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

Xue Wang [a], Sung Bae Kim [a], Dongwoo Khang [b], Hyug-Han Kim [c], Chang-Joon Kim [a,*]

[a] Department of Chemical Engineering and RIGET, Gyeongsang National University, Jinju, Republic of Korea
[b] Department of Medicine, Gachon University, Incheon, Republic of Korea
[c] Department of Chemistry, Dankook University, Cheonan, Republic of Korea

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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

* Corresponding author at: Department of Chemical Engineering and RIGET, Gyeongsang National University, 501 Jinju-daero, Jinju, Gyeongnam 660-701, Republic of Korea.
E-mail address: cj_kim@gnu.ac.kr (C.-J. Kim).

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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

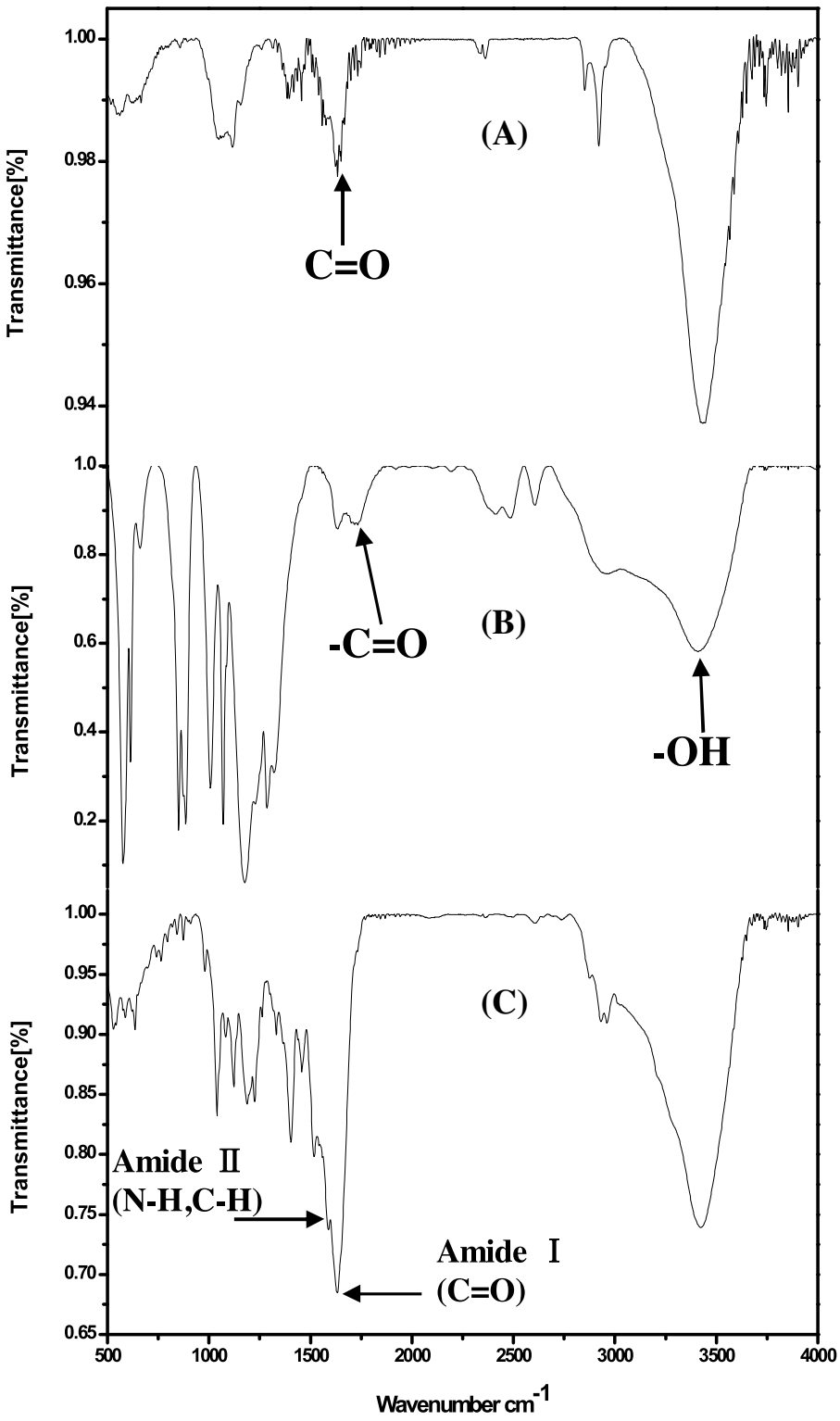

Fig. 1. FTIR spectra of (A) MWCNTs, (B) acid-treated MWCNTs, and (C) GOx-MWCNTs.

temperature. The spots were visualized using a reagent containing cerium and molybdate: 25 g phosphomolybdic acid, 10 g Cerium (IV) sulfate, and 60 mL H_2SO_4 in 1000 mL of distilled water [27]. Quantitative determination of gluconic acid formed as well as residual glucose in reaction mixture containing GOx-MWCNTs was performed. Gluconic acid concentration was measured using the D-gluconic acid/D-glucono-δ-lactone assay kit (Megazyme, Wicklow, Ireland). Briefly, the assay involved the phosphorylation of D-gluconic acid into D-gluconate-6-phosphate by adenosine-5′-triphosphate (ATP) and gluconate kinase (GCK), followed by the oxidative decarboxylation in the presence of nicotinamide-adenine dinucleotide phosphate ($NADP^+$) by 6-phosphogluconate dehydrogenase (6-PGDH) to ribulose-5-phosphate with the formation of NADPH. The amount of NADPH formed in this reaction is stoichiometric with the amount of D-gluconic acid in sample, and was measured by the increase in absorbance at 340 nm using

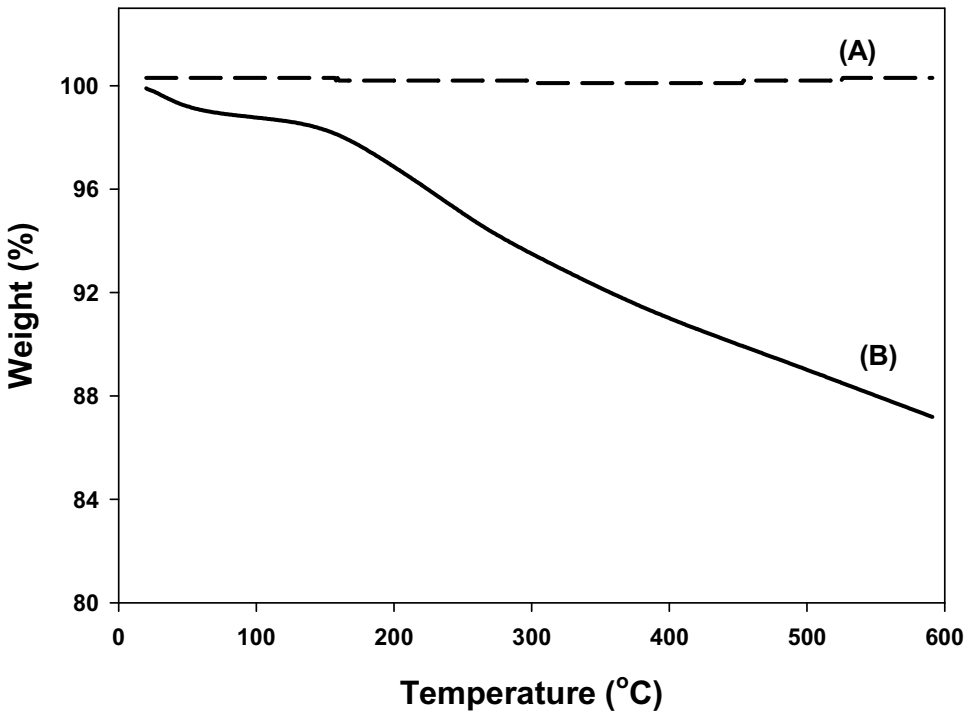

Fig. 2. TGA curves of (A) MWCNTs and (B) acid-treated MWCNTs.

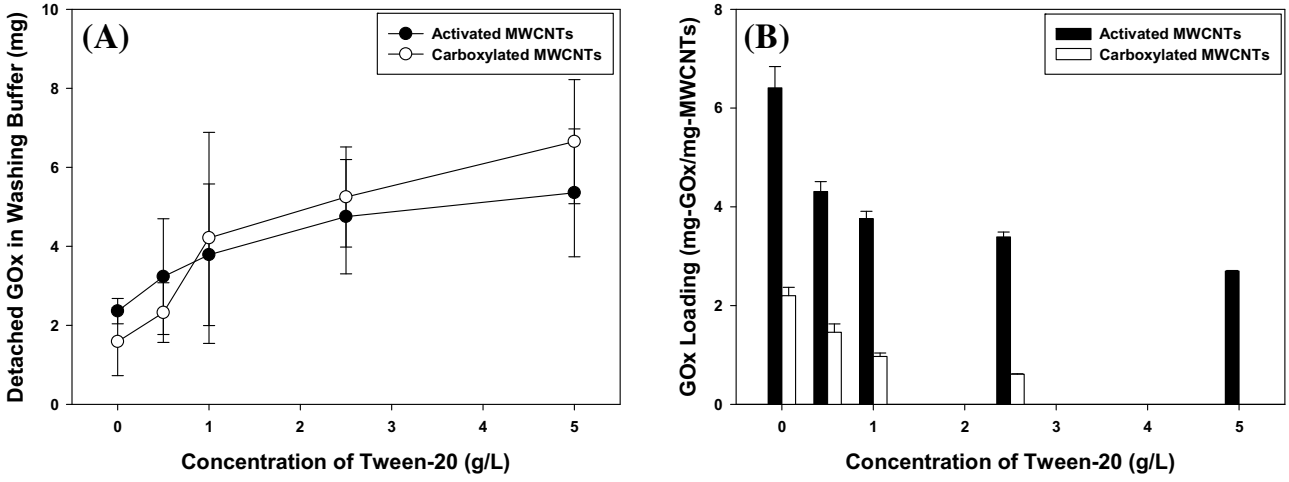

Fig. 3. The effect of Tween-20 on the adsorption of GOx on MWCNTs. The GOx was immobilized onto carboxylated MWCNTs (open symbols) and MWCNTs activated with EDC/NHS (filled symbols). The detached GOx by washing (A) and GOx loading (B) are shown.

UV/Vis spectrophotometer (Hewlett-Packard, Palo Alto, CA, USA). The assay mixture contained buffer (pH 7.6), $NADP^+$, ATP, 6-PGDH, GCK, and supernatant of reaction mixture. The concentration of residual glucose was measured by hexokinase method using the commercial assay kit (GDL Korea, Gyeonggi, Korea). In principle, glucose is converted into 6-phosphogluconate by hexokinase (HK) and glucose-6-phosphate-dehydrogenase (G6PDH). During these reactions, equimolar amount of NAD^+ is reduced to NADH. The increase in absorbance at 340 nm was measured and this increase is directly proportional to the glucose concentration. Assay mixture contained Tris-buffer (pH 7.5), ATP, HK, G6PDH, NAD^+, and supernatant of reaction mixture.

To examine the effect of Tween-20 on the activities of free- and immobilized GOx, a coupled-enzyme reaction, using horseradish peroxidase and *o*-dianisidine, was applied. The method is briefly described here. The assay mixture was prepared by mixing 2.5 mL of 1% *o*-dianisidine in 0.1 M potassium phosphate buffer (pH 6.0), 0.3 mL of 18% glucose solution, and 0.1 mL of 0.2 mg/mL of peroxidase. The mixture was saturated with oxygen by bubbling air for 30 min before use. The GOx solution was prepared in a buffer without (control) or with Tween-20 (5 g/L). Upon the addition of 0.1 mL of 0.1 mg/mL of GOx solution into the mixture, assay was started. The immobilized GOx washed by buffer with or without Tween-20 was dispersed in MES buffer. The suspension (40 μL) was dropped on a glass surface (0.6 cm × 3 cm, Paul Marienfeld GmbH & Co. KG, Germany) and then dried for 12 h at 4 °C. The glass coated with the immobilized GOx was immersed in 2.9 mL of the assay mixture in a spectrophotometer cuvette. The increase in the absorbance at 460 nm was measured once per minute using a UV/Vis spectrophotometer, and then the specific activity was calculated from the initial linear portion of the curve and GOx content.

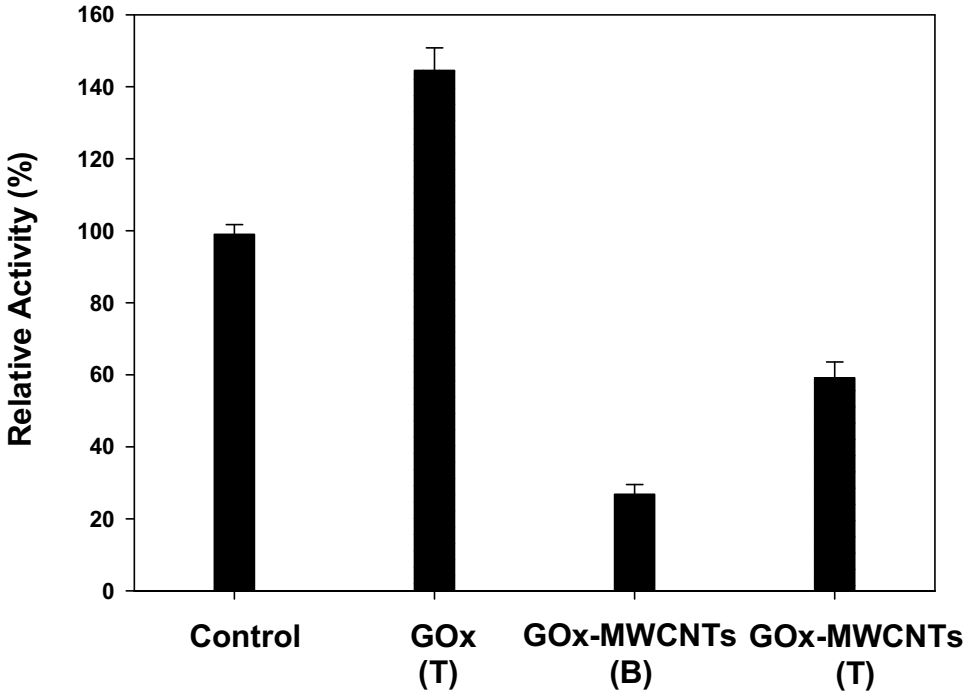

Fig. 4. Effect of Tween-20 on the activities of free- and immobilized GOx. Control and GOx (T) represent free GOx incubated in the absence and presence of Tween-20, respectively. The GOx-MWCNTs (B) and GOx-MWCNTs (T) are immobilized GOx washed by buffer only and buffer containing 5 g/L of Tween-20, respectively.

2.5. Electrode preparation and electrochemical measurement

Working electrodes were prepared according to the previously described procedure [28–30]: stock solutions of PVI-*Os*-dme-bpy (5 mg/mL), MWCNTs or GOx-MWCNTs (10 mg/mL), and PEGDGE (10 mg/mL) were mixed at a ratio of 4:4:1 (v:v:v). The mixture (5 μL) was then dropped on the surface of screen-printed carbon electrodes (SPCEs) with a diameter of 4.0 mm and then dried for 12 h at room temperature. The SPCEs were prepared by screen-printing on a flexible polyester film with hydrophilic carbon ink (Electrodag 423SS from Acheson, Port Huron, Mi, USA) using a screen printing machine (BS-860AP, Bando, Gyeonggi, South Korea) [31]. A platinum wire and Ag/AgCl (saturated KCl) were used as the counter and reference electrodes, respectively. Phosphate-buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.0) supplemented with glucose was used as the electrolyte. Electrochemical measurements were carried out using cyclic voltammetry by potential scanning from −0.2 V to 0.6 V at a scan rate of 10 mV/s in a Faraday cage with a CH Instruments model 660d electrochemical workstation (CH Instrument, Inc. Houston, USA) interfaced to a computer.

2.6. Analyses

TGA was performed using a Q600 thermo-gravimetric analyzer (TA Instruments, Delaware, USA) following the procedure described by Gao et al. [32] and Jimeno et al. [33]. Weighted MWCNTs were placed in aluminum crucibles, and an empty aluminum crucible was used as the reference. The samples were heated over a temperature range 20–600 °C at a heating rate of 20 °C/min under a nitrogen atmosphere with a flow rate of 60 mL/min. The reductions in the weight of MWCNTs and acid-treated MWCNTs were measured. All the samples were dried under vacuum before analysis. Fourier transform infrared (FTIR) spectra were obtained using a Vertex 80 V spectrometer (Bruker, USA). The surface morphologies of MWCNTs or GOx-MWCNTs were examined in ambient condition by tapping-mode atomic force microscope (Nanoscope IV Multimode AFM, Bruker, USA) with silicon AFM probe (Nanoworld, Switzerland) at the Korean Basic Science Institute (Jeonju, Korea). Small amount of samples was taken and then diluted with deionized water. The sample solutions were dropped on fresh mica surface and left to dry at ambient condition prior to analysis. AFM scans (2 × 2 μm) were performed on three surface positions and two representative images were obtained for each sample. The thickness of each sample was determined using Nanoscope V531r1.

Table 1
Effects of NHS, EDC and GOx concentrations on enzyme loading.

Run	Coupling	Activation		GOx loading (mg-GOx/mg-MWCNTs)
	GOx (mg/mL)	NHS (mM)	EDC (mM)	
1	0.6	0	0	0.09 ± 0.02
2	0.6	0	26	0.36 ± 0.03
3	0.6	4.3	0	0.48 ± 0.02
4	0.6	4.3	5.2	0.60 ± 0.04
5	0.6	4.3	26	0.75 ± 0.02
6	0.6	86.8	26	0.89 ± 0.03
7	1.7	86.8	26	1.25 ± 0.10

For entire experiment, the amount of immobilized enzyme was estimated by the difference between the amount of enzyme into the reaction mixture and enzyme remaining in the supernatant after the coupling reaction and desorbed during washing cycle [34]. The amount of enzyme was determined by measuring the protein content of enzyme solution using a commercial kit (Thermo Scientific, Rockford, IL, USA) following the protocol supplied by the company. The GOx loading was expressed as the amount of immobilized enzyme per weight of MWCNTs (mg-GOx/mg-MWCNTs). Alternatively, the procedure described by Gooding et al. [35] was also applied to measure the GOx loading of the optimized electrode to confirm the assay by the above-mentioned method: briefly, GOx-MWCNTs derived from 1 mg of MWCNTs were soaked in 10 mL of solution containing 8 M urea and 0.05 M KCl for 2 h. The concentration of the released flavin adenine dinucleotide (FAD) was measured using a fluorescence spectrometer (LS-50B, PerkinElmer,

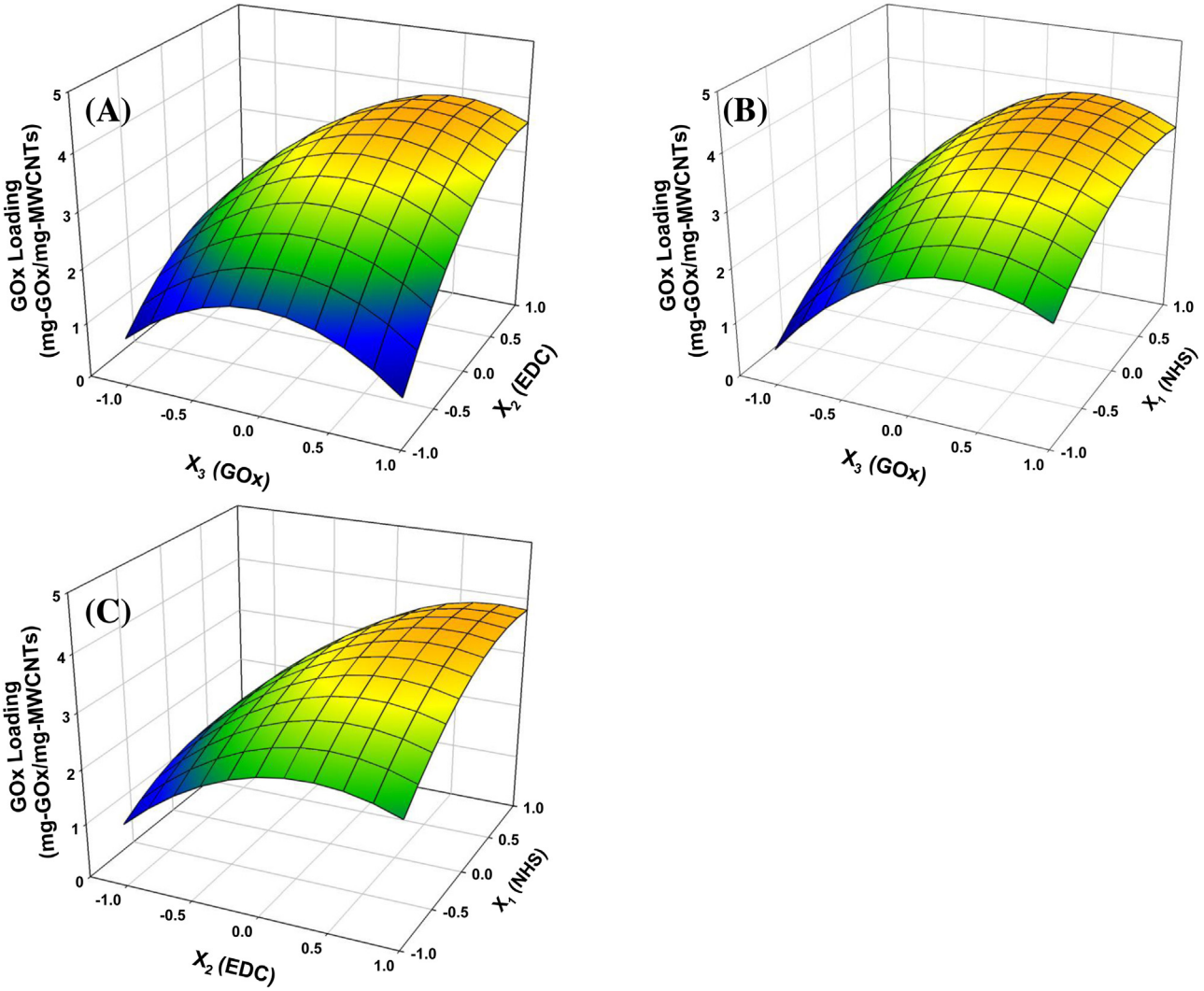

Fig. 5. The surface response plot showing the effect on Y (GOx loading) of (A) X_2 (EDC) and X_3 (GOx) at X_1 (NHS) = 0.68, (B) X_1 (NHS) and X_3 (GOx) at X_2 (EDC) = 0.62, and (C) X_1 (NHS) and X_2 (EDC) at X_3 (GOx) = 0.6.

USA), and the measured concentrations were related to the amount of immobilized GOx. Fluorometric measurements were performed at an excitation wavelength of 375 nm, and the fluorescence intensity was measured at 520 nm. The calibration curve prepared from a standard solution of FAD was linear up to 60 nM.

3. Results and discussion

3.1. Characterization of acid-treated MWCNTs using FTIR and TGA analysis

Because of the chemical inertness of MWCNTs, it is essential to create reactive groups on the surface for further modification [15]. The carboxyl groups on the MWCNTs provide an ideal anchoring point for the covalent immobilization of enzyme using EDC/NHS [15]. It has been demonstrated that sonicating MWCNTs in a mixture of acid solution opens the tube caps and forms carboxyl groups at the defect sites along the side walls [36].

Fig. 1 shows the FTIR spectra of MWCNTs and acid-treated MWCNTs. The appearance of sharp peaks at 1642 cm^{-1} indicates the presence of carbonyl groups (C═O) of the quinine type units along the side walls of the nanotubes [37], and its intensity decreased after acidification. Two noticeable peaks appeared at 1737 and 3409 cm^{-1} on the acid-treated MWCNTs, corresponding to the carbonyl (C═O) and hydroxyl (OH) stretching bands of carboxyl groups, respectively [8,37–39]. This clearly indicates that carboxyl groups were successfully formed on the surface of MWCNTs by the acid treatment.

Because the efficiency of covalent immobilization of GOx depends on the degree of MWCNTs carboxylation, TGA was performed to determine the amount of carboxyl groups on the surface of carboxylated MWCNTs. Fig. 2 shows the TGA curves of MWCNTs and carboxylated MWCNTs. If a high amount of amorphous carbon is present, appreciable weight loss should occur below 400 °C. However, no detectable weight loss of (air-oxidized) MWCNTs was observed, indicating only a few organic impurities such as amorphous carbon and high thermal stability [32,33,37]. Notably, the carboxylated MWCNTs demonstrated a considerable decrease in the weight at temperatures >190 °C. The weight loss of carboxylated MWCNTs was attributed to the decomposition of carboxyl group into CO_2 [32,33]. Based on the TGA data of the MWCNTs and carboxylated MWCNTs, the degree of carboxylation of MWCNTs was calculated. In the temperature range 200–480 °C, the weight loss of carboxylated MWCNTs was 9.1 ± 0.9%, which corresponds to 0.6 ± 0.1 mmol of carboxyl group per gram of carboxylated MWCNTs according to the relationship described by Gao et al. [32]. This value is similar to the data reported by Gao et al. [32].

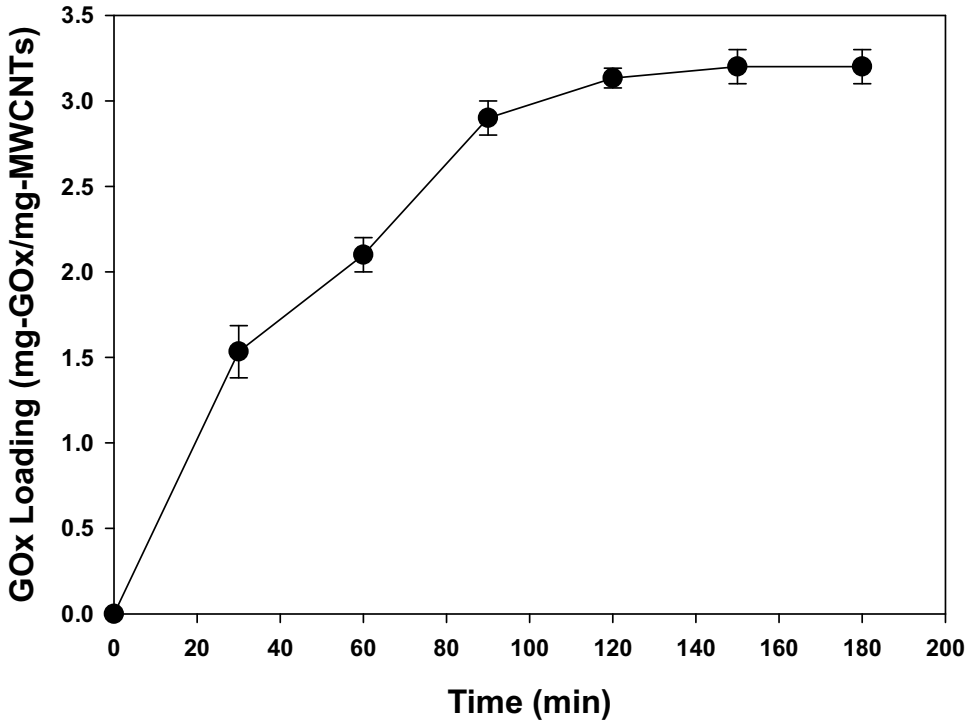

Fig. 6. Time profiles of GOx immobilization on MWCNTs activated with EDC/NHS under the optimal conditions.

Table 2
Experimental runs of the Box–Behnken design for maximizing loading of glucose oxidase.

Runs	X_1		X_2		X_3		Y (mg-GOx/mg-MWCNTs)	
	Coded	Uncoded (mM)	Coded	Uncoded (mM)	Coded	Uncoded (mg/mL)	Experiment	Calculation
1	+1	500	+1	60	0	6	3.6	3.3
2	+1	500	−1	20	0	6	1.7	2.0
3	−1	80	+1	60	0	6	2.5	2.4
4	−1	80	−1	20	0	6	1.0	1.2
5	+1	500	0	40	+1	10.5	3.3	3.2
6	+1	500	0	40	−1	1.5	1.8	1.6
7	−1	80	0	40	+1	10.5	1.9	1.7
8	−1	80	0	40	−1	1.5	1.7	1.4
9	0	290	+1	60	+1	10.5	3.3	3.3
10	0	290	+1	60	−1	1.5	1.3	1.4
11	0	290	−1	20	+1	10.5	1.5	1.1
12	0	290	−1	20	−1	1.5	1.4	1.1
13	0	290	0	40	0	6	3.4	3.5
14	0	290	0	40	0	6	3.5	3.5
15	0	290	0	40	0	6	3.4	3.5
16	0	290	0	40	0	6	3.6	3.5
17	0	290	0	40	0	6	3.6	3.5

X_1, NHS concentration; X_2, *EDC* concentration; X_3, GOx concentration; *Y*, GOx loading.

3.2. Effects of EDC, NHS, and GOx concentrations on immobilization

EDC and NHS are good crosslinkers for GOx immobilization. In general, one of the EDC double bonds reacts with a carboxyl group to form an amine-reactive *O*-acylisourea intermediate, which is prone to hydrolysis to regenerate the acid group, and the rearrangement of *O*-acylisourea forms unreactive *N*-acylisourea. The reaction of NHS with *O*-acylisourea affords NHS-ester, which is more reactive towards primary amines and more resistant to hydrolysis, and it also undergoes minimal formation of *N*-acylurea [40]. Therefore, NHS is usually used with EDC to increase the efficiency of EDC-mediated enzyme immobilization. The formation of NHS-ester is critically affected by pH; the carboxyl groups have poor reactivity to EDC/NHS at pH < 4.5, and NHS-esters are more susceptible to hydrolysis at alkaline pH values [18–20,41]. The amidation reaction of NHS-ester with amino group is known to be most efficient at the pH values between 6 and 8; amino groups exhibit poor nucleophilicity to NHS-ester at low pH, because of the formation of protonated amino groups, but amino groups have strong nucleophilicity, whereas NHS esters are unstable at high pH [18–20,41].

In this study, a two-step immobilization procedure was used: activation of carboxylated MWCNTs (NHS-ester formation) and coupling of GOx onto MWCNTs via the NHS-ester. The carboxylated MWCNTs were reacted with EDC/NHS at pH 6.0 for 3 h. The coupling reactions were performed at two pH levels (pH 6.0 and pH 7.4) for 3 h to investigate the effect of pH. A slightly higher amount of GOx was immobilized at pH 6.0 than at pH 7.4 (data not shown). Based on these experiments, the activation of carboxylated MWC-

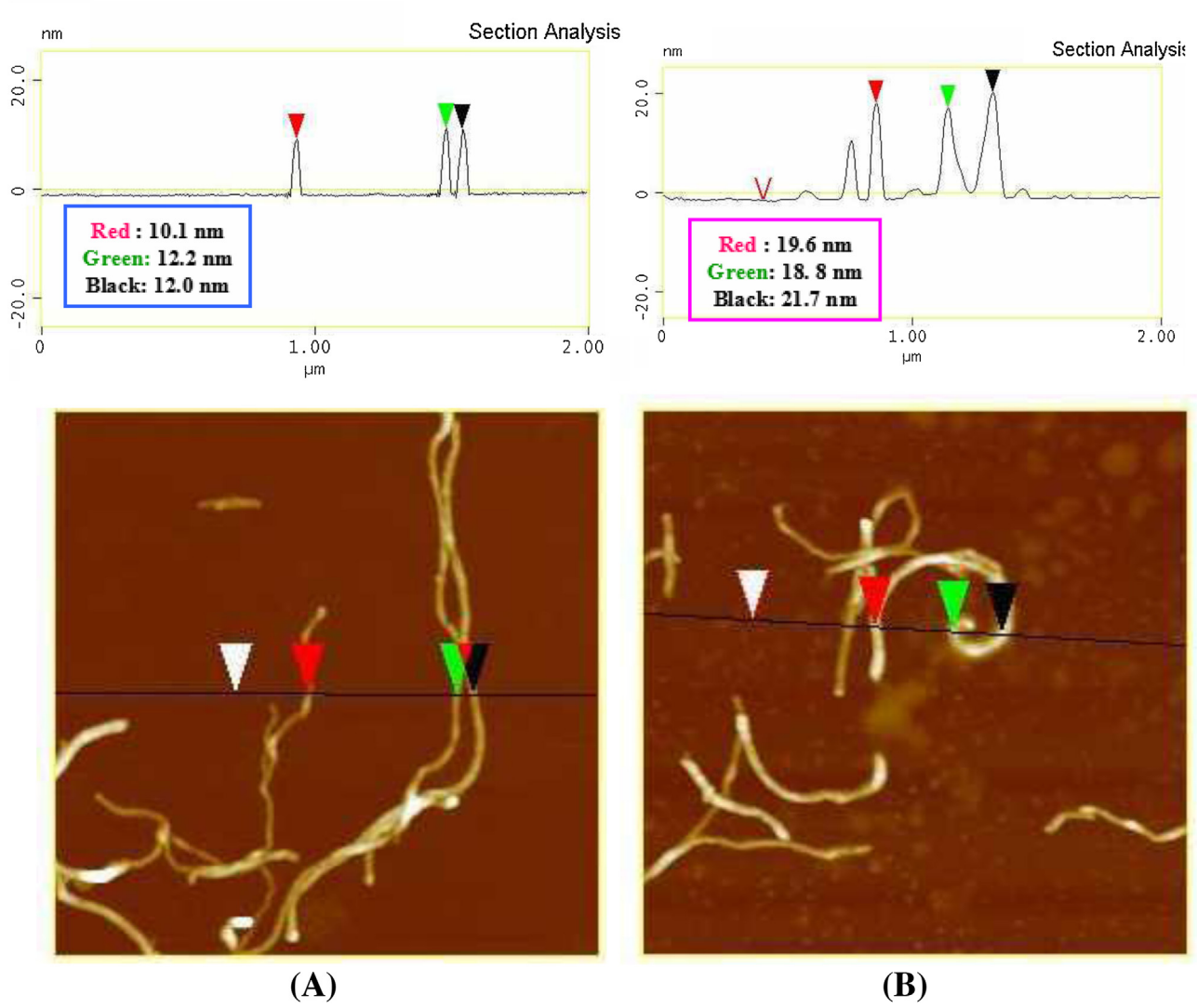

Fig. 7. AFM images of (A) MWCNTs and (B) GOx-attached MWCNTs. Scan range was 2 × 2 μm.

NTs and the coupling reaction were performed at pH 6.0 for 3 h in later experiments.

In addition to pH, the concentrations of EDC, NHS, and GOx affect the immobilization efficiency [18–20,40,41]. Therefore, preliminary experiments were performed to examine their effects. The carboxylated MWCNTs were activated with different concentrations of EDC and NHS and then reacted with 0.6 mg/mL of GOx. As shown in Table 1, a very small amount of GOx was attached to MWCNTs without activation (Run 1), but a four-fold greater amount was attached when MWCNTs were activated by only EDC (Run 2). A comparable amount of GOx was also attached to MWCNTs treated with only NHS (Run 3). A greater amount of GOx was attached when MWCNTs were activated by EDC in the presence of NHS (Run 4) than by EDC or NHS only. Furthermore, the activation of MWCNTs with increasing concentrations of EDC and NHS increased the attachment of GOx (Runs 5 and 6). A further increase in the attachment of GOx was also observed when a GOx solution of a higher concentration (1.7 mg/mL) was added to the activated MWCNTs (Run 7).

According to Wang et al. [40], a small amount of anhydride and *N*-acyl urea was formed, and most of the carboxyl groups still remained unreacted with 5 mM EDC and 10 mM NHS. They described that NHS-ester formation was dominated when the concentrations increased to 100 mM EDC and 200 mM NHS. However, they did not observe any significant improvement in the NHS-ester formation and the activation efficiency decreased at very high concentrations (500 mM EDC and 1000 mM NHS) because of the precipitation of the reactants on the surface, thereby blocking the surface reaction. Together, this demonstration and the experimental results clearly indicate the need for optimizing concentrations of these materials in the activation and the coupling reaction for maximizing the covalent attachment of GOx on the surface of MWCNTs.

3.3. Removal of physically adsorbed GOx using Tween-20

Though protein immobilization using EDC/NHS has often been performed, previous studies have not included clear evidence for covalent attachment. Furthermore, it has been argued that proteins can physically adsorb onto MWCNTs in the EDC/NHS-mediated immobilization [15]. Therefore, it was necessary to investigate whether the observed GOx immobilization occurred through covalent attachment or physical adsorption. The inclusion of Tween-20 in the post-reaction washes or its addition to the reaction mixture is known to effectively remove any physically adsorbed proteins during immobilization [42]. Accordingly, the effect of Tween-20 was examined for removing physically adsorbed GOx: the carboxylated MWCNTs were activated by EDC (47 mM) and NHS (165 mM) and then reacted with GOx (3.5 mg/mL). After separa-

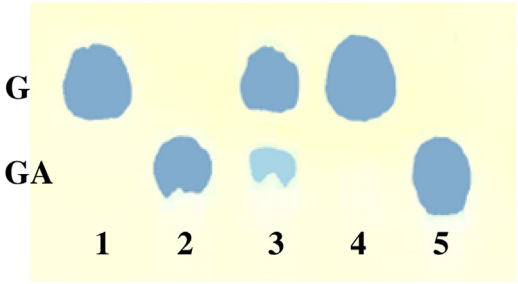

Fig. 8. TLC analyses of the reaction products by GOx-attached MWCNTs; Lane 1, glucose standard (2 mg/mL); lane 2, gluconic acid standard (2 mg/mL); lane 3, mixture of glucose (1 mg/mL) and gluconic acid standards (1 mg/mL); lane 4-5, the reaction mixture in the presence of MWCNTs without GOx (control) and GOx-attached MWCNTs after incubation at 37 °C for 3 h, respectively. G and GA represent glucose and gluconic acid, respectively.

tion, GOx-MWCNTs were washed with buffer containing different concentrations (0–5 g/L) of Tween-20, and GOx detachment was monitored. As shown in Fig. 3, an increase in the detachment of GOx was observed from GOx-MWCNTs as the concentration of Tween-20 increased. GOx loading was only 2.7 ± 0.1 mg-GOx/mg-MWCNTs after washing with buffer containing 5 g/L of Tween-20, whereas a value of 6.4 ± 0.4 mg-GOx/mg-MWCNTs was observed after washing with buffer only. For comparison, GOx immobilization was also performed in the same conditions except that the carboxylated MWCNTs were not activated by EDC/NHS. GOx loading was 2.2 ± 0.2 mg-GOx/mg-MWCNTs after washing with buffer only, and it decreased by increasing the concentration of Tween-20 in the washing buffer, leading to the complete removal of GOx at 5 g/L of Tween-20. Proteins are generally adsorbed through hydrophobic interaction, and these proteins can be effectively removed by washing with buffer containing Tween-20 in the range of 1–5 g/L [42–45]. Overall, GOx can be attached on the carboxylated MWCNTs by physical adsorption without activation, and only 42% of bound GOx can be covalently attached, even when MWCNTs were activated by EDC/NHS. The results also show that only covalently bound GOx can be obtained by washing GOx-MWCNTs with buffer containing 5 g/L of Tween-20 after the EDC/NHS-mediated immobilization.

Even though Tween-20 is a biocompatible detergent, the amount of detergent that remained after washing may adversely affect the enzyme activity. Enzyme assays were performed to examine the effect of Tween-20 on the activities of immobilized GOx washed with buffer only (GOx-MWCNTs (B)) or buffer containing 5 g/L of Tween-20 (GOx-MWCNTs (T)). The result is shown in Fig. 4, in which the relative activity represents the ratio of specific activity of GOx-MWCNTs to the specific activity of the control, free GOx incubated in the absence of Tween-20. The activity of GOx-MWCNTs (B) was 27 ± 3% of the control activity. It has been generally accepted that the reduction in the activity of immobilized enzyme is possible, because of the internal and external mass transfer, and/or a shift in the optimal condition required for the substrate conversion [46]. Notably, the activity of GOx-MWCNTs (T) was 2.2-fold higher than that of GOx-MWCNTs (B), indicating that the addition of Tween-20 in washing buffer can significantly enhance the activity of GOx-MWCNTs. A 44 ± 6% increase in the GOx activity was also observed, when free GOx was incubated in the presence of Tween-20 (167 μg/mL) for comparison (GOx-T). The enhancement of enzyme activity by Tween-20 was previously demonstrated for the reactions by cellulase [47,48], lipase [49], and 1,2-diacylglycerol phosphocholine transferase [50]. These studies explained that the enzymes were stabilized, and biospecific adsorption of enzyme onto substrate increased in the presence of Tween-20. This is the first demonstration of the positive effect of Tween-20 on free- and immobilized GOx, and further study is required to elucidate the role of Tween-20 on reaction mediated by glucose oxidase.

This result clearly indicates that Tween-20 did not have any detrimental effect on the activity of the enzyme, and the use of Tween-20 during enzyme immobilization is key to minimize the physical adsorption of the enzyme to MWCNTs without loss of enzyme activity. Accordingly, GOx-MWCNTs were washed with buffer containing 5 g/L of Tween-20 after immobilization in the following experiments.

Table 3
Estimated regression coefficients and significance test for the second-order model.

Sources	Sum of squares	D.F.*	Mean squares	F-value	Prob. > F
Model	15.0991	9	1.6777	29.01	<0.0001
$\mathbf{X_1}$	1.4281	1	1.4281	24.69	0.0016
$\mathbf{X_2}$	3.1375	1	3.1375	54.25	0.0002
$\mathbf{X_3}$	1.8915	1	1.8915	32.71	0.0007
$\mathbf{X_1X_2}$	0.0506	1	0.0506	0.88	0.3806**
$\mathbf{X_1X_3}$	0.4422	1	0.4422	7.65	0.0279
$\mathbf{X_2X_3}$	0.8281	1	0.8281	14.32	0.0069
$\mathbf{X_1^2}$	1.5233	1	1.5233	26.34	0.0014
$\mathbf{X_2^2}$	2.9494	1	2.9494	51.00	0.0002
$\mathbf{X_3^2}$	2.8484	1	2.8484	49.25	0.0002
Error	0.4049	7	0.0578		
Corrected Total	15.5040	16			

* D.F.: Degree of freedom.
** Not significant.

3.4. Determining the optimal concentrations of NHS, EDC, and GOx using a statistical approach

As mentioned above, increase in the concentrations of EDC, NHS, and GOx in immobilization concomitantly increased the GOx attachment on MWCNTs. Therefore, experiments were performed to optimize these three factors according to the Box–Behnken design (BBD). The design matrix and corresponding results from separate experiments are listed in Table 2. The regression analysis of the experimental data was performed using a SAS program, which produced a second-order polynomial equation. The overall formulated model equation for GOx immobilization is as follows:

$$Y = 3.500 + 0.423X_1 + 0.626X_2 + 0.486X_3 - 0.510X_1^2 - 0.772X_2^2 - 1.022X_3^2 + 0.333X_1X_3 + 0.455X_2X_3$$

where Y is GOx loading, and X_1, X_2, and X_3 are the coded values of concentrations of NHS, EDC, and glucose oxidase, respectively.

Table 3 shows the verification of the model based on the results of an F-test and ANOVA. The value of $P_{model} > F$ was <0.0001, indicating that the results produced by the model were highly significant. The coefficient of determination (R^2) was 0.974, when the goodness of fit of the model was tested, indicating that only 2.6% of the total variations were by chance, and thus were not explained by the model. All the terms of model equation were statistically significant ($P < 0.05$) except X_1X_2. The accuracy of the model was also verified by the data listed in Table 2, indicating that the observed and predicted values for the immobilization were almost equal. The signs of the coefficients of the factors in the model equation indicated their relative effects, in which the positive sign for X_1, X_2, and X_3 indicates that a higher response can be obtained if the values of these factors are greater than those of the center point. In addition, the presence of the positive interactions between X_1 and X_3 as well as X_2 and X_3 represents the synergistic relationship. The surface response plot was drawn based on the model equation, which clearly shows the effect of the three factors on GOx immobilization as shown in Fig. 5. These plots indicate that the maximal amount of GOx was attached when the carboxylated MWCNTs were activated with 430 mM of NHS and 52 mM of EDC

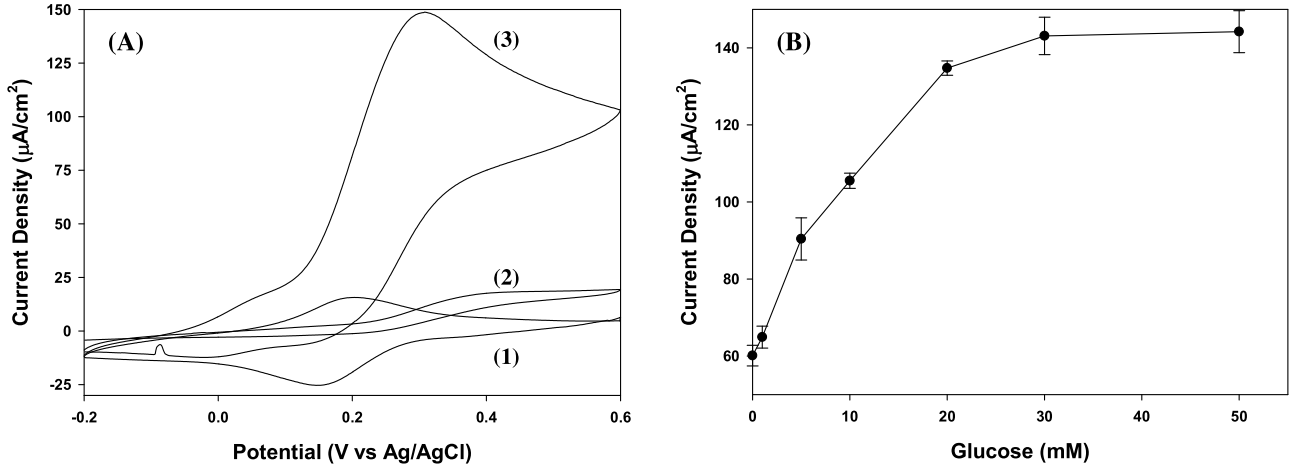

Fig. 9. (A) Cyclic voltammograms of GOx-MWCNT-SPCEs in PBS solution supplemented with 30 mM of glucose; (1) MWCNT-SPCEs, (2) 0.24- GOx-MWCNT-SPCEs (0.24 mg-GOx/mg-MWCNTs), (3) 3.3-GOx-MWCNT-SPCEs (3.3 mg-GOx/mg-MWCNTs). (B) Current density at 0.3 V (vs. Ag/AgCl) for 3.3-GOx-MWCNT-SPCEs (3.3 mg-GOx/mg-MWCNTs) as a function of glucose concentration. All the electrodes were prepared by drop coating of mixture (GOx-MWCNT (or MWCNT)- PVI-*Os*-dme-bpy—PEGDGE) onto the SPCEs. Scan rate was 10 mV/s. Each curve represents the average of triplicate measurements.

and then reacted in 8.7 mg/mL of GOx solution. Experiments were performed in triplicate under these conditions. The observed GOx loading was 3.3 ± 0.3 mg-GOx/mg-MWCNTs. This result shows a good agreement between the experimental and simulation results. Next, the reaction time for coupling was determined in terms of the attached amount of GOx under the optimal conditions. The coupling reaction was almost finished in 2 h and that prolonging the reaction time up to 3 h slightly increased the attachment of GOx (Fig. 6).

Enzyme loadings were reported to be in the range 1.0–2.0 pmol per cm^2 of gold electrode [35], 0.24 mg per mg of SWCNTs [16], and 2.5 mg per mg of SWCNTs [34] when GOx was covalently immobilized on the electrodes via EDC/NHS chemistry. The covalent immobilizations of GOx onto gelatin-multiwalled carbon nanotube modified glassy carbon electrode (3.88 nmol–GOx/cm^2 electrode) and poly(glycidyl methacrylate)-Si(111) hybrids (0.23 mg-GOx/cm^2 electrode) were also demonstrated [9,51]. Considering that the molecular weight of glucose oxidase is 160 kDa [21], the maximum GOx loading (3.3 ± 0.3 mg-GOx/mg-MWCNTs) obtained in this study corresponds to 21 nmol of GOx per mg of MWCNTs. This is one of the highest amounts of enzyme, which was immobilized by covalent binding to our knowledge. It was important to find whether the loading value was obtained correctly or not. As mentioned above, GOx loading was estimated by measuring the difference in the protein concentration of the solution before and after the coupling reaction. An alternative method was also applied for comparison. As GOx is a dimer, one mole of glucose oxidase has two moles of the cofactor, FAD. According to this principle, therefore, immobilized GOx is denatured, and then the amount of released FAD is related to the amount of immobilized GOx. It was observed that 2.8 mg of GOx was immobilized on the surface of 1 mg of MWCNTs, which was in fairly good accordance with the value estimated by the protein analysis of the supernatant.

For 1 mg of acid-treated MWCNTs, 600 nmol of carboxyl groups was formed, which can supply enough space to accommodate 21 nmol of GOx. Therefore, a high amount of GOx can be immobilized on MWCNTs by optimizing the concentrations of NHS, EDC, and GOx. The enzyme loading obtained in this study was 14- and 1.3-folds greater than those obtained by Kim et al. [16] and Min et al. [34], respectively. In previous studies, the concentrations of EDC, NHS or glutaraldehyde, and applied GOx were not optimized even though they critically affect the enzyme immobilization. In contrast, in this study, a high amount of GOx was covalently bound on MWCNTs if the carboxylated MWCNTs were activated by the presence of 52 mM of EDC with 430 mM of NHS at pH 6.0 for 3 h and then reacted with 8.7 mg/mL of GOx at pH 6.0 for 2–3 h.

3.5. Characterization of immobilized GOx using FTIR and AFM analysis

FTIR analysis was performed for the obtained GOx-MWCNTs under the optimal conditions. As shown in Fig. 1(C), the FTIR spectrum shows prominent peaks at 1639 and 1532 cm^{-1}, which are generally assigned to amide I (C═O) and amide II (N—H and C—H), respectively [14,52]. The appearance of these newly formed amide bonds clearly indicates the successful covalent attachment of GOx onto MWCNTs. The surface morphology of this sample was further examined by AFM. Fig. 7 shows the AFM image of GOx-MWCNTs (B) in comparison to that of MWCNTs without GOx (A). The region of MWCNTs has a height range of 10–12 nm (11.4 ± 1.2 nm), whereas the region of GOx-MWCNTs has a height range of 19–22 nm (20.0 ± 1.5 nm). The difference (8.6 ± 1.7 nm) between the two regions is the height of the attached GOx molecules. As the size of GOx is 5 nm [14], the results indicate that the MWCNTs surface is fully covered with GOx.

3.6. Biochemical and electrochemical analysis of immobilized GOx

The reaction product of the immobilized GOx was analyzed by TLC, in which the formation of gluconic acid from glucose was detected. The spots of authentic samples of glucose and gluconic acid are visible in the first and second lane of Fig. 8, respectively. The appearance of gluconic acid in the reaction mixtures incubated in the presence of MWCNTs but without GOx (control) or GOx-MWCNTs was identified by comparing spot migrating at the same position as gluconic acid. A clear gluconic acid spot was observed in the presence of GOx-MWCNTs (fifth lane), whereas only glucose spot without gluconic acid was observed in the presence of MWCNTs only (control, forth lane). The quantitative analysis of the reaction mixture incubated in the presence of GOx-MWCNTs revealed that 14.3 ± 1.4 mM of glucose remained, and 7.9 ± 1.3 mM of gluconic acid was produced. This result indicates that 37.8% of glucose was consumed, and most of it converted into gluconic acid by immobilized GOx. This result clearly shows that the immobilized GOx has a high activity for oxidizing glucose into gluconic acid.

Electrochemical analyses were performed using cyclic voltammetry. The osmium redox polymer, PVI-*Os*-dme-bpy was used as a mediator, and thus the oxidation of this complex served as an indicator for investigating the electrochemistry of electrodes loaded with different amounts of GOx. Fig. 9(A) shows the CVs for the MWCNTs (MWCNT-SPECs) and MWCNTs loaded with different amounts of GOx (GOx-MWCNT-SPECs) in PBS solution containing 30 mM glucose. The MWCNT-SPECs (Fig. 9(A)(1)) exhibits the oxidation and reduction peaks corresponding to the osmium (II/III) transition at 0.2 V (versus Ag/AgCl) and is similar to the redox potential observed for PVI-*Os*-dme-bpy in 10× PBS solution (pH 7.2) with 0.14 M NaCl shown in the supplementary information (Fig. A). As glucose was catalyzed by immobilized GOx, the electrical signal was monitored. The catalytic anodic current was observed at a potential of 0.3 V (versus Ag/AgCl) for the GOx-MWCNT-SPECs whereas no cathodic peak current was observed, which was likely because of the fast generation of reduced mediators by the catalytic enzyme reaction. This sigmoidal shaped cyclic voltammograms are characteristics of catalytic oxidation of glucose by the enzyme with electron transfer mediated by the osmium redox polymer from the enzyme active site to the electrode [53]. Notably, 3.3-GOx-MWCNT-SPECs with the highest amount of GOx (Fig. 9(A)(3)) showed the peak current densities of 148 $\mu A/cm^2$, which was much higher than those of the 0.24-GOx-MWCNT-SPECs with smaller amounts of GOx. The amperometric response of 3.3-GOx-MWCNT-SPECs to glucose was determined at 0.3 V (versus Ag/AgCl), and the result is shown in Fig. 9(B). The anodic current density significantly increased with increasing glucose concentration up to 30 mM, and then it leveled off for higher concentrations. This asymptotic curve is a typical response to high concentration of glucose by the CV analysis of immobilized GOx, because enzyme is saturated with glucose at high concentrations.

These results demonstrate that the immobilized GOx retains bio- and electrocatalytic activity. Current generation increased by increasing amount of GOx immobilized on the electrode, and thus the immobilized GOx electrode developed in this study will contribute to improve the performance of biosensor and biofuel cells.

4. Conclusions

This study demonstrates that GOx can be covalently attached to MWCNTs, without physical adsorption, by EDC/NHS-mediated immobilization. The covalently bound GOx amounted to 3.3 mg-GOx/mg-MWCNTs, which is one of the highest achieved values to our knowledge. This was accomplished by optimizing the concentrations of EDC, NHS, and GOx. The Box–Behnken design method was a useful approach to determine the optimal conditions. Washing the immobilized GOx with buffer solution containing Tween-20 was a key step to minimize the physical adsorption of GOx during the immobilization process. The TGA revealed the successful carboxylation of MWCNTs. The FTIR and AFM analyses clearly showed the covalent bonding between GOx and MWCNTs, and GOx covering the entire surface of MWCNTs. The biochemical analysis showed that the immobilized GOx possesses high activity for the conversion of glucose into gluconic acid. The electrode covered with GOx-MWCNTs obtained under the optimal conditions generated a current density of 148 $\mu A/cm^2$, which is much higher than that generated by the electrode covered with a smaller amount of GOx. This study will contribute to solving problems associated with miniaturization of bio-electronic devices such as biofuel cells or biosensors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2016.03.016.

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