Pittsburgh Tissue Engineering Initiative

Annual Progress Report: 2009 Formula Grant

Reporting Period

July 1, 2010 – December 31, 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.

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

Preliminary results have shown that the gold microfiber electrodes have a high surface area that is available for enzyme immobilization. The results have also shown that, in the presence of chemical mediators, Glucose oxidase (GOX) can produce electrons that can feed into the gold microfibers and generate potential and current. The necessity for the presence of the chemical mediators of electron transfer is problematic if the electrodes were to be implanted in the body as the mediators are not bound to the electrode and would therefore diffuse away, and further most if not all of the efficient mediators are toxic to cells. We have been working to bypass the necessity of chemical mediators by creating a direct connection between the enzyme active site and the gold microfibers of the electrode. We had initially tried to attach GOX directly to the gold via thiol intermediates. While this resulted in GOX attached to the surface (as seen by enzyme activity assays (data not shown), there was little or no electrical connection. Connecting active surface groups of GOX to the surface of the gold did not place the enzyme active site in a proper orientation to transfer electrons to the electrode. Carbon nanotubes (CNT) are known to conduct electricity and have been used as supports for enzymes in electrodes. The research in this project period thus centered on attaching CNT to the gold microfibers followed by attaching GOX to the CNT. The basic construction of the electrodes is shown in Figure 1.

Deposition of CNT on gold microfibers was accomplished through electrophoretic deposition. CNTs are exceedingly hydrophobic and adhere tightly to one another especially in the presence of water. Even deposition of CNT on a surface from a suspension requires a homogeneous suspension of individual CNT. Several methods were investigated to achieve a suspension of CNT. The protocol that yielded the best suspension involved the addition of the detergent triton X100 to the CNT suspension followed by several hours treatment with a sonic probe. The sonic energy dispersed the CNT and the Triton X100 acted to keep them apart and aided in the deposition onto the gold fibers. Figure 2 shows a picture of a gold microfiber electrode before and after CNT deposition. The fibers are evenly coated with few obvious clumps of CNTs.

To confirm that the morphology of the electrodes was not significantly altered by the deposition of the CNT, samples of the electrode/CNT combinations were subjected to electron microscopy. SEM images showed that the gold microfibers were intact and that there were no significant areas of accumulation of CNT. The even coating of the fibers was confirmed by transmission electron microscopy (Figure 3). The images clearly show a layer of nanotubes about 100 nm thick covering the gold microfiber.

The final part of the electrode was the enzyme GOX. The experiments conjugating GOX to the CNT are preliminary but encouraging. The enzyme was incubated with the activated CNT and the electrodes were washed and tested for electrochemical and enzymatic activity.

The response of the electrode to various concentrations of glucose was evaluated in an oxygen saturated PB solution (0.1 M, pH 7.0). When the solution is saturated with oxygen a large reduction peak of oxygen is observed (curve b in Fig 4A) along with the reduction of the FAD cofactor in GOX. When glucose is added GOX consumes the dissolved oxygen (glucose oxidation) which results in the decrease of peak current (curve c to I in Fig 4A). The data (as replotted in Fig. 4B) show that as the glucose concentration increases reduction peak decreases and the decrease is linearly proportional to the glucose concentration up to 6 mM which is the physiological glucose range. The system showed a sensitivity of 390 µA mM^{-1} (143 µA $mM^{-1}mg^{-1}$) which is the highest observed for a GOX electrode system with direct electron transfer capability. This suggests the enzyme specificity is maintained on the electrode system while maintaining direct electron transfer.

Conclusions

We have fabricated a hybrid gold/carbon nanotube electrode that has a high surface area capable of binding to active enzymes. When glucose oxidase is conjugated to the electrode, current is produced using glucose as the electron source. These results demonstrate that a glucose powered biofuel cell can be developed that will not only provide power but that will be directly responsive to glucose concentration.

Experimental

Electrodes were fabricated by a four step process:
(1) Electrospinning: A high voltage power source (Gamma High Voltage Research, Inc, Fl), syringe pump (World Precision Instruments, Inc., Fl) and a rotating paddle shaped collector made of gold wire (0.5 mm dia.) were combined in an electrospinning system. Dimethylformamide containing PAN 8% (w/w) and $HAuCl_4$ 10% (w/w) was heated at 80 ^oC until the solution was clear. A 20 kV was applied to the PAN/ $HAuCl_4$ solution while being ejected from a syringe fitted with a 21 G blunt needle (Small Parts, Inc, WA) at a flow rate of 0.3 ml/h. As the charged polymer solution travels from the tip to the collector 8 cm away, the whipping motion resulted in a reduction in fiber diameter as well as evaporation of DMF. The fibers were dried under vacuum to remove any trace solvent and stored in a desiccator until further use.

(2) Electroless deposition of gold onto electrospun fibers: The dried electrospun fiber was soaked in sodium borohydride solution (1 mM) to reduce the Au^{3+} in the electrospun fiber to Au^0. Once reduced the electrospun fibers were washed in distilled water to remove sodium borohydride. The crystalline gold present in and on the fiber was utilized as nucleating agent for electroless deposition of gold nanoparticles. The fibers were dipped in a $HAuCl_4$ solution (0.1 mg/ml) containing hydroxyl amine (0.02 mg/ml). This process was typically repeated in order complete coating of gold nanoparticles on the electrospun fiber.

(3) Electrophoretic deposition (EPD) of CNTs onto gold fibers: Our design exploited the conductivity of the gold-coated electrospun fiber as a base upon which to perform electrophoretic deposition of CNTs. Commercial CNTs with carboxyl functionalities at the end of the CNTs and at defect sites along the length of the tubes (0.5 mg/ml) were dispersed in water along with TX-100 as surfactant (1.0 mg/ml) by sonication. The combination of surfactant and sonication gave a highly dispersed CNT solution with ink-like consistency. The negative charge (carboxyl functionalization) on the CNT was then utilized to disperse the CNTs under DC voltage (electrophoresis) and deposit them on the oppositely charged gold fiber. Controlling the voltage, time and distance between electrodes during EPD (allowed us to uniformly coat of CNTs on the fiber surface. We found that 20V applied to the sensor material (positive) held in the middle of parallel copper plates (negative), gave an electrode gap of 0.5 cm and EPD was performed for 30s. The CNT-coated gold electrospun fiber was then dried under vacuum at room temperature before use.

(4) Enzyme Immobilization: Enzyme was covalently coupled to the CNTs via the carboxyl groups present on the CNTs using the coupling reagents N-(3-dimethylaminopropyl) -N´-ethyl carbodiimide hydrochloride (EDC)/ N-hydroxysuccinimde (NHS). We soaked the fiber in a solution of EDC/NHS (molar ratio of EDC (50 mM) to NHS = 1:1) in acetonitrile for 30 minutes. This was followed by drying the electrode in an inert atmosphere before immersing it in a 500 µl enzyme (10 mg/ml) in phosphate buffer (0.1 M, pH 7.0) for 18 hours at 4 °C. The resultant GOX electrode was washed with buffer until no enzyme was found in the washings, and then characterized for electrochemical and enzyme activity.

Electrode morphology

Scanning electron microscopy (SEM) was used to characterize the morphology of the fiber. The reduced electrospun fibers and the gold fibers were coated with carbon for imaging, whereas fibers with CNT coating were sputter coated with gold for imaging. Imaging was performed at 10 kV acceleration voltage using a JEOL 6330F SEM. Transmission electron microscopy (TEM) was used to evaluate the CNT coating on the fibers and to evaluate the presence of enzyme on the CNT surface. TEM imaging was performed at 40kV acceleration voltage using JEOL 1100. Imaging was performed at the University of Pittsburgh Center for Biologic Imaging.

Electrochemical Bioactivity

Electrochemical analysis of the microelectrodes was performed using a CH Instrument (CHI660) electrochemical work station. The setup was a three electrode electrochemical cell: (i) a reference electrode (Ag/AgCl) saturated with KCl, (ii) a counter electrode (0.5 mm Pt wire) and (iii) electrode fabricated was used as the working electrode. The electroactive surface area was estimated by chronocoulometry using 0.1 mM ferricyanide in 0.1M KCl. The electrochemical activity was characterized using cyclic voltammetry in phosphate buffer solution (0.1M, pH 7.0). All the electrochemical measurement was conducted under ambient conditions.

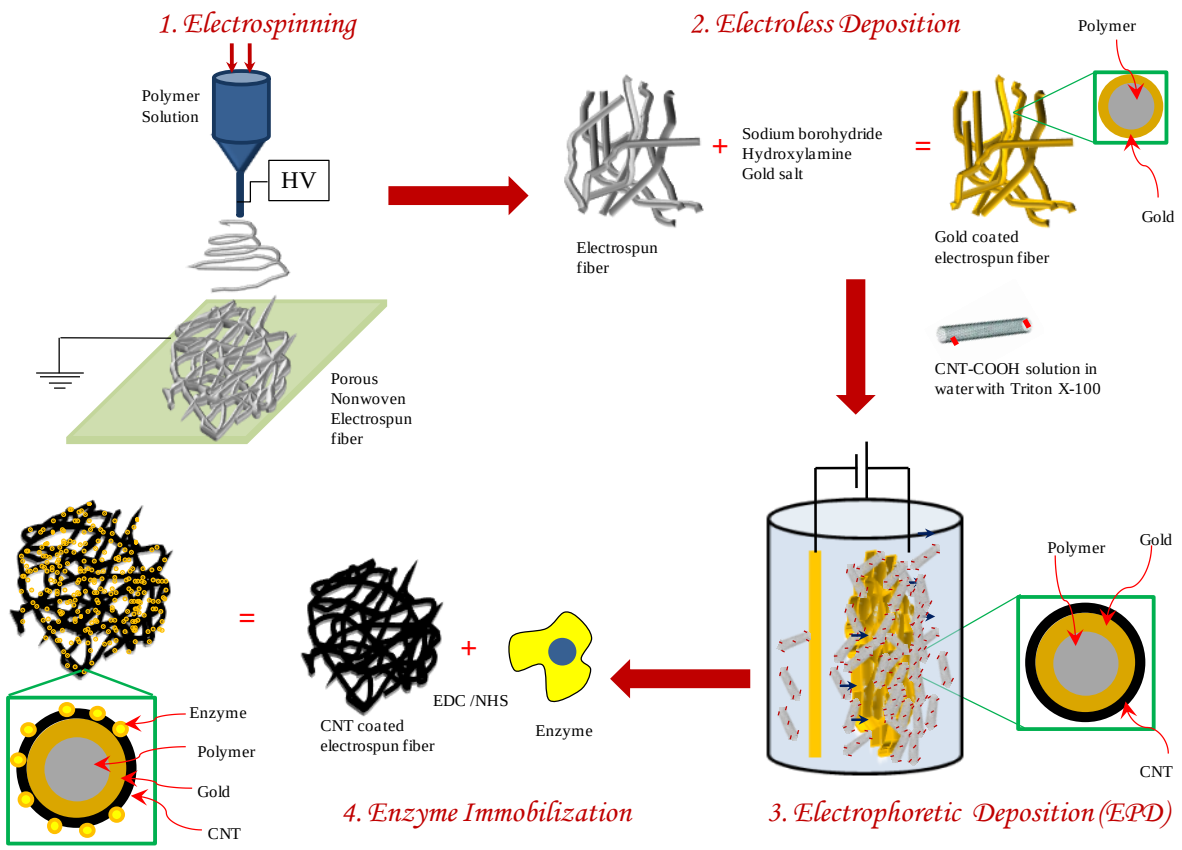

Figure 1. Schematic of electrode fabrication.

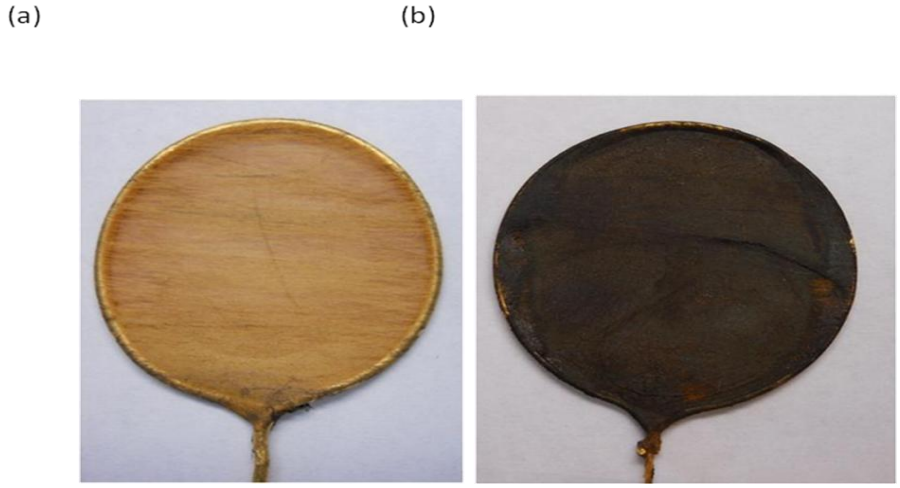

Figure 2. Photographs of gold fiber paddle before (a) and after (b) CNT coating by electrophoretic deposition

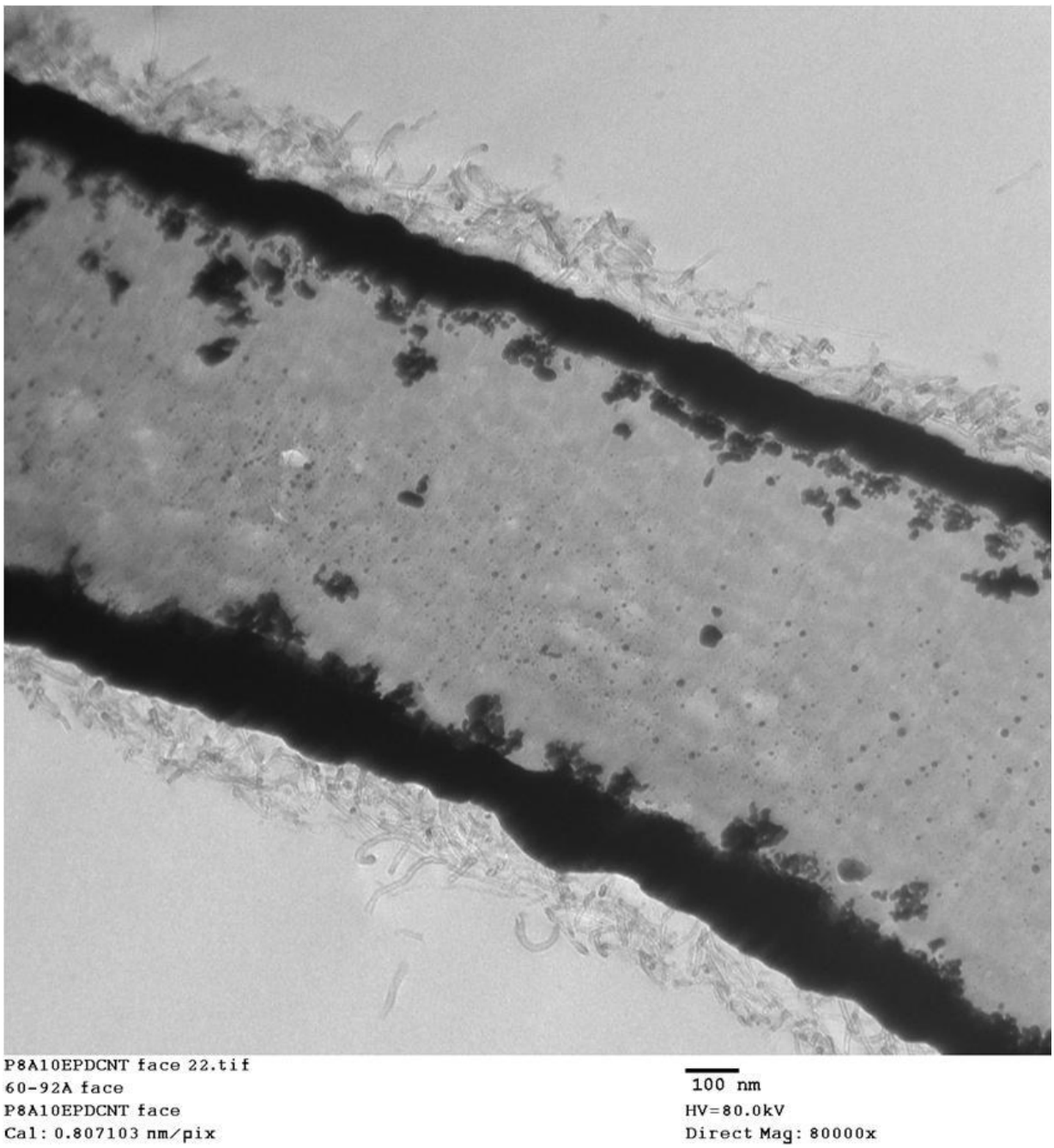

Figure 3. TEM image of a gold microfiber. The structure of the fiber consists of a layer of CNT on a layer of gold each about 100nm thick, covering the approximately 1 micron diameter polymer fiber.

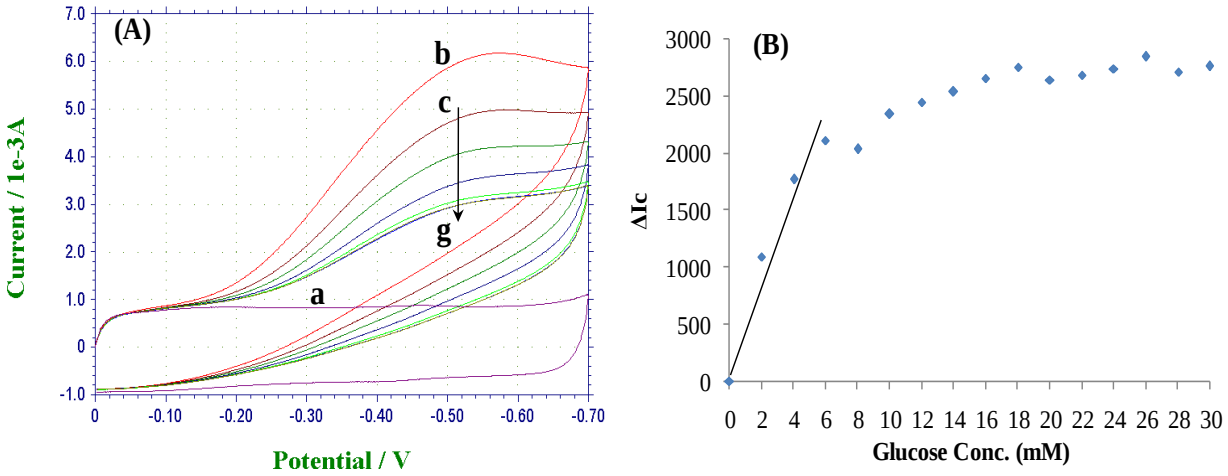

Fig4. (A) Cyclic voltammograms of electode in 0.1M phosphate buffer, pH 7.0 at a scan rate of 100 mV/s in the presence of different concentration of glucose: (a) Ar-saturated without glucose, (b) O_2-saturated without glucose, and (c-g) with of 2,4, 10,16,24 and 30 mM glucose**;** (B) calibration curve corresponding to the peak currents measured at -436 mV at different glucose concentration**.**