Electrochemical Properties of Seamless Three-Dimensional Carbon Nanotubes-Grown Graphene Modified with Horseradish Peroxidase

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ABSTRACT

Horseradish peroxidase (HRP) was immobilized through sodium dodecyl sulfate (SDS) on the surface of a seamless three-dimensional hybrid of carbon nanotubes grown at the graphene surface (HRP-SDS/CNTs/G) and its electrochemical properties were investigated. Compared with graphene alone electrode modified with HRP via SDS (HRP-SDS/G electrode), the surface coverage of electroactive HRP at the CNTs/G electrode surface was approximately 2-fold greater because of CNTs grown at the graphene surface. Based on the increase in the surface coverage of electroactive HRP, the sensitivity to H₂O₂ at the HRP-SDS/CNTs/G electrode was higher than that at the HRP-SDS/G electrode. The kinetics of the direct electron transfer from the CNTs/G electrode to compound I and II of modified HRP was also analyzed.

KEYWORDS: carbon nanotubes, graphene, hybrid, horseradish peroxidase, biosensing

1. INTRODUCTION

Seamless three-dimensional (3D) sp² carbon-based nanomaterials, such as foam-like graphene [1, 2] and carbon nanotubes-grown graphene (CNTs/G) hybrid

materials [3, 4], have recently attracted great attention not only because of their high electrical conductivity but also their high surface area per unit planar/footprint area. Additionally, since charge carriers are movable in all three dimensions without significant contact resistance, such seamless 3D sp^2 carbon-based nanomaterials are expected to provide electrode materials for high power fuel cells as well as highly sensitive sensors, compared with seamless 2D sp^2 carbon-based nanomaterials, such as graphene. Also, because of the flat surface of its backside, the CNTs/G hybrid has the potential for the development of flexible and wearable biosensors for physiological monitoring.

Redox enzymes, such as horseradish peroxidase (HRP) and glucose oxidase (GOx), are widely used for electrochemical biosensors. However, they are generally difficult to directly communicate with flat metal electrodes due to the deeply embedded active site in the thick insulating polypeptide layer. Meanwhile, it is known that CNTs and graphene flakes can communicate electrochemically with redox enzymes directly, because of their small nanostructure with high conductivity [5–11]. Such carbon nanomaterials have therefore been expected to be utilized for the development of enzyme-based electrochemical biosensors. However, properties of CNTs and graphene emerge only in one direction for CNTs and planar direction for graphene due to their one- and two-dimensional structures, respectively [12–14]. Therefore, controlling the orientation of those carbon nanomaterials is necessary. Additionally, there are also some non-trivial problems, such as the aggregate formation and significant contact resistance among CNTs and graphene flakes.

Toward the development of highly sensitive enzyme-based electrochemical biosensors without the above problems, we previously constructed CNTs/G hybrid electrodes modified with GOx [15] or heme peptide (HP) [16], which has a similar structure to the active center of peroxidase and has often been used for H_2O_2 sensing [17–19]. However, the catalytic activity of HP is lower than that of HRP and therefore HRP-based biosensor would be more sensitive compared to one based on HP. Thus, it is of interest in using the full HRP for H_2O_2 sensing. In the present work, we used HRP-modified CNTs/G electrodes and studied the kinetics of electron transfer from the CNTs/G hybrid to peroxidase. Based on the previous report by Yan et al., we used surfactant sodium dodecyl sulfate (SDS) to immobilize HRP at the CNTs/G [6]. Furthermore, based on the direct reduction of $HRP(P)Fe^{4+}$ -oxygen complexes (P:

porphyrin ring), such as compound I and II, in the presence of H_2O_2 at sufficiently higher potential relative to the formal potential of the $\text{HRP(P)Fe}^{2+/3+}$ couple [20, 21], we compared catalytic reduction currents of H_2O_2 at the HRP-modified CNTs/G electrode with those at HRP-modified graphene electrode (Figure 1). Thus, we confirmed the superiority of CNTs/G hybrid films in electrochemical biosensing, compared with graphene films.

“Here Fig. 1”

2. EXPERIMENTAL

2.1. Preparation of CNTs/G Films

CNTs/G hybrid was prepared according to the previous report [16]. Briefly, a copper (Cu) foil ($\sim 6.5 \text{ cm}^2$ and $25 \text{ }\mu\text{m}$ thick), which is a catalyst and substrate for graphene growth, was placed inside a quartz tube (ca. 3 cm in inner diameter and ca. 75 cm long) and annealed at $1030 \text{ }^\circ\text{C}$ under flowing Ar (190 sccm) and H_2 (10 sccm) at atmospheric pressure in an electric furnace (Lindberg/Blue MTM Mini-MiteTM Tube Furnace, Thermo Scientific). At this time CH_4 (5 sccm) was introduced in the tube for 9 min to grow graphene followed by cooling of the furnace to room temperature in flowing Ar/ H_2 atmosphere. Subsequently, the graphene-formed Cu foil was decorated with 1.5 nm thick iron film, which acted as catalysts for CNTs growth, using an e-beam evaporator (Temescal BJD-1800, Technical Engineering Services). The graphene/Cu foil with the iron film was placed back in the quartz tube and heated to $750 \text{ }^\circ\text{C}$ in flowing Ar (100 sccm) and H_2 (50 sccm) atmosphere. After the temperature was stabilized at $750 \text{ }^\circ\text{C}$, a 15 sccm flow of C_2H_2 was supplied in the tube for 5 min, followed by cooling of the furnace to room temperature in flowing Ar/ H_2 atmosphere. The graphene film formed on the backside of CNTs/G/Cu foil was removed by O_2 plasma, followed by etching of the Cu foil in a 1 M FeCl_3 aqueous solution to obtain the CNTs/G hybrid. The CNTs/G hybrid was cleaned with 5% HCl solution to completely remove the Cu foil and the iron nanoparticles. Note that we also obtained the graphene film using the same procedure. Fundamental properties of CNTs/G and graphene films were reported previously [15, 16].

2.2. Preparation of HRP-Modified Electrodes

A glassy carbon (GC) electrode (3 mm in diameter, ALS Co., Ltd) was polished with 1 and 0.05 μm alumina slurries on a polishing cloth and then thoroughly rinsed with distilled water, followed by sonication in 2-propanol and distilled water, respectively. After drying with a high-purity nitrogen stream, the CNTs/G or graphene was transferred to the GC electrode surface and then kept at 50 $^{\circ}\text{C}$ in an electric oven for 1 h to be firmly attached on the GC surface. Subsequently, the CNTs/G and graphene electrodes were immersed in either A) pH 7, 67 mM phosphate buffer (PB) containing 0.5 mM HRP (Sigma-Aldrich) with 20 mM SDS for 12 h at 4 $^{\circ}\text{C}$ to obtain the HRP-SDS/CNTs/G and HRP-SDS/G electrode, respectively, or B) pH 7, 67 mM phosphate buffer (PB) containing 0.5 mM HRP (Sigma-Aldrich) for 12 h at 4 $^{\circ}\text{C}$ to obtain the HRP/CNTs/G and HRP/G electrode, respectively.

2.3. Electrochemical Measurements

Electrochemical measurements were performed with a potentiostat Versa STAT (Princeton Applied Research, USA) in 67 mM phosphate buffer (pH 7.4) in a batch system. A Ag|AgCl|KCl (sat.) and a coiled platinum wire were used as reference and counter electrodes, respectively. The catalytic activity of HRP-SDS/CNTs/G and HRP-SDS/G electrodes toward H_2O_2 reduction was evaluated by amperometric measurements. After the working electrode was polarized at +150 mV and a steady-state current was obtained, a H_2O_2 solution was added into the electrolyte solution. From the steady-state current obtained here, the catalytic activity of HRP at the CNTs/G and graphene electrodes was determined.

3. RESULTS AND DISCUSSION

3.1. Electrochemical Characterization

Cyclic voltammetry (CV) were performed at the G, CNT/G, HRP/G, HRP/CNTs/G, HRP-SDS/G and HRP-SDS/CNTs/G electrodes in deaerated PB. As shown in Figure 2A, there was no apparent background redox peak at both the CNT/G and graphene electrodes. Similarly, the HRP/CNTs/G and HRP/G electrodes also did not show redox peak (data not shown). On the other hand, redox peaks appeared at both the HRP-SDS/G and HRP-SDS/CNTs/G at approximately -320 mV (vs. Ag|AgCl), corresponding to $(\text{P})\text{Fe}^{2+/3+}$. These findings are similar to reported by Yan et al. [6] and as in their case can be ascribed to properties of the SDS for facilitating protein

electrochemistry and the structural properties of CNT and the 3D morphology of the CNT/G electrode. We also evaluated cetyltrimethylammonium bromide (CTAB) for the immobilization of HRP in the phosphate buffer (pH 7.4). However, no redox peaks of (P)Fe^{2+/3+} were clearly observed. Since CTAB is a cationic surfactant and the isoelectric point of HRP is 8.9, HRP might not be immobilized through CTAB probably due to the electrostatic repulsion between CTAB and HRP. We therefore used only SDS modification for HRP immobilization for further studies.

The redox peak current (j_p) for both electrodes was proportional to the scan rate (ν) below at least 1.5 V s⁻¹ (Figure 2B), indicating that the redox reaction was a surface controlled process. In addition, the redox peak current at the HRP-SDS/CNTs/G electrode was approximately 2-times larger than that at the HRP-SDS/G electrode. This is in accordance with the previously reported ~2.5-fold larger apparent electroactive surface area for the CNTs/G film compared to graphene because of CNTs grown at the graphene surface [16]. Since the peak current was proportional to the scan rate below at least 1.5 V s⁻¹ (Figure 2B), the surface coverage Γ of electroactive HRP at both CNTs/G and graphene electrodes was determined from the equation,

$$I_p = n^2 F^2 \Gamma \nu / 4RT \quad (1)$$

where n is the number of electrons transferred ($n = 1$), F is the Faraday constant, ν is the scan rate, R is the gas constant, and T is the temperature. Based on equation 1, Γ was estimated to be $\sim 4.3 \times 10^{-11}$ mol cm⁻² for the HRP-SDS/CNTs/G electrode and 2.1×10^{-11} mol cm⁻² for the HRP-SDS/G electrode. On the basis of the reported Γ value of 5×10^{-11} mol cm⁻² for the HRP monolayer-modified basal plane pyrolytic graphite (BPPG) electrode through didodecyldimethylammonium bromide [22], we hypothesize that HRP molecules formed a monolayer at the graphene and CNTs/G surfaces.

“Here Fig. 2”

As mentioned above, the present redox reaction of HRP is a surface controlled process. In addition, as the scan rate increased, the peak-to-peak separation (ΔE_p) also gradually increased. To obtain the kinetic parameter for HRP redox, the apparent heterogeneous electron transfer rate constant k_{app} was therefore calculated according to the Laviron's theory [23]. The anodic and cathodic peak potential E_{pa} and E_{pc} can be

represented as follows:

$$E_{pa} = E^0 - (2.3RT/anF)\log(\alpha/m) \quad (2)$$

$$E_{pc} = E^0 + \{2.3RT/(1-\alpha)nF\}\log\{(1-\alpha)/m\} \quad (3)$$

$$m = RTk_{app}/F\nu \quad (4)$$

where α is the transfer coefficient. Based on the plots of E_{pa} or $E_{pc} - E_0$ versus $\log \nu$, α can be determined from the slopes of the anodic and cathodic processes (Figure 3) to be about 0.50 ± 0.02 and 0.41 ± 0.04 for HRP-SDS/CNTs/G and HRP-SDS/G, respectively. According to the equations 2–4, ΔE_p can be obtained by the following equation, which is accepted above 200 mV:

$$\Delta E_p = \{2.3RT/(1-\alpha)anF\}\{\alpha\log(1-\alpha) + (1-\alpha)\log\alpha - \log(RT/nF) - \log k_{app}\} + \{2.3RT/(1-\alpha)nF\}\log \nu \quad (5)$$

Based on this equation, k_{app} was determined to be $19.2 \pm 2.5 \text{ s}^{-1}$ for the HRP-SDS/CNTs/G electrode and $15.7 \pm 2.3 \text{ s}^{-1}$ for the HRP-SDS/G electrode, respectively. The difference in k_{app} can be ascribed to a small amount of defects and/or graphene edge sites in the CNTs/G hybrid. It is known that heterogeneous electron transfer reactions of redox species at edge-oriented pyrolytic graphite (EOPG) electrodes are generally faster than those at BPPG electrodes [24–27]. In addition, it is known that the heterogeneous electron transfer reaction rate is accelerated at carbon nanofibers with stacked graphene, which provided a high density of the edge site at their surfaces [28, 29]. As reported previously, the existence of defects and/or graphene edge sites in the CNTs/G hybrid were confirmed from Raman spectroscopy, whereas the graphene film formed a monolayer sheet with very few defects and edge sites [16]. Thus, the heterogeneous electron transfer rate of HRP might be facilitated at the CNTs/G in comparison with the graphene. Meanwhile, the k_{app} value obtained here was larger than that for previously reported HRP-SDS/CNTs (3.5 s^{-1}) [6] and HRP-Nafion/G (4.63 s^{-1}) [30] electrodes. The present CNTs/G and graphene electrodes were basically the seamless structure, whereas the previous CNTs and graphene electrodes consisted of their nanotubes and nanoflakes assembled on the GC electrode. Compared with the assembled structure, the seamless structure might facilitate the electron transfer to some extent.

“Here Fig. 3”

3.2. Catalytic Reduction of H_2O_2

Figure 4 shows typical cathodic current responses (Δj) to H_2O_2 at CNTs/G and graphene electrodes, on which HRP was immobilized via SDS. The cathodic current at the HRP-SDS/CNTs/G and HRP-SDS/G electrodes was significantly larger than that at CNTs/G and graphene electrodes without HRP. This considerably enhanced current responses at enzyme modified electrodes can be credited to electron transfer from CNTs/G and graphene electrodes to HRP. The possible reaction mechanisms are proposed as follows [20, 21].

where compound I and II are oxidized complexes of HRP, such as $[(\text{HRP})\text{Fe}^{4+}=\text{O}]^{+\bullet}$ and $[(\text{HRP})\text{Fe}^{4+}-\text{OH}]^+$, respectively. Further, as shown in Fig. 4, the cathodic current at the HRP-SDS/CNTs/G and HRP-SDS/G electrodes increased and then leveled off, as the concentration of H_2O_2 increased. The initial linear increase of current as a function of H_2O_2 concentration suggests the reaction 6 to be rate determining. On the other hand, the subsequent plateauing of current points to reaction 7 and/or 8 to be rate determining. It is to be noted, here the current response was not influenced when the electrolyte solution was stirred, an indication the catalytic cathodic currents obtained at both HRP-SDS/CNTs/G and HRP-SDS/G electrodes were kinetically controlled. Therefore, all electroactive HRP molecules should contribute to the current. The cathodic current response at the HRP-SDS/CNTs/G electrode was about 2-fold larger than that at the HRP-SDS/G electrode, reflecting that the surface coverage of electroactive HRP at the former is about 2 times larger than that at the latter, as mentioned above.

“Here Fig. 4”

3.3. Analysis of Reaction Kinetics

Based on the above findings that cathodic current was not diffusion controlled and was proportional to the surface coverage of electroactive HRP molecules, the rate constants for the three reactions (equations 6 to 8) can be calculated using the equation 9 [20, 21, 31],

$$i = 2F\Gamma/[1/k_1C_S + (k_2 + k_3)/k_2k_3C_H] \quad (9)$$

where k_1 , k_2 , and k_3 are the rate constant of reactions 6-8, C_S and C_H are the H_2O_2 and proton concentration in the bulk solution, respectively. At low H_2O_2 concentrations (linear range of current vs. H_2O_2 concentration plot in Fig. 4), when almost all HRP is in ferric state, the current response is determined by the rate of reaction 6 and therefore given by the equation [20, 21]

$$i_1 = 2F\Gamma k_1 C_S \quad (10)$$

Based on this equation, the rate constant k_1 for HRP-SDS/CNTs/G and HRP-SDS/G electrodes were estimated to be approximately 370 and 150 $M^{-1} s^{-1}$, respectively. Based on the fact that HRP was immobilized through SDS at essentially the same geometrical structure of sp^2 bonded carbon, we predicted the k_1 value at the HRP-SDS/CNTs/G and HRP-SDS/G electrodes to be almost the same. The reason for a larger k_1 at the HRP-SDS/CNTs/G is unclear. A plausible explanation could be an enhanced conformational stability of HRP on the nanofiber structure of CNTs grown at the graphene compared with the flat graphene. Meanwhile, k_1 for the HRP-SDS/CNTs/G electrode was larger than that for CNTs/G electrode modified with HP (ca. 120 $M^{-1} s^{-1}$) reported previously [16] because of the difference in molecular complexity. However, the k_1 values obtained here were much smaller than the value for dissolved HRP ($1.8 \times 10^7 M^{-1} s^{-1}$) [32]. A reason is the likely denaturation of HRP at CNTs/G and graphene due to its strong adsorption via SDS.

At higher H_2O_2 concentrations (plateau region of current vs. H_2O_2 concentration plot in Fig. 4), assuming that all the HRP molecules are existing as compound I or II and the reaction is not controlled by proton diffusion but the enzymatic reaction, the cathodic current i_{23} is given by the equation [20, 21]

$$i_{23} = 2F\Gamma k_2 k_3 C_H / (k_2 + k_3) \quad (11)$$

Based on the equation 11, the $k_2 k_3 / (k_2 + k_3)$ value at the HRP-SDS/CNTs/G electrode was calculated to be about $7.6 \times 10^5 M^{-1} s^{-1}$ which was slightly larger than that at the HRP-SDS/G electrode (ca. $6.2 \times 10^5 M^{-1} s^{-1}$). In addition, the values of the apparent heterogeneous rate constant, $C_H k_2 k_3 / (k_2 + k_3)$, at pH 7.4 were estimated to be $\sim 3.0 \times 10^{-2}$ and $2.5 \times 10^{-2} s^{-1}$ for HRP-SDS/CNTs/G and HRP-SDS/G electrodes, respectively. This difference is likely due to the existence of the small amount of defects and/or edge sites in the CNTs/G, as mentioned above. Actually, the $k_2 k_3 / (k_2 + k_3)$ values obtained here were one order of magnitude smaller than that obtained at pH 7.0 for the HRP-adsorbed pyrolytic graphite electrode reported by Razgus et al. ($6.6 \times 10^6 M^{-1} s^{-1}$)

[33]. In contrast, the obtained values were larger than that at a HRP-adsorbed graphite powder-coated electrode ($5.6 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$) [20]. For the graphite powder coating, a binder is often required for the fabrication of such graphite electrodes. It is known that the binder sometime interferes with the direct electron transfer between enzymes and graphite powder [20]. In the present work, no binder was required for the preparation of CNTs/G modified electrodes. Thus, the direct electron transfer from HRP to the CNTs/G might be accelerated, though the edge sites of graphene were virtually absent. However, the k_1 value for CNTs/G was significantly small, as mentioned above. Due to this, the lower detection limit of H_2O_2 for the HRP-SDS/CNTs/G electrode ($\sim 10^{-5} \text{ M}$) was five orders of magnitude larger than that for the HRP-adsorbed graphite powder-coated electrode ($\sim 2 \times 10^{-10} \text{ M}$) [20]. By improving this parameter, we strongly believe that CNTs/G could be applied for the development of a highly sensitive electrochemical H_2O_2 biosensor. For example, the surface modification of the CNTs/G with metal nanoparticles [34, 35] and metal nanosheets [36] might help retain enzymatic activity.

4. CONCLUSION

The electrochemical properties of the CNTs/G electrode modified with HRP through SDS were investigated to explore the enhancement of the sensitivity to analyte H_2O_2 because of the increase in the surface coverage of electroactive HRP at the CNTs-grown on graphene surface. Compared with the HRP/G electrode, the catalytic current for H_2O_2 reduction at the HRP/CNTs/G electrode increased by a factor of about 2. The surface coverage of HRP was about $\sim 4.3 \times 10^{-11} \text{ mol cm}^{-2}$ at the CNTs/G electrode. The rate constant k_1 was estimated to be $\sim 370 \text{ M}^{-1} \text{ s}^{-1}$. The overall rate constant of electron transfer reaction $k_2k_3/(k_2 + k_3)$ and the apparent heterogeneous rate constant $C_Hk_2k_3/(k_2 + k_3)$ at pH 7.4 were $\sim 7.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $3.0 \times 10^{-2} \text{ s}^{-1}$, respectively. Thus, we hypothesize that the CNTs/G hybrid would be a good candidate as the conductive nanoscaffold for enzymes toward the development of highly sensitive electrochemical biosensors.

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FIGURE CAPTION

Figure 1. Schematic illustration of a CNTs/G and G electrodes modified with HRP.

Figure 2. (A) CVs of graphene, CNTs/G, HRP/G, and HRP/CNTs/G electrodes in a 0.067 M phosphate buffer solution (pH 7.4) at scan rate of 100 mV s^{-1} . (B) Relationship between anodic and cathodic peak currents of HRP/G and HRP/CNTs/G electrodes and scan rate.

Figure 3. Dependences of $E_p - E^0$ of redox couple for HRP/G and HRP/CNTs/G electrodes on the logarithm of scan rate (Laviron plots) in the 0.067 M phosphate buffer solution (pH 7.4).

Figure 4. Cathodic current densities of H_2O_2 reduction at the graphene, CNTs/G,

HRP/G, and HRP/CNTs/G electrodes in the 0.067 M phosphate buffer solution (pH 7.4)
at +150 mV vs. Ag|AgCl

Figure 1

Figure 2A

Figure 2B

Figure 3

Figure 4

G – graphene; CNT – carbon nanotube; HRP – horseradish peroxidase; SDS – Sodium dodecyl sulfate