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PAPER

A promising dehydrogenase-based bioanode for a glucose biosensor and glucose/O_2 biofuel cell

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A new type of dehydrogenase-based amperometric glucose biosensor was constructed using glucose dehydrogenase (GDH) which was immobilized on the edge-plane pyrolytic graphite (EPPG) electrode modified with poly(phenosafranin)-functionalized single-walled carbon nanotubes (PPS-SWCNTs). The PPS-SWCNT-modified EPPG electrode was prepared by electropolymerization of phenosafranin on the EPPG electrode which had been previously coated with SWCNTs. The performance of the GDH/PPS-SWCNT/EPPG bioanode was evaluated using cyclic voltammetry and amperometry in the presence of glucose. The GDH/PPS-SWCNT/EPPG electrode possesses promising characteristics as a glucose sensor: a wide linear dynamic range of 50 to 700 μM, low detection limit of 0.3 μM, fast response time (1–2 s), high sensitivity (96.5 μA cm^{-2} mM^{-1}), and anti-interference and anti-fouling abilities. Moreover, the performance of the GDH/PPS-SWCNT/EPPG bioanode was tested in a glucose/O_2 biofuel cell. The maximum power density delivered by the assembled glucose/O_2 biofuel cell could reach 64.0 μW cm^{-2} at a cell voltage of 0.3 V with 40 mM glucose.

Introduction

The quantitative measurement of glucose is very important for clinical purpose in the analysis of human blood.[1] Glucose is an attractive target, because it is not only an important biomarker for diabetes but also a kind of fuel for biofuel cells. Some analytical methods have been developed over the years for the determination of glucose. An amperometric enzyme-based biosensor is one of the best choices for biochemical analysis due to its good selectivity, sensitivity, rapid response, miniature size, and reproducible results.[2] The good selectivity is attributed to the specific catalysis by the enzyme.

In most cases, NAD^+ (the oxidized form of β-nicotinamide adenine dinucleotide (NADH))-dependent enzyme-based biosensors require NADH as reagent for their functioning. Electrode modification with carbon nanotubes (CNTs) has been shown to produce electrocatalysis for NADH electrooxidation, thus allowing the preferable construction of dehydrogenase enzyme-based electrochemical biosensors.[3–5] Enzyme biosensors based on CNTs-composite electrodes, they have been found to possess improved analytical performance as a consequence of the unique properties of CNTs with the well-known advantages of composite electrode designs such as simple renewability and low background current.[6] Many recent studies have aimed at the functionalization of CNTs with various molecules using covalent[7,8] or non-covalent bond formation approaches[9,10] in order to obtain desired properties. The covalent functionalization is based on the formation of chemical bonds between CNTs and functional molecules, and the non-covalent functionalization is based on adsorption, wrapping, π-stacking, hydrophobic/π–π interactions, *etc.* Among the many different strategies to functionalize CNTs, electrochemical functionalization is an efficient, clean, and versatile alternative.[11] Fabrication of CNT/conducting polymer composites has become of great interest, since the CNTs can improve the electrical and mechanical properties of polymers. It has been demonstrated that the CNT/conducting polymer composites possess the original properties of the individual components with a synergistic function.[12–14] For example, single-walled carbon nanotube (SWCNT)/poly(3-octylthiophene) composites have been shown to have good photovoltaic properties,[15] and CNT/polyaniline composites have been found to be good materials in batteries with a high charge–discharge capacity, low charge voltage and high discharge voltage.[16] So far, several conducting polymers such as polypyrrole, polyaniline, polyphenothiazine, polythiophene and their derivatives have been used to fabricate CNT/polymer composites.[17–21] However, there are limited studies on the CNT/poly(phenazine) dye composites,[22] although poly(phenazine) dyes are widely used as redox mediators in the construction of electrochemical sensors and biosensors.[23–26] Phenazine dyes display a much lower redox potential at neutral pH than analogous phenothiazine and phenoxazine dyes.[23–26] An assembly of CNTs and poly(phenazine)

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where the surface of CNTs acts as a support of the redox mediator as well as an electronic conducting network may exhibit an effective electronic interaction and a faster charge transfer that can facilitate oxidation of analytes.

The present work aims at developing a dehydrogenase-based glucose biosensor by immobilizing glucose dehydrogenase (GDH) on the surface of a poly(phenosafranin) (PPS)-functionalized SWCNT nanocomposite. Previously we have reported the electrocatalytic oxidation of NADH by the basal-plane pyrolytic graphite (BPPG) modified with PPS film prepared by electropolymerization of phenosafranin (PS) which is an *N*-substituted phenazine with a half-wave potential of −0.458 V at pH 7.0.[27] We have also reported an excellent electrocatalysis of PPS-functionalized SWCNT-modified edge-plane pyrolytic graphite (EPPG) electrode for the NADH oxidation.[26] Here, by immobilizing GDH onto the PPS-SWCNT nanocomposite film, a dehydrogenase-based glucose (GDH/PPS-SWCNT/EPPG) biosensor was fabricated. The obtained results show that the GDH and PPS-SWCNT nanocomposite-based bioanode possesses an excellent electrocatalytic activity for glucose oxidation and thus functions as a sensitive and selective electrochemical biosensor of glucose in the presence of interfering biomolecules. Furthermore, the GDH/PPS-SWCNT/EPPG electrode was successfully applied as the bioanode of a glucose/O_2 biofuel cell.

Experimental section

Chemicals and reagents

Single-walled carbon nanotubes (SWCNTs) with a diameter of 1.2–1.5 nm and a length of 2–5 μm were purchased from Aldrich (Tokyo, Japan). Phenosafranin (PS) was purchased from Acros Organics (Geel, Belgium). Oxidized form of β-nicotinamide adenine dinucleotide (NAD^+, Oriental Yeast Co. Ltd., Tokyo, Japan) was used as received. Uric acid (UA) and ascorbic acid (AA) were purchased from Kanto Chemicals (Japan). Glucose dehydrogenase (GDH) from *Bacillus* sp. and 25% glutaraldehyde (GA) solution in water were purchased from Wako (Japan). Bovine serum albumin (BSA), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and a thermophilic laccase (30.6 U mg^{-1}) from *Thermus thermophilus* HB 27 were purchased from Sigma (USA). Phosphate buffer solution (PBS; 0.1 M, pH 6.0 and 7.0), prepared using sodium dihydrogen phosphate and disodium hydrogen phosphate (Kanto chemicals, Japan) was used as the supporting electrolyte for electrochemical experiments. The solutions throughout this work were always prepared using deionized water from a Milli-Q water system (Millipore, Japan). An edge-plane pyrolytic graphite (EPPG) (Bioanalytical Systems Inc. (BAS); 3 mm in diameter) disk was used as the working electrode. All of the other chemicals were of analytical grade and were used without further purification.

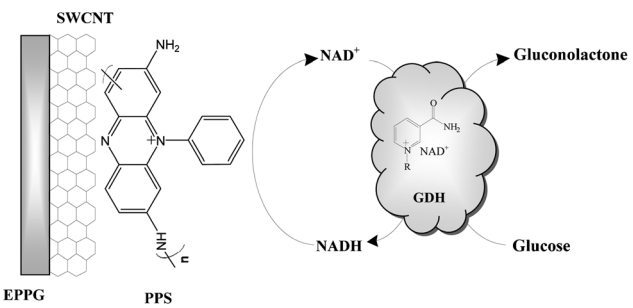
Apparatus and electrochemical measurements

Cyclic voltammetric and amperometric measurements were carried out with an ALS CHI 750Cz electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) using a conventional two-compartment three-electrode system with the prepared bioanodes as working electrodes, a spiral Pt wire as counter electrode and $Ag|AgCl|KCl_{(sat)}$ as reference electrode. The amperometric measurements of the fabricated glucose sensor were performed in 0.1 M PBS, stirred at *ca.* 300 rpm using a magnetic stirrer. A personal computer was used for data storage and processing. Electrolyte solutions were de-aerated by bubbling Ar gas (99.99%) for at least 30 min prior to electrochemical measurements. All the measurements were carried out at room temperature (25 ± 1 °C).

Procedures

Preparation of glucose bioanode. The EPPG electrodes were first carefully polished with emery paper and then transferred to 0.1 M H_2SO_4 solution and a potential scan was repeated between −0.2 and 1.4 V at 100 mV s^{-1} until a stable cyclic voltammetric response was attained. 1 mg of the SWCNTs was dispersed in 1 mL of DMF to form a 1 mg mL^{-1} SWCNT suspension solution with the aid of ultrasonic agitation. The SWCNT-modified EPPG (SWCNT/EPPG) electrode was prepared by casting 30 μL of the SWCNT suspension on the EPPG electrode surface and air-drying the cast suspension solution. A film of PPS was prepared by immersing the SWCNT/EPPG electrode in 0.2 M H_2SO_4 solution containing 0.5 mM PS for 10 min and then by repeating the potential scan between −0.5 and 1.3 V at 50 mV s^{-1}. The thus-fabricated PPS-SWCNT-modified EPPG electrode is hereinafter abbreviated as PPS-SWCNT/EPPG electrode. Finally, glucose dehydrogenase (GDH) was immobilized by a cross-linking reaction using glutaraldehyde (GA) in bovine serum albumin (BSA) matrices; for attaching the enzyme layer, typically 10 μL of GDH–BSA–GA mixture (5 μL of 20 mg mL^{-1} GDH solution in 0.1 M PBS (pH 7.0) + 2.5 μL of 1 wt% BSA solution in 0.1 M PBS (pH 7.0) + 2.5 μL of 1 wt% GA in water) was placed on the PPS-SWCNT/EPPG electrode and then air-dried at room temperature for at least 1 h. The electrode assembly fabricated (named as GDH/PPS-SWCNT/EPPG electrode) is schematically shown in Scheme 1.

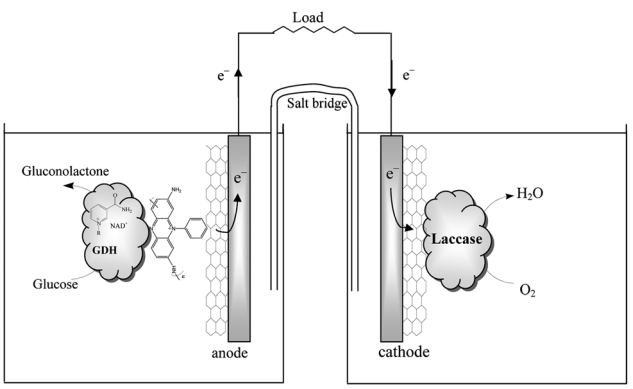
Preparation of laccase biocathode. Prior to coating, the bare EPPG electrode was cleaned in the same way as mentioned above. A methylene green-functionalized SWCNT-modified EPPG (MG-SWCNT/EPPG) electrode was prepared by casting 8 μL of an almost homogeneous suspension of a MG-SWCNT adduct on the clean EPPG electrode and air-drying the cast suspension at room temperature. According to the preparation



Scheme 1 Schematic of the GDH-based composite electrode for the electrocatalytic oxidation of glucose.

procedure of the MG-SWCNT-modified carbon fiber electrode,[28] the laccase/MG-SWCNT/EPPG biocathode was prepared as follows: a 20 μL of the purified laccase was mixed with 5 μL of BSA (1 wt%) to give a laccase–BSA mixture. Then 6 μL of the prepared laccase–BSA mixture was spread evenly on the MG-SWCNT/EPPG electrode with a microsyringe and then cross-linked with 2 μL of 1 wt% GA. The laccase was purified[29] typically by dissolving 50 mg of laccase in 500 mL of 0.10 M phosphate buffer (pH 6.0) and transferring the mixture into a dialysis bag. The dialysis bag was immersed into phosphate buffer and the buffer was stirred for 30 min. The bag was then transferred into a fresh phosphate buffer and dialysis allowed to proceed for one day at 4 °C. The solution left in the bag was saturated with ammonium sulfate to give a suspension that was centrifuged for 10 min. The obtained precipitate was dissolved in a minimal amount (*ca.* 500 mL) of 0.10 M phosphate buffer (pH 6.0) saturated with ammonium sulfate. The resulting suspension was then centrifuged for 10 min and the supernatant was discarded. The precipitate was dissolved in a minimal amount (*ca.* 300 mL) of 0.10 M phosphate buffer and the obtained solution was transferred into a micro test tube and concentrated by centrifuging for 5 min. The resulting solution of laccase (concentration not defined) was stored at 4 °C in a refrigerator and used for electrochemical measurements. The laccase/MG-SWCNT/EPPG electrode was dried at ambient temperature and rinsed with water before use. When not in use, all the modified electrodes were stored at 4 °C.

Cell design. A two-compartment cell (Scheme 2) was constructed to investigate the performance of the GDH/PPS-SWCNT/EPPG electrode as the bioanode of a glucose/O_2 biofuel cell. For assembling the glucose/O_2 biofuel cell, the laccase/MG-SWCNT/EPPG electrode was used as the cathode for O_2 reduction and the GDH/PPS-SWCNT/EPPG electrode as the anode for oxidation of glucose. Both the electrodes were placed into a two-compartment cell with a salt bridge and each half-cell containing 20 mL of electrolyte solution. The bioanode was immersed into 0.1 M PBS (pH 6.0) containing 40 mM glucose and 10 mM NAD^+ and the biocathode was immersed into 0.1 M PBS (pH 6.0) containing 0.46 mM ABTS. The cell performance was evaluated at ambient temperature.



Scheme 2 The setup of a glucose/O_2 biofuel cell composed of the GDH/PPS-SWCNT/EPPG bioanode and the laccase/MG-SWCNT/EPPG biocathode.

Results and discussion

Electrocatalytic characterization of the GDH/PPS-SWCNT/EPPG electrode for glucose oxidation

The GDH/PPS-SWCNT/EPPG electrode was electrochemically characterized by cyclic voltammetry in 0.1 M PBS (pH 7.0) containing 10 mM NAD^+ in the absence and presence of glucose (Fig. 1). In the presence of glucose (20 mM), a large anodic current was observed with a very low onset potential of −0.15 V *vs.* Ag|AgCl|$KCl_{(sat.)}$ (voltammogram a), in contrast to the case in the absence of glucose (voltammogram b). Here it should be noted that no oxidation of glucose occurred at the PPS-SWCNT/EPPG electrode (without GDH) in 0.1 M PBS (pH 7.0) containing 10 mM NAD^+ and 20 mM glucose (voltammogram c). These results clearly demonstrate that the electrocatalytic oxidation of NADH to NAD^+ takes place smoothly *via* an electron transfer mediation of the molecular assembly (PPS-SWCNT) composed of PPS mediator and SWCNTs and that the GDH immobilized on this molecular assembly, as expected, functions well as a dehydrogenase enzyme for glucose. Consequently, the observed excellent electrocatalysis of the GDH/PPS-SWCNT/EPPG electrode for the oxidation of glucose allowed us to use it as a glucose biosensor. The electrocatalytic current of this electrode for glucose oxidation was found to depend on the loading amount of GDH and the NAD^+ concentration (Fig. 2); 10 μL loading of the GDH–BSA–GA mixture (see Experimental section) and 10 mM NAD^+ were typically used in the application of the electrode as a glucose sensor and the bioanode of a glucose/O_2 biofuel cell (mentioned below).

Amperometric glucose biosensor

Fig. 3 displays the typical steady-state catalytic current–time response of the GDH/PPS-SWCNT/EPPG electrode for the successive injection of 50 μM glucose in 0.1 M PBS (stirred at 300 rpm). In this case, the electrode potential was kept at 0.0 V. The detection potential is 400 mV lower than that used

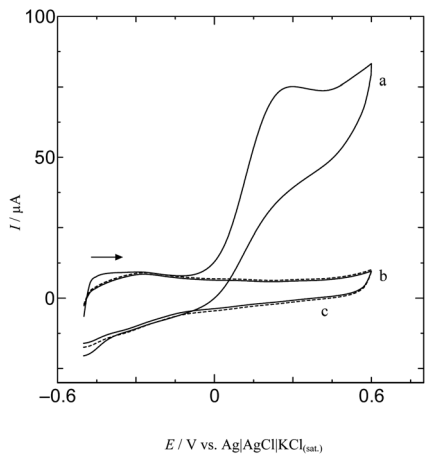


Fig. 1 CVs obtained at the GDH/PPS-SWCNT/EPPG electrode in 0.1 M PBS (pH 7.0) containing 10 mM NAD^+ in the presence (curve a) and absence (curve b) of 20 mM glucose. Curve c (dotted line) is CV of the PPS-SWCNT/EPPG electrode in 0.1 M PBS (pH 7.0) containing 10 mM NAD^+ and 20 mM glucose. The scan rate was 5 mV s^{-1}.

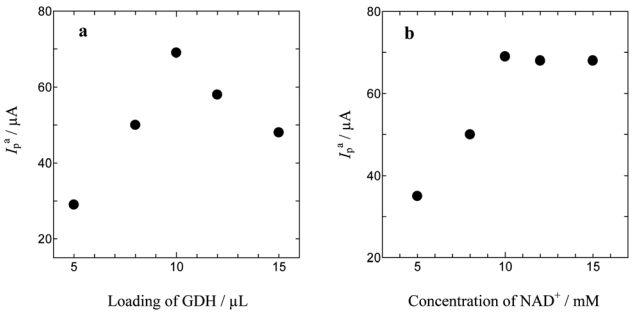


Fig. 2 Effects of the GDH loading (a) and the concentration of NAD^+ (b) on the electrocatalytic response of the GDH/PPS-SWCNT/EPPG electrode for the glucose oxidation in 0.1 M PBS (pH 7.0) in the presence of 20 mM glucose. In every case, the anodic peak current (I_p^a) values were estimated at 5 mV s^{-1}.

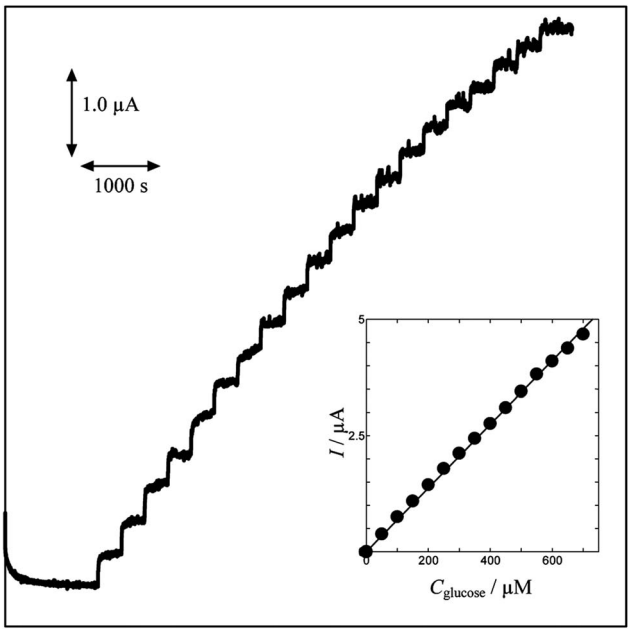


Fig. 3 Current–time response of the GDH/PPS-SWCNT/EPPG electrode for the successive addition of 50 μM glucose at 0.0 V in 0.1 M PBS (pH 7.0) containing 10 mM NAD^+. The solution was stirred with a magnetic stirrer at 300 rpm. The inset shows the plot of the steady-state current against glucose concentration.

previously[30] for the CNT-CHIT-GDI-GDH/GC composite electrode (CHIT: chitosan; GDI: glutaric dialdehyde). The response time was within 1–2 s. Such a fast amperometric response is indicative of a fast oxidation of NADH in the overall electrochemical and enzymatic reaction at the modified electrode under investigation. The response was linear over a wide glucose concentration range of *ca.* 50–700 μM (inset of Fig. 3). This dynamic range is much broader than those obtained using glucose oxidase (GOx)-immobilized Ag/CNT/(CHIT) film (0.5–50 μM)[31] and GC/CNT-CHIT-GDI film (5–300 μM)[30] electrodes. The sensitivity of the electrode was also determined to be 96.5 μA cm^{-2} mM^{-1} from the slope of the *I vs.* $C_{glucose}$ plot (inset of Fig. 3) which is higher than those obtained with the GC/CNTs/(PTBO(poly-toluidine blue O))/GOx electrode (14.5 mA M^{-1} cm^{-2})[32] and the Au/GOx/ZnONT electrode (21.7 mA M^{-1} cm^{-2}).[33] The limit of detection (LOD) was estimated to be 0.3 μM which is much lower compared to those of other typical glucose biosensors reported previously.[30,34]

It is well known that some electroactive species in serum such as ascorbic acid (AA) and uric acid (UA) may influence the performance of glucose biosensors.[35,36] The effect of these common interfering species on the sensor response was evaluated and the result is demonstrated in Fig. 4, in which amperometric responses are given typically for the relevant physiological levels (0.5 mM) of glucose, AA and UA. These responses were obtained at 0.0 V by a batch addition of each interfering species after the addition of glucose and finally again by adding 0.25 mM glucose. The addition of UA and AA did not substantially change the signal for glucose. This highly selective response for glucose is attributed to the low detection potential (0.0 V).

The operational stability of the GDH/PPS-SWCNT/EPPG sensor was also examined by a continuous measurement of 0.5 mM glucose at an applied potential of 0.0 V over a period of *ca.* 30 min. Fig. 5 shows a considerably stable amperometric response with a 10% signal drift demonstrating a considerable resistance against so-called electrode fouling. Also, the proposed bioanode showed excellent long-term stability which was evaluated by examining the response of the bioanode for 20 mM glucose concentration, at every 15 days, over a period of 1 month. When the bioanode was not being used to evaluate its stability, it was stored in a dry condition in a refrigerator (4 °C). During the first 2 days, the anodic current response decreased by about 1% and in the next 2 weeks the current response decreased by about 5% of its initial response, and 8% after 1 month. The observed stability of the PPS-SWCNT-based biosensor may be probably due to the surface coating of the three-dimensional network of SWCNTs by the PPS which prevents the denaturation of the enzyme or its desorption from the electrode surface. In addition, a large surface area of the SWCNTs allows a large amount of GDH to be immobilized within the carbon nanotubes assembly, resulting in a CNT-based enzyme reservoir.

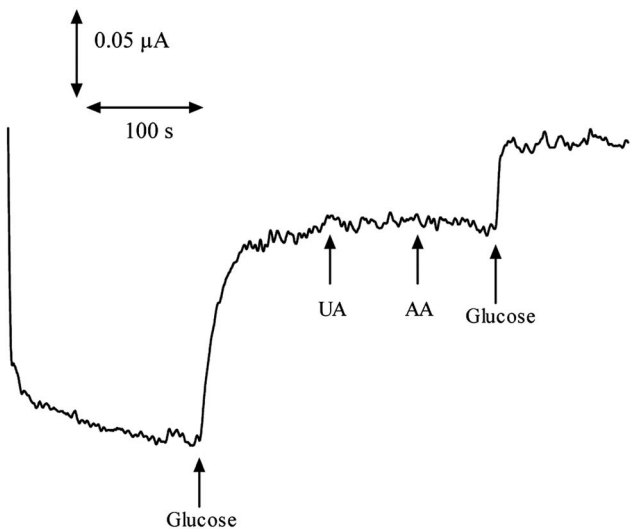


Fig. 4 Amperometric current–time curve depicting the effect of coexisting UA and AA on the measurement of glucose at the GDH/PPS-SWCNT/EPPG electrode. 0.5 mM glucose, 0.5 mM UA, 0.5 mM AA and 0.25 mM glucose were successively added.

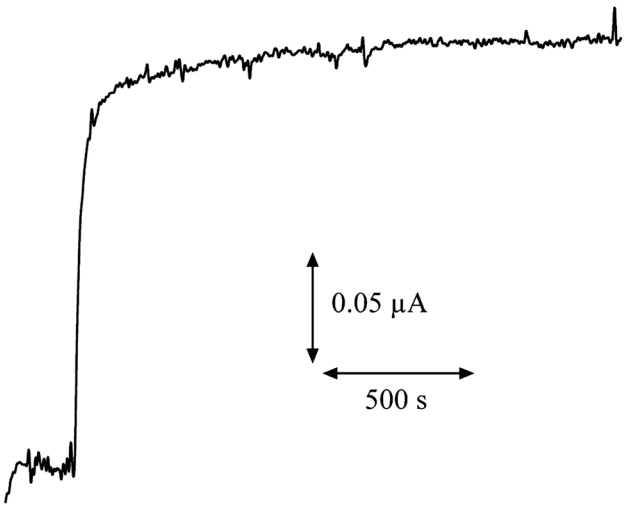


Fig. 5 A continuous recording of the current response of the GDH/PPS-SWCNT/EPPG electrode in 0.1 M PBS (pH 7.0) containing 10 mM NAD^+ and 0.5 mM glucose at an operating potential of 0.0 V. Other experimental conditions are the same as in Fig. 3.

Performance of the assembled glucose/O_2 biofuel cell

A glucose/O_2 biofuel cell was constructed by assembling the as-prepared GDH/PPS-SWCNT/EPPG bioanode and the laccase/MG-SWCNT/EPPG biocathode. The cell voltage V_{cell} was measured as a function of the cell current density j_{cell}, and the power output density P_{cell} given by $j_{cell} \times V_{cell}$ was determined. The polarization curve and the relationship between P_{cell} and j_{cell} (Fig. 6) were obtained for the glucose/O_2 biofuel cell, in which the GDH/PPS-SWCNT/EPPG bioanode and the laccase/MG-SWCNT/EPPG biocathode were immersed in 0.1 M PBS containing 10 mM NAD^+ and 40 mM glucose and in 0.1 M PBS containing 0.46 mM ABTS (O_2-saturated), respectively, and the anodic and cathodic compartments were separated by a salt bridge (0.1 M PBS). The open circuit voltage (OCV) was found to be *ca.* 0.82 V and the maximum power density (P_{cell}^{max}) was estimated as 64.0 μW cm^{-2} at 0.3 V. The obtained value of P_{cell}^{max} is larger compared with the previously reported values (2.1 and 15.8 μW cm^{-2}) for the BFCs composed of GDH/CNT-MDB(meldola's blue) and GOD/CNTs-HA(hydroxyapatite) bioanodes, respectively, and a laccase-based biocathode.[37,38] Although from the practical application point of view, the performance of the prepared glucose/O_2 biofuel cell remains to be improved, this newly developed PPS-SWCNT-based nanocomposite support for GDH immobilization may be applicable to the immobilization of other enzymes for preparing various bioelectrocatalyst electrodes.

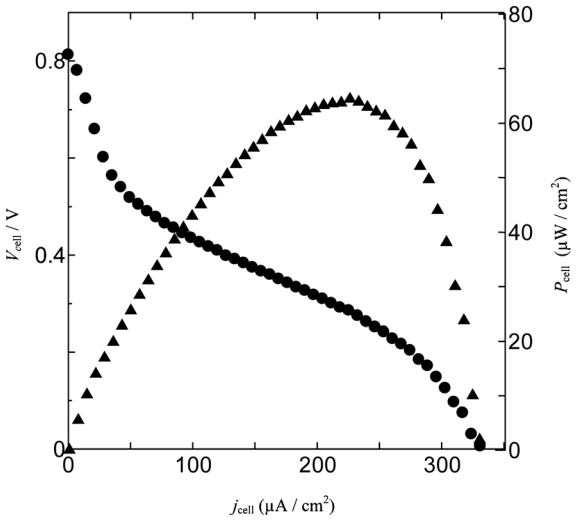


Fig. 6 Polarization curve (●) of the assembled GDH/PPS-SWCNT-based glucose/O_2 BFC in 0.1 M PBS (pH 6.0) anolyte containing 10 mM NAD^+ and 40 mM glucose and 0.1 M PBS (pH 6.0, O_2-saturated) catholyte containing 0.46 mM ABTS and the dependence of the power output (▲) on the current density.

Conclusions

A dehydrogenase enzyme-based glucose sensor, in which the enzyme was immobilized on a PPS-functionalized SWCNT nanocomposite-modified EPPG electrode, was successfully constructed. The molecular assembly of SWCNTs and PPS redox polymer enabled a significantly enhanced electrocatalytic oxidation of NADH, and the GDH/PPS-SWCNT/EPPG electrode was found to electrocatalyze the oxidation of glucose. The fabricated glucose biosensor showed a high sensitivity for glucose (96.5 μA cm^{-2} mM^{-1}) with a linear concentration dependence in the range of 50 to 700 μM at a detection potential of 0.0 V. This sensor also showed a preferential detection of glucose without interferences from the usual interfering substances such as UA and AA. Moreover, the GDH/PPS-SWCNT/EPPG electrode is promising as the bioanode in a glucose/O_2 biofuel cell.

Acknowledgements

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