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Electrochemical Detection of Cardiac Biomarker Troponin I at Gold Nanoparticle-Modified ITO Electrode by Using Open Circuit Potential

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Gold nanoparticles (GNPs) have been electrodeposited on indium tin oxide (ITO) and applied to detect molecular interaction between antigen and antibody by measuring open circuit potential (OCP). As a capture protein for immunoassay, specific monoclonal antibody against human cardiac troponin I (cTnI) was modified on the GNP coated ITO electrode surface by self-assembly and characterized by electrochemical techniques. This is a simple, stable, and repeatable approach. The modified electrode can be used to detect human cTnI. In this study, we adopted a new strategy to get an electrical signal due to the enzyme-based immunecatalytic reaction by measuring the changes of OCP. This novel approach can be applied to a simplified and miniaturized diagnostic system. A linear dependence of OCP changes according to cTnI concentrations is observed in the range of concentration from 1 to 100 ng/ml. Because the clinical borderline of cTnI between normal and patient is 0.5 to 2.0 ng/ml, our results indicate that the new approach using OCP changes has a great potential for clinical diagnostic application.

Keywords: Gold nanoparticles, ITO electrode, open circuit potential, cardiac troponin, biosensor

1. INTRODUCTION

A rapid and accurate diagnosis of acute myocardial infarction (AMI) is crucial for saving lives. Three specific protein markers, including cardiac troponin I (cTnI), creatine kinase-MB isoenzyme (CK-MB) and myoglobin are found in cardiac muscle. They and their complex are released to blood

circulation soon after onset of AMI. Therefore, early accurate detection of these biomarkers is essential for the successful medical treatment of AMI disease. Especially, cTnI, cardiac muscle protein (29 kDa), will release in the bloodstream when cardiac muscle is damaged or dead. The concentration of cTnI starts to rise rapidly within 3–4 h after onset of AMI [1, 2] and remains to be raised up to 4–10 days [3, 4]. Therefore, the content of cTnI in serum has a close relation with the progress degree of this disease.

In a recent previous study, it was reported that the clinical borderline of cTnI between normal and patient is 0.5 to 2.0 ng/ml. [5]. Whereas, normal levels of cTnI in patients are around 10 ng/ml, and it goes up to a range from 20 to 550 ng/ml when AMI occurred in patients [6]. Therefore, the early stage detection method which can measure very low concentration of cTnI with high sensitivity, are needed for the early diagnosis of AMI and progress management of patients.

In a clinical application field, enzyme immunoassays which are based on a selective antigen–antibody binding and labeled enzyme, is one of the widely used conventional detection principle [7]. In this analysis method, the antigen or antibody is labeled by horseradish peroxidase (HRP) enzyme depending on the analysis format and the activity of labeled HRP enzyme will be measured to get a signal due to the molecular interaction between antigen and antibody. As a detection method, diverse approaches including fluorimetric [8-13], luminometric [14-18], colorimetric [19-21], and amperometric detection [22] are widely used. Among them, the amperometric detection has attracted attention in recent years, because of its high sensitivity, fast response time, and wide linear range [23]. Because of these attractive merits of electrochemical detection method, it was considered as a prime candidate for the development of potable smart immunosensors. However, there is still remained some requirement including laboratory equipments with suitable instrument, standard electrode, and skillful person. Therefore, a simple and smart detection principle which is coupled to molecular recognition, are required for the construction of more advanced smart diagnostic system. At this point of view, we have interest in the open circuit potential (OCP) as a new detection principle. OCP has been used to evaluate the property of chemical adsorbing electrode such as a reactive gas or polymer [24-26]. Moreover, among the wide range of electrochemical techniques, OCP measurement has estimated as an efficient and suitable tool for quality assessment of biochemically modified electrode and electrochemical system [27-29]. Therefore, one can use OCP monitoring as the analytical technique for the characterization of the key structural element of recently reported miniaturized reference electrodes [30-32].

In recent years, gold nanoparticles (GNPs) have been extensively studied in electroanalytical chemistry because of their unique properties, such as good biocompatibility, high surface-to-volume ratio, and ease of synthesis [33]. Furthermore, they have been used as base materials for the immobilization of HRP, and provide an environment similar to the native environment of enzyme [34].

In this work, a high-sensitive cardiac troponin sensor is reported based on HRP-labeled probe to detect human cTnI which is highly associated with damage of cardiac muscle and progress degree of AMI. Linker molecules including cystamine and glutaraldehyde were firstly chemically adsorbed on the GNP-modified indium tin oxide (ITO) electrode through self-assembly method. A capture protein, anti-troponin mAb was immobilized on the GNP-modified ITO electrode (GNP-ITO) surface with linker molecules. The target antigen, human cTnI would be captured by it through immune binding

reaction. The HRP labeled secondary mAb which has specific binding site against to another part of human cTnI would interact with antigen in a sandwich way. Finally, H_2O_2 electroreduction current catalyzed by HRP was measured amperometrically in the presence of hydroquinone (HQ) as the mediator. Through measuring the changes of OCP due to the H_2O_2 electroreduction current, the concentration of cTnI can be determined. The simple manipulation procedure, high selectivity, low-cost, and fast response are the main features of proposed cardiac troponin sensor.

2. EXPERIMENTAL SECTION

2.1. Reagent and materials

HRP enzyme (E.C.1.11.1.7) was obtained from Thermo Fisher Scientific Inc. (MA, USA). Human cardiac troponin I (cTnI) and monoclonal antibody against to cTnI was purchased from Meridian Life Science (ME, USA). The conjugation between HRP and anti-troponin mAb performed by using HRP conjugation kit (Pierce® Conjugate Purification Kit-44920, Thermo Fisher Scientific Inc) and resulted in a HRP conjugated anti-troponin mAb.

All other chemicals were of analytical reagent grade. HQ, hydrogen peroxide (H_2O_2), gold chloride trihydrate ($HAuCl_4.3H_2O$), cystamine, glutaraldehyde, $K_3Fe[CN]_6$, Na_2HPO_4 and NaH_2PO_4 were purchased from Sigma-Aldrich (USA). A phosphate buffer solution (PBS) was prepared as previously described [35]. All solutions were prepared with deionized water of 18 MΩ purified from a Milli-Q purification system. All experiments were carried out at room temperature.

2.2. Surface modification

GNP-ITO electrode was prepared as previously reported [36]. Briefly, ITO glass was cut to a rectangular shape with size of 1 x 3 cm and ultrasonicated in ethanol, and deionized water for 10 min consequently.

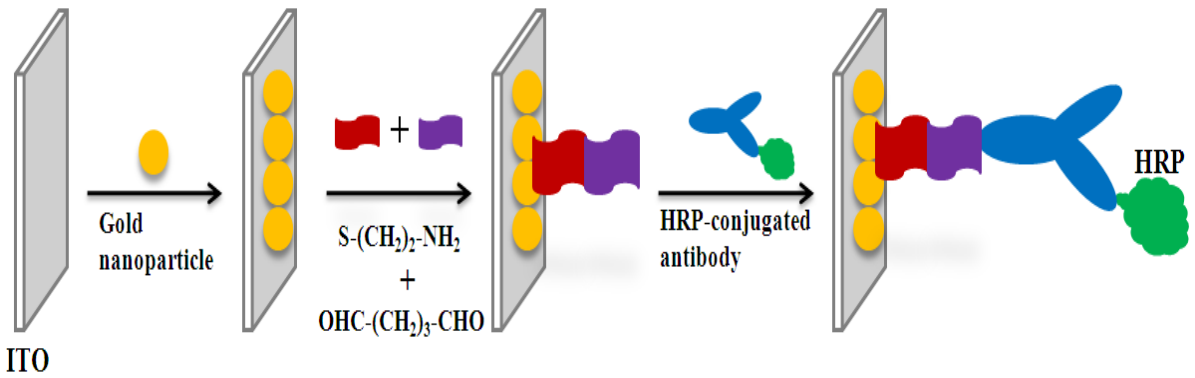

Figure 1. Scheme of modification process of gold nanoparticle-modified ITO electrode for the immobilization of HRP-conjugated antibody.

The cleaned ITO was then subjected to the electrochemical deposition of GNPs through the cyclic sweeping of the potential from -0.2 to -1.3 V for 20 cycles at 50 mV s^{-1} in 0.1M PBS (pH 7.0) containing 0.1 mM $HAuCl_4$. The prepared GNP-ITO electrode was then thoroughly rinsed with water for the further modification. To introduce linker molecules with amine residue, GNP-ITO electrode surface was treated with 20 mM cystamine for 30 min at room temperature. After rinsing with deionized water, 4 % glutaraldehyde was treated to the surface of GNP-ITO electrode for 30 min at room temperature, to introduce aldehyde residue which can bind amine residue of protein surface. The modification process is shown in Fig. 1.

2.3. Electrochemical measurements

Electrochemical experiments were performed with a CHI 430A electrochemical workstation (CH Instruments, Inc., TX, USA). A conventional three-electrode system was used, where GNP-ITO electrode or modified electrode, a platinum wire, and a Ag/AgCl electrode were used as working, counter, and reference electrodes, respectively. All potentials in this work referred to this reference electrode. The electrochemical measuring set-up is shown in Fig.2.

To confirm the chemical modification and enzyme immobilization on the GNP-ITO electrode, the measurement of cyclic voltammograms (CV) were performed in an electrolyte solution containing 5 mM $K_3Fe(CN)_6$ at a scan rate of 100 mV/s.

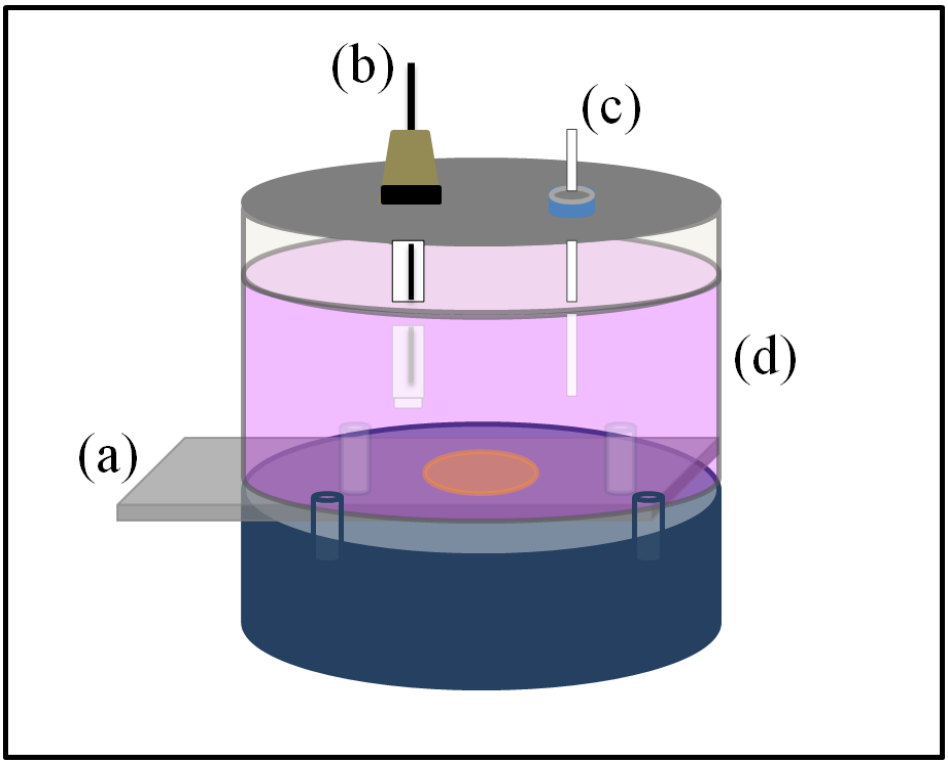

Figure 2. Schematic representation of measuring set-up for cyclic voltammograms (CVs) and OCP (Open circuit potential). (a) working (GNP-ITO), (b) reference (Ag/AgCl), and (c) counter (Pt wire) electrodes and (d) electrochemical cell .

The catalytic effect of immobilized enzyme were confirmed by measuring the CV of GNP-ITO electrode with or without HRP enzyme in a PBS buffer (pH 7.4) solution consisting of 0.5 mM HQ and 0.5 mM H_2O_2 at a scan rate of 100 mV/s. HQ and H_2O_2 were added as an intermediator molecule and substrate material respectively. HQ is an excellent electron mediator, which efficiently reduces the working potential and eliminates the effects of various electroactive substances in real sample [37]. Therefore, it has been successfully used in numerous peroxidase-based amperometric biosensors to detect hydrogen peroxide.

OCP measurement was performed by using CHI 430A electrochemical workstation (CH Instruments, Inc., TX, USA) with two electrode mode including working and reference electrodes. OCP change due to the different concentration of immobilized enzyme was measured for 50 sec. To confirm the change of OCP due to the catalytic activity of enzyme, HRP conjugated antibody was immobilized onto the surface of GNP-ITO electrode and the OCP change of GNP-ITO electrode was measured according to the concentration of HRP conjugated antibody.

2.4. Sensing of human cardiac troponin I by GNP-ITO electrode

Capture protein, which is a monoclonal antibody against to cTnI, was immobilized onto the surface of GNP-ITO electrode by drop and incubation process. Capture antibody was treated with a concentration of 200 μg/mL and incubated for 1 hr at room temperature. After immobilization of capture antibody, BSA solution with concentration of 1 mg/ml was used as a blocking agent. After rinsing with PBS buffer solution, target protein, cTnI was introduced into the electrode surface with different concentration from 1 to 500 ng/mL in PBS (pH 7.4) solution. After incubation with target protein, cTnI, the electrode surface was rinsed three times in PBS solution. Finally, HRP-conjugated anti-troponin Ab was introduced to interact with cTnI which was captured by probe protein with different concentration. After incubation with HRP-conjugated anti-troponin Ab for 1 hr, the electrode surface was rinsed and prepared for the measurement of OCP.

3. RESULTS AND DISCUSSIONS

3.1. Effect of surface modification

Because the cyclic voltammetry of ferricyanide is a valuable and convenient tool to monitor the barrier of the modified electrode, it was chosen as a marker to investigate the changes of the electrode behavior after immobilization of each molecule. Figure 3 shows CVs of differently modified electrodes in a 5mM ferricyanide solution. Well-defined typical CVs of ferricyanide are observed at the case of Au-coated ITO electrode (Fig. 3a). The redox peak decreased (Fig. 3b) after the electrode coated with linker chemicals, as the linker chemicals can hinder the diffusion of ferricyanide toward the electrode surface. After immobilizing process of HRP enzyme, a remarkable redox current decrease was observed as in Fig. 3b. This is due to the fact that HRP enzyme acts as a barrier in the electron transfer procedure.

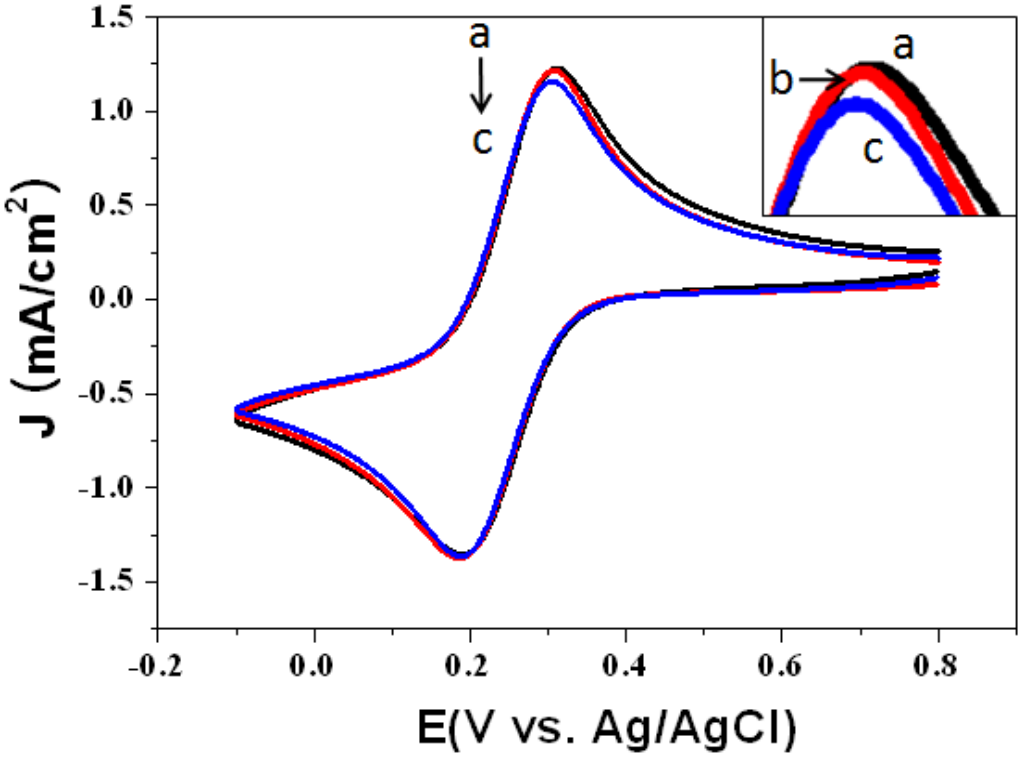

Figure 3. Cyclic voltammograms (CVs) of GNP/ITO (a), Cys-glu/GNP/ITO (b), and HRP/GNP/ITO (c). CVs of each condition were measured in 5 mM $K_3Fe[CN]_6$. In inserted figure, a decreased oxidation current was observed after immobilizing HRP. Scan rate is 100 mV/s.

3.2. Characterization of enzyme-modified electrode by cyclic voltammetry

The electrochemical behaviour of the HRP enzyme-immobilized GNP-ITO electrode in the absence and presence of 0.5 mM H_2O_2 in 0.1 M PBS (pH 7.4) containing 0.5 mM HQ was investigated using cyclic voltammetry. Figure 4 shows the CVs of the HRP enzyme-immobilized GNP-ITO electrode in pH 7.4 PBS containing 0.5 mM HQ at the scan rate of 100 mV/s. In comparison with the bare GNP-ITO electrode (Fig. 4a), a pair of well defined redox peaks appeared with a relatively large decrease of the peak currents, and the anodic and cathodic peak potentials were shifted slightly negatively and positively, respectively. Because HQ is an excellent electron mediator, the CVs of the bare GNP-ITO electrode in pH 7.4 PBS containing 0.5mM HQ shows high redox peak current. However, the CVs of the HRP enzyme-immobilized GNP-ITO electrode show the decrease of the peak currents, the shifts of anodic and cathodic peak position, and the peak broadening. The broadened oxidation and reduction peaks reflect a slow heterogeneous charge transfer on the surface of enzyme-immobilized electrode.

Meanwhile, the redox peak current remarkably increased at the HRP enzyme-immobilized GNP-ITO electrode, when 0.5 mM H_2O_2 was added (Fig 4b, and 4c). These results indicate that not only the HQ could effectively shuttle electrons between the redox center of HRP and the electrode surface, but also the current increase was mainly due to the catalytic reduction effect of HRP.

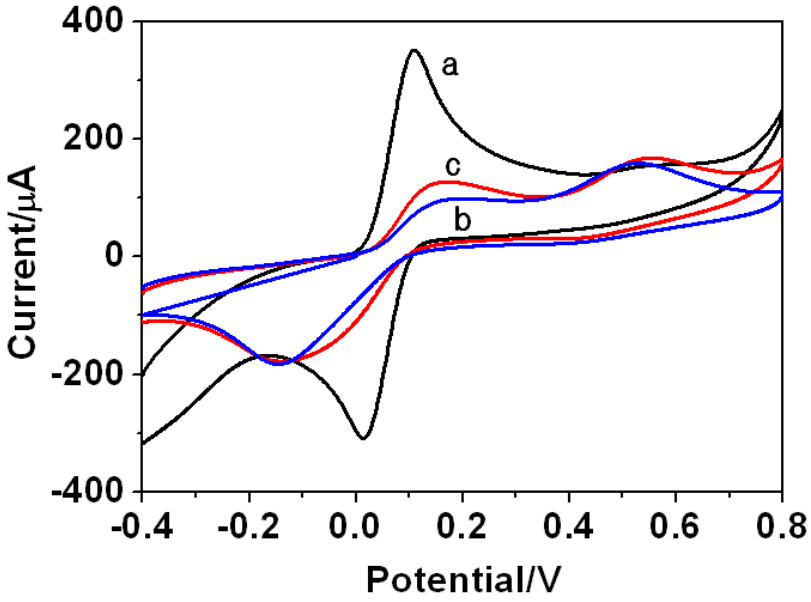

Figure 4. Cyclic voltammograms recorded, with a scan rate of 50 mV/s, in 0.1 M pH 7.4 PBS containing 0.5 Mm HQ at the bare GNP/ITO (a), and HRP/GNP/ITO (b, c) in absence (a, b) and presence (c) of 0.5 mM H_2O_2.

3.3. Measurement of open circuit potential (OCP)

After the immobilization of HRP-conjugated antibody onto the surface of GNP-ITO electrode, open circuit potential of this electrode system was measured in 0.1 M pH 7.4 PBS solution containing 0.5 mM HQ and 0.5 mM H_2O_2.

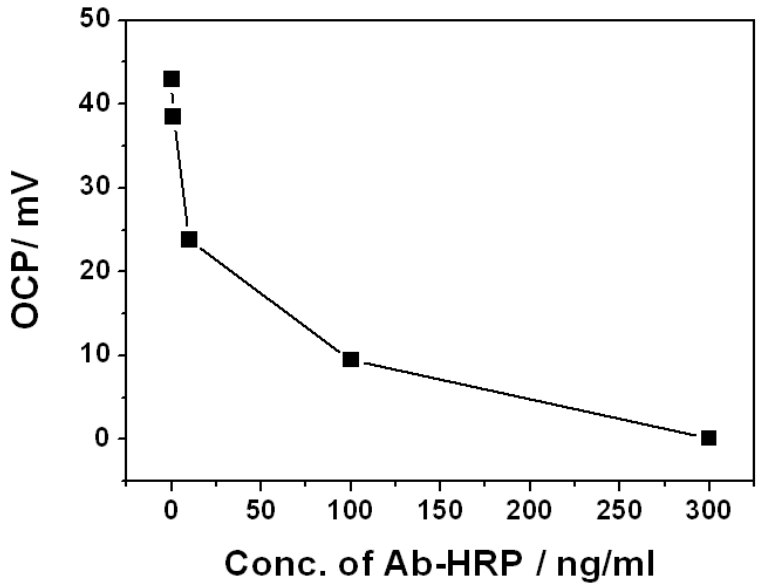

Figure 5. Effect of enzyme immobilization with different concentrations GNP-ITO electrodes were modified by HRP-conjugated Ab with different concentrations (0, 5, 10, 100, and 300 ng/ml). The OCP of two electrode system measured at the condition with 0.1 M pH 7.4 PBS containing 0.5 mM HQ.

Figure 5 shows the effect of the catalytic reduction of HRP on the surface of GNP-ITO electrode by measuring of OCP. Without any bias voltages, a potential between enzyme-modified GNP-ITO electrode and reference electrode was measured by two electrode mode. In general, the OCP reflect not only the electric properties of device without bias potential but also the potential change due to the oxidation or reduction of immobilized organic and inorganic molecules on the electrode surface. In figure 5, OCP of enzyme-modified electrode decreased sharply with increasing the concentration of immobilized HRP enzyme to the maximal value of 10 ng/ml, and then tended to be steady with further increases in the immobilized HRP concentration. These results indicate that OPC change on the GNP-ITO electrode reflect the concentration of immobilized HRP enzyme.

3.4. Sensing of human cardiac troponin I by OCP measurement

Using the sandwich method, molecular recognition between cTnI and HRP enzyme-conjugated secondary mAb was investigated by measuring OCP changes of electrode. In figure 6, the OCP of enzyme-modified electrode decreased gradually with increasing the concentration of cTnI. These OCP changes reflect the concentration of immobilized HRP-conjugated mAb which depend on the concentration of cTnI.

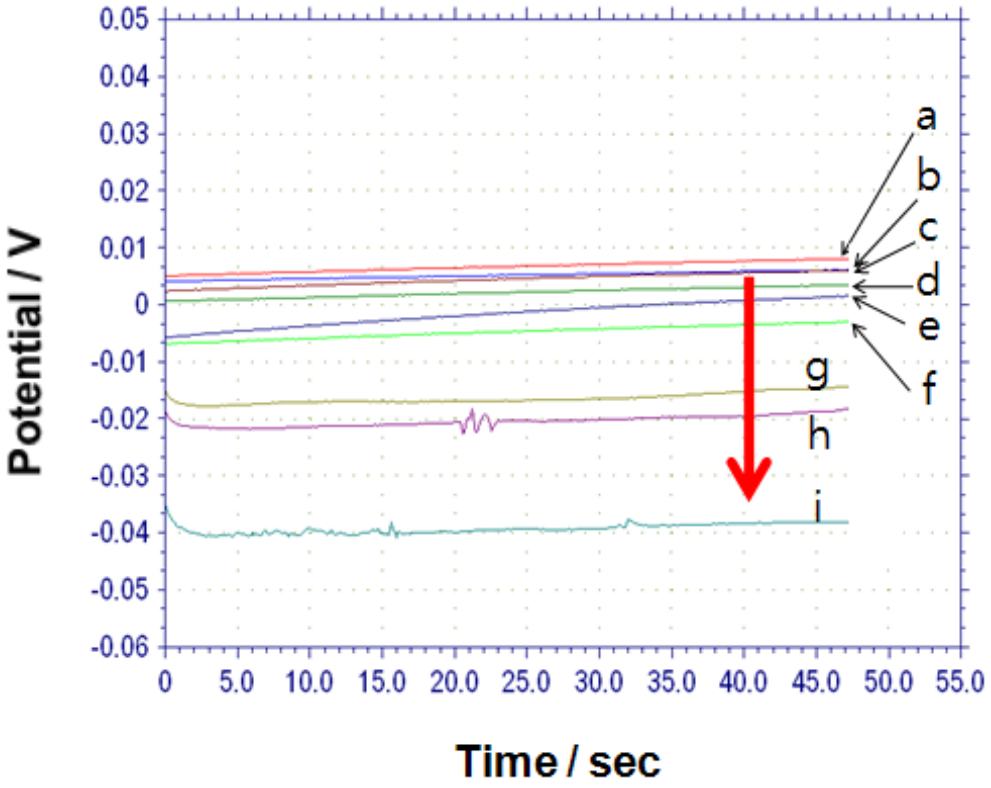

Figure 6. OCP-time curve for the interaction between HRP-conjugated anti-cTnI mAb and cTnI with different concentrations. Each line including from 'a' to 'i', indicates the different concentrations of cTnI (0, 1, 5, 10, 50, 100, 200, 300, and 400 ng/mL), respectively. The OCP of two electrode system measured at the condition with 0.1 M pH 7.4 PBS containing 0.5 mM HQ.

The relative value of OCP changes is shown in figure 7. When the concentration of cTnI is at the range from 0 to 100 ng/ml, OCP changes increased slowly. On the other hand, at the range from 100 to 500 ng/ml, OCP changes markedly increased with increasing the concentration of cTnI. In figure 7, a linear dependence of OCP changes according to cTnI concentrations is observed in the range of concentration from 1 to 100 ng/ml. In a previous study, it was reported the clinical borderline of cTnI between normal and patient is 0.5 to 2.0 ng/ml [5]. Moreover, it is well known that the normal levels of cTnI in patients are around 10 ng/ml, and it goes up to range from 20 to 550 ng/ml when AMI occurred in patients [6]. Therefore, our results indicate that the measurement of OCP changes based on the enzyme-modified electrode and sandwich method shows a potential for detecting the concentration of cTnI. Although this electrode system cannot shows a measuring possibility with very high sensitivity at low concentration of cTnI, however it shows meaningful signals at a clinical concentration ranges of cTnI.

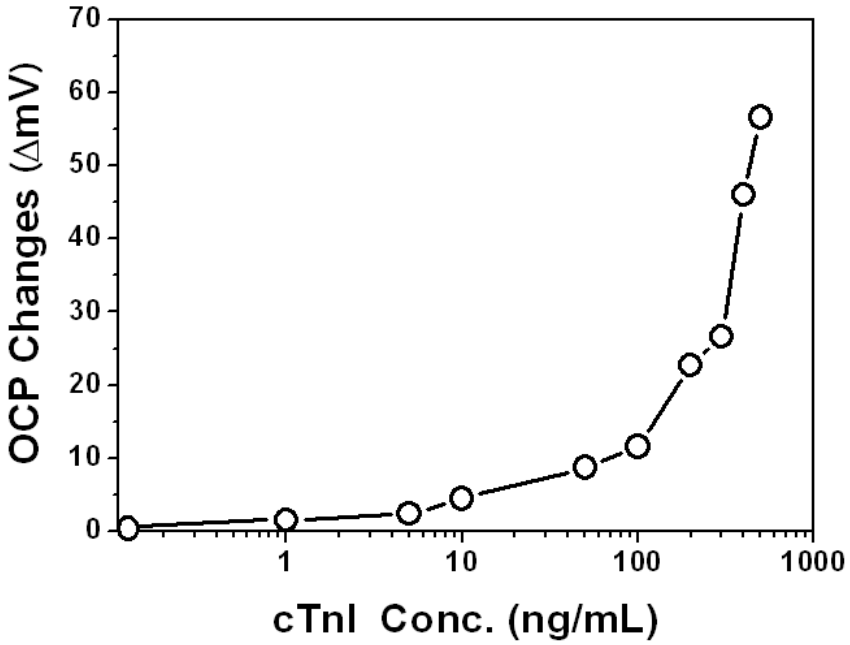

Figure 7. The plot of OCP changes according to the concentration of cTnI. The OCP of two electrode system measured at the condition with 0.1 M pH 7.4 PBS containing 0.5 mM HQ.

4. CONCLUSION

In this study, we fabricated GNP-ITO electrode and applied it to detect molecular interaction between cTnI and HRP-conjugated anti-troponin mAb. First of all, we estimated the application possibility of OCP as a transducer from enzyme catalytic reaction to electric signal, using the HRP enzyme immobilized electrode. Our results show that the OCP measurement can apply to detect the enzyme catalytic reaction as a signal transducer. When we applied it to detect cTnI, the linear dependence of OCP changes according to cTnI concentrations is observed in the range of

concentration from 1 to 100 ng/ml. Moreover, it shows a noticeable OCP changes at the concentration range from 100 to 500 ng/ml. On the basis of these results, we find that the measurement of OCP changes based on the enzyme-immobilized electrode system can apply to biosensor including cardiac biomarker detection system.

In conclusion, we confirmed the application potential of new approach based on the OCP measurement and expect that this new approaches will be applied to a simplified and miniaturized diagnostic system as a simple and smart detection method.

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