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Layer-by-layer assembly of enzymes and polymerized mediator on electrode surface by electrostatic adsorption

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Abstract

Through layer-by-layer adsorption (LBL) technique, multilayer film was prepared from enzymes, diaphorase (DI) and glucose-6-phosphate dehydrogenase (G6PDH) and polyelectrolytes. The adsorption interface morphology was directly observed by atomic force microscopy, and the immobilization amount and layer thickness were characterized from quartz crystal microbalance which showed formation of nanoscale multilayer structure and linear mass increase which depended on alternate adsorption cycles. In order to construct a new mediated bi-enzyme biosensor system, polymerized mediator, DI and G6PDH were immobilized on carbon electrode surface by using LBL method. Electrochemical experiments indicated highly efficient electron transfer by the polymerized mediator. Two enzymes kept their activities after immobilization, and the electrode immobilized by mediator and enzymes showed sensitive response to glucose-6-phosphate in the presence of free NAD^+, and high stability during long period of storage.
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Keywords: Layer-by-layer adsorption; Bi-enzyme biosensor; Quartz crystal microbalance; Oxidation of NADH

1. Introduction

As an analytical tool with fast detection speed, high selectivity and sensitivity, enzymatic biosensors have been extensively applied in clinical, food and environmental areas. Design and preparation procedure, especially immobilization procedure is the key to construct this kind of biosensors, because enzymes should be stabilized and easy to be contacted by substrates, and the immobilization procedure should be beneficial to electron transfer between enzymes and electrode surface by redox mediator [1].

Different approaches have been utilized to immobilize enzymes on modified electrode surface, and layer-by-layer adsorption (LBL) technique based on electrostatic force between polyelectrolytes and proteins has received great attention and interests. As a novel technique proposed by Decher and Kunitake, LBL has been introduced to fabricate multilayer films, and it represents a promising and environmental preparation method because no complicated instruments or chemical reactions are involved, and ultrathin multicomponent architecture can be constructed only by alternate adsorption in cationic and anionic polyelectrolytes [2,3]. Information about the structure of these multilayer assembles constructed by using LBL methods has been measured by various kinds of instruments, such as quartz crystal microbalance (QCM), UV, AFM, SEM and, etc. and mass-controlled LBL sequential adsorption technique was reported by using QCM to monitor the immobilization procedure [4–10]. It has been proved that LBL method is a simple and elegant way for preparation of ultrathin films with defined composition and uniform thickness in nanoscale.

In recent years, LBL technique shows much more applications on biosensor area. Alternate adsorption in various enzymes and synthetic or nature polyions on electrode surface has been used to fabricate biosensor systems. Finely controlled multilayer films were constructed by enzymes and redox polyelectrolytes, and enzymes retained their activities in multilayer assembly formed by using LBL method [11–13].

As it is well known $NAD(P)^+$-related dehydrogenases consist of the largest oxidoreductase group, and electrochemical oxidation of NAD(P)H in efficient and mild condition will provide a basis for construction of many kinds of amperometric enzymatic biosensors dependent on

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the specific dehydrogenase used [14,15]. Because high overpotential and serious side reactions are always involved in direct oxidation of NAD(P)H on bare electrode surface, mediator is necessary which not only decrease the high potential largely but also accelerate electron transfer. NAD(P)H oxidizing enzymes were reported which represented a more efficient way in combination with relevant mediator [16–20]. Several bi-enzyme biosensor systems have been developed on the basis of electrochemical oxidation of NAD(P)H [21,22].

In previous works, we have been engaged in deep investigation of enzymatic conjugation reactions related to regeneration circle of $NAD(P)^+$/NAD(P)H. Polymerized coenzyme were successfully synthesized by sodium alginate and $NAD(P)^+$ [23,24], and used in coupling reactions between ferredoxine-$NADP^+$ reductase and glutathione reductase [25].

Herein we reported the fabrication of a bi-enzyme biosensor on modified carbon electrode based on LBL method by using two enzymes, diaphorase (DI) and glucose-6-phosphate dehydrogenase (G6PDH). QCM was used to quantitatively analyze multilayer formation, and atomic force microscopy (AFM) was used to investigate the interface morphology after immobilization. Oxidation of NADH by free and immobilized mediator and DI and its conjugation with immobilized G6PDH were studied by electrochemical measurements.

2. Materials and methods

2.1. Materials

Sodium alginate (Alg-Na, molecular weight 23,000), 1,1′-carbonyldiimidazole (CDI), and polyethyleneimine (PEI, molecular weight 60,000–80,000) were purchased from Nacalai Tesque Co. (Kyoto, Japan). Ferrocene carboxylic acid, 3-mercaptopropionic acid (3-MPA) and lipoamide dehydrogenase (EC 1.8.1.4) (diaphorase, DI) were obtained from Sigma Chemical Co. (MO, USA). Glucose-6-phosphate dehydrogenase (G6PDH EC 1.1.1.49 from *Leuconostoc mesenteroides*) was purchased from Oriental Yeast Co. Ltd (Tokyo, Japan). NADH and NAD^+ were obtained from Kohjin Co. Ltd (Tokyo, Japan). Double deionized water was used for the preparation of Tris–HCl buffer (pH 7.5) containing 3.3 mM $MgCl_2$.

2.2. Synthesis of polymerized mediator

In 5 ml methanol, 35 μmol of ferrocene carboxylic acid was added, followed by 105 μmol of CDI. The mixture was stirred for 60 min at room temperature. Then 15 ml of 10% PEI was added, and the solution was stirred for another 60 min. Finally the resulting solution was dialyzed against distilled water for 12 h, and the polymerized mediator obtained was referred to as PEI-Fc.

2.3. Preparation of multilayer film on electrode by using LBL method

The basal plane pyrolytic graphite electrode (PG) was cleaned by 0.1 M H_2SO_4. Then electrode modification was achieved by electrochemical oxidation in nitric acid solution containing 2.5% $K_2Cr_2O_7$. A potential of +2.2 V vs. saturated calomel electrode (SCE) was applied to the bare electrode dipped into the above solution for 10 s, and negative charges were introduced to the electrode surface during this operation. In assembly of multilayer structure by using LBL method, PEI-Fc and PEI were used as polycations, and DI, G6PDH and Alg-Na as polyanions. The modified electrode was first immersed in PEI-Fc solution (5.0 mg/ml) for 20 min, thoroughly rinsed with water. Then it was immersed in Alg-Na solution (5.0 mg/ml) for another 20 min, and rinsed again to construct one layer of mediator. The multilayer mediator film was constructed by repeating the above procedure, and the electrode was referred to as PG/PEI-Fc_{n1}. The multilayer of DI and G6PDH were immobilized by alternate adsorption in PEI solution (1.0 mg/ml) and enzyme solution (1.0 mg/ml) under 4 °C repeatedly. The biosensor fabricated was referred to as PG/PEI-Fc_{n1}/DI_{n2}/$G6PDH_{n3}$ (n_1, n_2 and n_3 denoted the layer number of polymerized mediator, DI and G6PDH, respectively).

2.4. Characterization of multilayer film

QCM measurements were carried out on quartz crystal analyzer, (Seiko, QCA917). Gold-coated resonator electrodes (geometrical area 0.16 cm^2) for QCM were soaked in 'piranha' solution (3:7 volume ratio of 30% H_2O_2 and 98% H_2SO_4) for 5 min followed by washing with pure ethanol and water. The cleaned gold surface of resonator was immersed in 10 mM 3-MPA ethanol solution for 12 h (followed by thorough rinse) to create a negatively charged self-assembled monolayer for further LBL immobilization steps which were the same as for PG electrode. QCM experiments were operated in ex situ operation mode, in which both of the two sides of resonator were in touch with solution, and after each adsorption step, the resonator was rinsed and dried under high-purified nitrogen, followed by measuring the frequency.

For the observation of film surface after adsorption by LBL, Au electrode was used as substrate. The change of the substrate surface before and after adsorption of polyelectrolytes and enzyme was observed by atomic force microscopy (AFM, Shimadzu SPM-9500).

2.5. Electrochemical measurements

Electrochemical measurements were carried out on potentiostat (Hokuto Denko, HA-301) and function generator (HB-104) with an X-Y recorder (Pantos Co., Model U-335). A three electrodes system was used, with

the 3 mm diameter PG as the working electrode, SCE as the reference electrode and Pt as the counter electrode. All experiments were carried out in Tris–HCl buffer (pH = 7.5) containing 3.3 mM $MgCl_2$ at room temperature, and the buffer was bubbled with high-purity nitrogen for about 5 min before electrochemical measurement to eliminate the influence of dissolved oxygen. For amperometric experiments, the potential of working electrode was set at +0.6 V vs. SCE.

3. Results and discussion

3.1. Characterization of multicomponent films by QCM

According to the Sauerbrey equation, relationship between frequency shift and area mass density for one cycle adsorption was listed in Eq. (1). Where F_0 is the fundamental resonant frequency (9×10^6 Hz), μ_q is the shear modulus of the quartz (2.947×10^{11} g cm^{-1} s^{-2}), ρ_q is the density of the quartz (2.648 g cm^{-3}) and A is the electrode area (0.16 cm^2)

$$\Delta F = -\frac{2F_0^2}{(\mu_q \rho_q)^{1/2}} \frac{\Delta m}{A} \quad (1)$$

By taking into account the characteristics of quartz resonators, the adsorbed mass can be calculated from the frequency shift by Eq. (2), and 1 Hz frequency shift corresponds to a mass increase of about 0.9 ng

$$\Delta F = -1.83 \times 10^8 \frac{\Delta m}{A} \quad (2)$$

The QCM data was also used to estimate the thickness, d (cm) of multilayer films by Eq. (3), where ρ is the density of adsorbed materials (1.2 g cm^3 for polyelectrolytes and 1.3 g cm^3 for enzymes) [3], and A is the area of QCM electrode. Because adsorption occurred in both sides of QCM electrode, total mass increase should be divided by 2 [6]

$$d = \frac{\Delta m}{2A\rho} \quad (3)$$

The assembly of multilayer structure was accompanied by alternate adsorption in oppositely charged polyelectrolytes and enzymes solutions. At pH 7.0, PEI and PEI-Fc are positively charged, and Alg-Na, DI and G6PDH are negatively charged. The multilayer formation was investigated by QCM. Au electrode modified with 3-MPA self-assembled monolayer was alternate adsorbed in PEI/Alg-Na, PEI-Fc/Alg-Na, PEI/DI and PEI/G6PDH solution sequentially. The final assembly of the multilayer film was referred to as Au/$(PEI/Alg\text{-}Na)_2(PEIFc/Agl\text{-}Na)_2(PEI/DI)_2$ $(PEI/G6PDH)_2$. After each adsorption step, the frequency of Au electrode was measured and frequency shift was shown in Fig. 1. Negative linear progression of frequency shift was found to depend on adsorption cycles between 4 pairs of oppositely charged polyelectrolytes and enzymes, which indicated multilayer assembly could be prepared reproducibly by LBL method. From Eq. (3) and QCM data, the thickness of DI and G6PDH layers were estimated as 5.8 and 5.0 nm, and these data were close to the enzyme diameters (DI 6.4 nm and G6PDH 6.3 nm) which were calculated from molecular weight by assuming enzymes as rigid spherical molecules [26]. Typically, immobilization of enzyme is very complex, as pointed out by Lvov, and in some cases, the aggregation of enzyme during LBL procedure resulted in multilayer in one adsorption step [3]. In our work, QCM data showed monolayer enzyme adsorption occurred if the interface was considered as plane after immobilization.

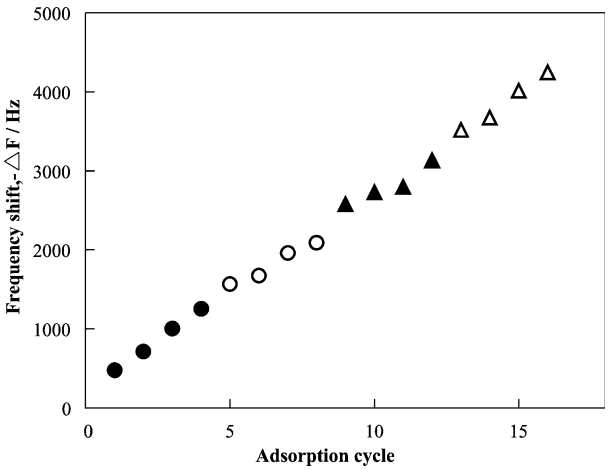

Fig. 1. Frequency shift ($-\Delta F$) due to cycles of alternate adsorption in PEI/Alg-Na (●), PEI-Fc/Alg-Na (○), PEI/DI (▲) and PEI/G6PDH (△) solutions.

3.2. Atomic force microscopy

In order to identify the immobilization by LBL and clarify the surface smoothness after alternate adsorption, the interface was viewed by AFM, and observed images were shown in Fig. 2. Fig. 2(A) is the image of bare gold electrode surface and Fig. 2(B) is the image of surface after alternate adsorption in PEI and DI solution. A relatively smooth surface is found for bare gold electrode, and after one bilayer immobilization, it has been changed to rough surface which confirmed that the adsorption step by LBL was a complicated procedure, and enzyme aggregation did occurred.

3.3. Electrochemical behavior of PEI-Fc

The synthesized polymer PEI-Fc was used as mediator in present research, and its electrochemical behavior and oxidation of NADH by free PEI-Fc and DI were first investigated on bare PG electrode, shown in Fig. 3.

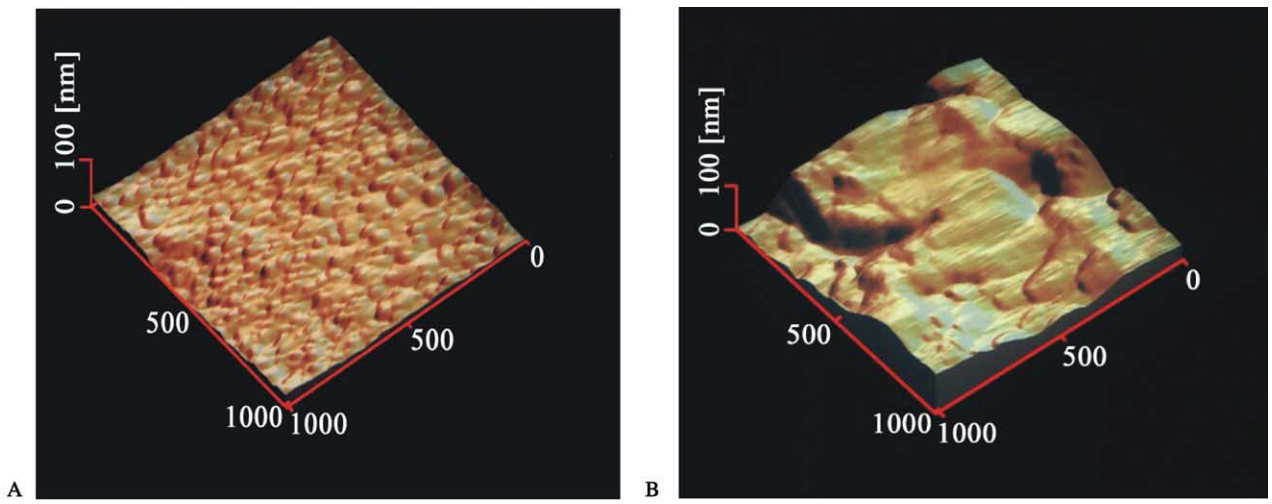

Fig. 2. AFM images of bare Au electrode (A) and after one bilayer adsorption of PEI/DI on Au electrode first assembled by 3-MPA (B).

Fig. 3(A) is the cyclic voltammogram of 0.5 mM PEI-Fc in buffer solution. It was found that anodic and cathodic peaks were at +0.286 and +0.235 V separately, which indicated the ferrocene groups bound to the backbone of PEI. After addition of NADH and DI, the anodic peak increased dramatically, and cathodic peak slightly decreased as shown in Fig. 3(B). It is confirmed that the oxidation of NADH by PEI-Fc and DI, and PEI-Fc was an efficient mediator to shuttle electrons between the electrode and DI.

3.4. Oxidation of NADH by immobilized PEI-Fc and DI

Oxidation of NADH by immobilized PEI-Fc and DI was further investigated. Mediator film was constructed by alternate immobilization of PEI-Fc and Alg-Na. By using free DI in buffer, the influence of layer number of PEI-Fc was studied and shown in Fig. 4. With only one PEI-Fc layer as mediator, the electrode gave high current response (820 nA for 2 mM NADH), which indicated the high mediation efficiency of immobilized PEI-Fc. But with the layer number of PEI-Fc increased, the current response against NADH decreased. This might be attributed to the diffusion of DI and NADH across the multilayer mediator film.

On the basis of 5 layers of PEI-Fc, DI was immobilized and the layer number of DI was studied and shown in Fig. 5. With the layer number of DI increased from 1, 2 and 5, the current response to NADH increased. The enhanced response should be attributed to the incorporation of more DI layers. Ten layers of DI gave nearly the same response as 5 layers. However, for 15 layers of DI, the response extremely decreased. Generally, NADH oxidation rate is accelerated by much more amount of enzyme and mediator. But for more layer of DI, the contact between enzyme

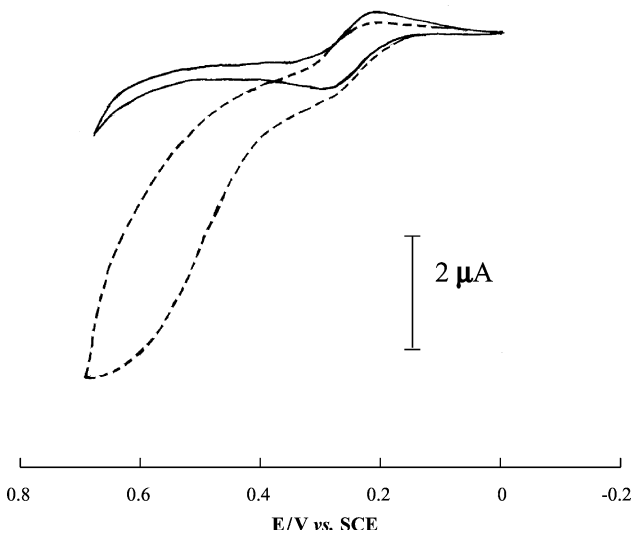

Fig. 3. Oxidation of NADH by free PEI-Fc and DI. Cyclic voltammograms of 0.5 mM PEI-Fc (A, —) and A plus 5 units DI and 2 mM NADH (B, - - -). Scan rate: 10 mV s^{-1}.

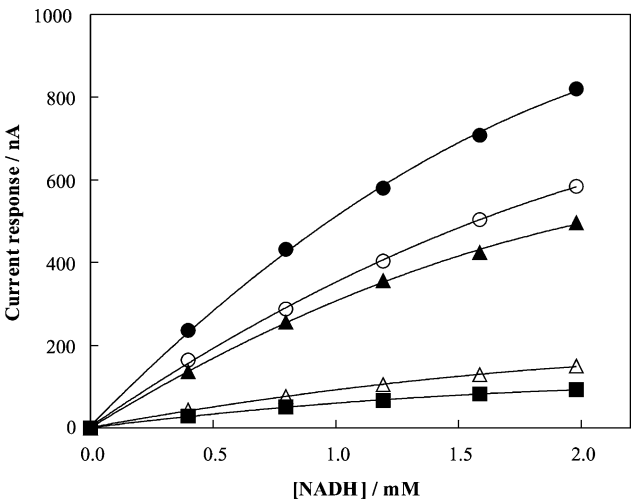

Fig. 4. Influence of layer number of PEI-Fc. Current response of PG/PEI-Fc_1 (●), PG/PEI-Fc_2 (○), PG/PEI-Fc_5 (▲), PG/PEI-Fc_{10} (△) and PG/PEI-Fc_{15} (■) to NADH.

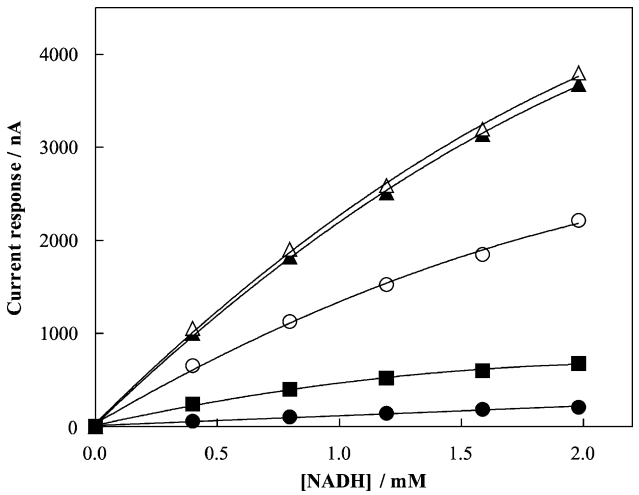

Fig. 5. Influence of layer number of DI. Current response of PG/PEI-Fc_5/DI_1 (●), PG/PEI-Fc_5/DI_2 (○), PG/PEI-Fc_5/DI_5 (▲), PG/PEI-Fc_5/DI_{10} (△) and PG/PEI-Fc_5/DI_{15} (■) to NADH.

and mediator was not efficient enough, and NADH diffusion across the DI layers increased which resulted in the low response for the electrode with 15 layers of DI.

3.5. Construction of bi-enzyme biosensor and the influence of concentration of NAD^+

In order to fabricate a bi-enzyme biosensor, 5 layers of PEI-Fc, 2 layers of DI and 2 layers of G6PDH were immobilized on the electrode by LBL alternate adsorption. In the presence of free NAD^+, the electrode gave current response to G6P. As coenzyme, NAD^+ participated and was regenerated during the enzymatic reactions catalyzed by DI and G6PDH, and the influence of NAD^+ concentration was studied and shown in Fig. 6. The current response was enhanced by increasing the concentration of NAD^+.

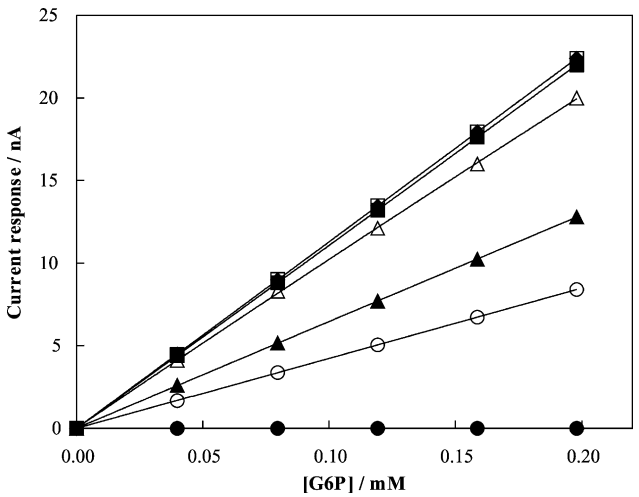

Fig. 6. Influence of concentration of NAD^+. Current response of PG/PEI-Fc_5/DI_2/$G6PDH_2$ to G6P in the presence of 0.004 mM (●), 0.06 mM (○), 0.12 mM (▲), 0.20 mM (△), 0.70 mM (■), 1.40 mM (□) and 2.0 mM (◆) NAD^+.

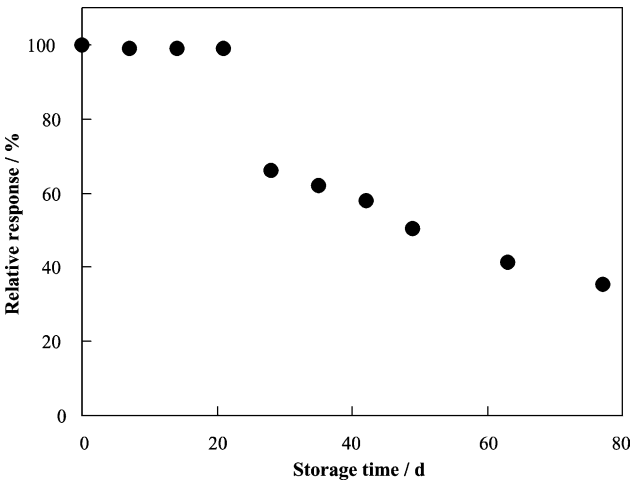

Fig. 7. Long-term stability of the bi-enzyme biosensor.

But when the concentration of NAD^+ exceeded 0.7 mM, there was no apparent increased current response. This confirmed that conjugation reactions happened on electrode surface which resulted in the current response to G6P, and a bi-enzyme biosensor could be constructed by immobilized polymerized mediator (PEI-Fc), DI and G6PDH.

3.6. Long-term stability

Long-term stability of the bi-enzyme biosensor was measured and shown in Fig. 7. When not in use, the electrode was stored in buffer under 4 °C. No obvious decrease in response to G6P was observed for first 3 weeks. This confirmed that both of DI and G6PDH kept their activities, and electrostatic force between polyelectrolytes and enzymes was strong enough to keep the multilayer architecture stable. After 20 days, relative response decreased continuously and slowly, which might be because enzymes began to lose their activities. But the biosensor still retained 40% of initial response after 75 days.

4. Conclusion

By using LBL method, multicomponent ultrathin film was constructed, including two enzymes (DI and G6PDH) and polyelectrolytes. QCM and AFM measurement confirmed the formation of nanoscale multilayer architecture. Electrochemical behavior of the synthetic polymerized mediator showed high performance in transferring electron between DI and electrode. Reagentless biosensor system was fabricated by immobilizing multilayer assembly including mediator and two enzymes on modified carbon electrode. The biosensor showed high sensitivity to G6P in the presence of free NAD^+, and high stability after long storage period, which confirmed that the incorporation of immobilized polymerized mediator, DI and G6PDH could result in integrated and stable biosensor system with efficient mass diffusion and electron transfer. This research work

demonstrates that LBL method is an excellent technique to assembly ultrathin film, especially for immobilization of enzymes, and oxidation of NADH by immobilized DI and polymerized mediator can provide a basis for the construction of specific biosensor system by utilizing NAD^+-related enzymes.

Acknowledgements

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