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Enzyme adsorption, precipitation and crosslinking of glucose oxidase and laccase on polyaniline nanofibers for highly stable enzymatic biofuel cells

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ABSTRACT

Enzymatic biofuel cells have many great features as a small power source for medical, environmental and military applications. Both glucose oxidase (GOx) and laccase (LAC) are widely used anode and cathode enzymes for enzymatic biofuel cells, respectively. In this paper, we employed three different approaches to immobilize GOx and LAC on polyaniline nanofibers (PANFs): enzyme adsorption (EA), enzyme adsorption and crosslinking (EAC) and enzyme adsorption, precipitation and crosslinking (EAPC) approaches. The activity of EAPC-LAC was 32 and 25 times higher than that of EA-LAC and EAC-LAC, respectively. The half-life of EAPC-LAC was 53 days, while those of EA-LAC and EAC-LAC were 6 and 21 days, respectively. Similar to LAC, EAPC-GOx also showed higher activity and stability than EA-GOx and EAC-GOx. For the biofuel cell application, EAPC-GOx and EAPC-LAC were applied over the carbon papers to form enzyme anode and cathode, respectively. In order to improve the power density output of enzymatic biofuel cell, 1,4-benzoquinone (BQ) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were introduced as the electron transfer mediators on the enzyme anode and enzyme cathode, respectively. BQ- and ABTS-mediated enzymatic biofuel cells fabricated by EAPC-GOx and EAPC-LAC showed the maximum power density output of 37.4 $\mu W/cm^2$, while the power density output of 3.1 $\mu W/cm^2$ was shown without mediators. Under room temperature and 4 °C for 28 days, enzymatic biofuel cells maintained 54 and 70% of its initial power density, respectively.

1. Introduction

Enzymatic biofuel cells are energy conversion devices that could efficiently convert the chemical energy of biofuels into electrical energy using enzymes as biocatalysts [1]. They can operate under mild condition such as a neutral pH and an ambient temperature [1,2]. Enzymatic biofuel cells have a great potential to be used as a portable and uninterrupted power source for the various medical, environmental and military applications by using the fuels such as glucose, which are commonly available to biological and environmental systems [3–7]. However, despite of promising application of enzymatic biofuel cells, their low power density and short lifetime, both of which linked to low loading and poor stability of enzymes, have been identified as two critical issues that need to be addressed [8]. As a potential solution, nanobiocatalytic approaches, in which enzyme are incorporated into nanostructured materials, have been employed to provide enhanced the loading and stability of enzymes [9]. In particular, polyaniline nanofibers (PANFs) are very interesting supporting materials because they can offer a large surface area with nanofiber matrices as well as high electron conductive property [10]. Moreover, PANFs can be easily and economically synthesized when compared to other nanostructured materials such as electrospun nanofibers, nanoparticles, carbon nanotubes and mesoporous materials. Because of these promising properties, PANFs have been

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employed to immobilize and stabilize various enzymes on PANFs [11–13].

In the present study, we immobilized glucose oxidase (GOx) and laccase (LAC) on PANFs via the enzyme adsorption, precipitation and crosslinking (EAPC) approach, together with the enzyme adsorption (EA) and enzyme adsorption and crosslinking (EAC) approaches as controls, to fabricate enzymatic biofuel cells. The anode is consisted of GOx immobilized in the form of EAPC (i.e., EAPC-GOx), while the cathode is consisted of LAC immobilized in the form of EAPC (i.e., EAPC-LAC). We investigated the effect of mediators on each electrode, and evaluated their biofuel cell performances in terms of power density and long-term stability by using glucose as the fuel. Based on our knowledge, it is first time to fabricate and successfully operate enzymatic biofuel cells by utilizing both the enzyme anode and enzyme cathode in the form of EAPC.

2. Materials and methods

2.1. Materials

Laccase (LAC) from *Trametes versicolor*, glucose oxidase (GOx) from *Aspergillus niger*, syringaldazine, methanol, β-D-glucose, horseradish peroxidase (HRP), 3,3′,5,5′-tetramethylbenzidine (TMB), glutaraldehyde solution (GA, 25%), ammonium sulfate, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,4-benzoquinone (BQ), Nafion® solution (5 wt%), aniline and ammonium persulfate were purchased from Sigma (St. Louis, MO, USA). Carbon papers (CPs) and Nafion® 117 membrane were purchase from Fuel Cell Store (Boulder, CO, USA).

2.2. Synthesis of polyaniline nanofibers

Polyaniline nanofiber was synthesized by initiating polymerization of aniline in acidic condition using ammonium persulfate as an initiator [10]. First, 9 M aniline monomer solution and 0.1 M ammonium persulfate solution were prepared in 1 M HCl. Both aniline and ammonium persulfate solutions in HCl were mixed and shaken using 200 rpm at room temperature for 24 h. After a completion of the polymerization reaction, PANFs were centrifuged down, washed using DI water excessively for 3 times, suspended in DI water and stored at 4 °C until use.

2.3. Immobilization of LAC and GOx on PANFs

PANFs were used for the immobilization of LAC and GOx in three different enzyme immobilization methods: EA, EAC, and EAPC. PANFs were washed with 100 mM phosphate buffer (PB) solution (pH 7.0) for 3 times prior to the immobilization processes. Immobilized LAC in the form of enzyme adsorption on PANF (i.e., EA-LAC) was prepared by mixing the 2 mg of PANF with the LAC solution (10 mg/ml) in 100 mM PB (pH 6.5) under the shaking condition at 150 rpm for 1 hr. For the preparation of immobilized LAC in the form of enzyme adsorption and crosslinking on PANF (i.e., EAC-LAC), the glutaraldehyde (GA) as the chemical crosslinking agent was introduced to make a final concentration of 0.5% (w/v) to the EA-LAC sample under the shaking condition at 50 rpm and 4 °C for 17 h. To prepare the immobilized LAC in the form of enzyme adsorption, precipitation and crosslinking on PANF (i.e., EAPC-LAC), the ammonium sulfate solution was introduced into the 100 mM PB solution (pH 6.5) containing both LAC and PANF to make a concentration of 50% (w/v). In the presence of the ammonium sulfate salt, the free LAC (i.e., LAC that is not adsorbed over PANF surface) was precipitated out to form the enzyme aggregates. After shaking at 200 rpm for 30 min, the GA solution was added into the mixture to make a concentration of 0.5% (w/v) to chemically crosslink the precipitated LAC aggregates over the surface of PANF at 4 °C for 17 h. To cap un-reacted aldehyde groups, the samples were shaken at 200 rpm in 100 mM Tris–HCl buffer (pH 7.4) solution for 30 min and the samples were excessively washed for 3 times with the 100 mM PB solution (pH 6.5). EA-LAC, EAC-LAC and EAPC-LAC were stored in 100 mM PB solution (pH 6.5) at 4 °C until use. The EA-GOx, EAC-GOx and EAPC-GOx were also prepared by following the same protocols that were used for the immobilization of LAC on PANF as described above.

2.4. Activity and stability measurement of immobilized LAC and GOx on PANFs

The activity was calculated from the time-dependent change of absorbance, and the stabilities of samples were checked by measuring the residual activity time-dependently after incubation in buffer solution at room temperature. The measurement of LAC activity was based on the oxidation of syringaldazine [14]. Syringaldazine (7.8 mg) dissolved in methanol (10 ml) with a final concentration of 0.216 mM. 100 μl of the solution containing the immobilized LAC on PANFs (0.1 mg/ml) was mixed with 800 μl of 100 mM PB solution (pH 6.5) and the mixtures were heated at 30 °C for 10 min. This heated mixture was added with 100 μl of syringaldazine solution (0.216 mM) and the absorbance at 530 nm (A_{530}) was measured by using UV spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan).

The activity of immobilized GOx on PANFs was measured by GOx assay [15]. The measurement of activity needs a reaction cocktail containing TMB and glucose solution. Reaction cocktail was made of 12 ml of TMB solution (0.576 mg/ml) and 2.5 ml glucose solution (110.1 mg/ml). To measure activity of immobilized GOx, 890 μl of reaction cocktail was mixed with 10 μl HRP solution (3.798 mg/ml). Then, 100 μl of the solution containing the immobilized GOx on PANFs (1 μg/ml) was added to 900 μl of the mixed solution. The absorbance of immobilized GOx was measured at 655 nm (A_{655}) by using UV spectrophotometer.

2.5. Preparation of enzyme electrodes

Carbon papers (CPs, thickness of 370 μm, 0.44 g/cm^3) were treated with acid before use. In a typical preparation, 2 cm × 2 cm squares of CPs was added to an acid solution composed of H_2SO_4 (98%, 30 ml) and HNO_3 (70%, 10 ml) for overnight at room temperature under a stirring condition. Then, acid-treated CPs were washed with distilled water, dried at vacuum condition and stored at room temperature until use. To prepare the GOx-based anode, the immobilized GOx on PANF sample was mixed with Nafion® solution (final conc. 0.5 wt%) and this mixture was stored at 4 °C for 1 h. Acid-treated CPs (0.332 cm^2) was soaked into the mixture for 10 min, followed by drying at ambient conditions. After drying, the prepared GOx-based enzyme anode was stored in 100 mM PB solution (pH 7.0) at 4 °C. For the LAC-based cathode, ABTS (30 mM, 3.3 mg) was added to the mixture of Nafion® and immobilized LAC on PANF sample. When the LAC-based cathode containing ABTS was dried, it was stored in 100 mM PB solution (pH 7.0) at 4 °C. Since ABTS has high solubility in aqueous solution, washing was not carried out [16].

2.6. Biofuel cell operation measurement

The electrochemical measurements were performed by using Bio-Logic SP-150 (Knoxville, TN, USA). The performance of enzymatic biofuel cells was measured by circulating 200 mM glucose solution with and without 10 mM BQ in 100 mM PB solution (pH 7.0) at the flow rate of 0.4 ml/min within the GOx-based anode. For the LAC-based cathode, the air-breathing structure was used to utilize the ambient air as its oxygen source. The Bio-Logic SP-150 was used to measure the current and voltage outputs of biofuel cell by 3 min interval under the various load conditions. The power density (μW/cm^2) was calculated by multiplying current and voltage and then divided by surface of electrode (0.332 cm^2).

3. Results and discussion

3.1. Immobilization of LAC and GOx on PANFs

Fig. 1 shows schematic illustrations for the immobilization of enzymes (LAC and GOx) in three different enzyme immobilization methods: EA, EAC, and EAPC. The scanning electron microscope (SEM) images of PANFs, EA, EAC and EAPC are shown in Fig. 2 for both GOx and LAC samples. The nanofiber morphology of EA and EAC samples was fairly similar with pristine PANFs (SEM image of pristine PANFs is not shown), whereas EAPC showed remarkably thicker nanofibers revealing the enzyme coating layer over the surface of PANFs. By checking twenty samples of nanofiber images, the average thicknesses of EA-LAC, EAC-LAC and EAPC-LAC were estimated to be 61 ± 6, 82 ± 7 and 115 ± 6 nm, respectively, while those of EA-GOx, EAC-GOx and EAPC-GOx were 75 ± 5, 91 ± 7 and 142 ± 15 nm, respectively. The thickness of the enzyme coating layer for EAPC samples increased significantly than those of EA and EAC samples due to their improved enzyme loading induced by the ammonium sulfate assisted enzyme precipitation step to form the enzyme aggregates and its subsequent chemical crosslinking step. Since the only difference between EAC and EAPC samples was the addition of the enzyme precipitation process for EAPC sample, the SEM data clearly indicates that the enzyme precipitation process is a critical step in order to form the thick enzyme-coating layer over the supporting material.