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Controlled drug delivery from polypyrrole powered by enzymatic bioelectrodes

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Intelligent Polymer Research Institute, ARC Centre of Excellence for Electromaterials Science

Controlled drug delivery from polypyrrole powered by enzymatic bioelectrodes

Willo Grosse
Bachelor of Nanotechnology (Honours)

A thesis submitted in fulfilment of the requirements for the award of the degree Doctor of Philosophy

from
University of Wollongong

June 2013
I, Willo Maree Grosse, declare that this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Chemistry, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Willo Grosse

Date: 5\textsuperscript{th} July 2013
Abstract

Galvanically coupling enzymatic bioelectrodes with drug loaded conducting polymers to develop a controlled drug delivery system is investigated. The system is designed to be a novel and sophisticated approach to in vivo drug delivery for the treatment of epilepsy. There is a suite of anti-epileptic drugs (AEDs) that are extremely effective at suppressing seizure activity however, their use is restricted due to debilitating side effects. As such, it is of interest to develop novel drug release methods to deliver the AEDs directly to the target area in the brain and in small doses to minimise, and ideally eliminate, the side effects. The significance of using an enzymatic power source lies in its ability to operate off simple sugars naturally present in the human body. Additionally, the on/off switching behaviour of the conducting polymer, polypyrrole (PPy), can be accessed to release the drug when the oxidation state of the polymer is changed. By exposing the glucose oxidase (GOx) bioelectrode to glucose in solution, the catalytic current generated can be harnessed to charge the polymer and instigate drug release on cue.

Experiments were carried out to develop and characterise GOx bioelectrodes based on a direct electron transfer (DET) mechanism facilitated by carbon nanomaterials, namely multi-walled carbon nanotubes (MWNT) and reduced graphene oxide (rGO).

Chapter 3 investigates the development of solvated graphene electrodes (SGE), a hydrogel-like system based on graphene, with immobilised GOx. It is proven through biological protein and kinetic assays that the GOx is stable within the graphene structures and the electrodes can be reproducibly fabricated. Through electrochemical techniques, including cyclic voltammetry, impedance, amperometry and fourier transform alternating current voltammetry, it was revealed, however, that the enzyme is not electrically wired to the solvated graphene matrix, and as such, cannot directly transfer the electrons generated from the oxidation of glucose to the nanostructured electrode. As a result of its inability to access the catalytic current, no useable charge is generated and the structures cannot be used to power drug release from a conducting polymer scaffold.
In Chapter 4, aqueous dispersions of rGO and MWNT are fabricated through a modified chemical reduction method. The significant advantage of the method developed is the omission of any stabilizing compound or organic solvent to obtain stable rGO-MWNT dispersions. Significantly biological entities, such as GOx, can be successfully incorporated into the dispersion. These dispersions were characterised using XPS, SEM, zeta potential and particle size measurements which showed that the dispersion stability is not sacrificed with the addition of GOx, and significantly, the electrical properties of the rGO and MWNTs are maintained. In this study, rGO acts as an effective dispersing agent for MWNTs and does not affect the solubility or electroactivity of the GOx. Bioelectrodes fabricated from these rGO-MWNT-GOx dispersions were characterised electrochemically to test their feasibility in facilitating direct electron transfer (DET) from the redox centre of the enzyme to the electrode. The DET results showed that the specific catalytic current generated at an optimized rGO-MWNT-GOx electrode was 72 µA/µg GOx, which is 3 times more efficient than other literature values for similar systems. The remarkable specific catalytic current can be attributed to the use of purified enzyme, the efficiency of charge transfer within the rGO-MWNT composite and the ability of the electrode to facilitate direct electron transfer. The optimised electrode developed and characterised in Chapter 4 is used in Chapter 5 to power the release of drug from PPy.

The release of the anti-inflammatory drug, Dexamethasone-21-phosphate (DEX), and the anti-convulsant drug, Fosphenytoin (FOS), from PPy films are characterised in Chapter 5. This work investigates, for the first time, the controlled release of FOS from a conducting polymer and galvanically connects the optimised rGO-MWNT-GOx bioelectrode (presented in Chapter 4) to drive the release, also the first of its kind. When the two half cells are connected and the conditions are changed in the bioelectrode cell from 0 mM to 150 mM glucose, there is an increase in the charging current generated, as a result of the additional catalytic current. A corresponding increase in the rate of FOS released was measured in the release cell from 24 to 47 ng/mL. This result shows that the concept of galvanically coupling enzyme-based bioelectrodes with drug loaded conducting polymers to fabricate a controlled drug delivery device is valid.
TO ACCOMPLISH GREAT THINGS, WE MUST DREAM AS WELL AS ACT

- Anatole France
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During the course of this thesis I have asked myself on many occasions, Why? I say it now with a smile on my face because it has become much clearer for me. I was always told to surround myself with good people, to be independent and to seek happiness, and I was lucky enough to find this at IPRI/ACES. To answer my own question, one of the reasons I embarked on this adventure was because of two brilliant and charismatic people, Gordon and Simon.

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<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)</td>
</tr>
<tr>
<td>AC</td>
<td>Alternating current</td>
</tr>
<tr>
<td>AED</td>
<td>Anti-epileptic drugs</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BOD</td>
<td>Bilirubin oxidase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CNT</td>
<td>Carbon nanotube</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>CV</td>
<td>Cyclic voltammetry</td>
</tr>
<tr>
<td>DC</td>
<td>Direct current</td>
</tr>
<tr>
<td>DET</td>
<td>Direct electron transfer</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethasone-21-phosphate disodium salt</td>
</tr>
<tr>
<td>DF</td>
<td>Dilution factor</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalography</td>
</tr>
<tr>
<td>EIS</td>
<td>Electrochemical impedance spectroscopy</td>
</tr>
<tr>
<td>$E_{pa}$</td>
<td>Anodic peak potential</td>
</tr>
<tr>
<td>$E_{pc}$</td>
<td>Cathodic peak potential</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FcMeOH</td>
<td>Ferrocene methanol</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FOS</td>
<td>Fos-phenytoin</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform – infrared spectroscopy</td>
</tr>
<tr>
<td>FTACV</td>
<td>Fourier transform alternating current voltammetry</td>
</tr>
<tr>
<td>GCE</td>
<td>Glassy carbon electrode</td>
</tr>
<tr>
<td>GO</td>
<td>Graphene oxide</td>
</tr>
<tr>
<td>GOx</td>
<td>Glucose oxidase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>MET</td>
<td>Mediated electron transfer</td>
</tr>
<tr>
<td>MWNT</td>
<td>Multi-walled carbon nanotube</td>
</tr>
<tr>
<td>NT3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>OCP</td>
<td>Open circuit potential</td>
</tr>
<tr>
<td>PANi</td>
<td>Polyaniline</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PEDOT</td>
<td>Poly(3,4-ethylenedioxythiophene)</td>
</tr>
<tr>
<td>PEGDGE</td>
<td>Polyethylene glycol diglycidyl ether</td>
</tr>
<tr>
<td>PEI</td>
<td>Poly(ethyleneimine)</td>
</tr>
<tr>
<td>PPy</td>
<td>Polypyrrole</td>
</tr>
<tr>
<td>PTh</td>
<td>Polythiophene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PTS</td>
<td>Para-toluene sulfonic acid</td>
</tr>
<tr>
<td>QCM</td>
<td>Quartz crystal microbalance</td>
</tr>
<tr>
<td>$R_{ct}$</td>
<td>Charge transfer resistance</td>
</tr>
<tr>
<td>rGO</td>
<td>Reduced graphene oxide</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SGE</td>
<td>Solvated graphene electrode</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-visible</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
<tr>
<td>ZRA</td>
<td>Zero-resistance amperometry</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 The treatment of epilepsy

In 2012 the World Health Organization (WHO) published that over 50 million people worldwide suffer from epilepsy\(^1\). The International Bureau of Epilepsy defines an epileptic seizure as “a transient occurrence of signs and/or symptoms due to abnormal and excessive neuronal activity in the brain”. Epilepsy is defined as a disorder of the brain characterised by an enduring predisposition to generate epileptic seizures\(^2\). Figure 1.1 shows a typical Electroencephalography (EEG) signal for a normal patient (a) and a patient undergoing an epileptic seizure (b).

![Figure 1.1](image.png)

**Figure 1.1** (a) An EEG signal for a normal patient and (b) an EEG signal for a patient undergoing an epileptic seizure. Note the labels to the right of each graph represent the bipolar EEG channels selected for analysis. Adapted from Ref\(^3\).

Seizures can significantly affect a person’s quality of life and, of the 50 million epilepsy sufferers worldwide, only 70% are responsive to current treatment options leaving 15 million people to cope with a life of uncontrolled seizures. The most common treatment involves the administration of anti-epileptic drugs (AED) that patients take orally at least once a day. If AEDs are ineffective and the seizures are too severe to go untreated, there are alternate, but higher risk, options including surgery to remove the brain tissue most active during seizures (termed the epilepsy
focus), stimulation of the vagus nerve (using a device which continuously sends electrical pulses through the entire brain as a counter stimulation), or, especially in children, the ketogenic diet (a diet that encourages the production of ketones to replace sugar as the main energy source for the brain).

There is a suite of AEDs that are commonly used to treat epilepsy with many patients responding well to a specific drug, while others respond better to a combination of drugs. There are also a few select anti-epileptic medications that are potent and pose the risk of worsening the condition, among other side effects. Some of these types of medications are restricted and usually administered intravenously. Although AEDs generate debilitating side effects, they can be extremely effective at suppressing severe seizure activity.

If the AEDs are delivered directly to their intended site of action in the body, then undesirable actions on other parts of the body, resulting in side effects, will be minimised. Also to be considered are parameters like the potency of the drug (i.e. the minimum dose to achieve the desired response) and the therapeutic window (i.e. the difference between the minimum effective level and the dose at which the drug becomes toxic). Consequently, a controlled drug delivery device of this type could increase the portion of the afflicted population that can be treated through AEDs and avoid the more risky treatments.

1.2 Thesis overview

This thesis aims to develop a prototype of an implantable controlled drug delivery device designed specifically for the treatment of epilepsy. It will investigate the release of clinically relevant medications from intelligent material constructs and, ultimately, integrate an implantable power source of an enzyme-based bioelectrode. This project is primarily concerned with the fabrication and characterisation of electrodes for the intended application and attention will be paid throughout the thesis to outline the significance of chosen methodologies and materials to the ultimate visionary goal – to treat epilepsy.
1.3 Current state of drug delivery devices

The concept of controlling the delivery of therapeutics in the human body has been investigated since the 1960’s\textsuperscript{8}. Traditional drug delivery methods include the application of ointments and creams to the surface of the skin\textsuperscript{9}, the use of capsules or tablets to be taken either orally or inserted into the gastrointestinal tract, or direct administration into the systemic circulation by intravenous injection\textsuperscript{10}. Although systemic methods, those drug delivery systems that administer drug to the entire body, are still the most commonly used drug delivery routes, they have their limitations. Systemic delivery can result in the presence of a large concentration of drug in the circulatory system that can adversely affect the rest of the body because the drug reaches toxic levels, as shown in Figure 1.2 (a). Although the procedure can be optimised, by reducing the amount administered to ensure the level of drug reaches the minimum effective level threshold but does not reach a toxic level, as shown in Figure 1.2 (b), the drug in the circulatory system passes through the body and quickly drops below the effective level. Hence it is necessary in systemic administration to frequently readminister the drug to maintain the minimum effective drug concentration. In the case of AEDs that are intended to act on brain tissue, the concentration of drug penetrating the blood brain barrier is significantly less than that in the circulatory system\textsuperscript{7,11}. For this reason, daily systemic dosing is required to maintain a therapeutic concentration of drug in the target area\textsuperscript{12}, which also presents patient adherence issues\textsuperscript{13}. Hence, it is of interest to develop new methods of delivering drugs to address these problems and maintain a constant level of drug, as shown in Figure 1.2 (c).
Targeted delivery of therapeutics presents the following advantages over traditional drug delivery methods:\(^{13}\):

- Improved efficiency
- Smaller targeted doses sufficient to elicit treatment
- Reduced side effects, and
- Enhanced patient compliance

Many controlled drug delivery devices are on the market for a range of applications with the majority based on the release of drug from polymer constructs (for a comprehensive review see Table 1.1 on page 6). For many years the focus was on inert polymers, for example silicone, which require surgery to remove the polymer skeleton after the drug is fully released. More recently, biodegradable polymers have attracted attention for this application because of their ability to release drug during their breakdown and for the breakdown products to naturally be excreted from the body. Naturally occurring polymers like peptides, nucleic acids and...
polysaccharides, such as alginate, collagen and chitosan, as well as a suite of synthetic polymers have been used for these applications and are listed by Pillai et al.\textsuperscript{15}.

Although polymeric materials form the majority of controlled drug delivery devices, recent research has focused on modifying polymer properties by using existing and novel manufacturing techniques to more accurately control the release of drug. For example, Halliday \textit{et al.} outline a range of manufacturing techniques such as electrospinning, casting, 3D printing, spray drying and particulate formulations\textsuperscript{7}. The formation of novel polymer structures through advanced manufacturing techniques allows new uses of pre-existing polymer materials which have already undergone Food and Drug Administration (FDA) approval, and hence, offer a less hindered route to commercialisation than using new materials.

There is also a suite of new material composites that release drug in response to external stimuli such as infrared light, magnetic fields and ultrasound, as listed by Timko \textit{et al.}\textsuperscript{16} For example, gold nanoparticles coated with a drug loaded polymer (poly(N-isopropylacrylamide)) can absorb near-infrared light and convert the light energy into heat. As the underlying gold nanoparticles heat up past a critical temperature, the polymer matrix collapses and releases the drug (Figure 1.3). This type of on-cue drug release offers a distinct clinical advantage over systems that release drugs passively or through natural breakdown in the body. These remotely triggered delivery systems allow the control of dose, timing and duration of drug release and offer more advanced treatment options\textsuperscript{16}.

![Figure 1.3](image-url)  
**Figure 1.3** Macroscale poly(N-isopropylacrylamide)-gold nanoparticle composites collapse when heated beyond a critical temperature, expelling drug (green spheres). Adapted from Ref\textsuperscript{6}.  

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\textsuperscript{5} Page 5
Table 1.1 categorizes controlled drug delivery systems into four general classifications, namely, diffusion controlled, water penetration controlled, chemically controlled and regulated systems. Moreover, it outlines the mechanism of drug delivery and examples of commercially available devices. The table is designed to give an overview of the current delivery methods for all applications, not specifically for the treatment of epilepsy, which still utilise systemic delivery methods.

In addition to examples given in Table 1.1 there is an intense research drive to advance existing devices and to develop novel intelligent drug delivery systems to respond to in vivo stimuli, identified as self-regulating\textsuperscript{17} or closed-loop systems\textsuperscript{18}. In the case of treating epilepsy, this would mean delivering a drug in a timely manner in response to the onset of seizure activity. Figure 1.1 (b) shows that a seizure starts in less than one second and, consequently, a device designed to treat epilepsy is required to have a switching speed in a similar time frame. In order to do this, the prediction\textsuperscript{19} or detection\textsuperscript{3} of a seizure would have to be built into the drug delivery device, a concept which is outside the scope of this thesis but a consideration worth mentioning.

Conducting polymer scaffolds have been extensively researched for their application in controlled drug delivery systems\textsuperscript{20}, which will be discussed in the following section. The research in this thesis will look at the biologically relevant conducting polymer polypyrrole (PPy), for its ability to release AEDs on-cue and in a timely manner for application in a drug delivery device to treat epilepsy.

### Table 1.1 Classification of controlled drug delivery systems including the mechanism of release and examples of commercially available products. Adapted and updated from Ref\textsuperscript{14}

<table>
<thead>
<tr>
<th>Type of drug delivery system</th>
<th>Mechanism of release and commercial examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion Controlled</td>
<td>Diffusion of drug through a membrane from a reservoir</td>
</tr>
<tr>
<td>• Reservoir</td>
<td><em>E.g. Ocusert: for the treatment of glaucoma</em>\textsuperscript{21}</td>
</tr>
<tr>
<td>• Bulk</td>
<td><em>E.g. Mirena: Intrauterine device releases contraceptive medication</em>\textsuperscript{22}</td>
</tr>
<tr>
<td></td>
<td>Diffusion of drug through a bulk polymer</td>
</tr>
<tr>
<td></td>
<td><em>E.g. Nexplanon: a subdermal rod implant that releases contraceptive medication by diffusion through a bulk ethylene vinylacetate copolymer</em>\textsuperscript{23}</td>
</tr>
</tbody>
</table>
Chapter One | Introduction

| Water penetration controlled | The osmotic transport of water through a semi permeable membrane activates an osmotic pump and hydrostatic pressure builds up in the system, forcing the drug out. E.g. OROS systems by ALZA corp.
 | Osmotic | E.g. OROS systems by ALZA corp.
 | Swelling | Water uptake by a multilayered polymer construct, which releases enclosed drugs at a rate dependent on the swelling rate of the polymer layers. E.g. Geomatriz by Skypharma.

| Chemically controlled | Based on polymer systems and involves either pure polymer degradation, or a combination of degradation and bulk diffusion. E.g. Resomer® by Sigma-Aldrich is a polymer blend of poly(glycolide) (PGA) and poly(lactide) (PLA) whose by-products are biodegradable.
 | Bulk degradation | E.g. Resomer® by Sigma-Aldrich is a polymer blend of poly(glycolide) (PGA) and poly(lactide) (PLA) whose by-products are biodegradable.

| Regulated systems | Drug delivery controlled by charge transfer as a function of the conducting polymer oxidation state. E.g. To the author’s knowledge no commercial examples exist (see section 1.4 for more details).
 | Magnetic, infrared or ultrasound | External application of magnetic field, infrared or ultrasound to the device to instigate drug delivery. E.g. Diffucaps by Eurand.
 | Chemical | Enzyme-substrate reactions. E.g. Zuplenz: Oral strip made of a cellulose derivative that is broken down in the mouth by enzymes and designed to treat nausea and vomiting after chemotherapy.
 | Electrochemically (Conducting polymers) | Drug delivery controlled by charge transfer as a function of the conducting polymer oxidation state. E.g. To the author’s knowledge no commercial examples exist (see section 1.4 for more details).

1.4 Polypyrrole for controlled drug delivery

1.4.1 Introduction to conducting polymers

Alan MacDiarmid, Alan Heeger and Hideki Shirakawa won the Nobel Prize in Chemistry (2000) for their work on conductive polymers since their discovery in the 1970’s. The discovery that some polymers, traditionally classed as insulating materials, could conduct electricity saw the expansion of a new and exciting field of scientific research. The most practically useful and stable conducting polymers that have emerged in the last few decades of study are based on polypyrrole (PPy), polythiophene (PTH) and polyaniline (PANI). These materials have since found applications in energy storage, photovoltaics, electrochromics,
electromechanical actuation, separation membranes, corrosion protection, chemical sensors, and, of course, controlled delivery devices. Polypyrrole will be studied extensively in this thesis; therefore, it will be introduced in the following sections.

1.4.2 Electrochemical polymerisation

The synthesis of conducting polymers can be achieved through chemical or electrochemical methods, the latter of which was utilised in this study and will be discussed in detail. A three-electrode electrochemical cell is generally used for polymerisation and comprises of a reference electrode, an auxiliary (or counter) electrode and a working electrode (discussed in more detail in Chapter 2 – section 2.2.3.2). The working electrode is the conducting substrate where the polymer film will ‘grow’. The oxidation of pyrrole monomer in solution can be achieved electrochemically by applying a constant current (galvanostatic), constant voltage (potentiostatic) or changing voltage (cyclic voltammetry) to the working electrode. In the monomer solution it is critical to have a suitable doping anion that is incorporated into the insoluble polymer product to balance the charges generated on the polymer during oxidation (discussed in section 1.4.3). The result of this process is a conducting film of polypyrrole doped with the anion on the working electrode. This is shown schematically in Figure 1.4.

![Figure 1.4 Schematic of the electrochemical oxidation of pyrrole monomer in the presence of an anion (A) to form doped polypyrrole, where n is the number of monomer units per dopant anion and m relates to the polymer chain length that determines molecular weight. Adapted from Ref](image-url)

---

8 | Page
Initially, polymerisation starts in solution and eventually oligomers (several monomer units) adsorb onto the working electrode surface when they reach a given molecular weight and become insoluble. These sites are known as nucleation sites and subsequent polymerisation spreads from these points on the electrode surface until the entire working electrode is covered. Sadki et al detail the mechanism of polymerisation for polypyrrole extensively in their review paper. Pyrrole monomer is partially soluble in aqueous mediums making it suitable to use in biomaterial applications because biomolecules, which can be incorporated as anions (A⁻) (Figure 1.4), are usually water-soluble. In electrochemical polymerisation of pyrrole, many experimental parameters can effect the properties of the final film, including dopant/monomer concentrations, temperature, substrate type, solvent, electrodeposition protocol, different dopants and growth time. In this thesis, galvanostatic electrochemical deposition was used to prepare polypyrrole films and the process was monitored through measuring charge (A.s) and potential (V) as a function of time. The charge passed was monitored to ensure that the same amount of polymer was deposited for each sample. Fonner et al. show that the correlation between the charge passed and the amount of polypyrrole deposited is a linear relationship. The voltage channel also gave information on the quality of the film deposited. Polypyrrole oxidises at approximately +0.8 V (vs. Ag|AgCl) and if the growth potential exceeds +1.0 V it indicates the formation of an over-oxidised polymer that will have insulating electrical properties. The monitoring of the growth process is critical to troubleshoot and identify problems with the system.

1.4.3 Structure and electrical conductivity

The fundamental difference between traditional insulating polymers and conducting ones is the conjugation found in conducting polymers that allow charge delocalisation. Conjugation is the repetition of single and double bonds between carbon atoms, as highlighted for polypyrrole in Figure 1.5.
The conjugation of conducting polymers allows the overlapping of \( \text{sp}^2 \)-hybridised orbitals, which is critical for electrical conduction. In its neutral (undoped) state, polypyrrole is a moderate insulator because these overlapping \( \text{sp}^2 \) orbitals are full. This is represented in band theory (Figure 1.6 a) with a large band gap of 4.0 eV for polypyrrole, as calculated using valence-effective Hamiltonian methods. The band gap of a material is the difference between the conduction band (highest occupied molecular orbital, HOMO) and the valence band (lowest unoccupied molecular orbital, LUMO), which is the energy required for an electron to transition across this gap. This transition defines the electrical properties of material. The addition or subtraction of charge from these orbitals is the act of doping, and is the mechanism by which polypyrrole becomes electrically conducting. For polypyrrole, \( p \)-type doping is achieved through oxidation of the polymer in the presence of a ‘doping’ anion, which is incorporated into the structure to balance charge (Figure 1.4). \( p \)-type doping results in the formation of a polaron (a positive charge) that is a charge carrier delocalised across, on average, 4 monomer units in the backbone (represented in Figure 1.7 as a red positive charge). When another polaron is generated it is energetically favourable to form a bipolaron, or a pair of polarons. This is shown schematically in Figure 1.7.
Figure 1.6 Band structure evolution upon doping of polypyrrole (a) undoped, (b) low doping level with non-interacting bipolarons present of the polymer chain, (c) intermediate doping level where bipolaron bands form in the gap and (d) high doping levels. Adapted from Ref

At a low doping level there is very little interaction between bipolarons along the polypyrrole backbone. This is represented in band theory as the appearance of bipolaron states in the gap (Figure 1.6 b). As the doping level is increased to an intermediate level the bipolarons start interacting and form bipolaron bands in the gap (Figure 1.6 c). At high doping levels bipolaron bands merge with the conducting band and the valence band, and the band gap is sufficiently low for the material to be classed as conducting (Figure 1.6 d).
Figure 1.7 The formation of polarons and bipolarons on a polypyrrole chain; where (a) represents the undoped state, (b) represents the formation of a single polaron and (c) represents a bipolaron on the polypyrrole backbone. Polaron can be identified by the red positive charge in this figure.

1.4.4 Electroactivity and drug release mechanism

The term ‘electroactive’ is given to a material that is responsive to electrical stimuli. Conducting polymers are electroactive and can be reversibly switched between the oxidised (conducting) and reduced (insulating) states. During electrochemical switching there is movement of dopants (or counter ions) to balance the charge.
induced on the polymer backbone. Figure 1.8 shows that when the polymer is in an oxidised state, the anion is electrostatically attracted to the positive charge (polaron or biopolaron) on the polymer backbone. Additionally, when the polymer is in a reduced state the positive charge is removed and the relatively negative polymer expels the anion. The mechanism of charge balance is dependent on the size and hence mobility of the doping ion, which for the case of polypyrrole is an anion. If the doping anion is very large it is sterically restricted from being expelled from the polymer matrix upon reduction, and, as such, cations from solution migrate into the structure to balance the charge. If the doping anion is sufficiently small it is expelled from the structure during reduction, again, to balance charge. The latter mechanism is represented in Figure 1.8 and underpins the process of drug release utilised in this thesis because the doping anions (introduced in section 1.4.5) are relatively small. Significantly, the doping anion for this system can be a therapeutic drug. During redox cycling conducting polymers also undergo other property changes that may contribute to the release of the doping ion. These changes include swelling/deswelling, colour changes and changes in mechanical properties and will be discussed in more detail in Chapter 5. It is impossible to separate these changes to understand their individual involvement in the release mechanism.

\[
\begin{align*}
\text{Oxidised} & : [\text{H} \text{N} \text{H}]_n^+ \text{A}^- \\
\text{Reduced} & : [\text{H} \text{N}]_m^0 + \text{A}^- \\
\end{align*}
\]

Figure 1.8 Mechanism of charge balance by anion movement (in the case of a small anion) upon oxidising and reducing doped polypyrrole. Adapted from Ref\(^{51}\)
1.4.5 Controlled drug release

Polypyrrole has been extensively researched for its application in controlled delivery due its ability to incorporate biomolecules and therapeutic drugs, its stability in aqueous environments\textsuperscript{52} and its biocompatibility\textsuperscript{53}. Drug delivery from conducting polymers differs from controlled delivery devices already on the market (see Table 1.2) in that it is dependent on electrical stimulation; additionally, it offers enhanced control, through its electroactivity, to facilitate the on/off release of drug, a property critical for the proposed device to treat epilepsy.

The therapeutic drugs chosen for this thesis, to take the place of anion A$^-$ in Figure 1.8, were the pro-drugs dexamethasone-21-phosphate disodium salt (DEX) and Fos-phenytoin (FOS), whose structures are shown in Figure 1.9. DEX is an anti-inflammatory drug that has been studied extensively as a dopant for polypyrrole\textsuperscript{54–56}. Delivering an anti-inflammatory drug directly to the site of trauma can treat the damage induced on implantation of the proposed brain device. Alternatively, FOS is a pro-drug AED currently used to suppress severe seizures in clinical settings and is delivered intravenously\textsuperscript{57}. Its wider use is prohibited due to its debilitating side effects, including severe allergic reactions, aplastic anaemia, increased suicide risk, irregular cardiac rhythms and deliria\textsuperscript{58} however, if a targeted delivery method is discovered these side effects can be significantly reduced, if not eliminated\textsuperscript{7}. Both these molecules have a phosphate anionic side group, making them appropriate to use as a dopant for polypyrrole (Figure 1.9).
Figure 1.9 Chemical structure of (a) Dexamethasone-21-phosphate disodium salt and (b) Fosphenytoin.

Table 1.2 provides an extensive list of literature that investigates the release of therapeutic agents from polypyrrole under various stimulation protocols. Since Zinger and Miller first studied the use of polypyrrole as a release substrate in 1984, many other groups have studied it for a range of drug delivery applications, including the treatment of schizophrenia, neural electrode coatings and implantable microchips for controlled drug release. The number of publications in this area significantly increased around the year 2000 and continues to interest scientists looking at novel ways to deliver drugs in vivo. One of the primary concerns with implanting drug loaded polypyrrole structures is the need to power them from inside the body. This underpins the next section of this introduction dealing with implantable power sources.
Table 1.2 Literature summary of molecular release from polypyrrole electrodes under the stimulation protocols tabulated. Their intended application is also identified.

<table>
<thead>
<tr>
<th>Year</th>
<th>Molecule released</th>
<th>Stimulation Protocol</th>
<th>Application</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1984</td>
<td>Glutamate (neurotransmitter)</td>
<td>Constant potential (-1.0 V for 120s)</td>
<td>Implantable electrode</td>
<td>59</td>
</tr>
<tr>
<td>1992</td>
<td>Adenosine triphosphate (ATP)</td>
<td>Constant potential (potential step from +0.6 to -0.6 V)</td>
<td>Controlled drug delivery</td>
<td>63</td>
</tr>
<tr>
<td>1994</td>
<td>Acetylsalicylate</td>
<td>Constant potential (-1.0 V)</td>
<td>Controlled drug delivery</td>
<td>64</td>
</tr>
<tr>
<td>1994–1996</td>
<td>Adenosine triphosphate (ATP)</td>
<td>Constant potential (Polarised at +0.6 V, then the film was reduced by stepping the potential down to 0V then to -0.6 V)</td>
<td>Ionic drug and biomolecule release systems</td>
<td>65,66</td>
</tr>
<tr>
<td>1997</td>
<td>Chlorpromazine (used to treat schizophrenia)</td>
<td>Pulsed potential (between +0.2 V and -0.8 V for 2 minute intervals)</td>
<td>Feasibility of QCM as measuring technique</td>
<td>67</td>
</tr>
<tr>
<td>1998</td>
<td>Salicylate (anti-acne drug), naproxen (anti-inflammatory drug), nicoside (vitamin-B) and tosylate (model molecule)</td>
<td>Constant potential (Polarised at +0.5 V, then the film was reduced by stepping the potential down to -0.5 or -0.8 V )</td>
<td>Ion gate membrane</td>
<td>52</td>
</tr>
<tr>
<td>2000</td>
<td>Adenosine triphosphate (ATP)</td>
<td>Constant potential (-0.7 V)</td>
<td>Drug delivery device</td>
<td>68</td>
</tr>
<tr>
<td>2001</td>
<td>Sulfosalicylic acid</td>
<td>Constant potential (-0.5 V)</td>
<td>Controlled drug release</td>
<td>69</td>
</tr>
<tr>
<td>Year</td>
<td>Material/Compound</td>
<td>Method</td>
<td>Application</td>
<td>Page</td>
</tr>
<tr>
<td>------</td>
<td>------------------</td>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>2002</td>
<td>Dexamethasone-21 Phosphate disodium salt (DEX)</td>
<td>Constant potential (-0.7 V)</td>
<td>Controlled drug release</td>
<td>70</td>
</tr>
<tr>
<td>2005</td>
<td>Heparin (anti-coagulant)</td>
<td>Constant current</td>
<td>Controlled drug release</td>
<td>71</td>
</tr>
<tr>
<td>2006</td>
<td>Hexacyanoferrate (model drug)</td>
<td>Constant potential (Polarized at +0.5 V then stepped to increasingly negative potentials (0, -0.1, -0.3, -0.5 and -0.7 V for 10 s and back)</td>
<td>Feasibility study of a measuring technique</td>
<td>72</td>
</tr>
<tr>
<td>2006</td>
<td>Nerve growth factor</td>
<td>Constant potential (0.1 V for 2 hours)</td>
<td>Nerve conduits and neural probes</td>
<td>60</td>
</tr>
<tr>
<td>2006</td>
<td>Biotin and neural growth factor</td>
<td>Constant potential (3 V, applied for 30 sec or 150 sec)</td>
<td>Neural electrode coatings</td>
<td>73</td>
</tr>
<tr>
<td>2006</td>
<td>Dexamethasone-21 Phosphate disodium salt (DEX)</td>
<td>Cyclic voltammetry between -0.8 and +1.3 V</td>
<td>Neural electrode coatings</td>
<td>56</td>
</tr>
<tr>
<td>2006-2010</td>
<td>Neurtrophic protein neurotrophin-3 (growth factor)</td>
<td>100 µs biphasic current pulses at 250 Hz and at a current density of ± 1 mA/cm².</td>
<td>Cochlear implant</td>
<td>51,74–76,77</td>
</tr>
<tr>
<td>2007</td>
<td>Sodium salicylate and sodium naproxene</td>
<td>Constant potentials (between -0.4 and -0.7 V)</td>
<td>Drug-eluting membrane for coronary stents</td>
<td>78</td>
</tr>
<tr>
<td>2007</td>
<td>Adenosine triphosphate (ATP)</td>
<td>Cyclic voltammetry (between 0 and -1.1 V)</td>
<td>Measurement feasibility study</td>
<td>79</td>
</tr>
<tr>
<td>Year</td>
<td>Drug Combination</td>
<td>Methodology</td>
<td>Application</td>
<td>Page</td>
</tr>
<tr>
<td>------</td>
<td>------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>2009</td>
<td>Sulfosalicylic acid (anti-acne prodrug) and Adenosine triphosphate (ATP)</td>
<td>Constant potential (-0.5, -0.8 and -1.0 V)</td>
<td>Implantable microchip for controlled drug delivery</td>
<td>61</td>
</tr>
<tr>
<td>2009</td>
<td>Flourescein (model drug) and Dexamethasone-21 phosphate disodium salt (DEX)</td>
<td>Constant potential (-0.5 or -2.0 V)</td>
<td>Controlled drug release</td>
<td>80,81</td>
</tr>
<tr>
<td>2009</td>
<td>Sulfosalicylic acid</td>
<td>Electric field (0.0 or +1.0 V)</td>
<td>Transdermal drug delivery</td>
<td>82</td>
</tr>
<tr>
<td>2010</td>
<td>Dexamethasone-21 Phosphate disodium salt (DEX)</td>
<td>Cyclic voltammetry (between -0.8 and +0.9 V)</td>
<td>Neural electrode coatings</td>
<td>54</td>
</tr>
<tr>
<td>2010</td>
<td>Risperidone (Anti-psychotic)</td>
<td>Constant potential (-0.6 or +0.6 V)</td>
<td>Implantable drug delivery device</td>
<td>83,84</td>
</tr>
<tr>
<td>2010</td>
<td>Ibuprofen (anti-inflammatory)</td>
<td>Diffusion</td>
<td>Drug delivery from nanoparticles</td>
<td>85</td>
</tr>
<tr>
<td>2011</td>
<td>Antibiotics (penicillin/streptomycin) and Dexamethasone-21 phosphate disodium salt (DEX)</td>
<td>Cyclic voltammetry (-1.0 to +1.0 V at 100 mV/s)</td>
<td>Controlling cellular behaviour for orthopaedic applications</td>
<td>55</td>
</tr>
<tr>
<td>2011</td>
<td>Adenosine triphosphate (ATP)</td>
<td>Constant potential (-0.8 V)</td>
<td>Controlled release from nanowires</td>
<td>86</td>
</tr>
<tr>
<td>2011</td>
<td>6-cyano-7-nitroquinoxaline-2,3-</td>
<td>Cyclic Voltammetry (-1.0 to +0.8 V)</td>
<td>Implantable neural electrode arrays</td>
<td>87</td>
</tr>
<tr>
<td>Year</td>
<td>Compound Description</td>
<td>Electrochemical Method</td>
<td>Application/Comment</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-----------------------------------------------</td>
<td>-------------------------------------------</td>
<td>---------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>Risperidone (anti-psychotic)</td>
<td>Constant potential (-0.6 or +0.6 V)</td>
<td>Treatment of schizophrenia</td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>Dexamethasone-21 phosphate disodium salt (DEX)</td>
<td>Cyclic Voltammetry (-1.0 to +1.0 V)</td>
<td>Orthopaedic implants</td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>Naproxen (anti-inflammatory)</td>
<td>Constant potential (-0.6, -0.4, -0.2, 0.0, +0.2 and +0.4 V)</td>
<td>Controlled drug release</td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>Praziquantel and trichlorofon (anti-schistosomiasis agents)</td>
<td>Constant potential (OCP, -0.3, -0.4 and -0.5 V)</td>
<td>Treatment of Schistosomiasis</td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>Fluorescein (model drug)</td>
<td>Electric field (-0.5 or -1 V applied across two electrodes)</td>
<td>Drug delivery from nanoparticles</td>
<td></td>
</tr>
<tr>
<td>2013</td>
<td>Risperidone (anti-psychotic)</td>
<td>Constant potential (+0.6 or -0.6 V) or alternating pulsed potential (±0.6 V; 0.5 Hz)</td>
<td>Implantable drug delivery device</td>
<td></td>
</tr>
<tr>
<td>2013</td>
<td>Adenosine triphosphate (ATP) or Dexamethasone-21 phosphate disodium salt (DEX)</td>
<td>Cyclic voltammetry (-0.9 to +0.6 V)</td>
<td>High drug loading implantable delivery</td>
<td></td>
</tr>
</tbody>
</table>
1.5 Implantable power sources

1.5.1 Current state of implantable power sources

Implantable electronics have attracted significant research and commercial attention over the last few decades. All electronic circuits need a power source and thus a fundamental requirement of a bionic device is a power source that is mobile with the patient, for example a battery. Bionic devices currently on the market include pacemakers, deep brain stimulation devices and cochlear implants. Each of these devices have large power requirements and as such need specialised power sources that are either implanted in the body (housed within the pulse generator for pacemakers and deep brain stimulators) or work externally (in the ear piece for the cochlear implant) as shown in Figure 1.10. Batteries that are currently used for these devices use Lithium ion or zinc air technology. Some of the requirements of an implantable battery are long operation lifetimes, small size, high energy density and for the chemical reaction to produce no gas. The cochlear implant uses batteries that are housed in the external ear piece, and hence, are easily replaced by the patient when the battery expires. Pacemakers and deep brain stimulation devices, however, use implantable power sources and require invasive surgery once the battery’s lifetime expires to replace them. New research in this field aims to increase the lifetime of power sources to avoid additional high-risk surgery. In the past more hazardous battery components were used, like radioactive plutonium, but their implantation was discontinued when their risks were fully discovered. The nuclear battery, for example, has a lifetime of >19 years and showed no increase in the rate of cancers with it’s patients (which was the main concern). There were, however, many regulations for disposing of the battery after use. Most importantly, if the battery casing failed, plutonium is extremely toxic and just 1 µg in the blood stream could be fatal. For these reasons the risk was considered too high for widespread use of nuclear batteries as implantable power sources.
In the above mentioned implantable power sources, internal components are isolated from the *in vivo* environment by protective casings. There is, however, a growing demand for completely implantable power sources that do not need to be replaced or pose serious risk to the patient. Theoretically, this could be achieved through power sources that are not isolated, but operate by consuming fuels naturally replenished in the body. If the power source interacted with the internal environment it would have to be engineered to be completely biocompatible, to pose a lower toxicity risk, to last longer than current battery technologies (ideally the lifetime of the patient) and to provide sufficient power to operate the device.
(approximately 100 mW for a cochlear implant and just tens of microwatts for a conducting polymer film).

1.5.2 Bioelectrodes

1.5.2.1 Biofuel cells

A conventional fuel cell converts chemical energy into electrical energy through electrochemical processes. The cell is comprised of a cathode, where reduction occurs, and an anode, where oxidation occurs, with an electrolyte separating the two components. The distinguishing factor between batteries and fuel cells is that a battery operates off the energy stored within the casing, and when it ‘dies’ the battery is either discarded or needs to be recharged. Fuel cells, however, rely on fuel from the external environment and will operate as long as the fuel is supplied.\textsuperscript{98}

Biofuel cells operate in a similar way to fuel cells but biocatalysts replace metal catalysts\textsuperscript{99} at the electrodes. These biologically modified electrodes are called bioelectrodes. Bioelectrodes are electrode materials modified with living organisms and designed to generate power. They function by exploiting biochemical pathways to transform chemical energy into electrical energy using renewable natural fuels, like simple sugars found in the body.\textsuperscript{100} They are considered ideal for implantable devices because they operate most efficiently at biological temperatures and conditions, and the electrodes do not need to be isolated from physiological medium.\textsuperscript{101} The active component of a bioelectrode is usually enzymes or microorganisms, which work to extract and transfer electrons from the fuel molecule and convey them to the underlying electrode material. One major advantage of bioelectrodes is that they are based on naturally occurring cofactors, all of which can be broken down by natural metabolic pathways. The two major advancing technologies that utilise bioelectrodes are biofuel cells and biosensors.\textsuperscript{102}

The use of enzymes offers great benefits in catalytic activity, specificity and optimal performance at physiological temperatures. They do however demonstrate some barriers in terms of lifetime, low power density and, for implantable systems,
biofouling; where the accumulation of biological material on the electrode surface in vivo could isolate the electrodes\textsuperscript{104,105}. 

![Diagram of a biofuel cell](image)

**Figure 1.11 Schematic of a biofuel cell used to convert a fuel (glucose) to electricity.** Ferrocenemethanol (FcMeOH) is a redox mediator for the enzyme glucose oxidase (GOx) that works at the anodic electrode, while another enzyme bilirubin oxidase (BOD) works at the cathodic electrode to complete the electrical circuit.

A schematic of a typical biofuel cell using two well-documented enzymes, glucose oxidase (GOx) and bilirubin oxidase (BOD), is shown in Figure 1.11. The anodic component uses GOx to catalytically oxidise glucose in solution (the fuel) to produce glucolactone and H\textsubscript{2}O\textsubscript{2} by-products. The electrons gained by the enzyme during this reaction are subsequently transferred to the anode through a redox mediator, ferrocene methanol (FcMeOH). The electrons are driven by the potential difference between the two electrodes and are conveyed through the external circuitry. The cathode component is based on BOD, which accepts electrons from the cathode and uses them to reduce molecular oxygen to water. The circuit also contains a supporting electrolyte to aid in electron transfer. In biofuel cells the
limiting electron transfer process is generally associated with the cathodic side due to the tendency of enzymes, appropriate for this application, to have redox centres buried deep within their tertiary structure. Because the electron tunnelling distance is large, the electron transfer rates are hindered and the power density of the biofuel cell is ultimately limited $10^6 - 10^8$. Biofuel cells based on the oxidation of glucose by glucose oxidase have been studied extensively for application as implantable power sources since the fuel (glucose) is naturally present in the human body and is replenished through diet and healthy metabolic processes $^{109}$.

1.5.2.2 Biosensors

A biosensor is an analytical device that can operate on the same principles as an enzyme-based bioelectrode. Essentially taking the GOx anode component of the above biofuel cell and calibrating the current generated as a function of glucose concentration, a glucose-sensitive biosensor can be realized. Biosensors are characterised based on the sensitivity to the analyte, the detection range and the response time, and also benefits from the selectivity of enzymes to a particular analyte. It is common practice to immobilise the enzyme onto the electrode for biosensor applications and is used for measuring blood glucose (sugar) levels. The concept of immobilisation will be discussed in section 1.5.5.2. There are a number of commercially available glucose biosensors based on the amperometric response of glucose oxidase including elegance® CT-X12 (Convergent Technologies, Germany) and MiniMed® Paradigm Revel™ (Medtronic, USA) $^{110,111}$. The area of enzymatic glucose biosensors has been extremely active over recent decades with researchers investigating new electrode materials, genetically modified enzymes and avenues to immobilise redox mediators to form stable biosensors $^{112-115}$. Enzymatic biosensor literature is relevant to this thesis because it incorporates methods and materials common for bioelectrodes, whether their application is in biofuel cells or biosensors.
1.5.2.3 Bioelectrodes - A new approach

For enzymatic biofuel cells the process at the anode, usually involving the use of the well-documented enzyme glucose oxidase (section 1.5.4), is the most efficient electron transfer process, while the electron transfer at the cathode is the rate-limiting step, which ultimately lowers the power density of the final biofuel cell. In biosensors, the cathode component of a biofuel cell is essentially eliminated and the literature describes the output of a single bioelectrode based on its access to fuel. Hence it may be possible to use the anode component of the biofuel cell, or similarly the single bioelectrode of an enzymatic biosensor, to supply power to a low power consumption device, eliminating the need for traditional power sources.

Moulton et al. galvanically coupled a drug doped conducting polymer to a biodegradable magnesium electrode to control the release of a therapeutic drug. As the magnesium alloy corroded in biological conditions, the potential difference between the electrodes and the current generated drove the release of dexamethasone (DEX) from a polypyrrole (PPy) film (Figure 1.12). Magnesium is a highly corrosive metal and even with protective coatings the material has a limited lifetime and, therefore, limited electrons to power the release of drug. Using the same concept, a single enzymatic bioelectrode could be used to replace the magnesium electrode in this system, and if it generates enough power to control the release of drug, it could be a promising alternative to increase the lifetime of the drug delivery device and eliminate the need for traditional power sources.
1.5.3 Introduction to enzymes

Enzymes are defined as proteins that catalyse chemical reactions and are central to every biochemical process in the body. They demonstrate a high degree of specificity for their particular substrate, which is a direct function of their protein structures. Enzymes work by forming a region in their protein structure, called the active site, which is lined with amino acids in such a conformation that they bind the substrate. The enzyme’s catalytic activity depends on the integrity of their native protein conformation, because it ensures this active site is available to substrate binding. If an enzyme is denatured, the catalytic activity can be destroyed. Enzymes are classified by the type of reaction that they catalyse and these are summarised in Table 1.3.117.

Table 1.3 International Classification of Enzymes. Adapted from Ref117

<table>
<thead>
<tr>
<th>No.</th>
<th>Class</th>
<th>Type of reaction catalysed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oxidoreductases</td>
<td>Transfer of electrons</td>
</tr>
<tr>
<td>2</td>
<td>Transferases</td>
<td>Group transfer reactions</td>
</tr>
</tbody>
</table>
Hydrolases
Hydrolysis reactions by the transfer of functional groups to water

Lyases
Addition of groups to double bonds, or formation of double bonds by removal of groups

Isomerasenses
Transfer of groups within molecules to yield isomeric forms

Ligases
Formation of C-C, C-S, C-O and C-N bonds by condensation reactions coupled to ATP cleavage

1.5.4 Glucose oxidase

Glucose oxidase (GOx) is a class 1 oxidoreductase enzyme that catalyses the oxidation of β-D-Glucose to D-glucono-1,5-lactone and hydrogen peroxide by using molecular oxygen as an electron acceptor (Figure 1.13 a). GOx is a dimeric protein consisting of two identical peptide chain subunits (shown in light and dark blue, Figure 1.13 b) with two flavin adenine dinucleotide (FAD) cofactors buried 13 Å within each protein shell (shown in pink, Figure 1.13 b). FAD is a redox centre that is responsible for extracting the electrons during the oxidation of glucose and in some cases genetic modification of the enzyme has increased its sensitivity, selectivity and electrocatalytic current for biosensor applications.

The GOx used in this study is produced by Aspergillus niger and has a molecular weight of 160 kDa. GOx has been used extensively in biosensor and biofuel cell research because of its stability, its specificity and its high electrocatalytic activity. Like all enzymes, GOx has specific environmental conditions that need to be optimised to achieve the most efficient catalytic activity. For example GOx works optimally at 37°C but will denature at temperatures higher than 40°C. Similarly, its optimum pH range is 4–7 and outside of this range the activity drops significantly. This becomes very important during practical application in bioelectrodes. Additionally, the purification of GOx can significantly enhance the catalytic current generated up to three-fold compared to the un-purified equivalent. Gao et al. measured that commercial powders contained about 20% of non-protein compounds, which will have a detrimental effect on the efficiency of the
bioelectrodes produced. A purification protocol was used in this thesis and is outlined in detail in Chapter 2.

![Figure 1.13](a) The reaction scheme for the catalytic oxidation of glucose by glucose oxidase. (b) A schematic of the 3D structure of glucose oxidase, indicating the identical protein subunits (dark and light blue) and the FAD redox centres (pink). Adapted from Ref\textsuperscript{121}

1.5.5 Mechanism of electron transfer

As an oxidoreductase enzyme, GOx transfers electrons during the oxidation of glucose. It is these electrons that are extracted from the biochemical pathway and conveyed to an electrode that allows a current to be generated from a bioelectrode. There are two mechanisms by which the electrons can be collected, direct electron transfer (DET) or mediated electron transfer (MET), as shown in Figure 1.14. These two mechanisms will now be discussed in detail.
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1.5.5.1 Mediated electron transfer (MET)

MET utilises a redox active species to shuttle the electrons from the enzyme to the electrode. A redox mediator may be required if the redox centre is buried too deep within the quaternary structure to be able to directly communicate with the electrode and, in some cases, can enhance the rate of electron transfer of the bioelectrode. For a redox mediator to be suitable for a specific enzyme, the difference in redox potential between the mediator and the enzyme must be non-zero so there is a driving over-potential\(^\text{125}\). Ideally, for the GOx system, the mediator will have a redox potential more positive than the oxidation potential of GOx, which is approximately \(-450\) mV (vs. Ag\(\mid\)AgCl, for neutral pH)\(^\text{124}\). The redox mediator used in this thesis was Ferrocene methanol (FcMeOH), which has an oxidising potential of approximately +200 mV (vs. Ag\(\mid\)AgCl). In some cases, diffusion limits can hinder the effectiveness of a mediator and for the purpose of biosensors, where it is optimal to have all components confined to the surface of the electrode, a ‘free’ mediator may not be ideal. For this reason many research groups have looked into immobilising the mediator onto the electrode to not only increase

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**Figure 1.14** Alternate electron transfer mechanisms. Direct electron transfer (DET) from the active site of the enzyme to the electrode material, also known as the tunnelling mechanism, and mediated electron transfer (MET) via a redox mediator. Adapted from Ref\(^\text{122}\).
the mediator concentration at the electroactive surface area but also to avoid
diffusional limitations. Materials used for this purpose include conducting
copolymers and redox mediator polymers but one major disadvantage affecting
the efficiency of these systems is the tendency of the matrix to hinder substrate
diffusion to the immobilised enzyme. Mano et al. fabricated an Os-redox polymer
hydrogel to address this issue and achieved efficient electron transfer rates using
this system.

1.5.5.2 Direct electron transfer (DET)

Direct electron transfer involves direct communication between the enzyme and
the electrode material. This usually entails the immobilisation of the enzyme at the
electrode surface to satisfy a small electron tunnelling distance (Figure 1.14). The
DET mechanism bypasses some of the limitations associated with MET outlined
above and simplifies the overall reaction pathway to generate a more efficient
electron transfer process. DET systems are restricted to a monolayer of enzyme in
intimate contact with the electrode due to the globular structure of the protein shell
and this limits the number of catalytic centres accessible to the electrode. To
address this problem research has been directed at the application of nanostructured
electrodes to immobilise GOx, and bioaffinity chemistry to favourably orient the
enzymes with respect to the electrode surface. Nanostructured electrodes offer an
extremely large electrode surface area available to bind enzymes and not
surprisingly have proven to outperform flat electrodes in terms of current density
for DET systems. Literature concerning electronic coupling of GOx directly to
electrodes covers a large range of materials. This thesis is concerned with
allotropes of carbon for this application, in particular multi-walled carbon
nanotubes and graphene, and the following section is dedicated to outlining the
current state of the literature in this area.
1.5.6 Materials for bioelectrodes

1.5.6.1 Allotropes of carbon

Carbon exists in a number of allotropes: diamond, graphite, fullerenes, amorphous carbon, carbon nanotubes, and graphene, most of which are shown in Figure 1.15, where BC8 represents a thermodynamically stable form of carbon at high pressures\textsuperscript{129}. Some of these forms of carbon have been investigated for their use as biomaterials, and more recently for their application in bionic devices, in particular carbon nanotubes (CNTs) and graphene\textsuperscript{31}. Properties that make CNTs and graphene appropriate for such applications include their long-range $\pi$-conjugation for electrical conductivity, incredible mechanical and thermal properties, low density and biocompatibility. It is still under debate whether these nanomaterials are toxic in the body with concern for their use \textit{in vivo}\textsuperscript{130}.

![Allotropes of carbon](image)

\textbf{Figure 1.15 The allotropes of carbon. Adapted from ref\textsuperscript{31}}
1.5.6.2 Carbon for bioelectrodes

Carbon nanotubes (CNTs) have been investigated for their ability to facilitate direct electron transfer (DET) in bioelectrodes\textsuperscript{112}. It has been hypothesized that the reason they are so effective is that the CNTs enter the protein shell of the enzyme to make electrical contact directly with the redox centre, without affecting the affinity of the active site to bind to its fuel substrate. In 2002, Guiseppi-Elie \textit{et al.} described the phenomenon as a long sharp needle piercing a balloon without popping it\textsuperscript{131}. Since then, other forms of carbon materials have proven to be able to facilitate DET just as effectively, including the new wave of literature in the area of graphene. As such the hypothesis about piercing the balloon seems to be less appropriate. It could, however, be attributed to the copious quantities of edge plane defects that are present in both allotropes, which have also been associated with high levels of electroactivity\textsuperscript{132–134}.

Functionalization has played a major part in the processability of CNTs and graphene. This is particularly the case for CNTs because of their insolubility in aqueous systems due to a lack of hydrophilic functional groups. It is critical for these materials to be processed in an aqueous environment for bionic applications to increase their biocompatibility; especially for enzymes whose environment greatly alters their catalytic activity\textsuperscript{101}. A simple method that has been widely adopted to disperse carbon nanotubes in water is through the use of surfactants or dispersing agents\textsuperscript{135}. Although these dispersing agents can be in the form of biopolymers like chitosan or gellan gum to increase the biocompatibility of the composite material, the addition of such polymers is generally detrimental to the electrical properties before a percolation threshold is reached\textsuperscript{136}. On the other hand, graphene can be readily dispersed in aqueous mediums due to oxygen functionalised basal plane defects, in addition to peripheral functional groups\textsuperscript{137}. Figure 1.16 shows the chemical reduction of graphene oxide (GO) to reduced graphene oxide (rGO) by hydrazine and represents the reduction method utilised in this thesis. Its name, graphene oxide (GO), is given due to the large amount of oxygen in this form of graphene. The processability of GO, and its more conducting form rGO as aqueous
based systems make them appropriate to use in bionic applications, without the loss of electrical properties. It is reported that rGO is more conducting than its predecessor GO because the reduction process removes some of the oxygen functional groups and restores conjugation in the basal plane structure, subsequently enhancing the electrical properties (shown in Figure 1.16). Graphene and CNTs will be discussed in more detail in Chapters 3 and 4 in reference to their ability to facilitate DET with GOx.

Table 1.4 summarises major contributions to the field of carbon nanomaterials in enzymatic bioelectrodes, but is not exhaustive. It includes DET systems only and reports on work done exclusively with GOx in order to narrow down the literature field to research relevant to this thesis. The existing method of comparing the output of bioelectrodes uses the current density (current per cm$^2$), although this introduces some calculation errors. Electrodes produced in a lab environment are usually smaller than 1 cm$^2$ and to scale the current density up to this dimension by calculation may not reflect the real current density of a manufactured device. Additionally, it is difficult to calculate the actual area with the use of nanomaterials that have extremely large electroactive surface areas. As such, it is most common to use the dimensions for the entire electrode rather than the area of the nano-features, which also skews the real current density value. A more accurate measure of comparison in this field of bioelectrodes is the current generated per microgram of

![Graphene Oxide (GO) and Reduced Graphene Oxide (rGO)](image_url)

**Figure 1.16** Structural changes of graphene after hydrazine reduction. The reaction scheme shows the structure of graphene oxide (GO) and reduced graphene oxide (rGO).
biocatalyst immobilised. This gives an indication not only of the actual current output, but also of the efficiency of electron transfer and activity of the biocatalyst in the given system. This data is represented in Table 1.4 and provides a context for which the electrodes presented in this thesis will be discussed. In 2012 Alwarappan et al. reported the highest specific catalytic current for a bioelectrode in this field at 25 \( \mu \text{A/\mu g GOx} \) using a graphene electrode material\textsuperscript{138}. Interestingly, they didn’t use any other insulating support materials, which can hinder the bioelectrode output.

Table 1.4 Literature summary of specific catalytic current generated by glucose oxidase bioelectrodes based on carbon allotrope direct electron transfer systems.

<table>
<thead>
<tr>
<th>Carbon material</th>
<th>Current per biocatalyst ((\mu \text{A/\mu g GOx}))</th>
<th>Other materials</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWNT</td>
<td>0.08</td>
<td>Cellulose</td>
<td>139</td>
</tr>
<tr>
<td>MWNT</td>
<td>0.003</td>
<td>Platinum-MWNT-Alumina-coated silica nanocomposite</td>
<td>140</td>
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<td>MWNT</td>
<td>3.3</td>
<td>Nafion®</td>
<td>106</td>
</tr>
<tr>
<td>Mesoporous carbon</td>
<td>0.06</td>
<td>Nafion®</td>
<td>119</td>
</tr>
<tr>
<td>GO</td>
<td>0.12</td>
<td>Chitosan matrix</td>
<td>141</td>
</tr>
<tr>
<td>MWNT</td>
<td>0.05</td>
<td>Toray® carbon paper substrate with polyethylenimine support</td>
<td>142</td>
</tr>
<tr>
<td>MWNT</td>
<td>0.5</td>
<td>Nafion®</td>
<td>124</td>
</tr>
<tr>
<td>MWNT</td>
<td>0.01</td>
<td>Toray® carbon paper substrate with Nafion® support</td>
<td>143</td>
</tr>
<tr>
<td>MWNT</td>
<td>0.008</td>
<td>Nitrogen doped MWNT with Nafion® support</td>
<td>144</td>
</tr>
<tr>
<td>Exfoliated GO</td>
<td>0.42</td>
<td>Chitosan and platinum nanoparticles</td>
<td>145</td>
</tr>
<tr>
<td>rGO</td>
<td>&gt;0.001</td>
<td>-</td>
<td>146</td>
</tr>
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<td>MWNT</td>
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<td>Mesoporous carbon</td>
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<td>Platinum nanoparticles, cross-linked gelatin</td>
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<tr>
<td>MWNT</td>
<td>&gt;0.001</td>
<td>Electro-oxidised polypyrrole in the presence of functionalised MWNT and GOx</td>
<td>150</td>
</tr>
<tr>
<td>MWNT</td>
<td>0.5</td>
<td>Alumina-coated silica with Nafion® support</td>
<td>151</td>
</tr>
<tr>
<td>MWNT</td>
<td>0.08</td>
<td>Nafion®</td>
<td>152</td>
</tr>
<tr>
<td>MWNT</td>
<td>3.3</td>
<td>Sol-gel matrix</td>
<td>153</td>
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<tr>
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<td>0.1</td>
<td>Platinum nanoparticles on gold electrodes with Nafion® support</td>
<td>154</td>
</tr>
<tr>
<td>rGO</td>
<td>0.009</td>
<td>Polyvinylpyrrolidone, polyethyleneimine functionalised ionic liquid</td>
<td>155</td>
</tr>
<tr>
<td>rGO</td>
<td>1.44</td>
<td>Gold nanoparticles and chitosan</td>
<td>156</td>
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<tr>
<td>Sulfonated GO &amp; MWNT</td>
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<td>Gold nanoparticles, Nafion®</td>
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<td>Polymeric ionic liquid</td>
<td>158</td>
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<tr>
<td>Graphene platelets</td>
<td>0.05</td>
<td>Chitosan</td>
<td>159</td>
</tr>
<tr>
<td>rGO</td>
<td>0.008</td>
<td>Ionic liquid</td>
<td>160</td>
</tr>
<tr>
<td>Exfoliated GO</td>
<td>0.01</td>
<td>-</td>
<td>161</td>
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<table>
<thead>
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<th>&gt;0.001</th>
<th>-</th>
<th>162</th>
</tr>
</thead>
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<td>rGO &amp; MWNT</td>
<td>0.25</td>
<td>Nafion®</td>
<td>163</td>
</tr>
<tr>
<td>rGO &amp; CNT</td>
<td>&gt;0.001</td>
<td>-</td>
<td>164</td>
</tr>
<tr>
<td>rGO</td>
<td>25</td>
<td>-</td>
<td>138</td>
</tr>
<tr>
<td>Graphene quantum dots</td>
<td>1</td>
<td>-</td>
<td>165</td>
</tr>
</tbody>
</table>

*Data presented in this table was extracted from the given references. The specific current was calculated by either taking the maximum current generated with the lowest GOx loading (if it wasn’t specified), or the anodic peak height at 50 mV/s again with the specified (or lowest) GOx loading. If GOx was adsorbed onto the electrode surface the calculation was based on the soaking solution concentration.

1.6 Overview and thesis aims

The aim of this thesis is to develop an operational drug delivery system powered by a naturally occurring biofuel, glucose. This thesis reports on the development of GOx bioelectrodes and their specific catalytic current in reference to the literature presented in Table 1.4. These electrodes are based on chemically reduced graphene oxide and multi-walled carbon nanotubes. A GOx bioelectrode will be used exclusively to power the release of therapeutic drug from a conducting polymer scaffold. This will be the first prototype for a completely implantable drug delivery device that will operate off glucose. Throughout the results and discussion chapters, reference will be made to the practical treatment of epilepsy, which is the intended application for this device.

Specifically, the objectives of this thesis were to:

- Develop aqueous carbon nanomaterial dispersions and fabrication techniques suitable to incorporate GOx without destroying the integrity of the enzyme.
- Engineer and characterise GOx bioelectrodes using the above-mentioned dispersions. **Chapter 3** investigates the fabrication of solvated graphene bioelectrodes while **Chapter 4** focuses on a simple drop casting method to investigate the efficiency of an rGO-CNT composite material to facilitate direct electron transfer from immobilised GOx.

- **Chapter 5** examines the feasibility of using the composite electrode presented in Chapter 4 to power the release of drug from a polypyrrole structure. This is shown schematically in Figure 1.17 with the red box highlighting the GOx bioelectrode side, and the purple box showing the drug loaded conducting polymer side. Chapter 6 specifically targets the overall aim of this thesis to develop an operational drug delivery system powered by glucose.

![Diagram](image)

**Figure 1.17** Cell design used in this thesis to power the release of drug from a conducting polymer film using an enzymatic bioelectrode.
2. General experimental

2.1 Overview

This chapter aims to outline materials and characterisation techniques that were common throughout the results presented in this thesis. It is also an opportunity to introduce the protocol used to purify the glucose oxidase; a critical milestone achieved for the foundations of this work.

2.2 Experimental techniques

2.2.1 UV-Visible spectrophotometry

When a molecule absorbs a photon of light the energy of the molecule increases and it is said to be in an excited state. This is unique for the UV-visible region of light with other regions causing rotational motion or vibrations initiated by microwave and infrared light, respectively. Some substances dissolved in solution will absorb a certain wavelength of white light and the intensity of this absorbance is directly proportional to the concentration according to Beer-Lambert’s Law:

**Equation 2.1 Beer-Lambert’s Law**

\[ A = \varepsilon l c \]

where \( A \) is the measured absorbance, \( l \) is the path length of the cell, \( c \) is the concentration of analyte in mol/L and \( \varepsilon \) is the molar extinction coefficient (the absorbance of a 1 M solution of analyte).

The absorbance of a dissolved substance is linear with concentration as long as the concentration is not too high to cause shadowing effects if the molecules are in the same optical path. A calibration curve can be constructed based on a standard series with solutions of known concentrations and a linear relationship can be fitted. From
this relationship the concentration of an unknown sample can be measured. The experiments in this thesis were conducted using the Shimadzu UV-1601 spectrophotometer with UVprobe (v.2.10) software. Specific experimental protocols will be introduced in the relevant chapters.

2.2.2 Scanning electron microscopy

Scanning electron microscopy (SEM) was performed using the JEOL Cold Field Emission Gun Scanning Electron Microscope. The SEM was operated at an accelerating voltage of 5 kV and secondary electron images were taken with a semi-in-lens detector at a working distance of 8 mm and a spot size setting of 8. Specific sample preparation for the different electrodes will be outlined in the relevant experimental sections in the following chapters.

2.2.3 Electrochemistry

2.2.3.1 Electrochemical theory

During an electrochemical experiment two types of processes occur at the electrode that contribute to the overall current generated or stored, depending on the electrode potential. Faradaic processes occur when an analyte in solution undergoes oxidation or reduction at the electrode and involves the transfer of charge from solution to the electrode material. Faradaic processes are governed by Faraday’s 1st law that states that the amount of a substance that undergoes electrolysis at an electrode is proportional to the current generated. Non-Faradaic processes do not involve a transfer of charge as such but are defined by the formation of an electrical double layer at the electrode surface. This storage of charge depends on the potential of the electrode and it’s electroactive surface area, and is also known as the capacitive current.
2.2.3.2 Electrochemical cell

For all electrochemical characterisation techniques a 3-electrode cell configuration was used as schematically represented in Figure 2.1. A platinum mesh counter electrode and a Ag|AgCl reference electrode were used in each experiment however, the working electrode and nature of the electrolyte was varied depending on the type of experiment.

![Figure 2.1 Schematic representation of the 3-electrode cell used to electrochemically characterise electrodes](image)

If the working electrode was a conducting polymer film the experiment was performed at room temperature. Degassing of the electrolyte using argon was only necessary if the reducing potential used was greater than -0.4 V (vs. Ag|AgCl), the potential region where oxygen reduction would interfere with the results. If the
working electrode was an enzyme-loaded bioelectrode the degassing step was critical to remove the oxygen that would otherwise compete with the glucose substrate. A 15 minute degassing procedure was implemented before each experiment and an argon gas blanket was maintained for the duration of each electrochemical characterisation technique. Additionally, for the analysis of an enzyme loaded bioelectrode the electrochemical cell would be kept at 38°C (using a water bath) to maximise enzyme activity.

2.2.3.3 Cyclic voltammetry

Cyclic voltammetry (CV) is an electrochemical technique where an applied potential is swept at a constant rate between two limiting potentials and the current generated is measured. For a non-faradaic experiment, i.e. when no redox active species is present in solution or associated with the electrode, the CV generated will have a rectangular current response and demonstrate sharp charging and discharging behaviour (Figure 2.2 a).
Figure 2.2 (a) A schematic representation of a CV showing a typical non-faradaic response, and (b) A CV of graphene-carbon nanotube composite electrode submersed in a mediator electrolyte showing a Faradaic response.

When a redox active species is present in solution the CV generated will have a distinct set of peaks associated with the oxidation and reduction of that species (Figure 2.2 b). At the beginning of the potential sweep only non-Faradaic currents flow, provided that the initial potential is sufficiently more negative than the formal potential of the redox species (point 1). As the potential sweep approaches the formal potential of the redox species oxidation starts to occur at the electrode
surface and Faradaic current begins to flow (point 2). At the oxidation peak potential the system is instantaneously oxidising the redox species and the process is limited by the diffusion rate of the reduced redox species in solution to the electrode surface (point 3). Point 4 indicates the potential at which the scan is reversed and the oxidised species produced in the previous scan starts to undergo reduction as the scan approaches the formal potential of the redox couple (point 5). For a completely reversible redox system the oxidation and reduction peaks should have identical peak heights, widths and overall shape, and also indicates efficient electron transfer between the redox species and the electrode. The anodic ($I_{pa}$) and cathodic ($I_{pc}$) peaks in a CV will be separated by approximately 59 mV as given by the Nernst relationship (Equation 2.2) for single electron transfer processes, with the peak height ratio being close to 1. The shape and relative separation of peaks in a CV can also give some indication if the redox process is surface confined, which will be of interest when investigating the electrochemistry of the enzyme-loaded bioelectrodes where the glucose oxidase is immobilised on the electrode structure.

**Equation 2.2 The Nernst Equation**

$$E = E^0 + \frac{0.059}{n} \log \frac{C_O^s}{C_R^s}$$

where $E^0$ is the formal redox potential of the redox species, $C^s$ is the concentration of electroactive species at the electrode surface in either the reduced (R) or oxidised (O) state at the electrode, $E$ indicates the potential measured between the electrodes and $n$ is the number of electrons transferred.

### 2.2.3.4 Open circuit potential

Open circuit potential (OCP) is a technique that measures the potential difference between the working and counter electrodes without applying any current. There is an initial relaxation period as the electrodes establish their electrical double layers before coming to a stable state. An open circuit potential experiment plots the potential as a function of time. All experiments were performed in 50 mM PBS and generally a potential reading was taken every 0.5 seconds until a stable potential...
was observed. CH Instrument potentiostat and CHi software were used for all OCP experiments.

2.2.3.5 Electrochemical impedance spectroscopy

Ohm’s law defines resistance (\( R \)) as the ratio between voltage (\( E \)) and current (\( I \)) according to Equation 2.3. This is a well-known relationship, however, it is limited to an ideal resistor and in the real world, circuit elements exhibit much more complex behaviours.

Equation 2.3

\[
R = \frac{E}{I}
\]

Resistance - \( R \) and Impedance - \( Z(\omega) \) are similar in that they are a measure of the ability of a circuit to resist the flow of electrical current however, impedance is not limited to an ideal resistor. Electrochemical impedance spectroscopy (EIS) is an AC technique whereby a small sinusoidal perturbation (i.e. 5 mV) is applied to the electrode potential and the current through the circuit is measured. Experimentally, the electrode potential is initially measured through OCP, as outlined in section 2.2.3.4. The potential and current can be expressed as complex functions as given in Equation 2.4 and Equation 2.5, respectively. Consequently, the impedance measured can be represented as a complex number containing a real and an imaginary part, as shown in Equation 2.6\(^{167} \).

Equation 2.4

\[
E_t = E_0 \exp(j\omega t)
\]

where \( E_t \) is the potential at time \( t \), \( E_0 \) is the amplitude of the signal, \( j \) is the imaginary unit and \( \omega \) is the radial frequency.
Equation 2.5

$$I_t = I_0 \exp(j\omega t - \phi)$$

where $I_t$ is the response current at time $t$, $I_0$ is the amplitude of the signal, $j$ is the imaginary unit, $\omega$ is the radial frequency and $\phi$ is the phase shift between voltage applied and the response current.

Equation 2.6

$$Z(\omega) = \frac{E_t}{I_t} = Z_0 \exp(j\phi) = Z_0 (\cos \phi + jsin \phi)$$

where $Z(\omega)$ is the impedance, $E_t$ and $I_t$ are the potential and current at time $t$, $Z_0$ is the magnitude of impedance, $j$ is the imaginary unit and $\phi$ is the phase shift.

A Nyquist plot is a representation of the resulting data that plots the real (x-axis) and imaginary (y-axis) part of the impedance, with each point on the plot representing the impedance at one frequency. A Nyquist plot is schematically represented in Figure 2.3. The point at which the plot intercepts the x-axis first ($R_1$) is a measure of the solution resistance $-Rs$, while the next intercept ($R_2$) indicates the charge transfer resistance $-R_{ct}$ and is calculated by finding the difference between the two intercepts (i.e. $R_{ct} = R_2 - R_1$). The charge transfer resistance of an electrode gives an indication of the conductivity of the material. The specific parameters used for each EIS experiment will be outlined in the respective chapters.
2.2.3.6 Amperometry

Amperometry is an electrochemical technique used to investigate the sensitivity, response time and performance of enzyme-loaded bioelectrodes. A current-time ($i$-$t$ curve) response is measured as a function of an applied voltage and usually the applied voltage is an oxidising potential. When the generated current stabilizes at the applied voltage for a bioelectrode system, glucose is spiked into the electrolyte and the response recorded is analysed as the catalytic current. Specific experimental parameters will be outlined in the relevant chapter sections.

2.2.3.7 Ferrocene methanol as a mediator for glucose oxidase

In some of the above-mentioned electrochemical techniques, in order to characterise the enzyme-loaded bioelectrodes effectively a mediator had to be incorporated into the system. Ferrocene methanol (FcMeOH) is a redox mediator that can be used to characterise glucose oxidase systems\[168. A mediator works to shuttle electrons from the oxidation of glucose, at GOx, to the electrode and in this case the mediator is in solution. The CV shown in Figure 2.4 (black trace) represents data collected for a

---

Figure 2.3 A schematic representation of a typical Nyquist plot.
glassy carbon electrode (GCE) submersed in PBS with FcMeOH (5 mM) and saturated glucose (150 mM) in solution. It shows characteristic reversible redox behaviour of FcMeOH on a flat electrode. When GOx is added to solution (green trace) the CV takes on a hysteresis shape and shows a plateau current that is a function of mass transport limitations; i.e. the mediator diffusing to the electrode. The increase in magnitude of the oxidative current represents the catalytic current generated by the oxidation of glucose by the enzyme. This type of response demonstrates that FcMeOH is an effective mediator for glucose oxidase.

It is also important to note that the glucose used in all experiments was prepared at 1 M concentration as a stock solution in 50 mM phosphate buffer solution (PBS) and allowed to mutarotate for 24 hours before use. Mutarotation is a critical step used to allow the α- and β-anomers of D-glucose to equilibrate.

Figure 2.4 Cyclic voltammograms of a glassy carbon electrode (3 mm diameter) in PBS with FcMeOH (5 mM) and saturated glucose (150 mM) with (green trace) and without (black trace) glucose oxidase in solution.
2.2.3.8 Fourier transform alternating current voltammetry

Faradaic currents generated during an electrochemical experiment are usually non-linear and the mathematical translation of such complex terms is generally avoided in theoretical modelling of experimental results. In electrochemical impedance spectroscopy (EIS) for example, a small AC perturbation is superimposed on top of a constant DC potential and this allows the assumption of a linear faradaic response. As a result, simplified equivalent circuits can be used to simulate the results. In fourier transform alternating current voltammetry (FTACV) a large AC perturbation is superimposed on top of a sweeping DC potential (i.e. a cyclic voltammogram) and through mathematical algorithms the analysis of the resulting data can provide a wealth of information on the contributing electrode mechanisms, for example, it can separate the capacitive and faradaic contributions. The waveform ($E_t$) of a single sine wave of amplitude $\Delta E$ and frequency $\omega$ at time $t$, superimposed onto a DC potential ramp ($E_{dc}$) is given in Equation 2.7.

**Equation 2.7**

$$E_t = E_{dc} + \Delta E \sin(\omega t)$$

Such a waveform will generate a response that contains both DC and AC contributions, the latter of which is represented in a series of harmonics, namely first ($\omega$), second ($2\omega$), third ($3\omega$) etcetera. In order to analyse the data generated, the resulting current shown in Figure 2.5 (a), generated from a FTACV experiment, is transformed from the time domain to the frequency domain through the use of a Fourier transform (FT) algorithm (process indicated by the blue arrow). From the power spectrum shown in Figure 2.5 (b) the individual components (i.e. DC, first harmonic, second harmonic etc) can be separated through the implementation of an inverse FT procedure (process indicated by the coloured arrows to each component)\textsuperscript{170,171}. 


Figure 2.5 Large-amplitude ac cyclic voltammogram obtained for reduction of 0.5 M ferrocenemonocarboxylic acid on a glassy carbon electrode: (a) total current and (b) the log power spectrum after Fourier transformation. (i), (ii), (iii), (iv) and (v) show the components highlighted by coloured boxes after inverse Fourier transformation and represent the DC, first harmonic, second harmonic, third harmonic and fourth harmonics, respectively. Adapted from Ref\textsuperscript{170}

The resulting current data is represented in the time domain and can provide information on the faradaic and non-faradaic current contributions. The double layer capacitive contributions are generally linear systems, as given by Equation 2.8, and as such, will only be represented in the DC and first harmonic components. The capacitive current is highlighted in Figure 2.5 (ii) and (iii) using circles to identify the presence and absence of the non-faradaic background component, respectively. If the capacitance is non-linear, which can occur for higher electroactive surface area electrodes, residual non-faradaic current is observed in higher harmonics. The non-linear faradaic contributions are represented in the higher order harmonics in the absence of the capacitive current. Hence, the data can
be used qualitatively to determine if any faradaic current is generated for electrode systems where the capacitive current is large and might swamp the relatively small faradaic response. Alternatively, and if the faradaic response is clear in the higher harmonics, electrochemical simulation can be used to extract kinetic parameters for the given electrode system.

**Equation 2.8**

\[ i = \nu C_d \]

where \( \nu \) is the scan rate in a cyclic voltammogram, \( i \) is the anodic or cathodic current and \( C_d \) is the electrode double layer capacitance.

### 2.3 Materials

**2.3.1 Glucose oxidase**

**2.3.1.1 Purification protocol**

Glucose Oxidase *aspergillus niger* (GOx) was purchased commercially from Sigma and 100 mg was dissolved in 2.5 mL of Buffer 1 (from Table 2.1). The buffers were prepared using potassium phosphate monobasic and dibasic salts (Sigma) with the desired pH and ionic strength precisely calculated. Diammonium sulphate (Sigma) and sodium chloride (Sigma) were added according to Table 2.1. All buffers were sonicated and filtered through a 0.22 \( \mu \)m membrane before use.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 1</td>
<td>50 mM Phosphate Buffer (pH7.5) + 3 M diammonium sulphate</td>
</tr>
<tr>
<td>Buffer 2</td>
<td>50 mM Phosphate Buffer (pH7.5)</td>
</tr>
<tr>
<td>Buffer 3</td>
<td>50 mM Phosphate Buffer (pH8)</td>
</tr>
<tr>
<td>Buffer 4</td>
<td>50 mM Phosphate Buffer (pH8) + 1 M sodium chloride</td>
</tr>
</tbody>
</table>
The GOx in Buffer 1 solution was loaded onto a disposable PD-10 desalting column (GE Healthcare) followed by another 5 mL of Buffer 1. Approximately 4.5 mL of the eluent was collected and syringe filtered using a 0.22 µm membrane before loading onto a high-performance liquid chromatography (HPLC) column.

HPLC is an analytical technique used to separate a mixture into its different components. It uses high pressure to force a liquid mobile phase through an enclosed column containing a porous stationary phase. Samples are loaded onto the column in the mobile phase and interact with the column depending on the sample properties and the type of column\textsuperscript{166}. The eluent is collected in fractions and monitored by UV-Vis for protein elution (see 2.2.1 for an introduction to UV-Vis).

During these experiments the HPLC system was kept in a cool room at 4°C and the enzyme purification involved two separations using different types of columns.

1. A hydrophobic column (Hi Trap Phenyl HP column – 1 mL)
2. An anion exchange column (Hi Trap QFF column - 1 mL)

Initially the 4.5 mL of GOx collected from the desalting column was loaded onto a pre-equilibrated hydrophobic column using Buffer 1. Hydrophobic columns work by attracting hydrophobic regions of proteins under high salt aqueous conditions (i.e. Buffer 1). When the salt concentration is dropped (i.e. the addition of Buffer 2) the forces of attraction between the hydrophobic proteins and the column are weakened and the proteins are eluted based on their relative hydrophobicity\textsuperscript{172}. Figure 2.6 (a) shows the successful binding of GOx onto the column as indicated by the strong yellow colour characteristic of GOx. Buffer 2 was loaded according to the gradient profile shown in Figure 2.6 (b) and the volume fraction eluents were collected in 1 mL aliquots.
Figure 2.6 (a) A photograph showing glucose oxidase (yellow) bound to the hydrophobic column, and (b) is a schematic representation of the buffer gradient profile utilised between Buffer 1 & 2.

The collected aliquots were monitored by UV-vis at 280 nm to identify which fractions contained GOx. This is represented by the blue trace in Figure 2.7. The green trace represents the gradient profile introduced in Figure 2.6 (b) while the brown trace is a measure of the conductivity of the column. The red markings show the 1 ml aliquots collected. It is evident that the GOx comes off the column between 36–49 mL and these fractions were collected for further purification. There are small peaks on the blue trace between 23–27 mL and 55–58 mL, which represent contaminant protein. It is specified on the Sigma technical sheet when GOx was purchased that the material may contain contaminants including amylase (≤ 0.5%), catalase (≤ 2 Sigma units/mg solid), galactose oxidase (≤ 3%), glycogenase (≤ 0.5%), invertase (≤ 0.5%) and maltase (≤ 2%). It is for this reason that the purification step is required to achieve a high specific activity of GOx. The fractions collected from the hydrophobic column were concentrated to 5 mL using a 10,000 molecular weight centrifugal filter unit (Merck-Millipore) and put through another syringe filter in preparation for loading onto the anion exchange column.
Figure 2.7 The fractions collected from the hydrophobic phenyl column (red) according to the elution of protein as measured at 280 nm by UV-vis (blue). The green and brown trace show the buffer gradient program used and the conductivity of the column, respectively.

The anion exchange column was equilibrated with Buffer 3 before the GOx sample was loaded. The elution gradient of Buffers 3 & 4 is represented schematically in Figure 2.8 (a) and the actual elution of GOx detected at 280 nm is represented by the blue trace in Figure 2.8 (b). The green trace represents the elution gradient, also shown in Figure 2.8 (a), and the brown trace is the column conductivity.
Figure 2.8 (a) A schematic representation of the buffer gradient program used for the anionic exchange column between Buffer 3 & 4, and (b) the fractions collected from the anionic exchange column (red) according to the elution of protein as measured at 280 nm by UV-vis (blue). The green and brown trace show the buffer gradient program used and the conductivity of the column, respectively.

Fractions 24 to 44 mL were collected as the protein containing aliquots. The spike in protein absorption in the first few fractions can be attributed to column overloading. These fractions were collected and passed through the column again.
The protein containing aliquots were again concentrated using a 10,000 molecular weight centrifugal filter unit down to the smallest volume possible to remove as much Buffer 4 as possible. The purified GOx was then resuspended in phosphate buffer (50 mM, pH 5) for characterisation and storage.

2.3.1.2 Characterisation

2.3.1.2.1 Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to confirm that the GOx was sufficiently purified. SDS-PAGE separates proteins based on their molecular weight and there was a clear band at 160 kDa corresponding to the molecular weight of GOx indicating that the purification was successful.

2.3.1.2.2 Storage

16 batches of GOx were purified using the protocol (2.3.1.1) and after gel electrophoresis they were combined to make one large batch. 1.5 mL aliquots were stored in epindorf tubes at -80°C while one 1.5 mL aliquot was kept at 4°C for further characterisation and use.

2.3.1.2.3 Concentration

The concentration of GOx in solution can be calculated using UV-Visible spectrophotometry. GOx has a characteristic absorption spectra with the protein shell showing a strong absorbance at 280 nm and the FAD redox active centre absorbing at 450 nm. If the intensity of the FAD peak at 450 nm is 10x less than the protein peak at 280 nm it indicates that the enzyme is in its native state (i.e. two FAD centres per protein shell, as discussed in Chapter 1). This was evident when
the purified GOx was characterised during UV-Vis and the concentration was calculated using the Beer-Lambert Law (Equation 2.9).

**Equation 2.9**

\[ A = \frac{e l c}{10} \]

where \( A \) is the measured absorbance (at 280 nm), \( l \) is the path length of the cell (cm), \( c \) is the concentration of analyte (mg/mL), \( e \) is the extinction coefficient (\( e = 16.7 \) for a 1% solution of GOx) and the adjustment factor of 10 is used to express the concentration in units of mg/mL.

From the absorption of the purified GOx sample it was calculated that the concentration of GOx in the stock solution is 0.5 mg/mL. This was also confirmed through protein assay experiments using the bicinchoninic acid (BCA) Pierce Assay (2.3.1.2.5).

2.3.1.2.4 Catalytic activity

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay is used to study enzyme kinetics and can quantify the activity of glucose oxidase in a given sample. To briefly describe the reaction mechanism, GOx catalyzes the oxidation of glucose to glucolactone and when the reduced form of the enzyme is converted back to the oxidized form it transfers two protons and two electrons to molecular oxygen to generate hydrogen peroxide (reaction scheme 1). Horseradish peroxidase (HRP) catalytically reduces \( \text{H}_2\text{O}_2 \) and this process is coupled to the oxidation of ABTS (reaction scheme 2), which undergoes a corresponding colour change. The oxidation of glucose can therefore be measured by monitoring the generation of \( \text{ABTS}^{(\text{Ox})} \) at 405 nm.
In an ABTS assay, a reaction cocktail of 180 µM ABTS, 150 mM Glucose and 2U/mL of HRP in phosphate buffer (50 mM, pH 7.4) was prepared on ice. 300 µL of reaction mixture was added to each required well in a 96-well plate and equilibrated at 37°C before 10 µL of enzyme sample was added and briefly stirred. The calibration curve was measured in the range of 1 – 0 mg/mL GOx. The absorption is measured continuously at 405 nm for 5 minutes and the slope of the linear region (ΔDO) is used to calculate the enzyme activity, as in Equation 2.10.

**Equation 2.10**

\[
\text{Activity (U.mL}^{-1}) = \frac{(\Delta DO_{\text{test}} - \Delta DO_{\text{blank}}) \times V_{\text{final}} \times DF}{\xi_{\text{ABTS}} \times V_{\text{initial}}}
\]

where \(\Delta DO_{\text{test}}\) and \(\Delta DO_{\text{blank}}\) are the linear increase in absorbance measured for the sample of interest and the enzyme free buffer, respectively. \(V_{\text{final}}\) is the final volume of the assay solution in mL. DF is the dilution factor of the enzyme stock solution, \(\xi_{\text{ABTS}}\) is the molar extinction coefficient for ABTS and is 36.8 mM/cm, and \(V_{\text{initial}}\) is the volume of enzyme solution added to the assay in mL. Note that one unit of activity is defined as the amount of enzyme that catalyzes the conversion of 1µmole of substrate per minute.

The measured activity for the purified GOx is 450 U/mg while the non-purified GOx measured an activity of 23 U/mg (~ 20 times less). The purified GOx was used for all experiments in this thesis and from now on if the abbreviation ‘GOx’ appears it refers to the purified material.
2.3.1.2.5 Pierce BCA protein assay

The Pierce BCA protein assay was utilized to determine the protein concentration in a solution. It depends on two steps. Firstly the well-known biuret reaction where Cu$^{+2}$ is reduced to Cu$^{+1}$ by protein in an alkaline environment, and secondly, the purple coloured reaction product is formed when two molecules of bicinchoninic acid (BCA) chelate with one cuprous ion (Cu$^{+1}$). The Cu(BCA)$_2$ complex exhibits a strong absorbance at 562 nm and is sensitive to 20-2000 µg/mL protein. The reaction takes place at 37°C over 30 minutes and a calibration curve was generated using both glucose oxidase and bovine serum albumin (BSA) standard series. The protocol from Thermo Scientific was followed precisely$^{174}$.

2.3.2 Graphene

2.3.2.1 Dispersion preparation

Graphene Oxide (GO) was synthesized from natural graphite powder using a modified Hummers method$^{175,176}$. The carbon material was received as a thick paste and weighed out to make a 0.5 mg/mL solution in milliQ water. This solution was pulse sonicated for 1 hour using a Branson Digital Horn Sonifier (500W) at 30% amplitude (2 seconds ON, 1 second OFF) in an ice bath to dissipate the heat generated. For the graphene-carbon nanotube composite electrodes fabricated and characterised in Chapter 4, the above dispersion was used as the starting material and the experimental section (4.3) describes the specific protocol followed. For the solvated graphene electrodes presented in Chapter 3 the following reduction process was used.

The chemical reduction of GO to the more conducting form reduced graphene oxide (rGO) by hydrazine was described in Chapter 1 (1.5.6.2). The experimental protocol followed was first introduced by Li et al. and describes the critical ratio of hydrazine:GO (7:10) to achieve a stable rGO dispersion$^{177}$. For these experiments, 10 mL of 0.5 mg/mL GO$_{(aq)}$ dispersion was diluted with 10 ml of milliQ water.
µL of Hydrazine (35 wt%) and 70 µL of ammonia solution in water (28 wt%) were added and the mixture was shaken for a few minutes immediately before being held at 95°C in a water bath for 1 hour. Larger quantities of rGO aqueous dispersion were prepared by scaling up the above ratios. Aggregates that form on the surface during the reduction process were removed by careful decanting and the stable 0.25 mg/mL rGO dispersion was stored under nitrogen gas and kept in an airtight shot bottle.

2.3.2.2 Dispersion characterisation

2.3.2.2.1 Optical microscopy

A drop of rGO dispersion was cast onto a glass slide with a glass cover sheet placed on top for qualitative optical analysis using a Leica optical microscope. An opaque white plastic sheet was placed underneath the glass slide to achieve a representative image, as it was difficult to distinguish dispersion features with the microscope stage behind the sample.

2.3.2.2.2 Particle size analysis and zeta potential

Zeta potential and particle size analysis were performed using the Zetasizer Nanoseries (Malvern Instruments). A dilute rGO sample was added to a universal dip cell quartz cuvette and the sample was analysed. The mechanism of particle size analysis is considered in detail in Chapter 3 (3.4.3.4) to support the discussion around experimental results.
2.3.2.2.3 pH

The pH of the rGO dispersion was measured using a TPS pH Meter (WP-80) after it had been calibrated using known pH standards. The pH of the rGO dispersion was >10.

2.3.2.2.4 Electrode fabrication

Specific details on electrode fabrication are outlined in the respective experimental sections of each results and discussion chapter.

2.3.3 Dexamethasone 21-phosphate disodium salt

Dexamethasone is a corticosteroid that inhibits the release of substances that cause inflammation in the central nervous system. Dexamethasone 21-phosphate disodium salt is a pro-drug of dexamethasone and the presence of the phosphate group gives the structure an anionic charge, allowing it to act as a dopant during the electrochemical growth of conducting polymers, as described in Chapter 1 (1.4.5). Figure 2.9 (a) shows the chemical structure of dexamethasone, the insoluble form of the drug, and Figure 2.9 (b) shows dexamethasone-21-phosphate disodium salt, the soluble anionic form. For the experiments carried out in this thesis the pro-drug dexamethasone-21-phosphate disodium salt was used. Henceforth, if a reference is made to dexamethasone it will refer to the pro-drug chemical structure (Figure 2.9 b) and will be given the abbreviation DEX.

![Chemical structures](image)

Figure 2.9 The chemical structure of Dexamethasone (a) and its pro-drug Dexamethasone-21-phosphate disodium salt (b).
2.3.4 Fos-phenytoin

Fos-phenytoin is a strong anti convulsant drug administered to epileptic patients intravenously as discussed in Chapter 1 (1.4.5). The chemical structure of Phenytoin is shown in Figure 2.10 (a) and the pro-drug form Fos-phenytoin is shown in Figure 2.10 (b). Fos-phenytoin was formulated to address the solubility issues of Phenytoin that requires a basic solution with ethylene glycol for intravenous injection. The addition of the phosphate group not only increases the drug’s solubility effectively decreasing drug administration time, a critical quality when seizures need to be controlled quickly; but the anionic nature also makes it appropriate to use as a dopant for conducting polymer structures. Fos-phenytoin (Figure 2.10 b) is used in this thesis and is given the abbreviation FOS.

![Figure 2.10 The chemical structure of Phenytoin (a) and its pro-drug Fos-phenytoin (b).](image)

2.3.5 Polypyrrole

2.3.5.1 Instrumentation

Electrochemical polymerisation of polypyrrole and controlled release experiments were performed using an eDAQ potentiostat/galvanostat with eDAQ Chart software (v2.0.7).
2.3.5.2 Electrochemical polymerisation

Pyrrole monomer (Merck, >99%) was distilled prior to use and stored under nitrogen at -20°C. A 0.2 M aqueous solution of pyrrole was used for all electrochemical polymerisation experiments with varying dopant anion concentrations and growth conditions that will be outlined in the respective chapters. The dopants used were para-toluene sulfonic acid (PTS), dexamethasone-21-phosphate disodium salt (DEX) or Fos-phenytoin (FOS). A 3-electrode electrochemical cell was used for all growth experiments and is shown schematically in Figure 2.11 (a). Gold mylar (CP Films Inc., USA) was used as a substrate for polymer growth (working electrode) and was washed in three steps before use; these steps included detergent water, ethanol and finally milliQ water before being dried using a nitrogen stream. Platinum mesh was used as a counter electrode and positioned directly opposite the gold mylar working electrode, as shown in Figure 2.11, and a Ag|AgCl reference electrode completes the configuration. This 3-electrode set up was used to galvantically grow polypyrrole (PPy) onto gold mylar electrodes for controlled release studies. For PPy-FOS growth the volume of this cell was reduced to cater for the limited amount of drug available and it’s relative cost. The specifics of this experiment are outlined in Chapter 5 (5.3).

Figure 2.11 (b) shows a PPy-DEX film on a gold mylar substrate directly after growth. All films were rinsed extensively with MilliQ water to remove any loosely bound dopant and excess monomer.
Figure 2.11 (a) A schematic of the 3-electrode electrochemical cell used to grow the conducting polymer films, and (b) a photograph of an as formed PPy-DEX film.

2.3.5.3 Controlled release experiments

Calibration curves were initially established for DEX and FOS using UV-Vis (2.2.1) so that when drug release was detected from the polypyrrole films it could be quantified. For controlled release experiments a 3-electrode cell was designed in a UV-Vis quartz cuvette to completely fit inside the UV-vis instrument chamber (Figure 2.12). The electrodes were connected to a potentiostat that would apply a potential to the polymer film and the drug release would be simultaneously measured in the release cell. It was critical to stir the release solution in order to get an accurate drug release reading and this was achieved using a flea stirrer bar inside the cuvette that was agitated by a small motor with a large stirrer bar attached, as represented in Figure 2.12. This was necessary to disperse the drug that would otherwise take a long time to diffuse away from the working electrode and give a real time representation of how much drug was released as a function of time at the given potential.
2.3.5.4 Drug release from polypyrrole powered by an enzyme bioelectrode

The protocol used to fabricate enzyme bioelectrodes will be outlined in the experimental section of Chapters 3 and 4. Additionally, an extensive description of the experimental set up used to characterise the release of drug from polypyrrole powered by an enzyme bioelectrode can be found in Chapter 5.
3. Solvated graphene bioelectrodes

3.1 Chapter aims

This chapter aims to investigate the feasibility of incorporating purified glucose oxidase into solvated graphene electrodes and to characterise the performance of the resulting bioelectrode. The results are analysed in the context of powering an implantable controlled drug delivery device for the treatment of epilepsy, as described in Chapter 1.

3.2 Classification and terminology

Slaughter et al. define hydrogels as “three-dimensional networks formed from hydrophilic homopolymers, copolymers or macromers (preformed macromolecular chains) cross-linked to form insoluble polymer matrices”\(^\text{178}\). Adhering to this definition, the graphene electrode structures presented in this chapter do not fall under the traditional classification of ‘hydrogel’ as they are not formed from polymers, even though they retain a high percentage of water similar to gels. The electrodes presented in this chapter are fabricated from aqueous dispersions of graphene (that DO NOT contain any surfactants or other polymeric dispersing agents), which are suction filtrated similar to a ‘buckypaper’, however, the filtration is stopped prematurely to retain water within the structure. They will be from here on referred to as solvated graphene electrodes (SGE).

3.3 Introduction

In Kopecek's 2007 review on hydrogel biomaterials, he outlines the many applications of the water–swollen materials including advanced tissue engineering, synthetic extracellular matrix, implantable devices, biosensors, controlled drug
delivery, separation systems, smart microfluidics, and energy-conversion systems\textsuperscript{179}. Coincidentally, their inherently soft mechanical properties make water-swollen materials especially suitable for implantation due to their similarities with natural human tissue\textsuperscript{180}. A critical challenge with implantable biomaterials, such as hydrogels, is the ability to incorporate functionalities so they are responsive to the surrounding environment\textsuperscript{181}. Hydrogel materials have been investigated for their application in biosensors and biofuel cells\textsuperscript{100,101} with the intention of supplying charge to low power consumption \textit{in vivo} devices.

The objective of this chapter is to fabricate a solvated graphene electrode, comparable to traditional hydrogels, with the additional bonus of exceptional electrical properties. The aim is to incorporate glucose oxidase (GOx) and facilitate direct electrical communication between the underlying graphene electrode and the immobilised GOx.

Traditionally, hydrogels were fabricated from cross-linked polymer networks and research is still being directed at these materials\textsuperscript{182}. With the rise in popularity of carbon nanotubes and graphene, considerable work has focussed on adding conducting fillers to polymer matrices to achieve conducting hydrogels\textsuperscript{183}. The difficulty with using conducting fillers is reaching a percolation threshold that will allow electrical conductivity by overcoming the insulating properties of the polymer matrix\textsuperscript{184,136}. Using an alternative approach, researchers investigated conducting polymers, such as Poly(3,4-ethylenedioxythiophene) (PEDOT), to create conducting polymer-hydrogel composites with graphene fillers so the material electrical properties are enhanced by the conducting polymer matrix\textsuperscript{185}.

Hydrogels can be used as a supporting matrix for the immobilisation of biocatalysts, such as enzymes or microbes, within the scaffold. This ability has benefits over conventional non-hydrated electrodes in that the reactants and products of the catalytic process can easily diffuse in and out of the structure\textsuperscript{186,187,185} and continuously supply the required fuel for the reaction. A significant amount of research interest is directed at achieving high energy density hydrogel biosensors and biofuel cells.
The major challenge in fabricating such complex electrodes is to use materials and processes that are not detrimental to the activity of the enzyme to be incorporated into the electrodes. Lehr et al. investigated the fabrication of a GOx-epoxy hydrogel cross-linked with polyethylene glycol diglycidyl ether (PEGDGE) and observed a decrease in GOx activity with curing time, which they attributed to the high crosslink density either denaturing the enzyme or restricting substrate mobility\textsuperscript{188}. Jang et al. looked at the activity of enzymes as a function of covalent immobilisation on silica nanoparticles within a hydrogel matrix and showed a 60% decrease in enzyme activity if the enzymes were not anchored to the nanoparticles\textsuperscript{189}. Once the enzymes are successfully immobilised within a hydrogel their activity can be retained due to the formation of a stable microenvironment of suitable solvent or buffer within the porous network of the hydrogel material\textsuperscript{190}.

It was hypothesized that some of the complications associated with traditional hydrogels for applications in biofuel cells and biosensors may be overcome if the insulating polymer matrix was eliminated and a hydrogel-like structure could support the enzyme based solely on the highly conducting nanomaterial, graphene.

There are many fabrication processes reported in the literature to fabricate ‘hydrogels’ from graphene. A common fabrication method relies on hydrothermal processes\textsuperscript{191} where a reducing agent is added to a graphene oxide dispersion. For example, Sheng et al. added sodium ascorbate to the GO dispersion and then the mixture was heated to > 90°C for 1.5 hours for sufficient reduction. Gelation occurs upon cooling\textsuperscript{192} (Figure 3.1). As enzymes are sensitive to heat, pH and a suite of chemicals, hydrothermal processes such as this are not appropriate for maintaining enzyme activity during the fabrication process.
Sheng et al., who utilised the sodium ascorbate reduction method above, discussed that the same gelation can occur at 37°C if the reaction is left for > 30 hours, however, the half life of native enzyme is only 7-8 hours in solution\textsuperscript{128}. As a result, there would be a significant reduction in enzyme activity over this time period. Another graphene gelation mechanism involves increasing the GO concentration up to 30 mg/mL for the fabrication of a hydrogel at room temperature\textsuperscript{193}. Unfortunately, the electrical properties of this gel were not characterised. A similar method investigated by Qiu et al. showed that electrical conductivity could be achieved for a GO-CNT composite\textsuperscript{194}.

Figure 3.2 represents the electrode fabrication procedure used in this chapter. In 2011, Yang et al. showed that during vacuum filtration, a reduced graphene oxide (rGO) dispersion undergoes gelation at the solid-liquid interface forming a hydrogel of ~ 92 wt% water, composed completely of the conducting rGO. Figure 3.2 (a) illustrates the gelation process as the rGO sheets order themselves into a highly structured assembly during filtration. Figure 3.2 (b) shows the resulting hydrogel being handled. In theory, as long as the enzyme added to these dispersions did not cause aggregation or inhibit the gelation mechanism of partial π-π stacking, this could be a feasible method of immobilising an enzyme within a graphene hydrogel matrix for biosensor applications.
Figure 3.2 (a) A schematic representation of the filtration and gelation process, (b) a photograph of an as-formed hydrogel and (c) an SEM cross section micrograph of a freeze dried hydrogel. Scale bar: 1µm. Adapted from Ref. 195.

Figure 3.2 (c) shows a SEM micrograph of the cross section of a freeze dried gel. If the hydrogel were formed in the bulk solution one would see a randomly cross-linked 3D structure, however, the highly uniform layered structure observed supports the hypothesized gelation mechanism. The rGO sheets cannot be fully packed down to form graphite because the sheets are highly solvated, which causes intersheet electrostatic repulsions between water molecules. Competing with the repulsive forces are the attractive forces of partial π-π stacking and this delicate balance gives the hydrogel material its extraordinary electrical and mechanical properties. It is the most conducting hydrogel recorded with a conductivity measured at 0.87 S/cm with the highest tensile strength reported at 75 MPa, compared to 0.01 to 10 kPa for other hydrogel materials of similar hydration195. Solvated graphene electrodes have also been tested for their application as a supercapacitor and show exceptional charge/discharge rates with a measured capacitance of up to 273 F/g.196.

In addition to the above-mentioned characteristics, the filtration process presented in Figure 3.2 (a) has been chosen to fabricate biosensors in this thesis due to its extreme versatility. The simple filtration method allows the addition of functional molecules, like glucose oxidase. Filtration also demonstrates control over film thickness by adjusting the volume of the dispersion. Additionally, there is no
insulating polymer filler present to interfere with the electrical properties of the final electrode. The resulting hydrogel is porous to reaction reactants and products and is a soft material that is relevant for the intended application of an implantable device.

3.4 Experimental

3.4.1 Materials

Reduced graphene oxide (rGO) dispersions and purified glucose oxidase (GOx) were used as described in Chapter 2. A 1.0 M stock solution of D-(+)-glucose was made up in phosphate buffer solution (PBS) (50 mM, pH 7.4) prepared from potassium phosphate monobasic and dibasic salts (Sigma). This solution was allowed to mutarotate\textsuperscript{197} for 24 hours and stored at 4°C when not in use. Ferrocene methanol (FcMeOH) (97%, Sigma) was made up to 5 mM using the same buffer. A standard series (0.016 to 2.5 mg/mL) of bovine serum albumin (BSA) (minimum 96% electrophoresis, Sigma) was prepared in milliQ water for use in the biological assay experiments. The QuantiPro BCA protein assay kit (Sigma) was used to perform the Pierce BCA assay. Peroxidase, Type II: From Horseradish (Sigma) and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma) were used in the ABTS enzyme kinetic assay.

3.4.2 Electrode fabrication

A micro filtration system was set up as shown in Figure 3.3 (a) with a 0.05 µm pore size mixed cellulose filter paper. The Buchner flask was connected through a safety collection flask to a Vacuubrand 80 mbar vacuum pump. 20 mL of 0.25 mg/mL rGO aqueous dispersion was transferred into the glass filtration set up using a glass syringe to minimise aggregation. The amount of enzyme added to the dispersion was calculated as a weight % of the amount of rGO present and the samples
analysed in this study were 20%, 10%, 5%, 2% and 0%. The labelling system, SGE-GOx (5%), will be used for these samples and the percentage value will indicate the weight % of GOx to the weight of graphene in the sample. The required amount of GOx was made up to 2 mL with milliQ water; a procedure also required to minimise aggregation, before the GOx was loaded into the rGO dispersion and gently mixed by syringe mixing. Syringe mixing is the process by which a solution is drawn into a pipette tip and expelled again. This is repeated a number of times and the volume should be sufficient to instigate effective mixing. For example, for the 22 mL rGO-GOx dispersion used in this study, a 2 mL pipette was used for syringe mixing. The syringe mixing served two purposes, firstly to ensure complete transfer of the entire enzyme contents into the dispersion, and secondly to disperse the GOx evenly throughout the solution to be filtered. Filtration was performed at room temperature and stopped at a predetermined point to identify the meniscus level immediately before the solvent was completely drawn through. This was critical to ensure the SGE structure was maintained in its hydrated state.

The filtrate was collected and refrigerated for future bioassay characterisation while the sample was washed with milliQ water and stored in 50 mL of PBS (50 mM, pH 7.4) in the refrigerator at 4°C. The SGEs were sliced into 0.5 x 0.5 cm (0.25 cm²) square samples for electrochemical and bioassay characterisation.
For electrochemical characterisation, a 0.25 mm$^2$ section of solvated graphene was sandwiched between two pieces of gold mylar as illustrated in Figure 3.4. The active surface area of each gold mylar strip was isolated using adhesive tape and the assembly was secured together using parafilm that wrapped around the electrode sandwich, leaving the bottom of the electrode exposed for electrochemistry.
3.4.3 Characterization methods

3.4.3.1 Water content

Solvated graphene sections were cut and weighed using a high precision microbalance. The sections were subjected to freeze drying using the CHRIST Alpha 2–4 LSC freeze dryer for 48 hours and the samples were weighed again with the mass loss calculated to be the water content as a percentage of the original hydrated mass (Equation 3.1).

Equation 3.1

\[
\text{Water content (\%)} = \frac{W_H - W_D}{W_H}
\]

where \( W_H \) is the hydrated weight of the solvated graphene sample and \( W_D \) is the dehydrated weight measured after freeze drying.
3.4.3.2 Scanning electron microscopy

Scanning electron microscopy (SEM) (JEOL Cold Field Emission Gun Scanning Electron Microscope) was performed on the freeze-dried samples, providing information on the cross-sectional structure of the material. The specimens were sputter coated with 10 nm of platinum using the Edwards sputter coater before analysis.

3.4.3.3 Optical analysis

The Leica Optical Microscope was used to characterise dispersion properties by depositing two drops of dispersion onto a glass slide and covering it with a glass cover slip. The microscope was used at x10 magnification to qualitatively observe the formation of aggregates and dispersion stability.

3.4.3.4 Particle size

Particle size analysis using the Zetasizer Nanoseries-ZS particle analyser (Malvern Instruments) was conducted to quantitatively analyse dispersion stability as a function of enzyme loading. Dynamic light scattering (DLS) is a technique where particles in solution scatter a laser beam at different intensities due to their Brownian motion. The translational diffusion coefficient can then be extracted and used to calculate the relative particle size using the Stokes-Einstein relationship (Equation 3.2).
The Stokes–Einstein relation where $D_h$ is the hydrodynamic diameter (or particle size), $D_t$ is the translational diffusion coefficient (given by DLS), $k_B$ is Boltzmann’s constant, $T$ is the temperature and $\eta$ is the dynamic viscosity.

The challenge with analysing graphene dispersions using this technique is that the theory is based on the solution containing homogenous spherical particles. The graphene dispersions however, are composed of sheets of rGO that are somewhere between flat and randomly wrinkled structures in solution depending on their level of purity\textsuperscript{161} and hence the data generated through this particle size analysis technique is only relevant for comparative studies within each experimental data set. In order to understand the extent of agglomeration of the graphene particles more thoroughly and to support particle size analysis the polydispersity index (PDI) was also measured. PDI is a measure of the particle size distribution within a sample with an ideal spherical, narrow monomodal system having a PDI < 0.1. The values presented in this thesis are much larger than this and can be attributed to the non-spherical nature of graphene in solution. The PDI values presented in this chapter will also be comparatively studied relative to the immediate data set.

3.4.3.5 Biological assays

Biological assays were used to determine the amount of enzyme immobilised within the solvated graphene electrode (SGE) samples and also how much was collected in the filtrate during electrode fabrication. Assays were also performed on the sample soaking solution (identified in Figure 3.3) to establish if the enzymes are sufficiently entrapped within the SGE samples (i.e. how much leaks out into the soaking solution). A kinetic assay was used to determine if the enzymes survived the processing and remain in their biologically active conformation. All biological
assays used a UV-vis plate reader to measure the absorbance and control the temperature of the experiments.

3.4.3.5.1 Pierce BCA protein assay

The Pierce BCA protein assay was utilized to determine the protein concentration in a sample. The protocol published in the Thermo Scientific technical sheet was followed precisely\(^{174}\). The Pierce BCA protein assay was presented in detail in section 2.3.1.2.5 of this thesis.

3.4.3.5.2 Glucose oxidase/peroxidase kinetics (ABTS assay)

The ABTS assay is used to study enzyme kinetics and can quantify the activity of glucose oxidase in a given sample. The ABTS assay was introduced in detail in section 2.3.1.2.4 of this thesis. For SGE analysis, the samples were soaked in the assay reaction cocktail and at each time point the solution was pipette transferred out into another 96-well plate for analysis in the plate reader. Afterwards it was returned to the same well with the SGE sample and incubated at 37\(^{\circ}\)C until the next time point where the procedure was repeated. At each time point the solution was mixed using the pipette to ensure all the reaction products were collected for analysis\(^{198}\).

3.4.3.6 Electrochemistry

All electrochemistry was performed in a 3-neck round bottom flask submersed in a thermo-coupled water bath maintained at 38\(^{\circ}\)C, as shown in Figure 3.5. Before each experiment the electrolyte was degassed using argon or nitrogen gas for a minimum of 15 minutes, and during electrochemical experimentation a constant gas blanket was used to eliminate oxygen. Platinum mesh was used as the counter electrode and all experiments were performed with reference to an Ag|AgCl
reference electrode (3 M NaCl). Each neck of the cell was stoppered with a rubber plug to secure the electrodes in place and to minimise oxygen contamination. Experiments were performed in a faradaic cage to minimise noise.

![Schematic of 3-electrode cell](image)

**Figure 3.5** Schematic representation of the 3-electrode cell used for electrochemical characterisation of rGO-GOx solvated graphene electrodes (SGE).

### 3.4.3.6.1 Electrochemical impedance spectroscopy

Electrochemical impedance measurements were conducted using a CH instruments potentiostat with accompanying software. Experiments were performed in PBS (50 mM, pH 7.4) or PBS with 5 mM FcMeOH. For the PBS experiments, the open
circuit potential was measured for each electrode before hand until a stable value was reached and this potential was used as the applied DC voltage for the impedance measurements. The FcMeOH experiments were driven at the oxidation potential of FcMeOH (+0.28 V vs. Ag|AgCl). All samples were measured over the frequency range 100 kHz to 0.1 Hz at an AC amplitude of ±5 mV.

3.4.3.6.2 Cyclic voltammetry

Direct electron transfer (DET) and mediated electron transfer (MET) mechanisms of electron transfer were introduced in Chapter 1 (1.5.5). Cyclic voltammetry was used to identify if DET peaks could be observed for the immobilised GOx over the capacitance of the SGEs and also to establish if the electrodes could facilitate MET with FcMeOH. The parameters for each experiment will be outlined when the data is presented in the results and discussion section.

3.4.3.6.3 Amperometry

Current vs. time curves were utilised to measure the amperometric response of the immobilised GOx in the SGEs. Experiments were conducted for 33 minutes with the first 10 minutes utilised to stabilise the electrode, before spiking the relevant glucose concentration. Usually, amperometric studies would show a sequential addition of glucose with a current increase corresponding to the glucose concentration. Due to complicated mass diffusion and electron transfer kinetics in the SGE system, it was necessary to measure each glucose concentration individually. Figure 3.6 represents a typical GOx loaded SGE response to a spike in glucose and identifies the data analysis method employed. Briefly, the baseline current was subtracted from the highest current measured and this was taken as the catalytic current generated from that electrode ($\Delta i$) at the given glucose concentration. Experiments were done in triplicate.
3.4.3.6.4 Fourier transform alternating current voltammetry

Fourier transform alternating current voltammetry (FTACV) experiments were performed using a custom built FTACV instrument at Prof. Alan Bond’s electrochemistry research group at Monash University in Melbourne, Australia. The theory of this technique was introduced extensively in Chapter 2 (2.2.3.8). A three-electrode cell was used for all experiments similar to that presented in Figure 3.5 and controlled at 38°C under nitrogen gas. A highly concentrated buffer of 0.5 M PBS (pH 7.4) was used as the electrolyte to minimise solution resistance. Experiments were performed at a frequency of 9 Hz and amplitude of 120 mV.

3.5 Results and discussion

3.5.1 Electrode fabrication and storage

An attempt was made to fabricate a SGE using graphene oxide, i.e. before the reduction step removed a portion of the oxygen groups. However, this approach
was unsuccessful due to the large amount of oxygen containing functionalities causing electrostatic repulsions, which dominate the interaction and inhibit the formation of a stable gel. The balance between attractive and repulsive forces that underpin the stability of SGEs was postulated by Yang et al.. Their group suggests that partial π-π stacking is responsible for the forces of attraction while the oxygen functionalities, and hence the tightly packed water molecule layer, prohibit the sheets from being fully compact. Hence, if the repulsive forces are large compared to the attractive forces, the graphene will not pack into a SGE structure. The motivation behind trying to fabricate a SGE out of GO arises from concerns associated with the integrity of the glucose oxidase (GOx). Factors that could affect the GOx integrity, and ultimately, its catalytic activity, include survival through the filtration process and denaturation caused by the high pH value from the added ammonia (used for stabilization) or the hydrazine (used for chemical reduction). If the SGE could be fabricated using GO it would be unnecessary to expose the enzymes to these chemicals. Unfortunately, this was not feasible and the ultimate advantage of the reduction step is that it warrants a more electrically conductive final product.

Another attempt was made to avoid exposing the enzymes to the harsh reducing chemicals by filtering the enzyme solution on top of an already formed SGE. It was believed that the enzyme may penetrate into the SGE, however, SEM analysis showed that the enzyme actually packed down on top of the graphene electrode, as shown in Figure 3.7. Ideally, the enzyme will be evenly distributed throughout the structure to ensure efficient electrical wiring to the graphene sheets.
An attempt was also made to reduce the pH of the dispersions to a level suitable for the enzymes using dilute acetic acid. Figure 3.8 (a) shows the relative activity of the purified GOx over the range pH 2 – 10.5, as measured through an ABTS kinetic assay, and it is evident that the optimal range for glucose oxidase activity is pH 4 - 7. An rGO dispersion has a pH ~ 10 due to the addition of ammonia for stabilisation. When dilute acid is added to the dispersion there is immediate and visible aggregation caused by the ionization of carboxylic acid functional groups. This can be seen in Figure 3.8 (b) where (i) is the stable rGO dispersion (pH 10) and (ii) is the same dispersion after the pH was gently lowered (final pH ~6.5). It is evident that extensive aggregation has occurred.
When electrolytes are added to an electrostatically stabilised dispersion the repulsion forces are suppressed due to electrical double layer screening, as discussed in Chapter 2. If the double layer is hindered too much the dispersion will agglomerate and destabilise. Based on the same theory, the SGEs undergo volumetric changes in the thickness direction when the ionic strength of the soaking solution changes, as the repulsive forces are dependent on the ionic strength of the solution\textsuperscript{195}. Significantly, Yang et al. reported that the as-formed SGE can be stored in buffer without disruption of the SGE stability. The ability to store the SGEs in buffer is of relevance to this study because enzymes are stored in buffers to maintain their activity at a relevant pH. In this way storage in a buffer solution was critical.
3.5.2 Dispersion properties

3.5.2.1 Optical analysis

It was critical to understand if the addition of enzymes and their supporting buffer salts would cause aggregation in the dispersions, as this may affect SGE formation. To avoid instabilities, the enzyme that was added to the dispersion before filtration was made up to 2 mL with milliQ water to dilute the salts, with the exception of the 20% sample which required 2 mL of enzyme stock solution and hence no dilution. Optical analysis of the dispersions showed qualitatively that for each GOx loading, no significant aggregation is observed, with Figure 3.9 (a) showing an example of the highest tested GOx loading in an rGO dispersion, namely rGO-GOx (20%). It is evident that no aggregates are present in the optical micrograph. The qualitative analysis is based on comparison to an aggregated example at the same magnification (Figure 3.9 b).

Figure 3.9 Optical analysis of rGO dispersions, with micrograph (a) showing an rGO-GOx (20%) dispersion, while micrograph (b) shows an example of an aggregated system (rGO – pH 6.5). Scale bars represent 1000 µm.

3.5.2.2 Particle size analysis

The enzyme-loaded dispersions were characterised by particle size analysis to further examine the degree of aggregation, which may not be detected through
qualitative optical analysis. As mentioned in the experimental section of this chapter, the data generated by this technique can only be used comparatively within experimental data sets, and is not accurate as an absolute value. Taking this into consideration, on analysis of the nominal number average particle size data there is very little difference, and certainly within experimental error, between rGO dispersions with different GOx loadings (Figure 3.10). Theoretically, aggregated particles would be detected as larger particles as they would have significantly slower Brownian motion compared to freely dispersed graphene sheets. This result would suggest that there is no significant increase in particle size, and hence, aggregation, with the addition of GOx and salts. The large error bars arise due to the fact that rGO is not a spherical shape and therefore, as the sheet moves under Brownian motion, light interacting with the faces and edges of the sheets result in large variations in scattered light intensity. These variations produce large discrepancies in average particle size values.

Figure 3.10 also graphs the polydispersity index (PDI) of the samples and a significant increase in PDI for the 20% GOx loaded sample compared to the other samples was noted. PDI gives an indication of the size distribution of particles within a solution, suggesting a larger range of particle sizes in the 20% sample. This may be attributed to the fact that during the addition of GOx there was no dilution of the salts, alternatively the system may have reached a critical salt threshold, which would initiate the onset of aggregation. In addition, the enzyme may tend to agglomerate together when added to the dispersion, which would also be detected as large particle sizes.

The degree of aggregation for the tested samples was not considered a concern for these experiments, especially since there was no significant decrease in dispersion quality through optical analysis (Figure 3.9), and as such electrodes were fabricated for all tested GOx loadings.
3.5.3 Electrode characteristics

A series of SGEs were fabricated using rGO (pH 10) with different loadings of enzyme, namely 0%, 2%, 5%, 10% and 20% (as a weight % of rGO). For each GOx loading, three samples were freeze dried and weighed to determine their percentage hydration. There was very little discrepancy between the values and interestingly no trend between the different enzyme loadings. It was calculated that each SGE in the series contained 96.7 ± 0.09% water.

To determine the distribution of enzymes throughout the final SGE-GOx sample a cross sectional analysis was performed using SEM. Figure 3.11 shows an rGO-GOx (2%) sample and it is evident that the enzymes form discreet layers within the
structure. A similar structure was observed for all enzyme loadings. These layers tend to encourage delamination of the graphene sheets and this may be detrimental to the conductivity of the final electrode. On closer analysis of the interface between the enzyme and graphene, it is evident that there is some physical interaction between the two components (Figure 3.11 b), which may be critical for direct electron transfer (DET).

Figure 3.11 SEM micrographs of an SGE-GOx (2%) electrode sample at (a) low (scale bar 1 \(\mu\)m) and (b) high (scale bar 100 nm) magnification showing discreet layers of enzyme and delaminated graphene sheet bundles.

The tendency of the GOx to agglomerate into these discreet layers is not surprising given that there may be hydrazine and ammonia associated with the graphene sheets. Tung et al. suggest the formation of hydrazinium compounds consisting of negatively charged carbon nanotubes surrounded by N\(_2\)H\(_5\)\(^+\) ions\(^{199}\) and this may be similar for the negatively charged graphene sheets, as supported by data extracted from the pierce BCA biological assay. The BCA assay relies on the reduction of Cu\(^{2+}\) to Cu\(^{1+}\) through interactions with certain amino acids on the protein shell of glucose oxidase; however, as hydrazine is a strong reducing agent it overrides the reaction with protein and gives a strong indication of where the hydrazine is in the filtration process. No protein or hydrazine was detected in the filtrate or the soaking solution (Figure 3.3) for the formed electrodes using this assay. Furthermore the SGEs gave a very strong response to the assay, even when no
protein was present, indicating that the hydrazine is still associated with the graphene sheets and may support the formation of the suggested hydrazinium compounds in solution. After the electrodes were stored in PBS (pH 7.4) the pH of the electrode was tested to be approximately pH 7 and the filtrate had a pH of 10 indicating that the ammonia does not remain in the SGE structure.

3.5.4 Biological assays

For all enzyme-loaded SGE compositions, no GOx was detected in the PBS (pH 7.4, 50 mM) soaking solution, not only from the BCA protein assay, where the concentration could have been below the detection limit of the test, but also pH 7.4 with the ABTS activity assay which should have detected all active enzyme. This indicates that once the enzyme-loaded SGEs are formed, the GOx remains immobilised within the structures.

3.5.4.1 Peirce BCA protein assay

The Pierce BCA assay gave interesting results in response to the hydrazine present in the samples; however, the collected filtrates were still too dilute for the sensitivity of this assay. The hydrazine has an overriding effect on the reduction of the copper, and hence the amount of GOx immobilised within the SGEs was inconclusive.

3.5.4.2 Glucose oxidase/peroxidase kinetic assay (ABTS)

Given that the data from the protein assay was inconclusive in determining how much enzyme is entrapped in each SGE-GOx electrode, the results presented in Figure 3.12 were extrapolated from the ABTS kinetic assay calibration curve. Briefly, the enzyme activity measured during the ABTS assay for each SGE
concentration (i.e. 0, 2, 5, 10 and 20% GOx loading) was calculated against the standard curve to determine the amount of active GOx present (in mg) in the assay. Based on the initial active enzyme loading (also in mg) a percentage value was extrapolated to give the results in Figure 3.12. As such the data is not only a representation of how much active GOx is immobilised in the SGE structures, but also how much of the initial enzyme loading mass is active.

Figure 3.12 The amount of enzyme retained in each SGE-GOx electrode composition as a percentage of the initial loading.

Figure 3.12 represents the amount of enzyme retained in each SGE-GOx electrode as a percentage of the initial loading amount. Interestingly a similar amount is observed for 2%, 5% and 10% samples. When the enzyme loading is increased to 20% there is a much larger retention of enzyme and one explanation for this is the tendency of GOx to agglomerate within the structure, forming large particulates that cannot be filtered out. This data is consistent over multiple repeats of the experiment within the standard deviation represented in Figure 3.12, and coupled with the consistency in water content of 96.7 ± 0.09% among all SGE-GOx
electrode compositions; it demonstrates good reproducibility for electrode fabrication.

The ABTS kinetic assay was chosen to determine if the enzymes were still biologically active after the electrode fabrication process; i.e. if GOx survived the high pH environment and also the filtration step. Figure 3.13 shows the enzyme activity for each SGE-GOx electrode composition and the positive result indicates that the enzymes retain some of their activity (also supported by Figure 3.12). Plotted on the same graph is the amount of enzyme calculated in each assay experiment and there is some correlation between the amount of enzyme present and the amount of units of activity that were measured through the kinetic assay. The amount of GOx measured per assay sample was calculated from the retention value (given in Figure 3.12) and the size of the SGE-GOx sample used for the assay, i.e. 0.25 cm$^2$, as a function of the total area (and loading) of the as-formed SGE-GOx electrode (17.4 cm$^2$).

Given that the purified enzyme measures an enzyme activity of 4.5 U/mL it is evident that there is a decrease in enzyme activity during the electrode fabrication process; which may be caused by exposure to hydrazine and extreme basic conditions.
3.5.5 Electrochemical characterisation

3.5.5.1 Cyclic voltammetry

Cyclic voltammograms were used initially to determine if a DET signal could be detected from the electrochemical oxidation and reduction of glucose oxidase, without the need for a mediator. Glucose oxidase undergoes redox chemistry at $E^\circ = -0.46 \text{ V}^{12+}$ (vs $\text{Ag|AgCl}$) and as such reversible peaks should be observed around this potential when the active site of the enzyme is wired to the conducting material. Figure 3.14 shows the voltametric response for an rGO-GOx (10%) sample with and without glucose present, green and black trace, respectively. It is evident that no defined redox peaks are observed. This may be due to the enzyme not being directly wired to the graphene in this configuration. Other literature has
shown a DET response using rGO-GOx\textsuperscript{146} however, the electrodes are not in a hydrated form such as the SGEs. Additionally, the capacitive response of the rGO may be too large to detect the relatively small faradaic response of the GOx.

![Cyclic voltammogram of an SGE-GOx (10%) sample cycled at 10 mV/s in PBS (black trace) and PBS + 150 mM glucose (green trace) at 37°C under an argon blanket from -0.65 to -0.35 V (vs. Ag|AgCl)](image)

**Figure 3.14** Cyclic voltammogram of an SGE-GOx (10%) sample cycled at 10 mV/s in PBS (black trace) and PBS + 150 mM glucose (green trace) at 37°C under an argon blanket from -0.65 to -0.35 V (vs. Ag|AgCl)

In order to determine if the GOx remained catalytically active within the SGEs, 0.5 mM FcMeOH was added to a PBS electrolyte solution (pH 7.4, 50 mM) to demonstrate if a mediated electron transfer (MET) response could be measured for a SGE-GOx (10%) sample. FcMeOH undergoes a reversible one-electron redox reaction and successfully shuttles electrons for GOx, as indicated in Equation 3.3\textsuperscript{200}.

On a planar GCE electrode FcMeOH undergoes redox chemistry with an $E^\circ$ value of 0.15 V\textsuperscript{201} (vs. Ag|AgCl). Mediated electrochemistry on the SGE-GOx electrodes is represented in Figure 3.15 and shows a non-reversible redox process with a significant shift in $E^\circ$ to ~0.52 V (black trace). When 150 mM glucose is added to the system (green trace) the sigmoidal shape representative of a mediated electron transfer response is only slightly obvious and on analysis a small increase in catalytic current is observed, but for some samples it was not measurable. All data was analysed at a scan rate of 10 mV/s on the 10\textsuperscript{th} cycle of a series of potential sweeps for each electrode. Before this analysis the electrochemistry was stabilised.
with 20 cycles at 100 mV/s, 20 cycles at 50 mV/s and finally 10 cycles at 10 mV/s for each electrode. This was determined to be sufficient cycling to generate stable and representative electrochemistry for data analysis. Cycling the electrode between the relevant potentials before analysis is critical to ensure both the oxidised and reduced forms of the mediator are present to produce reproducible cyclic voltammograms. This system conditioning is widely used in electrochemistry. The peak height was measured relative to the extrapolation of the capacitive baseline current, as represented by the dashed lines in Figure 3.15, and the catalytic current is the difference between the peak heights before and after glucose is added.

**Equation 3.3**

\[
\text{GOx}_{(\text{Red})} + \text{FcMeOH}_{(\text{Ox})} \rightarrow \text{GOx}_{(\text{Ox})} + \text{FcMeOH}_{(\text{Red})} \\
\text{FcMeOH}_{(\text{Red})} \rightarrow \text{FcMeOH}_{(\text{Ox})} + e^{-}
\]

**Figure 3.15** Cyclic voltammogram of an SGE-GOx (10%) sample cycled at 10 mV/s in FcMeOH/PBS (black trace) and FcMeOH/PBS + 150 mM glucose (green trace) at 37°C under an argon blanket from 0 to 0.8 V (vs. Ag|AgCl)
The CV’s and their respective peak heights were inconsistent between samples of the same rGO-GOx composition and it was difficult to draw conclusions from the data. The discrepancies may arise from the electrode configuration (Figure 3.4), as it is difficult to secure good electrical contact with a soft SGE in order to test its electrochemical properties. The configuration used was the most effective of the samples tested, however, it still consisted of a crude assembly procedure with little control over how effectively the gel and the gold mylar were in electrical contact. This could lead to large contact resistances and be detrimental to the electrical properties of the working electrode. Additionally, it was thought that FcMeOH may not penetrate into the SGE sufficiently to mediate the shuttle of electrons from GOx to rGO, however, in a study conducted on poly(ethylene glycol) hydrogels, Jeerage et al. suggest that for a small neutral species like FcMeOH it is reasonable to assume that the concentration of species within the hydrogel is equivalent to the concentration in the external electrolyte, given that the pore sizes of the hydrogel are sufficiently big and do not physically obstruct its mobility. Yang et al. outline for similar SGEs, as used in this study, small ions are not hindered by pore size.

It is inconclusive from cyclic voltammograms if the enzyme is catalytically active and hence an alternative form of amperometry was used to characterise the system electrochemically.

### 3.5.5.2 Amperometry

Initially, the I-t curve was used to monitor the change in current with sequential additions of glucose. This is a common method used to characterise the sensitivity, range and response time of a biosensor, however, it was difficult to do this for the SGE-GOx electrodes as the response time was several minutes and the increase in current was not a stepwise response, but rather a slow deviation from the baseline current. Figure 3.16 shows an example of the amperometric response recorded for an SGE-GOx (5%) electrode sample with 5 mM of glucose added to the 0.5 mM FcMeOH in PBS solution at 600 seconds. It took this sample roughly 5 minutes to
respond to the glucose addition, even though it was mixed thoroughly, and the maximum current (minus the baseline current) was recorded. From this data it is evident that the enzyme shows some catalytic activity, however, the transfer of charge within the SGE and across the SGE-gold mylar interface seem to be the limiting factors, given that the FcMeOH can penetrate into the SGEs unrestricted. This hypothesis will be explored further through electrochemical impedance analysis in the following section.

Figure 3.16 An example of the amperometric response measured when an i-t curve experiment is done on a SGE-GOx (5%) sample. The spike at 600 seconds represents the addition of 5 mM glucose to the electrochemical cell (vs. Ag|AgCl).

Analysis of the amperometric data generated from testing the range of enzyme-loaded SGEs with a 5 mM spike of glucose showed that there was very little trend between the amount of enzyme present in the SGE and the maximum catalytic current generated. In fact, the error bars associated with this data set prohibit any meaningful conclusions to be drawn (Figure 3.17). It is interesting to note that the sample with no GOx present (i.e. 0%) generates the highest maximum current (although not statistically different) and it suggests there is a significant amount of baseline drift within the system which was completely housed in a faradaic cage and
grounded to minimise experimental error. The disruption of the electric double layer at 600 seconds when the glucose is spiked into the system causes a slight shift in the baseline current, which is shown in Figure 3.16, and this may be the source of discrepancy. Care was taken to minimise the disruption by gentle and quick pipette mixing and every precaution was taken during data analysis to utilise the ‘new’ baseline for corrections, however, the ability of charge to pass through the hydrated electrode and its contact resistance with the gold mylar was difficult to control and may play a significant role in the errors observed.

![Figure 3.16](image.png)

**Figure 3.16** The maximum catalytic current generated as a function of GOx loading as measured through amperometric experiments in response to the addition of 5 mM of glucose for SGE’s.

Similarly, when different enzyme loadings were analysed, namely 0%, 5% and 10%, and spiked with different amounts of glucose it is difficult to draw conclusions from the resulting data (Figure 3.18). A new sample was used for each experiment and the data presented is the average of a minimum of 3 samples for each glucose concentration and enzyme loading. The data presented in Figure 3.18 was normalised against an SGE-GOx (0%) reference data series at 0 mM glucose (i.e. PBS) and is identified by an asterisk (*). There is a significant increase in catalytic current for both SGE-GOx (5%) and SGE-GOx (10%) when 0.5 mM glucose is added, however this increase is not observed for samples of the same composition at
higher glucose concentrations. This effect is not an artefact of laboratory procedure as the experiments were carried out one SGE-GOx composition at a time and the glucose additions were in an intentionally random order. It is difficult to rationally explain the increase in catalytic current for both samples at 0.5 mM glucose. There is also an unexplained decrease in catalytic current for the SGE-GOx (5%) and SGE-GOx (10%) samples with increasing glucose concentration from 0.5 mM. The amperometric results are inconclusive as to whether the enzyme is measurably catalytically active. The fact that in most cases there is an increase in ‘catalytic’ current for samples with enzymes present may support the activity of the enzyme (as quantified through biological assays) however, the lack of trends in the data set may suggest that the enzyme is in fact not efficiently electrically wired to the graphene electrode material.

![Graph showing catalytic current vs glucose concentration](image)

**Figure 3.18** The maximum catalytic current generated as a function of glucose concentration for SGE-GOx electrode samples 0%, 5% and 10% GOx loading. Asterisk (*) indicates the baseline data series used to normalise the data.
3.5.5.3 Electrochemical impedance spectroscopy (EIS)

The charge transfer resistance ($R_{ct}$) measured through EIS is a representation of the electrical resistance within the SGE plus the contact resistance between the sample and the gold mylar electrode. For this system it will be difficult to separate the two. Figure 3.19 (a) shows a typical Nyquist plot for a SGE sample and the method used to measure the $R_{ct}$ of each sample. The results presented in Figure 3.19 (b) represent at least 3 repeats for each data point. For SGEs produced by Yang et al., they reported $R_{ct}$ in the range of tens of ohms where for this system the resistances are measured in the range of hundreds of ohms (Figure 3.19). Yang et al. configured their SGEs using platinum foil and used the device as a two-electrode cell, rather than the working electrode in a three-electrode cell, as was the case for the experiments in this thesis. Since the experimental procedure used in this thesis is based on Yang’s initial results it may suggest that the contact resistance between the SGE and the gold mylar is the biggest contributor to the electrode $R_{ct}$.

Nevertheless, trends in $R_{ct}$ values between the different enzyme loading compositions can be analysed. Figure 3.19 shows the $R_{ct}$ measured for each enzyme loading with the PBS profile representing data generated when the electrode was held at open circuit potential in a PBS (pH 7.4, 50 mM) solution and the FcMeOH trace shows similar data for the electrodes held at an oxidising potential for FcMeOH (0.28V vs. Ag|AgCl). The PBS trace is representative of the internal resistance of the electrode and shows that there is very little statistical difference between samples from SGE-GOx (0%) to SGE-GOx (10%), however, SGE-GOx (20%) has a lower $R_{ct}$ than the other compositions. This may be related to a larger amount of supporting salts being added to the electrode when GOx was added which would decrease the repulsive forces between the rGO sheets, forming a more compact electrode and ultimately aiding charge transfer. In the mediated system (FcMeOH) the electrodes generally exhibit lower charge transfer resistances which is not surprising given the system is being driven by the oxidation of FcMeOH in solution. This also tells us that the shuttle of electrons from FcMeOH to rGO is not the limiting factor in this system and supports the discussion in section 3.5.5.1 to
explain the inconclusive cyclic voltammetry results. The EIS results also support the amperometric response of SGE’s (section 3.5.5.2), which hypothesize that the errors observed may be due to a large charge transfer resistance within the SGE itself and between the SGE sample and the gold mylar.

![Figure 3.19](image)

Figure 3.19 (a) A Nyquist plot for a SGE-GOx (20% GOx loading) sample indicating the method used to calculate the charge transfer resistance ($R_{ct}$). (b) Charge transfer resistance of SGE-GOx electrodes as a function of enzyme loading.
It is difficult to draw conclusions from other electrochemical techniques including cyclic voltammetry, amperometry and impedance on the contribution of the immobilised GOx to the current generated. As such, Fourier transform alternating current voltammetry (FTACV) was used to separate and identify the faradaic GOx current from the capacitive current.

3.5.5.4 Fourier transform alternating current cyclic voltammetry

FTACV, which was introduced in chapter 2, is a technique that extracts faradaic and capacitive currents separately. Essentially, this allows the analysis of the individual contributions of the GOx faradaic response and the rGO capacitive current, and ultimately, will determine if the enzyme is directly wired to the SGE. Briefly, the technique relies on applying an AC perturbation superimposed on a DC cyclic voltammogram and data is extracted through Fourier transform algorithms. The data is converted from the frequency domain to the time domain for analysis and represents a response that contains a DC contribution as well as a series of responses at the fundamental, second, third and higher harmonics. If the GOx is not electrically wired to the graphene the harmonics will be very similar to the control sample when no enzyme is present. Alternatively, if the GOx is electrically wired to the graphene, features will be visible in the higher order harmonics. Figure 3.20 shows the results for SGE-GOx sample at 20% GOx loading (green traces) and the control profile of SGE with no enzyme present (black traces). Figure 3.20 (a) shows the DC component, which is exactly the same as a cyclic voltammogram (CV) and correlates to previously presented CV electrochemistry for similar samples (Figure 3.14). It is evident that when enzyme is present smaller cathodic and anodic currents are observed and this may be due to the delamination observed previously in SEM analysis (Figure 3.11). Figure 3.20 (b) and (c) show the first and second harmonics, respectively, and information on the electrical wiring of the enzyme cannot be extracted due to the large capacitive current still present in the profile. This capacitive contribution is identified in Figure 3.20 (c) with red arrows and equates to 10-15 µA for SGE-GOx (0%) and 3-10 µA for SGE-GOx (20%). A
harmonic can be used to determine the GOx contribution when this capacitive current is close to zero, as in Figure 3.20 (d). This residual capacitive response is common for electrodes with large non-linear capacitive contributions like the SGE’s presented in this piece of work.

Figure 3.20 FTACV data for SGE-GOx (0%) and SGE-GOx (20%) samples showing (a) the DC component, and the (b) first, (c) second, (d) third, (e) fourth and (f) sixth harmonics in the time domain. Experiments were performed at 9Hz and 120 mV in 0.5M PBS at 38°C under nitrogen.
The third and fourth harmonics represented in Figure 3.20 (d) and (e), respectively, show more defined harmonics for the SGE-GOx (20%) sample compared to the control case with no enzyme present (black trace), as indicated by blue and red asterisks groups. This response can be attributed to the faradaic contribution from the immobilised enzyme however, the weak response may suggest that most of the enzyme is not electrically wired or not accessible by glucose. It is possible that this response may be attributed to the surface GOx that is accessible to glucose in solution; however, the positive biological assay results suggest that the glucose is penetrating into the SGE structure and also confirms that the enzyme shows reasonable enzyme activity for the amount of GOx initially immobilised. For comparison, the same experiments were performed on an optimised rGO-MWNT-GOx-PEi electrode, introduced and characterised in Chapter 4 of this thesis. Figure 3.21 (a) and (b) show well-defined third and fourth harmonics, respectively. This system also represents 70% less GOx immobilised per geometric area than the SGE-GOx (20%) sample and shows a much clearer faradaic response. The rGO-MWNT-GOx-PEi system showed a clear direct electron transfer (DET) response through cyclic voltammetry electrochemistry (section 4.4.2) and the data in Figure 3.20 is shown here purely for comparative analysis with the SGE electrodes. Figure 3.20 (f) shows the sixth harmonic and it is evident that no faradaic response is measured, also supporting claims that the GOx is not effectively wired to the SGE.

It can be concluded from FTACV experiments that although some GOx is wired to the SGE to show a faradaic response in the third and fourth harmonics, the response is small indicating that most of the GOx is not effectively wired. The electrodes do not respond reproducibly to environmental increases in glucose concentration, as discovered through CV and amperometric electrochemistry, and as such, will be inappropriate to use in biofuel cells, biosensors and for the purpose of driving the release of drug from a conducting polymer scaffold.
Figure 3.21 FTACV data for optimised rGO-MWNT-GOx-PEi electrodes showing the (a) third and (b) fourth harmonics for comparison with SGE electrodes presented in Figure 3.20. Experiments were performed at 9Hz and 80 mV in 0.5M PBS at 38°C under nitrogen.

3.6 Conclusions

Solvated graphene electrodes (SGE) loaded with GOx were characterised to determine their final composition and structure, as well as their ability to facilitate direct electrical communication with the immobilised enzyme. The SEM micrographs suggest good physical interaction between the immobilised GOx and the rGO sheets however, does not show conclusively that the enzyme is electrically wired to the SGE. The CVs performed in PBS did not show a DET signal, suggesting that the GOx is not electrically wired however, this is also inconclusive as the capacitive current could be swamping the relatively small faradaic response from the enzymes. To determine if the enzymes remain catalytically active a mediator (FcMeOH) was added to the system to bypass the direct wiring mechanism, since the FcMeOH will shuttle electrons from the enzyme to the electroactive surface of the electrode. Unfortunately the resistance within the system prohibits the detection of meaningful catalytic data from cyclic voltammetry. Mediated amperometry was used to determine the maximum current generated when a given amount of glucose was spiked into the electrochemical system and again the results were inconclusive. Fourier transform ac voltammetry showed conclusively that the electrical communication between the immobilised GOx and the SGE scaffold was poor, as determined by analysing the higher order harmonics, which distinguish faradaic currents from non-faradaic components.
From electrochemical techniques the data was convincing in suggesting that the enzymes are not sufficiently electrically wired to the SGE. Biological assay experiments showed that the immobilised enzymes remain catalytically active. For the given application of powering a controlled drug delivery device the SGE-GOx electrodes will be inappropriate given that there is no significant catalytic current generated in the presence of glucose, which is the controlling factor for the drug release experiments. Even if a suitable catalytic current was generated the response time seems to be in the order of several minutes, which is unacceptable for epilepsy management. It is critical for seizure activity to be treated in the first couple of seconds due to the quick onset of a seizure as outlined in Chapter 1 (1.1).

### 3.7 Acknowledgements

The author gratefully acknowledges Dr Anita Quigley for contributions to the execution and analysis of the protein assay experiments and Dr Tony Romeo of the Electron Microscopy Facility at the Australian Institute of Innovative Materials for SEM assistance. Additionally, thank you to Professor Alan Bond, Dr Alexandr Simonov and Ms Kiran Bano of Monash University for critical help with Fourier transform alternating current voltammetry. The author would also like to thank Dr Dan Li and Dr Sanjeev Gambhir for invaluable discussions around all things graphene.
Chapter Four | Reduced graphene oxide-carbon nanotube composite bioelectrodes

4. Reduced graphene oxide-carbon nanotube composite bioelectrodes

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4.1 Introduction

Unique electrode architectures have been fabricated from processable solutions of carbon nanotubes. These include fibres, films and hydrogels, the formation of which usually requires a surfactant or organic solvent to suspend the highly insoluble carbon nanotubes. The addition of surfactants has detrimental effects on the electrical properties of the final electrodes and whilst organic solvents provide an alternative dispersing media to surfactants, these have limitations in terms of being inappropriate to use with biological systems. Therefore it would be highly beneficial for carbon nanotubes to be suspended in an aqueous solution, without the need for such surfactants or organic solvents.

Recently, graphene has been praised as a new nanomaterial with the potential for large scale production in aqueous media. Graphene oxide (GO) is a highly dispersible form of graphene due to its surface functionalities, with chemical reagents, high temperatures and electrochemical methods widely used to reduce GO into a more conducting form with less basal plane oxygen-containing functionalities. Of significant importance is that the conducting reduced GO (rGO) is stable in an aqueous dispersion and as such has the versatility of being amenable to a range of solution processing techniques.

Graphene oxide-carbon nanotube (GO-CNT) composites have been the subject of significant research interest with studies aimed at characterising the enhanced
electrical properties\textsuperscript{211,199,212,215} of the composites or modelling the complex interaction between the two allotropes of carbon\textsuperscript{214,215,216}. It has been shown that graphene has a higher electroactive surface area than carbon nanotubes\textsuperscript{212,217,218}. However, carbon nanotubes can effectively bridge\textsuperscript{219} graphene sheets resulting in improvements in sheet resistivity\textsuperscript{199} for composite materials. It is still unclear as to how graphene and CNTs interact on a molecular level in solution with some reports suggesting the graphene sheets wrap around carbon nanotubes\textsuperscript{217} while others hypothesise that carbon nanotubes inhibit the aggregation of suspended graphene sheets\textsuperscript{212}. The formation of Schottky barriers play a significant role in determining the electrical properties of the final composite, regardless of what type of graphene or carbon nanotube system is being employed\textsuperscript{219,220}. To date the reduction of GO-CNT composites has been performed chemically\textsuperscript{208}, electrochemically\textsuperscript{196} or through heat treatment\textsuperscript{217,221}, and in most cases this reduction is implemented after electrode fabrication or in the presence of stabilising agents such as polyethyleneimine or hydrazine\textsuperscript{222}.

Carbon nanotubes have been extensively used in electrode structures for enzymatic biosensors\textsuperscript{223,148,145} and biofuel cells\textsuperscript{106,142,100}. The high aspect ratio and good electrical properties makes them ideal candidates for use with bio-catalysts due to their ability to access the enzyme’s active site and facilitate direct electron transfer (DET)\textsuperscript{148,151}. Graphene has also received some attention in this regard\textsuperscript{141,146}. However, to our knowledge composite graphene-carbon nanotube electrodes have not been investigated for use in biological applications. Essentially, rGO’s ability to act as an effective dispersing agent for the MWNTs and the enzyme in an aqueous system allows the fabrication of novel bioelectrodes.

Organic solvents have been used to stabilise rGO-CNT suspensions\textsuperscript{109}, however, these systems are unsuitable for biological applications unless the solvent is completely removed before biological entities are incorporated. It is therefore useful to have a technique that allows for biologically relevant components to be integrated during the solution-processing phase of electrode fabrication. It has been extensively shown that cells\textsuperscript{224,225} respond to electrical stimuli and enzymes\textsuperscript{146} can
be electrically wired to conducting materials, and as such achieving an intimate connection between the biological entity and the materials is critical for the performance of advanced medical devices. The ability to solution process dispersions of conducting materials together with biological entities could facilitate this intimate connection. Additionally, these dispersions allow for existing solution phase fabrication techniques, including fibre fabrication for nerve regeneration and printable implantable electronics, which could open the door to a new range of bioelectrodes.

### 4.2 Chapter aims

In this chapter a novel method for the successful formation of aqueous rGO-MWNT dispersion without the use of other stabilising agents is reported. In addition, it is shown that it is possible to incorporate the enzyme glucose oxidase (GOx) into the dispersion without compromising the biological activity of the enzyme. The electrodes formed from these rGO-MWNT-GOx solutions achieve very efficient DET signals and outperform many of the previously reported rGO and MWNT based enzyme electrodes in terms of specific catalytic current (see Table 1.4). An aqueous based “one-pot” processing strategy for the development of biologically compatible carbon electrodes will have a profound effect on the development of advanced electrodes for biomedical applications.

### 4.3 Experimental

#### 4.3.1 Materials

Graphene Oxide (GO) was synthesized from graphite by a modified Hummers method and suspended in MilliQ water before being chemically reduced using hydrazine, as described in Chapter 2. Multi-walled carbon nanotubes (MWNT) (purchased from NanoAmor at 99.9% purity) were used as received without further
treatment. Purified glucose Oxidase (GOx) was used as outlined in Chapter 2. A 1 mg/ml solution of poly(ethyleneimine) (SigmaAldrich) was prepared in an aqueous 0.5 M solution of NaCl (SigmaAldrich). A 1 M solution of D-(+)-glucose in PBS (pH 7.4, 50 mM) was prepared and allowed to mutarotate for 24 hours at 4°C before use. Glassy Carbon Electrodes (GCE) with a diameter of 3 mm were used for all experiments.

4.3.2 Dispersion properties

4.3.2.1 Dispersion preparation

MWNTs were weighed out and added to 10 mL of GO (0.5 mg/mL) in water with the following weight% of MWNTs; 0 %, 10 %, 33 %, 50 %, 85 %, 90 % and 100 %. In order to suspend the MWNTs effectively, the GO-MWNT solution was subjected to horn sonication (Branson Digital Sonifier – 500W) for 1 hour (30% amplitude, 2 sec ON, 1 sec OFF) in an ice bath to form a series of stable composite dispersions. Each dispersion was chemically reduced by taking 10 mL of composite dispersion and adding 10 mL of MilliQ water, 10 µL of 35 wt% hydrazine and 70 µL of 28 wt% ammonia solutions. The mixture was then shaken vigorously for 5 minutes and left for 1 hour at 95°C for reduction to take place. The final concentration of rGO was 0.25 mg/mL after reduction and the weight fraction of MWNTs for each sample is given by the following labelling system throughout the rest of this thesis. rGO-MWNT_{10} represents a rGO-MWNT dispersion containing 10 weight% MWNTs or rGO-MWNT_{90} to represent a rGO-MWNT dispersion containing 90 weight% MWNTs and so on. Additionally, when the label rGO is used it signifies that no MWNTs are present and similarly, when MWNT is used it signifies that no rGO is present.
4.3.2.2 Dispersion characterisation

4.3.2.2.1 X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) was used to confirm that the chemical reduction of graphene oxide (GO) to reduced graphene oxide (rGO) was successful in the presence of carbon nanotubes. Deconvolution of the C1s peak showed characteristic binding energies for the different elemental states and gave information on the extent of chemical reduction for each sample.

4.3.2.2.2 Optical microscopy

A drop of each rGO-MWNT dispersion was cast onto a glass slide with a glass cover sheet placed on top for qualitative optical analysis using a Leica optical microscope. To test if the dispersions remained stable when enzyme is present, the GOx loading used for electrode fabrication was added to each dispersion and analysed optically as described above. An opaque white plastic sheet was placed underneath the glass slide to achieve a representative image since it was difficult to distinguish dispersion features with the microscope stage behind the sample.

4.3.2.2.3 Scanning electron microscopy

Scanning electron microscopy (SEM) was performed using the JEOL Cold Field Emission Gun Scanning Electron Microscope. The SEM was operated at an accelerating voltage of 5 kV and secondary electron images were taken with a semi-in-lens detector at a working distance of 8 mm and a spot size setting of 8. Samples were prepared by drop casting the dispersions onto aluminium foil and imaged from directly above.
4.3.2.4 Zeta potential and particle size analysis

Zeta potential and particle size analysis was performed on each dispersion before and after the addition of enzyme using the Zetasizer Nanoseries (Malvern Instruments). A dilute sample was added to a universal dip cell quartz cuvette and the sample was analysed. Zeta potential studies did not show significant variation or trend between samples and this is attributed to relatively good dispersion properties. The data presented in this chapter deals with particle size analysis only.

4.3.2.5 Fourier transform infrared analysis (FTIR)

The dispersions were dried at 80°C under vacuum overnight before FTIR analysis. An IRPrestige-21 Fourier transform infrared spectrophotometer (Shimadzu) was used to analyse the functional groups for each sample. KBr powder was used and the scanning was performed in the range of 4000–400 cm⁻¹.

4.3.3 Electrode properties

4.3.3.1 Electrode fabrication

Glassy carbon electrodes (GCE) of 3 mm diameter were polished using a series of alumina powder slurries with various particle sizes, namely 1 µm, 0.3 µm, 0.1 µm and 0.05 µm. The electrodes were then subjected to bath sonication in MilliQ water for 10 minutes, before being dried by a nitrogen stream. 10 µL of the above rGO-MWNT dispersions was added to 10 µL of purified GOx (0.5 mg/mL in 50 mM PBS, pH 7.4 – unless otherwise stated) and gently mixed before drop casting 5 µL onto a clean GCE. These electrodes were placed in the refrigerator at 5°C and left until dry before a final layer of poly(ethyleneimine) (PEI) (5 µL of 1 mg/mL in 0.5 M NaCl) was drop cast to entrap the enzyme, and again allowed to dry at 5°C.
4.3.3.2 Electrode characterisation

4.3.3.2.1 Electrochemical impedance spectroscopy

A series of electrodes were prepared in a similar fashion to section 4.3.3.1 for electrochemical impedance spectroscopy (EIS) analysis, however the rGO-MWNT aqueous dispersions were drop cast without the GOx and PEI layers. Once dry, the electrodes were electrochemically tested in 10 mM ferrocenemethanol (FcMeOH) in PBS (50 mM, pH 7.4) to characterise the charge transfer resistance of each rGO-MWNT composition. EIS was performed at an oxidising potential for FcMeOH (+0.28V vs. Ag|AgCl) in the frequency range 0.1 to 100000 Hz at an AC amplitude of 5 mV.

4.3.3.2.2 Catalytic response

All enzyme electrochemistry tested with glucose in solution (henceforth referred to as the catalytic response), was performed at 37°C with a PBS (pH 7.4, 50 mM) supporting electrolyte under a blanket of argon gas. Initially the dissolved oxygen was removed from the electrolyte by bubbling argon for at least 15 minutes. Cyclic voltammetry was used to characterise the electrodes both with and without enzyme for 20 cycles at 50 mV/s, which was sufficient to achieve stable electrochemistry before the sweep rate was dropped to 10 mV/s for steady state analysis. The electrochemical cell used was introduced in Chapter 2 (2.2.3.2) and the specific conditions will be outlined in the results and discussion of this chapter.
\section*{4.4 Results and discussion}

\subsection*{4.4.1 Dispersion and electrode properties}

\subsubsection*{4.4.1.1 X-ray photoelectron spectroscopy}

MWNTs were dispersed by sonication in an aqueous dispersion of GO prior to the reduction process. GO-MWNT samples were characterized by X-ray photoelectron spectroscopy (XPS) before and after hydrazine reduction to quantify the ratio of carbon to oxygen containing groups in the final dispersion. It is evident in Figure 4.1 (a) and (b) that there is a significant decrease in C-O content (286 eV) relative to the C=C/C-C peak (284 eV) after chemical reduction of a GO dispersion\cite{228,192}. This shows the extent of oxygen defects removed from the GO sheets that contribute to the number of sp² bonded carbon sites, directly affecting the electrical resistivity of the material\cite{229,230}. The peak at 288 eV corresponds to C=O groups, which are the remaining oxygen containing groups responsible for keeping the rGO dispersion electrostatically stable\cite{177}.
The same characterisation was performed on a GO-MWNT$_{50}$ dispersion (data not shown), however, the carbon-carbon peak at 284 eV dominated the spectra and the carbon to oxygen ratio was difficult to determine. Using XPS analysis it is inconclusive whether reduction of GO occurs in the presence of MWNTs.

4.4.1.2 Fourier transform infrared analysis

FTIR analysis (Figure 4.2) suggests that chemical reduction in fact does occur with the disappearance of the broad carboxylic acid band at ~ 3100 cm$^{-1}$ after reduction.
for samples with and without MWNTs present, as well as the feature at 1724 cm⁻¹ which corresponds to a C=O stretching vibration peak that also diminishes after reduction. The disappearance of the oxygen containing group peaks indicate that chemical reduction has occurred in the presence of MWNTs.

4.4.1.3 Electrochemical impedence spectroscopy

It has been reported that rGO has a higher conductivity than GO, and therefore, electrochemical impedence spectroscopy (EIS) was used to investigate the electrical properties (namely the charge transfer resistance - $R_{ct}$) of films formed from GO-MWNT dispersions before and after the chemical reduction treatment. A decrease in the $R_{ct}$ after chemical reduction of a film formed from a GO-MWNT dispersion indicates that the GO has been reduced to its more conducting rGO form. For the GO dispersions with 0 wt% MWNTs, the $R_{ct}$ before and after chemical reduction was $166 \pm 31$ Ω and $108 \pm 2$ Ω, respectively. This indicates that rGO is...
significantly more conducting than GO suggesting that reduction of GO has occurred. Figure 4.3 shows that for all wt % MWNTs, the rGO samples exhibit a lower $R_{ct}$ compared to the same samples before reduction, suggesting that reduction has occurred in the presence of MWNTs and highlighting the fabrication of a conducting composite dispersion. There is a notable decrease in $R_{ct}$ before reduction (GO profile, Figure 4.3) as the amount of MWNTs was increased from 0 wt% to 33 wt% due to the MWNT's inherently high conductivity. There is no statistical difference for samples that have higher MWNT contents than 33 wt% in this data set, suggesting the percolation threshold has been reached.

![Figure 4.3 Charge transfer resistance as a function of MWNT loading before (GO) and after (rGO) chemical reduction.](image)

4.4.1.4 Scanning electron microscopy & optical microscopy

The reduced dispersions were drop cast onto aluminium foil for SEM analysis (Figure 4.4). The rGO (Figure 4.4 a) exhibits the characteristic wrinkle features of drop cast graphene. As the MWNT content is increased from rGO-MWNT$_{10}$ (Figure 4.4 b) through to rGO-MWNT$_{90}$ (Figure 4.4 e), the MWNT features become dominant with some rGO wrinkles still visible. For MWNT samples (Figure 4.4 f), characteristic features of entangled MWNTs are evident and it seems
to be a less homogenous film compared to when rGO is present. The insets in Figure 4.4 are the optical micrographs of the respective dispersions used to form the films for SEM. The optical micrographs demonstrate the quality of each dispersion ratio and it is evident that rGO is very effective as a dispersing agent given that when no rGO is present (inset Figure 4.4 f) MWNT aggregation is obvious. It was also observed that the qualities of the dispersions before and after chemical reduction were identical thus highlighting the attractive nature of this rGO-MWNT dispersion synthesis route.

![Figure 4.4 SEM micrographs of rGO-MWNT composite dispersions at various weight % MWNTs drop cast on to Al foil. Inset: Optical images for each dispersion. White scale bars represent 1 µm for SEM images and black scale bars represent 1 mm for optical images. (a) rGO, (b) rGO-MWNT₁₀, (c) rGO-MWNT₃₃, (d) rGO-MWNT₈₅, (e) rGO-MWNT₉₀ and (f) MWNT.](image)

The dispersion properties were also characterised by SEM and optical microscopy after the addition of the glucose oxidase (GOx) enzyme to the dispersions (Figure 4.5). The images in Figure 4.5 show the films formed from rGO-MWNT₁₀ with (a) and without (b) the addition of GOx and rGO-MWNT₉₀ with (c) and without (d) GOx addition. It is evident in both samples that rGO and MWNTs form a homogenous film (Figure 4.5 a and c) and when the enzyme is present, the biological entity is incorporated into the homogenous film (Figure 4.5 b and d).
Carbon nanotube features are more predominant in the rGO-MWNT\textsubscript{90} sample, as expected, and show thorough interaction between the carbon nanomaterials and the enzyme with MWNTs protruding through the characteristic features of GOx. The optical micrographs (inset Figure 4.5) show that dispersion quality is not sacrificed when enzymes are present. These results give some indication that firstly, the enzyme does not destabilize the dispersion, and secondly, that there seems to be extensive physical interaction between the enzyme and the carbon materials which may facilitate DET.

Figure 4.5 SEM micrographs of rGO-MWNT and rGO-MWNT-GO\textsubscript{x} composite dispersions drop cast on to Al foil. Inset: optical images for each dispersion. White scale bars represent 200 nm for SEM images and black scale bars represent 1 mm for optical images. (a) rGO-MWNT\textsubscript{10}, (b) rGO-MWNT\textsubscript{10}-GO\textsubscript{x}, (c) rGO-MWNT\textsubscript{90} and (d) rGO-MWNT\textsubscript{90}-GO\textsubscript{x}.

4.4.1.5 Zeta potential and particle size analysis

Zeta potential and size analysis was carried out on rGO dispersions of various weight % MWNTs and each demonstrated similar and reproducible zeta potentials averaged at -45.6 mV, all within an average standard deviation of 9.6 mV. Zeta
potential values more negative than $-30$ mV are considered to represent sufficient electrostatic repulsion to ensure a stable dispersion\textsuperscript{232}. There was no trend in zeta potential in relation to the amount of MWNTs present, which indicates the entire rGO-MWNT concentration range represents electrostatically stable systems. Particle size analysis gives more quantitative information given that through optical analysis the MWNT sample was obviously not a stable dispersion (inset Figure 4.4 f). The particle size distribution analysis showed good consistency for all samples from rGO through to rGO-MWNT\textsubscript{90}, which had average polydispersity index (PDI) values for day 1 and day 50 of $0.27 \pm 0.04$ and $0.32 \pm 0.06$, respectively (Table 4.1). These relatively large PDI values arise from the nature of the analysis which assumes spherical particles, with an ideal spherical, narrow monomodal system having a PDI < 0.1. The dispersions, however, are composed of relatively flat sheets of rGO and cylindrical particles of MWNTs so the following particle size data analysis is only relevant for comparison within this experiment. It is evident that there is a higher PDI value for the 50-day particle size analysis compared to day 1 which indicates there is an increase in particle size distribution and may indicate the onset of dispersion destabilisation, however, on analysis of the number average particle size there is no statistical difference between day 1 and day 50. There is no observable evidence of agglomeration after 50 days of standing and in conjunction with particle size analysis this suggests that the dispersions, where rGO is present (i.e. rGO to rGO-MWNT\textsubscript{90}), are stable for over 50 days. The poor dispersion quality of the MWNT sample described previously was confirmed through particle size analysis, which showed a PDI value of 1.000 from day 1. This result highlights the necessity of rGO to act as a dispersing agent for the highly insoluble MWNTs.

There is an interesting drop in nominal number average particle size between rGO and rGO-MWNT\textsubscript{10} at both day 1 and day 50. It is unclear as to why the particle size is larger for rGO alone but may be attributed to the way the graphene sheets and the CNTs interact in solution, which is still a topic of debate in the literature. Some authors suggest the sheets can wrap around the CNTs\textsuperscript{217} while Shin \textit{et al.} suggest the CNTs are attached to the edges and surfaces of the graphene sheets\textsuperscript{233}.
When GOx was added to the rGO-MWNT composite dispersions on day 1 there was no statistical variation in number average particle size between before and after enzyme addition, 180 ± 80 nm and 164 ± 31 nm, respectively. This indicates that enzymes do not cause instabilities in the rGO-MWNT dispersions.

Table 4.1 Nominal number average particle size and PDI data collected at day 1 and day 50 for the various compositions of rGO and MWNTs

<table>
<thead>
<tr>
<th>Dispersion</th>
<th>Day 1</th>
<th>Day 50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nominal Number Average Particle size (nm)</td>
<td>PDI</td>
</tr>
<tr>
<td>rGO</td>
<td>339 (± 172)</td>
<td>0.26</td>
</tr>
<tr>
<td>rGO-MWNT₉⁺</td>
<td>119 (± 71)</td>
<td>0.35</td>
</tr>
<tr>
<td>rGO-MWNT₃³</td>
<td>133 (± 68)</td>
<td>0.26</td>
</tr>
<tr>
<td>rGO-MWNT₅₀</td>
<td>152 (± 78)</td>
<td>0.27</td>
</tr>
<tr>
<td>rGO-MWNT₈₅</td>
<td>168 (± 86)</td>
<td>0.26</td>
</tr>
<tr>
<td>rGO-MWNT₉₀</td>
<td>166 (± 79)</td>
<td>0.23</td>
</tr>
<tr>
<td>MWNT</td>
<td>1603 (± 1603)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

4.4.2 rGO-MWNT-GOx electrode fabrication and characterisation

The aqueous nature of the rGO-MWNT dispersions makes them amenable to incorporation of enzymes for use in biological applications. Graphene and carbon nanotubes have been investigated separately for their ability to facilitate direct electron transfer (DET) in enzymatic systems. Here we test the validity of using a composite of these materials to achieve an enhanced enzymatic catalytic response. In order to investigate the ability of the composite electrodes to facilitate DET with immobilised GOx, the enzymes were mixed in the given composite dispersion and
drop cast onto a polished GCE, immobilised with a thin layer of PEI and electrochemically tested in PBS (pH 7.4, 50 mM) at 38°C under an argon blanket.

The pH of all the rGO-MWNT dispersion was measured to be 10.0 and it was unknown if the GOx would remain in its active state in films formed from the basic dispersions. We have observed a significant decrease in the enzyme activity when moving from a pH of 7 to 10 (Figure 3.8 a). This decrease in activity is attributed to a change in the enzyme structure. Cyclic voltammetry (Figure 4.6) performed on these rGO-MWNT-GOx electrodes in PBS (pH 7.4) clearly show a pair of well defined redox peaks with anodic peak (E_{pa}) and cathodic peak (E_{pc}) at -455 mV and -470 mV respectively, corresponding to 1.5 mV for \( \Delta E_p \) and an \( E^\circ \) of -0.463 V (vs. Ag|AgCl). The \( E^\circ \) value of GOx incorporated into the rGO-MWNT electrode is in accordance with the typical characteristics of GOx electrochemistry in neutral pH solution. This result demonstrates that despite GOx being incorporated into an rGO-MWNT dispersion at pH 10, it still maintains its electroactivity in films formed from these dispersions. When GOx is drop cast directly onto GCE (i.e., no nanomaterial present) no peaks are evident (Figure 4.6 d) which is to be expected as many publications have investigated the relationship between carbon nanomaterials and their capacity to facilitate DET compared to bare electrodes with no nanomaterials. Figure 4.6 shows other cyclic voltammogram profiles for rGO, rGO-MWNT_{90} and MWNT bioelectrode samples (Figure 4.6 a, b & c, respectively). A large capacitive response is observed for the rGO containing samples, attributed to graphene's high capacitance. The ability to communicate with immobilised enzymes is evident due to the oxidation and reduction peaks, which indicate the oxidation of GOx (FAD) to GOx (FADH_2) and its subsequent reduction. The MWNT sample shows a slightly more resistive response and less defined redox peaks, contrary to other literature where an obvious GOx redox response on CNT drop cast films is evident. This may be the result of the unstable nature of the dispersion with MWNTs preferring to agglomerate together rather than interact with the enzyme, however, it is still unclear.
The stability of the rGO-MWNT_{10}-GOx electrodes were characterised for a period of time under constant potential cycling at 10 mV/s in PBS (50 mM, pH7) between -0.7 to -0.2 V (vs. Ag|AgCl). It was evident that without the immobilizing PEI layer being present the electrode lost 100% of its DET signal after 1 hour, however, when a PEI layer is used to secure the components, the electrode retains 73% of its anodic peak height after 4 hours of cycling. This result highlights the importance of the PEI layer to keep the enzyme immobilised.

Enzymatic catalytic current can be analysed through the addition of glucose to the PBS solution and monitoring the change in anodic peak ($I_{pa}$) height (after subtraction of the appropriate capacitive background current) (Figure 4.7 a). This data provides information on the effectiveness of each composite electrode to facilitate DET, with larger changes indicating enhanced electron transfer efficiency from the FAD redox centre of the GOx to the composite electrode material. The catalytic current is plotted for each composite electrode (Figure 4.7 b) with

Figure 4.6 Cyclic voltammograms of electrodes (a) rGO-GOx (b) rGO-MWNT_{10}-GOx, (c) MWNT-GOx and (d) GOx immobilised with PEI with no carbon nanomaterials present, in PBS at 37˚C under argon at a scan rate of 10 mV/s (vs. Ag|AgCl).
significant variation associated with the higher weight % MWNT samples and more reproducible catalytic currents recorded for low weight % MWNT. The variable response with increasing MWNT content can be attributed to the increase in film heterogeneity, which may be detrimental to the direct wiring of GOx. Catalytic analysis was not possible for the MWNT sample since the $I_{pa}$ was undetectable.

![Graph showing I vs. E for different conditions](image)

**Figure 4.7** (a) An example of an increase in $I_0$ in response to 150 mM glucose in solution for an rGO-MWNT–GOx sample vs. Ag|AgCl and (b) the catalytic current as a function of wt% MWNTs.
The observed catalytic responses presented in Figure 4.7 indicate that the glucose can access the enzyme and that this enzyme is electrically wired to the electrode. These values can be directly compared to analyse the efficiency of each rGO-MWNT ratio to facilitate DET. In this case the largest reproducible catalytic response came from the rGO-MWNT\textsubscript{10} composition, although no observable trends evolved as a function of the rGO-MWNT ratio. The rGO-MWNT\textsubscript{10} system was subsequently used to determine the optimal GOx loading in terms of current generated per amount of GOx used (\(\mu\text{A}/\mu\text{g}\)). The average specific catalytic current for our best performing electrode (rGO-MWNT\textsubscript{10}-GOx) composite, with an optimised enzyme loading of 0.002:1 with respect to rGO (i.e., GOx: rGO), was 72 \(\mu\text{A}/\mu\text{g}\) GOx at 50 mV/s. Other literature values for DET systems to date, using electrodes prepared using the same drop cast method, are identified in Chapter 1 (Table 1.4) and some of the more efficient systems report 25 \(\mu\text{A}/\mu\text{g}\) of GOx for a graphene-GOx composite\textsuperscript{138}, 1 \(\mu\text{A}/\mu\text{g}\) of GOx for graphene quantum dot-GOx electrodes\textsuperscript{165}, 0.5 \(\mu\text{A}/\mu\text{g}\) of GOx for a MWNT/CTAB/GOx/Nafion composite\textsuperscript{124} and 0.12 \(\mu\text{A}/\mu\text{g}\) GOx for a Graphene Oxide/Chitosan/GOx electrode\textsuperscript{184}, at the same scan rate. This equates to approximately a 3-fold increase in efficiency for the electrodes prepared in this manuscript compared to the closest literature value. The rGO-MWNT\textsubscript{90}-GOx and rGO-GOx samples measured 65 and 64 \(\mu\text{A}/\mu\text{g}\) of GOx, respectively, also demonstrating an enhanced specific catalytic performance. The significant improvement in specific catalytic current for the rGO-MWNT composite electrodes compared to other literature values can be attributed to the higher activity achieved through enzyme purification and the ability of the composite to facilitate efficient direct electron transfer.

### 4.5 Conclusions

We have optimised the process of fabricating stable solutions of highly conducting rGO and MWNTs in an aqueous system, without the need for additional stabilising agents. Dispersion quality was studied before and after the addition of the enzyme...
glucose oxidase, and it was determined through SEM, optical microscopy, zeta potential and size analysis that the dispersions remain stable for all rGO-MWNT composites with MWNT content between 10 and 90 wt%. MWNTs alone did not form stable dispersions under any conditions due to their extremely low solubility in water. This result highlights how effectively rGO acts as a dispersing agent for the otherwise insoluble MWNTs. The optimal bioelectrodes were determined to be rGO-MWNT_{10}-GOx which produced the largest reproducible catalytic response for the compositions tested, and upon optimisation of enzyme loading proved to out-perform other literature values by a factor of approximately 3 for specific catalytic current generated. The exceptional specific performance of the electrodes is attributed to the efficiency of the composite materials to facilitate direct electron transfer and the high specific activity of the purified enzyme. The ability to fabricate aqueous dispersions of highly conducting materials in conjunction with biological entities, like enzymes, provides opportunities for implementing other solution processable techniques to form a range of bioelectrodes

### 4.6 Acknowledgements

The author would like to acknowledge Mr Joffrey Champavert from University of Montpellier 2, France for his preliminary experiments and Dr Sanjeev Gambhir for supplying the starting material, graphene oxide.
5. Controlled drug delivery from polypyrrole: powered by enzyme-based bioelectrodes

5.1 Overview

This chapter investigates the controlled release of therapeutic drugs from polypyrrole films, powered by the rGO-MWNT-GOx-PEi bioelectrode developed in Chapter 4, by galvanically coupling the two materials. A long-term aim is to produce an implantable device for localised controlled release of therapeutic drugs within the brain to treat epilepsy. The galvanic device presented in this chapter represents a proof of concept prototype for such a purpose.

5.2 Introduction

As discussed in chapter 1, the development of controlled drug delivery devices is an active area of research since many drugs currently on the market when administered by traditional methods, such as orally or intravenously\(^{16,255}\), can cause severe side effects. In the treatment of epilepsy for example, the use of Fosphenytoin (FOS) is restricted because of debilitating side effects, but otherwise it is an incredibly effective anti-convulsant drug. When FOS is delivered intravenously, 15-20 mg FOS/kg\(^57\) weight (of the patient) is used to suppress a seizure. This is delivered systemically around the whole body so the amount of FOS reaching the brain may be different to the blood stream concentration. Walton et al. suggest that phenytoin experiences rapid entry into the brain due to high lipid solubility, rapid dissociation from the injection serum proteins and substantial binding to multiple brain constituents, all of which cause the drug to be maintained...
within brain tissue and establish a higher concentration than the rest of the blood\textsuperscript{236}. As such, a novel method for its delivery could see it used more widely and effectively.

A promising area of controlled drug delivery relies on the intrinsic properties of conducting polymers and their ability to change oxidation state under a given electrical stimulus. Polypyrrole (PPy) will be discussed in this chapter and Scheme 1 (a) shows the process of dopant (A\textsuperscript{-}) incorporation during electrochemical polymerisation. Scheme 1 (b) shows the release of this dopant when the oxidation state of the polypyrrole backbone is changed. It has been shown that at a reducing potential the anionic dopant is expelled from the conducting polymer structure, as discussed in Chapter 1 (1.4.5). Significantly, a wide range of molecular dopants can be incorporated into a polypyrrole film including antibodies, enzymes, whole living cells, growth factors and of relevance here, therapeutic drugs\textsuperscript{237,51}.

The therapeutic drugs studied in this chapter were dexamethasone-21-phosphate disodium salt (DEX) and Fosphenytoin (FOS). Their chemical structures are shown in Figure 5.1. These particular drugs were specifically chosen due to the presence of
the phosphate group, which imparts a negative charge on the molecule making them suitable to act as a dopant in the electrochemical polymerisation of polypyrrole, effectively taking the place of A⁻ in Scheme 1.

![Chemical structure of Dexamethasone-21-phosphate disodium salt and Fosphenytoin.](image)

**Figure 5.1** Chemical structure of (a) Dexamethasone-21-phosphate disodium salt and (b) Fosphenytoin.

DEX reduces inflammation in the central nervous system while FOS works as a strong anti-convulsant. Both of these drugs have relevance in the desired application, given that a brain implant will cause some trauma during implantation and a drug such as DEX could be locally administered to reduce an inflammatory response. Additionally, reducing seizure activity from within the brain may be achieved by the controlled delivery of FOS directly to the target area.

The incorporation of therapeutics drugs into polypyrrole, including DEX, has been extensively characterised in recent literature. To the author’s knowledge, this is the first report of using FOS as a dopant for a conducting polymer. Studies have shown that a nanostructured electrode architecture can enhance the release of drug compared to a flat electrode substrate. The release can also be tailored according to the stimulation protocol used. Svirskis *et al.* show the difference in the release of para-toluene sulfonic acid (PTS) from a polypyrrole film between applying a constant oxidising potential, a constant reducing potential and a pulsed potential protocol. The results are shown in Figure 5.2 and demonstrate that a substantial amount of PTS is release under a pulsed program, however the biggest
difference was observed between applying a constant oxidising or reducing potential.

![Graph showing PTS release under different stimulation protocols.]

**Figure 5.2** Para-toluene sulfonic acid release from PPy films under the application of different stimulation protocols, where the 120s and 500s labels correspond to the time frame of the experiment. Adapted from Ref\(^8^3\)

Of interest is the increased amount of PTS released when the sample was held in a reduced state. This confirms other literature findings that suggest that during reduction the negative charge induced on the polymer backbone would generate repulsive forces on the anionic dopant ions and cause them to be expelled from the material\(^2^8\). The contraction and swelling of polypyrrole upon oxidation and reduction due to the movement of ions and water molecules is also well documented and contributes to the release of the dopant molecules\(^5^1\). Since the above mechanisms occur concurrently, it is difficult to distinguish their relative contribution to the release of the dopant, which explains why in Figure 5.2 dopant is still released during oxidation and pulsed stimulation. These are the basic mechanistic principles behind the data presented in this chapter. It is, however,
widely accepted that electrical stimulation greatly enhances the release of dopant molecules compared to unstimulated (passive) release\textsuperscript{61,54,90}.

Although a significant amount of research has gone into characterising the release of therapeutic drugs from conducting polymer electrodes with the promise of completely implantable controlled drug delivery devices, few studies address the issue of supplying power to the device. Some studies that investigate conducting polymer coatings on already established devices, like the cochlear ear implant or deep brain stimulation electrodes, claim the use of the existing power supply to command the controlled release. Meanwhile, Moulton \textit{et al.} and Ru \textit{et al.} separately investigated galvanically coupling a magnesium electrode to a polypyrrole film so that when the Mg rapidly corrodes it charges the polymer and instigates release\textsuperscript{86,116}. It was observed however, that it was difficult to stop the decomposition of the Mg electrode once initiated, and for the ultimate application of controlled drug delivery from CPs, it is desirable to have the ability to access the switchability of the conducting polymer system.

Traditional power sources for implantable devices largely include Lithium battery technologies\textsuperscript{239}. Currently, Li batteries power cardiac pacemakers and research is invested in prolonging the life of the batteries to minimise invasive battery replacement surgery. Although the current technology is adequate, there is a desire to avoid unnecessary surgery and develop novel systems to power implantable devices from inside or outside the body. Interest in wireless power transmission to power implantable devices\textsuperscript{240} has gained momentum in recent years with cases reported where blood pressure sensing implants\textsuperscript{241} and small silicon drug reservoir devices\textsuperscript{242} have been powered wirelessly. There has also been some interest in human body energy scavenging where, for example, energy is harvested using piezoelectric generators that take advantage of human body movements to power implanted devices\textsuperscript{243,244}. Although promising, there is still some way to go to make these technologies a feasible reality.
Biofuel cells have been investigated for implantable power source applications for many years due to their ability to operate off blood sugars and dissolved oxygen already present and naturally replenished in the human body, as discussed in Chapter 1. Low power density limitations have prohibited biofuel cells from practical application and generally the limiting factor in the biofuel cell is the cathodic electrode, which is extremely inefficient. The anodic side of a biofuel cell, however, has proven to be very efficient in converting reactants to products and harnessing the electrons produced in the process. I have shown this for the rGO-MWNT-GOx-PEi electrodes characterised in Chapter 4. It is the aim of this chapter to utilize the catalytic output of the bioelectrode developed in Chapter 4 to power the drug release from a polypyrrole film by galvantically coupling the two components, much the same as the studies that used magnesium corrosion to power drug release.

5.3 Experimental

5.3.1 Materials

Pyrrole monomer (Merck, > 97%) was distilled prior to use and stored under nitrogen at -20°C. Dexamethasone-21-phosphate disodium salt (DEX, Sigma-Aldrich, >98%), para-toluene sulfonic acid (PTS, Merck) and Fosphenytoin (FOS, Sigma, ≥98%) were all used as received and stored at 4°C. Phosphate buffer solution was prepared in MilliQ water at a concentration of 50 mM (pH 7.4). Enzyme bioelectrodes were used as characterised in Chapter 4 and the rGO-MWNT-GOx-PEi (10%) composition with a GOx loading of 1.25 µg was used for all experiments. Gold mylar (CP Films Inc., USA) was used as a substrate for polymer growth and was washed in three steps with detergent water, ethanol and finally milliQ water, before being dried using a nitrogen stream.
5.3.2 Electropolymerisation

Polypyrrole films were electrochemically grown onto gold mylar electrodes by galvanostatic deposition using an eDAQ potentiostat/galvanostat with eDAQ Chart software (v2.0.7). The exact working area of the gold mylar electrode was controlled using adhesive tape. 0.2 M pyrrole was prepared in MilliQ water with PTS, DEX or FOS acting as a dopant. Table 5.1 outlines the optimised growth parameters used for each dopant. Justification for the different growth parameters will be outlined in the results and discussion section.

Table 5.1 Growth conditions for polypyrrole films with various dopants.

<table>
<thead>
<tr>
<th>Dopant</th>
<th>PTS</th>
<th>DEX</th>
<th>FOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (mM)</td>
<td>50</td>
<td>5</td>
<td>2.5</td>
</tr>
<tr>
<td>Electrode Area (cm²)</td>
<td>2</td>
<td>2</td>
<td>0.125</td>
</tr>
<tr>
<td>Current Density (mA/cm²)</td>
<td>2</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Charge Passed (C/cm²)</td>
<td>0.18</td>
<td>1</td>
<td>0.24</td>
</tr>
<tr>
<td>Approx. Growth Time (mins)</td>
<td>1.5</td>
<td>33</td>
<td>5</td>
</tr>
</tbody>
</table>

During deposition, the potential was monitored vs. a Ag|AgCl reference electrode to ensure the conducting polymer film was grown in the correct potential range. Typically, polypyrrole grows at approximately +0.8 V however, if the potential exceeds +1.1 V the film’s electrical conductivity will be sacrificed due to over oxidation of the polypyrrole\textsuperscript{247}. If the potential exceeded +1.1 V the experiment was terminated and the solution was refreshed before proceeding with a new electrode. For the PPy-FOS samples it was critical to replenish the growth solution between each electrode to maintain consistency and sample quality, this will be discussed in results and discussion (5.4.2). The polymerisation solution for PTS and DEX was
degassed for 15 minutes with nitrogen before growth; however, the FOS growth solution was not degassed due to the small volume (as restricted by the price and quantity of the drug).

After growth, all films were washed extensively with milliQ water to remove excess monomer and loosely bound dopant. For the PPy-PTS and PPy-DEX films the samples were cut in half to form two 1 cm² electrodes, and the PPy-FOS samples were also halved to make two 0.0625 cm² electrodes.

5.3.3 Drug release characterisation

5.3.3.1 Cyclic voltammetry

Initially, the samples were characterised through cyclic voltammetry to identify the oxidation and reduction potentials of the drug loaded conducting polymer films. Fresh samples were used for each experiment and the films were not re-used for release experiments after CV analysis. The specific parameters for each experiment will be identified when the data is presented; typically, a three-electrode electrochemical cell was established using a platinum mesh counter electrode and a Ag|AgCl reference electrode in PBS (50 mM, pH7.2). An eDAQ potentiostat/galvanostat was used with eChem software (v2.0.7) for analysis.

5.3.3.2 UV-visible spectroscopy – a method for drug detection

UV-visible spectroscopy (UV-Vis) is a quantitative technique used to determine the concentration of an analyte in solution, as described in Chapter 2 (2.2.1). The release of DEX or FOS from polypyrrole films was monitored using UV-visible spectroscopy. Standard calibration solutions of DEX and FOS were measured and the linear range for each was determined to be 1 to 250 µM and 0.5 to 50 µM, respectively, at their relevant wavelengths (given in Table 5.2). The standard
curves were used to calculate the concentration of drug released during experimentation and the results were subsequently converted into the mass of drug released.

5.3.3.3 In-situ UV-Vis release experiments

A three-electrode cell was designed to fit inside the UV-Vis chamber in order to characterise the release of drugs from the polypyrrole films during electrochemical stimulation. The real time in-situ analysis of drug release was monitored using the absorption function of the UVprobe software, which was programmed to take a spectrum from 600 to 190 nm at each time interval to be analysed later at a wavelength appropriate for the drug being analysed. Table 5.2 identifies the respective absorption wavelengths for each drug used.

<table>
<thead>
<tr>
<th>Dopant or drug</th>
<th>Absorbance wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEX</td>
<td>242 nm$^{54}$</td>
</tr>
<tr>
<td>FOS</td>
<td>210 nm$^{246}$</td>
</tr>
</tbody>
</table>

The in-situ three-electrode cell is schematically represented in Figure 5.3. The cell was arranged in a 3 mL quartz cuvette for DEX with the gold mylar-polypyrrole working electrode and the Pt mesh counter electrode resting flush against opposite frosted cuvette walls. The transparent cuvette sides were aligned with the direction of the spectrophotometer beam, which was not obstructed by any of the cell components. A Ag|AgCl reference electrode was suspended in the middle to complete the electrochemical cell and PBS (50 mM, pH7.2) was used as the release solution. A small motor modified with a large stirrer bar was placed inside the UV-Vis chamber (but outside the release cuvette as shown in Figure 5.3) to facilitate
gentle solution mixing by magnetically moving the stirrer bar inside the cuvette. This was done for the duration of all release experiments to assist the diffusion of the released drug from the working electrode surface to the bulk solution in order to take representative spectra.

![Figure 5.3 Schematic of the three-electrode cell designed for in situ electrochemical stimulation and recording of UV-vis spectra.]

For FOS experiments, the release solution volume was reduced to 200 µL due to the smaller film size. The release was done in a separate cell and the 200 µL aliquot was then transferred to a 500 µL quartz cuvette for UV-vis analysis. The transfer of solution in these experiments limited the time points to 1 minute intervals.

The electrochemical cell electrodes were connected to a potentiostat and eDAQ software was used to apply an oxidising or reducing potential to the working electrode. The current and charge profiles were recorded while simultaneously collecting absorbance data from the UV-vis. The absorbance results were converted to a mass of drug released using a calibration curve and the charge data was converted to represent the charge passed per square centimetre in order to...
accurately compare different samples. All the results were plotted against time to obtain release profiles.

5.3.4 Controlled drug delivery device assembly and characterisation

Figure 5.4 Schematic of the electrode configuration used to characterise the release of drug from a PPy film as stimulated by the output of the bioelectrode anodic cell.

The device prototype utilised to characterise the feasibility of powering the release of drug from a polypyrrole film using a working GOx bioelectrode is schematically represented in Figure 5.4. The rGO-MWNT-GOx-PEi (10%) electrode used in these experiments was fully characterised in Chapter 4 and for the remainder of this discussion will be referred to simply as the bioelectrode. A 3-electrode electrochemical cell was used in the bioelectrode cell, and during the release
experiments, the current and potential of the bioelectrode|Pt couple were measured using the zero-resistance amperometry (ZRA) function of the eDAQ electrochemical system.

Zero-resistance amperometry (ZRA) is a nonperturbative electrochemical technique used to measure time dependent current and potential profiles of a galvanic cell. By placing a ZRA in the electrical circuit, the galvanic current and potential can be measured against a Ag|AgCl reference electrode. Measurements were made at 0.5 points per second and the program was set to accumulate data for the duration of the release experiments. ZRA experiments were performed using eDAQ software and the integral of the current was simultaneously calculated (i.e. the charge passed). The charge profile was converted to charge passed per square cm for each sample for ease of comparison between samples.

In order to power the release of drug, the PPy film was connected to the Pt counter electrode that collects electrons produced by the bioelectrode in the anode cell. This current is directly wired to charge the PPy film and the release cell, located inside the UV-Vis, was gently stirred and measured for drug release. A 3 M Ag|AgCl salt bridge connected the two cells. The data from these experiments was plotted as a function of time and the amount of drug released was analysed with reference to the current and potential generated by the bioelectrode|Pt couple measured by ZRA.

The system was tested at 0 mM and 150 mM glucose concentration. These conditions were chosen to represent the absence of fuel (0 mM) and the saturation concentration (150 mM). A more biologically relevant glucose concentration would be 5 mM, however, the bioelectrode did not respond consistently to this concentration. The release data was compared to the passive release of drug from PPy (i.e. when the two cells were disconnected) and natural elution of drug was recorded.
5.4 Results and discussion

5.4.1 Dexamethasone release characterisation

DEX was originally chosen for drug delivery experiments as it is a commonly used anti-inflammatory drug\textsuperscript{66} and release from PPy had been described previously\textsuperscript{116}. A series of PPy-DEX samples were grown on clean gold mylar according to the parameters given in Table 5.1. Cyclic voltammograms for the deposited materials show broad but defined oxidation and reduction peaks at +0.28 V and -0.28 V (vs. Ag|AgCl), respectively (Figure 5.5). When PPy is in the reduced state (-0.28 V), according to Scheme 1, it will release the doping anion, which in this case is DEX. The potentials marked on Figure 5.5 (a) represent the voltages selected for \textit{in-situ} stimulated release characterisation and the experiment was set up as described in Figure 5.3. In theory, when the PPy is held in a reduced state it should release a larger amount DEX compared to when it is not fully reduced (i.e. at 0 V). -0.55 V was also tested to see if more DEX was released when the film is reduced further than the reduction potential.

Figure 5.5 (b) shows the DEX release profiles according to the potential applied to the PPy film and as a function of time. Over 24 hours, no significant plateau in drug release was observed indicating that there is enough drug incorporated into the films to continuously release for that time frame. There is also no significant initial burst release, which is common for drug release from conducting polymers\textsuperscript{48,55}, however it does tend to release at a faster rate for the first 5 hours. There is some correlation between the amount of drug released and the charge density, however, the large error bars associated with this data indicate that the control over the release of drug is not reproducible. In all stimulated cases, the amount of drug released is greater than the passive release profile (Figure 5.5 b - grey profile).
Chapter Five | Controlled drug delivery from polypyrrole: powered by enzyme-based bioelectrodes

Figure 5.5 (a) CV characterisation of a PPy-DEX film in PBS (pH 7.4 under Nitrogen at 100 mV/s) (b) Release (solid) and charge (dashed) profiles of DEX release from PPy-DEX films when stimulated at -0.55 V (purple), -0.28 V (green) or 0V (black) vs Ag|AgCl. Passive release is shown in grey. DEX release was monitored by in-situ UV-Vis at 242 nm.

Thompson et al.\textsuperscript{51} described a two layer approach where a PPy-PTS prelayer was deposited before a PPy-NT3 (Neurotrophin) layer. Such an approach demonstrated
more homogenous film growth as well as enhanced incorporation of NT-3 in the final layer compared to the equivalent system with no prelayer present. It was hypothesized that the advantage of the two-layer system may be due to the increased surface roughness or the shielding of the gold surface from the biomolecule. The same approach was adopted for the PPy-DEX samples in this work. A thin PPy-PTS layer was prepared on clean gold mylar, according to the parameters outlined in Table 5.1, and after a thorough wash and dry, the PPy-DEX film was grown on top. Figure 5.6 (a) shows the electrochemistry of the PPy-PTS-DEX electrode with broad oxidation and reduction peaks at +0.2 V and -0.4 V, respectively.
Figure 5.6 (a) CV characterisation of a PPy-PTS-DEX prelayer film in PBS (pH 7.4 under Nitrogen at 100 mV/s) (b) Release (solid) and charge (dashed) profiles of DEX release from PPy-DEX films with a PPy-PTS prelayer when stimulated at -0.6 V (purple), -0.4 V (green) or 0V (black) vs Ag|AgCl. DEX release was monitored by in-situ UV-Vis at 242 nm.
Release profiles were obtained at the potentials marked in Figure 5.6 (a) and the results are presented as a function of time in Figure 5.6 (b). The error bars indicate that when a PPy-PTS prelayer is present the results are much more reproducible than when no prelayer is present (as shown in Figure 5.5). Thompson et al. hypothesize that the presence of the prelayer improves polymer growth and may contribute to a more homogenous release profile\textsuperscript{51}. The maximum amount of DEX is released when stimulated at the reduction peak potential (-0.4 V). When stimulated at a more reducing potential (i.e. -0.6 V) the polymer sample experiences more charge (purple charge profile), however not as much DEX is released in the over-reduced state (purple release profile). When stimulated at a more oxidising potential (i.e. 0 V) there is not as much charging current (black charge profile) and a negligible amount of DEX is released (black release profile). From this data it is evident that the applied potential governs the extent of DEX release from PPy, rather than the amount of charge passed. The results presented agree with the results and mechanism proposed by Svirskis et al. who reported an enhanced release response when the polypyrrole film was held at a reducing potential\textsuperscript{83}. These results suggest that the controllable switching behaviour of drug release from conducting polymers is a direct function of the change in oxidation state of the polymer backbone and the corresponding ion exchange mechanism to balance the charge\textsuperscript{250}. An on-off switching feature such as this is desirable for an implantable drug delivery device to demonstrate control over the release of therapeutic drug.

While the anti-inflammatory drug DEX has significance in novel drug delivery devices it was of interest to find a suitable anti convulsant drug to make the experiments relevant to the treatment of epilepsy.

5.4.2 Fosphenytoin release characterisation

Fosphenytoin (FOS) is a water soluble pro drug which has an anionic phosphate group (Figure 5.1 b) making it suitable to use as a dopant in polypyrrole electrodes for controlled drug release\textsuperscript{56}.
UV-vis spectrophotometry was used to detect the release of FOS. Figure 5.7 shows the absorption spectra of FOS and it is evident that peak absorption occurs around 200 nm however, this wavelength overlaps with the absorption spectrum of the quartz cuvette (190-200 nm). Cwik et al. have prepared a calibration curve for FOS by measuring its absorption at 210 nm\textsuperscript{210}. Subsequently, this wavelength was used for the detection of FOS.

Initial experiments using the PPy-PTS-DEX samples showed that the output of the rGO-MWNT-GOx-PEi bioelectrode was insufficient to charge and instigate release from films of 2 cm\textsuperscript{2}. As such it was decided to use 0.125 cm\textsuperscript{2} PPy-FOS electrodes and grow much thinner films (as outlined in Table 5.1) so the device could be assembled effectively. The motivation behind monitoring the release of FOS from the PPy films for just 10 minutes compared to the 24 hour release profiles analysed for PPy-PTS-DEX arises from the proposed application. An epileptic seizure can last for anywhere between a few seconds to minutes at a time\textsuperscript{251}, so it was important to characterize the release of FOS in this critical time frame.

![UV-vis spectra of FOS and PTS in PBS (50 mM, pH7.2) from 190-400 nm.](image)

**Figure 5.7** UV-vis spectra of FOS and PTS in PBS (50 mM, pH7.2) from 190-400 nm.
PPy-FOS films were prepared both with and without a PPy-PTS prelayer and there was negligible improvement in the release profile when the prelayer was present. Additionally, PTS absorbs around 200 nm (Figure 5.7) and given that the PPy-FOS film is thin it would be difficult to assign the absorbance reading at 210 nm to FOS alone, when PTS could also be released and skew the results. For these reasons it was decided not to use a prelayer for further experiments.

The electrochemistry of PPy-FOS thin films in Figure 5.8 (a) shows two defined redox couples (labelled \(A/A_1\) and \(B/B_1\)). Street et al. suggest that the reason polypyrrole usually has one set of redox peaks is the short conjugation length, which only allows space for one polaron state. As discussed in Chapter 1, polypyrrole usually incorporates approximately one doping anion per four pyrrole units, however, this will depend on the efficiency of the doping molecule to effectively balance the charge on the polymer backbone. Thus it may be the case that the conjugation lengths in the PPy-FOS system are long enough to support doubly oxidised states (bipolarons) and account for the two oxidation states observed. This may also reflect the efficiency of FOS to act as a dopant. One feature of FOS that may contribute to the doping efficiency is the presence of two aromatic functional groups (Figure 5.1 b) that could pi stack with the aromatic polypyrrole backbone and restrict charge mobility. It is widely documented that the properties of PPy change dramatically depending on the type of dopant and the growth conditions used.
Figure 5.8 (a) CV of a PPy-FOS film in PBS (50 mM, pH 7.4 under Nitrogen at 10 mV/s) from -0.8 to 0.6 V (vs. Ag|AgCl) (b) Release profiles (bar graph) of FOS out of the PPy-FOS films stimulated at +0.2 V (black), -0.4 V (green) and passive release (purple) measured by UV-Vis at 210 nm. Charge profiles (line graph) for +0.2 V (solid) and -0.4 V (dashed) measured by ZRA. Error bars represent standard deviation (*p=0.03).
From Figure 5.8 (a), the oxidation peak around +0.2 V and the reduction peak at -0.4 V were selected for FOS release experiments, shown in Figure 5.8 (b). The charge generated per square cm was very similar for both stimulation potentials and can be related back to the nearly identical anodic and cathodic currents realized in the cyclic voltammogram. A thinner conducting polymer film allows the entire film to charge quicker when a potential is applied, allowing faster switching times. The release profiles in Figure 5.8 (b) show the release of FOS from PPy when stimulated at different potentials, namely 0.2 V (oxidising) and -0.4 V (reducing), in comparison with a passive release profile (i.e. when no electrical stimulation is applied). The results show no statistical difference between the passive release and the release measured at -0.4 V however, a statistically significant increase (*p=0.03) was observed when the film was stimulated at +0.2 V. The opposite of this response was observed for the PPy-PTS-DEX system presented in Figure 5.6. A complex mechanism describes the release of a dopant from a conducting polymer, as outlined in Chapter 1, and for thin films the amount of charge injected may influence the release of the dopant more than the applied potential does. An explanation for the observed release profiles could be that the reduction potential selected is over-reducing the PPy, which may not release as much dopant, as discussed previously for the PPy-PTS-DEX samples in Figure 5.6 (b).

In general, conducting polymers as bioactive molecular delivery systems suffer from several major limitations; (i) Passive loss of loaded drug molecules by diffusion, (ii) loss of loaded drug molecules as a result of anion exchange with the surrounding environment, (iii) inherently low drug loading levels, (iv) poor control and unpredictability of the binding and release kinetics, and finally, (v) low diffusion coefficients of the released drug through dense semi-crystalline polymer fibrils resulting in poor release kinetics. It is a combination of these features that contribute to the large error bars associated with the controlled release of molecules from conducting polymers like polypyrrole, as observed for the PPy-FOS system in Figure 5.8 (b). To understand the underlying mechanisms for this system, the FOS release was powered by an enzymatic bioelectrode (according to Figure
5.4) and experimental parameters like bioelectrode potential and charge output, and FOS release were measured simultaneously.

### 5.4.3 Controlled drug delivery powered by a bioelectrode

The following experiments were set up according to Figure 5.4 with the bioelectrode output measured simultaneously with the release of FOS from PPy. Initial experiments using the PPy-PTS-DEX samples showed that the output of the rGO-MWNT-GOx-PEi bioelectrode was insufficient to charge and instigate release from films of 1 cm², hence the following study was carried out with PPy-FOS films of 0.0625 cm² as a pilot study. It was hypothesized that by changing the conditions for the enzyme in the anodic cell, the bioelectrode output would increase and, if enough power was generated (approximately 10 uW for a PPy-FOS sample), could instigate the delivery of FOS in the release cell. Figure 5.9 (a) shows the charge generated from bioelectrodes under different anodic cell conditions when connected to the PPy-FOS sample in the release cell. It is evident that when glucose is present, a larger charging current is generated compared to open circuit potential (OCP) when no glucose is present. The immobilised GOx can contribute this extra catalytic current through the oxidation of glucose (1.5.4). Figure 5.9 (b) shows the corresponding release profile of FOS in the connected release cell and demonstrates that an increase in the rate of FOS released is observed when the glucose concentration increases from 0 mM to 150 mM in the bioelectrode cell. Linear extrapolation of the release profiles show that there is an increase in the FOS release rate from 20 ng/min to 35 ng/min when the conditions in the bioelectrode cell are changed. There is a statistically significant difference between the release profiles at the 10 minute time point (*p=0.02).

While a more positive potential was recorded in the presence of glucose, there was no statistical difference between the values for the cell potential (Figure 5.9 a). This supports the hypothesis that for thin films, the charging current will significantly affect the release of dopant, while the cell potential plays a less critical role. Due to
experimental limitations, data could not be obtained within the one minute time frame to determine if a clinically relevant amount of FOS can be immediately (< one second) and reproducibly released from a PPy-FOS film. This is a critical experiment to do to understand if the system can operate in the time frame relevant for epileptic seizure activity. A more sophisticated experimental set up will be established in the laboratory for future research in this area to detect drug release with appropriate detection sensitivity at short time frames.
Figure 5.9 (a) The bioelectrode charge output (bar graph) at 0 mM (black) and 150 mM (green) glucose concentration in the bioelectrode half-cell, when the bioelectrode cell and the release cell are galvanically coupled. Cell potential profiles (line graph) for 0 mM (black) and 150 mM (green) glucose concentration measured by ZRA (b) The release of FOS from PPy-FOS measured at 0 mM (black) and 150 mM (green) glucose concentrations in response to the bioelectrode cell charge output. Error bars represent standard deviation (*p=0.02).
Figure 5.9 represents an encouraging result for the feasibility of fabricating a completely implantable controlled drug delivery device. If the necessary control over the rate and amount of drug released can be achieved using the power generated from a bioelectrode, a first prototype of the device can be developed. The next stage of experimentation involved addressing some of the specific requirements for a device to treat epilepsy.

The previous experiments were performed with a minimum of 3 fresh polymer samples measured for each data point however, in the proposed application we envisaged a single polymer film to undergo a series of on-off switches in response to repetitive seizure activity. Hence, it was of interest to change the conditions in the anodic cell during experimentation and monitor the changes in bioelectrode output alongside any changes in the rate of FOS release from the PPy-FOS film. Figure 5.10 shows the response of the system maintained at OCP (i.e. 0 mM glucose) up to 10 minutes before a spike of 150 mM glucose was added to the anodic cell. Figure 5.10 (a) shows the charge and voltage output of the bioelectrode while (b) shows the corresponding release profile as detected by UV-vis.
Figure 5.10 The two half-cells are connected and no glucose is present in the bioelectrode cell for the first 10 minutes. At 10 minutes, the bioelectrode cell is spiked to a glucose concentration of 150 mM. (a) Bioelectrode charge output (squares) and measured cell potential (diamonds) and (b) the corresponding release of FOS from PPy-FOS in response to the above mentioned bioelectrode output.
From the bioelectrode output graph presented in Figure 5.10 (a), there is a slight increase in the slope of the charge profile which can be attributed to the contribution of the catalytic current and a corresponding increase in potential was observed. A significant change in the rate of FOS release is observed in Figure 5.10 (b) in response to the change in bioelectrode conditions presented in Figure 5.10 (a). Linear extrapolation of the release profiles show that prior to addition of glucose the passive release rate was 24 ng/min ($R^2 = 0.969$), and upon the addition of glucose the release rate doubled to 47 ng/min ($R^2 = 0.992$). Error bars tend to be larger after the glucose is added indicating inconsistency in sample response to the change in bioelectrode output. The data represents an average of 4 identical samples grown under the same conditions. The large errors may be explained by the limitations of drug release from conducting polymers outlined previously (5.4.2). Variability may also be introduced during film growth due to the concentration of monomer and dopant decreasing with consecutive film growth. It was not feasible to use a new solution for each sample because of the limited supply of materials. Instead, after each sample was prepared the growth solution was put back into the stock solution and after gentle mixing a new growth solution of the same volume was taken out. This was a necessary step to ensure the most consistent film growth, because if the solution was not refreshed, the next film showed poor electrode coverage with inconsistent growth. It could be that the FOS is used up in the growth procedure and if the solution is used again there is limited availability of the doping anion, hindering the formation of a homogenous film. An attempt was made to increase the FOS concentration to address this issue however inconsistent films resulted when the FOS concentration in the growth solution exceeded 2.5 mM when all other parameters were kept constant (Figure 5.11).
Figure 5.11 Photographs taken of PPy-FOS samples with different concentrations of FOS in the growth solution. Other growth parameters remained consistent.

Figure 5.12 shows the consequence of leaving the PPy-FOS film in a passive release state (i.e. not connected to the bioelectrode) before connecting the two cells. The bioelectrode output and the FOS release profile were monitored in the same way as the previous experiment. At the 10 minute mark the electrical connection was made between the two cells and 150 mM of glucose was added to the bioelectrode cell. Although there was very little change in the voltage recorded, a large charging current was generated (Figure 5.12 a). There was an increase in FOS released as a result of this charging current from 20 to 35 ng/min (Figure 5.12 b). Large error bars associated with this data set indicate variability in the release of FOS between samples, even when in a passive release state (i.e. 0-10 minutes). This may again be attributed to the limitations of the conducting polymer release system and the depletion of FOS in the growth solution between consecutive samples.
Figure 5.12 (a) Bioelectrode charge output (squares) and measured cell potential (diamonds) where the anodic cell and the release cell are not galvanically coupled until after 10 minutes. Before this the release cell is recorded in a passive release state and after the 10 minute time point the two cells are connected and 150 mM glucose is immediately spiked into the anodic cell, and (b) the corresponding release of FOS from PPy-FOS in response to the above mentioned bioelectrode output.
Critical for the efficient operation of a drug delivery device in response to an epileptic seizure is the instantaneous release of FOS from the polypyrrole system. In further prototypes of the device additional circuitry may be included so that the bioelectrode can charge a capacitor and when a seizure is detected the capacitor could discharge onto the polymer film and initiate the release of drug. This concept will be discussed further in Chapter 6 however, the results presented above demonstrate the proof of concept for the given materials.

In the development of such a sophisticated device it is difficult to know how much drug is \textit{clinically relevant} when delivering the drug directly to the brain. Current methods of delivering FOS are restricted due to the debilitating side effects it causes and is usually administered intravenously\textsuperscript{57}, as discussed in Chapter 1. Large amounts of drug are given so that it can pass throughout the whole body and for enough to penetrate the blood brain barrier and have a therapeutic effect. It is therefore critical to take the dosage amount into consideration when developing an implantable drug delivery device. If too much is delivered or if the drug leaks out it could have toxic effects on the brain. This is a major unknown requirement in this line of study and once a suitable device is developed, animal studies will be done to improve the prototype to deliver the appropriate amount of drug.

\section*{5.5 Conclusions}

The control over the release of DEX from polypyrrole films was greatly enhanced when a PPy-PTS prelayer was incorporated into the electrode structure. The prelayer may improve polymer growth by shielding the gold mylar from the DEX and also may contribute to a higher surface area and better foundations for PPy-DEX growth. When stimulated at a reducing potential a larger amount of DEX was released compared to stimulation at an oxidising potential. This result aligns with other findings in literature. The 2 cm\textsuperscript{2} film of PPy-PTS-DEX film could not
be powered by the given bioelectrode (characterized in Chapter 4) because the bioelectrode output was not sufficient to instigate release.

Fosphenytoin (FOS) is a strong anti-convulsant drug, whose release from conducting polymer electrodes was not previously reported. The release of FOS from polypyrrole thin films was subsequently quantified. PPy-FOS samples were galvanically coupled with the anodic cell containing an rGO-MWNT-GOx-PEi bioelectrode (previously characterised in Chapter 4) and the conditions were changed from 0 mM to 150 mM glucose concentration in the anodic cell. There was an increase in the charge output of the bioelectrode when glucose was present due to the additional current generated by the oxidation of glucose. The measured potential of the whole device did not change dramatically under these conditions, however, an increase in the amount of FOS measured in the release cell was observed. This was a promising result to prove that such a device is feasible.

For the proposed application of the treatment of epilepsy, release of the drug has to be rapid to quickly respond to the onset of a seizure. It would also be beneficial if a single polymer film could be switched on and off and demonstrate a repeatable release profile. Experimental limitations did not allow the measurement of FOS release in less than one-minute time intervals, however, with a modified experimental set up this could be achieved. Thus it was not conclusive in this study if a therapeutic amount of FOS can be instantaneously released from the PPy electrodes. Data points at 1 minute indicate that approximately 20 ng of FOS is released in the first minute and it is difficult to put this into context given the absence of information regarding what a therapeutic amount of FOS delivered directly to the brain is. Experiments were performed to determine if a single polymer film could be switched on in response to the catalytic bioelectrode output through the addition of glucose to the anodic cell. It was determined that an increase in release rate from 24 to 47 ng/mL was observed in response to a switch between OCP and high glucose concentration.
6. Conclusions and future work

6.1 Conclusions

6.1.1 Overview

The aim of this thesis was to evaluate the validity of using enzyme based bioelectrodes to power the release of drug from conducting polymer scaffolds with the intention of developing a device to treat epilepsy. Chapters 3 and 4 were concerned with the fabrication and characterisation of two separate bioelectrodes based on carbon nanomaterials, whilst Chapter 5 investigated the performance of a drug delivery device prototype. The following sections give a brief overview for each body of work, and future work considerations are proposed.

6.1.2 Solvated graphene bioelectrodes

Solvated graphene electrodes (SGE) loaded with glucose oxidase (GOx) were characterised to determine their final composition and structure, as well as their ability to facilitate direct electrical communication with immobilised enzyme. Protein and kinetic biological assays performed on the as formed solvated graphene electrodes (SGE) showed that the glucose oxidase (GOx) enzymes are both present and active within the structure. It was also shown that the electrode fabrication procedure was very reproducible through water content and GOx retention studies. Various electrochemical studies were performed on the electrodes including cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), amperometry and Fourier transform alternating current voltammetry (FTACV). It was concluded from electrochemical analysis that the enzymes are not efficiently electrically wired to the graphene and as such cannot directly transfer the electrons generated from the oxidation of glucose to the nanostructured electrode. Scanning electron micrographs show that in the presence of GOx the graphene sheets delaminate due
to aggregation of the enzyme into discreet layers. This aggregation of the GOx may explain why very little direct electron transfer is observed and offer an explanation to the relatively high resistances recorded through EIS. Amperometry showed irreproducible catalytic current responses to the addition of glucose to the electrolyte solution. When an electrode showed a positive result it’s response time was on the order of minutes, making it unsuitable for real-time reaction to an epileptic seizure. Additional circuitry could be incorporated into the device to remove this requirement (discussed further in future work) however; the inconsistent catalytic response to glucose renders these electrodes unsuitable as bioelectrodes.

6.1.3 Reduced graphene oxide - carbon nanotube composite bioelectrodes

A materials feasibility study was performed to determine the stability of rGO-MWNT composite dispersions and showed that rGO effectively acts as a dispersing agent for MWNTs. Traditionally, surfactants have been used to disperse carbon nanotubes in aqueous systems due to their insolubility, however this is usually detrimental to the electrical properties of the resulting electrode. For the dispersions presented in this thesis, two highly conducting forms of carbon nanomaterial were suspended in water without any insulating surfactants, namely, carbon nanotubes and graphene. The dispersions remained stable for > 50 days and did not form aggregates even with the addition of GOx, as indicated by particle size analysis. Electrodes were fabricated from the dispersions and show well-defined direct electron transfer redox peaks during cyclic voltammetry. The small (15 mV) separation between anodic and cathodic peak potentials indicated a surface confined fast electron-transfer process. When glucose was added to the system an increase in anodic peak height corresponded to the catalytic current generated from the oxidation of glucose by GOx. Upon optimisation of the rGO:MWNT ratio and the GOx loading it was determined that the specific catalytic current generated (µA/µg of GOx) was 3-fold higher than the closest literature value for similar drop-cast
systems. The optimised bioelectrode presented in this chapter was utilised in the following chapter to power the release of drug from conducting polymer scaffolds.

6.1.4 Controlled drug delivery from polypyrrole: powered by enzyme-based bioelectrodes

The release of therapeutic drugs Dexamethasone (DEX, an anti-inflammatory) and Fosphenytoin (FOS, an anti-convulsant) were characterised from the conducting polymer polypyrrole (PPy). Polypyrrole was selected as a drug release scaffold due to its biocompatibility and its capability to switch between oxidation states and, by charge balance mechanisms, control the release of the drug dopant. When the PPy-FOS films were connected to the rGO-MWNT-GOx bioelectrodes (Chapter 4) there are observable changes in the rate of release of FOS that can be directly related to changes in the bioelectrode output fuelled by the oxidation of glucose by GOx. From a materials science perspective, the concept of galvanically coupling enzyme based bioelectrodes with drug loaded conducting polymers to fabricate a controlled drug delivery device prototype was validated. Optimisation of the prototype is required to make it suitable for effective epilepsy treatment and some considerations for this proposal will be discussed in section 6.2.

6.2 Future work

This thesis successfully showed from a materials perspective that the delivery of anti-epileptic drug can be powered by an enzyme-based bioelectrode. The concept was validated with the intention of fabricating an implantable device to treat epilepsy. For a technology like this to be realized and implemented, some key issues need consideration. This section aims to outline not only what can be done from a materials development approach to advance the technology, but also to discuss other avenues to achieve the ultimate goal.
To achieve immediate release of drug from a conducting polymer film it requires an immediate large charging current. This type of response is difficult to achieve reproducibly using an enzyme-based bioelectrode and as such, additional circuitry may be required to attain the desired response. For example, the enzymatic bioelectrode could continuously work to charge a capacitor and when a seizure occurs the capacitor could discharge and provide the relevant power to drive drug release. There is also the limitation of the enzyme-based bioelectrode lifetime. It is difficult to determine the longest operation time from the literature because many papers report either storage lifetime or intermittent lifetime where the electrode is refrigerated between experiments. This is far from the conditions an enzyme-based bioelectrode would encounter in vivo. In this thesis, the electrodes presented in Chapter 4 showed that approximately 30% of the catalytic current was lost in the first 4 hours of electrochemical cycling when maintained at a simulated in vivo environment. Ideally the implant will last a few years, if not the patient’s entire lifetime and as such the longevity of such electrodes has been a major target of research for many years however, it is fundamentally limited by the enzyme’s lifetime. One alternative would be to anchor an electrode to the inside of the intestines or bowel. In this environment the electrode would experience a constant flow of microbes naturally present in the intestinal tract, and as such, if the electrons produced by the microbial processes could be harnessed, they could be used to power a controlled drug delivery system or other implantable bionic device. The biocatalyst, in this case, would be naturally replenished and negate the issue of bioelectrode lifetime.

Another materials science consideration is the ability of conducting polymer scaffolds to repeatedly deliver a clinically relevant amount of anti-epileptic drug. A limited amount of drug can be incorporated into a conducting polymer film, as well as their ability to reproducibly release the same amount of drug with consecutive stimulations, and this will limit the lifetime of the final device. Too address this issue, a microelectrode array could be used that has around 100 small conducting polymer films, which can be individually addressed one at a time. This would allow each microelectrode to release its entire contents but would limit the amount of
treatable seizures to the number of microelectrodes present. For a person who has frequent seizures this would not be suitable. Alternatively, a reservoir system could be adopted where the conducting polymer acts like a gate or valve. This introduces the added risk of releasing toxic levels of drug if the reservoir bursts, but may address one aspect of the issues related to device longevity.

Additionally, seizure detection (or prediction) will be a critical component of the final device. Recent advances in this technology from our collaborators at St Vincent’s hospital in Melbourne, and backed by American company NeuroVista, have successfully predicted the onset of seizure activity with high likelihood seizure prediction sensitivity between 65 and 100%\(^{257}\). In the 2013 analysis, Cook et al. tested 11 patients for the first in-human study of it’s kind and plan to up scale the testing population to gain a better understanding of seizure generation. In parallel studies, which have subsequently been patented, it has been shown that seizures can be controlled through counter electrical stimulation\(^{258}\). Once the accuracy of predicting seizures improves the combination of these two features in one unit will possibly see the first real device in this area enter the market.

6.2.1 Solvated graphene – an interesting discovery

The Bradford protein assay was used to characterise the solvated graphene electrodes presented in Chapter 3 and was implemented to determine the amount protein present in the hydrated electrodes. The following section will introduce the Bradford protein assay followed by the results observed during experimentation and suggestion of a practical use for the material.

6.2.1.1 Bradford protein assay introduction

The Bradford Reagent can be used to determine the concentration of protein in solution and is a standard assay widely used by cellular biologists. The driving mechanism of the assay relies on the formation of a complex between the dye,
Brilliant Blue G, and the protein. The protein-dye complex causes a shift in the absorption maximum of the dye from 465 to 595 nm, correlating to the disappearance of a brown colour and the appearance of a blue colour, and the amount of absorption at 595 nm is proportional to the protein concentration\textsuperscript{259}.

6.2.1.2 Bradford protein assay results

When the solvated graphene electrodes (SGEs) were tested, the brown colour of the Bradford reagent was observed to disappear over time and failed to produce the blue colour change as expected. It was determined that the Bradford reagent was complexing with the protein and as such was also becoming immobilised within the solvated graphene electrode. This presented an interesting discovery that if a protein, or other molecule, is immobilized within the solvated graphene structure and an appropriate complexing agent is in its immediate environment, the agent will be effectively extracted from solution. The large surface area of graphene and the high water content could lend this material to applications of extraction, given that binding agents can be filtered and entrapped in the structure, and the process is scalable and cost effective.
7. References


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