\(E^\circ = +0.35\) V vs. Ag/AgCl, Med\(_2\)), as O\(_2\)-reducing cathodes. We combine such cathodes with glucose oxidizing GOx anodes, mediated by [Os(4,4\(^{\prime}\)-dimethoxy-2,2\(^{\prime}\)-bipyridine)\(_2\)(polyvinylimidazole)\(_{10}\)Cl\(_2\)]\(^+\), (\(E^\circ = -0.05\) V vs. Ag/AgCl, Med\(_1\)), and compare power output for these membrane-less EFCs under pseudo-physiological conditions assembled on glassy carbon (GC) and graphite disk electrodes. Recent efforts have focused on increasing the stability of the biocatalytic films through coupling to surface modified electrodes. For example, diazonium salt chemistry can introduce functional groups to carbon electrode surfaces for covalently anchoring of enzymes (Pellissier et al., 2008), mediator (Boland et al., 2008a & 2009a) or co-immobilized redox polymer and enzyme (Boland et al., 2008b & 2009b, Jenkins et al., 2009) or DNA (Hajdukiewicz et al., 2010). In this report we thus compare covalently anchored enzyme and
redox polymer films on surface derivatized graphite electrodes to those prepared with underivatized graphite, to evaluate EFC performance with respect to stability.

3.2 Experimental Section

3.2.1 Materials

Synthesis of the redox polymers was achieved by others in our laboratory by adapting literature procedures (Kober et al., 1988, Forster and Vos, 1990), using (NH₄)₂OsCl₆ (Aldrich) as starting material to prepare the cis-Os(4,4'-dimethoxy-2,2'-bipyridine)₂Cl₂ and cis-Os(4,4'-dichloro-2,2'-bipyridine)₂Cl₂ complexes, which were then complexed, via ligand substitution reaction in ethanol/water solvent, to a previously pre-synthesized polyvinylimidazole (PVI) polymer. Poly(ethylene glycol)diglycidyl ether (average Mn ~ 526) was purchased from Sigma - Aldrich. GOx and MvBOD were obtained from Sigma – Aldrich and ThLacc was donated by VTT Technology, Finland (K. Kruus). Unless otherwise stated all other chemicals were obtained from Sigma- Aldrich. All buffers were prepared from solutions of the selected base then adjusted to the desired pH using solutions of the acid.

3.2.2 Apparatus

Teflon-shrouded GC electrodes (3 mm diameter, IJ Cambria), and graphite disc electrodes (3 mm diameter), formed by shrouding graphite rods (Goodfellow) in glass tubes using heat-shrinkable tubing and establishing an electrical connection to copper rods (Farnell) at the rear with silver epoxy resin (Farnell), were used as working electrodes. Cyclic voltammetry was carried out with a CHI 650 potentiostat, using a GC or graphite electrode, Ag/AgCl and platinum wire as working, reference and counter electrodes, respectively (IJ Cambria). Membrane-less EFCs were assembled by insertion of anode and cathode into a compartment-less electrochemical cell containing 5 mL of electrolyte solution. The anode and cathode were externally connected through a resistance box (IET Labs) over a resistance range of 5 MΩ to 1 kΩ, and the voltage between the electrodes measured with a multimeter (Keithley) for each load.

3.2.3 Preparation and modification of electrodes

Electrodes were prepared, based on previously reported ratios of redox polymer to enzyme, (Boland et al., 2009b, Kavanagh et al., 2009) by depositing a drop (12 µL) containing the enzyme (6 µL of either GOx of 10 mg/ml (1500 U/ml), ThLacc 390 U/ml or
MvBOD 95 U/mL), the redox polymer (3 µL of a 8–10 mg/ml solution/suspension in water) and poly(ethylene glycol) diglycidyl ether (3 µL of a 15 mg/ml solution in water) as a crosslinker onto 3 mm diameter GC or graphite electrodes, followed by at least 24 h drying of the film. Unless otherwise stated, current and power densities were measured at 37 °C in buffered solutions containing 0.15 M NaCl, 0.1 M glucose and saturated O₂. Modification of the graphite electrodes by introduction of amine functional groups was achieved by electrochemical reduction of the diazonium cation generated in situ from p-phenylenediamine (Baranton and Bélanger, 2005, Boland et al., 2009b). Briefly, 8 mM of NaNO₂ was added into a 10 mM acidic solution (HCl) of p-phenylenediamine to generate in situ the diazonium cation. The solution was kept in complete darkness and in an ice bath (approx. 4 °C) and allowed to react for 5 min under argon and stirring. Surface derivatization was carried out by electrochemical reduction, in the diazonium cation-generating solution, by scanning from 0.4 V to −0.4 V vs. Ag/AgCl at 20 mV s⁻¹ for four cycles. The resulting modified electrodes were removed and rinsed with acetonitrile and then water, followed by ultrasonication for 1 min to remove any loosely bound species. Electrodes were then rinsed again with copious amounts of water and dried under a stream of argon.

3.3 Results and Discussion

3.3.1 Enzymatic fuel cell design

A simplified model of the enzymatic fuel cell design is shown in Fig. 3.1A. At the anode, glucose is oxidized by glucose oxidase (GOx) to yield gluconolactone with an osmium redox polymer, Med₁, to shuttle electrons from the otherwise insulated FAD/FADH₂ active site to the electrode surface. The electrons then pass through the circuit where they are transferred, via Med₂, to the multicopper oxygenase active site (ThLacc or MvBOD) where they are recombined with protons in the presence of O₂ to form H₂O. In order for current to flow a potential difference (ΔE) must be established between the anode and cathode, primarily influenced by the difference in redox potential of anode and cathode electron transfer mediators, Med₁ and Med₂. Modification of 2,2′-bipyridine (bpy) ligands of a redox polymer [Os(bpy)₂(polyvinylimidazole)₁₀Cl]⁺ (Forster et al., 1990) with electron donating/withdrawing groups in the 4,4′-positions allows manipulation of the redox potential of the Os(II/III) redox transition in the 4,4′-positions allows manipulation of the redox potential of the Os(II/III) redox transition to address electron transfer to/from enzymes. The redox polymers Med₁ and Med₂ (Fig.3.1B) are selected to facilitate thermodynamically favourable transfer of electrons from the GOx active site (E° = −0.35 V vs. Ag/AgCl) (Swoboda and
Massey, 1995, Swoboda and Massey, 1996, Gregg and Heller, 1991a, Gregg and Heller, 1991b, Heller et al., 1992) to the T1 Cu of multicopper oxygenases ($E^\circ = +0.57$ V $\text{vs.} \, \text{Ag/AgCl}$ for $\text{ThLacc}$ (Shleev et al., 2005) +0.4 V $\text{vs.} \, \text{Ag/AgCl}$ for $\text{MvBOD}$ (Xu et al., 1996) whilst maintaining a potential difference between the anode and cathode. The redox polymer, Med$_1$, has been previously reported on as a mediator for oxidation of glucose by GOx (Taylor et al., 1995) and, more recently, by pyranose dehydrogenase (Zafer et al., 2010). It is selected as mediator at the anode, as its redox potential is 0.3 V more positive than that of the bound FAD cofactor providing a favourable thermodynamic driving force for electron transfer from the GOx active site (see chapter 2).

Figure 3.1 (A) Simplified schematic of glucose/oxygen EFC. GOx is used at the anode and either MvBOD or ThLacc at the cathode. Med$_1$ and Med$_2$ indicate anodic and cathodic mediators respectively. Enzyme active sites are represented by diamonds (♦) while osmium redox sites are represented by circles (●). (B) Structure proposed for the osmium redox polymers Med$_1$ and Med$_2$: $\text{[Os}(4,4\text{-XX}^\prime-2,2\text{-bipyridine})_2\text{(polyvinylimidazole)}_{10}\text{Cl}]^+}$ (X = OCH$_3$ (Med$_1$) or Cl (Med$_2$)).
We initially compare mediated ThLacc and MvBOD cathode performance in a membrane-less EFC assembly using the common GOx/Med$_1$-based bioanode. Cyclic voltammetry (CV) is used to evaluate bioelectrocatalytic current densities for glucose oxidation by anodes and oxygen reduction by cathodes. To simplify this initial comparison we elect to first use relatively smooth GC electrodes as a base for assembly of the bioelectrocatalytic film and report current and power densities with respect to the two-dimensional geometric area of the base GC disk.

### 3.3.2 Redox polymer/enzyme films on glassy carbon electrodes

The redox polymer, Med$_2$, has been used as a mediator for oxygen reduction by ThLacc (Jenkins et al., 2009) and provides a redox potential ~0.2 V more negative than that reported for the T1 Cu of ThLacc (Shleev et al., 2005). The optimum activity of GOx for glucose oxidation is at pH ~ 6 (Swoboda and Massey, 1995, Swoboda and Massey, 1996) while that of ThLacc for oxygen reduction is at pH ~ 4.5 (Shleev et al., 2005). Slow-scan CV responses of films of GOx/Med$_1$ and ThLacc/Med$_2$ on glassy carbon electrodes in the presence of 0.1 M glucose at pH of 4.5, 5.5 and 7.4 are presented in Fig.3.2. A steady-state current density of 50 µA cm$^{-2}$ is observed at pH 7.4 for glucose oxidation at GOx/Med$_1$ anodes in quiescent solutions. This compares well with a reported estimated current density of ~0.2 mA cm$^{-2}$ for oxidation of 32 mM glucose by films of GOx/Med$_1$ adsorbed on GC electrodes and rotated at 1000 rpm (Taylor et al., 1995). However, consistent with previous studies using GOx co-immobilized in redox hydrogels (Ohara et al., 1993, Pekel et al., 2003), a marked decrease in steady-state current density for glucose oxidation is evident for CVs recorded at lower pH values, attaining only 15 µA cm$^{-2}$ at pH 4.5. In contrast to the anode and in agreement with previous reports on fungal laccase activity (Jenkins et al., 2009, Barrière et al., 2004, Xu, 1997) the highest steady-state current density of 140 µA cm$^{-2}$ for oxygen reduction by the laccase-based cathode is observed at pH 4.5, with a marked decrease in current density obtained as pH approaches physiological conditions. This trend is unsurprising since ThLacc shares a $> 90\%$ sequence similarity to TvLacc, for which a similar trend with redox polymer mediation of oxygen reduction has been reported (Barrière et al., 2004) and, like the TvLacc laccase, is inhibited by the hydroxyl ion (Xu, 1997).

Power densities were recorded in a single membrane-less cell at pH of 4.5, 5.5 and 7.4 for assembled membrane-less EFC based on the GOx/Med$_1$ and ThLacc/Med$_2$ glassy carbon electrode anode and cathode. A maximum power density of 3.5 µW cm$^{-2}$ at 0.35 V is observed at pH 5.5, as this pH offers the best compromise between the optimal conditions for
anode and cathode current generation. At pH 4.5 and pH 7.4, power density is severely diminished by poor currents at the anode and cathode respectively, yielding only 36% and 68% of the maximum power density observed at pH 5.5.

**Figure 3.2** Cyclic voltammograms of GOx mediated GC anode and Thlacc mediated GC cathode in membrane-less EFC in the absence of glucose and oxygen at pH 7.4 (dashed line) and in the presence of 0.1 M glucose and saturated O₂ at pH 4.5 (dark black line), pH 5.5 (grey line) and pH 7.4 (black line), scan rate 5 mV s⁻¹. Cell conditions: 0.1 M phosphate buffer (pH’s 5.5 and 7.4) or citrate buffer (pH 4.5), 37 °C, 0.15 M NaCl.

As it may be desirable for an EFC to operate in physiological environments, for application to powering implantable systems, cathodes in a second EFC prototype were assembled using *Mv*BOD for oxygen reduction as this enzyme is reported to have optimal activity close to neutral pH (Xu *et al.*, 1996). When co-immobilized with Med₂ on GC electrodes, the *Mv*BOD cathode produced a steady-state current density of 35 µA cm⁻² in the presence of saturated O₂ under pseudo-physiological conditions, **Fig.3.3a**. When this cathode is coupled, in a membrane-less configuration, to the glucose oxidizing GOx/Med₁ anode, a maximum power density of 10 µW cm⁻² at a cell voltage of 0.25 V is obtained at pH 7.4, **Fig.3.3 b**. As expected based on the slow scan CVs, the maximum power density represents
an approximately five-fold increase over that obtained for the EFC based on the \( ThLacc/Med_2 \) cathode under similar conditions. However, maximum power was attained at a cell voltage that is 0.1 V less than that observed for the EFC based on the \( ThLacc/Med_2 \) cathode, presumably because the T1 Cu site for \( MvBOD \) has a redox potential ~0.17 V less positive than that reported for the T1 Cu site in \( ThLacc \) (Shleev et al., 2005). In any event, because of superior power density under pseudo-physiological conditions at pH 7.4, the \( MvBOD/Med_2 \) cathode was selected for further investigation, to probe the influence of surface roughness and anchoring chemistry on current/power generation and stability.

**Figure 3.3** a) Cyclic voltammograms of \( MvBOD \) mediated GC cathode in an EFC in the absence (dashed line) and presence (solid line) of saturated \( O_2 \), scan rate 5 mV s\(^{-1}\). b) Power curves of EFC at different pH. Cell conditions: 0.1 M glucose, saturated \( O_2 \), 37 °C, 0.15 M NaCl.
### 3.3.3 Redox polymer/enzyme films on graphite electrodes

One of the methodologies to improve power outputs of EFCs is to increase the available surface area of electrodes. Many approaches have been taken to achieve this, including utilization of carbon fiber (Mano et al., 2002), carbon nanotube (Gao et al., 2010), porous carbon (Liu et al., 2005), graphene (Liu et al., 2010) and mesoporous gold (Deng et al., 2008) electrodes. A simple method to probe the surface roughness effect on EFC performance is use of graphite in place of glassy carbon as base electrode material. For example, a typical surface roughness factor for graphite is around 5 (Jaegfeldt et al., 1983), higher than the typical roughness factor of 1.6 reported for GC electrodes (Weigel et al., 2007). Liu et al. (2005) have shown that utilization of porous carbon, instead of glassy carbon, electrodes as support for adsorbed GOx or fungal laccase can provide for an approximately ten-fold increase in the short-circuit current density in a Nafion-separated enzymatic fuel using solution phase mediators. The results obtained using slow scan CVs of graphite electrodes modified with films of GOx/Med₁ for the anode and MvBOD/Med₂ for the cathode show that a 10-fold and 5-fold increase in steady-state glucose oxidation (~0.5 mA cm⁻²) and oxygen reduction (~0.2 mA cm⁻²) current densities, respectively, are achieved when compared to films on glassy carbon (Fig.3.4). Confirmation of a relative surface roughness of 5 for the graphite electrodes over that for the GC electrodes can be estimated by comparing the capacitive currents of the CVs in Fig.3.4, in agreement with observations elsewhere (Jaegfeldt et al., 1983, Weigel et al., 2007). An oxygen reduction current density of 5 mA cm⁻² for MvBOD/Med₂ films adsorbed to carbon cloth was observed when the cathode was rotated at 1000 rpm under physiological conditions (Mano et al., 2002). This compares to current densities of 0.7 mA cm⁻² for similar films adsorbed to carbon fiber microelectrodes in quiescent solutions (Kim et al., 2003). It should be noted that current densities are quoted here, and elsewhere in the biofuel cell literature, with respect to the projected two-dimensional geometric area of the base graphite disk and as such, do not represent specific current densities.
Figure 3.4 Cyclic voltammograms of GOx mediated anode (left) and MBOD mediated cathode (right) on GC (solid line) and graphite (dashed line), scan rate 5 mV s\(^{-1}\), 0.1 M phosphate buffer pH 7.4, 0.1 M glucose, saturated O\(_2\), 37.5 °C, 0.15 M NaCl. Inset: maximum power density of EFC using i) GC and ii) graphite electrodes.

An estimate of osmium surface coverage (\(\Gamma_{\text{Os}}\)) for each film can be evaluated by integrating the charge passed (Q) where the film is comprehensively electrolysed. An osmium surface coverage of \(1.2 \times 10^{-9}\) mol cm\(^{-2}\) is obtained for both the anode and cathode films adsorbed on GC electrodes. This compares to \(\Gamma_{\text{Os}}\) of \(2.2 \times 10^{-8}\) mol cm\(^{-2}\) and \(1.5 \times 10^{-8}\) mol cm\(^{-2}\) for anode and cathode films adsorbed on graphite electrodes, respectively. Thus, although the same amount of Os redox polymer and enzyme are deposited on electrode surfaces the \(\Gamma_{\text{Os}}\) increases by approximately one order of magnitude at graphite electrodes over that for GC electrodes. It is feasible that the relative roughness of the graphite electrode, compared to the smoother GC, provides for improved retention of films on the rougher surface, a factor that has been reported on previously (Boland et al., 2009b). This propensity of graphite electrodes for retention of an increased amount of redox polymer at the surface may be a significant contributor to the increased currents observed for glucose oxidation and
oxygen reduction for films adsorbed on graphite over those adsorbed on GC, a point that merits further consideration.

EFCs were assembled using GOx/Med₁ and MvBOD/Med₂ biocatalytic films adsorbed to graphite electrodes and placed in 0.1 M potassium phosphate buffer electrolyte of pH 7.4 containing 0.1 M glucose at 37 °C. To extract information on power output, fuel cell polarization was performed under a variable resistance load periodically, with the electrodes stored in the cell at 37 °C between measurements. An initial maximum power density of 43 µW cm⁻² at 0.25 V was recorded for this EFC. This observed value is similar in magnitude to the 50 µW cm⁻² at 0.5 V power output (Kim et al., 2003) obtained for an EFC based on osmium redox polymers co-immobilized with the same enzymes as reported here under pseudo-physiological conditions, but with the films adsorbed on carbon cloth electrodes. In that case, power output was limited by the kinetics of anode electrocatalysis, as a glucose concentration of 15 mM was used. In contrast, for the present EFC, power output is limited by cathode electrocatalysis as oxygen reduction current density is half of that observed at the anode for glucose (0.1 M) oxidation, under these conditions.

Stability of maximum power output from the graphite-based EFC was evaluated by intermittent polarization, with results shown as a function of time in Fig. 3.6A. The EFC based on simple drop-coating, and film adsorption at underivatized graphite electrodes lost 90% of the maximum power output after 24 h and the remaining 10% after 48 h. Leaching of biocatalyst and redox polymer from films adsorbed at underivatized graphite electrode has been reported as a major factor in loss in bioelectrocatalytic signal for such films (Boland et al., 2009b, Jenkins et al., 2009). In order to investigate whether an improvement in stability of EFC power output could be obtained, graphite electrodes were derivatized to introduce functional groups to provide a platform for covalent anchoring of biocatalytic films, as reported on separately for half-cells, anodes or cathodes, of EFCs (Boland et al., 2009b, Jenkins et al., 2009). Previous reports indicate that such surface modification improves the stability of films of enzymes (Pellissier et al., 2008), osmium-based redox complexes or polymers (Boland et al., 2008a & 2009a) or co-immobilized redox polymer and enzyme (Boland et al., 2008b & 2009b, Jenkins et al., 2009) on carbon-based electrodes. Fig. 3.5a shows the CVs for electrochemical reduction of the aryldiazonium, generated in situ from 1,4 phenylenediamine, to graphite electrodes in N₂ saturated solution containing 8 mM NaNO₂, 10 mM 1,4 phenylenediamine and 0.5 M HCl at a scan rate 20 mV/s over four cyclic scans. Such a methodology is proposed to introduce surface arylamine functional groups on the graphite electrodes. These amine-functionalized graphite electrodes were used to
covalently anchor films of GOx/Med₁ and MvBOD/Med₂ to provide for glucose-oxidizing and oxygen-reducing anodes and cathodes Fig. 3.5 b. Such an approach yields a slight decrease in osmium surface coverage, to $1.9 \times 10^{-8}$ mol cm$^{-2}$ and $1.2 \times 10^{-8}$ mol cm$^{-2}$, compared to $2.2 \times 10^{-8}$ mol cm$^{-2}$ and $1.5 \times 10^{-8}$ mol cm$^{-2}$, representing a 14 % and 20 % decrease for anode and cathode films adsorbed on graphite electrodes, respectively. Corresponding bioelectrocatalytic current densities of 0.43 mA cm$^{-2}$ and 0.15 mA cm$^{-2}$ for glucose oxidation and oxygen reduction were observed using films of GOx/Med₁ and MvBOD/Med₂, respectively, anchored to amine-functionalized graphite electrodes. These current densities represent a slight decrease of 14 % and 25%, for anode and cathode, respectively, compared to that observed for films adsorbed to underivatized graphite electrodes. The decrease in current density mirrors well the decrease in redox polymer surface coverage for the electrodes, highlighting the effect of this factor in bioelectrocatalytic signals for these systems. A comparison of the stability of the derivatized graphite-based EFC power output with time, evaluated by intermittent polarization, with that obtained for films adsorbed on underivatized graphite is shown in Fig.3.6A with full power curves at each time interval for the EFCs shown in Fig. 3.6B for underivatized and Fig. 3.6C for derivatized graphite surfaces. Whilst the initial maximum power density is largely unaffected by the derivatization procedure, the EFC operated using the derivatized graphite to anchor the films retained 70 % of the initial maximum power after 24 h, compared to a just 10 % retention for the EFC based on underivatized graphite. Further decreases to 42 % and 25 % of initial maximum power are estimated after 48 h and 72 h, respectively.
**Figure 3.5a.** CVs for electrochemical reduction of the aryl diazonium salt, generated *in situ* from 1,4 phenylenediamine, at graphite electrodes in N$_2$ saturated solution containing 8 mM NaNO$_2$, 10 mM 1,4 phenylenediamine and 0.5 M HCl. Scan rate 20 mV/s with four cyclic scans. (Figure shows four scans with reduction peak of aryldiazonium salt at first scan and disappearance of peak subsequently).

**Figure 3.5b.** Simplified schematic representation of surface modification of graphite electrode and covalent attachment of enzyme and osmium redox polymer at graphite electrode.
Figure 3.6 (A) Maximum power density over time of assembled EFCs consisting of GOx/Med$_1$ anode and $Mv$BOD/Med$_2$ cathode on underivatized (■) and derivatized graphite (▲) electrodes, and individual power density curves on underivatized (B) and derivatized graphite (C) at 0h (■), 3h (∆), 6h (Ο), 24h (●), 48h (□), 72h (▲). Other conditions as in Figure 3.4.
Figure 3.7 a) CV recorded for bilirubin oxidase (BOD) drop coated over graphite electrode showing oxygen reduction catalytic current (black) and background scan (bare electrode) (red). (10 µl of BOD was drop coated over graphite electrode and incubated for 1h and then rinsed with distilled water before CVs). CVs were recorded in potassium phosphate buffer with oxygen as substrate at pH 7.4, 0.15 M NaCl, operated at 37°C. Scan rate 5mV/s. (Insert shows the first order derivative of the DET of BOD).

It has previously been postulated that a direct electron transfer (DET) of electrons from electrode through BOD to oxygen could be an explanation for high OCVs for these EFCs, that are larger than the potential differences between the two redox polymer mediators selected for the anode and cathode. For example, OCVs ~ 0.55 V irrespective of the potential of the redox polymers, are observed for the EFCs in this chapter. We thus sought to verify if a DET current for oxygen reduction could be responsible for such a large OCV. Electrocatalysis for oxygen reduction was observed for slow-scanned CVs recorded when BOD was drop coated at a graphite electrode Fig. 3.7. The current densities for oxygen reduction observed were on the order of ~ 25 µA cm⁻² an order of magnitude less than the current densities observed in the presence of redox polymer mediators. This appears to be due to the T1 copper site oriented on the electrode sufficiently close to permit electron transfer, with electrons injected into the T2/T3 cluster of the enzyme for reduction of oxygen (Ramírez et al., 2008). Based on first order derivative of BOD DET single redox couple at + 0.46 V
vs. Ag/AgCl is estimated for the T1 copper site of BOD, in agreement with that observed by others for this enzyme (Ramírez et al., 2008).

Similar EFCs, assembled by adsorption of osmium redox polymers and enzymes to carbon fiber microelectrodes, to those reported on in this chapter have been reported to yield an initial power density of 50 µW cm⁻² that dropped to 30 µW cm⁻², 60% of initial power, after two days of continuous operation (Kim et al., 2003). In this chapter introduction of amine functional groups to the surface of a graphite electrode can provide for anchoring chemistry to improve the stability of EFC power output when compared to an EFC operated using films adsorbed to underivatized graphite electrodes. The improved stability of the EFC based on surface-modified graphite is likely due to higher retention of bioelectrocatalytic films than at unmodified graphite, as reported on previously (Boland et al., 2008b & 2009b, Jenkins et al., 2009). Nonetheless, even using the surface-modified electrodes a gradual loss in maximum power density is evident (Fig.3.6). Previous reports, using surface-modified carbon electrodes to anchor redox complexes and polymers demonstrated that an [Os(bpy)₂(4-amino-methylpyridine)Cl]PF₆ complex attached to a pre-functionalized graphite electrode lost only 16% of redox signal, observed using cyclic voltammograms, over a period of 30 days (Boland et al., 2008a). Similarly, films of [Os(bpy)₂Cl(PVI)₁₀]⁺ anchored to surface-modified graphite electrodes retained up to 90% of redox signal over a 48 h period. This can be compared to retention of only 54% or 59% of bioelectrocatalytic signal over the same period for films containing the same redox polymer, but co-immobilized with GOx or MvBOD, respectively (Boland et al., 2009b). The loss in stability of the bioelectrocatalytic signal could be attributed to several factors, but is most likely related to degradation/inhibition of enzyme activity, as reported on by others for MvBOD, with irreversible de-activation occurring when the T1 Cu is in the oxidizing state (Kang et al., 2006), and for GOx, with deactivation by competitive inhibition by hydrogen peroxide, produced when reduction of oxygen as co-substrate occurs, competing with the redox polymer mediator for electrons (Kleppe, 1966, Bao et al., 2003). Improvements in EFC stability should target approaches for stabilization of enzyme activity by, for example, selection of dehydrogenases that do not produce peroxide (Yuhashi et al., 2005, Zafar et al., 2010), genetic engineering to improve enzyme stability (Okuda-Shimazaki et al., 2008) and use of stabilizers that protect enzyme activity such as modified Nafion membranes (Moore et al., 2004) or liquid crystal films (Rowinski et al., 2007).
3.4 Conclusions

At pH 7.4, negligible oxygen reduction currents are observed for GC electrodes coated with an adsorbed film of redox polymer and Thlacc, severely impeding its application in a potential EFC operating under physiological conditions. Use of MvBOD cathode improved electrocatalysis under physiological conditions providing power output of 10 µW cm\(^{-2}\) when combined with a GOx-based anode. A four-fold increase in power output is obtained by replacement of GC with graphite electrodes. The stability of this EFC power output is improved using surface-modified graphite electrodes to anchor the bioelectrocatalytic films. Here the immobilization of biocatalyst eliminated the need for a membrane to separate anode and cathode and largely improved the power densities and stability when compared to the solution phase EFC reported in chapter 2. Use of such an approach, combined with alternate enzymes and electrode materials, provides a route to prototype EFC for application to powering implantable systems over extended lifetimes to that previously possible.
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4. A membrane-less enzymatic fuel cell with layer-by-layer assembly of redox polymer and enzyme over graphite electrodes

4.1 Introduction

As mentioned in previous chapters, enzymes can be used as catalysts in fuel cells, oxidizing the fuel at the anode and reducing the oxidant at the cathode, to convert chemical energy to electrical power (Cracknell et al., 2008, Barton et al., 2004, Davis and Higson, 2007, Kendall, 2007). In particular, research has focused on glucose/O₂ enzymatic fuel cells (EFC), utilizing glucose oxidase (GOx) for glucose oxidation at the anode and laccase for O₂ reduction at the cathode, with potential application to powering implanted or portable electronic devices (Barrière et al., 2006, Barrière et al., 2004, Yan et al., 2006, Chen et al., 2001, Mano, 2008). Harnessing enzyme electron transfer activity can be achieved by direct electron transfer (DET) between the enzyme active site, typically embedded in an insulating protein shell, and the electrode. Though feasible for some enzymes (Coman et al., 2010) this approach can limit the current and power density in EFCs mainly due to low, sub-monolayer, coverage and inappropriate orientation of enzyme at the electrode surface (Ivnitski et al., 2006). In general, mediated electron transfer (MET) to/from enzyme active sites, for example using redox polymers, permits assembly of 3-dimensional, electrically-contacted films capable of producing current and power densities several orders of magnitude higher than that of their DET type counterparts (Barton et al., 2001, Jenkins et al., 2009, Heller, 2004, Palmore et al., 1999) although use of nanostructured surfaces can help promote DET to/from enzyme active sites for EFC applications (Zebda et al., 2011). Immobilization of redox polymer and enzyme at electrodes can ensure high EFC current and power densities, whilst preventing short circuiting of current-flow (Heller, 2004, Palmore et al., 1999). Redox polymers and enzymes have been immobilized on electrodes using covalent linkage (Battaglini et al., 2000), drop-coating (Kavanagh et al., 2008), hydrogel formation (Mano et al., 2002), and layer-by-layer adsorption (Calvo et al., 2001) approaches. Alternate layer-by-layer (LBL) electrostatic adsorption of charged polymers and enzymes is a simple method to build 3D electrocatalytic structures providing spatial distribution of redox polymer and enzymes (Calvo et al., 2001) mostly at gold electrodes pre-treated with thiol-based self-assembled layers. An advantage to using graphite electrodes instead of gold is that the need for thiol modification of gold to adsorb anionic or cationic layers is eliminated. In this chapter an LBL approach for the formation of alternate layers of mediating redox polymer and enzyme is investigated for application to assembly of
bioelectrocatalytic films on carbon electrodes. These electrodes will be investigated for use in EFCs, with a view to examining whether an improvement in EFC current density can be achieved by increasing the enzyme and osmium redox polymer loading at the electrode surface in a commensurate, layer-by-layer, fashion. Such an approach may improve the power output of an assembled EFC compared to the EFCs constructed based on crosslinking films assembled by drop-coating of solutions of enzyme and redox polymers on electrodes, as described in chapter 3.

4.2 Experimental Section

4.2.1 Materials

Synthesis of the redox polymers was achieved by others in the Leech research group by adapting literature procedures (Kober et al., 1988) using (NH₄)₂OsCl₆ (Aldrich) as starting material to prepare the cis-Os(4,4'-dimethoxy-2,2'-bipyridine)₂Cl₂ and cis-Os(4,4'-dichloro-2,2'-bipyridine)₂Cl₂ complexes, which were then complexed, via ligand substitution reaction in ethanol/water solvent, to a previously pre-synthesised polyvinylimidazole (PVI) polymer (Forster and Vos et al., 1990). Glucose oxidase (GOx) from (A. Niger) was obtained from Sigma – Aldrich and ThLacc was donated by VTT Technology, Finland (K. Kruus). Unless otherwise stated all other chemicals were obtained from Sigma-Aldrich. All buffers were prepared from solutions of the selected base then adjusted to the desired pH using solutions of the acid.

4.2.2 Apparatus

Graphite disc electrodes (3 mm diameter), formed by shrouding graphite rods (Goodfellow) in glass tubes using heat-shrinkable tubing and establishing an electrical connection to copper rods (Farnell) at the rear with silver epoxy resin (Farnell), were used as working electrodes. Cyclic voltammetry was carried out with a CHI 650 potentiostat, using a graphite electrode, Ag/AgCl (3 M KCl) and platinum wire as working, reference and counter electrodes, respectively (IJ Cambria). EFCs were assembled by insertion of anode and cathode into a compartment-less electrochemical cell containing 5 mL of electrolyte solution. The anode and cathode were externally connected through a resistance box (IET Labs) over a resistance range of 5 MΩ to 1 kΩ, and the voltage between the electrodes measured with a multimeter (Keithley) for each load.
Film assembly was monitored at each step with a quartz crystal microbalance (QCM, USI Japan) with 9 MHz QCM resonators (AT-cut, International Crystal Mfg). The gold resonators were first treated with 4 mM 3-mercaptopropionic acid in ethanol overnight to form negative monolayer surface to mimic the graphite surface. Films were assembled onto the negatively charged gold resonator surface and were dried in a stream of nitrogen before measuring the frequency change ($\Delta F$) (Rusling et al., 2000).

4.2.3 Enzyme activity

Glucose oxidase was obtained from Sigma – Aldrich (10 mg/ml stock solution was prepared in phosphate buffer pH 7.4) and *Trametes hirsuta* (ThLacc) was provided by VTT (Finland) as (3.6 mg/mL) stock solution in citrate buffer pH 5. Enzyme activity was calculated using spectrophotometric assays (using and Agilent 8453 diode array). Glucose oxidase activity was monitored at 460 nm, where the increase in absorbance is a result of the oxidation of dianisidine through a peroxidase coupled system, using an extinction coefficient of 11300 M$^{-1}$ cm$^{-1}$, in phosphate buffer pH 6 (Bergmeyer et al., 1974). The activity of the laccase was measured by monitoring the oxidation of 5 mM ABTS in 50 mM acetate buffer pH 4.5 at 420 nm over a period of 10 minutes using extinction coefficient of 36 000 M$^{-1}$ cm$^{-1}$ (Bourbonnais et al., 1998).

4.2.4 Methods

Electrodes were prepared, based on LBL self-assembly of redox polymer and enzyme (Calvo et al., 2001). For preparation of anode and cathode 10 µL solutions of redox polymers (8–10 mg/ml solution/suspension in water) were deposited onto electrodes to permit adsorption to graphite electrodes over 20 minutes, then rinsed with milli Q water. Enzymes solutions of 10 µL of either GOx of 10 mg/ml (1500 U/ml), or ThLacc (390 U/ml) were deposited to permit adsorption for 20 minutes, then washed with Milli Q water. Further layers were deposited by repeating these steps to form (polymer /enzyme)$_n$ films. Prepared films were dried for 12 h before testing electrochemically or used for EFC assembly. Unless otherwise stated, current and power densities were measured at 37 °C in 0.1M potassium phosphate buffered solutions containing 0.15 M NaCl, 0.1 M glucose and saturated O$_2$. 
4.3 Results and discussion

Most LBL assemblies are developed at gold electrodes although using graphite instead of gold electrodes eliminates the need for thiol modification to facilitate adsorption of the primary cationic layer (Gao and Yang, 2004, Zheng et al., 2004, Lee et al., 2005). Work reported in this chapter shows the first study of a fully assembled membrane-less EFC in which both the anode and the cathode use redox polymer - enzyme LBL assembly at graphite electrodes. Previously it was reported that an EFC based on crosslinked GOx or laccases with osmium redox polymer films at graphite (Barrière et al., 2006) and glassy carbon electrodes (Kavanagh et al., 2008) can be assembled. The redox polymers [Os(4,4′-dimethoxy-2,2′-bipyridine)$_2$(polyvinylimidazole)$_{10}$Cl]$^+$ (Taylor et al., 1995, Mano and Heller, 2003) $E'_{o'} = -0.05$ V vs. Ag/AgCl (Med$_1$) and [Os(4,4′-dichloro-2,2′-bipyridine)$_2$(polyvinylimidazole)$_{10}$Cl]$^+$ (Jenkins et al., 2009, Mano et al., 2003) $E'_{o'} = +0.35$ V vs. Ag/AgCl (Med$_2$) are selected to provide a voltage output in a glucose/O$_2$ EFC assembly, whilst facilitating a thermodynamically favourable transfer of electrons from the GOx active site ($E'_{o'} = -0.35$ V vs. Ag/AgCl) (Taylor et al., 1995) to the T1 Cu site of a Trametes hirsuta laccase (ThLacc, $E'_{o'} = +0.57$ V vs. Ag/AgCl) (Shleev et al., 2005). The redox potential of the T1 copper site in fungal laccase is ~ 0.2 V more positive than the redox potential of the T1 copper site in BOD, which was used in chapter 3. The use of a fungal laccase to investigate assembly by LbL of 3-dimensional films, rather than the BOD, was undertaken to attempt to provide for higher cell voltages of assembled EFCs.

Biocatalytic anodes and cathodes were assembled by contacting graphite electrodes alternately to redox polymer and enzyme (GOx 1500 U/ml or ThLacc 390 U/ml) solutions with a schematic of a self-assembled (Med$_1$/GOx)$_2$ film shown in Fig. 4.1(a). The isoelectric point of native GOx is 4.05, (Voet et al., 1981) whilst that for ThLacc is 4.2, (Shleev et al., 2004) ensuring negatively charged enzyme in the deposition solutions of pH 7.4 and 5.0 respectively (Calvo et al., 2001). Cyclic voltammetry in the absence of glucose for Med$_1$/GOx films, Fig. 4.1(b), display an Os (II/III) redox transition at −0.05 V vs. Ag/AgCl. Osmium surface coverages of $7.8 \times 10^{-9}$ mol/cm$^2$ and $1.6 \times 10^{-8}$ mol/cm$^2$ for films of (Med$_1$/GOx)$_1$ and (Med$_1$/GOx)$_2$, respectively, compare well to that of $8.3 \times 10^{-11}$ mol/cm$^2$ reported for LbL assembly of an LbL of (osmium redox polymer/GOx)$_2$ at a modified gold surface (Calvo et al., 2001).
Figure 4.1 a) Diagram showing layer by layer assembly of (Med₈/GOx)₂ films at graphite electrode. CVs of (Med₈/GOx)₁ (grey) and (Med₈/GOx)₂ (black) films in 0.1 M potassium phosphate buffer containing 0.15 M NaCl at 37 ºC, pH 7.4 in b) absence and c) presence of 0.1 M glucose. Scan rate 5 mVs⁻¹.

In the presence of 0.1 M glucose at pH 7.4 sigmoidal signals indicative of bioelectrocatalytic glucose oxidation, Fig. 4.1(c), yield current densities of 190 and 540 μAcm⁻² for (Med₈/GOx)₁ and (Med₈/GOx)₂ films, respectively. The 3 fold increase in current density for the (Med₈/GOx)₂ film over the (Med₈/GOx)₁ film, whilst only recording a 2 fold increase in osmium surface coverage could be due to a larger proportion of GOx molecules.
adsorbed in the second bilayer, further supported by monitoring of mass adsorbed for such systems at mercaptopropionic acid-treated gold electrodes Fig. 4.2 (Zhou and Rusling, 2001). A plot of frequency vs. sequential layers on gold resonators shows linear response suggesting regular and reproducible film growth.

Table 4.1 shows total mass adsorbed / unit area, thickness of \((\text{Med}_1/\text{GOx})_2\) and \((\text{Med}_2/\text{ThLacc})_2\) films on gold resonators obtained using a quartz crystal microbalance (QCM). In this approach, layer-by-layer films of osmium redox polymer and enzyme were formed electrostatically over negatively charged gold QCM resonators and were dried in stream of nitrogen before measuring the frequency change (\(\Delta F\)). The adsorbed mass and the nominal thickness were estimated using the Sauerbrey equation (Sauerbrey, 1959).

\[ \Delta F = -2f_0^2\frac{\Delta M}{A}(\mu\rho)^{1/2} \]  \hspace{1cm} (4.1)

The relationship between adsorbed mass, \(\Delta M\) (g), and frequency shift, \(\Delta F\)(Hz), can be estimated by taking into account the properties of quartz resonator used in this work of \(f_0\) the resonant frequency of the fundamental mode of the quartz crystal (8 MHz), \(\mu\) the shear modulus of quartz \((2.947 \times 10^{11} \text{ g cm}^{-1} \text{ s}^{-2})\), \(\rho\) the density of the crystal \((2.648 \text{ g cm}^{-3})\), and \(A\) the geometric area of the QCM electrode \((0.196 \text{ cm}^2)\). Thus, 1 Hz of frequency decrease corresponds to 1.35 ng of mass increase. QCM data were also used to estimate the nominal thickness of adsorbed layer for dry(Med/enzyme)\(_n\) films. The thickness, \(d\) (centimeters), can be expressed by (Zhou et al., 2002).

\[ d = \left(-3.4 \times 10^{-9}\right)\Delta F/\rho \]  \hspace{1cm} (4.2)

Current densities for glucose oxidation by \((\text{Med}_1/\text{GOx})_2\) films are comparable to the 600 \(\mu\text{Acm}^{-2}\) observed for a crosslinked system at miniaturised carbon fibre electrodes (Mano and Heller, 2003). A LbL approach used for \((\text{Med}_2/\text{ThLacc})_2\) films yields osmium surface coverage of \(6.9 \times 10^9\) mol/cm\(^2\), with lower surface coverage due to incomplete dispersion/dissolution of Med\(_2\) compared to Med\(_1\) in the coating solutions, as observed previously (Kavanagh et al., 2009). The osmium surface coverages are nonetheless higher than the picomoles/cm\(^2\) coverages reported for LBL assemblies of osmium redox polymer/laccase at modified gold surfaces (Scodeller et al., 2010).
Figure 4.2 QCM frequency shifts for alternate redox polymer – enzyme a)Med$_1$/GOx and b) Med$_2$/ThLacc adsorbed on gold resonators first coated with a monolayer of 3-mercaptopropionic acid rendering a negatively charged surface that mimics the self-assembly at graphite electrode.
Table 4.1 Total mass adsorbed/ unit area, thickness of (Med₁/GOx)₂ films and (Med₂/ThLacc)₂ films on gold resonators obtained using quartz crystal microbalance.

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mass of (Med₁/GOx)₂ films adsorbed per unit area (µg/cm²)</td>
<td>4.82 ± 0.77</td>
</tr>
<tr>
<td>Total nominal thickness of (Med₁/GOx)₂ films (nm)</td>
<td>14.32 ± 2.28</td>
</tr>
<tr>
<td>Total mass of (Med₂/ThLacc)₂ films adsorbed per unit area (µg/cm²)</td>
<td>5.08 ± 0.23</td>
</tr>
<tr>
<td>Total nominal thickness of (Med₂/ThLacc)₂ films (nm)</td>
<td>14.90 ± 0.70</td>
</tr>
</tbody>
</table>

Figure 4.3 Catalytic CVs of assembled EFC of (Med₁/GOx)₂ (left) and (Med₂/ThLacc)₂ (right) films in oxygen-saturated 0.1 M potassium phosphate buffer containing 0.15 M NaCl at 37 ºC, in the presence of 0.1M glucose. Scan rate 5 mVs⁻¹ (electrodes dried for 12 hours). Grey (pH 7.4) and black (pH 5.5).
A membrane-less EFC with $(\text{Med}_1/\text{GOx})_2$ as anode demonstrates steady state glucose oxidation current density of 510 $\mu$Acm$^{-2}$ and 240 $\mu$Acm$^{-2}$ at pH of 7.4 and 5.5, respectively, confirming higher enzyme activity at neutral pH (Fig.4.3) (Wiebel and Bright, 1971). In the same solution the $(\text{Med}_2/\text{ThLacc})_2$ cathode provides oxygen reduction current densities of 60 $\mu$Acm$^{-2}$ and 330 $\mu$Acm$^{-2}$ at pH of 7.4 and 5.5, respectively, reflecting the lower activity of ThLacc at neutral pH (Barrière et al., 2006, Barrière et al., 2004). The oxygen reduction current densities compare well to current densities of $\sim$ 40 $\mu$Acm$^{-2}$ for the crosslinked films on glassy carbon at pH 4.5 (Jenkins et al., 2009) and current densities of 280 $\mu$Acm$^{-2}$ for crosslinked films on graphite at pH 4.7 (Barrière et al., 2006). In addition films of Med$_2$ cross-linked with bilirubin oxidase on carbon cloth yield oxygen reduction current densities of $\sim$ 300 $\mu$Acm$^{-2}$ when rotated at 100 rpm (Shin et al., 2009).

The membrane-less EFC was polarized under variable load conditions in non-stirred oxygenated buffer in 0.1 M glucose, 0.15 M NaCl, at 37 °C to yield maximum power density of 103 $\mu$Wcm$^{-2}$ (at 0.35 V), at a current density of 380 $\mu$Acm$^{-2}$ in pH 5.5 buffer (Fig. 4.4a). A maximum power density of 40$\mu$Wcm$^{-2}$ (at 0.42 V), at a current density of 150 $\mu$Acm$^{-2}$ was observed when the membrane-less EFC operated in pH 7.4 buffer. These results are higher than that obtained in previous EFC studies using assembly of redox hydrogels at 6 mm graphite electrode, by 2.5 times (Barrière et al., 2006), or one magnitude improved power density compared with EFC based on Melanocarpus albomyces laccase cathode (Kavanagh et al., 2009). In chapter 3, the same Med$_1$ and Med$_2$ was used in redox hydrogel based EFC cross-linked with glucose oxidase and bilirubin oxidase for glucose oxidation and oxygen reduction respectively, which yielded a 43 $\mu$Wcm$^{-2}$ at 0.25 V at physiological conditions.

Consistent with other studies, (Chen et al., 2001, Kavanagh et al., 2008, Mano et al., 2002) an OCV of 0.65 V observed for EFCs is greater than the difference between the redox polymer mediator potentials (0.4 V), postulated to occur because of a DET for oxygen reduction at the T1 copper redox potential at the ThLacc cathode (Ramírez et al., 2008). Fig.4.3 provides further evidence that DET can contribute to the oxygen reduction current for laccases, as well as BOD. The relatively low coverage of osmium for the $(\text{Med}_2/\text{ThLacc})_2$ films may permit adsorption of ThLaccontO to the underlying graphite for DET to occur. As expected, independent cell polarization studies reveal that higher cathode current densities are sustained at pH 5.5 compared to pH 7.4 (Fig. 4.4b). The power density is an improvement on the 40 $\mu$Wcm$^{-2}$ at pH 5.5, and 16 $\mu$Wcm$^{-2}$ at pH 7.4, obtained using crosslinked redox hydrogels of GOx and laccase at graphite electrodes (Barrière et al., 2006) and the 12.6 $\mu$Wcm$^{-2}$ for an EFC based on LBL self assembly of enzymes and gold nanoparticles on planar gold.
electrodes reported by Deng et al. (Deng et al., 2008). The results also compare well with that obtained for an EFC using films of Med₁ with glucose oxidase and Med₂ with bilirubin oxidase on carbon fibre electrodes, which yielded 244 μWcm⁻² at 0.36 V under physiological conditions (Mano and Heller, 2003).

**Figure 4.4** a) Power and current density curve of an EFC composed of (Med₁/GOx)₂ and (Med₂/ThLacc)₂ films in oxygen-saturated 0.1 M potassium phosphate buffer containing 0.15 M NaCl at 37 °C, in the presence of 0.1 M glucose. Scan rate 5 mVs⁻¹ (electrodes dried for 12 hours). (open circle(pH 5.5), closed circle (pH 7.4). b) Cell behaviour during polarization of EFC. (Grey line – Cell Voltage, Black line - Cathode potential, Dashed line – Anode potential).
4.4 Conclusions

In conclusion we demonstrate that LbL assembly provides a facile method for production of redox polymer-based EFCs that can provide maximum power densities 40 \( \mu \text{Wcm}^{-2} \) at pH 7.4 or 103 \( \mu \text{Wcm}^{-2} \) at pH 5.5. Improvements may be achieved by using LBL assembly to form thicker films (> 2 bilayers) and by use of alternate enzymes and redox polymers. Such an approach can form high power density yielding EFCs. The highest power output reported in this chapter is 2 times higher when compared to EFCs reported in chapter 3. To further improve the power output at physiological conditions, laccase can be replaced with bilirubin oxidase or other multi-copper oxygenases that are active under these conditions.
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5. Redox polymer hydrogels containing enzymes on oxidized pyrolytic graphite as electrodes in enzyme-based fuel cells

5.1 Introduction

Construction of compartment-less, single chambered miniaturized EFCs could provide for future applications in powering implantable and small medical devices (Persson and Gortan, 1985, Katz et al., 1999). Membrane-less mediated EFCs constructed using films prepared by redox polymer crosslinking with glucose oxidase to provide a glucose oxidizing anode, and with a laccase or a bilirubin oxidase to provide an oxygen-reducing cathode, have been studied extensively (Mano and Heller, 2003, Barrière et al., 2004, Kim et al., 2003). Although these hydrogel modified electrodes perform well the response of systems based on simply adsorbing films to surfaces quickly diminishes (Barton et al., 2004, Kim et al., 2006). Several researchers have used modified carbon surfaces for covalent coupling of the redox species to improve stability. Allongue et al. and Brooksby and Downward have electrochemically modified carbon surfaces (glassy carbon, highly oriented pyrolytic graphite) via electrochemical reductive adsorption of aryldiazonium salts to covalently anchor redox molecules (Allongue et al., 1997, Brooksby and Downward, 2004). Most recently, carbon electrodes (Pellissier et al., 2008), and gold and carbon electrodes (Boland et al., 2009a) were functionalized using aryldiazonium salts bearing carboxylic acid groups. Redox hydrogels anchored at a surface modified electrode have been shown to improve the stability of the electrochemical response (Boland et al., 2009b).

Shin et al. have used quaternized[Os(4,4-dichloro-2,2-bipyridine)$_2$(polyvinylimidazole)$_{10}$Cl]$^+$ wired with BOD, and found it stabilized the Os redox signal compared to films of non-quaternized [Os(4,4-dichloro-2,2-bipyridine)$_2$(polyvinylimidazole)$_{10}$Cl]$^+$ because of, in their opinion, lower tendency for ligand substitution of the chlorine (Shin et al., 2009). Other than this, Bunte et al. have shown that the electrolyte used influences ferrocene based redox polymer stability (Bunte et al., 2010). They have suggested that irreversible uptake of the bivalent HPO$_4^{2-}$ upon oxidation contributed to decreased stability of the redox hydrogel at pH 7.4 compared to pH < 6 buffer.

In this chapter enzyme redox hydrogels electrodes were prepared with two different curing times, for 24 h and 48 h, of cross-linking using a poly(ethylene glycol) diglycidyl ether to study the influence over catalytic current and stability. Pyrolytic graphite (PG) electrodes have more C-O functionalities at which many redox species (esp. proteins) can adsorb, and so can display fast electron transfer (Blanford and Armstrong, 2006, Vaze and Rusling, 2002).
Coupling of a poly-L-lysine (PLL) film to oxidized PG, with subsequent reaction with enzymes provides films of excellent stability and biocatalytic activity, as reported on for films used in the oxidation of o-methoxyphenol (Guto et al., 2008). The application of such approaches may improve catalytic currents and stability of biocatalytic films for glucose/O₂ EFCs. Covalent attachment of poly-L-lysine to a carboxylated surface is thus investigated as a platform for anchoring the biocatalytic films in this chapter. Firstly, PLL films were covalently attached to electrochemically oxidized PG electrodes. Next, an epoxide crosslinker was used to covalently crosslink the enzyme and osmium redox polymer over the surface modified PG electrode (Jenkins et al., 2009, Boland et al., 2009b). Stability of such systems has been studied for glucose oxidation with the [Os(4,4-dimethoxy-2,2'-bipyridine)₂(polyvinylimidazole)₁₀Cl]⁺/glucose oxidase and [Os(2,2'-bipyridine)₂(polyvinylimidazole)₁₀Cl]⁺/bilirubin oxidase for oxygen reduction at physiological buffer conditions at 37 °C. Influence of surface-modified and control electrodes on catalytic current and stability improvement was studied for both glucose oxidation and oxygen reduction. In chapter 3, amine-functionalized graphite electrodes were used to covalently anchor biocatalytic films to improve the EFC stability. Nonetheless, even using the surface-modified electrodes a gradual loss in maximum power density was evident. Here in this chapter an effort has been made to form a stable biocatalytic films that contains enzyme and osmium redox polymer covalently cross-linked over modified PG electrode to provide an EFC.

5.2 Experimental Section

5.2.1 Materials

Synthesis of redox polymers was achieved by others in the Leech laboratory by adapting literature procedures (Kober et al., 1988). (NH₄)₂OsCl₆ (Aldrich) was used as the starting material to prepare the cis-Os(4,4'-dimethoxy-2,2'-bipyridine)₂Cl₂ and cis-Os(2,2'-bipyridine)₂Cl₂ complexes, which were then complexed via ligand substitution reaction in ethanol/water solvent, to a previously pre-synthesized polyvinylimidazole (PVI) polymer (Forster et al., 1990). Poly (ethylene glycol) diglycidyl ether (average Mn ~ 526) was purchased from Sigma - Aldrich. Glucose oxidase (GOx) from Aspergillus niger, MvBOD from Myrothecium verrucaria were obtained from Sigma – Aldrich. Unless otherwise stated
all other chemicals were obtained from Sigma- Aldrich. All buffers were prepared from solutions of the selected base then adjusted to the desired pH using solutions of the acid.

5.2.2 Enzyme activity

GOx activities were assayed spectrophotometrically using the peroxidase coupled oxidation of dianisidine (Bergmeyer, 1974) and BOD activities were measured spectrophotometrically by bilirubin oxidation to biliverdin (Murao and Tanaka, 1981).

5.2.3 Preparation of redox hydrogel electrodes

Pyrolytic graphite (PG) disks 6 mm diameter were attached to steel rods and shrouded in heat-shrink Teflon tubing. The disks were polished with p-400 grit silicon carbide paper (Buehler) and rinsed in Milli Q water then oxidized by scanning at 5 mV/s from 1.55 to 1.76 V vs. Ag/AgCl in aqueous 2.5% K$_2$Cr$_2$O$_7$/10% HNO$_3$ (Campbell et al., 2001). Next, 10 µl of freshly prepared 24mM EDC (in Milli Q water) was drop coated to the electrode surface for 20 minutes to activate the carboxylic group. After that, a 15 µl aqueous solution of poly(L-lysine) PLL (4mM lysine residues) and 5 µl of freshly prepared EDC solution was drop coated over the oxidized PG electrode and left for 6 hours at room temperature. Care was taken that the PLL was not allowed to dry by covering the electrode with moist glass vials. Following rinsing with MilliQ water, 10 µl of either the [Os(4,4'-dimethoxy-2,2-bipyridine)$_2$(polyvinylimidazole)$_{10}$Cl]$^+$ or [Os(2,2'-bipyridine)$_2$(polyvinylimidazole)$_{10}$Cl]redox polymer (8mg/ml), 10 µl of GOx (1400 U/ml) or BOD (95 U/ml), and 5 µl of poly(ethyleneglycol) diglycidyl ether (PEG) (15 mg/ml) (Ohara et al., 1993) was drop coated at the modified surface of the PG electrode. For control electrodes, PG electrodes that were not submitted to the oxidation procedure, nor to coupling of the PLL, were used to form physisorbed films of crosslinked redox polymer and enzyme. Electrodes following drop coating were dried for 24 or 48 h before testing for electrochemical properties. Unless otherwise stated, current and power densities were measured at 37 °C in 0.1M potassium phosphate buffered solutions with a pH 7.4 containing 0.15 M NaCl, 0.1 M glucose or saturated O$_2$.

5.2.4 Electrochemical measurements

Cyclic voltammetry (CV) and chronoamperometry experiments were carried out with a CHI 1040 multichannel potentiostat, using a PG, Ag/AgCl (3 M KCl) and platinum wire as working, reference and counter electrodes, respectively (IJ Cambria). The anode and cathode
of assembled single-compartment, membrane-less EFC cells were externally connected through a resistance box (IET Labs) over a resistance range of 5 MΩ to 1 kΩ, and the voltage between the electrodes was measured with a multimeter for each load.

### 5.3 Results and Discussion

#### 5.3.1 Characterization of redox polymer films over modified and control PG electrodes

**Fig.5.1** shows the proposed surface modification process at the PG electrode for the covalent attachment of an enzyme and osmium redox polymer. In this scheme the PG electrode was electrochemically oxidized and PLL was covalently attached by forming an amide group between polymer amine and EDC activated carboxylic acid groups introduced on the electrode through the oxidation process. Then the enzyme and osmium redox polymer were anchored covalently over electrode surface by crosslinking between amines of each, and PLL, using the PEG. **Fig.5.2** shows the linear sweep voltammogram recorded for the electrochemical oxidation of the PG electrode. The K₂Cr₂O₇ is a strong oxidizing agent that oxidizes the carboxylic functionalities over the electrode surface as a result a strong oxidation peak is evident from the linear sweep voltammetry. This creates more functional groups for more redox species to interact with the electrodes. CVs of scan rate dependent study for [Os(4,4-dimethoxy-2,2-bipyridine)₂(polyvinylimidazole)₁₀Cl]⁺(Med₁) /GOx over a modified and control PG electrodes were shown in **Fig. 5.3&5.4** respectively, under nitrogen. Similarly, CVs of scan rate dependent study for [Os(2,2-bipyridine)₂(polyvinylimidazole)₁₀Cl]⁺(Med₂) /BOD over a modified and control PG electrodes were shown in **Fig. 5.5&5.6** respectively, under nitrogen. CVs show signals for oxidation and reduction of the osmium metal center with a mid-point potential, used to estimate the formal potential of the Os(II)/(III) transition of −0.05 V vs. Ag/AgCl and E°⁺ = +0.22 V vs. Ag/AgCl for Med₁ and Med₂ respectively. The redox potential of Med₁ is thermodynamically favourable for the transfer of electrons from the GOx active site (E°⁺ = −0.35 V vs. Ag/AgCl (Gregg and Heller, 1991a, Gregg and Heller, 1991b,Heller et al., 1992,Taylor et al., 1995). Similarly Med₂ transfers electrons to the T1 Cu of multicopper oxygenases (Jenkins et al., 2009) E°⁺ = +0.4 V vs. Ag/AgCl for MvBOD (Xu et al., 1996). Redox polymer [Os(4,4′-dichloro-2,2′- bipyridine)₂(polyvinylimidazole)₁₀Cl]⁺ (Jenkins et al., 2009,Mano et al., 2003) E°⁺ = +0.35 V vs. Ag/AgCl, that has been previously used (and discussed in chapter 4 of this thesis), is less thermodynamically favourable as the redox potential is very close to that of MvBOD.
**Figure 5.1** Surface modification process at pyrolytic graphite electrode for covalent attachment of enzyme and redox polymer. (Blue figure - enzymes, red circles - redox polymers)

**Figure 5.2** Linear sweep voltammetry of the electrochemical oxidation of PG electrode in aqueous 2.5% K$_2$Cr$_2$O$_7$/10% HNO$_3$. 

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Figure 5.3 a) Scan rate dependent studies - CVs of the different scan rates of Med$_1$/GOx redox hydrogel over modified PG electrode in 0.1 M potassium phosphate, 0.15M NaCl, with no glucose under nitrogen at 37 °C. Scan rates: 5, 50, 100, 300, 500 mV/s. a) 24 h cured, b) 48 h cured.

Figure 5.4 Scan rate dependent studies - CVs of the different scan rates of Med$_1$/GOx redox hydrogel over control PG electrode in 0.1 M potassium phosphate, 0.15M NaCl, with no
glucose under nitrogen at 37 °C. Scan rates: 5, 50, 100, 300, 500 mV/s. a) 24 h cured, b) 48 h cured.

**Figure 5.5** Scan rate dependent studies - CVs of the different scan rates of Med/BOD redox hydrogel over modified PG electrode in 0.1 M potassium phosphate, 0.15M NaCl, with no glucose under nitrogen at 37 °C. Scan rates: 5, 50, 100, 300, 500 mV/s. a) 24 h cured, b) 48 h cured.

**Figure 5.6** Scan rate dependent studies - CVs of the different scan rates of Med/BOD redox hydrogel over control PG electrode in 0.1 M potassium phosphate, 0.15M NaCl, with no glucose under nitrogen at 37 °C. Scan rates: 5, 50, 100, 300, 500 mV/s. a) 24 h cured, b) 48 h cured.
glucose under nitrogen at 37 °C. Scan rates: 5, 50, 100, 300, 500 mV/s. a) 24 h cured, b) 48 h cured.

Figure 5.7 Plot of peak current in the absence of glucose with the square root of scan rate for the Medi/GOxredox hydrogel films on modified PG electrode cured for 24 h. Other conditions as in figure 5.3.

It has been shown that the curing time for the diepoxide cross-linking reaction can affect the response of films of redox polymers on electrodes (Lehr et al., 2010), with 24 h proposed to provide films of highest current, but longer curing times providing greater stability of films. In order to test which protocols can provide for optimal signals for use of such films in EFCs, two different curing times, 24 h and 48 h, for the crosslinking process were investigated. CVs of different scan rates ranging from 5 to 500 mVs⁻¹ both at modified and control electrode resulted in well-defined peaks for the Os(II)/(III) redox process. For all the redox polymer hydrogels, increasing the scan rate from 5 to 500 mVs⁻¹ yielded peak currents proportional to the square root of scan rate (ip vs. ν^{1/2}) shown in Fig.5.7, a feature characteristic of surface bound redox polymer films controlled by semi-infinite diffusion (Daum et al., 1980). To estimate electron exchange diffusion coefficients within the redox hydrogel polymer films, a charge transport related parameter (D^{1/2}C) was determined using the Randles-Sevcik equation, given in Chapter 1 (Bard and Faulkner, 2001).
Table 5.1 shows the comparison of the charge transport parameter ($D^{1/2}C$), catalytic current and surface coverage ($\Gamma_{os}$) values estimated for the modified and control redox hydrogels for both the redox polymers at 24 h and 48 h curing. From the observed values, $D^{1/2}C$ is very similar for the surface modified electrodes compared to control electrodes for both types, Med$_1$ and Med$_2$.

Table 5.1 Comparison diffusion coefficient ($D^{1/2}C$), catalytic current and surface coverage ($\Gamma_{os}$) values estimated for redox hydrogel at modified and control PG electrodes.

<table>
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<tr>
<th>Curing time (h)</th>
<th>Surface modified electrode</th>
<th>Control electrode</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Gamma_{os}$ (nmol cm$^{-2}$)</td>
<td>19</td>
</tr>
<tr>
<td>24</td>
<td>$D^{1/2}C$ ($\times 10^8$ mol cm$^{-2}$ s$^{-1/2}$)</td>
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<tr>
<td>Med$_1$/GOx</td>
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<td>360</td>
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<td></td>
<td>$\Gamma_{os}$ (nmol cm$^{-2}$)</td>
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<tr>
<td>48</td>
<td>$D^{1/2}C$ ($\times 10^8$ mol cm$^{-2}$ s$^{-1/2}$)</td>
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<td></td>
<td>i$_{cat}$ ($\mu$A cm$^{-2}$)</td>
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<tr>
<td>Med$_2$/BOD</td>
<td>$\Gamma_{os}$ (nmol cm$^{-2}$)</td>
<td>5</td>
</tr>
<tr>
<td>24</td>
<td>$D^{1/2}C$ ($\times 10^8$ mol cm$^{-2}$ s$^{-1/2}$)</td>
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</tr>
<tr>
<td></td>
<td>i$_{cat}$ ($\mu$A cm$^{-2}$)</td>
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<tr>
<td></td>
<td>$\Gamma_{os}$ (nmol cm$^{-2}$)</td>
<td>55</td>
</tr>
<tr>
<td>24</td>
<td>$D^{1/2}C$ ($\times 10^8$ mol cm$^{-2}$ s$^{-1/2}$)</td>
<td>20</td>
</tr>
<tr>
<td>Med$_3$/BOD</td>
<td>i$_{cat}$ ($\mu$A cm$^{-2}$)</td>
<td>588</td>
</tr>
<tr>
<td></td>
<td>$\Gamma_{os}$ (nmol cm$^{-2}$)</td>
<td>43</td>
</tr>
<tr>
<td>48</td>
<td>$D^{1/2}C$ ($\times 10^8$ mol cm$^{-2}$ s$^{-1/2}$)</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>i$_{cat}$ ($\mu$A cm$^{-2}$)</td>
<td>384</td>
</tr>
</tbody>
</table>
Measurements are taken at 37 °C in 0.1M potassium phosphate buffered solutions with a pH 7.4 containing 0.15 M NaCl, with or without (0.1 M glucose or saturated O2).

The curing time applied to the redox hydrogel has an influence over $D^{1/2}C$. With 24 h cured electrodes, the $D^{1/2}C$ for films of Med1 on modified and control electrodes is 2.2 and 2.4 times higher respectively compared to 48 h cured electrodes. For films of Med3 the shorter curing time results in $D^{1/2}C$ values 1.8 times higher than the longer curing time. Here, the obtained $D^{1/2}C$ values in table 5.2 are higher compared to a range of $10^{-9}$ mol cm$^{-2}$ s$^{-1/2}$ for films of Med3 conating DNA over gold microelectrodes reported previously (Hajdukiewicz et al., 2009). Bunte et al. have reported $D^{1/2}C$ value of $7.2 \pm 0.2 \times 10^{-9}$ mol cm$^{-2}$ s$^{-1/2}$ for films of a ferrocene redox polymer (Bunte et al., 2010). For this ferrocene redox polymer when the film thickness state was taken into account by measuring with an AFM (Atomic Fluorescent Microscope), for a dry and swollen redox hydrogel, apparent diffusion coefficients ($D_{app}$) of $6.7 \pm 0.7 \times 10^{-10}$ cm$^2$ s$^{-1}$ and $1.9 \pm 0.4 \times 10^{-9}$ cm$^2$ s$^{-1}$ respectively were obtained. In general, the $D_{app}$ in redox hydrogels varies in a range between $10^{-12}$ to $10^{-6}$ cm$^2$ s$^{-1}$ (Mano et al., 2006).

An estimation of total osmium surface coverage ($\Gamma_{Os}$) can be obtained for each film, evaluated by integrating the charge passed (Q) where the film is comprehensively electrolysed. Table 5.1 shows the surface coverage for the modified and control hydrogel electrodes cured at 24 h and 48 h estimated from integration of charge from the CVs at scan rates of 5 mV/s. A consistently slight increase in surface coverage was achieved with the surface modified hydrogel electrode compared to the control, for both films cured at 24 h and 48 h even though the same amount of osmium redox polymer was coated for preparation of all of the films. The electro-oxidation process for preparation of the PG prior to modification may have increased the number of functionalized carboxyl groups to then attach covalently PLL, which then retains a greater amount of redox polymer, compared to untreated PG electrodes. An increase in estimated surface coverage is observed for all of the films cured for 24 h compared to those cured for 48 h. This might be linked to the lower $D^{1/2}C$ parameters extracted for these films, as the hydrogel stiffness and thickness may increase with curing time, and the resulting highly crosslinked hydrogel might have slowed down the mobility of charge transport within the redox hydrogel (Lehr et al., 2010). However, the scan rate used to estimate coverage may not have been sufficiently slow to permit full electrolysis of all of the redox sites within the films, and the surface coverage results may not therefore be a reliable indicator of amounts of mediator on the electrode surfaces. In order to comprehensively
electrolyse the redox hydrogel films, a slower scan rate <5 mV/s could have been used, and it is therefore recommended in any future studies of such systems.

5.3.2 Catalytic properties of redox polymer films over modified and control PG electrode

To verify the catalytic properties of the films of redox hydrogels and enzymes on PG electrodes cyclic voltammograms were recorded in the presence of excess glucose (0.1M) for Med₁ cross-linked with GOx for the catalytic oxidation of glucose (Fig.5.8) and in the presence of saturated oxygen for Med₃ cross-linked with BOD for the catalytic reduction of oxygen (Fig.5.9). Firstly, slightly higher catalytic currents are observed for all the films prepared on surface modified electrodes compared to those prepared at the control electrodes. Improved catalytic currents might be due to provision of carboxyl functional groups formed at the modified PG electrode, that were used to anchor PLL that forms a support for coupling relatively more redox polymer and enzyme, than at the control electrode. Secondly, redox hydrogels cured for 24 h resulted in higher catalytic current density compared to the 48 h cured hydrogels. One reason for this might be an increase in hydrogel compactness or stiffness with curing time, which might have reduced the mobility of redox complexes/polymers hence decreasing the rate of charge transport. As the curing time increases so does the stiffness within the redox hydrogel electrodes, also possibly affecting enzyme conformation and activity. For example, Lehr et al. (2010) reported a decrease in enzyme activity in these redox hydrogels as the curing time increases, due to increase in stiffness of surrounding environment and by conformational changes of the enzyme.

Current density has been shown by others to depend on the redox polymer diffusion coefficient, which can be improved with long tethers between the redox centers and polymer backbone. For example, Mao et al. have compared two types of redox polymers that differ in the 13 atom spacers arms, and therefore in the resulting current density. PVP-[Os(N,N'-dialkylated-2,2’biimidazole)₃]²⁺ with a 13 atom long flexible tether gives a higher diffusion coefficient yielding a current density of 1.15 mA cm⁻² when compared to the PVI-[Os(4,4’-diamino-2,2’-bipyridine)₂Cl]²⁺ that yielded current density of 0.15 mA cm⁻² for glucose oxidation (at 15 mM glucose) using a vitreous carbon rotating disk electrode (Mao et al., 2003). This result is comparable with findings for our redox polymer Med₁, which when cured at 24h, with no spacers, yielded a current density of ~360 µA cm⁻² for glucose oxidation under non-stirred condition. In another report, Mano et al. have used a redox polymer with 8 atom long spacer arms (D of 7.6 ×10⁻⁷ cm⁻² s⁻¹) that yielded a current density
of 0.86 mA cm$^{-2}$ for reduction of oxygen: superior to that for both redox polymer without spacer arms and for platinum, electrocatalyzing the reduction of oxygen in pH 5 citrate buffer (Mano et al., 2006). In the work reported on here, Med$_3$ with no spacers and a redox potential that is 0.18V more negative than the BOD T1 substrate-oxidizing redox center yielded a current density of 588 µA cm$^{-2}$ for oxygen reduction at pH 7.4 under non-stirred conditions. Improvements in current densities could have been achieved for glucose oxidation and oxygen reduction with 24 h surface modified electrode in two possible ways. Firstly, stirring the electrolyte enhances mass transfer to and through the hydrogels (Merchant et al., 2010, Shin et al., 2009), and secondly current densities could also have been improved by the inclusion of spacer arms.

**Figure 5.8** Catalytic CVs of the Med$_i$/GOx, in presence of 0.1 M glucose, under nitrogen in 0.1 M potassium phosphate, 0.15M NaCl, at 37 °C. Scan rate 5 mV/s. (solid line – Surface modified PG, dashed line – control PG) (blue - 24h and red - 48 h cured electrodes)
Figure 5.9 Catalytic CVs of Med$_3$/BOD, over PG electrode in saturated oxygen in 0.1 M potassium phosphate, 0.15M NaCl, at 37 °C. Scan rate 5 mV/s. (solid line – Surface modified PG, dashed line – control PG) (blue - 24h and red - 48 h cured electrodes)

Improvement in the cell voltages of EFC could be achieved by use of redox polymers for oxygen reduction that have more positive redox potentials. The ability of the redox polymer, [Os(4,4-dichloro-2,2-bipyridine)$_2$(polyvinylimidazole)$_{10}$Cl]$^+$ (Med$_2$) ($E^o$ = +0.35V vs. Ag/AgCl, just 0.05V negative to BOD redox active center) to catalyze oxygen reduction for films formed at PG electrodes was therefore also investigated. Such films yielded a current density of ~ 100 µA cm$^{-2}$ at surface modified PG electrodes cured for 24h (Fig. 5.10) at 0.3 V vs Ag/AgCl. A $D^{1/2}$ for films of this polymer with BOD on the surface modified PG electrodes in the absence of oxygen was estimated as 2.4×10$^{-8}$ mol cm$^{-2}$ s$^{-1/2}$. From slow-scan CVs (5 mV/s) in the absence of oxygen, a surface coverage of 5×10$^{-9}$ mol cm$^{-2}$ is estimated (Fig. 5.11). The relatively low oxygen reduction current densities obtained with films of this redox polymer might be due to the decreased driving force, making electron exchange thermodynamically less favourable (Jenkins et al., 2009), indicating the trade-off between cell voltage and current/power densities for EFCs.
Figure 5.10 CVs shows the catalytic currents Med$_2$/BOD (black) and Med$_3$/BOD (grey) in 0.1 M oxygenated potassium phosphate, 0.15M NaCl, at 37 °C.

Figure 5.11 CV shows the scan rate dependent study for the Med$_2$/BOD modified electrode under nitrogen 0.1 M potassium phosphate, 0.15M NaCl, at 37 °C. Scan rates: 5, 50, 100, 300, 500 mV/s.
5.3.3 Operational stability of redox polymer films

Long-term operation of glucose oxidizing and oxygen reduction electrodes are important for EFC operations proposed as in vivo power sources (Barton et al., 2004). The operational stability is defined as the stability when the enzyme redox polymer electrodes are continuously monitored for catalytic efficiency, with the electrodes held at constant applied potentials. Fig. 5.12 shows the glucose oxidation current density vs. time for films of Med₁ cross-linked with GOx at both surface modified and control PG electrodes, when the working electrode is held at an applied potential of + 0.2 V vs. Ag/AgCl under nitrogen. A gradual decrease in current signal for glucose oxidation is evident in all types of electrodes. For the films prepared using 24 h curing of hydrogels, the current dropped by 60% and 63% for the surface modified and control hydrogel electrodes from an initial value of 287 and 218 µA/cm², respectively after 20 h of continuous operation. For the films prepared using 48h curing of hydrogels, the current dropped by 50% and 74% for the surface modified and control hydrogel electrodes respectively from an initial value of 108 and 51 µA/cm² over the same time period. It is evident that whilst surface modification improved the initial current density, it does not necessarily improve operational stability for these systems. In other reports, decreases in current over time are ascribed to Med₁ instability. For example, in the operational stability of lactate oxidase based electrodes a half-life of Med₁ was 2 h compared to 16 h for the [Os(4,4-dimethoxy-2,2-bipyridine)₂(polyvinyl pyridine)₁₀Cl]⁺polymer (Kenausis et al., 1996). Several other factors can influence the stability of redox hydrogel films. Previously, films of Med₃ anchored to surface-modified graphite electrodes retained up to 90% of the redox signal over a 48 h period. When the same redox polymer was co-immobilized with GOx only 54% of bioelectrocatalytic signal was retained over the same period (Boland et al., 2009b). Flexer et al. have shown that GOx inactivation at higher glucose concentration can also be a reason for catalytic current decay (Flexer et al., 2008).
Operational stability of the Med$_{3}$/GOx poised at +0.2 V vs. Ag/AgCl, in presence of 0.1 M glucose, under nitrogen, 0.1 M potassium phosphate, 0.15M NaCl, at 37°C. (solid line – Surface modified PG, dashed line – control PG) (blue - 24h and red - 48 h cured electrodes) Data is the average of 3 individual electrodes. Example of errors are provided for 0, 5, 10, 15 and 20 h data points, where the error bars are the standard deviation of the 3 data points.

Fig. 5.13 Shows the oxygen reduction current density vs. time for Med$_{3}$/BOD cross-linked with BOD at surface modified and control PG electrodes, poised at + 0.12 V vs. Ag/AgCl under saturated oxygen. It seems that the Med$_{3}$/BOD redox hydrogel may undergo an initial break-in (substrate diffusion and swelling) period when the current increases slightly as a function of time. In any event, the current response is considerably more stable than the glucose oxidation currents over the same time period. Catalytic currents with surface modified PG containing the 24 h and 48 h cured redox hydrogels are stable over the 20 h operational test period. However, the control hydrogel electrodes provide catalytic currents that, whilst of lower magnitude, are of similar stability over the same time period.
Mano et al. found that PAA PVI-[Os(4,4'-dichloro-2,2'-bipyridine)2Cl]^{+/2+} wired with *Trachyderma tsunodae* BOD (Mano et al., 2002) on carbon electrodes lost 5% current density compared to 10% of loss for the *Myrothecium verrucaria* BOD (Mano et al., 2002) wired carbon electrodes, per day (pH 7.4, 37°C 0.15 M NaCl). Shin et al. used quaternized Q-PVI-[Os(4,4'-dichloro-2,2'-bipyridine)2Cl]^{+/2+} to wire *Trachyderma tsunodae* BOD at pyrolytic graphite PG electrode, and found a loss of 7.2% in catalytic current per day (Shin et al., 2009). By comparison, oxygen reduction catalytic currents using the system proposed in this chapter are stable for at least 20h.

### 5.3.4 Operational stability of assembled EFCs

Chapter 3 reported on glucose/O$_2$EFCs based redox polymer films (Med$_1$ and Med$_2$) crosslinked with enzymes and with surface derivatized graphite electrodes to produce a power output of 43 $\mu$W cm$^{-2}$ at 0.25 V under physiological conditions. In that system cathode current densities were limiting, affecting EFC power output. In order to improve the power output, Med$_3$ with a more positive redox potential can be used at the cathode. For oxygen reduction...
reduction, Med$_3$/BOD yielded a 6 fold improved catalytic current when compared to Med$_2$/BOD (Fig. 5.9). Others have also reported on EFCs which yielded higher current densities for Med$_1$ compared to Med$_2$, when wired with BOD, or with a Melanocarpus albomyces laccase at carbon electrodes (Jenkins et al., 2009, Kavanagh et al., 2009). The combination of Med$_3$ and BOD, with glucose-oxidizing films to form an EFC is therefore an interesting system to examine for production of power under physiological conditions. The following sections report on such a study, using the surface modified PG in EFCs, with a view to comparing the results to those observed in previous chapters, and by others previously. Biocatalytic films covalently coupled to surface modified PG electrodes cured for 24 h were selected for EFC assembly, as these gave the highest currents when compared to the control PG electrodes.

Fig. 5.14 shows the catalytic currents of glucose oxidizing Med$_1$/GOx and the oxygen reducing Med$_2$/BOD electrodes in an assembled membrane-less single compartment EFC. The anode and cathode in an assembled FC showed similar magnitudes of catalytic current to that observed in individual half-cell studies in the previous section when placed in 0.1 M potassium phosphate, oxygen saturated at pH 7.4 containing 0.1 M glucose at 37°C. A maximum initial power density of 96 µW cm$^{-2}$ at 0.33 V was observed when the cell was polarized with external loads (Fig. 5.15). Here, Med$_3$/BOD at PG electrodes showed a 3 fold increase in cathode current densities when compared to Med$_2$/BOD at derivatized graphite electrodes reported in chapter 3, resulting in a 2-fold improvement in power density in the assembled EFC. Previously, Melanocarpous albomyces based EFCs, Med$_3$ also produced a higher power density of 53 µW cm$^{-2}$ at 0.21 V compared to the same enzyme immobilized with the Med$_2$ (Kavanagh et al., 2009).

In order to check EFC operational stability, Med$_1$/GOx electrode was poised at + 0.2 V vs. Ag/AgCl and the Med$_2$/BOD electrode was poised at + 0.12 V vs. Ag/AgCl in the same cell with saturated oxygen and 0.1 M glucose (Fig. 5.16). After 20 h of continuous operation, anode catalytic currents had dropped by 74%, whilst those for the cathode showed a stable response. After the 20 h period, the maximum cell power density, estimated from power curves and obviously limited by the anode, had dropped to 13 µW cm$^{-2}$ (Fig. 5.15). A comparison of slow-scan CVs recorded prior to and after the polarisation (i.e. 0 h and 20 h) showed an 83% loss in anode catalytic current, supporting the chronoamperometry data (Fig. 5.14). The slow-scan CVs recorded prior to and after the polarisation (i.e. 0 h and 20 h) for the cathodes verified that the signal is stable over this period.
Figure 5.14 Assembled EFC - Catalytic CVs Med$_1$/GOx and Med$_3$/BOD over modified PG electrode cured at 24h, in presence of 0.1 M glucose, saturated oxygen, 0.1 M potassium phosphate, 0.15M NaCl, at 37 °C. Scan rate 5 mV/s. (Grey line – 0h, black line- 20h). (Dashed line – no substrate at 0h).

Figure 5.15 Power density of assembled EFC with Med$_1$/GOx and Med$_3$/BOD over modified PG electrode cured at 24h. (Closed square – 0h and Open Square – 20h).
Figure 5.16 Operational stability in EFC of Med$_1$/GOx poised at +0.2 V vs. Ag/AgCl (dashed line) and Med$_3$/BOD poised at +0.12 V vs. Ag/AgCl (solid line) over modified PG electrode cured at 24h, in presence of 0.1 M glucose, saturated oxygen, 0.1 M potassium phosphate, 0.15M NaCl, at 37 °C. (Arrows indicate CVs and power curves recorded at 0 h and 20h)

In order to probe the reasons for the decrease in EFC power, a comparison of CV recorded initially, in the absence of substrate, with those recorded following removal of EFC electrolyte solution and replacement with fresh electrolyte containing no substrate, after the 20 h polarisation period was undertaken (Fig. 5.17). A major cause for anode current loss, from this study, was found to be a 72% loss of osmium signal (surface coverage?) from films of Med$_1$/GOx (Table 5.2). By comparison films of Med$_3$/BOD retained osmium coverage over the same period.
Figure 5.17 CVs with no substrate (diffusional peaks) for Med₁/GOX and Med₃/BOD recorded at 0 h (dashed line) and 20h (solid line) recorded during the EFC operation.

Table 5.2 Osmium surface coverage after 20 h of the surface modified electrodes of an EFC

<table>
<thead>
<tr>
<th>Surface coverage (Γₐₗₐₜ) EFC</th>
<th>Med₁/GOX</th>
<th>Med₃/BOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Γₐₗₐₜ (nmol/cm²)</td>
<td>0 h</td>
<td>20 h</td>
</tr>
<tr>
<td></td>
<td>0 h</td>
<td>20 h</td>
</tr>
<tr>
<td>18</td>
<td>63</td>
<td>61</td>
</tr>
<tr>
<td>5</td>
<td></td>
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</tr>
</tbody>
</table>
Under a fixed applied potential the anode catalytic current of a Med$_1$/GOx in EFC dropped by 14% more in an oxygen-saturated environment when compared to the electrode conditioned under nitrogen Fig.5.18. This may be due to the instability of Med$_1$ in presence of oxygen as reported elsewhere (Kenausis et al., 1996). Catalytic current loss might also be due to H$_2$O$_2$ production affecting GOx activity with the films (Kleppe, 1966). In an effort to cleave H$_2$O$_2$, a catalase was incorporated within redox polymer hydrogels, however, no improvement in stability was found (Binyamin and Heller, 1999).

Conclusions

Poly (L-lysine) was covalently bound to surface activated carboxylic group introduced at a PG electrode. Enzyme redox hydrogels were then covalently cross-linked with the surface modified PG electrode and each other using an epoxide cross-linker. These films showed an improved catalytic current for both glucose oxidation and oxygen reduction, using appropriate enzymes and redox polymer mediators, when compared to the redox hydrogels at the control, non PLL, non-oxidized, PG electrode. Redox hydrogel electrodes

**Figure 5.18** Comparison of operational stability in EFC of Med$_1$/GOx (black dashed line – presence of oxygen) to the Med$_1$/GOx (blue line – under nitrogen) over modified PG electrode cured at 24h, poised at +0.2 V vs. Ag/AgCl in presence of 0.1 M glucose, 0.1 M potassium phosphate, 0.15 M NaCl, at 37 °C.
cured for 24 hours resulted in a higher parameters characterizing charge transport diffusion ($D^{1/2}C$), higher surface coverages of osmium and improved catalytic currents over control electrodes. An EFC was assembled using 24 h cured surface modified redox hydrogel electrodes with Med$_1$ and GOx for the anode and Med$_3$ and BOD for the cathode, that resulted in a maximum power density of 96 µW cm$^{-2}$ at 0.33 V in physiological conditions. This power density is higher than that observed under similar conditions for films immobilized at derviatized graphite electrodes, in chapter 3. The power density is similar to that observed for the LbL films in chapter 4, but with that result obtained only under non-physiological conditions (pH 5.5). Electrochemical investigation of the response over time of the half-cell in an assembled EFC revealed that the anode was limiting the power output stability, as it lost redox polymer signal over time, while the cathode retained its signal.

In order to extend the operational lifetime of the EFC reported here, an alternate redox polymer that does not contain a labile chlorine ligand might be considered. Alternate enzymes to GOx such as pyranose dehydrogenase (Zafer et al., 2010) and cellobiose dehydrogenase (Tasca et al., 2008) that do not produce H$_2$O$_2$ and are insensitive to oxygen could be used in an anode. Selection of an efficient anodic redox polymer and alternate glucose oxidizing enzyme coupled to the stable cathode reported in the present investigation can extend the life of the operational stability in EFCs.
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6. Electrochemical characterization of *Geobacter sulfurreducens* and *Rhodoferax ferrireducens* biofilms on carbon electrodes

6.1 Introduction

A microbial fuel cell (MFC) can use electroactive bacteria (EAB) for the production of electricity. Bacteria able to reduce metals in anaerobic conditions, such as *Geobacter* sp, *Shewanella* sp, *Geothrix fermentans*, and *Rhodoferax ferrireducens*, transfer electrons through the outer membrane (Lovely, 2008, Gralnick and Newman, 2007, Coates et al., 1999, Shi et al., Finneran et al., 2003) to an electron acceptor. Among these EAB, the electrochemistry of *Geobacter sulfurreducens* has been intensively studied (Dumas et al., 2008a, Busalmen et al., 2010, Fricke et al., 2008). A range of mechanisms of electron transfer from bacteria to anode has been proposed and includes indirect transfer using low molecular weight electron shuttles (e.g. mediators) or electron shuttling proteins (e.g. cytochromes), or direct electron transfer from cell surface redox active proteins or via electron conductive “nano-wires” (pili) produced by the bacteria (Schröder, 2007). *Geobacter sulfurreducens*, a metal-reducing bacterium that is the focus of intense MFC research, has been proposed to be wired to the electrode (anode) via outer cell surface expressed c-type cytochromes during electricity generation (Bond and Lovely, 2003). Apart from *Geobacter sulfurreducens* many other EAB such as *Aeromonas hydrophila* (Pham et al., 2003) and *Geothrix fermentans* (Bond and Lovely, 2005) are found to be electrochemically active. Many studies of MFCs report dynamic behavior in terms of variations in cell potential or current under conditions of biofilm growth, or due to changes in operating conditions or parameters. However, the level of biochemical and biophysical activities and processes are subject to the prevailing growing conditions in an anode reducing community. Little is known or reported about the influence of physiological growing conditions on electrochemical voltammetric behavior.

In order to evaluate the electrochemical behavior of ERB induced to grow on electrodes, techniques such as cyclic voltammetry (CV), differential pulse voltammetry (DPV) and chronoamperometry have been used by researchers (Fricke et al., 2008, Richter et al., 2009, Katuri et al., 2011). In particular CV is a powerful technique as it can permit which measurement of the steady state response of the EAB as a function of anode potential. Voltammetric methods that can measure extracellular electron transfer from viable biofilms of *Geobacter* sp grown on electrodes (Richter et al., 2009, Srikanth et al., 2008) and *Shewanella* sp (Richter et al., 2009) have been reported. Voltammetry allows the evaluation of redox couple potentials and characteristic charge transfer properties based on sweeping
potentials forward and in reverse within a potential range. Using chronoamperometry, electrodes are constantly polarized at a particular potential, which can allow them to act as a sink for the bacteria to deposit electrons continuously while oxidizing substrate. Most of the ERB are only capable of oxidation of simple volatile fatty acids, with acetate being used as a model substrate for most fundamental studies of ERB biofilm responses on electrodes.

In this chapter, electrochemistry of *Geobacter sulfurreducens* and *Rhodoferax ferrireducens* have been evaluated and electrochemical properties are compared. *Geobacter sulfurreducens* is an extensively used model organism in electroanalytical analysis to aid understanding of electrochemical communication between EAB and anode (Fricke et al., 2008, Busalmen et al., 2008, Srikanth et al., 2008, Richter et al., 2010, Katuri et al., 2010). For example: the mechanism of electron transfer, behavior under different growth conditions and substrate availability, influence of applied potentials on biofilms activity and growth, the distinct roles of membrane bound cytochromes (omcB, omcE, omcST, omcZ and pilA) in the extracellular electron transfer, and response to short- and long-term changes in electron acceptor potential, have all been studied.

*Rhodoferax ferrireducens* isolated from aquifer sediments is highly unusual as it metabolizes sugars into CO₂ and transfers electrons to metals directly without any requirement of electrochemical mediators. When studied in an MFC, the recovery of electrons from glucose oxidation was 83% with *Rhodoferax ferrireducens* reportedly transferring electrons effectively to electrode (Chaudhuri and Lovely, 2003). Electrochemical behavior of *Rhodoferax ferrireducens* is still unreported though it has been studied in MFCs.

Taking this into consideration, electrochemical properties of biofilms of *Rhodoferax ferrireducens* induced to grow on electrodes under a fixed applied potential were studied using cyclic voltammetry and chronoamperometry to examine formal potentials of redox couples expressed and current generation profile under various applied potentials. Some of the research reported in this chapter, related to growth of the *Geobacter sulfurreducens* biofilms on glassy carbon electrodes, was conducted by Dr. Krishna Katuri, in collaboration with Saravanan Rengaraj.

### 6.2 Experimental Section

#### 6.2.1 Formation of *Geobacter sulfurreducens* biofilms

Custom built glassy carbon and graphite rod (≈ 2 cm²) (Goodfellow, UK) electrodes were used to form *Geobacter sulfurreducens* biofilms. These were made by shrouding glassy
carbon rods, of various dimensions, exposed in glass tubes using heat-shrink plastic tubing carbon (Alphawire, UK) and establishing an electrical connection at the rear with a 0.3 cm diameter copper rod (Farnell electronics, Ireland) and silver epoxy adhesive (Radionics, Ireland). Verification of electrical connectivity was established by cyclic voltammetry in buffer electrolyte. *Geobacter sulfurreducens* (ATCC 51573, from German Collection of Microorganisms and Cell Culture center) was used as a source of electro-active bacteria. The strain was sub-cultured in 100 mL air tight, rubber septa-sealed, anaerobic syringe bottles containing 70 mL of growth medium. The growth medium was prepared according to the protocol supplied by the culture center (http://www.dsmz.de, medium No. 826). The bacteria, prior to inoculation in the electrochemical cell, were cultured in fumarate-containing *Geobacter* growth medium for ~2 weeks (3 sub-cultures) and subsequently grown in the presence of growth medium and 10 mM acetate (initial concentration) under applied potential in electrochemical cells, with replenishment of acetate periodically when the current decreased to baseline levels. CVs were recorded after one hour following removal of the electrodes from the growth medium and replacement in new medium containing 10 mM acetate. All inoculations were carried out in a sterile anaerobic glove box (Coy Laboratory, USA) and incubations were performed at 30 °C in a sterilized controlled-temperature hot room. Biofilms were formed in duplicate under constant applied potentials (−0.2 V, 0 V, +0.2 V and +0.4 V vs. Ag/AgCl) using a multi-channel potentiostat (CHI-1030a, CH Instruments, USA) in a three electrode electrochemical cell using glassy carbon rods as working electrodes (anodes). Subsequent experiments using graphite rods were conducted for applied potentials of −0.4 V, 0 V and +0.6 V vs. Ag/AgCl.

**6.2.2 Formation of Rhodoferax ferrireducens biofilms**

Custom built graphite rod (~ 1 cm$^2$) (Goodfellow, UK) electrodes, constructed as previously described, were used as anodes to form *Rhodoferax ferrireducens* biofilms. *Rhodoferax ferrireducens* (DSMZ 15236) from (German Collection of Microorganisms and Cell Culture center) was used as a source of electro-active bacteria and grown under strictly anaerobic conditions in a defined medium (DSMZ 1001) at 21±1 °C in 100 ml air tight septa bottles with 70 ml of medium. Electron donor was 10 mM acetate with iron nitritotriacetic acid (Fe (III) – NTA) as electron acceptor. Both electron donor and acceptor were filter sterilized. After repetitive sub-culturing of the bacteria, the resulting cells were harvested by centrifuging at 10000 x for 10 min, 4 °C (Beckman-Coulter, USA). The resulting pellet was washed twice with sterile anaerobic saline solution (0.9 % NaCl solution) to remove
remaining Fe (III) – NTA. All inoculations were carried out in a sterile anaerobic glove box (Coy Laboratory, USA) and incubations were performed at room temperature 20 ± 1°C. Cell pellets were re-suspended with fresh medium and fed into the electrochemical cell to develop biofilms for electroanalysis. Biofilms were formed on duplicate electrodes under constant applied potentials (−0.2 V, 0 V, +0.2 V and +0.4 V vs. Ag/AgCl) using a multi-channel potentiostat (CHI-1030a, CH Instruments, USA) in an electrochemical cell.

### 6.2.3 Electrochemical cell design

Electrochemical cells consisted of 8 graphite or glassy carbon working electrodes (~1 or 2 cm² geometric area), platinum gauze (5 cm x 5 cm) as counter electrode and an Ag/AgCl electrode ((BioAnalytical Systems, USA) as reference inserted in a Teflon capped 100 ml glass cell. Gaps were sealed with silicon adhesive in order to maintain anaerobic conditions during biofilm growth.

### 6.2.4 Scanning electron microscopy analysis (SEM)

Electrodes were removed from the electrochemical cell and fixation undertaken by placing in the following solutions: a) 1% glutaraldehyde, 2% paraformaldehyde, 0.2% picric acid, 10 mM HEPES (pH 7.4) for 1 h, b) 50 mM NaN₃ for 1 h, c) 2% tannic acid for 1 h, d) 1% osmium tetroxide for 2 h, e) 1% thiocarbohydrazide for 30 min, f) 1% osmium tetroxide overnight, and g) 2% uranyl acetate for 2 h, while washing with 10 mM HEPES buffer (pH 7.4) between steps. Then samples were dehydrated in a graded series of aqueous ethanol solutions (10–100%) and finally oven dried (2 hrs at 40°C) to remove residual moisture. The dried samples were mounted over SEM stubs with double-sided conductivity tape and a thin layer of gold metal applied using an automated sputter coater (Emitech, K550) for 1 min. The biofilm samples were then examined using a SEM (Model 4700, Hitachi, Japan).

### 6.3 Results and discussion

Electrode reducing bacteria (ERB) are able to harvest electricity by oxidizing a substrate (electron donor) and transferring electrons to conductive surfaces. These bacteria can provide promising catalysts in MFC and in bio-electrochemical system (BES). One of the most efficient ERB studied to date is *Geobacter sulfurreducens*, a member of the metal-reducing Geobacteraceae family, which is capable of complete oxidation of acetate to carbon dioxide with the anode serving as the sole electron acceptor. Here *Geobacter sulfurreducens*
induced to grow on carbon electrodes by applying potential to study is electron transfer to the anode electrode. Glassy carbon and graphite electrodes have been used as different material sources for growth of the *Geobacter sulfurreducens* in a batch mode.

### 6.3.1 Electrochemistry of *Geobacter sulfurreducens* biofilms on glassy carbon

**Fig. 6.1** shows slow-scan CVs of a *Geobacter sulfurreducens* biofilm in the presence of acetate as substrate, at two time intervals following inoculation, with biofilm growth induced by application of a potential of 0 V vs. Ag/AgCl. A sigmoidal shaped CV is observed, indicative of catalytic oxidation of the substrate by the biofilm and heterogeneous electron transfer to the electrode. Examination of the first derivative CV for the biofilm (**Fig. 6.1.** inset A) reveals at least two distinct redox couples centered at −0.45V and −0.39 V vs. Ag/AgCl, also observed previously ([Fricke et al., 2008](#)) for wild-type *Geobacter sulfurreducens* biofilms, and postulated to be due to the presence of distinct cytochromes, further supported by recent CV analysis of biofilms of mutant *G. sulfurreducens*. These potentials can be compared to average (and non-turnover) midpoint potentials of solubilized multiheme cytochromes implicated in *Geobacter* electron transport such as the periplasmic cytochrome c A (PpcA) purified from *Geobacter sulfurreducens* (−0.37 V vs. Ag/AgCl) ([Lloyd et al., 2003](#)), PpcA expressed in *E. coli* (−0.34 V vs. Ag/AgCl) ([Pessanha et al., 2003](#)), and OmcB purified from *Geobacter sulfurreducens* (−0.39 V vs. Ag/AgCl) ([Magnuson et al., 2001](#)). The CV response of the biofilm recorded under acetate depleted conditions in culture medium again reveals at least two distinct redox couples, further supporting the likelihood that at least two redox species are present in the film (**Fig. 6.1.** inset B). Integration of the charge passed during electrolysis of the biofilm as slow scan rates can yield an estimated projected coverage of $\sim 4 \times 10^{15}$ electroactive moieties/cm$^2$ of geometric electrode area. For a film of estimated thickness, from SEM images **Fig. 6.1b**, of $\sim 3$ µm a rough approximation of $5 \times 10^9$ electrically connected electroactive moieties per bacterium can be evaluated.
Figure 6.1  a) Cyclic voltammogram (1 mV/s) of acetate (10 mM) oxidizing Geobacter sulfurreducens biofilms grown under applied potential at 0 V vs. Ag/AgCl on glassy carbon for 64 hours (grey line) and 474 hours (black line). Inset (A) is the 1st order derivative of the 64 hr biofilm and (B) the CV (1 mV/s) of biofilm under acetate depleted (~160 µM) growth conditions. b) SEM image of Geobacter sulfurreducens biofilms grown under applied potential at 0 V vs. Ag/AgCl on glassy carbon.

In contrast, the electrochemical behavior of Geobacter sulfurreducens grown under external electron acceptor limiting conditions, i.e. no potential applied or fumarate in growth medium, shows a single redox couple with a mid-point potential of −0.665 V (vs. Ag/AgCl electrode) in the early growth stages. The redox peak current scaling linearly with scan rate
indicates a bound redox species on the electrode surface (Fig. 6.2a). A sigmoidal shaped CV appears when 0 V is applied to control biofilms grown under open circuit Fig. 6.2 b.

Figure 6.2  a) Cyclic voltammetric response of Geobacter cells grown under no applied potential (control). b) Cyclic voltammogram (1 mV/s) of acetate (10 mM) oxidizing Geobacter sulfurreducens biofilms grown for 250 hours at open circuit (solid) and under applied potential at 0 V vs. Ag/AgCl on glassy carbon (dashed).

This peak for the single redox couple with a mid-point potential of −0.665 V (vs. Ag/AgCl electrode) for the control biofilms increases over time, with no evidence from slow-scan CV of a steady-state current for acetate oxidation. It was recently hypothesized that, if external electron acceptors are not immediately available in surrounding G. sulfurreducens growing environment, extracytoplasmic cytochromes (Finklestein et al., 2006) can act as
capacitors to store electrons in the periplasm and outer membrane. This phenomenon provides a potential short-term solution to the bacteria, an may explain the emergence of a different signal for biofilms held at open circuit, compared to those held at potentials permitting substrate oxidation.

Since the electrode, in inducing growth of the biofilm, acts as a sink of electrons for the bacteria, the role of applied potential on inducing biofilm electrochemistry is of interest (Schröder, U, 2007, Bond and Lovely, 2003, Fricke et al., 2008). Fig. 6.3 shows the amperometric and CV response of *Geobacter sulfurreducens* biofilms developed under different applied potentials (i.e. steady state polarization) in batch mode operation in the same cell using a multi-channel potentiostat to control the potential applied to individual electrodes versus a common Ag/AgCl reference. The CVs were recorded 5 hrs (A) or 13 hrs (B) after addition of new growth medium containing 10 mM acetate, with periodic replacement of medium over the course of the 250 hrs whenever the current dropped to baseline levels.

Chronoamperometry and CV reveal that increasing the applied potential to induce growth results in increased steady-state current density towards acetate oxidation. This is contrary to observations by Dumas et al. (Dumas et al., 2008a) for growth on stainless steel electrodes, where no growth is observed for applied potentials lower than 0 V vs. Ag/AgCl, but in agreement with reports from the Lovley group (Fricke et al., 2008). The observed increase in biofilm current density with applied potential may be due to enhanced adhesion, due to the increasing charge on the polarized electrode surfaces (Busalmen et al., 2008) and/or from the greater thermodynamic driving force established between the electrode and membrane bound cytochromes of *Geobacter sulfurreducens*. These cytochromes are thought to be the predominant electron carriers from the microbe to the electrode surface, resulting in a higher rate of electron transfer and thus an increased rate of metabolic growth of the bacteria to form thicker films. In addition, as the applied potential is increased the electrochemically active membrane-bound proteins might be over-expressed compared to those in biofilms grown at lower potentials. If this is the case, a higher density of redox species per unit area of electrode would be present which could be a possible reason for the observed higher catalytic current. A similar observation was reported by Busalmen et al. who found that bacterial cell growth of *Geobacter sulfurreducens* increased when 0.6 V instead of 0.1 V (vs. Ag/AgCl) was applied to graphite rods (Busalmen et al., 2008). However, no difference in the redox potential of the resulting films were found between this and other studies (Schröder, U, 2007, Finklestein et al., 2006, Fricke et al., 2008).
6.3.2 Electrochemistry of *Geobacter sulfurreducens* on graphite electrode

In order to extend the study, applied potentials close to acetate oxidation potential -0.4 V vs. Ag/AgCl and much more oxidizing, +0.6 V vs. Ag/AgCl, were used to grow *Geobacter sulfurreducens* biofilm on a graphite electrode with 10 mM acetate as substrate. In addition, *Geobacter sulfurreducens* biofilm grown on graphite electrodes under an applied potential of 0 V vs. Ag/AgCl were studied to allow comparison with the current densities obtained with glassy carbon electrodes. *Geobacter sulfurreducens* biofilms were as grown under different applied potentials for a period of 480 h, with replacement of substrate when the acetate oxidation currents decreased to baseline levels. CVs were recorded, under the substrate limited conditions at the end of the 480 h. Replacement of medium with a fresh growth medium was then undertaken and chronoamperometry and a cyclic voltammogram of the biofilms, after the 480 h of initial growth, recorded (Fig 6.4, 6.5 & 6.6). After addition of the new medium and acetate (10 mM), an increase in oxidation current was evident for all the electrodes. Chronoamperometry data shows that the *Geobacter sulfurreducens* biofilm grown
under 0 V (Fig. 6.5) applied potential showed maximum current densities ~ 5 A/m² when compared to the −0.4 V (Fig. 6.4) and 0.6 V (Fig. 6.6) applied potential with current densities closer to 0.1 A/m². A similar magnitude of current density of 5.5 A/m² was obtained with *Geobacter sulfurreducens* on a graphite rod grown under 0 V vs. Ag/AgCl reported previously (Katuri *et al.*, 2011). When compared to glassy carbon electrodes, current density is 8 times higher with a graphite electrode polarized at 0 V vs. Ag/AgCl. This may be due to the higher surface roughness factor of approximately 5 for graphite, versus 1.6 reported for glassy carbon (Weigel *et al.*, 2007). Electrode roughness allows more bacterial colonization for more efficient electron transfer, which could have contributed to the higher current densities with the graphite electrode. At the −0.4 V applied potential where a lower driving force exists for acetate oxidation (−0.48 V vs. Ag/AgCl oxidation potential for acetate) (Finkelstein *et al.*, 2006), a current density of 0.065 A/m² was obtained. This lower current density as a function of potential is as observed for biofilms grown on glassy carbon electrodes. At 0.6 V a maximum current density of 0.095 A/m² was documented.

Dumas *et al.* obtained 8 A/m² for acetate oxidation by biofilms of *Geobacter sulfurreducens* grown on a graphite electrode when polarized at +0.2 V vs. Ag/AgCl. This was compared to current densities of 2.4 A/m² for the same system grown on stainless steel (Dumas *et al.*, 2008a b). Yi *et al.* observed that an isolate designated as *Geobacter sulfurreducens* (KN400) grew more rapidly and produced a high current density of ~ 2.7 A/m² when compared to a wild strain which produced 1.5 A/m² when polarized at -0.4 V vs. Ag/AgCl (Yi *et al.*, 2009) on graphite electrodes. In our case catalytic currents are evident at - 0.4 V, but comparatively lower than for a biofilm grown under 0 V.
Figure 6.4 a) Amperometric response of *Geobacter sulfurreducens* 480 h biofilm (batch mode) grown on graphite electrode under -0.4 V vs. Ag/AgCl (Black arrow – addition of acetate (10 mM), Green arrow – CV recorded). b) CVs of (1mV/s) in acetate (10 mM), and acetate limiting conditions
Figure 6.5 a) Amperometric response of *Geobacter sulfurreducens* 480 h biofilm (batch mode) grown on graphite electrode under 0 V vs. Ag/AgCl (Black arrow – addition of acetate (10 mM), Green arrow – CV recorded). b) CVs of (1 mV/s) in acetate (10 mM), and acetate limiting conditions.
Figure 6.6 a) Amperometric response of *Geobacter sulfurreducens* 480 h biofilm (batch mode) grown on graphite electrode under 0.6 V vs. Ag/AgCl (Black arrow – addition of acetate (10 mM), Green arrow – CV recorded). b) CVs of (1mV/s) in acetate (10 mM), and acetate limiting conditions
CVs were recorded at 1 mV/s under substrate-limited conditions and after acetate addition (CV recorded at ~17 h after acetate addition where the current plateaued) to see the difference in the electrocatalytic response. Electrodes at −0.4 and 0 V produced a sigmoidal shaped catalytic CV starting at −0.5 V vs. Ag/AgCl confirming acetate oxidation, upon addition of acetate substrate. Catalytic currents produced in CVs are comparable to the currents produced using chronoamperometry. Maximum current densities of 0.12 A/m² and 4.7 A/m² where observed at applied potentials of −0.4 and 0 V vs. Ag/AgCl respectively from the slow scan catalytic CVs. Interestingly, electrodes polarized at +0.6 V vs.Ag/AgCl showed current production in chronoamperometry, but no evidence of a catalytic response was found up to potentials of 0.2 V. With the graphite electrode at 0 V vs. Ag/AgCl) poised potential, the biofilm grew much more rapidly and produced a higher maximum current than did biofilms developed on the electrodes poised at −0.4 V and +0.6 V. This demonstrated that the appropriate potential in the polarized electrodes directs the *Geobacter sulfurreducens* electron transport pathway, like bacteria triggers different pathway to use wide range of electron acceptor present in the nature.

*Geobacter sulfurreducens* has been shown to transfer electrons directly to the electrode under applied potential and has been extensively studied. In order to expand the knowledge to the new bacterial species, *Rhodoferax ferrireducens* an isolate from anoxic subsurface sediments (Finneran et al., 2003) as dissimilatory Fe (III) – reducing microorganism was considered for the study. This bacterium can grow at temperatures 4°C to 30°C with the optimum at 25°C. To determine whether it can directly transfer electrons to the electrode, growth of the *Rhodoferax ferrireducens* was induced by applied potential in an electrochemical cell at room temperature 20±2°C. Electrochemistry of the *Rhodoferax ferrireducens* is still unknown and this is the first preliminary study to show its electrochemical behavior.

### 6.3.3 Electrochemistry of *Rhodoferax ferrireducens* on graphite electrode

Similar to *Geobacter sulfurreducens*, *Rhodoferax ferrireducens* was analyzed for its expression of electron donating redox couples (membrane bound cytochromes?) as a function of different applied potentials (−0.2, 0, + 0.2 and + 0.4 V vs Ag/AgCl) to a graphite electrode within a similar electrochemical cell. *Rhodoferax ferrireducens* was cultivated in anaerobic air tight septa bottles with acetate as the sole electron donor and iron nitrilotriacetic acid (Fe
(III) – NTA) as the electron acceptor at room temperature 20 ± 1°C. Cells were harvested and pellets were washed with fresh saline water and inoculated in the electrochemical cell with acetate (10 mM) as the electron donor and a graphite electrode as the electron acceptor instead of nitrilotriacetic acid (Fe (III) – NTA) at 20 ± 1°C. The potential was applied to the electrode, with a duplicate for each potential and a control (non potentiostated electrodes ie., at open circuit) with constant stirring to avoid mass transfer limitations.

Interestingly, the electrochemistry of \textit{Rhodoferax ferrireducens} varied with respect to the applied potential. For example, \textbf{Fig. 6.7a} shows the chronoamperomteric response of biofilms grown under −0.2 V \textit{vs.} Ag/AgCl over a 400 h period, with additions of acetate, and replacement of media (leaving the bacteria to settle in the cell) periodically(indicated by the arrows). Current evolution was slow with almost 100 hours of a lag phase, and then showed a maximum current density of ~0.008 A/m² over a relatively short period, with the signal returning to current densities of ~0.002 A/m² over the remaining period, regardless of acetate or medium replacement. CVs were recorded at regular intervals to monitor the development redox couple electrochemistry during constant polarization. Slow scan rate CVs show the appearance of at least three types of redox couples \textbf{Fig. 6.7b}. Development of a redox couple was observed at −0.6 V \textit{vs.} Ag/AgCl, −0.27 V \textit{vs.} Ag/AgCl and at +0.4 V \textit{vs.} Ag/AgCl. It seems that redox couple at 0.4 V is related to either the medium or the bacterium in solution. It appears to be present in responses for all conditions.\textbf{Fig. 6.8a} shows the electrochemistry of biofilms grown under 0 V \textit{vs.} Ag/AgCl. A similar trend is seen here, with a 100 hours of a lag phase followed by a sharp increase in current thereafter reaching ~ 0.015 A/m² before the current dropped drastically. Similarly, three redox couples were expressed with higher peak currents emerging for the redox couple at −0.27 V \textit{vs.} Ag/AgCl when compared to the redox couple at −0.6 V \textit{vs.} Ag/AgCl and +0.4 V \textit{vs.} Ag/AgCl and to that for films developed at −0.2 V \textbf{Fig. 6.8b}. For the biofilms grown under +0.2 V \textit{vs.} Ag/AgCl, similar trends to that of the biofilm grown under 0 V \textit{vs.} Ag/AgCl were observed. \textbf{Fig. 6.9}. \textbf{Fig. 6.10a} shows the electrochemistry of the electrodes polarized at +0.4 V \textit{vs.} Ag/AgCl over the same period. A single redox couple pattern was observed initially under these conditions, that may be due to the presence of redox active moieties in the growth medium, as evident from the signal of electrodes in the medium at time 0 (\textbf{Fig. 6.10b}). Unlike \textit{Geobacter sulfurreducens}, no sigmoidal shaped CV was observed with the \textit{Rhodoferax ferrireducens}, even at a scan rate of 1 mV/s.
Figure 6.7  a) Chronoamperometry of *Rhodoferax ferrireducens* operated at -0.2 V vs. Ag/AgCl. b) CVs of *Rhodoferax ferrireducens* biofilm recorded at 1 mV/s, gray (96 h biofilm), blue (120 h biofilm), brown (250 h biofilm), saffron (274 h biofilm). Insert shows the CVs of medium with acetate 10 mM and *Rhodoferax ferrireducens* at the outset of the batch experiments (no biofilm) at 5 mV/s.
Figure 6.8  a) Chronoamperometry of *Rhodoferax ferrireducens* operated at 0 V vs. Ag/AgCl.

b) CVs of *Rhodoferax ferrireducens* biofilm recorded at 1 mV/s, gray (96 h biofilm), blue (120 h biofilm), brown (250 h biofilm), saffron (274 h biofilm). Insert shows the CVs of 0h biofilm (with fresh medium with acetate 10 mM and *Rhodoferax ferrireducens*) at 5 mV/s.
Figure 6.9 a) Chronoamperometry of *Rhodoferax ferrireducens* operated at + 0.2 V vs. Ag/AgCl. b) CVs of *Rhodoferax ferrireducens* biofilm recorded at 1 mV/s, gray (96 h biofilm), blue (120 h biofilm), brown (250 h biofilm), saffron (274 h biofilm). Insert shows the CVs of 0h biofilm (with fresh medium with acetate 10 mM and *Rhodoferax ferrireducens*) at 5 mV/s.
Figure 6.10 a) Chronoamperometry of *Rhodoferax ferrireducens* operated at + 0.4 V vs. Ag/AgCl. b) CVs of *Rhodoferax ferrireducens* biofilm recorded at 1 mV/s, gray (96 h biofilm), blue (120 h biofilm), brown (250 h biofilm), saffron (274 h biofilm). Insert shows the CVs of 0h biofilm (with fresh medium with acetate 10 mM and *Rhodoferax ferrireducens*) at 5 mV/s.
Slower scan cyclic voltammograms at 0.1 mV/s were recorded in the presence of acetate, in an attempt to verify if electrocatalysis can occur on the timescale of these experiments rather than at a scan rate of 1 mV/s. The CVs, apart that recorded for biofilms grown at an applied potential of 0.4 V vs. Ag/AgCl, showed slight steady state acetate oxidation currents, indicative of slow catalytic oxidation of the substrate by the biofilm (Fig. 6.11). A slow growth pattern of *Rhodoferax ferrireducens* might be the reason for low current values for acetate oxidation (Finneran *et al.*, 2003). Not all electrode reducing bacteria can efficiently oxidize acetate. For example, Xu and Lu (2011) reported a new exoelectrogenic bacteria *Citrobacter* sp which yielded very low current densities for oxidation of acetate when compared to other substrates such as glycerol, sucrose, lactose and glucose (Xu and Lu, 2011). In previously reported MFCs, Chaudhuri and Lovely (2003) and Liu *et al.* (2007) used *Rhodoferax ferrireducens* as a biocatalyst, providing glucose as a carbon source rather than acetate for the generation of electricity (Chaudhuri and Lovely, 2003, Liu *et al.*, 2007).

![Figure 6.11](image.png)

**Figure 6.11** Slow- scan cyclic voltammetry of *Rhodoferax ferrireducens* (144 h biofilm) at 0.1 mV/s.
In addition to glucose, *Rhodoferax ferrireducens* was also capable of producing current from other sugars, such as fructose, sucrose and xylose (Chaudhuri and Lovely, 2003). Current densities documented here with *Rhodoferax ferrireducens* induced to grow at different applied potentials are similar to the current densities observed for glucose oxidation in an MFC that uses *Rhodoferax ferrireducens* biofilms as an anode (Chaudhuri and Lovely, 2003). In order to explore electrocatalytic response of *Rhodoferax ferrireducens* it may be necessary to extend the study to other sugars, particularly with glucose.

### 6.3.4 Effect of applied potential on biofilms grown under different applied potentials

In order to check the influence of applied potential over redox couple expression, experiments were conducted by changing the applied potential for a short time at the same electrode. Fig. 6.12 shows the disappearance of a signal at a potential of −0.2 V when the applied potential at a biofilm was altered to −0.6 V for ~24 h, for biofilms previously grown at a potential of −0.2 V. The lower currents found in chronoamperometry experiments support the CV data (disappearance of one of the redox couples). This may be due to an applied potential range which is downhill from the oxidation potential of acetate, −0.48 V vs. Ag/AgCl (Finkelstein et al., 2006), and so sufficient driving force is not provided to enable acetate oxidation at this potential. A redox couple was detected centered at a potential of −0.71 V. Multiheme cytochromes are known to be expressed in adverse conditions to store electrons when suitable electron acceptors are absent (Bancroft et al., 1981, Katuri et al., 2010). These proteins might permit the bacteria to conserve energy in absence of electron acceptors. The presence of a peak at such negative potentials could provide a pathway for *Rhodoferax ferrireducens* to conserve energy. Or maybe, as a redox signal in this region of the voltammogram is present in all of the previous scans, regardless of applied potential to induce bacterial growth, it is simply a redox couple (bacterial) that can exchange electrons with the electrode, but is not involved in the substrate oxidation process.
Figure 6.12 Chronoamperometry of *Rhodoferax ferrireducens* biofilm at -0.2 V vs. Ag/AgCl (gray) and when the potential was switched to -0.6 V vs. Ag/AgCl (black), for biofilms grown under an applied potential of -0.2 V over the previous 672 h. Inset: CV recorded before and 24 h after the switch in potentials at a scan rate of 5 mV/s.

In another experiment an electrode that was polarized at 0 V had its potential switched to +0.4 V. The chronoamperometric current increased and a similar disappearance of the peak at -0.27 V vs. Ag/AgCl as that observed for a switch to -0.6 V was observed (Fig. 6.13).
Figure 6.13 CVs of *Rhodoferax ferrireducens* biofilm recorded at poised potential at 0 V vs. Ag/AgCl (gray) and the same electrode switched to 0.4 V vs. Ag/AgCl (black) at end of the experiment (672 h biofilm). (CV shows the disappearance of redox couple that follows the trend of biofilm developed at 0.4V applied potential). Scan rate 5 mV/s.

Control experiments without applied potentials (electrode placed in the same electrochemical cell, non potentiostated electrodes) did not show any expression of a redox couple. Induction of redox expression might also be possible (Fig. 6.14) as when these control electrodes were conditioned with an applied potential at -0.2 V, expression of a redox couple at around −0.27 V vs. Ag/AgCl appeared. These phenomena strongly support a hypothesis that *Rhodoferax ferrireducens* can suppress or express membrane bound proteins according to the potential held in the polarized electrodes. It shows that the electrode acts as an electron sink and the expression of the protein responsible for direct electron transfer is triggered according to the applied potential, mimicking the natural system.
At present the proteins expressed by *Rhodoferax ferrireducens* responsible for the redox signals are unidentified. However the redox couple at ~ −0.3 V vs. Ag/AgCl for biofilms of this bacterium on graphite electrodes can be compared to the redox potential of periplasmic cytochrome c (PpcA) purified from *G. sulfurreducens* (~−0.37 V vs. Ag/AgCl) (Lloyd *et al.*, 2003), PpcA expressed in *E. coli* (~−0.34 V vs. Ag/AgCl), (Pessanha *et al.*, 2003) and OmcB purified from *G. sulfurreducens* (~−0.39 V vs. Ag/AgCl) (Magnuson *et al.*, 2001). The membrane bound pathways composed by PpcA, OmcB and OmcS are responsible for reduction of iron by certain metalloreductases. The redox couple expressed from the biofilm grown under +0.4 V applied potential shows an oxidation peak at 0.76 V and a reduction peak 0.28V with (E_{1/2} = 0.53V vs. Ag/AgCl), similar to the reduction potentials of high potential acceptors such as vanadates (Ortiz – Bernad *et al.*, 2004). Similarly, *Geobactersulfurreducens* have shown to express a redox couple at higher redox potentials.
under higher applied potentials. It is clear that the applied potential directs the electron transfer pathway to electrodes in these ERB.

Fig. 6.15 shows the SEM image of *Rhodoferax ferrireducens* obtained from the electrode that was polarized at 0 V. The presence of rod shaped bacteria attached to the graphite electrode is obvious from the SEM image. The bacteria seem to be scattered over the graphite electrode with coverage much less than a monolayer unlike the *Geobacter sulfurreducens* that forms a thick biofilm. The cells were short, straight, rod shaped, approximately 5µm long. Generally biofilm formation influences the current production. It is evident from the SEM image of *Rhodoferax ferrireducens* that the lower current densities are due to low bacterial colonization over the electrode.

![Figure 6.15](image.png)

**Figure 6.15** Scanning electron microscopy of *Rhodoferax ferrireducens* (biofilms 672 h) grown under applied potential 0 V vs. Ag/AgCl. The electrode was switched to 0.4 V for 24 h on graphite electrode prior to SEM imaging.

### 6.4 Conclusions

In conclusion, it has been demonstrated that controlled growth of electroactive bacteria, such as *Geobacter sulfurreducens* and *Rhodoferax ferrireducens* can be induced by application of a fixed potential difference between the working and reference electrodes, resulting in films that show multiple redox transitions at potentials close to redox potentials reported for cytochromes. Potential control induced biofilm growth of
"Geobactersulfurreducens" on glassy carbon and graphite electrodes produced films capable of sustaining catalytic steady-state currents for acetate oxidation after short lag times. The current density scaled with applied potential, proposed to be through a combination of (a) increased adhesion of bacteria to the surfaces, and (b) a greater driving force for bacterial metabolism, possibly inducing increased biomass, and/or over-expression of redox-transferring moieties within the bacteria. Expression of a redox couple by "Rhodoferax ferrireducens" was also induced by applied potentials at the electrode. Slow growth and slow acetate oxidation resulted in low catalytic currents for this system. Currents indicative of acetate substrate oxidation were observed only at low scan rates of 0.1 mV/s in CV. Further investigation is required to determine electrocatalytic properties for oxidation of substances other than acetate, such as sugars (e.g., glucose), which have been used as a substrate for "Rhodoferax ferrireducens" in an MFC reported previously, that yielded high coulombic efficiency (Chaudhuri and Lovely, 2003).

In general, the aim of this work was to induce the growth of "Geobactersulfurreducens" and "Rhodoferax ferrireducens" under applied potential and to use voltammetry to try elucidate whether electron transfer from bacteria to electrode can occur. From observations, biofilms of "Geobactersulfurreducens" are superior for the generation of current through electrocatalytic oxidation of acetate, yielding higher current densities, when compared to "Rhodoferax ferrireducens". Catalytic currents increase after a short lag phase with "Geobactersulfurreducens". This could be advantageous for acclimatization at the anode or in microbial electrolysis cells for a quick startup of reactors.
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7. Conclusions and future directions

7.1 Enzymatic fuel cells

Enzymatic fuel cells utilize redox enzymes and mediators for oxidation/reduction reactions that facilitate electron transfer between substrate and electrode, converting chemical energy to electrical power. An EFC has a potential to provide power to implanted devices that oxidize/reduce the substrate in vivo. Due to enzyme specificity, membrane separation is not required for anode and cathode which makes it possible to build miniature devices. Performance of EFC mainly depends on the a) nature of biocatalyst, b) the connection of biocatalyst and mediator to the electrode surface, c) electron transfer kinetics between the substrate and electrode, d) the stability and reproducibility of the layer and the electrically active surface area. Development of electrodes based on biocatalyst immobilization, coupling chemistry and surface engineering could contribute to improvement of the stability of such systems for application to long term operation. Development of miniature semi-implantable glucose/O\textsubscript{2} systems to provide power for the lifetime of an implanted glucose sensor (typically <1 week) and be discarded after their first and only use is more realistic, thereby eliminating the need for longer term stability. This thesis attempts to evaluate different biocatalysts and mediators and looks at different methods of immobilization to improve the output and long term stability of enzyme electrodes.

Chapter 2 focuses on investigating the interaction of enzymes and mediators in solution phase. Rate constants for enzyme-mediator interactions were evaluated for different mediators of GOx. The methodology of evaluating enzyme-mediator interaction, based on a standardized method of measuring signals and calculating relative rate constants, allowing for the evaluation and screen of potential mediators with respect to GOx, were reported. A range of complexes of general formula Os(N-N)\textsubscript{2}Cl\textsubscript{2}, where N-N represents a bipyridine ligand, were characterized. The corresponding redox potentials of these complexes could be manipulated by substitution of electron withdrawing or electron donating groups in the 4 and 4' positions of the bipyridine ligand, creating a library of complexes that could potentially be used as mediators for a variety of different enzymes. Complexes with the general formula [Os(N-N)\textsubscript{2}(4-AMP)Cl].PF\textsubscript{6} and [Os(N-N)\textsubscript{2}(4-AEP)Cl].PF\textsubscript{6} displayed efficient electron transfer rates with GOx indicating that the addition of the 4-AMP and 4-AEP ligand leads to a favored interaction between the GOx and mediator. Mediator kinetics with wide range of redox potentials allowed for the selection of appropriate mediator for construction of prototype solution phase enzymatic fuel cells, again providing a means to rapidly evaluate
anode and cathode components of fuel cells. Despite the fact that solution phase kinetics differ from immobilized systems, estimation of the relative rate constants gives some insight into enzyme-mediator interactions and allows for a rapid screening process for evaluation of potential biocatalyst and mediators.

In order to assist with subsequent immobilization the initial complexes were coordinated, via ligand substitution of one of the chlorines, to polymeric backbones of poly(N-vinylimidazole). Chapter 3 focuses on an immobilization strategy based on electrodes modified with films of enzyme and osmium redox polymer, cross linked with poly (ethylene glycol) diglycidyl ether. Films of redox hydrogels and enzymes formed at graphite and glassy carbon electrodes were evaluated for operation in a membrane-less glucose/O₂ enzymatic fuel cell. At physiological conditions (pH 7.4) negligible oxygen reduction currents are observed for GC electrodes coated with an adsorbed film of redox polymer and Thlacc, severely impeding its application in a potential EFC operating under physiological conditions. Use of a BOD cathode improved electrocatalysis under physiological conditions providing power output of 10 µW cm⁻² when combined with a GOx-based anode. A four-fold increase in power output is obtained by replacement of GC with graphite electrodes demonstrating the effect of surface roughness on signals. Graphite electrodes derivatized to introduce amino groups, prepared by electrochemical reduction of aryl diazonium salts, for chemical anchoring the biocatalytic films retained 70% power at 24 h when used for anchoring the redox hydrogels and enzymes to construct EFCs, whereas fuel cells prepared without chemical anchoring to graphite retained only 10% of power over the same interval. Nonetheless, even using surface modified electrodes a gradual loss in maximum power density is evident over time.

Increasing the enzyme and mediator loading at electrode surface can enhance the biocatalytic currents at anode and cathode, as seen in chapter 3 by using graphite instead of GC. As an alternative approach, Layer- by –Layer self-assembly based on electrostatic adsorption of charged polymers and enzymes that forms a 3D electrocatalytic structure at graphite electrodes, is reported in chapter 4. A threefold increase in current densities were obtained with two bilayer assembly of [Os(4,4'-dimethoxy-2,2'-bipyridine)₂(polyvinylimidazole)_10Cl]⁺/GOx compared to one bilayer assembly. These anodes, in a membrane-less glucose/O₂ EFC, when combined with two bilayer assembly of [Os(4,4'-dichloro-2,2'-bipyridine)₂(polyvinylimidazole)_10Cl]⁺/laccase as a cathode, provided maximum power densities of 40 µWcm⁻² at pH 7.4 or 103 µWcm⁻² at pH 5.5. The LBL technique improved the EFC performance in terms of maximum power density when
compared to EFC assembled using physisorption of single layers on redox hydrogels and enzymes. It is expected that formation of more than two layers will yield higher power density EFCs. It would also be interesting to attempt to replace the acidophilic laccase used for the cathode in these studies with a BOD that is more active under physiological conditions, as shown in chapter 3.

Chapter 5 focused on examining the operational stability of redox hydrogels formed at oxidized pyrolytic graphite electrode compared to control electrodes that were not oxidized. A PLL support was covalently bound to the surface activated carboxylic group at the oxidized PG electrode. When appropriate enzymes and redox hydrogels were covalently cross-linked, using an epoxide crosslinker, with the surface modified PG electrode an improved catalytic current when compared to the redox hydrogels at control PG electrodes was obtained, for both glucose oxidation and oxygen reduction. Redox hydrogels that were cured for 24 hours at the modified PG electrode resulted in improved catalytic currents over those cured for 48 hours. Such an approach was thus selected to form a membrane-less EFC with \([\text{Os}(4,4'-\text{dimethoxy-2,2'-bipyridine})_2(\text{polyvinylimidazole})_{10}\text{Cl}]^{+}/\text{GOx}\) for glucose oxidation and \([\text{Os}(2,2'-\text{bipyridine})_2(\text{polyvinylimidazole})_{10}\text{Cl}]^{+}/\text{BOD}\) for oxygen reduction, yielding a maximum power density of 96 µW cm\(^{-2}\) at 0.33 V under physiological conditions, a threefold improvement on the power density over the systems described in chapter 3 and 4 under the same conditions. An attempt has been made to elucidate the reason for catalytic current loss. Polarization studies revealed that the anode was limiting the EFC stability as the signal for the anode osmium-based redox couple decreased as a function of time compared to that for the cathode osmium-based redox couple, which showed a stable catalytic current.

Stabilization of biocatalysts for the extended operation of EFC is one of the main obstacles for implementation of these implantable devices. Improvements in EFC can be achieved by replacing the anode mediator, which is the limiting factor for the stable EFC operation. An example shown by Mao et al. with their redox polymer PVP-\([\text{Os}(\text{N,N'}-\text{dialkylated-2,2'biimidazole})_3]^{2+/3+}\) can be used instead of the \([\text{Os}(4,4'-\text{dimethoxy-2,2'-bipyridine})_2(\text{polyvinylimidazole})_{10}\text{Cl}]^{+}\) which could improve the stability of the redox hydrogels, as the chlorine ligand exchange reaction, presumed to contribute to some of the decay in current, is eliminated (Mao et al., 2003). This proposed redox polymer with 13 atom spacer arms attached to the back bone of redox center that enhances collisional electron transfer, which could also yield a high catalytic current for glucose oxidation at the anode (Fig 7.1).
Figure 7.1 Structure of proposed anodic osmium polymer with a 13-atom flexible spacer between the polymer backbone and the osmium complex.

For an EFC operation with the fuel and the oxidant in the same solution, low sensitivity of the anode reaction to oxygen is very important. This issue has been experimentally investigated in chapter 5 that showed use of GOx at the anode revealed a 14% drop in anode catalytic currents in the presence of oxygen compared to the system without oxygen (under nitrogen). Alternative enzymes to GOx such as dehydrogenases (e.g., cellobiose dehydrogenase or glucose dehydrogenases) that do not produce hydrogen peroxide could be used to prevent this. It is anticipated that use of these proposed redox polymers and enzymes could improve the stability of EFC operation shown in chapters 3, 4 and 5.

7.2 Microbial fuel cells

Similar to the enzymes, certain bacteria can catalyze the electrooxidation of substrates and effect electron transfer to the electrode. This is the basis for catalytic current generation at anodes in MFCs for production of electrical power. In contrast to the enzyme electrocatalysis, catalytic currents increase as the bacteria grows as long as the substrate is provided. Bacteria can undertake direct electron transfer to the electrode without any addition/incorporation of artificial mediators. Chapter 6 examines the electrochemistry of
Geobacter sulfurreducens and Rhodoferax ferrireducens biofilms developed at electrodes under an applied potential. It is evident that bacterial electron transfer to the electrode is induced by the applied potential leading to different rates of growth in signal for catalytic oxidation of substrate. This is the first report showing that the Rhodoferax ferrireducens can be induced to growth by applied potentials and a hypothesis that they express different redox couple based on the environment.

The Geobactersulfurreducens films are superior for electrocatalytic oxidation of acetate yielding high current density when compared to Rhodoferax ferrireducens. This initial study reported with Rhodoferax ferrireducens needs further investigation with different substrates such as glucose in order to verify its electrocatalytic properties comparable to Geobactersulfurreducens.

Future technological applications of MFC are being explored. One such approach could focus on combining anaerobic digestion (AD) that treats wastewater with the production of biogas, with MFCs. The volatile fatty acids produced during the AD process can oxidized by the microbes at anodes in an MFC and can be used to drive the production of electricity. These bio-electrochemical systems that are driven by bacteria can also be used for microbial electrosynthesis. Microbial fuel cell cathodes can be used to synthesise bioproducts such as hydrogen peroxide, bioplastics from wastewater and CO₂. Hydrogen can be produced by adding power to the anode and cathode to generate hydrogen at the cathode.
References