Pittsburgh Tissue Engineering Initiative

Annual Progress Report: 2009 Formula Grant

Reporting Period

January 1, 2010 – June 30, 2010

Formula Grant Overview

The Pittsburgh Tissue Engineering Initiative received $12,224 in formula funds for the grant award period January 1, 2010 through December 31, 2010. 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 continue our development of a miniaturized biofuel cell, a device that can turn available substances like sugars and oxygen to energy, by using an assembly of biocatalysts and a special gold fiber electrode. The device will be designed to utilize fructose as the fuel, and turn the sugar into energy by a series of enzymatic reactions, using commercial enzymes as the catalytic elements. The process will be carried out on the surface of a new type of gold microfiber electrode, which will be developed. In this project continuation we would like to expand the fuel sources and study a biofuel cell based on fructose conversion into electrical energy by fructose dehydrogenase.

Anticipated Duration of Project

1/1/2010 - 12/31/2010

Project Overview

The research objectives are to develop a biofuel cell, with high current density, using fructose as the source of fuel. In addition, the anode and the cathode of the cell will be new high surface area gold fiber electrodes which will be fabricated using an electrospun gold-polymer scaffold.

This research program began in the previous grant, and this project is a continuance. The research will be divided into 3 parts: (1) fabrication of gold electrodes; (2) immobilization of the enzymes on the electrodes; and (3) optimization of experimental conditions for maximal current density. Gold electrodes will be prepared by a multi step process using a composite gold - polymer microfiber scaffold to construct the gold microelectrodes. First, electrospun gold salt-polymer fibers will be fabricated. Gold electroless deposition or direct electrochemical reduction will form a gold coating on the fiber. Increased surface area will also be attempted by dissolving the polymer core and creating gold hollow fibers. Characterization will be by scanning electron

microscopy (SEM) and transmission electron microscopy (TEM), X-ray diffraction (XRD), BET surface area and cyclic voltammetry (CV).

The enzymes to be used will be fructose dehydrogenase at the anode, and bilirubin oxidase or laccase at the cathode. The enzymes were chosen after examining their redox potentials to have the highest difference between the oxidizing and reducing enzyme in order to achieve the highest open circuit potential, and perform at acidic pH. There are two important features in the planned design: direct electrical communication of the enzymes with the electrode, without highly toxic electron mediators, and glucose as the only fuel source. Fructose will be oxidized by the PQQ cofactor in the enzyme, resulting in two electrons that will be transferred to the electrode. Fructose dehydrogenase, in contrast to the more popular glucose oxidase, does not use oxygen in the oxidation process, thus not depleting the oxygen from the solution; on the cathode, oxygen will be reduced by bilirubin oxidase or laccase to water, using two electrons. These processes will be probed using CV and by standard biochemical enzymatic assays to evaluate enzyme loading. Enzymes will be immobilized on the gold electrode using conventional cross linking techniques (thiol SAM with subsequent covalent cross linking, or direct physical adsorption).

The biofuel cell assembly will be constructed of a two- electrode cell connected through an external resistor load and a multimeter. The potential that will be developed between the anode and cathode will be measured.

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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 fructose as the single source of energy, and will transform the fructose to energy by using enzymes- nature's catalysts. The end product will be able to generate energy from fructose containing substances - such as fruit juices and high fructose corn syrup - a cheap commercial fructose source. It is highly desired to fabricate such devices that utilize commonly available fuel sources to produce energy. It is also possible to add another enzyme, invertase, to the anode and then use this anode to convert the endogenous glucose to fructose, thus applying the fuel cell in an implanted fashion. These power sources are not toxic like those commonly used in batteries, 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). The design proposed is better, 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, the proposed design will enable the production of a smaller device with higher power output, due to the novel high-surface area electrodes that we will use. Preliminary results show that the gold microfiber electrodes indeed have a high surface area that is available for enzyme immobilization. In addition, we have shown that redox enzymes can be immobilized on the surface and portray electron transfer capabilities with the gold fiber electrode.

Summary of Research Completed

A new form of high surface area bioelectrode, based on nanofibers of electrospun gold with immobilized fructose dehydrogenase was developed. The gold fibers were prepared by electroless deposition of gold nanoparticles on an electrospun poly(acrylonitrile)-$HAuCl_4$ fiber. The material was characterized using electron microscopy, XRD and BET, as well as cyclic voltammetry and biochemical assay of the immobilized enzyme. The surface area of the gold microfibers was 0.32±0.04 m^2/g. Fructose dehydrogenase was covalently coupled to the gold surface through glutaradehyde cross links to a cystamine monolayer. The enzyme exhibited mediated electron transfer directly to the gold electrode and catalytic currents characteristic of fructose oxidation in the presence of a ferrocene methanol mediator were observed. The limit of detection of fructose was 11.7 μM and the K_m of the immobilized enzyme was 5 mM. The microfiber electrode was stable over 20 cycles with a 3.05% standard deviation. The response time of the sensor was less than 2.2 seconds and reached half maximum value within 3.6 seconds. The sensor was proven to be accurate and precise in both serum and popular beverages containing high fructose corn syrup.

Results

Material characterization.
Electrospun fibers were imaged using SEM with both secondary emission and backscatter detectors to image both the topography and the difference in material density of the fiber. 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 subsequent phase separation. The gold chloride was reduced by $NaBH_4$, to deposit small metallic gold regions on the fibers. These metallic clusters served as nucleation seeds for the electroless gold deposition process that followed. Au^{+3} was reduced by hydroxylamine to form metallic gold. The deposited gold and growth of the micro crystals was observed microscopically (Figure 1B). From the SEM images we observed that the gold microcrystals were ordered and formed linear crystalline arrays. The proximity of the gold microcrystals indicated that the material was metallic. The XRD analysis (Figure 1C) showed four characteristic diffraction peaks corresponding to the (111), (200), (220), (311) and (222) planes of a face-centered cubic gold crystal. After the electroless deposition procedure, the mean diameter of the fibers in figure 1 was 990±490 nm. Fiber size was dependant of flow rate, voltage and solvent. Cross sectioned fiber TEM showed that the particles formed a crust around the polymer core, and were adherent

to one another, imply that the metallic surface would be conductive (Figure 1D). HR-TEM confirmed that the nanoparticles of the gold were 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 2.38 Å, and the lattice constant from the (111) planes was 4.12 Å, in good agreement with known dimensions.

A nitrogen adsorption isotherm was used to determine the bulk surface area of the fibers and was found to be 3.15 m^2/g. In comparison, the BET-measured surface area of the PAN fibers (8% w/v without gold chloride) was found to be 12.3 m^2/g, and the surface area of the as-spun material was found to be 0.7 m^2/g. The electrochemical surface area, which can be distinct from the physical surface area, measured by chronocoulometry using potassium ferricyanide as the probe was 0.32±0.04 m^2/g. A possible explanation for the difference between the BET and electrochemically measured surface areas could be linked to the difference in the medium that the measurement was performed in. BET was performed in the gas phase, while choroncoulometry was performed in solution. Both methods relied on the adsorption of a molecular probe to the surface (N_2 or ferricyanide). In solution, ferricyanide was solvated, and the molecular footprint of the probe on the gold surface was larger than that of the nitrogen, so fewer molecules were actually used to cover the surface than when in the gas phase. The surface area calculation by chronocoulometry takes into account only the diffusion coefficient of the probe and not the size of the probe, and hence the difference in the observed values.

Electrochemical characterization of the gold microfiber.
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:

$$I = kn^{\frac{3}{2}} FAD^{\frac{1}{2}} v^{\frac{1}{2}}$$

where k=2.6710^5 (C/mol $v^{-1/2}$) , n is number of transferred electrons, F = 96485 C/mole, A is the electrode surface area, cm^2, D= $6.7x10^{-7}$ cm^2/sec and v = scan rate.

As expected, the peak current increased with scan rate (Figure 2). The linearity of both anodic and cathodic peak current with the square root of the scan rate indicated that the redox process was governed by diffusion. The data also showed that the probes behaved in a similar manner with bulk gold electrodes and the nanofibers.

Enzyme immobilization on the fiber.
Fructose dehydrogenase (FDH) was immobilized on the gold fibers via glutaraldehyde crosslinking to a self-assembled monolayer of cystamine which served as the chemical anchor to the gold. The enzymatic activity of the immobilized FDH was assayed using a Prussian Blue assay directly on the enzyme-fiber assembly. The coverage of the enzyme electrode was found to be 0.36± 0.03 enzyme molecules/nm^2 averaged on three electrodes. In comparison, when the

same immobilization procedure was performed on a polished gold disc electrode of 2 mm diameter, 1.8±0.14 molecules of enzymes/nm^2 were detected (n=3). The density of the enzyme on the fiber electrode is likely reduced because only the outer layers of the fibers were immobilized. The total protein content of the electrode was assayed using a highly sensitive fluorescent assay. The gold fiber electrode contained on average 0.50±0.02 molecules of protein/nm^2, when the gold disc electrode contained 9.46±0.43 molecules of protein/nm^2**.** Compared to other published results, immobilization of FDH on the gold nanofibers resulted in doubling the amount of immobilized protein, and quadruples the amount of enzyme units immobilized, per cm^2. Using the fluorescent assay, we can estimate the amount of active enzyme that was retained on the surface and the ability of the gold fibers to stabilize the immobilized enzyme. When taking into account the specific activity of the soluble enzyme (426 u/mg), it is possible to estimate the level of expected activity based on the protein content. We calculated that the protein weight that was found for the gold microfiber electrode should produce approximately 2.5 molecules of FDH per nm^2 of electrode. In comparison, the protein weight found for a gold disk electrode should translate to 46.8 molecules of FDH per nm^2. This means that after immobilization of FDH on gold microfibers, 14.4% of the enzyme remains active, while immobilization on the flat gold disc surface resulted in less than 4% activity of the immobilized enzyme. A possible explanation of this improved activity retention may lie in the micro-structure of the gold fibers.

Electrochemical evaluation of FDH on gold fibers.
The electrochemical activity of FDH was measured using the catalytic cathodic current observed when the electron mediator was electrochemically regenerated by the PQQ active site after fructose oxidation. At high mediator concentrations the heme *c* prosthetic group of the enzyme is reoxidized in an internal redox process. The electron mediator used in our study was ferrocene methanol, a commonly used mediator for redox enzymes. At a ferrocene methanol concentration study $5x10^{-4}$ M the anodic current measured was found to be optimal. The FDH-gold fiber electrode exhibited a proportional response to fructose concentrations in the range of 0.1 mM - 3 mM (Figure 3). The calculated limit of detection was 11.7 μM, which was in the same range of reported values of ferrocene mediated fructose sensors (7 mM). The Michaelis constant (K_M) of the immobilized FDH on the fiber electrode was calculated from the electrode responses (figure 3, inset), and was found to be 5 mM, with I_{max} = 142 μA. This value was in good agreement with K_M for immobilized FDH in similar systems and was 50% less than the K_m of FDH measured in solution (typically, 10 mM).

The sensor response to fructose was also measured by the oxidative current resulting from the injection of 20 mM of fructose to a solution of buffer containing ferrocene methanol, at a constant potential of 0.2 V (vs. Ag/AgCl). The detection of fructose was observed within 2.2 sec of injection and reached its half maximal value after 3.6 seconds, and steady state plateau within 20 sec. (Figure 4). The response time observed was at least 2-4 times shorter than other reported response times.

The stability of the enzyme electrode was measured by repeatedly determining the current at 20 mM fructose concentration. The relative standard deviation for 20 successive measurements of 20 mM fructose was 3.05 %, a significant improvement on previously described FDH immobilized on gold electrodes, which were reported to reduce response after as much as a

single measurement cycle. The nanofibrous fructose sensor was tested in bovine calf serum and popular beverages. The test of the sensor in full serum was intended to show the application of the sensor in complex environments containing interferents like ascorbate and high concentration of proteins. The measurement in beverages was to show the practical application of the sensor in fructose containing media. The FDH sensor electrode was tested in undiluted bovine calf serum that was spiked with 20 mM fructose. The measured current decreased by 21% by changing the medium from MIB to the serum. The decrease in the current can be attributed both to the different pH (the serum is more basic than the MIB used, thus the sensor is performing far from its optimal pH) and to the presence of interfering materials.

Cyclic voltammograms of the FDH electrode challenged with several popular beverages containing high-fructose corn syrup were collected. The concentration of fructose was measured using our sensor and with a commercial fructose kit (F-20, sigma) (Table 1). The correlation between the fructose sensor and the commercial fructose kit demonstrated the precision and accuracy of the sensor.

Methods

Electrospinning of PAN/$HAuCl_4$ composite: a solution containing PAN 8% (w/w) and $HAuCl_4$ 10% w/w was prepared by dissolving the solids in DMF and heating at 80 ^oC until the solution was clear. Electrospinning was performed using a power source (Gamma High voltage, Fl), and syringe pump (World Precision Instruments, Fl). The electrospinning voltage was 20 KV, the polymer flow rate was 0.5 ml/hr, and the tip-to collector distance was 8 cm. The fibers were collected on aluminum foil (for material characterization) or on a gold rod (1 mm diameter, for the sensor preparation) rotated by a motor at 3000 rpm.

Fabrication of gold microfibers: The resulting fibers were first reduced by 1 mM $NaBH_4$ solution until no H_2 evolution was apparent, and then washed with dilute HCl and then H_2O. Next, gold was electrolessly deposited on the fibers by dipping the fibers in an aqueous solution containing 0.02 mg/ml hydroxylamine hydrochloride and 0.1 mg/ml $HAuCl_4$. The fibers were incubated in the solution for 10-30 minutes and then moved to a fresh solution. This was repeated 10 times. The resulting electrodes were electropolished by cycling 0-1.5 V (vs. Ag/AgCl) in 0.1 M H_2SO_4 until a constant cyclic voltammogram (CV) was observed, were rinsed thoroughly in water and stored dry until used.

Enzyme modification: Fibers were incubated in 0.1 M cystamine for 3 hr, washed with H_2O and then incubated for 1 hr in 6.25 % glutaraldehyde. The fibers were rinsed with water and then incubated in FDH, (5 mg/ml in phosphate buffer 0.1 M pH 7.0) overnight at 4^oC. The electrodes were rinsed thoroughly with phosphate buffer until no FDH activity could be assayed in the washing buffer (typically, 5 washes). FDH activity on the fibers was measured using a Prussian Blue assay. Protein assays were performed using the fluorescent measurement of the adduct of o-phthaldialdehyde to amino acids, using BSA for calibration.

Electrochemical characterization: electrochemical experiments were run on a CHI 660 electrochemical station, equipped with a standard 3-electrode cell. The auxiliary electrode was a Pt wire, and an Ag/AgCl electrode was used as a reference. Cyclic voltammetry to measure the

response to fructose was performed by scanning between 0-0.6 V versus Ag/AgCl at a scan rate of 20 mV/sec, after adding $5x10^{-4}$ M ferrocene methanol as a diffusional electron mediator. The electrochemical surface area of the gold microfibers was determined by chronocoulometry using 1 mM ferricyanide in KCl.

Physical characterization of the fibers: Scanning Electron Microscopy (SEM) was performed on carbon coated fibers, at 10 KV acceleration voltage using a JEOL 6330F SEM. TEM images were obtained on epoxy embedded samples that were sectioned, and imaged on JEOL JEM1210. A Micromeritics ASAP 2010 instrument, N_2 adsorption device was used to determine surface area. Fiber diameter and gold particle size distribution were measured using ImageJ software from SEM/TEM images, using at least 50 objects for reliable statistics.

Table 1: Fructose measurement using fructose electrode and commercial kit

	fructose sensor fructose mg/ml	F-20 kit fructose mg/ml
Coca - cola	22.7±	23. ±
Pepsi	21.8±	24.4±

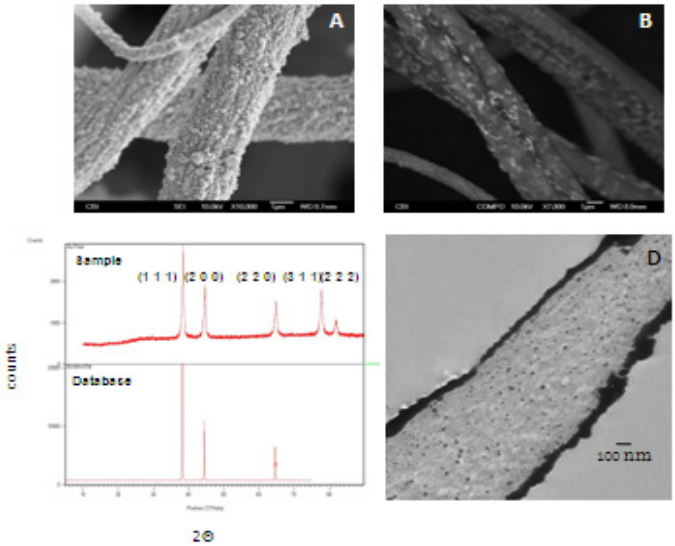

Figure 1. (A) SEM of as-spun PAN-$HAuCl_4$ polymer; (B) SEM of the same polymer after electroless deposition of gold; (C) XRD of the fiber after electroless deposition; (D) TEM of a cross section of the fiber through its length.

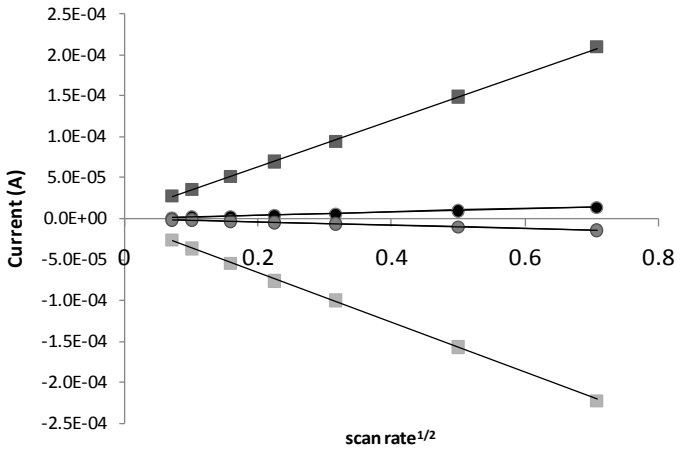

Figure 2. Anodic and cathodic currents as a function of the scan rate, measured on a gold fiber electrode (a) and on polished gold disc electrode (b) in 1 mM ferricyanide solution in 0.1 M KCl.

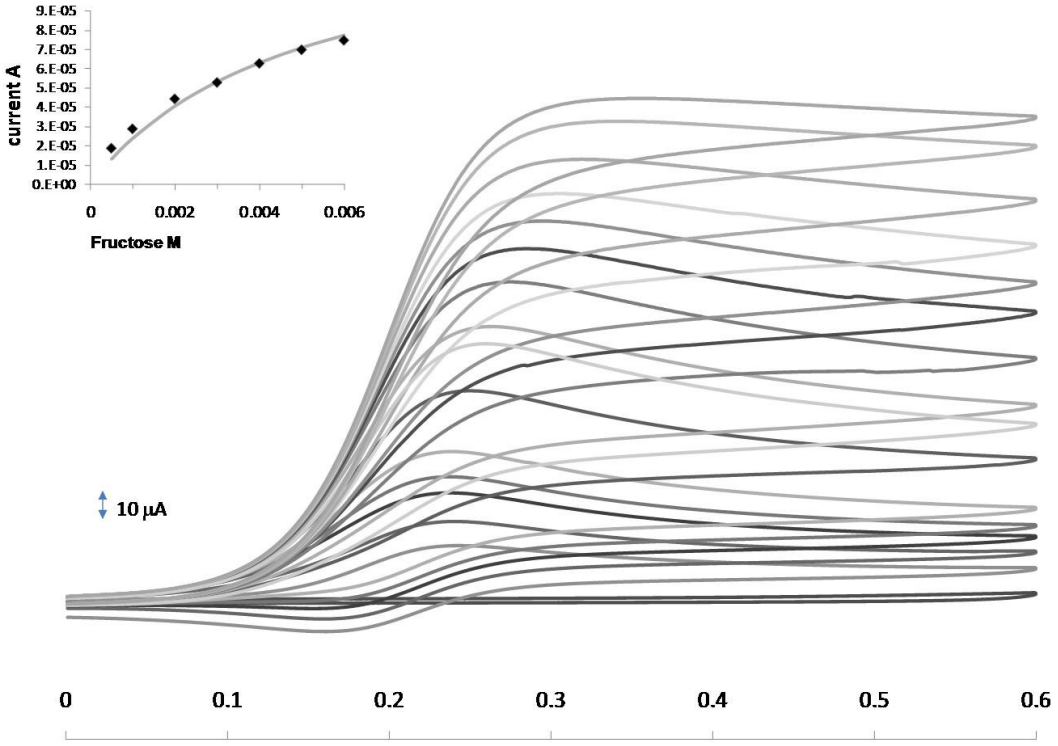

Figure 3. Cyclic voltammetry of FDH immobilized on gold fiber electrode, in McIlvaine buffer pH = 4.5, containing $5x10^{-4}$ M ferrocene methanol. Scan rate = 20 mV/sec. Concentrations are from 0.1 mM to 1.5 mM. Inset: Michalies-Menten kinetics of electrochemical activity of FDH as a function of fructose concentration.

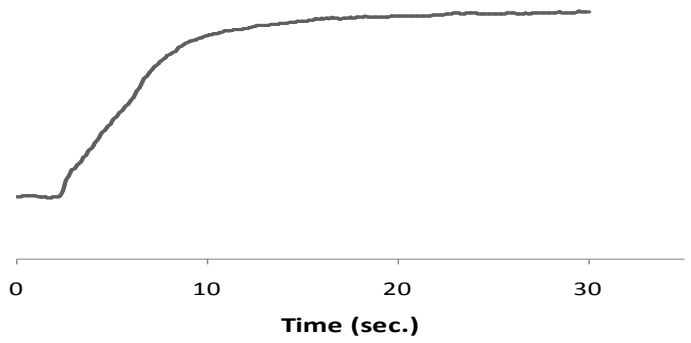

Figure 4. I-t curve of FDH response to 20 mM fructose injection into McIlvaine buffer pH = 4.5 containing $5x10^{-4}$ M Ferrocene methanol. Potential was held at 0.2 V vs. Ag/AgCl.