Nano-engineered Flavin-Dependent Glucose Dehydrogenase/Gold Nanoparticle-Modified Electrodes for Glucose Sensing and Biofuel Cell Applications

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ABSTRACT A three-dimensional composite consisting of the oxygen-insensitive flavin-dependent glucose dehydrogenase, GDH, and Au nanoparticles (NPs) is assembled on a Au surface using an electropolymerization process. The bis-aniline-cross-linked GDH/Au NPs composite reveals effective electrical contact with the electrode ($k_{et} = 1100\ s^{-1}$), and the effective bioelectrocatalyzed oxidation is driven by the enzyme/NPs matrix. The GDH/Au NPs-functionalized electrode is implemented as an amperometric glucose sensor, and it reveals superior functions when compared to an analogous glucose oxidase/Au NPs system. The O_2-insensitive GDH/Au NPs composite electrode was further used as an anode in a membraneless glucose/O_2 biofuel cell. The cathode in this system was composed of bilirubin oxidase cross-linked onto a carbon nanotube-modified glassy carbon electrode. The power output of the cell was 32 μW cm^{-2}.

KEYWORDS: biosensor · biofuel cell · glucose · Au nanoparticles · glucose dehydrogenase · electropolymerization

The electrical contacting of redox enzymes with electrodes is of fundamental significance for the development of amperometric biosensors and biofuel cell elements.[1−3] The effective electrical communication of redox enzymes with electrodes leads to high current outputs at low concentration of the substrates, thus leading to sensitive sensors and enabling their miniaturization for invasive or implant applications. Moreover, the efficient electrical contacting between enzymes and electrodes (that leads to high turnover rates) prohibits the perturbation in the performance of the sensors caused by interfering reagents that are being nonspecifically oxidized or reduced at the electrode. Specifically, an effective electrical contacting of enzymes with the electrodes competes with the natural activity of many oxidases catalyzing the oxidation of various substrates by molecular oxygen, which leads to oxygen-insensitive enzyme electrodes. Interestingly, the high turnover rates between redox proteins and electrodes are, also, important for designing biofuel cells.[4−10] The power output of a biofuel cell ($P = I \times V$) relates directly to the current generated between the anode and the cathode, which is, in turn, directly controlled by the effectiveness of the electrical contacting at the electrodes. Furthermore, numerous biofuel cells include anodes consisting of oxidases as biocatalysts that oxidize the fuel substrates, and biocatalytic cathodes that reduce molecular oxygen.[11] In these systems, the effective electrical wiring of the oxidases with the electrodes prevents the interfering biocatalytic oxidation of the fuel substrate by oxygen, thus enabling the assembly of membraneless, noncompartmentalized biofuel cells. Such biofuel cell elements can be used as implants that generate electrical power from biological fluids, *e.g.*, blood.[12] Different methods to electrically communicate enzymes with electrodes were developed, and these include the use of diffusional electron mediators,[13,14] the functionalization of the enzymes with electron relays,[15−18] and the immobilization of the enzymes in redox polymers.[19−21] Recently, a promising strategy to electrically contact a glycoprotein oxidase with an electrode through the removal of the glycosylating layer was reported.[22] Also, the alignment of redox enzymes on electrodes by the reconstitution of apo-enzymes on relay-cofactor monolayer-modified electrodes led to effectively wired enzyme electrodes that were unaffected by environmental interfering reagents.[23−25] While the advances in the electrical contacting of enzymes with electrodes led to the fabrication of new and superior biosensor technologies,

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challenging issues, such as the development of miniaturized implantable amperometric biosensors and high-power biofuel cells, are still ahead of us. The progress in the development of biofuel cells highlights the scientific impact of the fundamental studies in bioelectrochemistry on the construction of bioelectronic devices, and it encourages further research efforts. Since the first report on the effective electrical contacting of a monolayer-modified enzyme electrode, which led to an assembly of an integrated membraneless biofuel cell revealing a minute power output (4 μW cm^{-2}),[26] biofuel cell systems revealing power outputs corresponding to 700 to 800 μW cm^{-2} were reported.[27]

In the present paper, we describe the assembly of a highly efficient electrically contacted flavin-dependent glucose dehydrogenase, GDH, enzyme electrode and its use for glucose sensing. We also integrate the enzyme electrode as an anode in a biofuel cell consisting of bilirubin oxidase, BOD, assembled on carbon nanotubes, and acting as a cathode for the reduction of O_2 to water.

RESULTS AND DISCUSSION

The enzyme glucose dehydrogenase, GDH (E.C 1.1.99.10), is an oxygen-insensitive flavoenzyme. It was previously used for the electrochemical detection of glucose using $Fe(CN)_6^{3-}$ as a diffusional electron transfer mediator.[28] In the present study we have constructed an electrically contacted GDH/Au nanoparticle composite electrode using an electropolymerization process that was recently developed in our laboratory.[29] Glucose dehydrogenase was functionalized with thioaniline electropolymerizable groups, as demonstrated in Scheme 1A. Au nanoparticles (NPs), diameter ca. 3.5 nm, were functionalized with thioaniline (**1**), electropolymerizable groups, and mercaptoethane sulfonic acid (**2**) to enhance their stability and solubility in aqueous media. The thioaniline-functionalized Au NPs and the thioaniline-modified GDH were electropolymerized onto a thioaniline monolayer-modified Au electrode to yield the bis-aniline-cross-linked Au NPs/GDH composite, Scheme 1B. The bis-aniline bridges revealed a quasi-reversible redox wave at ca. 0.1 V vs SCE, at pH = 7.4. The resulting GDH/Au NPs cross-linked composite associated with the electrode exhibited electrocatalytic functions toward the oxidation of glucose. Figure 1A exemplifies the cyclic voltammogram observed upon the generation of the composite using 10 electropolymerization cycles and in the presence of glucose, 60 mM. An electrocatalytic anodic current at an onset potential of ca. −0.15 V is observed. Control experiments revealed that the anodic current was not generated in the absence of glucose or upon the interaction of glucose with the bis-aniline-cross-linked Au NPs composite that lacked the GDH. These control experiments imply that GDH incorporated in the Au NPs composite acts as the electrocatalyst for the oxidation of glucose.

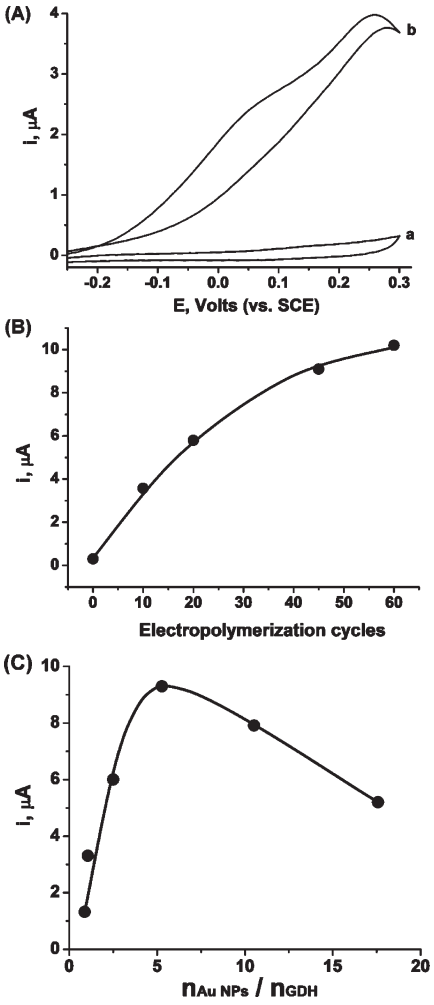
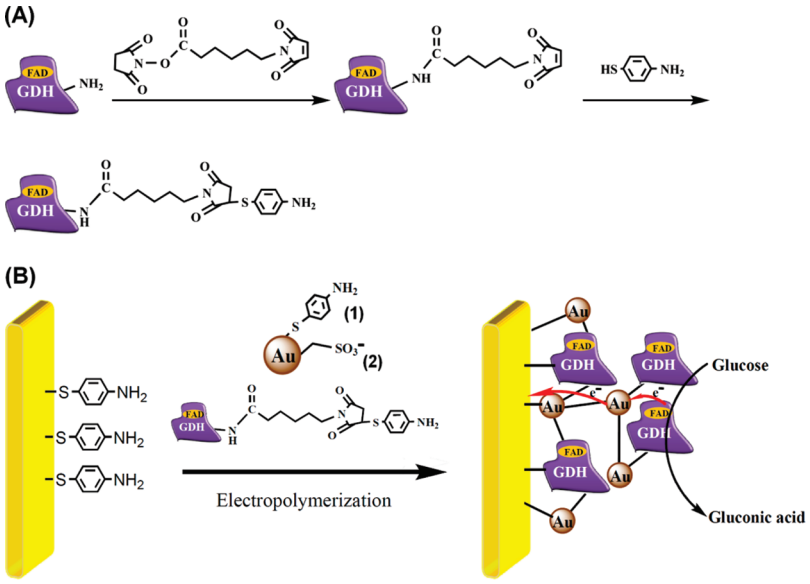


Figure 1. (A) Cyclic voltammograms corresponding to the bioelectrocatalyzed oxidation of glucose by the GDH/Au NPs composite-modified Au electrode. Glucose concentrations: (a) 0 mM; (b) 40 mM. Scan rate: 5 mV s^{-1}. The electrode was prepared by the application of 10 cyclic voltammetry scans between −0.1 and 1.1 V vs SCE and at a Au NPs:GDH molar ratio of 5.3:1.0. (B) Electrocatalytic anodic currents, at E = 0.3 V vs SCE, generated by the GDH/Au NPs composite-modified Au electrodes, prepared by the application of a variable number of cyclic voltammetry electropolymerization scans at a Au NPs:GDH molar ratio of 5.3:1.0. (C) Electrocatalytic anodic currents, at E = 0.3 V vs SCE, generated by the GDH/Au NPs composite-modified Au electrodes, prepared by the application of 60 cyclic voltammetry electropolymerization scans and different molar ratios of Au NPs:GDH (constant Au NPs content, 0.17 mg mL^{-1}). Data were recorded in a phosphate buffer solution (0.1 M, pH = 7.4) that included 60 mM glucose. In all experiments the scan rate was 5 mV s^{-1}.

The composition of the GDH/Au NPs-modified electrode was optimized. Figure 1B shows the electrocatalytic currents, at E = 0.3 V, generated by GDH/Au NPs-functionalized electrodes that were synthesized by the application of a variable number of electropolymerization cycles. As the number of electropolymerization cycles increases, the electrocatalytic anodic currents are intensified (implying higher GDH content), and they level off after ca. 60 cycles. The saturation of the electrode with the GDH/Au NPs composite is attributed



Scheme 1. (A) Modification of GDH with the electropolymerizable thioaniline functionalities. (B) Synthesis of the bis-aniline-cross-linked GDH/Au NP composite by the electropolymerization of the thioaniline-modified GDH and the thioanline-functionalized Au NPs on the thioaniline monolayer-functionalized Au electrode.

to the incorporation of insulating GDH components into the conductive Au NPs matrix. Upon the growth of the GDH/Au NPs layer, more insulator sites are formed, and these consequently prohibit the further growth of the composite. The effect of the ratio of the Au NPs:GDH components in the electropolymerization mixture on the electrocatalytic activity of the resulting composite was then examined, Figure 1C. The optimal bioelectrocatalytic activity of the matrix was observed for a molar ratio of Au NPs:GDH that corresponded to ca. 5:1. An activity assay of the composite that was electropolymerized under the optimized conditions indicated that the thioaniline-functionalized GDH exhibits 95% activity of the native enzyme. Assuming that the activity of the enzyme is unchanged upon being cross-linked in the composites, the coverage of the enzyme on the electrode is estimated to be 1.8×10^{-12} mol cm^{-2} (for the determination of the surface coverage of GDH, see Supporting Information, Figure S1).

The optimized bis-aniline-cross-linked GDH/Au NPs-functionalized electrode was then tested as a sensing platform for glucose. Figure 2A depicts the cyclic voltammograms corresponding to the electrode in the presence of variable concentrations of glucose. Evidently, the currents become intensified as the concentrations of glucose increase, and they level off at a concentration corresponding to ca. 260 mM. The resulting calibration curve is shown in Figure 2B. Also, the calibration curve corresponding to the amperometric responses in the concentration range significant for diabetic control is shown in Figure 2B, inset. The linear dependence between the electrocatalytic currents and the glucose concentrations suggests that the modified electrode could be used in the future by applying a single-point calibration process. The modified electrode, without any further stabilizing coating, operated without noticeable losses in its activity for three days. From the saturation current generated by the modified electrode, and by knowing the coverage of the enzyme on the electrode, the turnover rate of electrons between GDH and the Au surface was estimated to be ca. $k_{et} = 1100$ electrons s^{-1}.

The GDH/Au NPs matrix further demonstrates an oxygen-insensitive detection of glucose, Figure 2C. Interestingly, an analogous deposition of a bis-aniline-cross-linked glucose oxidase, GOx/Au NPs, composite (with an almost identical content of the biocatalyst) reveals that the GDH-containing matrix supports up to 2-fold higher bioelectrocatalytic functions, as compared with the GOx, Figure 3 (and Figure 3 inset). That is, the turnover rate between the GOx and the electrode in the GOx/Au NPs composite is ca. k_{et} = 500 electrons s^{-1},[29] less than half the value found for the GDH/Au NPs composite.

The insensitivity of the bis-aniline-cross-linked GDH/Au NPs composite-modified electrode to O_2, and the high turnover rate of electrons between the enzyme and the electrode, suggested that the electrode could be implemented as an anode in a membraneless glucose/O_2 biofuel cell. In fact, the FAD-dependent GDH was previously used as a biocatalytic material for the construction of a biofuel cell,[30] yet the resulting cell included the use of diffusional electron mediators. In our study, we combined the integrated, electrically contacted, bis-aniline-cross-linked GDH/Au NPs-functionalized anode with a bilirubin oxidase, BOD, O_2-reducing cathode to yield the biofuel cell that is schematically presented in Figure 4A. BOD was adsorbed on carbon nanotubes (CNTs) that were deposited onto a glassy carbon electrode, and the enzyme units were cross-linked by

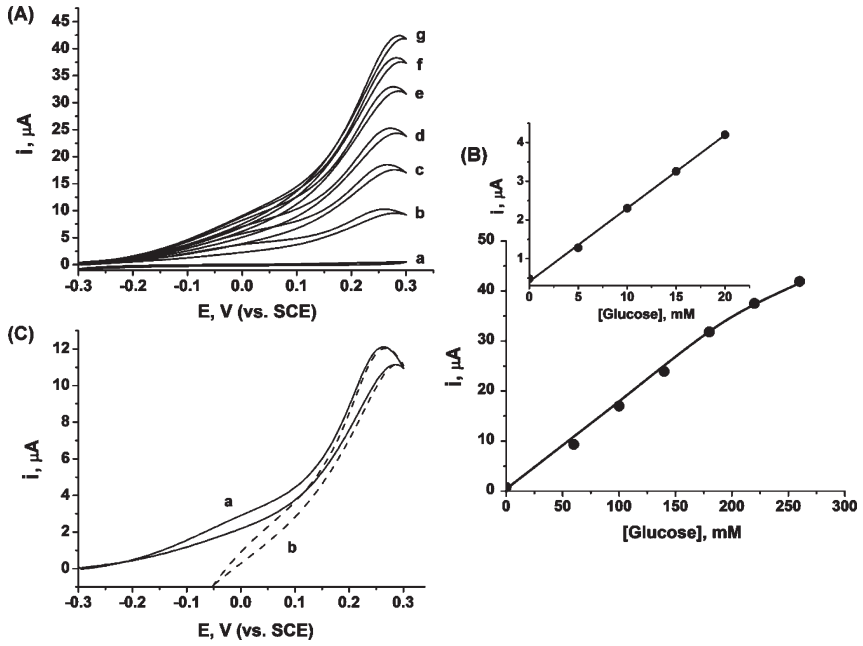


Figure 2. (A) Cyclic voltammograms corresponding to the bioelectrocatalyzed oxidation of glucose by the GDH/Au NPs composite-modified Au electrode, generated by the application of 60 electropolymerization scans and at a Au NPs:GDH molar ratio of 5.3:1.0, in the presence of variable concentrations of glucose: (a) 0 mM; (b) 60 mM; (c) 100 mM; (d) 140 mM; (e) 180 mM; (f) 220 mM; and (g) 260 mM. (B) Cyclic voltammograms corresponding to the bioelectrocatalyzed oxidation of glucose by the GDH/Au NPs composite-modified Au electrode in a phosphate buffer solution (0.1 M, pH = 7.4) that includes 60 mM glucose. Measurements were performed (a) under N_2 and (b) under air. (C) Calibration curve corresponding to the bioelectrocatalytic currents in (A), measured at $E = 0.3$ V vs SCE, for the different concentrations of glucose. Inset: Calibration curve corresponding to the bioelectrocatalytic currents measured at the lower glucose concentration range. In all experiments the scan rate was 5 mV s^{-1}.

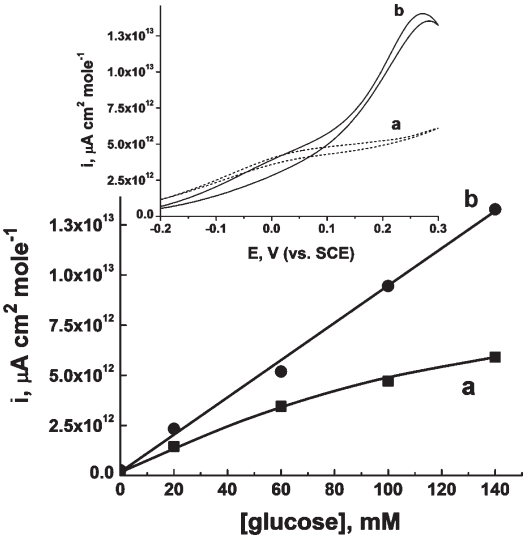


Figure 3. Calibration curves corresponding to the bioelectrocatalyzed oxidation of variable concentrations of glucose on (a) a bis-aniline-cross-linked GOx/Au NPs composite-modified Au electrode and (b) the bis-aniline-cross-linked GDH/Au NPs composite-modified electrode. Inset: Cyclic voltammograms corresponding to the bioelectrocatalytic oxidation of glucose, 140 mM, on (a) the GOx/Au NPs composite and (b) the GDH/Au NPs composite. Data were recorded in a phosphate buffer solution (0.1 M, pH = 7.4) at a scan rate of 5 mV s^{-1}. The surface coverage values of the GOx and the GDH enzymes on the composite electrodes are 1.8×10^{-12} and 2.0×10^{-12} mol cm^{-2}, respectively.

Sulfo-DSS (bis(sulfosuccinimidyl)suberate) to yield the integrated bioelectrocatalytic cathode. Previous studies have indicated that the adsorption of BOD onto CNTs led to partial unfolding of the protein and to the direct electrical contacting of one of the Cu^{2+}/Cu^{+} centers of the enzyme with the electrode.[31] The enzymatic assay of the BOD (see Supporting Information, Figure S2) associated with the glassy carbon/CNTs indicated a coverage of ca. 2×10^{-9} g cm^{-2}. Indeed, the resulting BOD/CNTs-functionalized electrode revealed an effective bioelectrocatalytic reduction of O_2 to H_2O, Figure 4B. The bioelectrocatalyzed oxidation of glucose by the bis-aniline-cross-linked GDH/Au NPs anode proceeds at an onset potential of ca. −0.1 V vs SCE (cf. Figure 2A), whereas the reduction of oxygen by the BOD/CNTs electrode proceeds at a potential of +0.4 V vs SCE. This provides a potential difference between the anode and the cathode that enables the operation of the biofuel cell. Figure 4C shows the discharge (polarization) profile of the biofuel cell, measured for different external resistances in air. Also, the power output of the cell is presented, indicating that the biofuel cell supports up to 32 μW cm^{-2}. This value is lower than the power output reported for other biofuel cell configurations.[27] One should note, however, that these high power outputs were obtained by the use of highly roughened surfaces and by the rotation of the electrodes, to overcome diffusion barriers.[9]

In conclusion, the present study has introduced the oxygen-insensitive glucose dehydrogenase as an effective bioelectrocatalyst for the assembly of glucose-sensing electrodes and for the construction of a membraneless

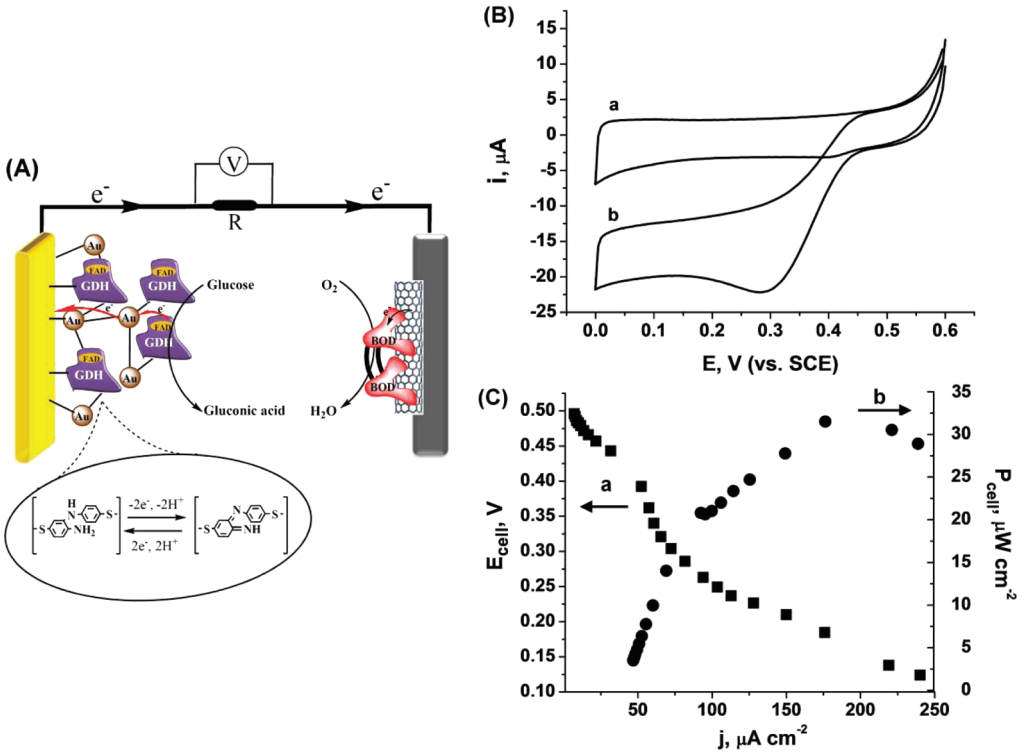


Figure 4. (A) Schematic presentation of the GDH/Au NPs composite/BOD-CNTs biofuel cell. (B) Cyclic voltammograms corresponding to the bioelectrocatalyzed reduction of O_2 on the BOD/CNTs-modified GC cathode. The electrolyte, phosphate buffer (0.1 M, pH = 7.4), was purged for 15 min with (a) N_2 and (b) O_2. (C) Cell discharge (polarization) curve (a) and the dependence of the cell power (b) on the current of the GDH/Au NPs composite/BOD-CNTs biofuel cell. Measurements were performed in air and using variable external resistances. The electrolyte was a phosphate buffer (0.1 M, pH = 7.4) that included glucose, 200 mM.

glucose/O_2 biofuel cell. The GDH biocatalyst was modified with electropolymerizable thionaniline units, and the electrosynthesized bis-aniline-cross-linked GDH/Au NPs composite revealed efficient electrical contacting with the electrode surface, resulting in an effective bioelectrocatalytic oxidation of glucose. This paper highlights a novel method to nano-engineer a hybrid composite of GDH and Au NPs that reveals effective electrical contacting with the supporting electrode. While this feature is a prerequisite to develop the new glucose sensor and the biofuel cell element, the practical application of these systems will certainly deserve further research efforts directed to the elucidation of the long-term stability, high-throughput reproducibility, and biocompatibility of the electrodes.

METHODS

Chemicals. FAD-dependent glucose dehydrogenase, GDH (E. C. 1.1.99.10), was obtained from Genzyme Diagnostics, UK. Bilirubin oxidase, BOD (E.C. 1.3.3.5, from *Myrothecium verrucaria*, activity of 5 U mg^{-1} solid), was purchased from Sigma. The enzymes were used without any further purification. Single-walled carbon nanotubes, SWCNTs, with an average diameter of ca. 2 nm and a length of ca. 50 μm, were purchased from Nanoport (Shenzhen, China). The SWCNTs were refluxed in 2.5 M HNO_3 for 10 h and were then washed in water and precipitated using a centrifuge to yield an average length of 2 μm. Bis(sulfosuccinimidyl) suberate and *N*-(maleimidocaproyloxy)-sulfosuccinimide ester were purchased from Pierce, USA. All other chemicals were purchased from Sigma/Aldrich and were used as supplied. Ultrapure water from a Nanopure (Barnstead) source was used throughout this work.

Nanoparticle Synthesis. Au NPs functionalized with 2-mercaptoethanesulfonic acid and *p*-aminothiophenol were prepared by mixing a 10 mL solution of $HAuCl_4$ (197 mg) in ethanol with a 5 mL solution of mercaptoethanesulfonate (42 mg) and *p*-aminothiophenol (8 mg) in methanol. The two solutions were stirred in the presence of 2.5 mL of glacial acetic acid in an ice bath for 1 h. Subsequently, 7.5 mL of an aqueous solution of 1 M sodium borohydride, $NaBH_4$, was added dropwise, resulting in a dark color associated with the presence of the Au NPs. The solution was stirred for one additional hour in an ice bath and then for 14 h at room temperature. The particles were successively centrifuged and washed (twice in each solvent) with methanol, ethanol, and diethyl ether. The average size of the NPs was estimated by TEM to be 3.6 $\pm$ 0.3 nm.

Modification of the Enzyme. A 3 mL solution of the FAD-dependent GDH in phosphate buffer (0.1 M, pH = 7.4) was treated with 52 μL of *N*-(maleimidocaproyloxy)sulfosuccinimide ester (12 mg mL^{-1}). Following the stirring of the solution for 40 min, it was mixed with 0.8 mL of *p*-aminothiophenol (thioaniline, 1.6 mg mL^{-1}) in ethanol, and the components were allowed to react for 2.5 h. The resulting solution was then eluted through a G-25 column (GE Healthcare) using phosphate buffer (0.1 M, pH = 7.4) as the eluent. The obtained purified, functionalized GDH solution was kept at 4 °C.

Modification of the Electrodes. Bis-aniline-cross-linked GDH/Au NPs composite-modified Au electrodes were prepared according to the following procedure: clean Au wires (effective surface

area 0.22 cm^2) were immersed for 10 h in a 10 mM solution of thioaniline in ethanol to yield the thioaniline-modified Au electrodes. The composite-modified electrodes were then synthesized by the electropolymerization of the thioaniline-functionalized Au NPs and the thioaniline-modified GDH on the thioaniline-modified Au electrodes, in a phosphate buffer (0.1 M, pH = 7.4) and by using a fixed number of repetitive cyclic voltammetry scans, ranging between −0.1 and +1.1 V (vs SCE). Scan rate: 100 mV s^{-1}.

BOD/CNT-modified glassy carbon (GC) electrodes were prepared according to the following procedure: SWCNTs, 10 mg mL^{-1}, were dispersed in DMF to yield a stable suspension. The dispersion, 5 μL, was deposited onto the GC electrodes to generate the CNT-modified GC electrode. A BOD solution (5 μL, 0.14 mg mL^{-1}) in a HEPES buffer (0.1 M, pH = 7.2) was deposited on the electrodes, and after 30 min, 1 μL of bis(sulfosuccinimidyl)suberate, 0.01 mg mL^{-1}, was added. The electrodes were then allowed to dry in air.

Instrumentation. All electrochemical measurements were performed using a PC-controlled (Autolab GPES software) potentiostat/galvanostat (μAutolab, type III). A graphite rod (d = 5 mm) was used as the counter electrode, and the reference was a saturated calomel electrode (SCE). GC electrodes (3 mm diameter) were purchased from Bioanalytical Systems (West Lafayette, IN). The GC electrodes were polished with an emery paper (#2000) and then by using 0.3 and 0.05 mm alumina slurries on a polishing cloth. Following their polishing, the electrodes were cleaned in an ultrasonic bath (ethanol and then water) for 10 min and were thoroughly rinsed with deionized water.

In order to construct the biofuel cell, the BOD/CNTs-modified GC cathode and the GDH/Au NPs composite-modified Au anode were placed into a 10 mL cell that was filled with phosphate buffer (0.1 M, pH = 7.4) containing glucose, 200 mM. The biofuel discharge (polarization) curves were recorded under air, at variable external resistances, and by using an electrometer (Keithley 617).

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Supporting Information Available: The enzymatic assays for the determination of the GDH content in the bis-aniline-cross-linked GDH/Au NP composite and the BOD content on the BOD/CNTs-modified GC. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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