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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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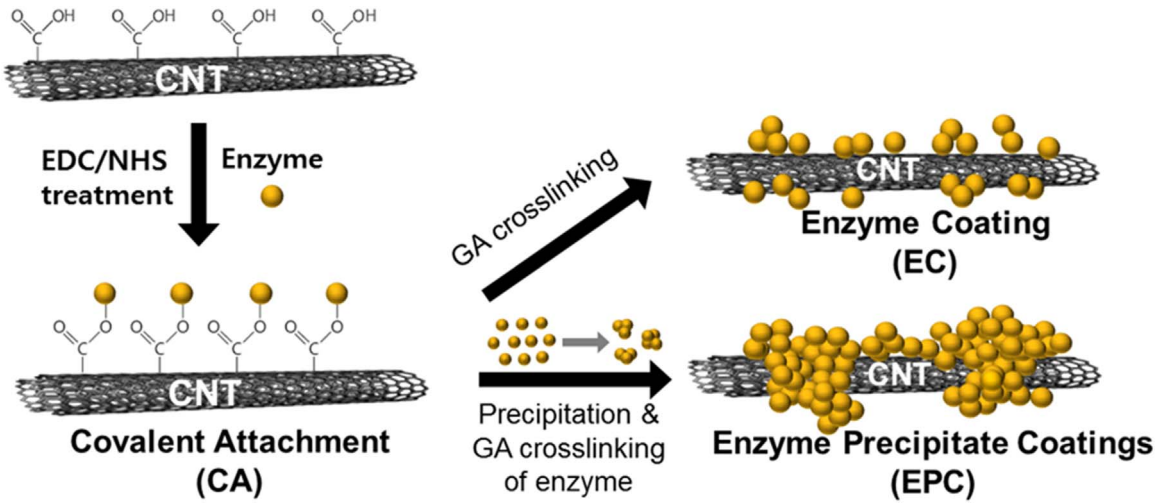

Fig. 1. Schematic illustrations of three different immobilization methods of pyranose oxidase (POx) on carbon nanotubes (CNTs).

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})