Pittsburgh Tissue Engineering Initiative

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2009 – December 31, 2009

Formula Grant Overview

The Pittsburgh Tissue Engineering Initiative received $15,697 in formula funds for the grant award period January 1, 2009 through December 31, 2009. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Novel Biofuel Cell Based on High Surface Area Enzymatic Microelectrodes - The purpose of this project is to develop a miniaturized biofuel cell, a device that can turn available substances like glucose to energy, by using an assembly of biocatalysts and a special high surface area electrode. The device will be designed to utilize glucose as the only fuel, and turn this into energy by a series of enzymatic reactions, using commercial enzymes as the combustion elements. The process will be carried out on the surface of a new type of electrodes, comprised of extremely high surface area gold microfibers, which will be developed. The high surface area will enable immobilization of a high concentration of enzymes and better diffusion of glucose and oxygen to and from the electrode. The final size of the device is expected to be small, i.e. less than 0.1 cm^3, and could be implanted in the body to power devices like insulin pumps or pace makers.

Duration of Project

1/1/2009 – 12/31/2009

Project Overview

The research objectives are to develop a mediator-less biofuel cell, that has a high current density, and uses glucose as the only source of fuel. In addition, the anode and the cathode of the cell will not be regular bulk electrodes which limit the amount of immobilized enzyme, but new, extremely high surface gold electrodes which will be fabricated using an electrospun gold-polymer scaffold.

The research will be divided into 3 parts: (1) fabrication of new gold electrodes; (2) immobilization of enzymes on electrodes; (3) optimization of experimental conditions for maximal current density.

The high surface area gold electrodes will be prepared by a multi stage process using a composite gold -polymer microfibers scaffold to construct the gold microelectrodes. The first

stage will be the fabrication of electrospun gold-polymer fibers. In the process, microfibers of polymer are produced by using high voltage to stretch a drop of a viscous polymer solution toward a grounded collecting plate. The polymer solution will contain a high percentage of gold salt precursors that will be distributed in the resulting fiber. Subsequent gold electroless deposition will form a gold coating on the fiber. The product characterization will be by scanning electron microscope (SEM), X-ray diffraction (XRD), and cyclic voltammetry (CV).

The enzymes used in this study will be catalase and glucose oxidase at the anode, and laccase at the cathode. Glucose will be oxidized by glucose oxidase to hydrogen peroxide and gluconolactone. Hydrogen peroxide will be oxidized by catalase to oxygen. The oxidizing process will have two outcomes: two electrons will be transferred to the anode and oxygen will be produced. This oxygen, in addition to the dissolved oxygen, will fuel the cathodic reaction, where it will be reduced by laccase to water, using two electrons. The enzymes were chosen after carefully examining their redox potentials to have the highest difference between the oxidizing and reducing enzyme in order to achieve the highest current density attainable.

Two important features of the design are illustrated: (A) the use of direct electrical communication between the enzymes and the electrode, without the use of highly toxic electron mediators, (B) the use of glucose as the only fuel source. These processes will be probed using CV as well as the standard biochemical enzymatic assays to evaluate enzyme loading. Enzymes will be immobilized on the gold electrode using conventional cross linking techniques (thiol self assembled monolayer with subsequent covalent cross linking).

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

The outcome of this research will be a miniaturized biofuel cell, a device that will essentially act as a power source made from a composite of biomaterials and metal. This power source will use available glucose as the single source of energy, and will transform the glucose to energy by using enzymes - nature's catalysts. The size of the device will be small enough to be implanted in the body and able to power a small device like an insulin pump. It is highly desired to fabricate such devices. Contrary to commonly used batteries, these implantable power sources are non-toxic; they do not contain any foreign materials that the body does not recognize and can, in principle, operate for extended periods of time (weeks up to several months). Preliminary studies by other groups have shown the feasibility of such devices to be able to generate power

that can operate a small electrical device such as a Sony Walkman. We believe that our design is superior, since it does not contain any toxic material, while most of the other designs rely on toxic electron mediators or toxic polymeric gels. In addition, we believe that our design will enable the production of a smaller device with higher power output, due to the novel high-surface area electrodes that we plan to use.

Summary of Research Completed

Novel Biofuel Cell Based on High Surface Area Enzymatic Microelectrodes

The goal of this project was to fabricate a biofuel cell that will be mediator-less and will have high power output (i.e., more than 100 $\mu W/cm^2$).
We proposed to reach this goal by combining two approaches:

1) Use Catalase as the working oxidizing enzyme – increase theoretical power density and increase oxygen concentration in the cell
2) Increase the enzyme concentration on both anode & cathode using high surface area micro-gold electrodes.

The use of Glucose Oxidase (Gox) together with catalase (Cat) will enable the biofuel cell to operate on glucose as the sole fuel source. The direct immobilization of catalase and laccase directly on micro wires will eliminate the use of electron mediators in their various forms and will render the system more simple and applicable for implantable applications.
The project was divided into four distinct segments:

1. Establishment of a reproducible process of fabrication of gold microfiber electrodes, and their characterization by electron microscopy.
2. Immobilization of redox enzyme on the electrode in a constant and reproducible fashion
3. Electrochemical characterization of anode and cathode half cells
4. Construction of a biofuel cell.

The progress made in the project will be reported using the above segments as guidance.

1. <u>Fabrication and characterization of gold microfibers</u>

In this project, we have succeeded to fabricate gold fiber by a three step process that included the electrospinning of a poly(acrylonitrile) polymer blended with gold chloride salt, then a step of reducing gold chloride to metallic gold by a mild reducing agent ($NaBH_4$) and them deposition of gold nanocrystals by electroless deposition. The resulting material was characterized using electron microscopy using scanning and tunneling electron microscopes, and also by X-ray diffraction and nitrogen adsorption.
Figure 1 shows the scanning electron microscope (SEM) image of the fibers after the electrospinning and after the electroless deposition process. The as-spun fibers contained defined domains of $HAuCl_4$ (Figure 1A). These domains were created during the evaporation of the solvent which resulted in partial crystallization of the gold chloride salt and phase separation. The gold chloride was reduced initially by $NaBH_4$, a process leaving small metallic gold regions on the fibers. These small metallic clusters served as the nucleation seeds for the electroless gold deposition process that followed. During the electroless deposition process, Au^{+3} was reduced by the mild reducer hydroxylamine to form metallic gold. In the presence of a nucleation seed (the gold particle in the fiber) the gold was deposited on the seed and growth of the gold microcrystal was observed (figure 1B, C). From the SEM images we observed that the gold microcrystal were

ordered, and formed linear crystalline arrays. The proximity of the gold microcrystals indicated that the material is metallic. The process of fabrication and characterization was repeated for several times to ensure that the resulting fibers are reproducible and exhibit the same properties for each batch.

Transmission electron microscopy (TEM) reveals more information on the microcrystalline nature of the gold crystals and their continuity. For TEM imaging the fibers were imbedded in epoxy resin and section to micro meter thin slices. Since the fibers have no particular order, we were able to image both vertical and horizontal sections of the fibers, figure 2. These images reveal that the gold crystals are deposited on the surface of the polymer core in a densely packed fashion and that the nanoparticles of the gold are crystalline and were deposited with no preference in orientation. This was expected since the fiber core was an amorphous polymer. The average size of the gold particles encrusting the polymer fiber was 93.1 ±27.7 nm. The remaining gold particles in the core of the fiber that were not exposed to the electroless deposition process remained small and had an average size of 22.3 ±6.6 nm. The d spacing that was measured for the gold crystals was d = 2.38 Å, and the lattice constant from the {111} planes was 4.12 Å, in good agreement with the parameters measured for gold crystals in the literature.
In order to assess the suitability of the fiber electrode as a platform for an electrochemical biosensor, we determined the electrochemical behavior of the gold microfiber. The reversible electron transfer of ferrocene methanol, a common reversible redox probe, was investigated on the gold microfiber. The data conformed to the Randles-Sevcik equation.
<u>In summary</u>: this milestone was completed successfully. The process was optimized to produce reproducible micron-sized fibers, with metallic and electrochemical characteristics of bulk gold.

2. <u>Immobilization of redox enzymes on the electrode</u>

Several redox enzymes were immobilized on the gold fibers using conventional crosslinking chemistry using a self-assembled monolayer of cystamine as the chemical anchor to gold. Redox enzymes like Glucose oxidase, fructose dehydrogenase, laccase and bilirubin oxidase were immobilized to the surface of the gold fiber. The activity of each enzyme was assayed after the immobilization to ascertain that the immobilization did not impair the biochemical activity of the enzyme.
<u>In summary</u>: this milestone was accomplished successfully. We have established an immobilization scheme that yields a stable, reproducible and active monolayer of enzyme on the gold electrode surface. It is, however, desired to increase the enzyme concentration, and this will be accomplished in the future by either using multi layers of enzymes or by further increasing the surface area of the gold fibers.

3. <u>Electrochemical characterization of anode and cathode half cells</u>

Each half cell that constructs the biofuel cells is essential an amperometric biosensor. For example, Gox immobilized on the gold fiber surface can function as a glucose sensor. As such, the response of the immobilized enzyme to glucose should be measured by recording the catalytic current resulting from glucose oxidation. An electron transfer mediator was needed to assist in shuttling the electrons between the active site and the gold fiber. When a Gox-immobilized electrode was challenged with increasing glucose concentrations, the resulting cathodic peaks were recorded, figure 3. The response of the enzyme electrode to glucose concentration was linear in the range of 0.2-30 mM glucose (figure 3, inset), which is also the

clinically relevant glucose concentration range. The current- glucose relationship was used to calculate the Michaelis constant of the immobilized enzyme. We found that the K_M = 10 mM. The limit of detection of glucose using this sensor was 0.1 mM. Similar experiments were performed on the other redox enzymes that were used in the study- fructose dehydrogenase, laccase and bilirubin oxidase. Catalase immobilization was different. We found that catalase adsorbs strongly to the gold fiber surface and no chemical immobilization is needed. When catalase is adsorbed on the gold fiber electrode and H_2O_2 is added to the buffer- it is possible to immediately detect O_2 bubbles formed on the surface. The anode design that is unique in this project is a combination of catalase- Gox immobilized on the surface of the gold fiber, where Gox will oxidize glucose to gluconic acid subsequently followed by the regeneration of the FAD active site by the reduction of O_2 to H_2O_2. H_2O_2 will then be consumed by catalase and transformed into O_2 and H_2O plus two electrons that will be transferred to the electrode. Figure 4 shows the amperometric response of catalase adsorbed on the gold fiber electrode. When only catalase is present, no special peaks are observed. Addition of Gox and glucose does not change the observed cyclic voltammogram. Only the saturation of the solution with oxygen, the co-substrate of Gox, starts the cascade of enzymatic reactions and leads to the appearance of the desired catalytic current with a peak at -0.4 V (figure 4, purple trace).
In summary: this milestone was accomplished successfully. All redox enzymes that were of interest to the study were characterized electrochemically using a 3-electrode half cell electrochemical set up. The response of each enzyme to its substrate was measured and analyzed in terms of limit of detection, linear response range, K_M, and ultimate buffer and mediator composition. Catalase was adsorbed on the surface of the electrode successfully and the direct electron transfer to the gold electrode was observed when Gox, glucose and O_2 were added to the enzyme.

4. Construction of a biofuel cell

The successful immobilization and electrochemical characterization of both oxidizing and reducing enzymes on the fold microfiber electrode led to the next step, namely to combine two half cells into a biofuel cell. The potential that is produced in the cell is measured in several external resistances loadings and the resulting current is calculated using Ohm's law. The power output is calculated from the potential and current at each resistance. The peak power is the maximal power density of the specific cell. In cases where a mediator is involved, a physical separation between the anode and the cathode is needed to avoid the poisoning of the enzymes by the other enzyme's mediator. The response curve of the biofuel cell is depicted in figure 5. The peak power output of this cell was a little over 6 μW, and when taking into account the surface area of the working electrodes, the power density was 20 $\mu W/cm^2$. This peak power output was measured at 0.5 V; the maximum current output was 13 μA. The power was constant for over 2 hours, and after 24 hours, only decreased by 20% in the power observed. Similar results are observed using laccase on the anode or Gox on the cathode.
In summary: preliminary results showing a biofuel cell from the enzyme electrodes that were developed is shown, the cell produced power for a prolonged time without the regeneration of the substrate and without a significant loss of activity. The power generation is lower than the desired goal ($100 \mu W/cm^2$).

During this project, we have succeeded to fulfill almost all the project goals as specified in the research plan. A platform for the immobilization of enzymes was developed in the form of

electrospun fibers that were transformed into gold microfibers. We have established the immobilization technique for all redox enzymes of interest in this study and have shown the ability to assay the enzymes both biochemically and electrochemically. The enzyme electrodes were analyzed as independent biosensors. A biofuel cell prototype was built and tested using pairs of anode and cathode enzyme electrodes that were developed. Each pair was characterized by it maximum power output and maximal voltage output. We have succeeded to achieve a maximum power output of 20 $\mu W/cm^2$, which is lower than what we anticipated. We plan to reach and even exceed our initial power output goal by increasing the amount of enzyme on the electrode, using a gold-carbon composite material, and increasing the available surface area of the fibers by using hollow fibers.

Figures

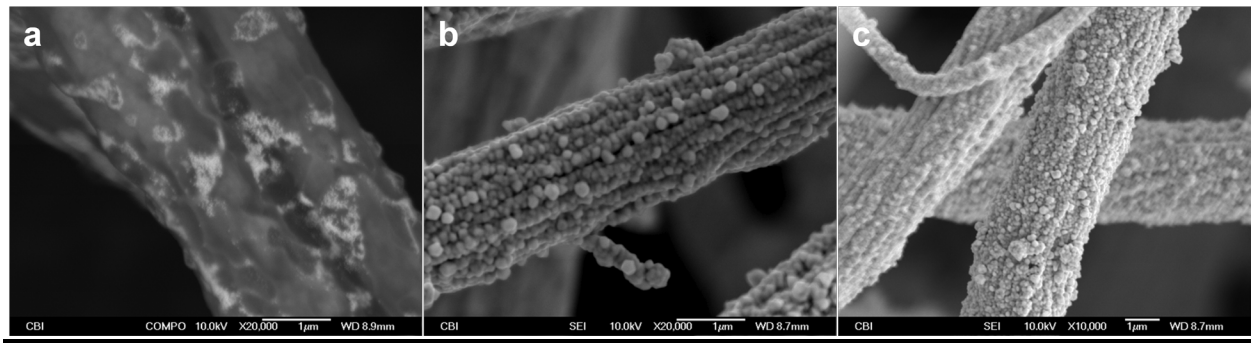

Figure 1: a- SEM of as-spun PAN-$HAuCl_4$ polymer; B- SEM of the same polymer after electroless deposition of gold using secondary emission detector; C: SEM of fiber after electroless deposition of gold using backscatter detector.

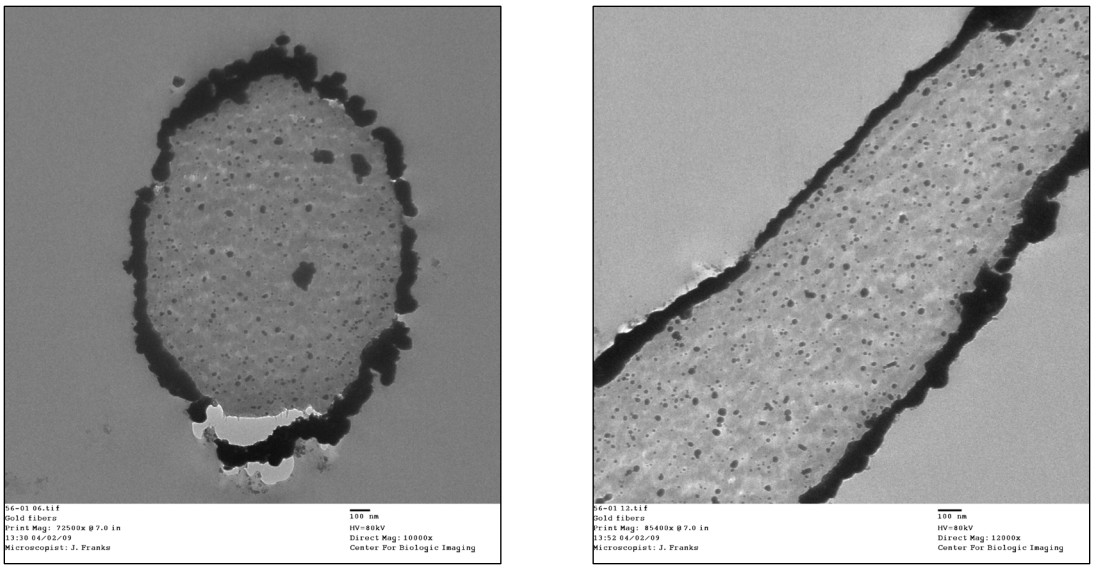

Figure 2: TEM images of horizontal (left panel) and vertical (right panel) cross section of a gold fiber.

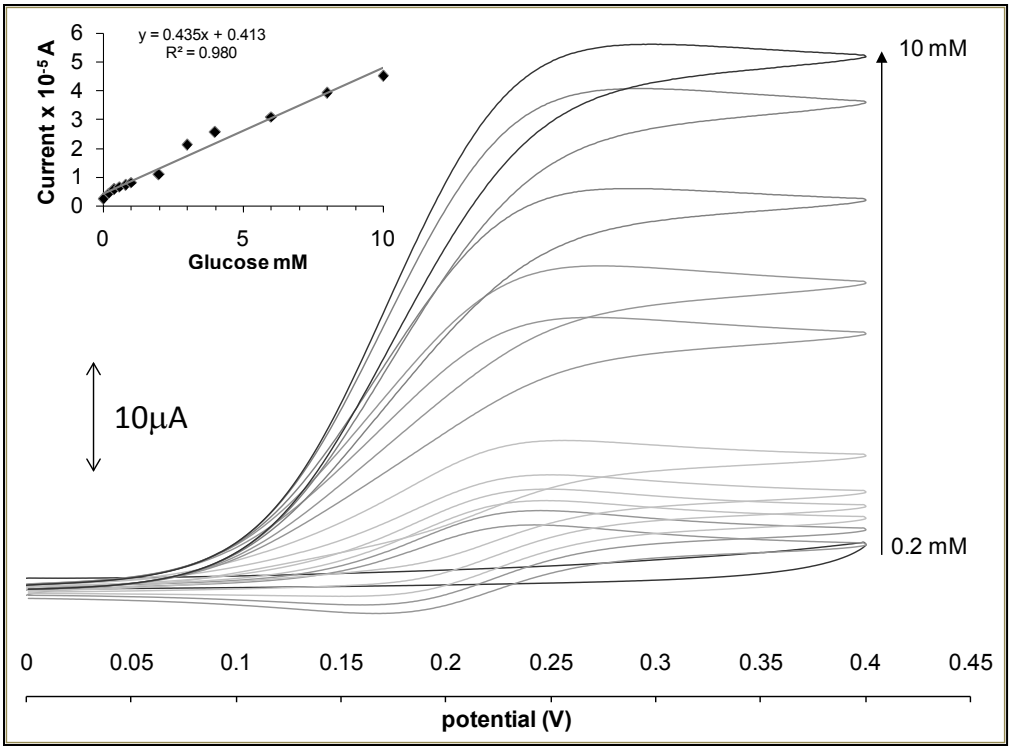

Figure 3: cyclic voltammetry of Gox immobilized on gold fiber electrode, in 0.05M phosphate buffer pH=7.0, containing $2.5x10^{-4}$ M ferrocene methanol. Scan rate = 5 mV/sec. Inset: the calibration graph of the response of the electrode to increasing glucose concentrations, measured for the peak of the cathodic peak.

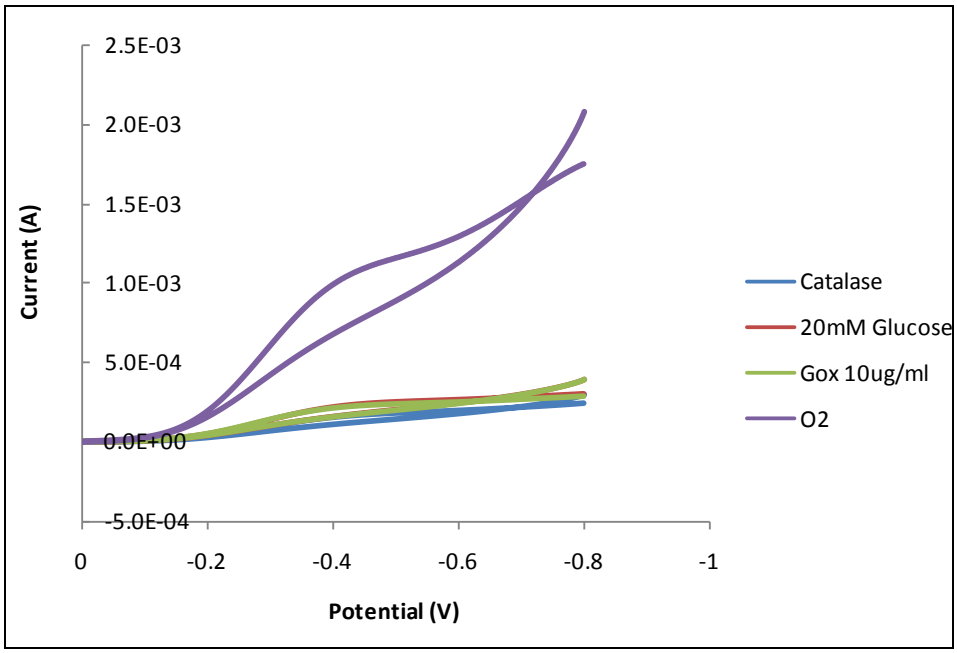

Figure 4: Cyclic voltammogram of catalase adsorbed on gold fiber. Blue trace- catalase in phosphate buffer 0.1 M pH = 7.0; green- addition of 10 mg/ml Gox; red: addition of 20 mM glucose; purple- same solution, after saturation with O_2. Scan rate = 100 mV/sec.

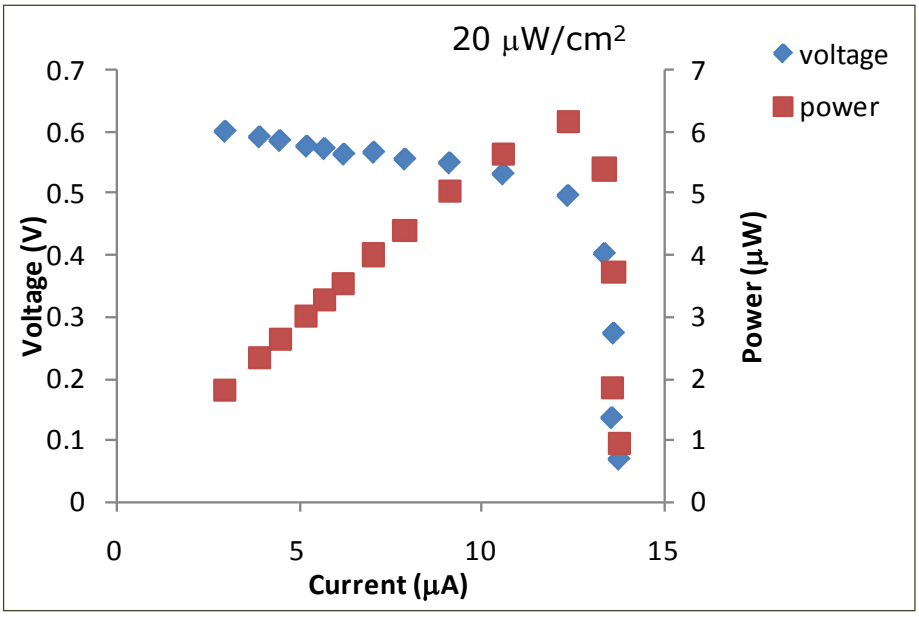

Figure 5: biofuel cell power output measurement. Anode: Fructose dehydrogenase on gold fibers, in McIlvain buffer pH = 4.5, with methylene blue 0.3 mM and fructose 0.2 M. cathode: bilirubin oxidase immobilized on gold fiber electrode, in phosphate buffer 0.1 M pH = 7.0, saturated with O_2, and 0.3 mM ABTS. ♦- voltage; ■- power.