Graphene Modified Electrode for the Direct Electron Transfer of Bilirubin Oxidase

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ABSTRACT
We fabricated graphene modified glassy carbon (Gr/GC) electrodes to enhance the direct electron transfer (DET) of bilirubin oxidase (BOD) by casting graphene-dispersed solutions with different concentrations. With a concentration of 2 µg µL⁻¹, a large increase was observed in the reduction current at the BOD-modified Gr/GC electrode, and the value was 74 times that of a BOD-modified GC electrode (unmodified with graphene). Moreover, with graphene modification the capacitance increase was much less than with other carbon materials including carbon black and graphite powder. These results demonstrate that a graphene nanostructure is suitable for achieving the efficient DET of BOD.

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1. Introduction

Graphene has attracted much interest because of its fascinating chemical and physical properties. These properties enable us to improve electrode performance by modifying conventional electrodes with this material. For example, a graphene modified electrode¹ has been investigated with a view to increasing its surface area and immobilizing enzymes to realize effective DET-type bioelectrocatalysis applicable to the study of biosensors or biofuel cells, as well as other nanocarbon materials such as carbon nanotubes,² carbon nanofiber³ and carbon cryogel.⁴ Because these nanostructures have the potential to enable an electron to access an enzyme directly from an electrode thanks to the reduction in the electron transfer distance between the redox centers and the electrode surface.² Moreover, graphene materials are also often used with other nanomaterials including nanoparticles and chitosan to achieve further DET enhancement.¹ These strategies are advantageous for the fabrication of such energy storage devices as biofuel cells because these devices need a large surface area for a high electrical output.³ However, such modification also brings about a significant capacitance increase, which is disadvantageous in terms of biosensor fabrication because the capacitive current interferes with the detection of trace amounts of analyte.⁶ Given that a graphene-based electrode with both a large signal and a low capacitive current was achieved solely by simple graphene modification, we can obtain significant insight into how to fabricate superior graphene-based biosensors easily by using various enzymes. Therefore, it is important to develop both graphene-modified electrodes without any other nanomaterials and their nanocomposites.

In this study we developed a graphene modified GC electrode that we realized simply by graphene modification with a much simpler fabrication process than that used for the above mentioned graphene nanocomposites. We investigated the effects of modification with different amounts of graphene with a view to achieving the efficient DET of bilirubin oxidase (BOD). We also compared the bioelectrocatalytic activities of BOD when co-immobilized with different carbon materials such as carbon black and graphite powders.

2. Experimental

Graphene dispersed solution (PureSheet™ (MONO), 0.05 µg µL⁻¹, average flake area of 10,000 nm²) was obtained from Nanointegris Inc. (USA). GC and highly oriented pyrolytic graphite (HOPG) electrodes (edge plane and basal plane), carbon black (CB, mean size 110 nm), and graphite powder (GP, mean size 3 µm) were purchased from BAS (Tokyo, Japan), Tokai Carbon (Aqua-Black, Japan), and Tsukuba Materials Information Laboratory, Ltd. (Japan), respectively. BOD was obtained from Amano Enzyme Inc. (BO “Amano” 3, Japan). Raman spectra were acquired using a Raman micro spectrometer (DXR Raman Microscope, Japan) with a 532 nm laser.

The electrode areas of all the electrodes used in the experiment were restricted by employing insulating tape with a 2 mm diameter hole. Gr/GC electrodes were prepared by casting 10 µL of graphene dispersed solution with various concentrations on GC electrodes, which were then thoroughly dried at room temperature. To adjust the graphene concentrations, 1.0 mL of the starting graphene solution (0.05 µg µL⁻¹) was concentrated by centrifugation (21,500 ×g, 30 min) and different volumes of the supernatant were removed. Finally we obtained a solution with various graphene concentrations (0.05–5 µg µL⁻¹). BOD modified electrodes were prepared by casting 10 µL of BOD solution (1 mg mL⁻¹ in 0.1 M phosphate buffer (PB, pH 7.0)) on the electrodes for 60 min, and then washing them with PB buffer.

Electrochemical measurements were performed using a potentiostat (Model CHI760B, CH Instruments). We used a three-electrode configuration throughout the experiment with Ag/AgCl (BAS) and Pt wire as reference and counter electrodes, respectively. All measurements were performed at room temperature.

3. Results and Discussion

We investigated the effect of the amount of graphene modification on the GC electrode as regards achieving efficient DET between BOD and the electrode surface by observing the
BOD-catalyzed oxygen reduction current. BOD belongs to an extensively investigated sub-group of multicopper oxidases.\textsuperscript{7} We performed a cyclic voltammetry (CV) measurement using the prepared BOD-modified Gr/GC electrodes [Fig. 1(a)]. The reduction currents began to increase at 0.5 V (vs. Ag/AgCl) and reached a steady state at 0.3 V. Interestingly, the magnitude of the reduction currents clearly increased depending on the amount of graphene. We estimated the dependence of the amount of graphene on the reduction current at a potential of 0.1 V (vs. Ag/AgCl) on CV measurements as shown in Fig. 1(b). In this study, graphene was modified on the GC electrode surface with efficient amounts of graphene to avoid exposing the GC surface.\textsuperscript{8} A sharp reduction current increase was observed when the graphene concentration exceeded 1 µg µL\textsuperscript{-1}, and the obtained reduction current was saturated at a concentration of 2 µg µL\textsuperscript{-1}, probably due to the amounts of the modified BOD. When the graphene concentration was 2 µg µL\textsuperscript{-1} (2Gr/GC), the reduction current was 74 times higher than that of the BOD/GC electrode. This result clearly indicates that graphene modification enhances the DET between the redox centers of BOD and the electrode surface with high efficiency. Such drastic increase of biocatalytic current could be attributed to the fact that the graphene orientation on the GC electrode was gradually changed from the lateral to the vertical orientation with increasing the amounts of the modified graphene.\textsuperscript{9} As a result, this change allowed to form a nanospace on the electrode surface suitable for enhancing DET. It is noteworthy that the DET of BOD in this study was greatly enhanced solely by changing the concentration of the modified graphene, which is quite different from the result obtained with graphene or other material composites (e.g., graphene/Au nanocomposite/chitosan).\textsuperscript{1} We also determined the capacitance values (C\textsubscript{0}) of these graphene-modified GC electrodes (without BOD), by using CV measurements at 0.25 V vs. Ag/AgCl in 1 M KCl (scan rate of 10 mV s\textsuperscript{-1}).\textsuperscript{10} The 2Gr/GC electrode exhibited C\textsubscript{0} values of 36.5 µF cm\textsuperscript{-2}, which was only twice that of a GC (17.7 µF cm\textsuperscript{-2}) electrode. Tang and co-workers previously reported that the C\textsubscript{0} value at a GC electrode modified with a reduced graphene sheet film was 13 times larger than that at a bare GC electrode.\textsuperscript{11} Interestingly, although we used equivalent amounts of graphene modification, the capacitance increase was suppressed in this study. This is presumably due to the difference in actual surface area as a result of the graphene size that they used (tens to several hundreds nm\textsuperscript{2}),\textsuperscript{12} which is smaller than ours (~10,000 nm\textsuperscript{2}). When the graphene concentration was 5 µg µL\textsuperscript{-1}, the C\textsubscript{0} value increased sharply up to 2110 µF cm\textsuperscript{-2}, which was 119 times larger than that of the GC electrode. From the standpoint of a high signal-to-noise ratio, 2Gr/GC exhibited the best performance for the DET of BOD among electrodes modified with various concentrations of graphene. Subsequent experiments therefore used a 2Gr/GC electrode.

We characterized the 2Gr/GC electrode using Raman spectroscopy to obtain microstructural information. Figure 2 shows the Raman spectra of the 2Gr/GC and other carbon material-based electrodes for comparison. Typically, the Raman spectrum of the graphene material has three prominent peaks at ~1350, ~1580 and ~2700 cm\textsuperscript{-1}, which are assigned to the D, G and 2D bands, respectively.\textsuperscript{2} The GC showed three peaks similar to those of a previous report,\textsuperscript{13} and therefore the 2Gr/GC spectrum overlaps three relatively similar peaks from the GC used as a supported substrate, which made it difficult to estimate the details. Nevertheless, a closer inspection revealed that the G band sharpened and increased in intensity compared with that of GC. The G band corresponds to the carbon sp\textsuperscript{2} vibrations of the domains in plane.\textsuperscript{12} This result indicated the presence of graphene on the GC electrode. Moreover, it is well known that the 2D band is sensitive to the number of graphene layers, whose peak is shifted to a higher wave number (blue-shift) as the number of graphene layers increases.\textsuperscript{12} Graf and co-workers also demonstrated that the 2D peak position for single-layer graphene was at 2678.8 ± 1.0 cm\textsuperscript{-1} and the 2D peaks were blue-shifted as they increased the number of graphene layers along with HOPG by using 532 nm laser excitation as in our study.\textsuperscript{14} Also in our study, the 2D band (2676 cm\textsuperscript{-1}) of the 2Gr/GC electrode exhibited a very similar peak position to that of the previous value obtained from the single graphene layer, and a lower peak position than that of HOPG.

**Figure 1.** (Color online) Typical CVs of BOD-catalyzed O\textsubscript{2} reduction on BOD-modified GC and Gr/GC electrodes with different amounts of graphene. Scan rate was +0.7 to 0 V. Scan rate was 20 mV s\textsuperscript{-1}. (b) Variation in reduction current at a potential of +0.1 V at Gr/GC electrodes as a function of graphene concentration, obtained from CVs.

**Figure 2.** (Color online) Typical CVs of BOD-catalyzed O\textsubscript{2} reduction on BOD-modified GC and Gr/GC electrodes with different amounts of graphene. Scan rate was 20 mV s\textsuperscript{-1}. (b) Variation in reduction current at a potential of +0.1 V at Gr/GC electrodes as a function of graphene concentration, obtained from CVs.
We speculated that such nano-sized graphene modification with many edge-plane surfaces contributed to the enhancement of the DET reaction due to the large adsorption of BOD in a similar way to previous works, which reported that edge sites promote the adsorption of some enzymes.\textsuperscript{15,16}

We compared the bioelectrocatalytic activities of BOD when co-immobilized with different carbon materials including GP and CB. These carbon materials were cast on the GC electrodes with the same condition as that of the 2Gr/GC electrode (casting volume of 10 \( \mu \) L of 2 \( \mu \) g/\( \mu \) L carbon dispersed solutions). Figure 3 shows the oxygen reduction currents catalyzed by BOD at bare GC and these carbon-modified GC electrodes (black bars). We also estimated the C\textsuperscript{0} values of these carbon-modified GC electrodes (Fig. 3, white bars). Large reduction peak currents were observed at the GP/GC, CB/GC and 2Gr/GC electrodes, which were respectively 223, 69 and 74 times higher than that at the bare GC electrode. However, the GP/GC and CB/GC electrodes exhibited large C\textsuperscript{0} values as well as large signal currents, which were 115 and 42 times higher than that at the bare GC electrode. These results showed that the contribution to achieving efficient DET at the GP/GC and CB/GC electrodes was low. In contrast, as described above, the 2Gr/GC electrode exhibited a very small increase in capacitance despite the fact that the amount of graphene modification was same to those of GP and CB. Taking account of these reduction current and C\textsuperscript{0} value results obtained at each carbon modified electrode, the 2Gr/GC electrodes offer a suitable biointerface for achieving efficient DET between BOD and an electrode surface while suppressing the increase in the capacitance, because graphene modification in this study may form a nanospace on a GC electrode whose size is approximately equivalent to the enzyme size for enhancing DET. Indeed, Martins and co-workers recently reported that the presence of graphene oxide at an enzyme/electrode interface might diminish the activation energy by decreasing the distance between the electrode surface and the enzyme.\textsuperscript{17} In addition, other electrode materials with nanostructured surfaces play an important role in achieving the efficient DET of enzymes.\textsuperscript{3,4,10,18} Further structural investigation such as surface wettability may give us significant information concerning the DET reaction.

4. Conclusion

We successfully developed a graphene modified GC electrode without any other nanomaterial modification to achieve the efficient DET of BOD while suppressing large increase in capacitance. This is unlike other carbon material modification results. We consider that our graphene modified electrode has the potential for use in developing a much simpler electrochemical enzyme biosensor platform based on the DET reaction such as therapeutic drug monitoring by using cytochromes P450.

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References