Biochemical Engineering Journal 112 (2016) 20–31

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular article

Optimization and characterization of covalent immobilization of glucose oxidase for bioelectronic devices

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ARTICLE INFO

Article history:
Received 23 October 2015
Received in revised form 22 March 2016
Accepted 31 March 2016
Available online 1 April 2016

Keywords:
Immobilized enzyme
Multi-walled carbon nanotubes
Optimization
Covalent immobilization
Biosensor
Biofuel cell

ABSTRACT

Enzyme electrodes are widely applied to miniature implantable bioelectronic devices such as biofuel cells and biosensors. The main obstacle associated with miniaturization is the reduced surface area of electrodes for the accommodation of enzymes, leading to poor power output or detection signals. This study aimed to maximize the loading of glucose oxidase (GOx) on the surface of multi-walled carbon nanotubes (MWCNTs), thereby enhancing the generation of electric power or sensing signals. Because the concentrations of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and glucose oxidase significantly affected the immobilization efficiency, these factors were optimized by the Box–Behnken design. The physically adsorbed enzyme was almost completely removed by washing the GOx-bound MWCNTs with buffer solution containing 5 g/L of Tween-20. Enzyme loading was found to be ~3.3 ± 0.3 mg-GOx/mg-MWCNTs under the optimal conditions (430 mM NHS, 52 mM EDC and 8.7 mg/mL GOx). The formation of carboxyl group on the surface of MWCNTs and the covalent bonding between GOx and MWCNTs, and immobilized GOx were observed by FTIR and AFM, respectively. The biochemical analysis showed that the immobilized GOx possesses high activity for the conversion of glucose into gluconic acid. The cyclic voltammetry data showed that the anodic current density of electrodes loaded with the highest amount of GOx was much higher than those of electrodes loaded with smaller amounts of GOx.

1. Introduction

In recent decades, there has been an increasing demand for small implantable biosensors or biofuel cells, causing less tissue damage or enabling patients to avoid surgery. Enzyme electrodes are one of the core components affecting the performance of these devices [1,2]. Electrode size should also be minimized for this purpose, which limits the space available to accommodate enzymes, thereby decreasing the power output or detection signals. The drawbacks associated with miniaturization can be overcome, if enzyme electrodes are fabricated to generate high electrical power or a large number of sensing signals per unit surface area of electrodes. To achieve this goal, electrode materials and immobilization methods should be improved to increase the enzyme loading on the electrode surface with strong binding [3]. Carbon nanotubes (CNTs) have recently attracted considerable attention in the field of bioelectronics, because they provide a large surface area for enzyme immobilization, better acceptable biocompatibility, chemical and electrochemical stability, and good electrical conductivity [4]. Many studies have shown that CNTs can be widely used as the modifying materials for the fabrication of biosensors and biofuel cells, in which the integration of CNTs into electrodes accelerated the electron transfer between electrode and enzymes [5–12]. CNTs were also used as effective electrical wiring/connectors between the electrodes and enzyme redox centers to overcome the kinetic barrier to electron transfer associated with a thick protein layer surrounding the redox center [13]. Noncovalent (adsorption) and covalent immobilization on the surface of CNTs have been reported for various enzymes [10,14–16]. Although physical adsorption methods are simple, and high enzyme loading can be achieved without the addition of reagents, immobilized enzymes can gradually be lost during use, because of weak bonding between the enzymes and the electrode surface [17]. Comparatively, covalent immobilization provides more durable attachment, and thus immobilized enzymes survive over a longer operation

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http://dx.doi.org/10.1016/j.bej.2016.03.016
1369-703X/

time [17]. Therefore, CNT-electrodes covered with enzymes by covalent attachment are suitable for miniaturized implantable devices. However, covalent immobilization requires the chemical modification of CNTs or enzymes, and enzyme loading and activity vary depending on the reaction conditions.

The coupling reaction mediated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) is one of the most attractive approaches for the covalent immobilization of enzymes onto the carboxyl-terminated surfaces in biosensors, because of its high conversion efficiency, mild reaction conditions, and excellent biocompatibility with a slight effect on the activity of target enzymes [18]. Many researchers immobilized enzymes on CNTs using EDC/NHS; however, extremely low enzyme loading was reported by this method. Although the immobilization efficiency is strongly affected by the pH, concentration of EDC and NHS, enzyme concentration, and reaction time [18], these factors for enzyme immobilization were not optimized. Therefore, there is significant room for the improvement in enzyme loading by immobilization using EDC/NHS. Recently, the EDC/NHS-mediated immobilization of biomolecules (amine or dopamine) on gold electrodes was reported, and the effects of EDC and NHS concentration, solution pH, reaction time, and biomolecule concentration on the immobilization efficiency were investigated by cyclic voltammetry [19,20].

This study aimed to maximize the loading of glucose oxidase (GOx) on the surface of multi-walled carbon nanotubes (MWCNTs) by covalent attachment to enhance the generation of electric power or sensing signals. GOx is a family of oxidoreductases that catalyze the oxidation of β-D-glucose to D-gluconolactone and hydrogen peroxide and is commonly used for biosensors monitoring blood glucose and biofuel cells that generate electricity [21]. The MWCNTs were carboxylated by acid treatment, and then GOx was immobilized on the surface of the carboxylated MWCNTs using EDC/NHS. The effects of pH, reaction time, and reactant concentration (EDC, NHS, and GOx) on the immobilization of GOx were investigated. The effect of anionic surfactant, Tween-20, on the removal of physically adsorbed GOx was also investigated. The response surface method was applied to determine the optimal concentrations of EDC, NHS, and GOx. Fourier transform infrared (FTIR) spectroscopy, atomic force microscopy (AFM), and thermogravimetric (TGA) analyses were performed to characterize the carboxylated MWCNTs and GOx-bound MWCNTs. Biochemical and electrochemical analyses were also performed to investigate the performance of immobilized GOx. We demonstrated that GOx were covalently attached to MWCNTs, without physical adsorption, by optimizing the EDC/NHS-mediated immobilization and washing step. The covalently bound GOx amounted to 3.3 mg-GOx/mg-MWCNTs, which is the highest achieved value reported so far. The electrode covered with this immobilized enzyme generated higher current density than those with smaller amount of GOx. This is the first report describing the optimization of EDC-mediated covalent immobilization of GOx on MWCNTs.

2. Materials and methods

2.1. Chemicals

Glucose oxidase from *Aspergillus niger* (product No. G0050) was purchased from TCI (Tokyo, Japan). Pristine MWCNTs (CM 250, purity > 95%) were purchased from Hanwha Chemical Co. Ltd. (Seoul, Korea). Reagent grade sulfuric acid and nitric acid were purchased from Junsei (Tokyo, Japan). *N*-(3-Dimethylaminopropyl)-*N*′-ethyl carbodiimide hydrochloride (EDC-HCl), *N*-hydroxysuccinimide (NHS), *N*-morholinoethane sulfonic acid (MES), Tween-20, poly(ethylene glycol) diglycidyl ether (PEGDGE, average molecular weight 500), horseradish peroxidase (product No. P6140), *o*-dianisidine, urea, and potassium chloride were purchased from Sigma-Aldrich (Missouri, USA). The osmium redox polymer, poly(*N*-vinylimidazole)-[*Os*(4,4′-dimethyl-2,2′-bipyridine)$_2$Cl]$^{+/2+}$ (PVI-*Os*-dme-bpy) was synthesized in our laboratory following previous report [22]. All the solutions were prepared in deionized water (1834 MΩ-cm) if not mentioned.

2.2. Carboxylation of MWCNTs

Pristine MWCNTs powder was placed in a crucible and heated in a closed muffle furnace (J-GAF, Jisico Co. Ltd., Seoul, Korea) at 400 °C for 2 h to burn off the amorphous carbon materials. Because the oxidation temperature of amorphous carbon contaminants is in the range 200–300 °C, most of the amorphous carbon is removed by oxidation upon heating to 350 °C [23–25]. The air-oxidized sample was assigned as MWCNTs. Then, 1 mg of the MWCNTs was dispersed in a glass tube containing 5 mL mixture of concentrated sulfuric acid and nitric acid (3:1, v/v) according to the published method [8,10,26]. This slurry was sonicated in an ultrasonic bath (160HT, Soniclean Pty Ltd, Seoul, Korea) for 2.5 h. The product was then centrifuged three times consecutively for 10 min at 1106 xg, and the pellet was washed with 20 mL of 100 mM MES buffer (pH 6.0) between each centrifugation. The resulting pellet was used as the immobilization matrix for the next step.

2.3. Optimization of conditions for covalent attachment of GOx on MWCNTs

The immobilization of GOx onto MWCNTs using EDC and NHS was performed according to the published procedures [15] with some modification: acid treated MWCNTs were suspended in 10 mL of 100 mM MES buffer (pH 6.0) containing different concentrations of EDC and NHS, and then the mixture was incubated at 25 °C for 3 h by shaking at 160 rpm. The slurry was centrifuged, and the resulting solid was rinsed thoroughly with the same buffer to remove unreacted EDC, NHS, and by-product urea. The activated MWCNTs were dispersed in 10 mL of 100 mM MES buffer (pH 6.0) containing different concentrations of GOx. After incubating the mixture at 160 rpm for 3 h at 25 °C, the GOx-bound MWCNTs (GOx-MWCNTs) were recovered by centrifugation and rinsed with MES buffer several times to remove any unbound or weakly bound GOx. The Box–Behnken design was applied to optimize the concentrations of EDC, NHS, and GOx. Seventeen experiments were performed according to this design. The regression analysis of the experimental data was conducted using SAS version 9.1 software (SAS Institute, Cary, NC, USA), and the optimal levels of the three factors were obtained by solving a second-order polynomial equation and analyzing the response surface contour plots.

2.4. Assay for determining the activity of immobilized GOx

1 mL of the mixture containing 23 mM of glucose and GOx-MWCNTs in 100 mM MES buffer (pH 6.0) was incubated at 37 °C for 3 h, and then the mixture was boiled for 10 min to denature the enzyme, thereby terminating the reaction. The assay was also performed with a control, prepared with the activated MWCNTs but without GOx. The activity of the immobilized GOx was determined by monitoring the generation of gluconic acid by thin-layer chromatography (TLC). The supernatant taken from the reaction mixture was spotted onto silica gel thin-layer sheets 60F_{254} (Merck, Daemstadt, Germany) and dried. Chromatograms were developed using an ascending solvent of 1-butanol-methanol-$CHCl_3$-25% ammonia solution (4:5:2:5, V/V/V/V) and dried overnight at room