

## Immobilization of Glucose Oxidase on Multi-Wall Carbon Nanotubes for Biofuel Cell Applications

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**Abstract** Glucose oxidase was immobilized on the carboxylated multi-wall carbon nanotubes (MWNT-COOHs) in the presence of a coupling reagent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. Significant amounts of glucose oxidase were also immobilized on MWNT-COOHs without the coupling reagent. Various conditions for the immobilization of glucose oxidase were optimized. Optimal pH for the maximal activity of the immobilized glucose oxidase shifted to 7 from the optimal pH of 6 for the maximal activity of free enzyme due to the carboxyl groups on the surface of MWNT-COOHs. An electrode of graphite rod with a diameter of 6 mm was fabricated using the immobilized glucose oxidase. The cyclic voltammetry study of the enzyme electrode revealed that the oxidation of glucose and subsequent transfer of electrons from the oxidation of glucose to the electrode were possible by the immobilized glucose oxidase without a mediator, implying that the enzyme electrode can be utilized for the development of biofuel cells.

**Key words:** Carbon nanotubes, glucose oxidase, immobilization

Biofuel cells utilize biocatalysts such as microorganisms or enzymes to convert chemical energy stored in various organic materials into electrical energy [1, 7, 12, 21]. Although biofuel cells utilizing microorganism electron transfer processes [12] or those coupling the hydrogen producing ability of microorganisms [13] with the conventional fuel cells are the earlier types and have long been explored, they suffer from several drawbacks, such as the requirement of certain size of complex system, poor reproducibility in their performance, limited power generation, and concerns on the safety issues associated with the microorganisms

inside the fuel cells [17]. On the other hand, biofuel cells utilizing enzymes are of increasing interest, since fuel cells for novel applications, such as miniaturized fuel cells of a few micrometers in diameter or fuel cells operating in a physiological solution, which can be implantable in the living body, can be developed [6, 15].

Typically, organic materials, such as glucose or methanol, are used as fuels for biofuel cells utilizing enzymes. They are oxidized to donate electrons catalyzed by redox enzymes such as glucose oxidase or alcohol dehydrogenase, respectively, at the anode, whereas molecular oxygen or H<sub>2</sub>O<sub>2</sub> are reduced to water catalyzed by laccase or peroxidase, respectively, at the cathode [5, 11, 16]. Among these enzymes, the oxidative catalysis of peroxidases has also been utilized in the removal of various environmental pollutants [19, 22]. In addition to soluble enzymes, various types of immobilized enzyme electrodes have recently been developed for biofuel cells; enzymes are integrated with conductive polymers [18], coupled to gold electrode through an electron transferring chemical bond [20], or coated on graphite powders which are sprayed on an electrode of a polymer sheet [17].

In an effort to explore a new type of enzyme electrode, we investigated the use of carbon nanotubes for the immobilization of glucose oxidase which is the most widely employed enzyme for biofuel cell applications. Carbon nanotubes were first discovered in 1991 and consist of one or several concentric tubules, each with a helically wound hexagonal honeycomb lattice. They are divided into multi-wall carbon nanotubes (MWNTs) of diameters from 2 to 25 nm and single-wall carbon nanotubes (SWNTs) of diameters of 1–2 nm, depending on the number of layers in the wall of the nanotubes [14]. Typically, carbon nanotubes are several micrometers in length. In particular, MWNTs are produced in large quantity to be available for many applications at cheap price. Due to several attractive properties they possess, such as high electrical conductivity, excellent chemical

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stability, and significant mechanical strength, carbon nanotubes have been explored for various potential applications as molecular electronic components [14].

Carbon nanotubes were also used for the immobilization of several proteins by adsorption, coating, or crystallization on the surface of MWNTs [2, 4, 8, 9]. To couple extrinsic molecules such as metals or proteins on carbon nanotubes, carboxylate groups can be introduced on the surface of the carbon nanotubes by the oxidation with strong acids such as sulfuric acids or nitric acids [3]. In this report, we describe the optimal conditions for the immobilization of glucose oxidase on MWNT-COOHs using a carbodiimide as a coupling reagent, and also results on the studies of the catalytic and electrical properties of the immobilized glucose oxidase for potential biofuel cell applications.

## MATERIALS AND METHODS

MWNTs with 95% purity prepared by the chemical vapor deposition method were purchased from Iljin Nano Tech (Korea). Glucose oxidase and other chemicals were purchased from Sigma and used as received.

### Carboxylation of MWNTs

MWNTs functionalized with carboxylic acid, MWNT-COOHs, were prepared according to the published method [2]. MWNTs (20 mg) were refluxed with 3 M HNO<sub>3</sub> (20 ml) for 12 h at 120°C. The MWNT-COOHs were washed with double distilled deionized water until the pH became nearly neutral, and then dried overnight at 80°C.

### Immobilization of Glucose Oxidase on MWNT-COOHs

One mg of MWNT-COOHs was mixed with various amounts of glucose oxidase and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in 1 ml of 0.1 M sodium phosphate buffer solution (pH 7.0). This mixture was shaken at 100 rpm, and then centrifuged to remove supernatant. Loosely bound glucose oxidase was removed from MWNT-COOHs by repeatedly washing the sample with the phosphate buffer and centrifuging until negligible activity of glucose oxidase was detected in the supernatant. Typically, six to seven repeated washings were required to completely remove the loosely bound enzyme from MWNT-COOHs.

### Measurement of the Activity of Immobilized Glucose Oxidase

To 1 ml of 0.1 M sodium phosphate solution (pH 7.0) containing 10 mM glucose, 1 mM guaiacol, and 50 µg of horseradish peroxidase, 1 mg of MWNT-COOHs with immobilized glucose oxidase was added. The activity of immobilized glucose oxidase was measured by determining the amount of guaiacol oxidized by horseradish peroxidase and H<sub>2</sub>O<sub>2</sub>; the latter is formed as a product from the

oxidation of glucose by the immobilized glucose oxidase. The reaction was terminated after 30 sec by adding 100 µl of 1 N HCl to lower the pH to approximately 2. The reaction mixture was centrifuged, and then 10 µl of the supernatant was analyzed by HPLC for the amount of remaining guaiacol. The activity of the immobilized glucose oxidase is represented as the number of micromoles of guaiacol oxidized per 1 min.

### HPLC Analysis

The concentration of guaiacol was analyzed using a high performance liquid chromatograph (HPLC) equipped with a Waters µBondapak C<sub>18</sub> column (3.9×300 mm) and a UV-visible detector. Guaiacol was detected at 280 nm. The mobile phase was an acetonitrile-water mixture (50:50, v/v) at a flow rate of 1 ml/min.

### Preparation of Enzyme Electrodes

An enzyme electrode for the electrochemical measurement of MWNT-COOHs with immobilized glucose oxidase was fabricated as follows. The surface of the graphite rod with a diameter of 6 mm was polished on a weighing paper. Approximately 1 mg of MWNT-COOHs with immobilized glucose oxidase was attached on the polished surface of the graphite rod by silver/epoxy resin. After drying the graphite rod in a vacuum chamber for 1 h at ambient temperature, the surface was covered with a dialysis membrane with a MWCO of 12,000, and the side of the electrode was sealed with an O-ring.

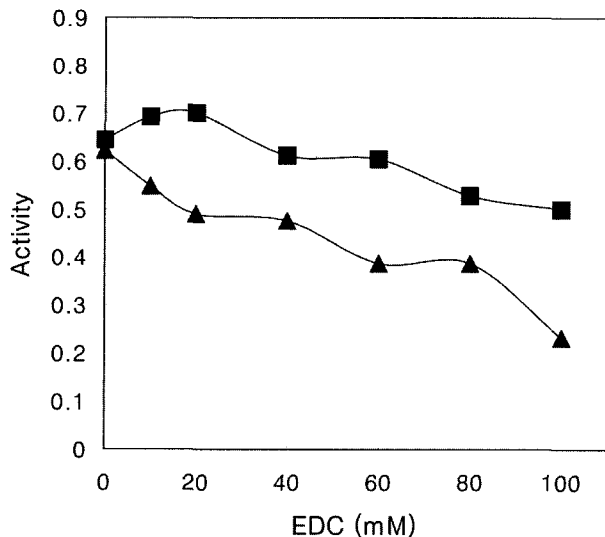
### Cyclic Voltammetry

Cyclic voltammograms were measured, using an EG&G potentiostat/galvanostat (Model 273A), at ambient temperature. A three-electrode cell containing 50 ml of 0.1 M potassium phosphate buffer (pH 7.0) with 20 mM KCl was used. The cell was purged with high purity nitrogen gas for 10 min prior to each measurement. The working electrode was the enzyme electrode on a graphite rod of a diameter of 6 mm, fabricated as described above. The counter and reference electrodes were platinum wire and a saturated calomel electrode, respectively. The scan rate was 50 mV/s.

## RESULTS AND DISCUSSION

### Immobilization of Glucose Oxidase on MWNT-COOHs

Immobilization of glucose oxidase on MWNT-COOHs is affected by various factors. In the present study, temperature, the concentration of coupling reagent (EDC), enzyme concentration in solution, and incubation time were optimized. Immobilization efficiency is represented as the activity of the final MWNT-COOHs samples with immobilized glucose oxidase. For immobilization, 1 mg of MWNT-COOHs was added to 1 ml of potassium

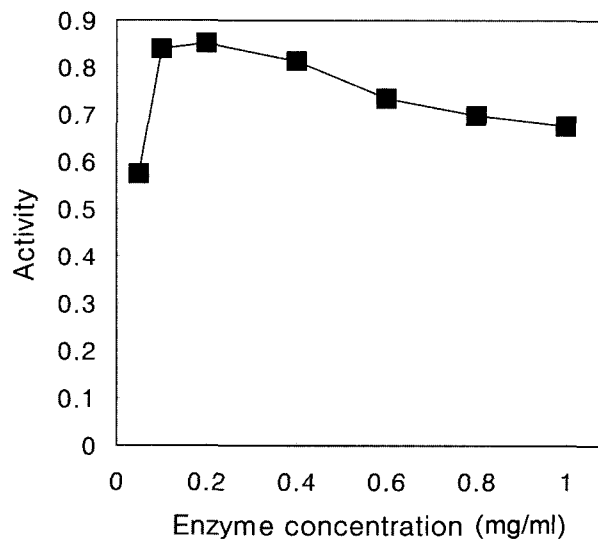


**Fig. 1.** Effects of EDC concentration on the immobilization efficiency of glucose oxidase on MWNT-COOHs at 4°C (■) and 25°C (▲).

Mixtures for immobilization contained 1 mg of MWNT-COOHs, 0.1 mg of glucose oxidase, and various concentrations of EDC in 1 ml phosphate buffer (pH 7) and were incubated for 3 h. The unit of activity is  $\mu\text{mole guaiacol/mg MWNT-COOHs}\cdot\text{min}$ .

phosphate buffer solution (100 mM, pH 7) containing 0.1 mg glucose oxidase and different concentrations of EDC, and the mixture was incubated for 3 h with shaking. EDC catalyzes the covalent coupling of carboxyl groups on the surface of MWNT-COOHs with amino-groups on the enzyme molecules.

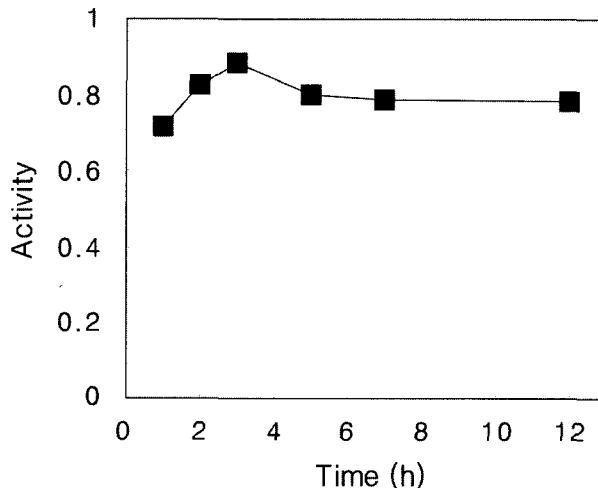
Figure 1 shows the effects of EDC concentration, ranging from 0 to 100 mM, at two different incubation temperatures of 4 and 25°C. Glucose oxidase was shown to be immobilized on MWNT-COOHs even in the absence of EDC, indicating that the coupling of glucose oxidase on MWNT-COOHs is either noncovalent or very facile, without the necessity of any coupling reagent. At 25°C, especially, higher concentrations of EDC inhibited enzyme activity to a greater extent. At 4°C, immobilization efficiency was enhanced with increasing concentration of EDC up to 20 mM, then decreased in the presence of higher concentration of EDC. Therefore, subsequent experiments for immobilization were performed in the presence of 20 mM EDC at 4°C. Figure 2 shows the change in the immobilization efficiency, when the concentration of glucose oxidase in the mixture for immobilization varied from 0.05 to 1 mg/ml. Immobilization efficiency was increased almost two-fold when the concentration of glucose oxidase increased from 0.05 to 0.2 mg/ml, and then decreased upon further increase of the glucose oxidase concentration. The decreased immobilization efficiency at high concentrations of glucose oxidase was unexpected, and its cause is far from being completely understood at this stage. One



**Fig. 2.** Effects of concentration of glucose oxidase on the immobilization efficiency of glucose oxidase on MWNT-COOHs at 4°C.

Mixtures for immobilization contained 1 mg of MWNT-COOHs, 20 mM EDC, and different amounts of glucose oxidase in 1 ml phosphate buffer (pH 7) and were incubated for 3 h.

possible reason would be that enzyme molecules at high concentrations may aggregate together, therefore, less number of enzyme molecules becomes available for the coupling with MWNT-COOHs. Finally, Fig. 3 shows the effects of incubation period on the efficiency of immobilization, which was maximal for the incubation period of 3 h; however, it decreased slightly if the incubation period became longer. The optimal conditions thus far obtained for the immobilization of glucose oxidase on



**Fig. 3.** Effect of incubation period on the immobilization efficiency of glucose oxidase on MWNT-COOHs at 4°C.

Mixtures for immobilization contained 1 mg of MWNT-COOHs, 0.2 mg of glucose oxidase, and 20 mM EDC in 1 ml of phosphate buffer (pH 7).

MWNT-COOHs include incubation of the mixture containing 1 mg of MWNT-COOHs, 0.2 mg of glucose oxidase, and 20 mM EDC in 1 ml phosphate buffer (pH 7) for 3 h at 4°C.

We compared the immobilization efficiency of glucose oxidase on MWNT-COOHs with that on unfunctionalized MWNTs by replacing MWNT-COOHs with MWNTs in the mixture for immobilization. The activity of the immobilized glucose oxidase on unfunctionalized MWNTs was nearly three times lower than that on MWNT-COOHs, indicating that carboxyl groups on MWNT-COOHs play a critical role in the immobilization of glucose oxidase.

These results indicate that glucose oxidase is immobilized on MWNT-COOHs by multiple modes of binding; physical adsorption without any specific interactions as revealed by the immobilization of glucose oxidase on unfunctionalized MWNTs, specific interaction between carboxyl group and proper polar or ionic groups of glucose oxidase, resulting in much higher immobilization efficiency on functionalized MWNT-COOHs, and covalent coupling of the enzyme molecules through EDC.

#### Characteristics of the Activity of Immobilized Glucose Oxidase

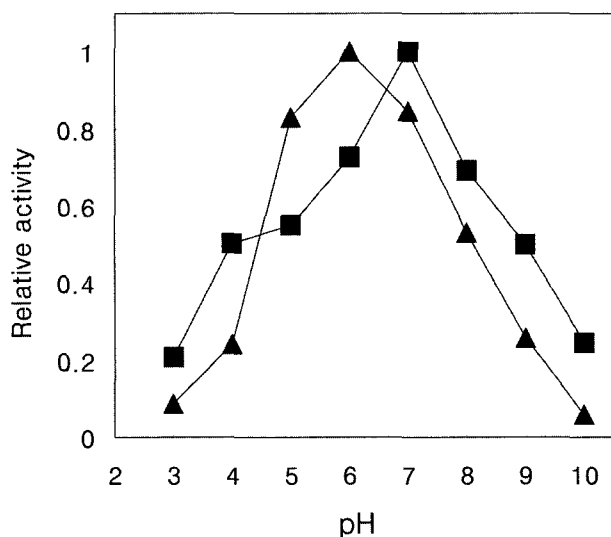
One of the pronounced effects of immobilization on the characteristics of enzyme activity is change of optimum pH of enzyme activity. This effect became more obvious when solid supports with polar or ionic groups on their surfaces were used for the immobilization of enzymes. MWNT-COOHs have carboxyl groups on the surface,

most of which are ionized in pH 7 aqueous buffer. When an enzyme is immobilized on an anionic solid possessing negative charges, the optimal pH for enzyme activity shifts toward alkaline due to the partitioning of hydrogen ions favoring the anionic solid surface in the microenvironment of the immobilized enzyme.

Figure 4 shows that the activity of soluble glucose oxidase was maximal at pH near 6. The activity of immobilized glucose oxidase on MWNT-COOHs, however, reached its maximal value at around pH 7, shifting slightly toward the alkaline side. This is in contrast to the immobilized enzyme on a nonionic support which shows the same optimal pH for the maximal activity as for free enzyme [10]. Another feature of the activity of immobilized glucose oxidase on MWNT-COOHs was that the activity profile over the pH range from 3 to 10 showed a shoulder on the acidic side of the maximal point, which was absent for the activity of soluble glucose oxidase. This feature in the activity of immobilized glucose oxidase on MWNT-COOHs indirectly indicates that immobilized glucose oxidase molecules on MWNT-COOHs experience heterogeneous microenvironments which might be due to the multiple modes of binding of enzyme molecules on MWNT-COOHs, as discussed above.

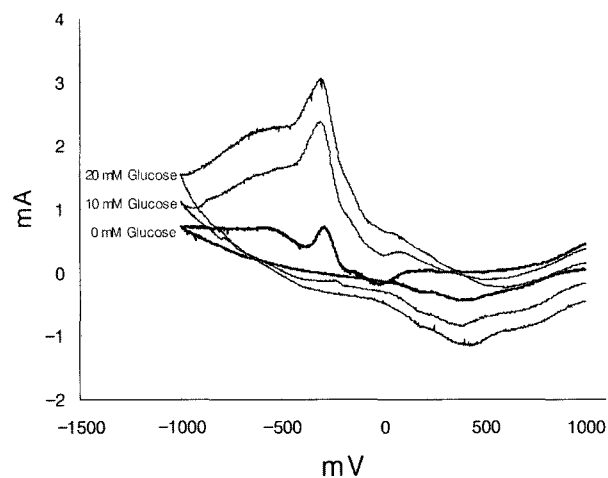
#### Electrochemical Study of Immobilized Glucose Oxidase

In order to apply enzyme electrodes for biofuel cells or biosensors, enzyme electrodes should catalyze the oxidation or reduction of the substrates, and then mediate the electron transfer into or out of the electrodes. Frequently, mediators of small molecules are added into the reaction mixtures to facilitate the electron transfer [16, 17]. We



**Fig. 4.** Effects of solution pH on the activity of soluble glucose oxidase (▲) and immobilized glucose oxidase on MWNT-COOHs (■).

Buffers (100 mM) used were sodium citrate for pH 3, sodium acetate for pH 4 and 5, sodium phosphate for pH 6 and 7, Tris-HCl for pH 8, sodium borate for pH 9, and glycine for pH 10.



**Fig. 5.** Differential cyclic voltammograms of the enzyme electrode with immobilized glucose oxidase on MWNT-COOHs.

The cyclic voltammogram for the graphite-rod electrode with MWNT-COOHs in the absence of glucose oxidase was subtracted from each cyclic voltammogram of the enzyme electrode to determine the differential cyclic voltammograms.

utilized cyclic voltammetry to test the possibility for biofuel cell applications of the enzyme electrode, manufactured as described in Materials and Methods, using immobilized glucose oxidase on MWNT-COOHs. Figure 5 shows that the electrode of immobilized glucose oxidase oxidizes glucose and transfers electrons generated by the oxidation of glucose to the graphite-rod electrode in the absence of any mediator. Cyclic voltammograms clearly imply that the increased current near 400 mV upon the increase of glucose concentration was due to the oxidation of glucose catalyzed by immobilized glucose oxidase on MWNT-COOHs.

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