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Enzyme precipitate coating of pyranose oxidase on carbon nanotubes and their electrochemical applications

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ABSTRACT

Pyranose oxidase (POx), which doesn't have electrically non-conductive glycosylation moiety, was immobilized on carbon nanotubes (CNTs) via three different preparation methods: covalent attachment (CA), enzyme coating (EC) and enzyme precipitate coating (EPC). CA, EC and EPC of POx on CNTs were used to fabricate enzymatic electrodes for enzyme-based biosensors and biofuel cells. Improved enzyme loading of EPC resulted in 6.5 and 4.5 times higher activity per weight of CNTs than those of CA and EC, respectively. After 34 days at room temperature, EPC retained 65% of initial activity, while CA and EC maintained 9.2% and 26% of their initial activities, respectively. These results indicate that precipitation and crosslinking steps of EPC have an important role in maintaining enzyme activity. To demonstrate the feasibility of POx-based biosensors and biofuel cells, the enzyme electrodes were prepared using CA, EC, and EPC samples. In the case of biosensor, the sensitivities of the CA, EC, and EPC electrodes without BQ were measured to be 0.27, 0.76 and 3.7 mA/M/cm^2, while CA, EC and EPC electrode with BQ showed 25, 25, and 60 mA/M/cm^2 of sensitivities, respectively. The maximum power densities of biofuel cells using CA, EC and EPC electrodes without BQ were 41, 47 and 53 μW/cm^2, while CA, EC and EPC electrodes with BQ showed 260, 330 and 500 μW/cm^2, respectively. The POx immobilization and stabilization via the EPC approach can lead us to develop continuous glucose monitoring biosensors and high performing biofuel cells.

1. Introduction

Enzyme-based biosensors and biofuel cells have attracted a great attention because they can potentially provide an improved sensitivity for both environmental and healthcare sensors, and an uninterrupted electrical power for various micro-devices (Kim et al., 2008; Minteer et al., 2012; Moehlenbrock and Minteer, 2008; Nichols et al., 2013). In particular, a continuous glucose monitoring based on enzyme biosensor has received a growing attention due to the ever-increasing number of diabetic patients (Heller and Feldman, 2008; Murphy et al., 2008; Rasmussen et al., 2016). Enzyme-based biofuel cells can generate electrical energy from biofuels such as sugars and alcohols, which are commonly available in biological and environmental systems (Ha et al., 2012; Meredith and Minteer, 2012; Osman et al., 2011). Due to their ability for utilizing these naturally available fuels, they can provide continuous and uninterrupted electrical energy, which are not possible for the battery technology.

However, the drawback of enzyme-based biosensors and biofuel cells is that the active site of enzyme is buried in their protein structure. Thus, it is difficult for the electrons to be transferred between the active site and the electrode, which limits their electrochemical performances. To enhance the electron transfer rate of enzyme-based biosensors and biofuel cells, various studies have been attempted to modify the enzyme structures and enzyme-based electrode designs (Courjean et al., 2009). For example, glucose oxidase (GOx) was deglycosylated to improve the direct electron transfer rate between the active sites and the electrode (Courjean et al., 2009). However, removal of glycosylation moiety from the surface of GOx by the chemical treatment can lower the initial activity and long-term stability of enzymes (Kalisz et al., 1991). In this standpoint, the use of intrinsically non-glycosylated

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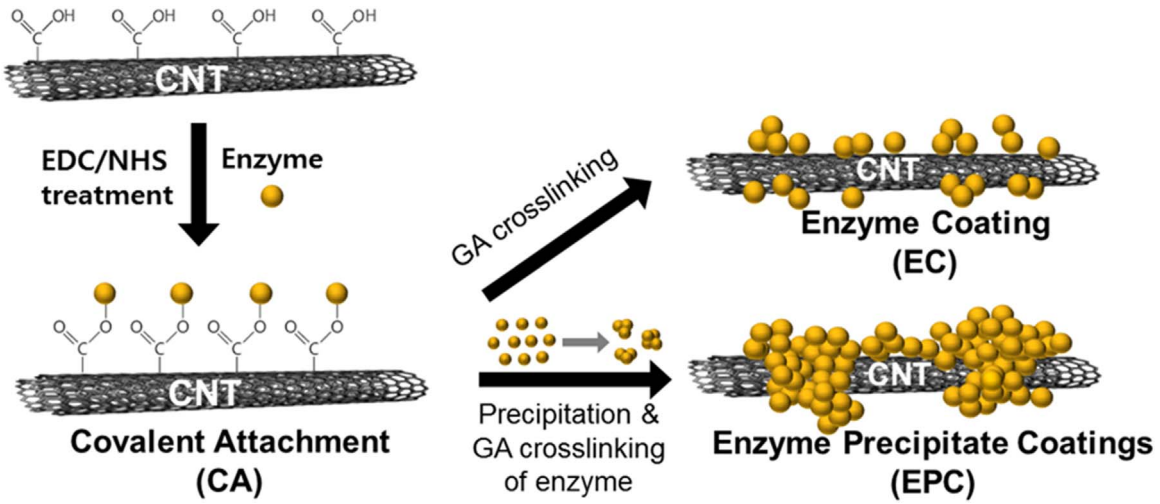

Fig. 1. Schematic illustrations of three different immobilization methods of pyranose oxidase (POx) on carbon nanotubes (CNTs).

enzyme such as pyranose oxidase (POx) can be a good alternative enzyme for various enzyme-based electrochemical devices (Spadiut et al., 2010; Spadiut et al., 2009; Tasca et al., 2007; Timur et al., 2006). POx produced from recombinant *E. coli* in a scalable fashion are not surrounded by electrically non-conductive glycosylation moiety (Giffhorn, 2000). In addition, the active site of POx is located closer to its outer surface than that of GOx (11 Å for POx versus 15 Å for GOx) (Courjean et al., 2009; Degani and Heller, 1987; Hallberg et al., 2004). Consequently, POx can offer a more efficient electron transfer between its active sites and electrode surfaces than that of GOx (Spadiut et al., 2010; Spadiut et al., 2009; Tasca et al., 2007; Timur et al., 2006). However, despite of these advantages, the eventual success of POx-based electrochemical applications requires the immobilization of POx to improve both its loading and stability.

For the past decades, various methods have been developed to improve the enzyme loading and stability (Kim et al., 2006, 2008; Minteer et al., 2012; Rasmussen et al., 2016). Especially, the utilization of electrically conductive nanomaterials such as carbon nanotubes (CNTs), polyaniline nanofibers and mesoporous carbons have been widely and successfully applied in enzyme-based electrochemical studies. By using these materials, the electron generation rate was improved by increasing the amount of enzyme loading, as well as the life-time is extended by stabilizing the immobilized enzymes in/on nanomaterials (Huang, 2006; Li et al., 2009; Minteer et al., 2012). Among those materials, CNTs are the most promizing candidate for enzyme-based electrochemical applications due to their high electron conductivity (Minteer et al., 2012; Wang, 2005). Consequently, CNTs have been used to immobilize and to stabilize various enzymes for different electrochemical applications such as biosensors and biofuel cells (Kim et al., 2011; Kwon et al., 2010; Lee et al., 2012; Minteer et al., 2012).

In the present work, we immobilized and stabilized POx on CNTs to investigate the electrochemical performance of POx-based biosensors and biofuel cells. To immobilize POx on CNTs, we applied an enzyme precipitate coating (EPC) method, which has a three-step process consisting of covalent attachment, precipitation, and crosslinking of enzymes. POx was also immobilized on CNTs via covalent attachment (CA) and enzyme coating (EC) methods for the comparative study. For the enzyme-based glucose biosensor and biofuel cell applications, glucose sensitivity and power density output were characterized by using EPC-POx, EC-POx and CA-POx-based electrodes. Based on their comparative studies, we investigated the effects of enzyme precipitation and crosslinking steps used in the EPC method on the electrochemical performances of POx-based electrodes.

2. Materials and methods

2.1. Materials

CNTs (multi-walled, 30 ± 15 nm in outer diameter and 1–5 μm in length, purity $> 95\%$) was purchased from Nanolab Inc. (Newton, MA, USA). Carbon papers and Nafion® membrane with Pt-cathode were purchased from Fuel Cell Store (Boulder, CO, USA). Pyranose oxidase from *Coriolus sp*. (POx, EC 1.1.3.10, expressed in *E. coli*), sodium phosphate, glutaraldehyde (GA, 25%), ammonium sulfate (AS), Tris–HCl, D-glucose, horseradish peroxidase, o-dianisidine dihydrochloride, Nafion® (5 wt%), 1, 4-benzoquinone (BQ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC) and 2-(N-morpholino) ethanesulfonic acid (MES) were purchased from Pierce (Rockford, IL, USA). N-hydroxysuccinimide (NHS) was purchased from Alfa Aeser (Ward Hill, MA, USA), respectively.

2.2. Acid-treatment of CNTs

CNTs (100 mg) were treated with the mixture of H_2SO_4 (7.5 mL of 18 M of H_2SO_4) and HNO_3 (2.5 mL of 16 M of HNO_3) solution at room temperature under shaking (200 rpm) for 12 h, which forms carboxyl groups on the CNT surface for the POx immobilization and increases the surface hydrophilicity for enhancing their miscibility in aqueous buffer solution. Acid-treated CNTs were washed with deionized water by using iterative cycles of centrifugation (10,000 rpm for 10 min), decantation and DI water washing. Excessively-washed CNTs were dried at 80 °C in a vacuum oven, and stored at room temperature.

2.3. Immobilization of pyranose oxidase (POx) on acid-treated CNTs

POx was immobilized on acid-treated CNTs via three different methods: covalent attachment (CA), enzyme coating (EC), and enzyme precipitate coating (EPC) (Fig. 1). For the preparation of EPC, 2 mg of acid-treated CNTs were suspended in the mixture of MES buffer (100 mM, pH 6.5), N-Hydroxysulfosuccinimide (NHS, 434 mM) solution, and N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 53.2 mM) solution (Jiang et al., 2004). After stirring for 1 h, CNTs were centrifuged and washed for two times with DI water. After 1 mL of POx solution (10 mg mL^{-1})

was added to the solution containing the functionalized CNTs, the mixture was shaken at 150 rpm at room temperature for 1 h. Ammonium sulfate solution was added into the solution containing POx and CNTs to make a final concentration of 40 wt% and the final mixture was further shaken at 150 rpm. After 30 mins, GA solution (final concentration of 0.5 wt%) was added to the mixture and incubated at 4 °C for overnight. Then, unreacted aldehyde groups were capped by incubating the samples in 100 mM Tris–HCl buffer (pH 7.4) for 1 h. After 3 times of washing with 100 mM sodium phosphate buffer (PB, pH 7.0), samples were stored in the 100 mM PB (pH 7.0) at 4 °C until use. For the preparation of EC, ammonium sulfate was replaced by 100 mM PB (pH 7.0). For the preparation of CA, ammonium sulfate and GA solution were replaced by 100 mM PB (pH 7.0).

2.4. Field emission scanning electron microscope (FE-SEM) analysis

The field emission scanning electron microscope (FE-SEM) images of POx-CNTs samples prepared by CA, EC and EPC methods were obtained by a Quanta FEG 250 (FEI, Oregon, USA). For the FE-SEM analysis, all the samples were washed by DI water to remove any salts and freeze-dried for 24 h. After platinum (Pt) coating, the samples were analyzed at an accelerating voltage of 15 kV.

2.5. Activity and stability measurements of POx on CNTs

The activities of POx-CNTs prepared by CA, EC, and EPC methods were measured by a previously reported GOx assay (Bergmeyer, 1974). To measure the activity, 10 μL of POx sample was mixed with 990 μL of reaction cocktail containing glucose (555 mM), o-dianisidine (0.21 mM), and horseradish peroxidase (6 units/mL) in 100 mM PB (pH 7.0). The increase of absorbance at 500 nm (A500) was measured using a UV spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan).

For stability measurement, the POx-CNT samples prepared by CA, EC and EPC methods were incubated in 100 mM PB (pH 7.0) at room temperature. The amount of each sample was determined by the amount of CNT (50 μg/mL), and an aliquot was removed from each sample and diluted 100 times for the measurement of residual activity at each time point. The relative activity was calculated from the ratio of residual activity to the initial activity of each sample.

2.6. POx on CNTs for glucose biosensor tests

Enzyme electrodes were fabricated on glassy carbon electrodes (GCE, 3 mm diameter, CH Instruments Inc., Austin, TX, USA). POx-CNTs prepared by CA, EC, and EPC methods at a final CNTs concentration of 2 mg mL^{-1} were dispersed in 0.5 wt% Nafion® solution. The suspension (20 μL) was dropped on the electrode surface and allowed to dry under ambient conditions for about 1 h. The enzyme electrodes were kept in 100 mM PB (pH 7.0) overnight before use.

An electrochemical workstation (CHI 760 C) from CH Instruments was used for an amperometric study of POx electrodes. A commercial standard Ag/AgCl electrode and a platinum wire electrode (CH Instruments, Austin, TX, USA) were used as the reference and counter electrodes, respectively. The diameter and length of the platinum counter electrode were 0.5 mm and 32 mm, respectively. For the amperometry study, 600 mV vs. Ag/AgCl was applied to the enzyme electrodes. After a stabilization of current response, 10 μL of 1 mM glucose solution was added into the 10 mL of 100 mM PB (pH 7.0) with and without 10 mM BQ and the current signal was obtained.

2.7. POx on CNTs for biofuel cell tests

Carbon papers (CPs) (Fuel Cell Store, San Diego, CA, USA) with a thickness of 0.37 mm and an area of 0.33 cm^2 were used to prepare the POx-based enzyme anode for the biofuel cells. The CPs were treated with 18 M of sulfuric acid and 16 M of nitric acid (v:v=3:1) followed by washing with DI water. The acid-treated CPs were immersed in the solution containing POx-CA, POx-EC, and POx-EPC samples (2 mg mL^{-1}) with 0.5 wt% Nafion for 10 mins and dried at room temperature for 1 h. CPs containing POx samples were kept in 100 mM PB (pH 7.0) overnight at 4 °C before use. A Nafion® membrane with a Pt-based cathode (i.e., a half membrane electrode assembly (MEA)) was purchase from Fuel Cell Store (Boulder, CO, USA). To assemble the biofuel cells, CPs containing POx samples were combined with this commercial half MEA using a homemade air-breathing cell fixture.

Measurements of biofuel cell performance such as polarization curves were carried out using a Bio-Logic SP-150 (Knoxville, TN, USA) at room temperature. The performance of POx-based anode enzymatic biofuel cells was measured by circulating 200 mM glucose solution with and without 10 mM 1, 4-benzoquinone (BQ) in 100 mM PB (pH 7.0) at a flow rate of 617 μL/min. For the polarization curve test, constant load discharge mode was used to obtain the correlation between potential and current. An external resistance box (Model 380,400, Extech, Waltham, MA, USA) was used to apply an external load to the cell. The power output of the enzymatic biofuel cells at each load was calculated from the stabilized current and potential, and power densities were calculated based on the geometric surface area of the anode.

3. Results and discussion

3.1. Immobilization of POx on CNTs

Fig. 1 schematically shows the immobilization of POx on CNTs via three different preparation methods: covalent attachment (CA), enzyme coating (EC) and enzyme precipitate coating (EPC). The CA sample was prepared by forming covalent bonds between POx and carboxyl-functionalized CNTs through EDC-NHS reaction. For the EC sample, the additional step for a glutaraldehyde (GA) treatment was introduced to form cross-linkages between POx molecules on CNTs. The EPC sample was prepared by covalent attachment, ammonium sulfate precipitation, and GA cross-linking of POx on CNTs.

The field emission-scanning electron microscope (FE-SEM) analyses of pristine CNTs, and POx-CNTs prepared by CA, EC and EPC methods were shown in Fig. 2. The FE-SEM images of POx-CNTs with CA and EC methods do not show apparent differences when compared to that of pristine CNTs, while the SEM image of EPC shows a thick enzyme coating layer over CNTs due to the presence of POx aggregates. By checking twenty spots of each image, the average thicknesses of pristine CNTs, and POx-CNTs with CA, EC and EPC methods were estimated to be 21 ± 4, 25 ± 4, 30 ± 5 and 64 ± 13 nm, respectively. The increased thickness of EPC compared to those of CA and EC indicates an improved POx loading on CNTs for EPC sample. It demonstrates that the ammonium sulfate precipitation and GA crosslinking processes of POx are two critical steps for increasing enzyme loading on CNTs during the POx immobilization procedures.

3.2. Activity and stability of immobilized POx on CNTs

The activity of immobilized POx toward the glucose oxidation was measured by monitoring the oxidation rate of *o*-dianisidine catalyzed by horseradish peroxidase. The activities of POx-CNTs

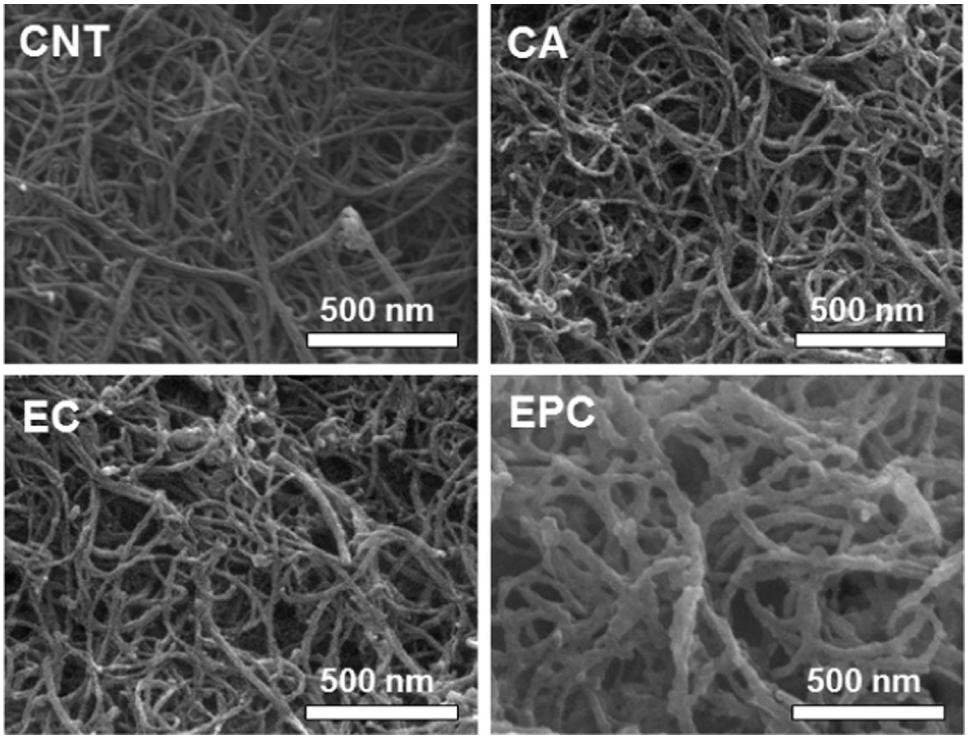

Fig. 2. The SEM images of pristine CNTs, CA, EC, and EPC.

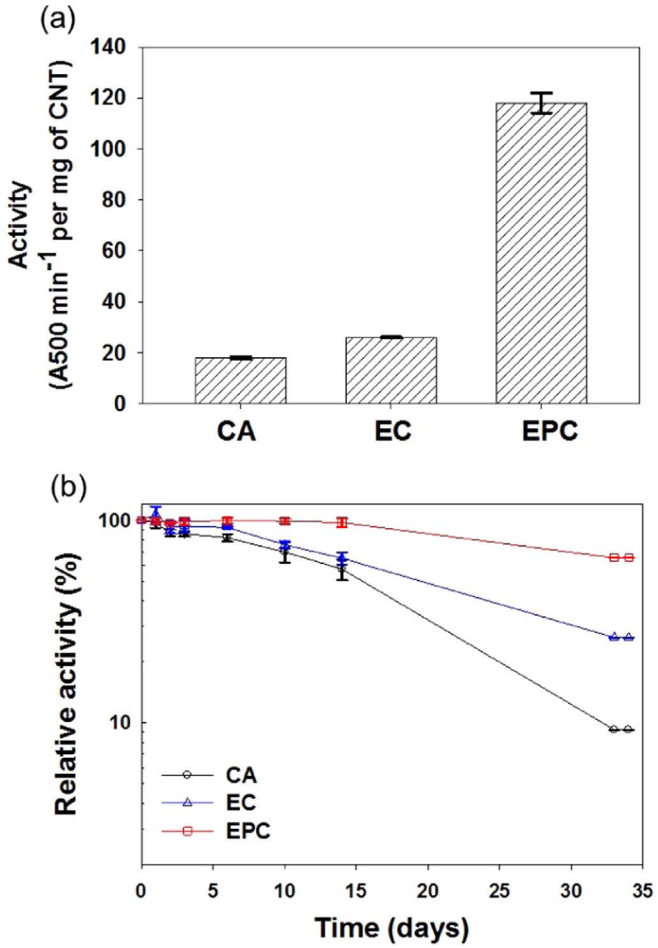

Fig. 3. (a) The activities of CA, EC, and EPC measured by oxidation of glucose. (b) The stabilities of CA, EC and EPC at room temperature under non-shaking condition.

prepared by CA, EC, and EPC methods were 18, 26, and 120 A_{500}/min per 1 mg of CNTs, respectively (Fig. 3a). The EPC sample resulted in 6.5 and 4.5 folds improvement of enzyme activity when compared to the CA and EC samples. The higher activity of EPC sample compared to that of CA and EC samples is a direct result of its enhanced enzyme loading over CNT by introducing both the precipitation and crosslinking steps in its immobilization process, as shown in FE-SEM images of Fig. 2.

The stabilities of POx-CNTs prepared by CA, EC and EPC methods were investigated by checking the time dependent activity of enzyme samples for 34 days at room temperature. As shown in Fig. 3b, EPC sample maintained 65% of its initial activity, whereas CA and EC samples showed 9.2% and 26% of their initial activities, respectively. The estimated half-life of EPC sample was 53.3 days, while those of CA and EC samples were 9.6 and 16.7 days, respectively. Thus, the half-life of EPC sample was 5.6 and 3.2 times longer than those of CA and EC samples, respectively. The higher stability of EPC than CA and EC can be explained by the multi-point cross-linkages of POx molecules presented in EPC sample. For EPC sample, precipitation by ammonium sulfate allows POx molecules to be closely packed. These closely packed enzyme molecules allow an efficient formation of multi-point enzyme cross-linkages over CNTs when the chemical cross-linker such as glutaraldehyde is introduced. These multi-point cross-linked POx aggregates over CNTs surface can efficiently inhibit not only the denaturation of enzyme molecules but also the detachment of enzyme molecules from the Supporting Materials (Mozhaev et al., 1990). Based on the comparison of activity and stability of POx-CNTs prepared by CA, EC and EPC methods, it can be concluded that the enzyme precipitation and cross-linking steps are critical for the improved loading and stability of immobilized enzymes.

3.3. Glucose biosensor tests of immobilized POx on CNTs

To investigate the glucose biosensor performance of POx, the enzyme electrodes were prepared by coating glassy carbon

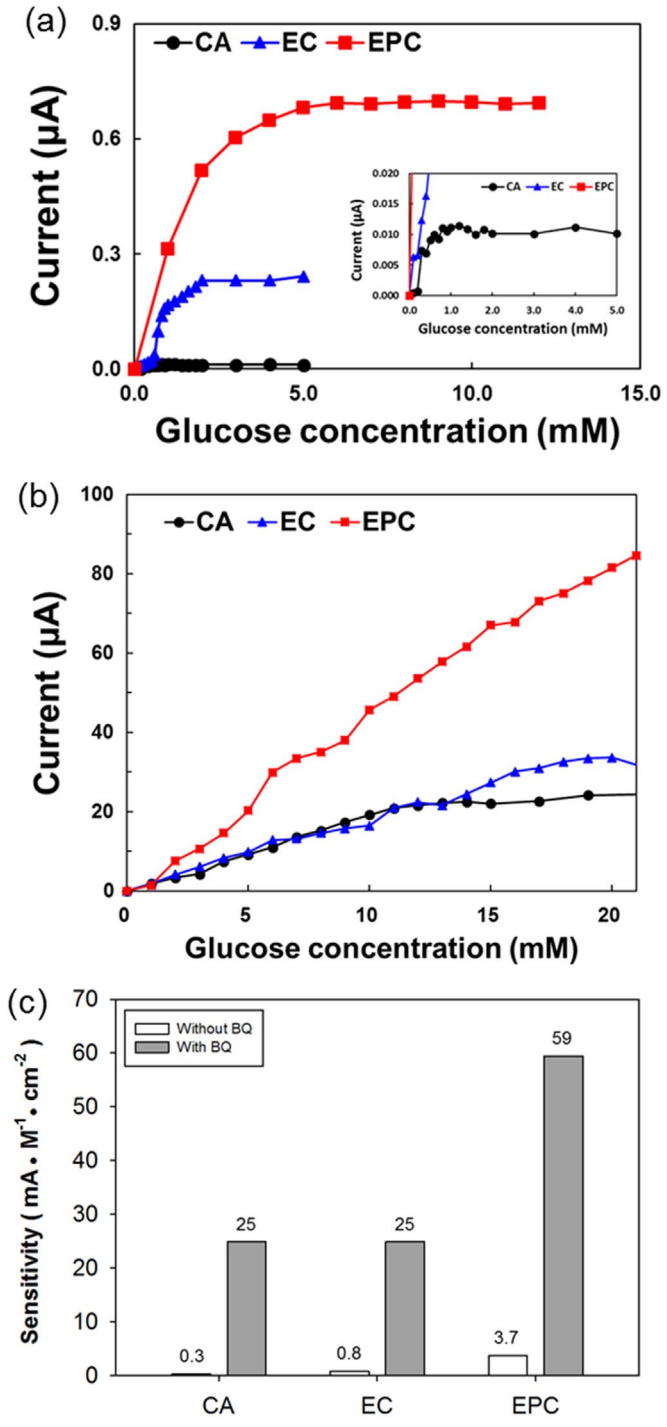

Fig. 4. Amperometric response to successive additions of glucose solution into the electrolyte of the POx-based electrode (a) without and (b) with mediator. The current response was measured under the operating potential of 600 mV vs. Ag/AgCl. The plot of current response versus the glucose concentration of CA is shown in the inset of Fig. 4a. (c) The sensitivity of CA, EC, and EPC without and with mediator.

electrodes with POx-CA, POx-EC, and POx-EPC samples in a presence of Nafion® binder. The current responses of CA, EC, and EPC electrodes without BQ (Fig. 4a) and with BQ (Fig. 4b) were obtained by successive additions of glucose to estimate the sensitivity of CA, EC and EPC electrodes from their slopes. As shown in Fig. 4c, the sensitivities of the CA, EC and EPC electrode without BQ were measured to be 0.27, 0.76, and 3.7 mA/M/cm^2, respectively. EPC electrode without BQ showed 13.6 and 2.8 times higher sensitivity than CA and EC electrode, respectively. The sensitivity of POx-based biosensor for the glucose detection can be mainly affected by (1) the amount of electron generation per surface area of the electrode and (2) the charge transfer efficiency of these electrons from enzyme active sites to electrode. The EPC electrode offers significantly high enzyme loading, which can be expected to generate a large number of electrons per surface area of the electrode. In the point of electron transfer efficiency, the use of non-glycosylated POx can be a better solution for enzyme biosensors than other glycosylated enzymes such as GOx. Non-glycosylated nature of POx allows its active sites to be located closer to the surface of enzyme, which leads to a shorter electron transfer distance between the enzyme active sites and electrode surface. This combination of both high enzyme loading offered by the EPC method and the efficient electron transfer offered by non-glycosylated POx leads to the high glucose sensitivity of the EPC-POx-based biosensor as shown in Fig. 4a.

The addition of BQ as a liquid mediator resulted in sensitivities of 25, 25 and 59 mA/M/cm^2 for biosensors with the CA, EC and EPC, respectively (Fig. 4c). The sensitivity of EPC was 2.3 times higher than those of CA and EC, respectively. In general, the sensitivity with BQ is much higher than one without BQ, because BQ molecules can easily diffuse into the active sites of enzymes and effectively mediate the electron transfer between the active sites of enzymes and electrode surfaces. However, it is very important to point out that the sensitivity of biosensors with EPC in both cases (without and with BQ) is much higher than that of biosensors with CA and EC. This higher sensitivity of biosensors with EPC is originated from its ability to form aggregated enzyme clusters via enzyme precipitation and crosslinking processes, which leads to its higher enzyme loading. With the higher enzyme loading, the biosensors with EPC can generate larger number of electrons in both cases without and with BQ.

We have further compared EPCs of GOx (EPC-GOx) and POx (EPC-POx) on CNTs (Table 1). Even though the spectrophotometric activity of EPC-POx was lower than EPC-GOx, the sensitivity (without BQ) of EPC-POx was 5.3 times higher than EPC-GOx. This higher sensitivity of EPC-POx could be explained by the efficient electron transfer offered by non-glycosylated protein structure of POx. Compared to the glycosylated protein structure of GOx, the active site of POx is located closer to its outer surface (11 Å for POx versus 15 Å for GOx) (Courjean et al., 2009; Degani and Heller, 1987; Hallberg et al., 2004). The charge transfer rate decreases exponentially as the separation distance between the active site and the electrode surface increases. Thus, the shorter electron transfer distance offered by the non-glycosylated POx can significantly enhance the glucose sensitivity of EPC-POx-based biosensor by improving its electron transfer rate. In order to validate the improved electron transfer rate for EPC-POx-based electrode compared to that of EPC-GOx-based electrode, we estimated their electron transfer rate constants from Laviron experiments (Laviron, 1979). The electron transfer rate constants of EPC-GOx and EPC-POx electrodes (without BQ) were 0.031 and 0.046 s^{-1}, respectively (Table 1). It indicates that glycosylated moieties of GOx hamper the electron transfer from the active site to the electrode,

Table 1
The comparison of spectrophotometric activity, glucose sensitivity, electron transfer rate constant between EPCs of GOx (EPC-GOx) and POx (EPC-POx).

	EPC-GOx	EPC-POx	Ratio (POx/GOx)
Spectrophotometric activity (ΔA_{500} min^{-1} per mg of CNTs)	980	120	0.12
Glucose sensitivity (mA M^{-1} cm^{-2})	0.69	3.7	5.3
Electron transfer rate constant (s^{-1})	0.031	0.046	1.5

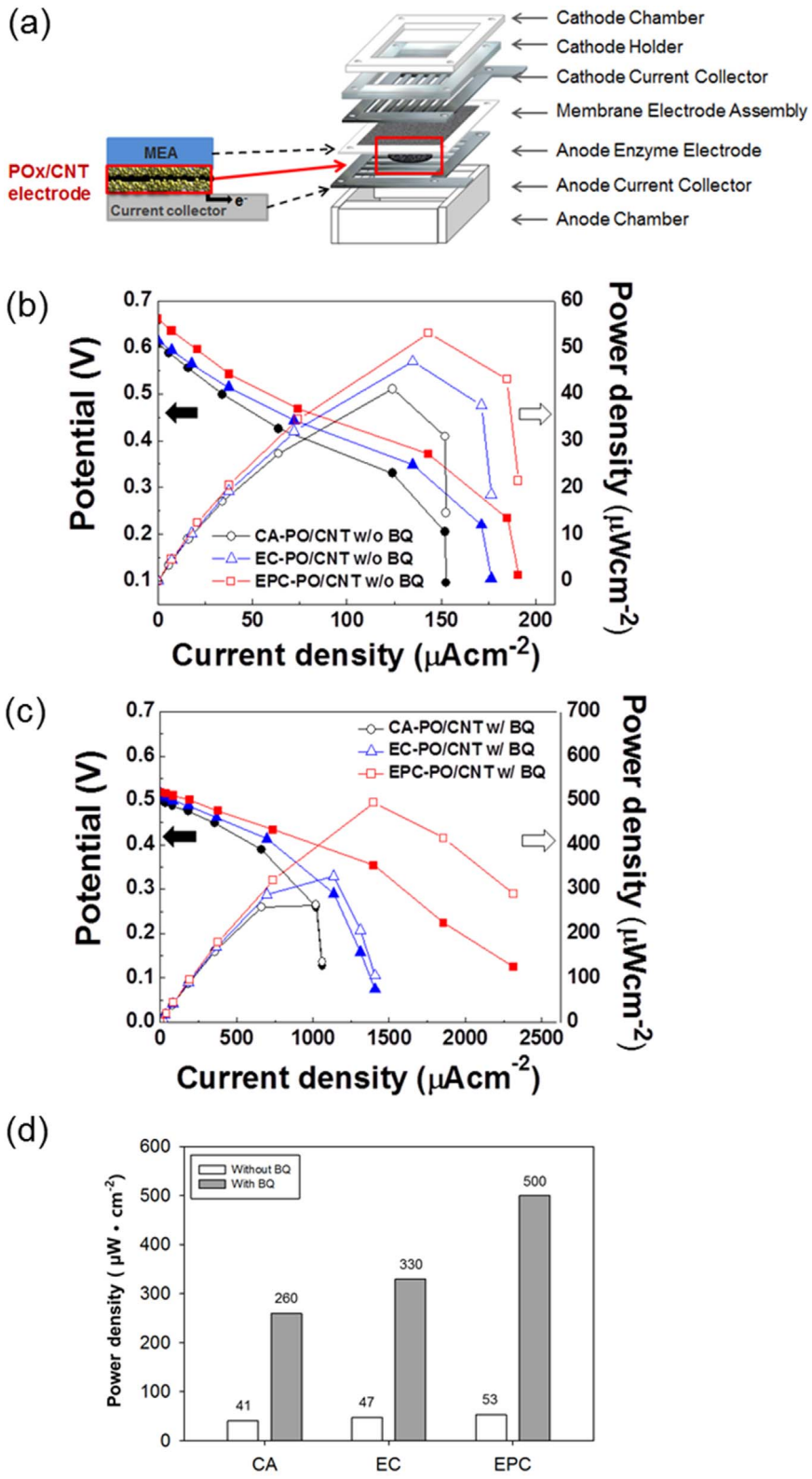

Fig. 5. (a) Schematic illustrations for the assembly of enzymatic biofuel cells comprising enzyme electrode. Polarization curves of CA, EC, and EPC electrodes (b) without and (c) with mediator. (d) The maximum power density of CA, EC, and EPC electrodes without and with mediator.

decreasing the sensitivity of EPC-GOx biosensors.

3.4. *Biofuel cells application of immobilized POx on CNTs*

The practical applications of biofuel cells are being hampered by the low initial power density and short lifetime. Among various factors, the low enzyme loading and poor enzyme stability are highly responsible for these low initial performance and lifetime issues of current biofuel cells, respectively. In this study, we have designed the enzyme anodes using POx via CA, EC, and EPC

methods for the biofuel cell applications. As indicated in Fig. 5a, the enzymatic biofuel cells consist of the anode chamber, anode current collector, anode enzyme electrode, proton exchange membrane (Nafion® 117), air-breathing Pt cathode, cathode current collector and cathode holder. During the cell operation, POx partially oxidizes a glucose molecule and generates two electrons at the C-2 position (Giffhorn, 2000). Fig. 5b and c show the cell polarization and power density curves of biofuel cells with CA-POx, EC-POx and EPC-POx-based enzyme anodes under the absence and presence of BQ, respectively. Their corresponding maximum power densities are summarized in Fig. 5d. According to Fig. 5d, the maximum power densities of biofuel cells with CA-POx, EC-POx and EPC-POx-based anodes under the absence of BQ were 41, 47 and 53 $\mu W/cm^2$, respectively. The addition of BQ as a liquid mediator improves the maximum power densities of biofuel cells similar to the biosensor results. Regardless of absence or presence of BQ, the biofuel cells with EPC-POx-based anode showed higher maximum power densities than that of CA-POx and EC-POx-based anodes. The reasons for achieving this higher maximum power density with EPC-POx-based anode can be explained in terms of its improved electron generation amount similar to EPC-POx-based biosensors. Since the EPC method offers a much higher enzyme loading on CNTs than CA and EC methods, a larger number of electrons would be generated per unit geometrical surface area of enzyme anode for the biofuel cell with EPC-POx-based anode. As the electron generation amount increases with EPC-POx-based anode, its power density output would be also improved when compared to that of CA-POx and EC-POx-based anodes. The power density output of biofuel cells can be further improved by adding CNTs or catalase. The addition of CNTs would increase the electron transfer rate constant of enzyme anode (Kim et al., 2015), while catalase can decompose hydrogen peroxide leading to the improved power output (Zebda et al., 2011).

4. Conclusions

In our present study, both POx-based biosensors and biofuel cells have successfully developed using the enzyme precipitate coating (EPC) approach. The EPC method offers the multi-point crosslinked POx aggregates over the CNTs surface via the enzyme precipitation and crosslinking steps. Due to a presence of these multi-point crosslinked POx aggregates, immobilization of POx on CNTs via EPC showed both improved enzyme loading and enzyme stability when compared to that of covalent attachment (CA) and enzyme coating (EC) methods. With the increased enzyme loading, the EPC-POx-based electrodes can generate a greater number of electrons per unit geometrical surface area of enzyme electrode. Furthermore, the non-glycosylated protein structure of POx should lead to the shorter electron transfer distance between the active site and the electrode surface. Consequently, the POx can offer the improved electron transfer rate compared to that of glycosylated GOx. This combination of high POx loading and improved electron transfer rate of POx lead to both the high glucose sensitivity of biosensor and high maximum power density of biofuel cell when EPC-POx-based enzyme electrodes are utilized. It is anticipated that the immobilization of POx on CNTs via the EPC protocol will create promizing opportunities in various electrochemical applications such as continuous glucose monitoring system and implantable biofuel cells.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.08.086.

References

Bergmeyer, H.U., Gawehn, K., Grassl, M., 1974. Methods of Enzymatic Analysis, Second ed. Academic Press Inc, New York, NY, pp. 457–458.
Courjean, O., Gao, F., Mano, N., 2009. Deglycosylation of glucose oxidase for direct and efficient glucose electrooxidation on a glassy carbon electrode. Angew. Chem. Int. Ed. 48, 5897–5899.
Degani, Y., Heller, A., 1987. Direct electrical communication between chemically modified enzymes and metal-electrodes. 1. Electron transfer from glucose oxidase to metal electrodes via eletron relays, bound covalently to the enzyme. J. Phys. Chem. 91, 1285–1289.
Giffhorn, F., 2000. Fungal pyranose oxidases: occurrence, properties and biotechnical applications in carbohydrate chemistry. Appl. Microbiol. Biotechnol. 54, 727–740.
Ha, S., Wee, Y., Kim, J., 2012. Nanobiocatalysis for enzymatic biofuel cells. Top. Catal. 55, 1181–1200.
Hallberg, B.M., Leitner, C., Haltrich, D., Divne, C., 2004. Crystal structure of the 270 kDa homotetrameric lignin-degrading enzyme pyranose 2-oxidase. J. Mol. Biol. 341, 781–796.
Heller, A., Feldman, B., 2008. Electrochemical glucose sensors and their applications in diabetes management. Chem. Rev. 108, 2482–2505.
Huang, J.X., 2006. Syntheses and applications of conducting polymer polyaniline nanofibers. Pure Appl. Chem. 78, 15–27.
Jiang, K.Y., Schadler, L.S., Siegel, R.W., Zhang, X.J., Zhang, H.F., Terrones, M., 2004. Protein immobilization on carbon nanotubes via a two-step process of diimide-activated amidation. J. Mater. Chem. 14, 37–39.
Kalisz, H.M., Hecht, H.J., Schomburg, D., Schmid, R.D., 1991. Effects of carbohydrate depletion on the strycture, stability and activity of glucose oxidase from aspergillus niger. BioChim. Biophys. Acta 1080, 138–142.
Kim, B.C., Zhao, X.Y., Ahn, H.K., Kim, J.H., Lee, H.J., Kim, K.W., Nair, S., Hsiao, E., Jia, H. F., Oh, M.K., Sang, B.I., Kim, B.S., Kim, S.H., Kwon, Y., Ha, S., Gu, M.B., Wang, P., Kim, J., 2011. Highly stable enzyme precipitate coatings and their electrochemical applications. Biosens. Bioelectron. 26, 1980–1986.
Kim, J., Grate, J.W., Wang, P., 2006. Nanostructures for enzyme stabilization. Chem. Eng. Sci. 61, 1017–1026.
Kim, J., Grate, J.W., Wang, P., 2008. Nanobiocatalysis and its potential applications. Trends Biotechnol. 26, 639–646.
Kim, J.H., Jun, S.A., Kwon, Y., Ha, S., Sang, B.I., Kim, J., 2015. Enhanced electrochemical sensitivity of enzyme precipitate coating (EPC)-based glucose oxidase biosensors with increased free CNT loadings. Bioelectrochemistry 101, 114–119.
Kwon, K.Y., Yang, S.B., Kong, B.S., Kim, J., Jung, H.T., 2010. High-performance biosensors based on enzyme precipitate coating in gold nanoparticle-conjugated single-walled carbon nanotube network films. Carbon 48, 4504–4509.
Laviron, E., 1979. General expression of the linear potential sweep voltammogram in the case of diffusionless electrochemical systems. J. Electroanal. Chem. 101, 19–28.
Lee, H.J., Kim, B.C., Oh, M.K., Kim, J., 2012. A sensitive and reliable detection of thrombin via enzyme-precipitate-coating-linked aptamer assay. Chem. Commun. 48, 5971–5973.
Li, D., Huang, J.X., Kaner, R.B., 2009. Polyaniline nanofibers: a unique polymer nanostructure for versatile applications. Acc. Chem. Res. 42, 135–145.
Meredith, M.T., Minteer, S.D., 2012. Biofuel cells: enhanced enzymatic bioelectrocatalysis. Annu. Rev. Anal. Chem. 5, 157–179.
Minteer, S.D., Atanassov, P., Luckarift, H.R., Johnson, G.R., 2012. New materials for biological fuel cells. Mater. Today 15, 166–173.
Moehlenbrock, M.J., Minteer, S.D., 2008. Extended lifetime biofuel cells. Chem. Soc. Rev. 37, 1188–1196.
Mozhaev, V.V., Melik-nubarov, N.S., Sergeeva, M.V., Šikšnis, V., Martinek, K., 1990. Strategy for stabilizing enzymes part one: increasing stability of enzymes via their multi-point interaction with a support. Biocatal. Biotransform. 3, 179–187.
Murphy, H.R., Rayman, G., Lewis, K., Kelly, S., Johal, B., Duffield, K., Fowler, D., Campbell, P.J., Temple, R.C., 2008. Effectiveness of continuous glucose monitoring in pregnant women with diabetes: randomised clinical trial. Br. Med. J. 337, 1–8.
Nichols, S.P., Koh, A., Storm, W.L., Shin, J.H., Schoenfisch, M.H., 2013. Biocompatible

materials for continuous glucose monitoring devices. Chem. Rev. 113, 2528–2549.
Osman, M.H., Shah, A.A., Walsh, F.C., 2011. Recent progress and continuing challenges in bio-fuel cells. Part I: enzymatic cells. Biosens. Bioelectron. 26, 3087–3102.
Rasmussen, M., Abdellaoui, S., Minteer, S.D., 2016. Enzymatic biofuel cells: 30 years of critical advancements. Biosens. Bioelectron. 76, 91–102.
Spadiut, O., Brugger, D., Coman, V., Haltrich, D., Gorton, L., 2010. Engineered pyranose 2-oxidase: efficiently turning sugars into electrical energy. Electroanalysis 22, 813–820.
Spadiut, O., Pisanelli, I., Maischberger, T., Peterbauer, C., Gorton, L., Chaiyen, P., Haltrich, D., 2009. Engineering of pyranose 2-oxidase: improvement for biofuel cell and food applications through semi-rational protein design. J. Biotechnol. 139, 250–257.
Tasca, F., Timur, S., Ludwig, R., Haltrich, D., Volc, J., Antiochia, R., Gorton, L., 2007. Amperometric biosensors for detection of sugars based on the electrical wiring of different pyranose oxidases and pyranose dehydrogenases with osmium redox polymer on graphite electrodes. Electroanalysis 19, 294–302.
Timur, S., Yigzaw, Y., Gorton, L., 2006. Electrical wiring of pyranose oxidase with osmium redox polymers. Sens. Actuator B – Chem. 113, 684–691.
Wang, J., 2005. Carbon-nanotube based electrochemical biosensors: a review. Electroanalysis 17, 7–14.
Zebda, A., Gondran, C., Le Goff, A., Holzinger, M., Cinquin, P., Cosnier, S., 2011. Mediatorless high-power glucose biofuel cells based on compressed carbon nanotube-enzyme electrodes. Nat. Commun. 2, 1–6.