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Stability improvement by crosslinking of previously immobilized glucose oxidase on carbon nanotube-based bioanode

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Abstract: Bioanode stability with glucose oxidase was enhanced significantly by covalent crosslinking without substantial enzymatic activity and affinity loss. Initially, glucose oxidase was immobilized by aldehyde groups on the electrode that was developed using ferrocenecarboxaldehyde, polyethyleneimine, multiwall carbon nanotubes, and carbon cloth for biofuel cell applications. The glucose oxidase half-life was extended by more than 4 times, from 27.2 to 124.7 h, after the electrode was crosslinked. Enzymatic kinetic parameters were determined for the crosslinked enzyme and they were compared to the noncrosslinked immobilized enzyme parameters on the electrode. The apparent substrate affinity of the crosslinked enzyme electrode was decreased (i.e. k_M was increased) by 16%, while the maximum reaction rate was decreased by only 3%, by the crosslinking process. Moreover, effects of the electrolyte type (i.e. buffer type) and concentration on the performance of the crosslinked enzyme electrode were evaluated and appropriate conditions were determined.

Key words: Glucose oxidase, enzyme immobilization, bioanode, enzyme fuel cell, enzyme stability, crosslinking

1. Introduction

Oxidoreductases (i.e. enzymes catalyzing oxidation/reduction reactions) could be utilized for electrochemical energy production by means of biofuel cells. However, limited stability and low current densities are the main difficulties for the real-world practice of enzymatic fuel cells. Many studies have been performed for increasing the current densities to improve the performance of enzymatic fuel cells. On the other hand, fewer studies have been performed on the enzyme stability enhancement of electrodes [1–5].

Glucose oxidase (GOx) (EC 1.1.3.4) is very popular among oxidoreductases for biofuel cell studies since both the enzyme and its substrate (glucose) are easily accessible in nature [5–8]. GOx is a flavoprotein that catalyzes the oxidation of glucose to gluconolactone and hydrogen peroxide. Dissolved oxygen in the reaction medium is utilized as the electron acceptor. GOx, which has a molecular weight of 160 kDa, is composed of a dimer of 2 equal subunits. The dimer comprises 2 flavin adenine dinucleotide (FAD) molecules (cofactors) that are not covalently connected to the main structure. FAD cofactors are essential for the oxidation-reduction reactions of the enzyme. On the other hand, the subunits of GOx dissociate along with the loss of cofactors in denaturing environments or during the reaction period. This process deactivates the enzyme [9]. Covalent immobilization is favorable to adsorption or other physical immobilization techniques when the stability of the immobilized enzyme is considered. Covalent immobilization techniques, with an additional crosslinking stage to prevent GOx dissociation, might further increase the enzyme stability. Therefore, multipoint covalent

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immobilization was reported as effective, since the enzyme molecule is bonded more tightly to preserve the 3-dimensional (3D) conformation of the protein [2,10–13]. Similarly, multilayered crosslinked enzymes were utilized and this method efficiently increased the enzyme stability on the surface of other enzyme molecules by preventing enzyme denaturation and leakage [14]. Kim et al. also stated a method by means of enzyme precipitates to produce stable GOx coatings on nanomaterials. The immobilization process introduced an enzyme coating step prior to the crosslinking. The crosslinked enzyme was found to be as effective and stable as the enzyme coating or covalently immobilized enzyme [2]. On the other hand, effective electrical conductance is essential from the enzyme active site to the electrodes for preparing a successful biofuel cell with a high current density. The active redox site (FAD) is deeply situated in the GOx protein conformational configuration that prevents the electrons from reaching the cathode surface [5,6]. Consequently, layer-by-layer precipitation or preparation of the enzyme aggregates might worsen the electron conductivity for reaching high current densities from the biofuel cell.

In recent studies, an electrode carrying active aldehyde groups for the immobilization of enzymes was developed using multiwall carbon nanotubes (MWCNTs), polyethylenimine (PEI), and carbon cloth [15]. The immobilized GOx kinetic parameters and electrochemical performance of the bioelectrode were also discussed [15,16]. In this study, immobilized enzyme stability was further improved by additional crosslinking of the enzyme on the electrode after the immobilization process. The proposed additional crosslinking process was optimized for maximum activity and stability aspects. In addition, effects of the buffer type and concentration on the performance of the enzymatic electrode were also discussed.

2. Materials and methods

2.1. Materials

The GOx (G2133-Sigma, type VII from *Aspergillus niger*), D-glucose (G8270-Sigma), branched PEI (BPEI) (482595-Aldrich), glutaraldehyde (G6257-Sigma-Aldrich), and the MWCNTs (724769-Aldrich) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The carbon cloth (EC-CC1-060) was obtained from ElectroChem Inc. (Woburn, MA, USA). Ferrocene carboxaldehyde (FcCHO, 98%) was purchased from Acros Organics (Thermo Fisher Scientific, Merelbeke, Belgium). The other reagents used were all of analytical grade. Distilled and deionized (DI) water was used in all of the experiments.

2.2. Preparation of the MWCNTs comprising anodes

A recently developed process was applied for the construction of MWCNTs containing carbon cloth electrodes [15]. The FcCHO solution, which was prepared by dissolving 0.4 g of FcCHO in 5 mL of methanol, was added slowly into 45 mL of 10% (w/w) BPEI in a methanol solution. The reaction medium was stirred (100 rpm) for 24 h to complete the reaction between the FcCHO and amine groups of the BPEI at 25 °C. As the next step, the solution was further treated with sodium borohydride [15,17]. The surface of the carbon cloth (5 cm^2) was uniformly covered with MWCNT paste using proper blades, which had been previously soaked in methanol. Next, 0.5 mL of the Fc-BPEI solution (including 40 mg/mL of BPEI-Fc in MetOH) was added to the MWCNT (10 mg, dry basis)-coated carbon cloth surface for the adsorption of the Fc-BPEI. A glutaraldehyde solution (25%, w/w) was employed to crosslink the adsorbed Fc-BPEI for 30 min at 25 °C. This procedure enabled not only strong fixing of the MWCNTs on the carbon cloth, but also reactive free aldehyde moieties for the enzyme immobilization [15,18]. The produced electrode was splashed extensively with DI water to eliminate unreacted glutaraldehyde and leftover chemicals from the preceding stages.

2.3. Glucose oxidase immobilization and further crosslinking

Electrodes with free aldehyde groups on the MWCNTs were placed into appropriately buffered (pH 5–7) enzyme solutions (i.e. 2 mL, 20 mg enzyme/mL) for the enzyme immobilization. The Schiff base reaction between the amine groups of the GOx and the aldehyde moieties on the electrode surface was performed for 24 h at 4 °C [15]. The amount of bound enzyme (i.e. protein) was determined using the Biuret method [19].

Immobilized GOx on the electrode was further crosslinked to improve stability. In this experimental set, 5-cm^2 enzyme electrodes were treated with 50 mL of 0.5%–2% (w/w) glutaraldehyde solutions for 24–72 h at 4 °C. After the process, the electrode was cleaned extensively with suitable buffer solutions to remove physically adsorbed glutaraldehyde. The resulting enzyme electrode was stored at 4 °C before use.

2.4. Glucose oxidase activity measurement by conventional and electrochemical methods and determination of kinetic parameters

Immobilized GOx catalytic activity (i.e. U: µmol glucose hydrolyzed/min/mg protein) was calculated by the measurement of the glucose concentration alteration over time in batch-wise reactors (i.e. the conventional method). A definite amount of immobilized GOx (i.e. 2.5 mg/cm^2, based on the total protein content) on the electrode (typically 1 cm^2) was put into the reactor with a substrate solution (200 mM glucose; 25 mL) at a suitable pH and temperature (typically 35 °C, pH 5) for the reaction. The reactor was stirred at 200 rpm for the reaction [15]. Half-cell electrochemical measurements were also performed for the GOx immobilized or immobilized-crosslinked electrodes (typically 1.0 cm^2) using the electrochemical method. A 3-electrode alignment was used. Platinum wire was employed as the counter electrode and a saturated Ag/AgCl electrode was used as the reference electrode. Typically, a cell volume of 50 mL was used at the preferred temperature and pH. Open circuit potential, chronoamperometry, linear sweep voltammetry, and short-circuit current measurements were used to determine the kinetic parameters of the immobilized enzyme on the electrode [16]. A potentiostat/galvanostat system (Gamry Instruments, Inc., Warminster, PA, USA; Reference 600R) was used for the electrochemical measurements.

3. Results and discussion

3.1. Immobilized enzyme stability improvement by crosslinking

Covalently immobilized GOx on the electrode was crosslinked covalently with glutaraldehyde under mild conditions by employing crosslinker concentrations of 0.5%–2% to obtain a more stable enzyme electrode. The crosslinking process duration was also considered as a parameter (Table 1). Enzymatic stability was determined by measuring the deactivation rate constant according to the first-order deactivation model [20].

The effect of the glutaraldehyde concentration on the enzyme stability was clearly observed and optimized (Table 1). As a result of 24 h of crosslinking using a low (0.5%) glutaraldehyde concentration, the stability increased by approximately 75% from 27.2 to 47.3 h. When the glutaraldehyde concentration was increased from 0.5% to 1%, stability enhancement reached 265% (71.9 h). However, a further increase in the glutaraldehyde concentration, from 1% to 2%, for the crosslinking process almost completely deactivated the enzyme. Some glucose (substrate) was also added to the crosslinking reaction medium for the protection of the 3D conformation of the active site of the enzyme with the substrate molecule inclusion. However, this idea did not provide the expected outcome. Consequently, a glutaraldehyde concentration of 1% (w/w) was determined as a favorable amount of crosslinker (Table 1).

Table 1. Effects of glutaraldehyde concentration and crosslinking reaction duration on the crosslinked GOx electrode half-life.

Glutaraldehyde concentration, %	**Crosslinking duration, h**	**Activity half-life of the electrode, h**
-	-	27.2 (half-life of the immobilized enzyme electrode without crosslinking*)
0.5%	24	47.3
1%	24	71.9
1% + 0.2 M glucose	24	Very low enzymatic activity
2%	24	Very low enzymatic activity
2% + 0.2 M glucose	24	Very low enzymatic activity
1%	48	124.4
1%	72	98.6

* Half-life of the free enzyme was 2.33 h [15].

Effects of the crosslinking reaction duration were also analyzed and 48 h of crosslinking was determined as optimum with the stability enhancement reaching 460% (i.e. from 27.2 to 124.4 h). The deactivation profiles of the immobilized vs. crosslinked enzymes are given in Figure 1. The deactivation constant of the crosslinked enzyme was calculated as 0.00557 h^{-1} ($t_{1/2}$: 124.4 h) as shown in Figure 1. Scanning electron microscopy (SEM) images were analyzed visually before (Figures 2a and 2b) and after (Figures 2c and 2d) the crosslinking process to perceive the effects of crosslinking. The crosslinked glutaraldehyde layer can be noticeably observed as expected on the electrode surface of the SEM image in Figure 2. Additional covalent crosslinking had been reported in the literature as effective for increasing enzyme stability by diminishing GOx dissociation and improving the 3D structure, since the enzyme bonded more tightly to preserve the conformation of the protein structure [2,10–13]. The results (half-life extended more than 4 times) of this study also showed that covalent crosslinking was useful for enhancing stability. Additionally, in the literature, multilayered crosslinked enzymes were also used to efficiently increase enzyme stability on the surface of other enzyme molecules by preventing enzyme denaturation and leakage [14]. On the other hand, effective charge transfer from the GOx active site to the electrodes was important for preparing a successful biofuel cell with a high current density. Layer-by-layer precipitation or preparation of the enzyme aggregates was not used in this study, since this method might worsen the electron conductivity for reaching high current densities from the biofuel cell.

3.2. Effects of crosslinking on optimum pH and kinetic parameters

Generally, substrate solutions should be buffered for stable pH to optimize enzymatic reaction performance and stability since the enzymes are amphoteric molecules comprising many acidic and basic moieties. pH may influence the reactivity of an enzyme's redox site. Additionally, if the enzyme reaction exchanges protons (i.e. modifies acidity) like GOx, additional behaviors may occur [21]. Therefore, electrical current density variation with the reaction medium pH was determined for the crosslinked electrode and the results were compared to the noncrosslinked enzyme electrode (Figure 3). It was observed that a more pH-unsusceptible electrode was obtained within the pH range of 4.5–7 by crosslinking of the immobilized enzyme. This result was valuable since the pH of the substrate solution would not be such an important parameter for the crosslinked electrode enzymatic activity.

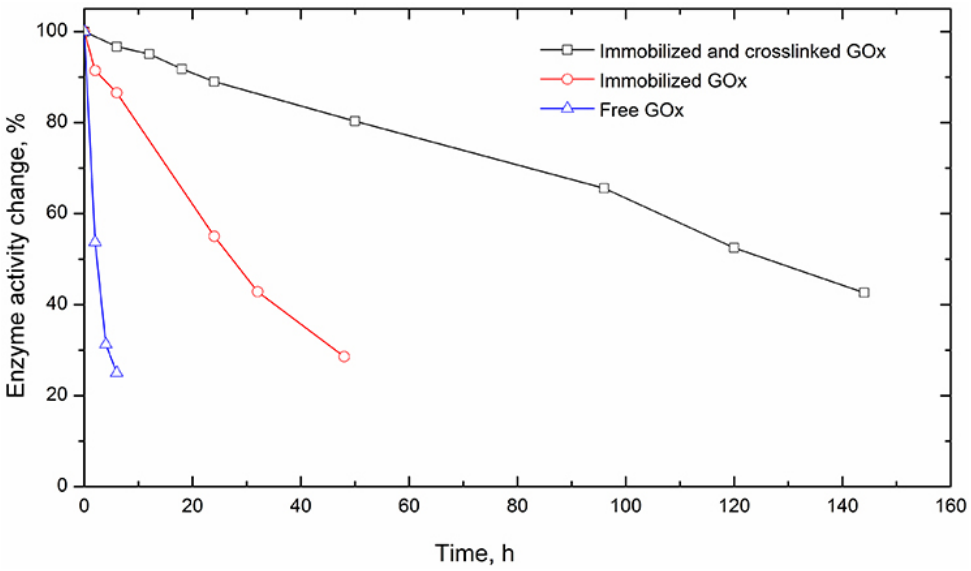

Figure 1. Enzymatic activity decrease over time for the free, immobilized, and crosslinked GOx (without any substrate limitation, pH 5, 35°C).

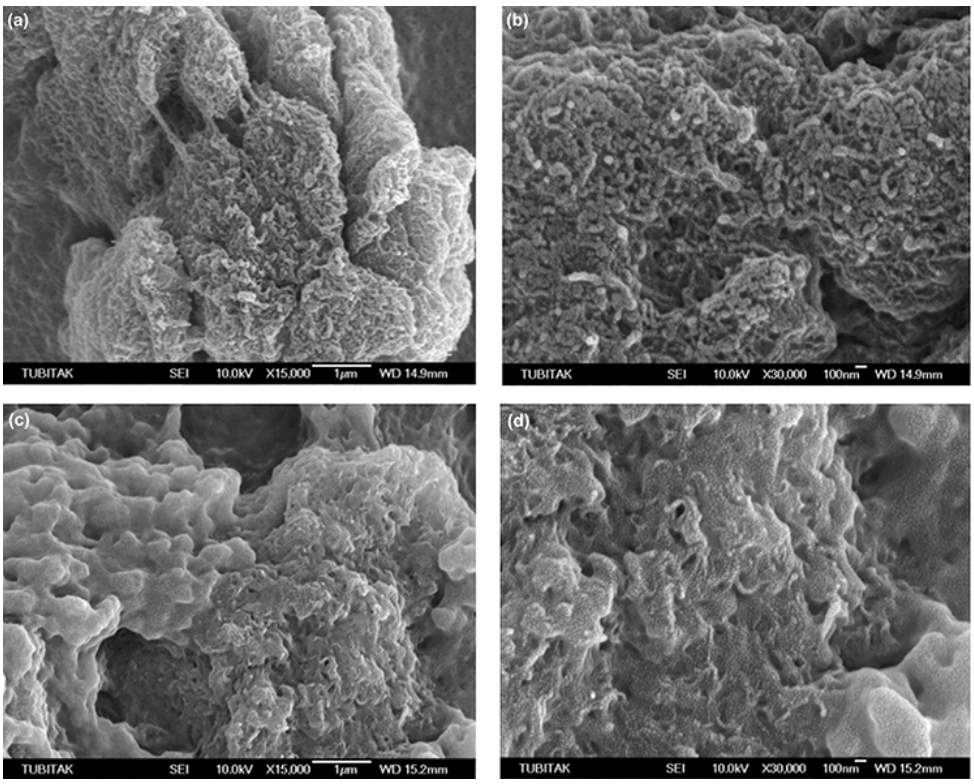

Figure 2. SEM images for the effects of crosslinking of GOx on the surface of the electrodes (a and b- GOx immobilized electrode at 15,000 and 30,000 magnification; c and d- GOx immobilized and crosslinked electrode at 15,000 and 30,000 magnification).

Some alterations of the kinetic parameters might be expected after covalent crosslinking due to undesirable conformational changes of the enzyme molecule and/or substrate diffusion limitations [9,21]. Accordingly, kinetic parameters of the immobilized and crosslinked glucose oxidase were calculated and compared by determining current densities for glucose concentrations between 10 and 120 mM, where the reaction medium pH and temperature were set at their optimum. The plateau values for both the crosslinked and noncrosslinked enzymes were reached after the initial glucose concentration of about 100 mM. Kinetic parameters (k_M and V_m) were calculated according to the Michaelis–Menten model from the data given in Figure 4. The results were acknowledged as very positive since only small changes were observed for V_m and k_M (Table 2) after crosslinking. These results were explained by the positive effects of both the previous covalent immobilization and mild conditions used for the crosslinking reaction (low temperature of 4 °C and 1% glutaraldehyde concentration).

3.3. Effects of buffer type and concentration on the enzyme anode and biofuel cell

Fuel cell cathode and anode compartments are generally separated by a membrane that must be electrically nonconductive but must enable effective proton transfer. Popular low-temperature fuel cell membranes (Nafion, etc.) are polymeric membranes with sulfonic acid groups in their structure. Platinum on carbon (Pt-C)-containing cathodes can easily be integrated onto these membranes for better physical contact and proton transfer by a simple hot-pressing process (typically at 100–120 °C for several minutes). However, such strong physical contact is difficult for enzymatic anodes to increase the proton transfer from the anode to the membrane, since hot pressing is not suitable because of enzyme deactivation. Therefore, proton transfer should be enhanced by supporting electrolytes (i.e. buffers).

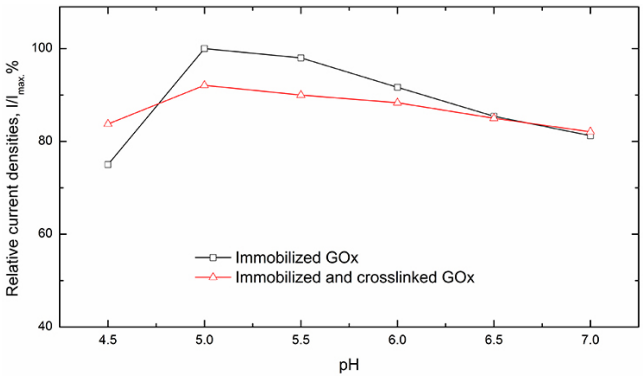

Figure 3. Variation of the relative anodic current densities (i.e. I/I_{max}) by the reaction medium pH (I_{max} = 2.24 mA/cm^2, fuel: 200 mM glucose solution, 35 °C).

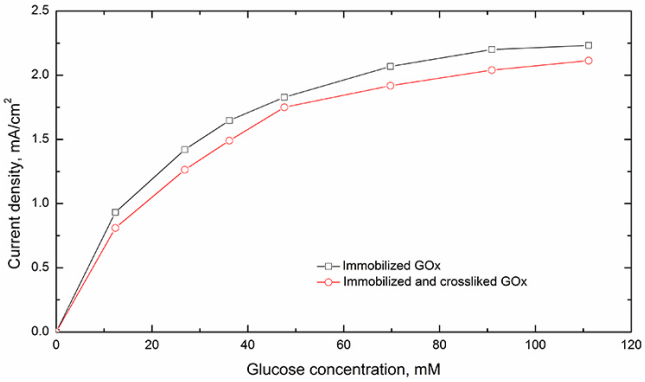

Figure 4. Variation of the anodic current densities (i.e. enzyme activities) by the glucose concentration for the immobilized GOx and crosslinked GOx electrodes to determine the kinetic parameters of Vm and kM (35 °C, pH 5.0, half-cell at 0.5 V polarization).

Table 2. Table 2. Kinetic parameters of immobilized GOx and crosslinked GOx electrodes (pH 5.0, 35 °C).

	k_M, mM	V_m, mA/cm^2	K_d, min^{-1}	Half-life, min
Immobilized GOx electrode	24.61	2.77	0.000425	1630
Immobilized-crosslinked GOx electrode	28.51	2.69	0.0000928	7464

In our recent studies, fast current density degradation was observed when a popular sulfonic acid type proton exchange membrane (Nafion 115) was used with the GOx immobilized anode to construct a whole biofuel cell [16,22]. This result was explained by the negative effect of the buffer cation exchange and accumulation within the membrane [16,22]. Moreover, Yeager et al. analyzed the selectivity of Nafion membrane and determined the affinity of cations by the membrane as $Cs^+ > Rb^+ > Ba^{++} > K^+ > Mg^{++} > Na^+ > H^+ > Li^+$ [23]. Therefore, the crosslinked GOx electrode anodic performance with different buffers was examined by a half electrochemical cell to observe the effect of the buffer cation type. It can be obviously observed in Figure 5 that buffers with different cations had only small effects on the anodic performance at the electrochemical half-cell without a membrane. This result showed that the buffer cation type was not a critical factor for the immobilized and crosslinked GOx enzymatic reaction. It can also be observed in Figure 5 that pH had little effect on the crosslinked-immobilized enzyme in the range of 4.5 to 6. The results were compatible with Figure 3, since the stability of the electrode was improved by crosslinking. The relative performances were in the range of 5% with 3 different buffer systems at altered pH values.

Next, the effect of the buffer concentration on the immobilized enzyme system was considered by determining the current densities in the half-cell electrochemical system and the results are given in Figure 6. The current density increased at first with an increase in the buffer concentration before reaching a plateau value. A further increase in the salt concentration resulted in a decrease in the current density. Therefore, a buffer concentration between 0.15 and 0.20 M was found as optimum for the current density. The observed current increase behavior was explained by the charge transfer resistance decrease to the electrode by increase of the ionic concentration at the electrode interfaces. The FAD cofactor of the GOx requires effective charge and electron transfer for proper enzyme reaction, as given in Eqs. (1)–(3) on the electrode [16,24].

$$\text{GOX (FAD)} + \text{glucose} \rightarrow \text{GOX (FADH}_2\text{)} + \text{gluconolactone} \quad (1)$$

$$\text{GOX (FADH}_2\text{)} + 2\text{Fc}^+\text{-PEI} \rightarrow \text{GOX (FAD)} + 2\text{Fc-PEI} + 2\text{H}^+ \quad (2)$$

$$2\text{Fc-PEI} \rightarrow 2\text{Fc}^+\text{-PEI} + 2\text{e}^- \quad (3)$$

Similar observations were reported in the literature with different explanations. Sakai et al. examined the effects of buffer concentration on enzyme performance and comparable results were reported. They explained the current density increase by more stable pH in the microenvironment with an increase in the buffer concentration. According to their results, after the optimum any further increase in the buffer concentration deactivates the enzyme [25]. Moreover, positive effects of ionic strength on GOx activity were reported by Voet et al. [26]. Finally, crosslinked GOx electrodes were integrated into biofuel cell hardware with Nafion 115 membrane and a conventional Pt-C cathode. It was expected to observe a reflection of improved performance of the anode obtained by crosslinking and optimization of the buffer concentration. However, the anticipated improvements were not detected in the complete biofuel cell system performance, as given in Figure 7. No current density increase with the buffer concentration—in fact, even some decrease—was observed with the biofuel cell application.

It can also be observed in Figure 8 that even a more stable immobilized enzyme anode was integrated into the biofuel cell and the current density performance decreased much faster than the enzyme deactivation rate given in Table 2. These results and our previous observations [16,22] were a clear indication of the membrane's cation exchange behavior and saturation with the cation of the buffer solution.

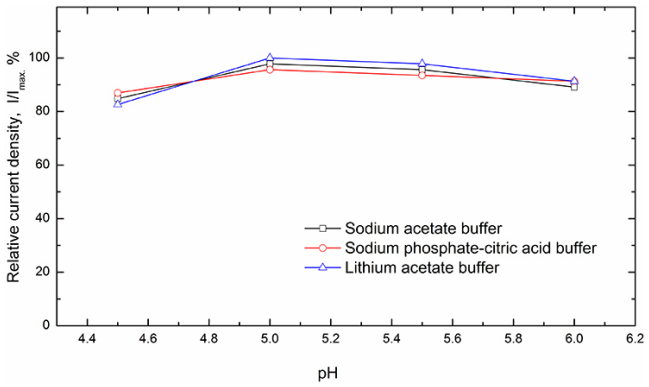

Figure 5. Effects of the reaction medium buffer type and pH on the relative anodic current densities of the crosslinked enzyme electrodes (fuel: 200 mM glucose solution, 35 °C, half-cell at 0.5 V polarization).

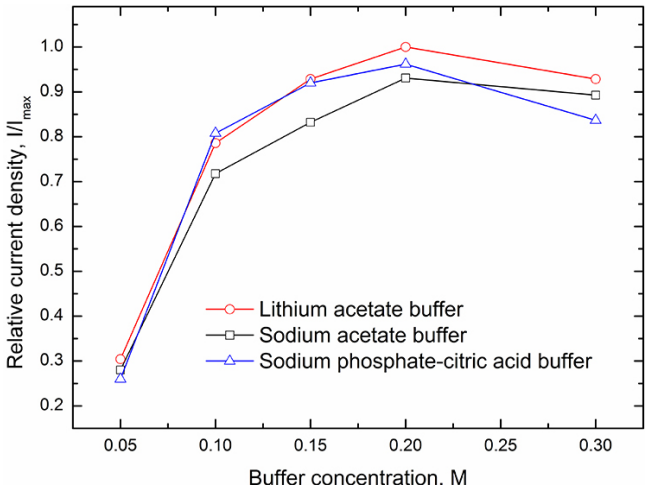

Figure 6. Effects of the reaction medium buffer type and concentration on the relative anodic current densities of the crosslinked enzyme electrodes (fuel: 200 mM glucose solution, 35 °C).

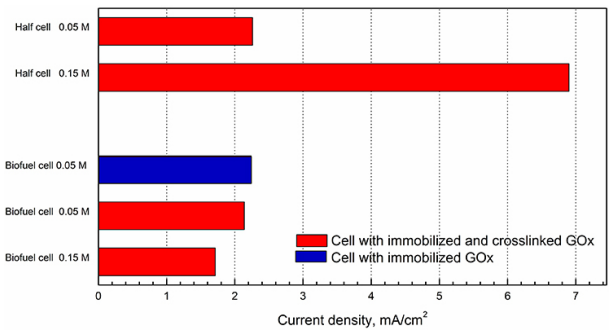

Figure 7. Initial (at time = 0) current densities obtained from the solitary GOx anode (at the half-electrochemical cell) and from the complete biofuel cell system at low (0.05 M) and high (0.15 M) buffer concentrations (fuels: 200 mM glucose solution for both the half-cell and complete biofuel cell as anode feed; oxygen used as cathode feed for the complete biofuel cell, 35 °C, pH 5).

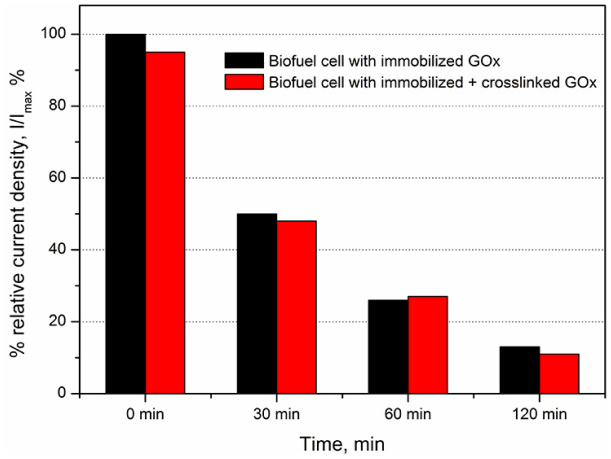

Figure 8. Immobilized and immobilized-crosslinked GOx bioanode complete fuel cell performance (current density) degradation over time (fuels: 200 mM glucose, solution (anode feed), oxygen (cathode feed), 35 °C, pH 5).

3.4. Conclusion

The GOx immobilized bioanode activity half-life was enhanced by more than 4 times, from 27.2 to 124.7 h, by additional crosslinking without considerable enzyme activity loss. The k_M and V_m values were almost completely conserved without a significant negative change after crosslinking. The buffer solution concentration was found as a more important parameter than the buffer solution cation type when only the anodic performance was considered in the half-cell electrochemical system. However, once the GOx anode was integrated to form

a complete biofuel cell, cation (Na^+ or Li^+ ions) exchange between the buffer solution and sulfonic acid type membrane was perceived as the main performance-determining process in the biofuel cell. Exchange and saturation of the membrane with the cations of the buffer solution and protons led to limited current density, even though other parameters were at their optimums. It was concluded that in order to maintain constant performance, alternative membranes are required with different proton transfer mechanisms than the perfluorosulfonic acid type membranes.

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