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Novel Architectures for Achieving Direct Electron Transfer in Enzymatic Biofuel Cells

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Novel Architectures for Achieving
Direct Electron Transfer in Enzymatic Biofuel Cells

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Materials Science & Engineering

by

Rita A Blaik

2015
ABSTRACT OF THE DISSERTATION

Novel Architectures for Achieving
Direct Electron Transfer in Enzymatic Biofuel Cells

by

Rita A Blaik

Doctor of Philosophy in Materials Science & Engineering
University of California, Los Angeles, 2015

Professor Bruce S. Dunn, Chair

Enzymatic biofuel cells are a promising source of alternative energy for small device applications, but still face the challenge of achieving direct electron transfer with high enzyme concentrations in a simple system. In this dissertation, methods of constructing electrodes consisting of enzymes attached to nanoparticle-enhanced substrates that serve as high surface area templates are evaluated.

In the first method described, glucose oxidase is covalently attached to gold nanoparticles that are assembled onto genetically engineered M13 bacteriophage. The resulting anodes achieve a high peak current per area and a significant improvement in enzyme surface coverage. In the second system, fructose dehydrogenase, a membrane-bound enzyme that has the natural ability to achieve direct electron transfer, is immobilized into a matrix consisting of binders and carbon nanotubes to extend the lifetime of the anode. For the cathode, bilirubin oxidase is immobilized in a carbon nanotube and sol-gel matrix to achieve direct electron transfer. Finally, a full fuel cell
consisting of both an anode and cathode is constructed and evaluated with each system described.
The dissertation of Rita A Blaik is approved.

Laurent G. Pilon

Yu Huang

Bruce S Dunn, Committee Chair

University of California, Los Angeles

2015
To my family, my friends, my love, and FAM.

Thank you.
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White Ground Ceramics ANAGPIC 2010

Gold-Coated M13 Bacteriophage as a Template for Glucose Oxidase Biofuel Cells with Direct
Electron Transfer ACS Nano. Article ASAP

INVITED TALKS

Angeles, CA.
California Polytechnic University, San Luis Obispo (2013). In conversation with Ruta Saliklis, Director of Exhibitions and Development at the San Luis Obispo Art Museum.

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Chapter 1. Objectives

The objective of this research is to investigate novel methods of constructing conductive nanomaterial-based architectures for biofuel cells. Nanomaterials are shown to be capable of achieving direct electron transfer, thus eliminating the use of mediators, allowing for a simplified design and a working potential closer to that of the redox potentials of the enzymes. Additionally, many of the methods discussed are able to hold more enzymes per unit area and stabilize enzymes for a longer operation time than other state-of-the-art designs. Multiple characterization techniques are used in order to quantify each step of the process, and electrochemistry is the primary method by which the performance of the cells is evaluated. A thorough understanding of the enzyme architecture and operation is key to achieving success with each method described.

This thesis is divided into six sections. First, an introduction to biofuel cells is given. Here, the difference between enzymatic and microbial fuel cells is discussed and the reasons for possibly using each type are explored. The design methods for each system are discussed and sustainability is also emphasized as the motivation for constructing biofuel cells. Lastly, electrochemical analysis and other major characterization methods used in this work are explained.

In the second section, a method of constructing a glucose oxidase biofuel cell anode with direct electron transfer is described. In this anode, an engineered bacteriophage is used as the templating mechanism to create a high-surface area substrate for glucose to covalently attach to. The procedure of assembling this anode is discussed, followed by a detailed characterization at
each step of the assembly process and electrochemical analysis of the anode using half-cell testing methods.

The third section looks at the stabilization of fructose dehydrogenase, a different enzyme that can be used at the anode with the natural ability to achieve direct electron transfer. Two different binders are explored in order to hold the enzyme in place and prevent denaturation, which can occur due to applied thermal or chemical stresses and results in the proteins losing their function. Fructose dehydrogenase anodes with carbon nanotubes providing the conduction pathway and either carboxymethyl cellulose or polyvinylidene fluoride as the binder are compared electrochemically and discussed, with special attention paid to long-term half-cell testing.

The fourth section discusses the preparation of a cathode by which to test the anodes against each other for full cell experiments. Based upon previous work by James Robert Lim, a bilirubin oxidase cathode is built that achieves direct electron transfer with carbon nanotubes and sol-gel chemistry. Discussed in detail is [1] the sol-gel synthesis and [2] the use of enzymes from different sources and how activity versus quantity of enzyme affects the performance of the system. Electrochemical characterization is again the primary means of analysis.

The fifth section compares the two anodes, glucose oxidase and fructose dehydrogenase, against each other in both a short-term and long-term study in order to evaluate which system might perform better for the most likely application of implantable devices. The sixth section then provides concluding remarks and suggestions for future research directions, both in the near and long term, with comparisons to other similar studies in the field. The goal of this thesis work is two-fold. The first objective is to provide a framework for the construction of nanoscale architectures in enzymatic biofuel applications, and the second is to provide a holistic
understanding of the design considerations taken throughout the work with the intended application of implantable devices.
Chapter 2. Introduction to Biofuel Cells

2.1 Fuel Cell Overview

A fuel cell is a device that converts chemical energy provided by a fuel source into electrical energy, which is done through a chemical reaction. Structurally, a fuel cell consists of a negatively charged anode electrode and positively charged cathode electrode, a catalyst that drives the reactions at each electrode, and an electrolyte that carries ions from one electrode to the other. However, fuel cells are distinct in that the chemical reaction (and hence electrical energy) can be sustained as long as fuel is provided continuously.

There are several kinds of fuel cells that each operate slightly differently, though the basic mechanism is generally the same (Figure 2.1). Positively charged hydrogen ions and electrons are generated at the anode from a hydrogen source. The electrons are driven from the anode to the cathode through an external circuit as direct current (DC) electricity. The hydrogen ions, which traveled through the electrolyte, combine with oxygen and the electrons that traveled through the circuit to form water at the cathode. The two most common fuel cell types are proton exchange membrane fuel cells (PEMFC) and solid oxide fuel cells (SOFC). PEMFCs operate at relatively low temperatures (below 100°C) but use precious metals such as platinum as the catalyst. SOFCs operate at very high temperatures (roughly between 500 and 1000°C) and thus do not require such expensive catalyst material. SOFCs are mainly suited for larger portable and stationary power generation, with some designs aiming for lower power portable devices. Alternatively, biofuel cells offer several distinct advantages over these fuel cells and other sources of both renewable and non-renewable energy.
2.2 Motivations and Markets

Biological fuel cells, commonly abbreviated as biofuel cells, have shown great promise as a green and sustainable source of alternative energy for implantable devices(3). Biofuel cells harness the energy production processes found in nature, converting the chemical energy of fuel and an oxidant into electrical energy by making use of biocatalysts to catalyze the oxidation and reduction reactions. They can utilize fuels from abundant sources such as plants, waste matter, blood glucose, and blood oxygen, and have significantly less environmental impact than traditional energy sources based on increasingly difficult to source fossil fuels(4). This allows for
novel approaches toward alternative energy and integration. Besides the use of benign and renewable fuels that are intrinsically non-polluting, biofuel cells offer size scalability, are efficient at room temperature, and are bio-compatible. This makes them particularly attractive for bioMEMS devices and implantable power supplies for biomedical devices such as cardiac pacemakers and cochlear implants (Figure 2.2). The typical battery for a pacemaker must be replaced every 10 years(5), putting patients in continual discomfort and risk, as well as incurring ongoing costs. Because the battery is typically the only part of the pacemaker with a known service life, it generally determines the service life of the implanted device itself(6).

Figure 2.2 In both a dual-chamber (A) and single-chamber (C) pacemaker, electrodes connected to a battery-powered pulse generator are inserted into the heart's right atrium and ventricle, stimulating the heart muscle (B). The majority of the weight and volume of the pulse generator is due to the battery. Image courtesy of the National Institutes of Health.

While market data for biofuel cells specifically is not available, the markets for both fuel cells and implantable devices are large and have strong predicted growth. A 2014 market
research report by MarketsandMarkets estimates that the global fuel cell market is expected to grow at a compound annual growth rate of 14.7% in the period from 2014 to 2019, going from approximately $2.61 billion to $5.20 billion(7). Similarly, a 2011 BCC Research analysis projects the global market for microelectronic medical implants, accessories, and supplies to grow to $24.8 billion by 2016, up from $15.4 billion in 2010(8).

2.3 Enzymatic versus Microbial Fuel Cells

Biofuel cells can generally be classified as either enzymatic or microbial. A microbial fuel cell (MFC) uses exoelectrogenic microorganisms as the catalyst, wherein the microbes release electrons upon forming a biofilm on the electrode surface. A specific advantage of MFCs is that the microbes and electrical current can also produce value-added chemicals in microbial electrolysis cells, such as H₂(9,10), or can drive water desalination (Figure 2.3) in microbial desalination cells(11). However, there are several challenges with MFCs that make them less suitable for implantable device operation, the most significant being the maintenance of the biofilm. A thicker biofilm is advantageous for macro-scale MFCs, as the dormant layers of bacteria beneath the surface can replace any dead or removed cells. In some cases, films as thick as 50µm can still produce electrons that transfer to the surface and contribute to the overall current production(12). During operation, this can decrease efficiency, resulting in a lower power output. From a design standpoint, thicker films are in opposition with miniaturization. Additionally, the biofilms require continuous maintenance to prevent clogging and infection(13,14,15).
Enzymatic fuel cells, which employ enzymes as the catalyst, operate like conventional and microbial fuel cells. A schematic of a typical enzymatic fuel cell is shown in Figure 2.4. Fuel is oxidized at the anode and the released electrons are driven through an external circuit, generating electric current. When the electrons reach the cathode, they combine with an oxidant and protons to generate a product. Enzymatic biofuel cells are an ideal candidate for supplying power to implantable devices and are relatively simple compared to microbial cells(16). As long as the enzymes remain functional, the cell requires little to no maintenance. Because there are no microbes, there is little chance of an immune response from the body that would damage the cell. The cells are also easily scalable and electron transfer is much simpler(17). Therefore, although the applications of enzymatic fuel cells are multi-fold, they have primarily been a candidate for implantable device applications(18).
Figure 2.4  Schematic of a typical glucose oxidase / bilirubin oxidase fuel cell, showing the series of oxidation and reduction steps that occur with each component at each electrode. The fuels at the anode and cathode are glucose and water, respectively, and the products are gluconolactone and water. Image courtesy of James Robert Lim.

2.4 Designs for Direct Electron Transfer

2.4.1 Design Motivations

According to a 2002 report from the California Department of Resources Recycling and Recovery, there were over 507 million batteries sold in California alone in the year 2001, only 0.55% of which were recycled(19). Batteries are considered hazardous due to the metals and/or other toxic or corrosive materials used, and e-waste is quickly becoming the largest segment of solid waste in the United States(20). Lithium batteries in particular, as a continually-rising market share of all batteries for portable devices(21), lack uniform regulatory policy on their disposal and contribute significantly to pollution and adverse human health impacts(22). Therefore, this work focuses on using renewable and non-toxic materials whenever possible and simplifying the designs of the cell to use a minimal amount of raw materials. The following sections describe in detail the fundamentals of methods used to make the electrodes described in subsequent chapters.
2.4.2 Genetic Engineering

Biological systems are an extraordinary example of complex units that can rationally combine to make a greater whole. Filamentous bacteriophages are excellent biological tools for bottom-up assembly due to their multiple peptide display systems that give them a tunable surface chemistry. This allows them to be used as simple building blocks for complex hierarchical structures. Additionally, bacteriophage can be used as a renewable, biocompatible material for biological and/or implantable systems. For example, the M13 phage infects and grows only in the male strains (displaying F-pili) of E. coli(23). Lysogenic phages such as M13 infect host cells by injecting its genetic material, which then hijacks the host metabolism in order to replicate itself. The replicated phage is then transported to the host cell membranes to be packaged and released(24,25). As an added advantage of renewability, the infection process does not kill the bacteria, and bacterial growth can continue for mass amplification(26).

Genetic engineering of phage allows for the development of specific functional surface peptide motifs, providing multiple possible attachment methods. Genetic engineering involves the manipulation of an organism's genome in order to produce enhanced or novel organisms(27). In genetics, an insertion is the addition of base pairs into a DNA sequence. By using insertion techniques, the various protein groups that make up a viral structure can be independently altered in order to modify the phage to suit a specific purpose. Phage display is a common research practice that employs the insertion technique, particularly for filamentous phage(28), and it is used to understand the connection of a protein with the gene sequence that encodes it. Libraries of these insertion-created displaying phages can then be screened for the property of interest by in vitro selection or biopanning(29). With these methods, genetic engineering has been described as the promise of “combinatorial chemistry on the cheap”(26).
2.4.3 Binders

A binder can be loosely defined as a material that contains or draws other adjacent materials together to form an interconnected unit. Often, this material takes the form of an organic, polymerizable monomer. Binders are a common component in systems containing nanotubes and other nanoparticles, such as lithium ion batteries (30) or electrochemical sensors (31), to aid in dispersion and deposition. Occasionally, several binders are used in conjunction with conductive additives to achieve a high conductivity; however, complex binder-binder and binder-active material interactions must then be considered (32).

Within the field of biologically compatible materials, a good binder should be able to dissolve within a biocompatible solvent, and then polymerize to consolidate the materials within the slurry. However, there can be several disadvantages to using binders (33), such as structural disintegration due to environmental changes, reduced electrical conductivity, and increased mass-transfer resistance (34). These drawbacks are important when considering both the binder and the quantity, and proper electrode design is critical to achieving high-power performance (35). Nonetheless, binders can offer a simple mechanism that uses materials well tested for biocompatibility (36, 37, 38, 39).

2.4.4 Sol-Gel Chemistry

The sol-gel process is a method of producing metal oxides (glass or ceramic) that involves the conversion of monomer precursors into a colloidal solution (sol) that then becomes an integrated network (gel) of either discrete particles or network polymers (Figure 2.5). As a versatile room-temperature synthesis method, sol-gel chemistry has become a common tool for
the encapsulation of biomolecules for a variety of applications(40). The controllable nature of the gelation process allows for a size-tunable architecture that can contain molecules of interest within the scaffold while still allowing for the diffusion of analyte molecules. As compared to other immobilization schemes such as adsorption and polymers, the sol-gel method allows biomolecules to retain a high functionality(41), a high storage stability(42), a high temperature stability(43), and a high chemical stability(44).

Figure 2.5  Schematic of the sol-gel process, as well as a variety of drying and processing methods which can produce various structures. Image courtesy of Lawrence Livermore National Laboratory.

For silicon dioxide sol-gels, a precursor of a metal alkoxide such as tetramethyl orthosilicate is combined with an acid catalyst to induce protonation (Equation 2.1). With the inclusion of water, a nucleophilic reaction replaces the alkoxide group with a hydroxide group,
otherwise known as hydrolysis (Equation 2.2). Finally, the production of siloxane bonds (Si-O-Si) occurs either by the condensation of a hydroxyl group and an alkoxide group (Equation 2.3) or two hydroxyl groups (Equation 2.4), forming the sol. A buffer solution is subsequently introduced to increase the pH and accelerate the condensation and gelation (Equation 2.5). Upon gelation, different processes for aging and drying may be used to produce gels with various morphologies such as xerogels or aerogels (Figure 2.5). This method of room-temperature gel synthesis has been previously explored for fuel cell applications as well(45).

\[
\text{Si} \left( \text{OCH}_3 \right)_4 + \text{H}^+ \rightarrow \text{Si} \left( \text{OCH}_3 \right)_3 \text{OCH}_4^+ \quad \text{(Eq. 2.1)}
\]

\[
\text{H}_2\text{O} + \text{Si} \left( \text{OCH}_3 \right)_3 \text{OCH}_4^+ \rightarrow \text{Si} \left( \text{OCH}_3 \right)_3 \text{OH} + \text{CH}_3\text{OH} + \text{H}^+ \quad \text{(Eq. 2.2)}
\]

\[
\text{Si} \left( \text{OCH}_3 \right)_3 \text{OH} + \text{HOSi} \left( \text{OCH}_3 \right)_3 \rightarrow \left( \text{H}_3\text{CO} \right)_3 \text{Si-O-Si} \left( \text{OCH}_3 \right)_3 + \text{H}_2\text{O} \quad \text{(Eq. 2.3)}
\]

\[
\text{Si} \left( \text{OCH}_3 \right)_3 \text{OH} + \text{CH}_3\text{OSi} \left( \text{OCH}_3 \right)_3 \rightarrow \left( \text{H}_3\text{CO} \right)_3 \text{Si-O-Si} \left( \text{OCH}_3 \right)_3 + \text{CH}_3\text{OH} \quad \text{(Eq. 2.4)}
\]

\[
\left( \text{HO} \right)_3 \text{Si-O-Si} \left( \text{OH} \right)_3 + \text{Si} \left( \text{OH} \right)_4 \rightarrow \text{SiO}_2\text{network} \quad \text{(Eq. 2.5)}
\]

2.5 Electrochemical Analysis

In what may be seen as divine foreshadowing, an Italian physician and anatomist by the name of Luigi Galvani predicted in 1791 both the connection between chemical reactions and electricity and electrochemistry in biological functions in a work entitled "De Viribus Electricitatis in Motu Musculari Commentarius". Electrochemistry is the study of chemical reactions that take place at the interface of an electrode and an ionic conductor, also known as the electrolyte. This takes the form of electricity that is generated by movements of electrons.
from one element to another in a redox reaction. Electrochemical methods may be utilized to reveal several key thermodynamic and kinetic properties of biofuel cell systems.

In order to evaluate each electrode individually, a standard half-cell analysis can be performed using a three-electrode setup as shown in Figure 2.6. The three electrodes, a working electrode, a counter electrode, and a reference electrode, are suspended in an electrolyte solution. Potential is applied at the working electrode with respect to the reference electrode, which is done in order to sufficiently change electron energy levels and induce redox reactions (Figure 2.7). By using a reference electrode, a controlled voltage can be maintained. Because the potentiostat places a high impedance between the working electrode and the reference electrode, no significant amount of current is allowed to pass between them. Current is therefore passed and measured between the working electrode and the counter electrode.

![Figure 2.6](image.png)

**Figure 2.6** A standard three-electrode setup for amperometry experiments. In addition to the three electrodes shown (working, reference, and counter), an inlet and outlet may be used that is external to the interior of the half-cell that to control the temperature by pumping a controlled fluid.
Figure 2.7 Representation of the results of positive or negative potentials being applied to a working electrode as energy levels. The application of a negative potential induces reduction of the solution and an oxidation of the electrode, and electrons flow from the electrode into the solution. The application of a positive potential induces oxidation of the solution, where electrons flow from the solution to the now-reduced electrode. For a complete glucose oxidase/bilirubin oxidase fuel cell, the electrons move from the most negative potential (glucose) to the most positive potential (the formation of water by bilirubin oxidase). Adapted from Bard(46).

With the 3-electrode setup, cyclic voltammetry and constant potential amperometry may be performed to evaluate properties such as reversibility, electroactive species concentration, and kinetic properties. In a cyclic voltammetry experiment, the voltage is swept linearly back and forth between two potentials and the resulting current is measured (Figure 2.8). By knowing the potential of the electroactive species versus a particular reference electrode, the performance of a fuel cell may be evaluated. As is convention, a positive current is assigned as oxidation and a negative current is assigned as reduction (Figure 2.9).
Figure 2.8  For a cyclic voltammetry sweep, the voltage is swept back and forth linearly over time at the working electrode between two potentials with respect to the reference electrode (Ag/AgCl) by the potentiostat to change the energy levels and induce oxidation and reduction reactions. Current is then passed between the working and counter electrode. Image courtesy of James Robert Lim.

The separation between the oxidation and reduction peak currents, also known as the peak-to-peak separation, indicates the electrochemical reversibility of the system. Equation 2.6 shows the equation for chemical reversibility ($\Delta E_p$) that is derived from the Nernst equation. In a one-step, one electron transfer system ($n=1$), $\Delta E_p$ is 57mV. Reversibility is universally considered to be within 70mV(47).
Figure 2.9  Example of a “duck shape” CV curve for a typical cyclic voltammetry sweep. At lower potentials (a), the electrons in the electrolyte have no affinity for the working electrode. At the oxidation peak (b), all available valence electrons have left the electrolyte and gone to the working electrode. On the sweep back to the initial potential, the potential at the working electrode becomes so negative, electrons leave and occupy all the valence electron molecular orbitals of the electrolyte. Image adapted from James Robert Lim.

\[ \Delta E_p = \frac{2.22RT}{nF} = \frac{57}{n} \]  (Eq. 2.6)

As opposed to cyclic voltammetry, constant potential amperometry (Figure 2.10) is performed by holding the potential at the working electrode (again, with respect to a reference electrode) constant and close to the onset potential of the electroactive species. Fuel is then added in steady increments over time and the subsequent current increase is measured. When testing a full cell, the setup is akin to the testing of a battery, where a load is applied and resulting voltage/current is measured.
In constant potential amperometry, the voltage is held steady near the potential of the redox species and fuel is incrementally added. The resulting current response is then measured.

In order to evaluate fuel cell performance, energy density and power density are the two most common metrics that are used. The gravimetric energy density is the amount of energy stored per unit mass. A component that has a high energy density can store a large amount of energy in its mass. The gravimetric power density is the maximum amount of power that can be supplied per unit mass. The combined energy density and power density values can be visualized in a Ragone plot as shown in Figure 2.11. On one end of the plot, components such as capacitors have a high power density but a low energy density, with the ability to quickly discharge a low amount of stored energy. Conversely, elements on the other end such as batteries have a high energy density but a low power density, with a high storage capacity that can only be slowly released.
The potential energy density of biofuel cells can be difficult to quantify, as the mechanism of electron transfer can vary. For example, the theoretical energy density frequently cited for glucose oxidase-based biofuel cells 4430 Wh/kg. This value is based on a thermodynamic calculation for the total oxidation of glucose to CO$_2$ by a 24-electron transfer, combining the anode and cathode reactions (Equations 2.7, 2.8, and 2.9). However, the available energy density of glucose with a glucose oxidase-based biofuel cell is 370 Wh/kg, based on the glucose oxidase mechanism of two-electron transfers that converts glucose into gluconolactone. This value is only 1/12 of the theoretical value of the total conversion to CO$_2$.

Figure 2.11  Ragone plot for energy storage devices, including the theoretical energy density for glucose (BFC).
Theoretical anode reaction: $C_6H_{12}O_6 + 24OH^- \rightarrow 6CO_2 + 18H_2O + 24e^- \quad \text{(Eq. 2.7)}$

Theoretical cathode reaction: $6O_2 + 12H_2O + 24e^- \rightarrow 24OH^- \quad \text{(Eq. 2.8)}$

Overall reaction: $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad \text{(Eq. 2.9)}$
Chapter 3. Glucose Oxidase Biofuel Cell Anode with Direct Electron Transfer

3.1 Motivation

Biofuel cell systems utilizing glucose oxidase (GOx) can catalyze the oxidation of glucose into electrical energy. A key element in this process is establishing a reliable and efficient electrical contact between the biocatalyst and the electrode surface. A significant challenge to this issue is posed by the large and complex structures of the enzyme, where the flavin adenine dinucleotide (FAD) redox center is deeply embedded in the protein. The location of the active site creates a spatial, steric separation between the electron donor (FAD) and the acceptor (the electrode surface), and thus represents a barrier to the electron transfer process. The relationship between the donor/acceptor separation distance and the electron transfer rate can be understood by electron-transfer theory, shown in Equation 3.1, which gives the rate of electron transfer between a donor/acceptor pair \( k_{et} \) as

\[
k_{et} \propto e^{-\beta(d-d_0)} e^{-\frac{(\Delta G^0 + \lambda)^2}{4RT\lambda}}
\]  

(Eq. 3.1)

where \( \Delta G^0 \) is the change in free energy associated with electron transfer, \( \lambda \) is the reorganization energy, \( d_0 \) is the van der Waals distance, \( d \) is the distance between the donor and the acceptor, and \( \beta \) is the electronic coupling coefficient.
One common approach which overcomes the electron transfer issue is the use of a mediator molecule to facilitate electron transfer, a process called mediated electron transfer (MET). However, MET is ultimately undesirable for a number of reasons. Most significantly, it lowers the total energy of the system. For instance, ferrocenemethanol, a common mediator used with GOx, has a potential of 0.210V versus Ag/AgCl compared to -0.340V for GOx. Since a more negative potential at the anode is desired (and a more positive potential is desired for the cathode), this mediator lowers the total working voltage achievable for a full fuel cell, as shown in Figure 3.1. The use of a mediator also introduces an additional constituent in the biofuel cell that needs to be considered for stability, biocompatibility, and toxicity. In the case of free mediator molecules in solution, the cell will also require membranes or compartments to separate the anode and cathode solutions, making the full cell more cumbersome and miniaturization difficult.

![Figure 3.1](image)  
Figure 3.1  Redox potentials of GOx/BOD biofuel cell components, showing the maximum potential without mediators and the working potential with mediators. Image adapted from James Robert Lim.
Several different nanomaterials\(^{(56, 57)}\) have been successfully explored for their ability to achieve direct electron transfer (DET) with GOx for both biofuel cell and biosensing applications. Some examples of such systems are shown below in Figure 3.2. These materials include primarily carbon nanotubes and graphene,\(^{(58)}\) where the enzymes are usually adsorbed, and gold\(^{(59)}\). In addition, relay-cofactor monolayers have been investigated where the enzymes are covalently tethered onto a surface. One of the early examples of this approach was achieved by Willner et al., who created an enzyme electrode via the reconstitution of apoGOx to a pyrroloquinoline quinone/FAD (PQQ/FAD) monolayer on a gold electrode\(^{(60)}\). While all of these designs produce successful fuel cell electrodes, there is still the possibility for developing electrode architectures with a more reliable attachment scheme, greater enzyme density, and that use more convenient materials. In cases where the enzyme and the electrode are not covalently linked, but merely adsorbed, the attachment is less robust and the steric position is non-optimal, generally resulting in a weakened electron transfer process\(^{(61, 62)}\). In addition, functionalized graphene and carbon nanotubes are very difficult to produce in single layers/strands of controlled length, and have a relatively low amount of functional groups available for covalent attachment as compared to their surface area. Monolayers, which provide a 2D morphology, have a major power limitation. In contrast, a 3D architecture can provide a much greater surface area per volume, and in turn greater enzyme density.
An attractive approach for generating novel high-surface-area fuel cell architectures is the use of viruses. Genetically engineered bacteriophages have recently gained attention for their promise in serving as a template for the assembly of conductive nanoarchitectures (66, 67). The M13 virus (Figure 3.3) is a filamentous bacteriophage comprised of a single stranded DNA enclosed in a cylindrical capsid composed of approximately 2700 copies of the pVIII protein, and contains 5 copies of the pIII protein at one end. The structure of the pVIII protein is oriented such that the surface of the shaft of the virus bears an amine group that can be covalently bound to carboxylic acid-functionalized materials. This phage has recently been explored for its
potential to be used in fuel cell applications. A recent study by the Belcher group focused on using virus-templated gold made through surfactant-mediated bio-mineralization of engineered M13 phage to make electro-catalysts for fuel cells(68). This process can be extended for enzymatic fuel cells, where the gold-coated viruses could then serve again as a template onto which enzymes can be attached.

Figure 3.3 M13 Bacteriophage composition. The green structures represent the pVIII proteins, and the blue structures represent the pIII proteins. Image courtesy of Wikimedia Commons.

In this chapter, a method is described to improve electrical contact between the redox proteins and the conductive support, thus leading to electrode architectures that are designed to achieve higher power output. Our approach is to build electrodes in which reconstituted glucose oxidase enzymes (recGOx) are covalently attached to gold nanoparticles, that are themselves assembled onto the M13 phage. In this way, we are able to construct large-scale, densely packed, conductive “nanomesh” electrodes for biofuel cell applications. This approach increases the available surface area for reaction and allows for significant improvements in enzyme surface loading and peak anodic current.
3.2 Experimental

3.2.1 Chemicals

The type 3 library M13 phage was purchased from New England Biolabs. The functionalized nanoparticles were purchased from Nanoprobes Inc. FAD-NH$_2$ was obtained as a custom synthesis from Chemtos following the procedure detailed by Bückmann et al.(69). The glucose oxidase Amplex® Red assay kit was purchased from Life Technologies. The electrode polish and pad were purchased as a kit from BASi. All other chemicals were purchased from Sigma-Aldrich.

3.2.2 Preparation of ApoGOx

ApoGOx was prepared via the partial unfolding of the GOx protein under strongly acidic conditions. 1mL of 20mg/mL GOx was added to saturated ammonium sulphate acidified to pH 1.7 with sulfuric acid. The solution was allowed to stir at 4°C for 2 hours, and was then rinsed by centrifuging at 5000 rpm for 15 minutes at 4°C with saturated ammonium sulphate (not acidified). The rinsing was repeated three times, and then resuspended in a final clear solution with 4mL of pH 5.5 McIlvaine PBS buffer solution.

3.2.3 Engineering of pIII Modified Phage

The engineered bacteriophage was prepared by a modified process from Huang et al.(70) Briefly, the genome of an engineered M13 bacteriophage bears insertions in gIII, which leads to motif expressions on pIII proteins. Through the use of a general biopanning technique by exposing the type 3-phage library to gold thin films, the peptide sequence HHQTLHR was selected as the antigold binding motif, where histidine has been shown to have a high affinity for
gold(71). AFM was then used to characterize the binding of the virus to a gold substrate. A silicon wafer with a gold coating of ~5nm was cleaned with piranha etch for 15 minutes and rinsed with ethanol. The virus solution was drop-cast (5x10^{10} phage/µL) and stirred with a slow rolling motion for one hour, then gently rinsed.

### 3.2.4 “Nanomesh” Preparation

The modified M13 phage was used as a template for gold nanoparticles containing approximately 10 carboxylate groups on its outer surface, where the amine-rich shaft (pVIII) of the phage is used as a binding site through EDC-NHS chemistry. An aqueous solution of 50µL of 30mM gold nanoparticles (1.4nm in diameter) was combined with 200µL of 5.76mg/mL EDC and 500µL of 3.28mg/mL NHS, each in pH 5.5 MES buffer. This mixture was stirred for 30 min, and then 50µL of 5x10^{10} phage/µL in pH 5.5 MES buffer was added. This solution was stirred at room temperature for an additional 2 hours. Before rinsing, 100µL of PEG/NaCl was added to aid in precipitation. After 20 minutes, the sample was centrifuged three times at 4°C and 20,000 rpm. The resulting nanomesh was then re-suspended in 300µL MES buffer pH 5.5 and allowed to sit overnight at 4°C.

### 3.2.5 Enzyme Attachment

The enzyme attachment to the gold nanomesh follows a similar EDC-NHS attachment scheme. To the previous phage/gold solution, 20µL of 5.76mg/mL EDC and 50uL of 3.28mg/mL NHS, each in pH 5.5 MES was added and stirred for 30 min. 25µL of 1mg/mL FAD-NH_2 in water was then added and stirred for 2 hours. 100uL of PEG/NaCl solution was added and after 20 minutes, the solution was centrifuged three times at 4°C and 20,000 rpm. The
phage/gold/FAD-NH$_2$ precipitant was then re-suspended in 300µL of pH 5.5 McIlvaine PBS buffer along with 50µL of the previously prepared 2mg/mL apoGOx, and allowed to sit overnight in order to form reconstituted GOx. AFM images were obtained courtesy of Adam Stieg and the Nano & Pico Characterization Lab at the California NanoSystems Institute.

3.2.6 Electrochemical Measurements

All electrochemical experiments were run with pH 5.5 McIlvaine PBS buffer solution in a 15mL cell vial purged of oxygen by bubbling in argon gas for at least 15 minutes prior to testing. Gold mesh working electrodes were cleaned before and after each experiment with piranha etch and rinsed with water and ethanol. All samples tested were allowed to attach to the gold substrate overnight before testing. The phage/gold control was prepared by using the EDC-NHS chemistry to attach the gold to the phage, and the resulting phage/gold nanomesh was then incubated in the refrigerator with a gold substrate to attach overnight. The phage/gold/FAD control was prepared by using the same EDC-NHS process to attach the phage/gold to the FAD. After the FAD attachment, the resulting phage/gold/FAD nanomesh was then incubated in the refrigerator with a gold substrate to attach overnight. The phage/gold/apoGOx control was prepared by adding the apoGOx to the phag e/gold mesh and allowing this to refrigerate overnight. The resulting phage/gold/apoGOx mesh was then incubated in the refrigerator with a gold substrate to attach overnight. The counter electrode used was platinum wire, and the reference electrode was Ag/AgCl. Platinum counter electrodes were polished using 3µm fine diamond polish and rinsed with ethanol in a sonicator for 10 minutes. CV scans were taken 10 times each from -0.7 to 0.7 V, both with and without glucose and at sweep rates of 100, 20, and 5mV/s. Amperometric measurements were taken at a potential of 0.3V.
3.2.7 Activity Assay

UV-Vis absorption was performed following the procedure detailed in the Life Sciences Amplex® Red kit (72).

3.2.8 Raman Spectroscopy

Samples were drop-cast onto a clean silicon wafer and allowed to dry overnight. Samples were evaluated with both 514nm and 633nm lines from an argon ion laser at 25mW and a helium-neon laser at 70mW, respectively. Wavenumber measurements were calibrated using the peak at 520cm$^{-1}$ taken from a silicon wafer. For the phage spectra at 514nm, 5 accumulations were averaged during a 30 second sample collection time following 5 minutes of photo-bleaching. For the FAD and the phage/gold/FAD-NH$_2$ sample spectra, 15 accumulations were averaged during a 1 second sample collection time. Raman data was obtained courtesy of Dan Wilkinson and with the assistance of Nathan Weiss.

3.2.9 TEM and EDX

For both TEM and EDX, phage/gold samples made using the “nanomesh” preparation were centrifuged and rinsed in deionized water. For EDX, 20µL of the sample was drop-cast a onto a clean silicon wafer and allowed to dry overnight. The collection time was 90s at an accelerating voltage of 15kV. For the TEM analysis, 20µL of the sample was drop-cast onto a copper grid and allowed to sit for 10 minutes before being wicked of excess moisture. The grid was then allowed to dry for at least 4 hours prior to placement in the microscope. EDX and HRTEM images were obtained with the assistance of Danielle Casillas.
3.2.10 Instrumentation

All UV-Vis work was performed on a Cary 100 (Agilent Technologies). TEM images were taken in a JEM1200-EX (JEOL) on a copper mesh grid (Ted Pella). All electrodes were purchased from BASi. Electrochemical measurements were recorded using a Bio-Logic VMP3 and a computer interface running EC-Lab V10.34. AFM images were taken using a Bruker Dimension Icon Scanning Probe Microscope. Raman spectra were acquired with a Renishaw InVia micro-Raman spectrometer running WiRE 3.3 software (Renishaw PLC). EDX measurements were obtained on a NovaNano (FEI) field emission gun variable pressure scanning electron microscope, coupled with energy-dispersive X-ray spectroscopy (VPSEM–EDS), and processed using NSS software (Thermo Scientific).

3.3 Results

Assembly of the electrode (Figure 3.4) involves covalent attachment of the enzyme glucose oxidase to gold-conjugated pIII-modified M13 bacteriophage, leading to an electrically contacted phage/gold/recGOx nanomesh composite electrode. The shaft of the phage, containing the pVIII protein, serves as the base onto which carboxylic acid-functionalized gold is covalently attached through EDC-NHS chemistry, creating a gold nanomesh substrate. Following this step, the FAD active site is attached to the gold nanomesh, also using EDC-NHS chemistry. Finally, the apoGOx is introduced and allowed to reconstitute with the FAD. The modified pIII protein is engineered so that the resulting phage/gold/recGOx complex can be anchored to a gold substrate. While EDC-NHS chemistry has been used previously to attach FAD to gold(73), it has not been known to be used to attach gold to phage.
ApoGOx was prepared using an acidified saturated ammonium sulfate solution to slightly denature the GOx protein and allow for the removal of the active site. The resulting apoprotein was checked for quality and concentration using ultraviolet–visible spectroscopy (Figure 3.5). The absorption band at $\lambda_{\text{max}}=280\text{nm}$ is intrinsic to GOx(74) and any structural change or degradation of the protein will cause a peak shift. The apoGOx was checked before each use with UV-Vis to ensure that the holoenzyme would still be functional and reconstitute successfully with the FAD. When the apoGOx degraded, a fresh solution was prepared.
Atomic force microscopy (AFM) was used to demonstrate attachment of the pIII protein on the engineered M13 phage to gold. Figure 3.6 shows the topographical scan of the engineered M13 phage attached onto a gold-coated silicon wafer both before (a) and after (b) the EDC-NHS gold attachment. Attachment of the phage to the wafer was achieved following a standard biopanning technique, where the engineered phage was drop-cast onto the wafer, placed onto a rocking machine for 1 hour, and then rinsed to remove any unattached phage. The orange lines on the scan represent the phage and the average length of the lines corresponds to the length of the phage, ~900nm. The modified pIII proteins show a high affinity for binding to gold as a result of the high concentration of histidine(75,76,77,78).
Figure 3.6  AFM showing phage affinity to a gold substrate before (a) and after (b) EDC-NHS chemistry. Samples were prepared by exposing the phage to the substrate for 1 hour followed by rinsing with deionized water to remove the unattached phage. The scale bar to the right shows the z-axis (top/down) absolute value from the starting point of the needle before it crosses the area being measured.

To construct an electrode that is highly conductive, the modified M13 phage, which contains many amine groups along the shaft, was first conjugated with 1.4nm gold nanoparticles functionalized with carboxylic acid groups using EDC-NHS chemistry. Figure 3.7 demonstrates the successful gold conjugation of the phage. The transmission electron microscopy (TEM) images are comparable to phage/gold hybrids reported using incubation (non EDC-NHS-linked) methods(79). The samples in Figure 3.7 were unstained, meaning that all the electron absorption and image contrast is coming from the gold nanoparticles. There appears to be a certain degree of aggregation or cross-linking of the phages, which is possible due to the multiple carboxyl groups on each gold nanoparticle. The 40nm width of the gold-coated phage measured via HRTEM also indicates that the phages are linked together to some degree. However, the AFM also appears to show a bundling of the phage both before and after gold attachment. Since it is possible that the gold may also be serving as a cross-linker between phage and forming a gold mesh as opposed to individual gold nanowires, we refer to the resulting structure as a “gold mesh”.
The HRTEM data was also used to calculate the amount of gold attached to the phage by image analysis, which is roughly 24wt%. The average size of the gold nanoparticles is 1.4nm, and the average size of the phage is 880x6.6nm. Therefore, the number of phage in the area measured was determined by the width of the strands in the image. The weight of the gold nanoparticles was calculated by determining the amount of gold in an average gold nanoparticle. The weight of the phage is approximately 16.3MDa(64). ImageJ software was used to calculate the number of gold nanoparticles in a given area, which was then extrapolated to the entire phage surface. The presence of gold on the phage was also chemically confirmed with EDX (Figure 3.7c), where the amount of gold, as determined by this method, was estimated to be approximately 19wt%. 
Figure 3.7 Gold-conjugated phage “nanomesh” using EDC-NHS chemistry imaged with TEM (a), in closer view (b), dark field HRTEM (c) and EDX elemental spectra (d). The TEM samples are unstained, with the gold nanoparticles providing the contrast.
The resulting gold nanomesh-like material was then used as the conductive base onto which N\textsuperscript{6}-(2-aminoethyl)-FAD, referred to here as FAD-NH\textsubscript{2}, was covalently attached as shown in Scheme 1A. Use of this tethered version of FAD as opposed to a “native” FAD allows for a greater distance between the redox molecule and the phage/gold substrate, and enables better reconstitution with the apoprotein(64). Raman spectroscopy was used to confirm the attachment between the phage/gold nanomesh and the FAD-NH\textsubscript{2} active site. After multiple rinses following the EDC-NHS attachment of the FAD-NH\textsubscript{2}, spectra were obtained (Figure 3.8), with the most prominent peaks identified in Table 3.1.

The Raman spectrum of the phage (Figure 3.8a) was taken using the 514nm emission from an argon ion laser. The peak at \(~1476\text{cm}^{-1}\) with a shoulder at \(~1448\text{cm}^{-1}\) can most likely be assigned to CH\textsubscript{3} and CH\textsubscript{2} deformation. The peak at \(~1278\text{cm}^{-1}\) with a shoulder at \(~1231\text{cm}^{-1}\), as well as the peak at \(~1654\text{cm}^{-1}\), is primarily assigned to amide modes(80). The FAD-NH\textsubscript{2} spectrum (Figure 3.8b) is in good agreement with spectra for FAD found in literature using comparable conditions(81). The Raman activity for FAD, determined using the 633nm emission from a helium-neon laser, is predominantly from vibrations of the tricyclic isoalloxazine ring(82). The dominant peak seen at \(~1350\text{cm}^{-1}\) is assigned to the stretching of the ring. The peak at \(~1232\text{cm}^{-1}\) is the N-H bending of the isoalloxazine ring, and the peaks in the \(~1500-1650\text{cm}^{-1}\) region are combinations of C=C and C=N stretching modes. The spectrum of the phage/gold/FAD-NH\textsubscript{2} composite (Figure 3.8c), also taken with the 633nm line, is dominated by the FAD-NH\textsubscript{2} peaks. Peaks that can be assigned specifically to the phage within the phage/gold/FAD-NH\textsubscript{2} spectrum can be extracted by subtracting the FAD-NH\textsubscript{2} spectrum from the phage/gold/FAD-NH\textsubscript{2} composite spectrum (Figure 3.8d).
Figure 3.8 Raman spectra of the engineered phage (a), FAD-NH\textsubscript{2} (b), phage/gold/FAD-NH\textsubscript{2} (c), and a subtraction of the FAD-NH\textsubscript{2} peak from the phage/gold/FAD-NH\textsubscript{2} peak to reveal the phage peaks, as compared to the phage alone (d). All samples were taken on a silicon wafer substrate. The phage samples were taken with the 514nm line and the FAD-NH\textsubscript{2} and phage/gold/FAD-NH\textsubscript{2} samples were taken with the 633nm line.

<table>
<thead>
<tr>
<th>Material</th>
<th>Peak</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage</td>
<td>~1476cm\textsuperscript{-1} (shoulder at ~1448cm\textsuperscript{-1})</td>
<td>CH\textsubscript{3} and CH\textsubscript{2} deformation</td>
</tr>
<tr>
<td>Phage</td>
<td>~1278cm\textsuperscript{-1} (shoulder at ~1231cm\textsuperscript{-1})</td>
<td>amide modes</td>
</tr>
<tr>
<td>Phage</td>
<td>~1654cm\textsuperscript{-1}</td>
<td>amide modes</td>
</tr>
<tr>
<td>FAD</td>
<td>~1350cm\textsuperscript{-1}</td>
<td>stretching of the isoalloxazine ring</td>
</tr>
<tr>
<td>FAD</td>
<td>~1232cm\textsuperscript{-1}</td>
<td>N-H bending of the isoalloxazine ring</td>
</tr>
<tr>
<td>FAD</td>
<td>~1500-1650cm\textsuperscript{-1} region</td>
<td>C=C and C=N stretching modes</td>
</tr>
</tbody>
</table>

Table 3.1 Raman peak assignments for the phage and FAD.
In order to evaluate the success of the reconstruction of the enzyme onto the electrode, an enzymatic activity assay using Amplex® Red (10-acetyl-3,7-dihydroxyphenoxazine) was performed. In this assay, the GOx reacts with D-glucose to form D-gluconolactone and hydrogen peroxide. In the presence of horseradish peroxidase, the hydrogen peroxide reacts with the Amplex® Red reagent and generates resorufin, which has a strong absorption at 560nm (Figure 3.9) with no interference from autofluorescence. The increase in absorption over time occurs because of the increasing concentration of resorufin. The phage/gold/recGOx composite shows equivalent activity to a native GOx control, indicating that successful reconstitution was achieved.

Figure 3.9 Amplex® Red activity assay during the first minute of the assay with 100mM glucose for the phage/gold/GOx electrode (red) as compared to a native GOx control (orange), a phage control (purple), and a gold nanoparticle control (blue). The assay was run at 560nm and at multiple time points, and showed equivalent activity between the phage/gold/GOx and control (2.3U/mL), with no activity for the phage and gold controls. Both the GOx control and the phage/gold/GOx samples reached saturation in approximately 30 minutes, when absorption reached a plateau.
Electrochemical characterization was carried out in half cells in order to better understand the kinetics of the electrode system, to determine the concentration of the enzyme on the electrode, and uncover any limitations associated with the materials being tested. The electrocatalytic oxidation of glucose by the phage/gold/recGOx composite was characterized using cyclic voltammetry. In this configuration, the voltage is swept linearly between -0.7V and 0.7V vs Ag/AgCl, and the resulting current is measured. At the working electrode containing the phage/gold/recGOx, potential is applied with respect to the reference electrode (in this case, Ag/AgCl) to change the energy levels and induce the attendant oxidation and reduction reactions.

Figure 3.10 shows cyclic voltammograms obtained at a sweep rates of 100mV/s and 1mV/s for the phage/gold/recGOx composite on a gold working electrode in comparison to a phage/gold control, a phage/gold/FAD control, and a phage/gold/apoGOx control, all on a gold substrate. All samples were exposed to a pH 5.5 McIlvaine buffer solution purged of oxygen. The phage/gold/recGOx composite shows a significant increase in current beginning at the FAD/GOx potential of -0.34V upon the addition of glucose, reaching 1.2mA/cm² at 0.7V, which is not seen with the controls. Appropriate and favorable comparisons to similar reported GOx DET systems are shown in Table 3.2. This experiment demonstrates that the fully reconstituted GOx is immobilized onto the electrode and that the FAD-NH₂ is responsible for the electrochemical response. The anodic peak does not appear to reach saturation, which is not unexpected due to the high enzyme concentration.
Figure 3.10  Cyclic voltammetry at 100mV/s (a) and 1mV/s (b) of the phage/gold/GOx without (red) and with (green) glucose, a phage/gold/apoGOx control without (light blue) and with (purple) glucose, a phage/gold/FAD control without (dark blue) and with (peach) glucose, and a phage/gold control without (orange) and with (gray) glucose. The addition of glucose in each sample created a final concentration of 50mM. Scans were taken in cells purged of oxygen with argon, in pH 5.5 McIlvaine PBS buffer solution, at 25°C. The insert for (a) shows a zoomed in view of the phage/gold/GOx compared to the phage/gold/apoGOx, both with glucose, between -0.5 and 0 V vs Ag/AgCl, where the phage/gold/GOx shows a current increase beginning at the GOx oxidation potential of -0.34V.
<table>
<thead>
<tr>
<th>Material, Attachment scheme</th>
<th>Scan rate (mV/s)</th>
<th>Glucose concentration (mM)</th>
<th>Enzyme surface coverage</th>
<th>Peak current per area (mA/cm²), Voltage (V)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>This work (Phage/gold, covalent by EDC-NHS)</td>
<td>100</td>
<td>50</td>
<td>4.74×10⁻⁸ mol/cm²</td>
<td>1.2, 0.7</td>
<td>----</td>
</tr>
<tr>
<td>rGO/AuNp composite, adsorbed</td>
<td>110</td>
<td>90</td>
<td>unknown</td>
<td>1.5, 0.17</td>
<td>84</td>
</tr>
<tr>
<td>PQQ/Gold, covalent coupling by soaking</td>
<td>5</td>
<td>80</td>
<td>1.7×10⁻¹² mol/cm²</td>
<td>1.0, 1.0</td>
<td>85</td>
</tr>
<tr>
<td>CNT/Carbon paper, adsorbed</td>
<td>100</td>
<td>20</td>
<td>0.7×10⁻¹² mol/cm²</td>
<td>0.5, 0.5</td>
<td>86</td>
</tr>
<tr>
<td>Hydroxyl fullerenes modified glassy carbon, adsorbed</td>
<td>50</td>
<td>unknown</td>
<td>8.3×10⁻¹¹ mol/cm²</td>
<td>0.18, -0.4</td>
<td>87</td>
</tr>
<tr>
<td>rGO/CNT dispersion</td>
<td>10</td>
<td>150</td>
<td>unknown</td>
<td>1.4×10⁻², -0.44</td>
<td>88</td>
</tr>
<tr>
<td>Modified glassy carbon, adsorbed then EDC-NHS</td>
<td>100</td>
<td>10</td>
<td>unknown</td>
<td>8.6×10⁻², -0.5</td>
<td>89</td>
</tr>
<tr>
<td>MoS₂ nanosheets/AuNp modified glassy carbon, adsorbed</td>
<td>100</td>
<td>unknown</td>
<td>2.41×10⁻¹² mol/cm²</td>
<td>7.9×10⁻², -0.45</td>
<td>90</td>
</tr>
<tr>
<td>nanoporous TiO₂/CNT</td>
<td>10</td>
<td>5</td>
<td>unknown</td>
<td>6.5×10⁻², 0.8</td>
<td>91</td>
</tr>
<tr>
<td>CNT, adsorbed</td>
<td>50</td>
<td>25</td>
<td>unknown</td>
<td>5.7×10⁻², -0.35</td>
<td>92</td>
</tr>
<tr>
<td>poly(L-arginine)/CNT, adsorbed</td>
<td>50</td>
<td>10</td>
<td>1.76×10⁻¹⁰ mol/cm²</td>
<td>3.9×10⁻², -0.4</td>
<td>93</td>
</tr>
<tr>
<td>poly(2,6-diaminopyridine)/CNT, covalent glutaraldehyde linker</td>
<td>100</td>
<td>8</td>
<td>unknown</td>
<td>3.3×10⁻², -0.4</td>
<td>94</td>
</tr>
<tr>
<td>AuNP modified graphene/CNT, electrostatic attraction</td>
<td>50</td>
<td>2</td>
<td>2.22×10⁻¹⁰ mol/cm²</td>
<td>unknown area</td>
<td>95</td>
</tr>
<tr>
<td>Mesocellular graphene foam, adsorbed</td>
<td>50</td>
<td>12</td>
<td>4.5×10⁻¹¹ mol/cm²</td>
<td>unknown area</td>
<td>96</td>
</tr>
</tbody>
</table>

Table 3.2 Comparison of peak current values and enzyme concentration for different GOx DET schemes taken with cyclic voltammetry. When not directly given, peak current per area was calculated by taking the peak current for the stated CV conditions and dividing by the stated surface area of the electrode. Not all reported systems reached peak current saturation or were scanned within the same voltage range. Enzyme concentration values given are either from the stated amount of GOx used or, when calculated by the authors, the enzyme surface concentration (Γ, given in mol/cm²).
The enzyme surface coverage $\Gamma$ was calculated according to Equation 3.2 below (97) where $Q$ is the charge involved in the reaction (obtained by integrating the anodic peak and dividing by the scan rate for coulombs of charge), $n$ is the number of electrons transferred (in this case, $n=2$), $F$ is the Faraday constant, and $A$ is the geometric area of the electrode. Using this method, we calculated an enzyme surface coverage on the electrode of $4.74 \times 10^{-8}$ mol/cm$^2$, a value significantly larger than other reported values with systems using non-covalent or monolayer approaches.

$$\Gamma = \frac{Q}{nFA} \quad \text{(Eq. 3.2)}$$

Figure 3.11 shows the amperometric response for successive 200$\mu$L additions of 4M of glucose into the original 15mL solution. No oxidation current was measured for apoGOx on the gold working electrode, confirming that the current response arises from the redox activity of the fully reconstituted GOx and also demonstrating direct electron transfer.
3.4 Conclusion

In summary, we have designed, fabricated, and operated an electrode consisting of glucose oxidase covalently attached to gold nanoparticles that are assembled onto genetically engineered M13 bacteriophage using EDC-NHS chemistry. The electrode was evaluated for the attachment of the various constituents by a combination of AFM, TEM, EDX, and Raman spectroscopy methods, while the activity of the enzyme and DET were evaluated using cyclic voltammetry, constant potential amperometry, and an activity assay quantified with UV-Vis. The resulting nanomesh-like architecture achieved a peak current per area of 1.2 mA/cm$^2$, which is greater than values reported for other DET attachment schemes. We have also achieved a high enzyme surface coverage that was calculated to be $4.74 \times 10^{-8}$ mol/cm$^2$, which is a significant improvement over most current GOx DET attachment methods.
Chapter 4. A Highly Stable Fructose Dehydrogenase Biofuel Cell Anode

4.1 Motivation

Fructose dehydrogenase is a heterotrimeric membrane-bound enzyme that catalyzes the oxidation of D-fructose into 2-keto-D-fructose. While fructose dehydrogenase enzymes are currently not readily available, it is possible to harvest them from multiple strains of Gluconobacter bacteria(98,99). Unlike the more commonly used glucose oxidase, it naturally allows for direct electron transfer (DET) bioelectrocatalysis, giving it an exciting advantage. The two active site/redox cofactors of FDH are PQQ (pyrroloquinoline quinone) and a cytochrome c-type heme(100). A heme is a cofactor consisting of a ferrous ion contained in the center of a large heterocyclic organic ring called a porphyrin, made up of four pyrrolic groups joined together by methine bridges. It is believed that FDH reacts with electrodes at the heme c subunit(101), however, no conclusive evidence has yet been shown(102).

While the DET ability of FDH allows for easy interfacial electron transfer, FDH is a very unstable enzyme that has been demonstrably difficult to immobilize(103). Therefore, a simple immobilization scheme that secures the enzyme in place and restricts motion would be particularly advantageous. While the sol-gel method has been shown to be an attractive option for enzyme encapsulation in biofuel cell applications and is discussed further in Chapter 5, this electrode preparation is not particularly suited to the FDH enzyme due to its limited pH stability and delicate membrane. Therefore, this chapter describes an alternative method to FDH encapsulation and stabilization.
Natural and non-reactive polymers have distinctive characteristics suited for enzyme immobilization, such as their availability and biocompatibility, which minimizes the possibilities of enzyme denaturation. Carboxymethyl cellulose (CMC), also known as cellulose gum, is a cellulose derivative with carboxymethyl groups (-CH2-COOH) bound to some of the hydroxyl groups of the glucopyranose monomers that make up the cellulose backbone. Aqueous solutions of CMC have also been previously used to disperse carbon nanotubes (104), where the long CMC molecules are thought to wrap around the nanotubes, allowing them to be more easily separated in water. While the higher solubility of CMC in water may make the matrix unstable in an aqueous fuel cell, its ability to disperse carbon nanotubes (CNTs) may allow for a matrix with more available surface area. Polyvinylidene fluoride (PVDF) is a low cost, non-reactive
thermoplastic fluoropolymer. PVDF artificial membranes are commonly used for western blots for the immobilization of proteins due to its non-specific affinity for amino acids, as well as for membranes in filtration devices. This immobilization ability, as well as their lower solubility in water, makes them an attractive candidate to immobilize high amounts of FDH within a stable matrix.

In this work, FDH-based anode electrode matrices were constructed using a mixture of either CMC or PVDF with carbon nanotubes in varying ratios. Long-term cyclic voltammetry studies were conducted on the most successful mixture to demonstrate using the binder as a means of preventing the FDH from denaturing and thus extending the lifetime of the half cell.

4.2 Experimental

4.2.1 Chemicals

D-Fructose dehydrogenase from Gluconobacter sp. was obtained by TOYOBO U.S.A., Inc. The activity of the enzyme as-obtained was 20U/mg-solid. The CMC had an average molecular weight of approximately 90,000 and was purchased from Sigma-Aldrich. The PVDF had an average molecular weight of approximately 534,000 and was also purchased from Sigma-Aldrich. The single-walled carbon nanotubes contain 1.0-3.0 atomic% carboxylic acid and were purchased from Carbon Solutions, Inc. The diamond electrode polish and pad were purchased as a kit from BASi.

4.2.2 Preparation of CMC Anodes

Solutions with weight ratios of 90/10, 50/50, 20/80, and 10/90 CMC/CNT solutions were prepared by dissolving the CMC in 250µL of deionized water and sonicationg for 1 hour, and then
adding the nanotubes. Simultaneously, gold working electrodes with a diameter of 3mm were polished with 3µm fine diamond polish and rinsed with ethanol in a sonicator for 10 minutes. 5µL of each solution was then pipetted separately onto a gold electrode and allowed to dry overnight. The CMC/CNT-coated electrodes were then soaked overnight in 1mL of a 1.5mg/mL solution of FDH in a pH 5.5 McIlvaine PBS buffer solution at 4°C.

4.2.3 Preparation of PVDF Anodes

Solutions with weight ratios of 10/90 and 20/80 PVDF/CNT were prepared by dissolving the PVDF in 250µL of deionized water and sonicating for 1 hour, and then adding the nanotubes. Simultaneously, gold working electrodes with a diameter of 3mm were polished with 3µm fine diamond polish and rinsed with ethanol in a sonicator for 10 minutes. 5µL of each solution was then pipetted separately onto a gold electrode and allowed to dry overnight. The PVDF/CNT-coated electrodes were then soaked overnight in 1mL of a 1.5mg/mL solution of FDH in a pH 5.5 McIlvaine PBS buffer solution at 4°C.

4.2.4 Electrochemical Measurements

All electrochemical experiments were run with pH 5.5 McIlvaine PBS buffer solution in a 15mL cell vial purged of oxygen by bubbling in argon gas for at least 15 minutes prior to testing. The counter electrode (also polished and rinsed before each use) was platinum wire, and the reference electrode was Ag/AgCl. CV scans were taken 4 times each from -0.2 to 0.5 V, both with and without 50mM fructose and at sweep rates of 100, 50, 20, 5, 10, and 1 mV/s. The long-term CV scan was taken for 500 cycles at 5mV/s and 100mM fructose.
4.2.5 Instrumentation

All electrodes were purchased from BASi. Electrochemical measurements were recorded using a Bio-Logic VMP3 and a computer interface running EC-Lab V10.34.

4.3 Results

The first binder to be tested was CMC in weight ratios of 90/10, 50/50, 20/80, and 10/90 CMC/CNT, each mixed and drop-cast onto a gold electrode to dry overnight. Of all these matrix compositions, the only ratio that both adhered to the gold substrate and did not dissolve and dissociate upon submersion into an aqueous solution was 10/90 CMC/CNT. Based on the initial assessment of the CMC compositions, PVDF matrices with weight ratios of 20/80, and 10/90 PVDF/CNT were then mixed and also drop-cast onto a gold electrode to dry overnight.

Figure 4.2 shows cyclic voltammograms obtained at a sweep rate of 5mV/s for 10/90 CMC/CNT, 20/80 PVDF/CNT, and 10/90 PVDF/CNT on a gold working electrode, each in a pH 5.5 McIlvaine buffer solution purged of oxygen and with 50mM fructose. The anodic oxidation peak begins to occur near the standard values reported for FDH of approximately 80mV vs Ag/AgCl(105). The 20/80 PVDF/CNT composite shows the highest peak current per area of 0.21mA/cm², compared to 0.13mA/cm² for 10/90 PVDF/CNT and 0.10mA/cm² for 20/80 CMC/CNT. The enzyme concentration surface concentration on the gold electrode (Γ) was calculated for 20/80 PVDF/CNT using Equation 2 (where in this case also, n=2) to be $1.15 \times 10^{-7}$mol/cm².
Figure 4.2  Cyclic voltammetry of the three best performing binder/CNT mixtures: 20/80 PVDF/CNT (green), 10/90 PVDF/CNT (blue), and 10/90 CMC/CNT (red). Cycles were taken upon the addition of fructose to a final concentration of 50mM. Scans were taken in cells purged of oxygen with argon, in pH 5.5 McIlvaine PBS buffer solution, at 25°C, and at a scan rate of 5mV/s.

In order to evaluate the long-term stability and immobilization ability of the 20/80 PVDF/CNT matrix compared to a control with carbon nanotubes alone, a CV experiment at 5mV/s, 500 cycles, and 100mM fructose was conducted. The results are shown in Figure 4.3, where although the peak current for the 20/80 PVDF/CNT matrix is lower, it also only demonstrates an approximately 15% reduction in peak current compared to a 30% reduction for the control of carbon nanotubes alone (no PVDF).
Figure 4.3  Long-term cyclic voltammetry test of FDH in a 20/80 PVDF/CNT matrix (red) versus a 100% CNT matrix with no PVDF (blue). The scan rate is 5mV/s and was run for 500 cycles and with 100mM fructose.

4.4 Conclusion

In summary, described is an electrode design consisting of fructose dehydrogenase captured in a matrix consisting of varying ratios of either carboxymethylcellulose or polyvinylidene fluoride to carbon nanotubes in order to immobilize the enzyme in a stable matrix. A long-term cyclic voltammetry study was conducted on the most successful mixture (20/80 PVDF/CNT) to demonstrate using the binder as a means of preventing the FDH from denaturing. It is therefore possible that this method of electrode preparation may extend the lifetime of a full FDH-based fuel cell. Peak current per area and enzyme surface coverage were found to be 0.21mA/cm$^2$ and $1.15\times10^{-7}$mol/cm$^2$, respectively.
Chapter 5. A Bilirubin Oxidase Sol-Gel Cathode with Direct Electron Transfer

5.1 Motivation

As discussed in previous chapters, direct electron transfer (DET) is a topic of interest both for fuel cells and electrochemical sensors. As opposed to mediated electron transfer (MET), DET offers a simple cell construction and higher power outputs by accessing the maximum possible potential of the enzymes. Many factors influence the success of a DET system. The current collector substrate must be highly conductive in order to achieve efficient electron transfer between the enzyme and the substrate to occur. The orientation of the enzyme on the substrate, as well as the ability of the substrate to access the active site(s) are also paramount. If a covalent link between the enzyme and the current collector is not established, then the distance cannot be greater than the electron tunneling distance and can be a limiting factor in electron transfer kinetics.

In the application of implantable devices, a number of enzymes have been considered for the oxygen reduction reaction at the cathode such as lacasses(106) and polyphenol oxidases(107). However, bilirubin oxidase (BOD) is of particular interest due to its availability, stability, and peak efficiency at pH values akin to the body. In a BOD cathode, electrons are directly transferred from the electrode surface to the type-1 copper site of BOD, and then transferred to the type-2/type-3 copper cluster, where they are used to reduce molecular oxygen into water(108). In a previous study by Lim et al(109), a sol-gel encapsulated BOD cathode was constructed to enhance the stability of the enzyme while also achieving DET. A TMOS-based gel was fabricated with nanotubes as the conducting component and polyethylene glycol (PEG)
as a dispersant for the nanotubes (Figure 5.1). This chapter describes a modified version of this method that was devised in order to further improve upon and elucidate the fabrication steps of the electrode.

![Figure 5.1](image.png)

Figure 5.1 Representation of the BOD fuel cell constructed by Lim et al. The sol-gel matrix encapsulates the bilirubin oxidase enzymes and is made conductive by multi-walled carbon nanotubes. Image courtesy of James Robert Lim.

5.2 Experimental

5.2.1 Chemicals

The Bilirubin oxidase from Myrothecium species with an activity of 2.51U/mg was generously donated by Amano Enzyme USA. Bilirubin oxidase from Myrothecium species with an activity of approximately 60U/mg was purchased from Sigma-Aldrich. Multi-walled carbon nanotubes with a purity of 95% were purchased from Sigma-Aldrich, and have an outer diameter of 20-30nm and an inner diameter of 5-10nm. The lengths of the nanotubes ranged from 0.5nm to 200µm. TMOS of 99% purity was purchased from Sigma-Aldrich, and 37% HCl was purchased from Spectrum Chemical. A 1X powder concentrate of Phosphate Buffered Saline,
was purchased from Fisher Scientific. The Gibco® brand of Dulbecco's phosphate-buffered saline (no calcium, no magnesium) was obtained from Thermo Fisher Scientific Inc.

5.2.2 Preparation of the TMOS Sol

For the TMOS sol, 7.6mL of TMOS was combined with 1.7mL of deionized water and 200uL of 0.04N HCl. This mixture was then quickly stirred and then sonicated in an ice bath for 15min to catalyze hydrolysis. The solution was then allowed to refrigerate for at least 24hrs to allow the hydrolysis to reach completion.

5.2.3 Gelation Studies

To study the effect of the various components of the sol-gel mixture on the gelation time, a series of gels with 80 or 150µL of buffer that was either unfiltered, industry-filtered, or laboratory filtered were tested along with a set of gels that either did or did not also include 20µL of PEG 600. All gels included 150µL of TMOS sol.

5.2.4 BOD Sol Gel Electrode

The initial BOD gel cathodes were constructed using 150µL of TMOS sol with 20µL of PEG 600. This mixture was added to 20µL of multi-walled carbon nanotubes (MWNTs) and stirred thoroughly to coat the carbon nanotubes in the TMOS/PEG solution. This was then combined with 150uL of pH 7.2 1x PBS buffer to raise the pH and induce condensation and gelation. After being allowed to gel in air for at least 10 minutes and up to 40 minutes, the electrodes were aged in pH 7.2 1x PBS solution for at least 24 hours at 4 °C.
For the final cathode construction, a BOD gel electrode was prepared by combining 150µL of TMOS sol with 15µL of PEG 600. This mixture was added to 15mg of MWNTs and stirred to combine. This was then combined with 150µL of pH 7.2 1x PBS buffer that either did or did not contain 30mg/mL BOD. Immediately after the addition and mixture of the buffer solution, the resulting paste was applied onto a gold mesh current collector which had been previously cleaned for 15 minutes with piranha etch and successive water and ethanol rinses. The electrode was allowed to gel in air for 10 minutes prior to aging in pH 7.2 1x PBS solution for at least 24 hours at 4 °C.

5.2.5 Activity Studies

For the activity studies, two gels with the same amounts of enzyme units were compared with each gel containing a different source amount of U/mg. The first BOD source used had an activity of 2.51U/mg and the second had approximately 60U/mg. To fabricate each electrode, 150µL of TMOS sol and 15µL of PEG 600 were combined and then added to 15mg of MWNTs. Each mixture was then combined with 150µL of pH 7.2 1x PBS buffer that contained either the 2.51U/mg or the 60U/mg BOD, where the total amount of activity in each electrode was then 11.5U. Immediately after the addition of the BOD solutions, the resulting paste of each BOD mixture was applied onto gold mesh current collectors which had been previously cleaned for 15 minutes with piranha etch and successive water and ethanol rinses. The electrodes were allowed to gel in air for 10 minutes prior to aging in pH 7.2 1x PBS solution for at least 24 hours at 4 °C.
5.2.6 Electrochemical Measurements

All electrochemical experiments were run with pH 7.2 1x PBS buffer solution in a 15mL cell vial. The buffer-filled vials were purged of oxygen by bubbling in argon gas for at least 15 minutes prior to testing for the control. The vials were then subsequently saturated with oxygen for at least 15 minutes prior to oxygen testing. The counter electrode used was platinum wire, and the reference electrode was Ag/AgCl. Platinum counter electrodes were polished using 3µm fine diamond polish and rinsed with ethanol in a sonicator for 10 minutes. CV scans were taken 10 times each from 0.1 to 0.7 V and at sweep rates of 100, 50, 20, 10, 5, and 1mV/s.

5.2.7 Instrumentation

EMD Millipore filters for the buffer solution made from the 1X powder concentrate were purchased from Fisher Scientific and have an average a pore diameter of 0.22µm. All electrodes were purchased from BASi. Electrochemical measurements were recorded using a Bio-Logic VMP3 and a computer interface running EC-Lab V10.34.

5.3 Results

The initial sol-gels created using the recipe created by Lim et al(106) did not successfully remain upon the mesh substrate. These gels were prepared by combining TMOS sol with PEG 600, MWNTs, and fresh unfiltered PBS buffer. Figure 5.2 shows an example of the initial gel experiments, where the majority of the condensation and gelation did not appear to occur until after depositing the electrode in the buffer solution before aging. It therefore appeared that one of the materials in the mixture was causing a large delay in the gelation.
Despite allowing for gelation times well over the recommended time 20 minutes, gelation would not successfully occur on the electrode. The sol has seeped out of the electrode to gel outside of the electrode.

For the sol-gel anodes, the time to gelation excluding the carbon nanotubes was studied in order to more easily evaluate gelation times. For the first set of mixtures, the same amount of TMOS sol (150µL) and PEG 600 (20µL) was used and varying types and amounts of PBS buffer were evaluated in order to determine whether the method of buffer filtration has a significant effect on gelation time. It is possible that crystals present in the unfiltered buffer may either prevent gelation by hindering network formation or encourage gelation by introducing nucleation sites. Table 5.1 shows the times to achieve a paste-like consistency and times to gelation for the various mixtures. Each condition was tested 5 times and the average values are shown. While there appears to be some degree of difference between the various types of buffers, there is not a significant advantage or disadvantage to using unfiltered buffer. What is clearly shown is that an
increase in buffer results in a decrease in gelation time, where using half of the amount of PEG results in an equal decrease of gelation time to about half.

<table>
<thead>
<tr>
<th>Unfiltered, 80uL</th>
<th>Unfiltered, 150uL</th>
<th>Filtered, 80uL</th>
<th>Filtered, 150uL</th>
<th>Gibco filtered, 80uL</th>
<th>Gibco filtered, 150uL</th>
<th>No PEG, 80uL, Filtered</th>
<th>No PEG, 150uL, Filtered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paste at 5.5 hours</td>
<td>Paste at 2.5 hours</td>
<td>Paste at 4 hours</td>
<td>Paste at 2 hours</td>
<td>Paste at 5 hours</td>
<td>Paste at 2.5 hours</td>
<td>Paste at 12 seconds</td>
<td>Paste at 5 seconds</td>
</tr>
<tr>
<td>Gel at 6.2 hours</td>
<td>Gel at 3.5 hours</td>
<td>Gel at 6.5 hours</td>
<td>Gel at 3 hours</td>
<td>Gel at 6 hours</td>
<td>Gel at 4 hours</td>
<td>Gel at 65 seconds</td>
<td>Gel at 34 seconds</td>
</tr>
</tbody>
</table>

Table 5.1 Comparison of paste and gelation times for various mixtures of gels. While there is a significant difference when additional buffer is added, no clear distinction can be made between filtered and unfiltered buffer.

In a second set of experiments, PEG 600 was removed from the mixture recipe to determine the effect of PEG on gelation time. The exclusion of PEG 600 resulted in a drastic decrease of time to gelation, which indicates that the PEG is the most significant barrier to network formation among all of the components. Based on these results, the amount of PEG was reduced from 20µL to 15µL and all buffers were filtered for subsequent experiments. Additionally, the amount of MWNTs was reduced from 20mg to 15mg, as the initial mixtures with MWNTs were found to be too dry to easily and evenly spread on the mesh substrate. The results of the final mixture are shown in Figure 5.3. As compared with earlier mixtures, this formula both spread easily on the current collector and gelled quickly onto the mesh substrate without any visible sol leakage upon submersion in the PBS buffer for aging.
Cyclic voltammetry (CV) experiments were conducted in order to evaluate the effectiveness of the BOD encapsulation and the conductivity of the MWNT-enhanced gel. Electrodes with and without 30mg/mL of BOD were placed in pH 7.2 1x PBS solution and electrochemically evaluated under both saturated argon and in saturated oxygen. Without the presence of BOD, the reduction wave does not exist when oxygen is added (Figure 5.4). Additionally, the curve appears resistive at faster sweep rates.
Figure 5.4  Cyclic voltammetry of a blank electrode exposed to saturated argon at 100mV/s (red) and 1mV/s (blue). The sweep rate was 1 mV/s and the sweep commenced at 700 mV vs. Ag/AgCl. Below is a zoomed-in view of the 1mV/s sweep.

Figure 5.5 shows the CV curves obtained for the gels that include BOD. Similar to the gels that do not any enzyme, there is no reduction wave when BOD is included but saturated
argon is pumped in and oxygen is removed. In gels with BOD that are pumped with saturated oxygen, a biocatalytic reduction current can be seen at approximately 450 mV. This value is consistent with the reported value of the redox potential of BOD, 460 mV(110) as well as other reported systems(102)(111). Using the geometric area of the mesh current collector and the peak current at the scan rate of 1mV/s, the peak current per unit area was calculated to be 125μA/cm². The enzyme concentration surface concentration on the gold electrode (Γ) was calculated using Equation 3.2 (in this case also, n=2) to be 3.24×10⁻⁴ mol/cm².

![Cyclic voltammetry of electrode containing BOD exposed to saturated argon (red) and saturated oxygen (blue). The sweep rate was 1 mV/s and the sweep commenced at 700 mV vs. Ag/AgCl.](image)

In order to distinguish the effects of enzyme activity versus enzyme quantity, two different gels were constructed using the same formulation, each using a BOD of a different amount of enzyme units (U) per mg, where the final amount of enzyme units in both gels were
the same, roughly 11 U. Therefore, one sample has a significantly higher amount of enzymes that are less active, and the other sample has a smaller amount of enzymes that are considerably more active. One U is defined as the amount of the enzyme that catalyzes the conversion of 1 μmol of substrate per minute. The results can be seen in Figure 5.6, wherein the gel which contains the higher amount of less active BOD has a much greater reduction peak than the gel with the lesser amount of more active BOD. This indicates that the reaction is not entirely “biocatalytic” in the sense that the reaction is not enhanced by more active enzymes (and also that the reaction is not being acted upon bilirubin, but instead molecular oxygen).

Figure 5.6  Cyclic voltammetry of two electrodes, each with 11.5U of BOD. The first gel contains BOD at 60U/mg and is shown under saturated argon (blue) and saturated oxygen (red). The second gel contains BOD of 2.51U/mg, and is also shown under argon (green) and oxygen (purple). The sweep rate was 1 mV/s and the sweep commenced at 700 mV vs. Ag/AgCl. A much more significant reduction wave can be seen for the gels containing less active (and thus more total) BOD.
5.4 Conclusion

In summary, described is an electrode design consisting of bilirubin oxidase immobilized in a TMOS-based sol-gel matrix consisting of multi-walled carbon nanotubes for conductivity, with PEG acting as a dispersant. The formula is a modified version of the method presented by James Robert Lim et al(106) in order to further understand and improve the gelation and construction process. A cyclic voltammetry study was conducted on the most successful of a series of mixtures to demonstrate direct electron transfer when exposed to oxygen. The system did not appear to be enhanced by more active enzymes. Peak current per area and enzyme surface coverage were found to be 125µA/cm² and 3.24×10⁻⁴ mol/cm², respectively.
Chapter 6. Full Biofuel Cell: Glucose Oxidase versus Fructose Dehydrogenase

6.1 Motivation

In the previous chapters, the methods for producing two enzymatic biofuel cell anodes and one cathode were discussed. Therefore, it is possible to utilize and compare these electrodes in full cells and evaluate them for applications of interest. In addition to the common engineering challenges of cost and scale-ability, there are additional considerations that must be made that are function-specific. In the case of implantable devices specifically, the benchmarks of performance in batteries, and by extension, biofuel cells, are power and stability (112). Therefore, when comparing the GOx and FDH systems discussed in Chapters 3 and 4, several factors must be considered.

The glucose oxidase system described previously achieves direct electron transfer with covalent enzyme attachment onto an engineered electrode surface of phage/gold nanomesh. The enzyme surface coverage and peak current per area were $4.74 \times 10^{-8}$ mol/cm$^2$ and 1.2 mA/cm$^2$, respectively. The fructose dehydrogenase system by comparison is much more simple as it achieves direct electron transfer naturally, and is therefore also much easier in construction. However, the peak current per area is much lower at 0.21 mA/cm$^2$ despite having a higher enzyme surface coverage of $1.15 \times 10^{-7}$ mol/cm$^2$. Therefore, on these data points alone, a clearly advantageous system cannot be established. In this chapter, the GOx and FDH anodes that were previously described in Chapters 3 and 4, respectively, are compared electrochemically as full fuel cells against the BOD cathode described in Chapter 4.
6.2 Experimental

6.2.1 Chemicals

For the glucose oxidase biofuel cell, the type 3 library M13 phage was purchased from New England Biolabs. The functionalized nanoparticles were purchased from Nanoprobes Inc. FAD-NH₂ was obtained as a custom synthesis from Chemtos following the procedure detailed by Bückmann et al(67). The glucose oxidase Amplex® Red assay kit was purchased from Life Technologies. The electrode polish and pad were purchased as a kit from BASi. All other chemicals were purchased from Sigma-Aldrich.

For the fructose dehydrogenase biofuel cell, D-Fructose dehydrogenase from Gluconobacter sp. was obtained from TOYOBO U.S.A., Inc. The activity of the enzyme as-obtained was 20U/mg-solid. The CMC had an average molecular weight of approximately 90,000 and was purchased from Sigma-Aldrich. The PVDF had an average molecular weight of approximately 534,000 and was also purchased from Sigma-Aldrich. The single-walled carbon nanotubes contain 1.0-3.0 atomic% carboxylic acid and were purchased from Carbon Solutions, Inc.

For the bilirubin oxidase biofuel cell, the Bilirubin oxidase from Myrothecium species with an activity of 2.51U/mg was generously donated by Amano Enzyme USA. Multi-walled carbon nanotubes with a purity of 95% were purchased from Sigma-Aldrich, and have an outer diameter of 20-30nm and an inner diameter of 5-10nm. The lengths of the nanotubes ranged from 0.5nm to 200µm. TMOS of 99% purity was purchased from Sigma-Aldrich, and 37% HCl was purchased from Spectrum Chemical. A 1X powder concentrate of Phosphate Buffered Saline, was purchased from Fisher Scientific.
6.2.2 Electrode Preparations

For the glucose oxidase biofuel cell, the electrode was prepared following the procedure detailed in Chapter 3. Briefly, the engineered M13 bacteriophage was conjugated with gold nanoparticles using EDC-NHS chemistry to create a gold “nanomesh”. Following the gold attachment, FAD-NH$_2$ was attached to the nanomesh also using EDC-NHS chemistry. This mixture was then attached to a clean gold foil current collector overnight at 4°C by the electrostatic binding with the engineering pIII protein of the phage. After this attachment, the apoGOx was added to reconstitute with the FAD-NH$_2$ and form a whole enzyme upon the current collector.

For the fructose dehydrogenase biofuel cell, the anode was prepared following the procedure detailed in Chapter 4. An 80/20wt% CNT/PVDF solution was first prepared by dissolving 2mg of PVDF in 250µL of deionized water and sonating for 1 hour or until the PVDF appeared to be dissolved. 8mg of nanotubes were then added and sonicated for an additional 15 minutes. Simultaneously, gold working electrodes (diameter = 3mm) were polished with 3µm fine diamond polish and rinsed with ethanol in a sonicator for 10 minutes. 5µL of the solution was then pipetted onto a gold electrode and allowed to dry overnight. The PVDF/CNT-coated electrodes were then soaked overnight in 1mL of a 1.5mg/mL solution of FDH in a pH 5.5 McIlvaine PBS buffer solution at 4°C.

For the bilirubin oxidase biofuel cell, the electrode was prepared following the procedure detailed in Chapter 5. The gels were prepared with a TMOS sol that consists of 7.6mL of TMOS, 1.7mL of deionized water, and 200µL of 0.04N HCl. This mixture was sonicated in an ice bath for 15min to and aged at 4°C overnight. The BOD gel electrode was then prepared by combining 150µL of TMOS sol with 15µL of PEG 600 and 15mg of multiwalled carbon nanotubes. This
mixture was then combined with 150µL of 30mg/mL BOD in pH 7.2 1x PBS buffer. The resulting paste was immediately applied onto a clean gold mesh current collector and allowed to gel in air for 10 minutes prior to aging in pH 7.2 1x PBS solution for at least 24 hours at 4 °C.

**6.2.3 Electrochemical Measurements**

Each cell was assembled in cell vials as shown in Figure 6.1. Anodes and cathodes were placed approximately 0.75 cm apart. The vials each contained pH 5.5 McIlvaine PBS buffer solution with oxygen bubbled in for at least 15 minutes prior to testing in order to reach saturation. The GOx/BOD cell contained a glucose concentration of 100mM, and the FDH/BOD cell contained a fructose concentration of 100mM. For each cell, loads were incrementally applied for an acquisition time of 5 seconds each at 500 kΩ, 350 kΩ, 100 kΩ, 50 kΩ, 10 kΩ, 7.5 kΩ, 5 kΩ, 3.8 kΩ, 2.5 kΩ, 1.5 kΩ, 1.2 kΩ, 1 kΩ, 950 Ω, 900 Ω, 850 Ω, 800 Ω, 750 Ω, 700 Ω, 650 Ω, 600 Ω, 550 Ω, 500 Ω, 450 Ω, 400 Ω, 350 Ω, and 300 Ω. The same loads were also applied again after approximately 2 months of incubation at 4 °C to assess the stability of each electrode preparation scheme. Each anode was tested with the same BOD cathode to reduce variability.

**6.2.4 Instrumentation**

All electrodes were purchased from BASi. Electrochemical measurements were recorded using a Bio-Logic VMP3 and a computer interface running EC-Lab V10.34.
6.3 Results

Both glucose oxidase and fructose dehydrogenase anodes were prepared for long-term studies in a full fuel cell with a bilirubin oxidase sol-gel cathode as shown in Figure 6.1. A GOx electrode was first constructed that was highly conductive with direct electron transfer. The shaft of the phage, consisting of the pVIII protein, contains amine groups onto which carboxylic acid-functionalized gold is covalently attached through EDC-NHS chemistry, creating a gold nanomesh substrate. The FAD active site of glucose oxidase is then attached to the gold nanomesh, also using EDC-NHS chemistry. Finally, the apoGOx is introduced to reconstitute with the FAD, creating a complete glucose oxidase enzyme electrode that is attached to gold-
decorated pIII-modified M13 bacteriophage. The pIII protein of the bacteriophage was engineered so that the resulting phage/gold/recGOx complex can be anchored to a gold substrate by electrostatic binding.

A fructose dehydrogenase electrode was then assembled by encapsulating fructose in a CNT/PVDF mesh adsorbed onto a gold substrate. The nanotubes ensure a highly conductive surface with multiple contact points for the heme-CNT electron transfer. The PVDF mesh creates a network to aid in the encapsulation and immobilization of the enzyme to prevent denaturation. A BOD gel electrode was prepared that immobilizes the enzyme in a sol-gel made conductive with multi-walled carbon nanotubes. An acid-catalyzed TMOS sol was first prepared and was then combined with PEG 600 and multi-walled carbon nanotubes. BOD in PBS buffer was then added both to increase the pH and induce condensation and gelation, as well as to introduce and encapsulate the enzyme within the gel matrix.

Both the GOx and FDH anode electrodes were compared in a full cell electrochemical test versus the BOD cathode, where a load is applied and the resulting current and voltage are measured at each load. From these values, both current density and power density / specific power can be calculated using the combined geometric surface area of both electrodes. The specific power output is a common metric used for determining fuel cell performance (113). Figure 6.2 shows the voltage and power density obtained for each cell as a function of current density. These curves were obtained by step-increasing the resistance applied across the fuel cell between 500 kΩ to 300 Ω. The cell voltage gradually decreased as the applied load was increased. The highest points on the curves indicate the maximum power density of each fuel cell. For the FDH/BOD and GOx/BOD fuel cells, this amounts to approximately 200µW/cm² and 350µW/cm², respectively.
The highest recorded current density value corresponds to the maximum achievable current density with zero resistance. At low current densities, the cell is mainly dependent on overvoltages and electron transfer kinetics. As higher current densities are reached, the limiting factors become mass transport kinetics[114] While the GOx/BOD cell has a lower maximum
current density than the FDH/BOD cell, the specific power is much higher due to the higher voltage window of the FDH and BOD enzymes.

The electrochemical test for each cell was also performed again after two months of incubation at 4°C to evaluate the stabilization and immobilization ability of each enzyme/scheme. The results are shown by the polarization and power curves in Figure 6.3. For the FDH/BOD and GOx/BOD fuel cells, the initial maximum power density values were 200µW/cm² and 350µW/cm², respectively. The new values of each system after incubation are 169µW/cm² and 259µW/cm². This corresponds to an FDH cell stability of approximately 84% stability, and GOx cell stability of 74%. Since the same cathode was used for testing against the anodes, the FDH anode therefore appears to be more stable than the GOx anode, which is likely a result of the enhanced binding mechanism of the PVDF as compared to the phage/gold nanomesh, which likely does not form as tight of a pore bind.

**6.4 Conclusion**

In summary, described are two fuel designs consisting of either a GOx or FDH anode as described in previous chapters, and a bilirubin oxidase immobilized in a TMOS-based sol-gel matrix cathode, also previously described. In order to assess the stability of each cell, the tests were performed 24 hours after preparation and after 2 months of incubation at 4°C. While the GOx system achieves a higher peak power density (350µW/cm² as compared to 200µW/cm²), the FDH system is more stable, maintaining 84% of its initial maximum power density as opposed to 74% for GOx.
Figure 6.3  Polarization curves by fuel cell load of both FDH/BOD (above) and GOx/BOD (below) biofuel cells as recorded after 2 months of incubation at 4°C.
Chapter 7. Conclusions and Suggestions for Future Research

With the rise of the global average life expectancy and a quickly aging population\(^{(115)}\), implantable power devices are an emerging biomedical market. From sensors to pacemakers to defibrillators, long-term implants have the significant challenges of low energy consumption, stability, and longevity\(^{(116)}\). A key element of an implantable device is the power supply. While batteries are a well-worn, reasonably well-performing, and widely understood technology, biofuel cells represent an exciting opportunity for a power supply that is highly biocompatible, easily renewable, and theoretically ever-lasting. Unlike a battery, which must be either recharged or replaced, a fuel cell will continue to run as long as the catalysts remain active and the fuel supply remains constant. This presents a distinct advantage for aging or at-risk populations in particular, as the procedures to remove and replace the power supply for the devices are invasive and present a degree of risk\(^{(117)}\). Additionally, it can save patients the cost of the replacement procedure, which can be prohibitively expensive for those who remain uninsured or poorly covered.

An additional concern of note that biofuel cells address is alternative energy. Climate change has been at the forefront of political and social discussions, and the broad scientific consensus is that the change in atmospheric CO\(_2\) levels and global temperatures are man-made\(^{(118,119)}\). The impact of climate change is potentially catastrophic, with rising sea levels possibly displacing and driving into poverty millions of people\(^{(120)}\). While the need for clean alternative energy sources is clear, there is no single technology that is yet available that can entirely replace coal or oil. Even for nuclear energy, the alternative energy source capable of the highest energy generation, one plant would need to have been built every 1.6 days for the next 45
years since 2004 to meet the projected global energy demands(121). Therefore, the future of power will undoubtedly come from a variety of sources.

Although the application that is emphasized in this thesis is implantable power supplies, biofuel cells may be utilized in any number of portable and MEMS devices. Significant government and industry efforts have put towards biofuel research(122). Enzymatic biofuel cells have the potential to fill the specific niche of supplying power to implantable devices that access the body’s own renewable fuel source. As such, there are many considerations that must be taken in the design of these cells, such as miniaturization, fuel access and diffusion, and biocompatibility. Through the novel utilization of well-known biochemistry and nanotechnology methods, new and improved architectures for the immobilization and direct electron transfer of enzymatic biofuel cells have been created. These methods were evaluated with multiple characterization techniques and electrochemically confirmed to achieve current and power densities that perform competitively against other techniques. The simplified, biocompatible designs in these electrodes make them potentially suitable for implantation in vivo.

A number of issues still must be addressed and improvements may be made before these devices are suitable for animal trials. A significant question raised in the construction of the full cells presented in this work is the anode/cathode separation, particularly for the FDH/BOD cell. A schematic of a new possible one-compartment in vitro full cell is shown in Figure 7.1, where the electrode tips face each other in order to minimize the distance between them for efficient hydrogen ion transfer. The opening at the top of the cell is to allow oxygen to bubble in for the BOD reaction at the cathode.
For an *in vivo* cell, the anode and cathode connect directly into the fuel source, which in most cases would be a blood vessel. Some recent progress has been made in constructing implantable power supplies that have been tested in a number of different animal trials\(^{(123,124,125,126,127)}\). An example is shown in Figure 7.2 of a GOx/laccase fuel cell implanted in the abdomen of a rat with leads coming out of the skull for measurements by Zebda et al\(^{(128)}\). In their design, the electrodes are composed of a CNT/enzyme/carbon paste mixture and are wrapped in a dialysis membrane, placed in a perforated silicone tube with a protective silicon layer, and then packed in dialysis bag that is sutured inside a Dacron\(^{®}\) sleeve. This system showed stability and no signs of an immune response for over 100 days.

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**Figure 7.1.** Design of a one-compartment fuel cell for both GOx/BOD and FDH/BOD systems.
Figure 7.2. A glucose oxidase/laccase fuel cell design implanted in the abdominal cavity of a rat by Zebda et al(119).

The most observable implantable device application for enzymatic biofuel cells is in powering a cardiac pacemaker, which is powered by lithium batteries that achieve 1µW of power and have lifetimes of approximately 10 years. The initial power achieved by both full cells described in Chapter 6 of this work (350µW/cm² and 200µW/cm² for the FDH/BOD and GOx/BOD fuel cells, respectively) are certainly able to achieve this task, though whether the lifetimes of these cells can also meet or exceed 10 years at physiological conditions are unknown. The temperature and pH of the body are not particularly ideal for the enzymes used and although some measure of immobilization is achieved, the degree to which these architectures protect the enzymes from the temperature, pH, and immune response elements of
the body are unknown. Figure 7.3 shows the stability of the fructose dehydrogenase used in Chapters 4 and 6, which is the least stable of all the enzymes used in this work. While the thermal stability of FDH is reasonably within the range of human body temperatures, the pH stability is extremely poor for most parts of the body except for regions such as the intestines and upper stomach (129).

Figure 7.3. Stability of the fructose dehydrogenase enzyme from Gluconobacter sp. Image courtesy of TOYOBO U.S.A., INC.

An ideal cell layout would likely consist of a structure akin to an interdigitated electrode as shown in Figure 7.4. This structure can be expanded into a three-dimensional layout to maximize the surface area to volume ratio. The glucose oxidase electrode can be made using the same method described in Chapter 3, with the gold foil surface being cut into the proper shape. Additionally, the M13 bacteriophage may be engineered differently, such that the pIII protein binds to a more cost-effective metal such as copper. If a fructose dehydrogenase anode is
desired, a drop-casting method may be used to attach the CNT/PVDF substrate, where a thin layer may be allowed to sit and dry before being dipped in an FDH solution for enzyme attachment. The BOD cathode will be more difficult to construct due to the difficulty in attaching a thin and defined layer of gel on the electrode. For this, an extrusion method may be devised in order to neatly pipe out the “paste” of the BOD/gel mixture before gelation occurs. Due to the relatively small voltages that biofuel cells produce, cells are usually placed together in series. However, as the amount of current in the cell will likely decrease over time as the enzymes degrade, placing cells in parallel or in a series/parallel combination may serve to preserve the current needed while achieving the desired voltages.

Figure 7.4. A schematic of an interdigitated electrode biofuel cell design.
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