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A novel enzyme-immobilization method for a biofuel cell

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ABSTRACT

Biofuel cells utilizing biocatalysts are attractive alternatives to metal catalyst-based cells because of environmentally friendly cells and their renewability and good operations at room temperatures, even though they provide a low level of electrical power. In this study, the effect of a novel enzyme immobilization method on anodic electrical properties was evaluated under ambient conditions for increasing the power of an enzyme-based biofuel cell. The anodic system employed in the cell contained a gold electrode, pyrroloquinoline quinone (PQQ) as the electron transfer mediator, lactate dehydrogenase (LDH), β-nicotinamide adenine dinucleotide (NAD^+) as the cofactor, and lactate as the substrate. The anodic electrical properties increased as a result of the novel enzyme-immobilization method. Furthermore, lactate, NAD^+, or $CaCl_2$, which can all influence enzyme activation, were used to prevent covalent bond formation near the active site of the LDH during enzyme-immobilization. Protection of the active site of the LDH using this novel enzyme-immobilization method increased its stability, which enabled to increase power production (142 $\mu W/cm^2$) in a basic enzymatic fuel cell (EFC).

1. Introduction

Minimized environmentally friendly biofuel cells, may prove to be attractive alternative energy sources for nano-microelectronic devices and biosensors [1–3]. EFCs are capable of functioning at moderate temperatures, and biocatalysts have been found to be useful for facilitating the transfer of electrons in biofuel cell [4]. Biofuel cells generate electrical energy from abundant substrates and can be constructed using a variety of methods [5–8]. However, different classes of oxidative biocatalysts (e.g., oxidases, dehydrogenases) require the application of different redox mediators to enable to transfer electrons from the substrate [9,10].

β-Nicotinamide adenine dinucleotide (NADH/NAD^+) is highly irreversible and results in large overpotentials in NAD^+-dependent enzyme fuel cells. In addition, mediated bioelectrocatalysis is essential for power production by EFCs process. In this process electron transfer proceeds through mediators such as PQQ, which are covalently linked to electrode via a self-assembled monolayer of cystamine in the presence of Ca^{2+} as a promoter [11]. In the EFC generated in this study, two anodic fuel-cell reactions involving NAD^+ electrocatalytic regeneration ($NADH + PQQ + H^+ \rightarrow NAD^+ + PQQH_2$ and $PQQH_2 \rightarrow PQQ + 2H^+ + 2e^-$) have been standardized [12]. In this system, the Ca^{2+} cation functions as a promoter for NADH oxidation and provides a favorable orientation of the NADH molecules for the redox process described above [11]. In addition, PQQ-dependent enzymes can be employed for the construction of EFCs [13]. However, when the majority of PQQ-dependent enzymes cannot transfer electrons [14] without additional electron transfer shuttles, redox mediators are required for power production by EFCs to occur [15–17].

A variety of electrode modification techniques have been used for the immobilization of enzymes. Of these techniques, covalent bonding methods are the most frequently utilized. Due to their profound bonding abilities, immobilized enzymes can be retained over a prolonged reaction period. However, the immobilization procedures are complicated and the enzyme activity is markedly reduced as a result of the immobilization process [18]. Although several reports [19–22] have suggested that the degree of activity loss is dependent on the type of enzyme utilized, these reports did not provide any reasons for this loss of activity.

In this study, the effect of a newly developed LDH-immobilization technique on the maintenance of LDH activity and the electrical properties of a basic EFC were investigated under ambient conditions (pH 7.0 and 25 °C of reaction temperature).

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2. Experimental

2.1. Reagents and materials

All materials were obtained from Sigma–Aldrich and utilized with no further purification. LDH from rabbit muscle (EC 1.1.1.27) was used as the anodic biocatalyst and *N*-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was used as the coupling agent, which was catalyzed for the formation of the amide bonds. NAD^+ was used as the cofactor for enzyme catalysis. The substrate used was L-(+)-lactic acid, and cystamine dihydrochloride was chemisorbed onto the Au electrode surface via the self–assembly method (SAM). In addition, *N*-(2-hydroxethyl) piperazine-*N*′-(2-ethanesulfonic acid) Hemisodium salt (HEPES buffer) was employed as the background solution in the PQQ immobilization experiment and potassium ferricyanide was utilized as a redox probe to determine the area of the electrode. Microperoxidase (MP-11) was used as the cathodic biocatalyst and the substrate used was H_2O_2.

2.2. Preparation of the LDH modified electrode

The Au electrode (2-mm diameter) was polished to a mirror-like finish using a 0.3 and 0.05 μm alumina slurry. Next, the electrode was sonicated and washed with absolute alcohol and triple-distilled water successively, after which it was dried at room temperature. The AU electrode was then soaked in a 0.1 M aqueous cystamine solution for 4 h. The disulphide group (S–S) of cystamine was active enough to allow chemisorption of the cystamine onto the Au electrode. The modified electrode was then thoroughly rinsed with water to remove the adsorbed compounds, after which the electrode was incubated for approximately 4 h in 0.003 M PQQ solution with 20 mM EDC (*N*-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride) in 0.01 M HEPES buffer (pH 7.0) [23]. The PQQ modified electrode was then rinsed with water to remove the absorbed PQQ. Next, the enzyme-modified electrode was prepared by covalent coupling of the enzyme to the PQQ-monolayer by the EDC coupling agent.

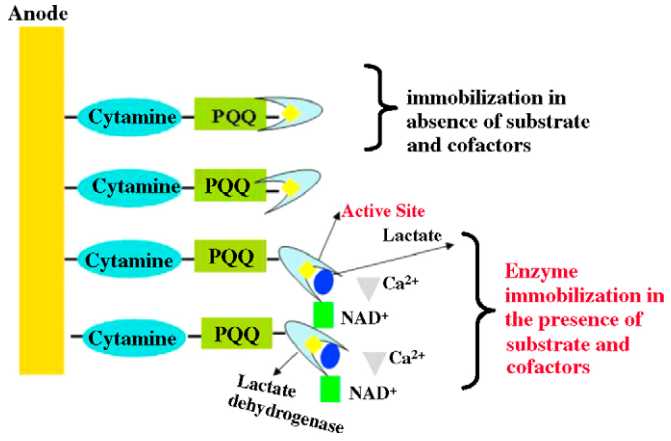
The LDH modified electrode was prepared via the covalent coupling of the enzyme to the PQQ-monolayer using an EDC coupling agent [24,25]. To accomplish this, a newly developed method was designed to immobilize the LDH onto a PQQ-modified anode electrode. Briefly, the PQQ-modified electrode was rinsed thoroughly with distilled water to remove the adsorbed compounds. Next, the electrode was incubated for approximately 4 h in 0.05 M EDC that contained LDH (283U/mg) and additives including lactate, NAD, or $CaCl_2$ in 0.1 M phosphate buffer (pH 7.0) to mask the area near the active site of the LDH (Scheme 1).

The LDH–PQQ modified electrode was prepared as described above and then rinsed in distilled water in order to remove the adsorbed LDH and other additives.

2.3. Preparation of a basic EFC

The anodic solution was comprised of 0.1 M tris buffer (pH 7.0) that contained 20 mM $CaCl_2$, 20 mM NAD^+ and 20 mM lactate [26]. To analyze the power curves of a basic EFC, the modified electrode that was prepared using the novel enzyme immobilization method was used as the anode in conjunction with a cathode (Scheme 2(a)).

The cathodic solution was comprised of 0.1 M phosphate buffer (pH 7.0) that contained 1.0 mM H_2O_2 as the substrate and (2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonate)-diammonium salt (ABTs) as the electron transfer mediator [27]. The cathodic electrode was prepared via the covalent coupling of the enzyme using



Scheme 1. Schematic of LDH immobilization method in presence of lactate, NAD^+, and $CaCl_2$ for the anode modification.

an EDC. To accomplish this, the cystamine modified electrode was rinsed thoroughly with distilled water to remove the adsorbed compounds and incubated for approximately 4 h in 0.05 M EDC that contained 10.0 μM MP-11 in 0.1 M phosphate buffer (pH 7.0) solution. Two electrodes separated by a Nafion membrane were then connected, after which the membrane was soaked in 5% Nafion solution and then dried at 4 °C for 4 h. The obtained electrode was then stored in a refrigerator at 4 °C until use.

2.4. Electrochemical measurements

The electrochemical measurements were performed using a Potantiostat/Galvanostat (WPG100, WonATech Ltd., Korea) and a conventional three-electrode cell. The Ag/AgCl electrode and a Pt foil were used as the reference electrode and the auxiliary electrode, respectively. The Au electrode was used as the substrate of the working electrode. Prior to measurement, oxygen was purged from the solution by bubbling with highly purified nitrogen for 30 min. In addition, a nitrogen environment was maintained in the cell, which was water-jacketed cell with a Teflon cover, throughout the duration of the experiment. All electrochemical measurements were conducted at an ambient temperature of 25 ± 1 °C (Scheme 2(b)) and all experiments were conducted in triplicate.

3. Results and discussion

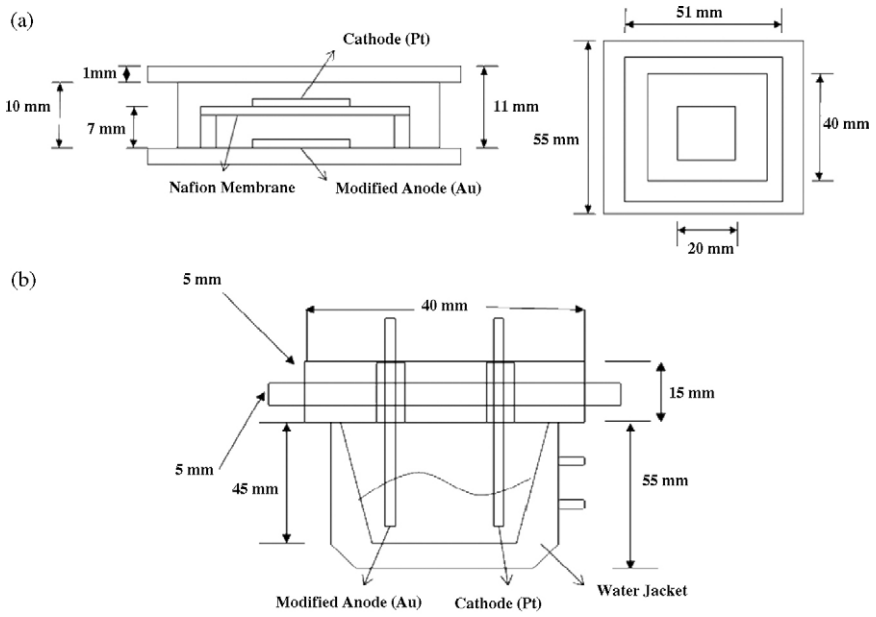
3.1. Modification of the anode electrode

A anode that contained a gold electrode, PQQ as the electron transfer mediator, LDH as a biocatalyst, NAD^+ as the cofactor, and lactate as the substrate, was designed under ambient conditions (pH 7.0 and 25 °C). Operation of this cell produced a typical catalytic current and an increased anodic current peak. In addition, LDH modified electrode resulted in an oxidation area and an anodic current peak (15 μA at 1.0 V) that was superior to those of the unmodified electrode (Fig. 1.).

It is believed that the location of the enzyme in a cell should be near the electrodes. This is because, although most enzymes are used as support for the electron substrates in the electrolyte, a portion of the enzymes near the electrode can easily transfer electrons to the electrode.

3.2. LDH immobilization

LDH immobilization on the anode electrode was conducted using a newly developed method of enzyme immobilization and



Scheme 2. Experimental design of biofuel cell. (a) A side and top view of a basic EFC set. The experiments were performed in a basic EFC at 25 °C and pH 7.0. (b) A biofuel cell with a water jacket. Cyclic voltammetrys were performed in a biofuel cell with a water jacket at 25 °C, pH 7.0, and 100 mV s^{-1} of scan rate.

the CV cycles were then evaluated in the PQQ-anode electrode. As shown in Fig. 2, LDH immobilization in the presence of all components, including lactate, NAD^+, and $CaCl_2$, resulted in a wider oxidation area and better anodic and cathodic current peak (181 μA at 1.18 V and 533 μA at −1.0 V, respectively) than those of traditional method.

The presence of lactate, NAD^+, and $CaCl_2$ results in significant masking near the active LDH site during immobilization onto the PQQ-anode electrode. This masking was attributed to an increase in the anodic peak value. The results observed when lactate and NAD^+ were used were similar to those obtained when all of the components were used because the electron-transferring location of NAD^+ was deeply buried within its complex structure near the active site of LDH. However, the CV results obtained when lactate and $CaCl_2$ were used were lower than those of the controls (the modified electrode without components). This is likely because the Ca^{2+} ion facilitated the formation of an ion-bridge between NAD^+ and LDH [11] and acted as a cofactor during the electron transfer. Therefore, these results indicate that Ca^{2+} is essential to the immobilization process and that it has an affinity for NAD^+, which was located near the active site of LDH.

The amino groups proximal to the active site of the enzyme become linked to the modified electrode during immobilization, which results in steric hindrance preventing the active sites from subsequently interacting with the substrates. This is because the electron-transferring unit of the enzyme is deeply buried within its complex structure, which makes efficient electrical communication between the electrode substrate and the enzyme biocatalyst difficult [28–30]. It has been reported that immobilized lipase activity was dramatically when fatty acid masked the active site of the lipase [31]. Therefore, it was attempted to use a substrate that allow the formation of covalent bonds between the modified electrode and the regions of the enzyme not involved in enzymatic function

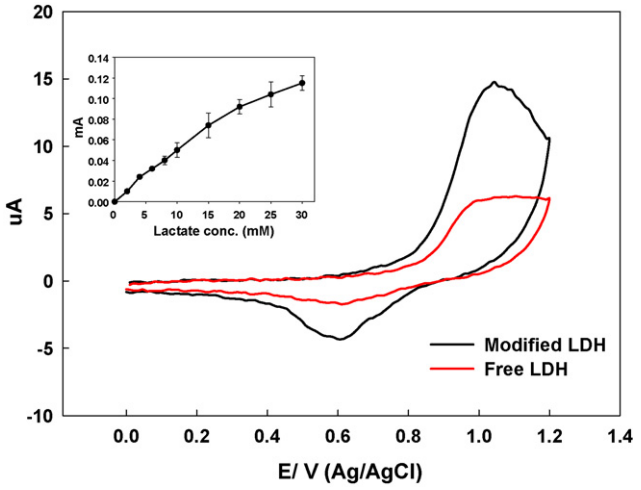


Fig. 1. The cyclic voltammetry of immobilized LDH. *Inset:* Calibration curve of electrocatalytic current at various lactate concentrations. The experiments were performed in a biofuel cell with water jacket at 25 °C, pH 7.0, and a scan rate of 100 mV s^{-1}.

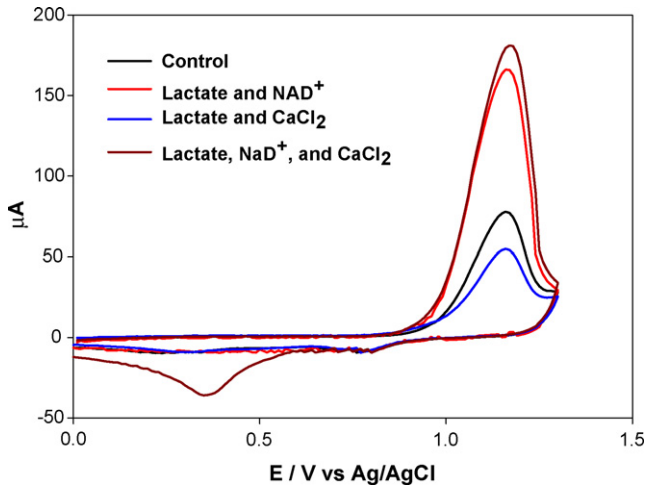


Fig. 2. Cyclic voltammetry following LDH immobilization in the presence of lactate, NAD^+, or $CaCl_2$ in 0.1 M tris buffer (pH 7.0). The experiments were performed in a biofuel cell with water jacket at 25 °C, pH 7, and a scan rate of 100 mV s^{-1}.

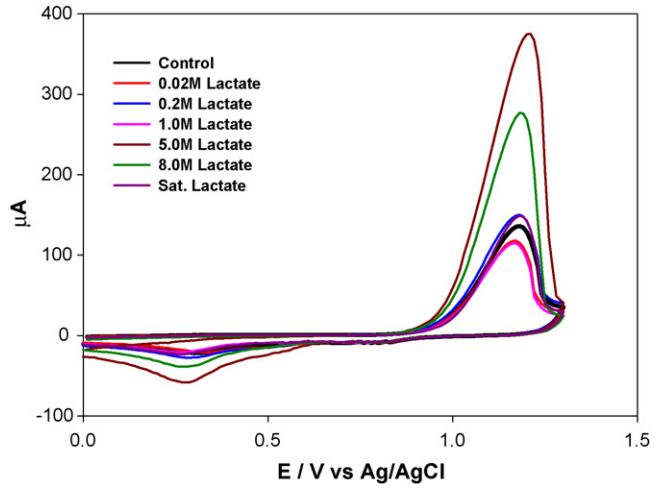


Fig. 3. Cyclic voltammetry following LDH immobilization in the presence of various concentrations of lactate in 0.1 M tris buffer (pH 7.0). The experiments were performed in a biofuel cell with water jacket at 25 °C, pH 7, and a scan rate of 100 mV s^{-1}.

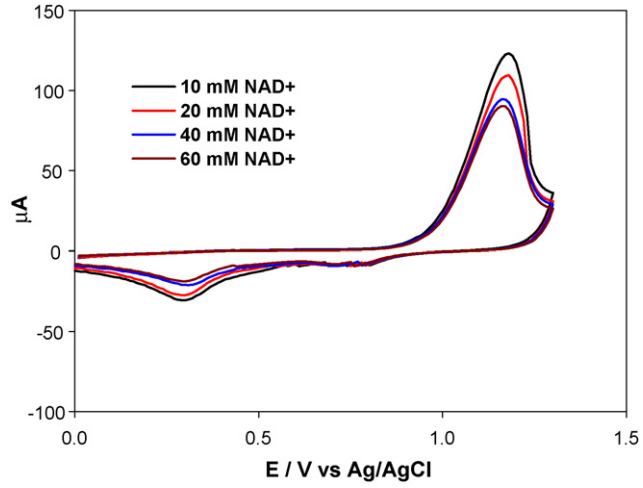


Fig. 4. Cyclic voltammetry following LDH immobilization in the presence of various concentrations of NAD^+ in 0.1 M tris buffer (pH 7.0). The experiments were performed in a biofuel cell with water jacket at 25 °C, pH 7, and a scan rate of 100 mV s^{-1}.

to prevent the immobilized enzyme from losing much activity. This seems to have led to wider oxidation area that was observed in the CV results.

3.3. Effects of the factors (lactate, NAD^+, and $CaCl_2$) on LDH immobilization

Fig. 3 shows the effects of various concentrations of components such as lactate, NAD^+, and $CaCl_2$ on LDH immobilization.

It was assumed that the use of a high concentration of lactate during the LDH immobilization process would results in the active site of LDH being tightly blocked. Fig. 3 shows the effects of masking the active LDH site using various lactate concentrations to immobilize the LDH with 10 mM NAD^+ and $CaCl_2$. The anodic peak value increased with increasing concentrations of lactate, with a maximum value of 375 μA at 1.21 V being observed when 5 M lactate was used; however, this value decreased when concentrations greater than 5 M were used.

These results suggest that high concentrations of the substrate interfered with its movement towards the active site of the enzyme. However, LDH immobilization was only slightly affected by changes in the concentrations of NAD^+ and $CaCl_2$ when compared with the presence of lactate. In addition, when higher concentrations of NAD^+ and Ca^{2+} were used, lower anodic peak values were generated by the CV cycle. For example, when LDH was immobilized onto the PQQ-anode electrode in the presence of 10 mM NAD^+ with 5 M lactate and 10 mM $CaCl_2$, the highest anodic peak (123 μA) observed during the CV cycle was obtained at 1.18 V (Fig. 4). However, the anodic peak was 237 μA at 1.20 V when LDH was immobilized onto the PQQ-anode electrode in the presence of 10 mM $CaCl_2$ (Fig. 5). These findings indicate that changes in the concentrations of NAD^+ and Ca^{2+} did not directly influence the anodic properties when compared with the results of lactate.

The results of this study revealed, it was identified that although NAD^+ and Ca^{2+} ions operate as factors that mask the active site of LDH and play important roles in the transfer of electrons from lactate, high concentrations of Ca^{2+} and NAD^+ can generate a shear effect near the active LDH site. Therefore, enzyme stability is required to increase the electrical properties of the cell, which is one of the serious disadvantages of the EFC [32]. In addition, it is likely that high concentrations of Ca^{2+} exert a pH effect on the buffer solution, leading to a reduction in its stability.

3.4. Power curves of an EFC containing the optimal modified anode electrode

The power curves of a basic EFC that contained the modified electrode generated using the novel enzyme immobilization method for the anode and an MP-11-modified electrode for the cathode were generated to identify the effect of the new method on the anodic electrical properties. This was accomplished by connecting the enzymatic anode and the cathode through external resistance. Fig. 6 shows the current-voltage relationship of a basic biofuel cell at different external resistances, as well as the respective power outputs of the cell.

The results demonstrated that the current density of the basic EFC was increased via the newly developed enzyme-immobilization method. When this method was used in a basic EFC and the concentration of components were optimized (5 M lactate, 10 mM NAD^+, and 10 mM $CaCl_2$), the maximum power density extracted from the cell corresponded to 1.42×10^{-1} mW/cm^2 (standard deviation, $\pm 9.42 \times 10^{-3}$ mW/cm^2). These results represent

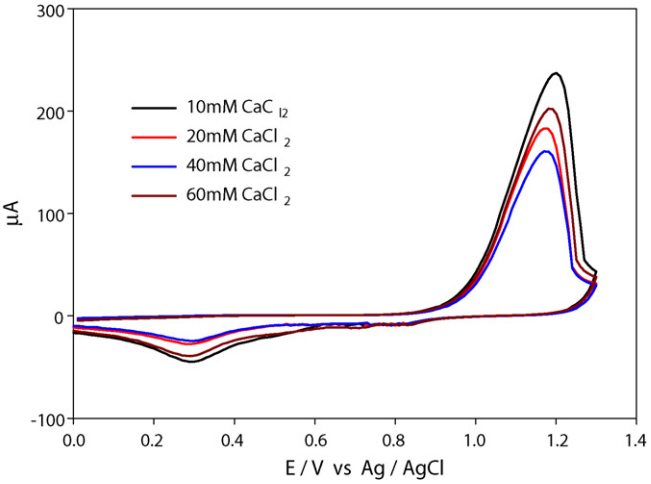


Fig. 5. Cyclic voltammetry following LDH immobilization in the presence of various concentrations of $CaCl_2$ in 0.1 M tris buffer (pH 7.0). The experiments were performed in a biofuel cell with water jacket at 25 °C, pH 7.0, and a scan rate of 100 mV s^{-1}.

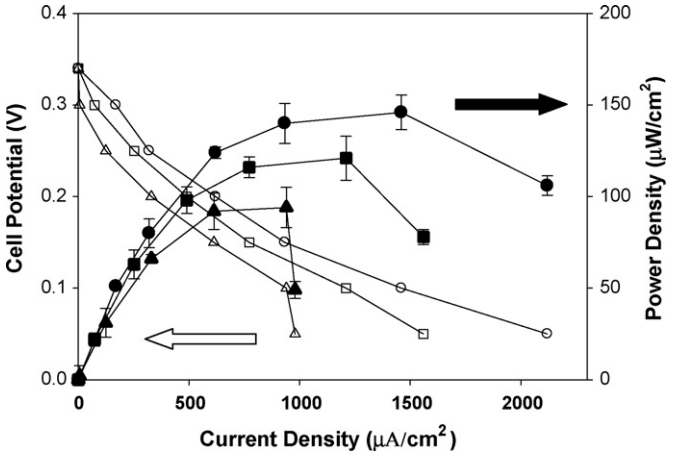


Fig. 6. Effect of a novel method of anodic modified-LDH immobilization on power curves in a basic EFC set. (●) LDH immobilization in the presence of lactate, NAD^+, and $CaCl_2$; (■) LDH immobilization without any component; (▲) Free LDH. All experiments were performed at 25 °C in 0.1 M tris buffer (pH 7.0).

a 52% increase over the results produced by the control EFC (LDH in electrolyte solution) and a 26% increase over the power density of the immobilized LDH, without considering the effect of masking near the active site. These findings indicate that the electron transfer between LDH and the electrode increased as result of the protection of the active site by the novel LDH-immobilization method developed here. To allow easy comparison of biofuel cells, microperoxidase was used as the cathode in an EFC that contained a low concentration of H_2O_2, even though the electrical power required to produce the H_2O_2 was substantially higher than the electrical power extracted from the cell. However, the use of the cathode electrode only as an electron acceptor in a basic biofuel cell would allow electrons from the anode to be collected, which would occupy the H_2O_2 reduction. Willner et al. showed that the cathode electrode (MP-11) catalyzes the H_2O_2 reduction using the electrons from anode ($NADH/NAD^+$) [33]. In addition, it has been suggested that modification of cathodic enzymes such as laccase [34] or MP-11 [31] using a substrate and an electron transfer mediator would result in the power density being increased. Therefore, many studies have been conducted to improve the electrical properties of EFCs using enzyme immobilization techniques to increase the stability of enzymes, which are also affected by temperature and pH in the EFC [35–37].

It is expected that this newly developed LDH immobilization method will be applied to the immobilization of other enzymes with weak stability.

4. Conclusion

An anodic system for an EFC was designed using a newly developed LDH immobilization method that utilizes lactate, NAD^+, and $CaCl_2$ under ambient conditions (pH 7.0 and 25 °C). The optimal concentrations of the lactate, NAD^+, and $CaCl_2$ were 5 M, 10 mM and 10 mM, respectively. In addition, it was found that the anodic modification in which LDH immobilization was conducted while masking the active site using substrates and cofactors improved the anodic electric properties of the EFC. These findings suggest that it is possible to apply other enzyme systems to the development of EFCs by adopting appropriate enzyme structures, buffer solutions, substrates, and cofactors. However, further research is needed to establish the appropriate cathodic modification and determine which enzymes should be employed to overcome complications such as enzyme stability, electron transfer, and power production.

Acknowledgement

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