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Direct electron transfer with enzymes on nanofiliform titanium oxide films with electron-transport ability

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ABSTRACT

Direct electron transfer (DET) from biomolecules to electrode is a process without electron-mediators, thus superior selectivity and sensitivity is expected in order to monitor electron transfer between electrode and biomolecules without any mediator interference. However, DET is difficult because a redox center which is an electron active center of proteins such as enzymes is buried deep. So, a unique electrode nanostructure to reach the redox center is a critical factor. Here we have systematically investigated terms for DET using various nanofiliformed electrode morphologies and enzyme concentrations. It is pointed out that the reaction site is below 100 nm, the ration amounts of adsorbed enzyme per surface area are below 1.0 are contributed to the DET. As a great application, we have developed a biosensor monitoring the hydrogen peroxide (H_2O_2) detecting capability from peroxidase directly. For the fabricated HRP/nTOF/Ti-electrodes observed the catalytic current value was linear according to the increase in the concentration of H_2O_2 up to 100 μM, which indicates a good potential for an H_2O_2 biosensor.

1. Introduction

The direct electron transfer (DET) between biomolecules and electrodes has been attracting increasing interest of the scientific community in recent years, in view of its high importance in fundamental science and in biological reactions, as well as its broad applications in energy systems, bioelectronics, and biosensors (Wei et al., 2002; Qiao et al., 2008; Tien et al., 1991; Cui et al., 2007; Salimi et al., 2007; Zhang et al., 2007; Bao et al., 2008; McKenzie and Marken, 2003). Although it is expected that DET can be exploited in various ways, it has mainly two critical limitations. First, the active site of redox enzymes is deeply buried in the protective protein matrix, so direct electron exchange between the redox center and an electrode can only occur under exceptional conditions. Second, the redox enzymes are too large and fragile to interact directly with a metallic electrode without being at least partly denatured. To induce DET, there are two requirements: (i) a nanostructure of the electrode to achieve contact directly with the redox center and (ii) a good matrix for immobilizing redox proteins. Nanomaterials with unique structures can induce DET due to their small size and novel physical, electronic, and chemical properties for electron-mediating the redox reaction (McKenzie and Marken, 2003). For example, carbon nanotube (CNT)- and gold-nanoparticle-based electrodes have enabled the study of electrochemistry of proteins and enzymes due to their unique electrical and structural properties (Ueda et al., 2011; Davis et al., 1997; Zhao et al., 2005; Xiao et al., 2003). However, it is very difficult to obtain the desired structural, electrical, and biocompatible properties for such electrodes as their fabricating process is very demanding and involves many complex steps. Therefore, there is a demand for new materials that have the desired electrical, structural, and biocompatible properties needed for fabricating one-spot electrodes for DET, and their use would solve the above problems.

In our previous studies (Lee et al., 2009, 2011) we managed to greatly improve the electric conductivity of nanofiliform titanium oxide films (nTOFs) using a wet corrosion process (WCP) with KOH solution. Synthesized nTOFs exhibit diverse structural, electrical, and biocompatible properties. Their geometry revealed filiform projections that were cross-linked nanotubular textures. As for electricity, the synthesized nTOFs showed good electrical properties. Considering the electrical transport ability and the chemical bonding structures, it is suggested that potassium exists regularly in TiO_2 layers as two phases (Ti–O–K and Ti–K components), so it acts as a dopant, even though it is not in the form of a conventional dopant. It is noteworthy that the obtained materials are titanium oxide films having just two components (Ti–O–K and Ti–O). Finally, Ti–O and Ti–O–K layers have good biocompatibility.

In this work, using synthesized nTOFs, which have potential for use as one-spot electrodes for DET, we systematically investigated the relation between nanoscale geometry and amounts of immobilized enzyme, horseradish peroxidase (HRP), for DET. We also applied a

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reduction catalyst enzyme-immobilized electrode to the fabrication of an H_2O_2 biosensor.

2. Materials and methods

2.1. Preparation of nanostructured titanium oxide films

Commercially pure Ti metal substrates (Ti: Ti > 99.8%, Nilaco Co., Tokyo, Japan) were used. Ti metal substrates of dimensions $10 \times 10 \times 1.0\ mm^3$ were polished with #400~#2000 SiC papers and washed with acetone and distilled water in an ultrasonic cleaner. Then they were subjected to alkali treatment by soaking in KOH aqueous solutions with concentrations of 1.0, 5.0, 10, 15, 20, and 25 mol/L at room temperature for 24 h. After the alkali treatment, the metal substrates were gently washed with distilled water.

2.2. Fabrication of bioelectrode

For fabricating the electrode, horseradish peroxidase (HRP, 100 mg/U) was immobilized onto nTOF electrodes dipped in the horse radish peroxidase (HRP, 165-10793, 100 mg/U, WAKO chemical) solution (concentrations: 1000, 1500, and 2000 U/mL) for 12 h (phosphate buffer solution, pH 7.0) at 4 °C. Changes in the surface structure, shape, and size of the nTOFs were observed using a field-emission scanning electron microscope.

2.3. Characterizations

Changes in the surface structure, shape, and size of the Ti specimens were observed using a field-emission scanning electron microscope (FE-SEM: Model JSM-6500F, JEOL Co., Tokyo, Japan). Amounts of adsorbed HRP were measured with micro-BCA Protein Assay Reagent (Pierce, Rockford, IL). The HRP-immobilized nTOF samples were immersed in a mixture of 0.03 g/dL BPF and 0.045 g/dL bovine serum albumin (BSA) in phosphate buffered saline (PBS, pH 7.4 and ion strength: 0.15 M) for 60 min at 37 °C and then rinsed with 500 mL of fresh PBS twice by the stirring method (300 rpm for 5 min). The immobilized HRP was detached in sodium dodecyl sulfate (SDS) (1 wt% in water) by sonication for 20 min, and the HRP concentration in the SDS solution was determined by using the micro-BCA Protein Assay Reagent method. By using the concentration of the standard HRP solution, the amount of adsorbed HRP was calculated. Ar gas adsorption/desorption experiments were performed using an Autosorb-1 analyzer (Quantachrome Instruments). The surface area was calculated using the Brunauer–Emmett–Teller method: BET equation. For observing the performance of electron transfer, an electrochemical measurement was performed with an electrochemical workstation. The fabricated nTOFs/Ti-electrode and HRP-immobilized HRP/nTOFs/Ti-electrode were used as the working electrodes. The surface area of pretreatment of KOH was 0.07 cm^2. A platinum wire was used as the counter electrode, and a KCl-saturated Ag/AgCl electrode was used as the reference electrode.

3. Results and discussion

3.1. Morphology of electrode surface

Fig. 1 shows the FE-SEM images of the surfaces of Ti metal treated with 5.0, 15, and 25 mol/L-KOH solutions, respectively. Network structures were synthesized on the Ti metal surface; however, the morphology of the network structure was different based on those of structures treated with an aqueous concentration of KOH. As concentration of KOH solution was increased, the diameter of the aggregated filiform projections (AFPs) increased. In this paper, we defined that an AFP is a reaction site of DET. The diameters of AFPs of 1.0, 15, and 25 mol/L-KOH treated Ti specimens were shown to be 35, 400 and 1800 nm. Fig. 1(e, f) shows a schematic illustration of HRP immobilized on KOH-1.0 mol/L treated Ti (e) and KOH-15 mol/L treated Ti (f). Overall the surface shows similar AFPs, however, the diameter and shape of AFPs are difference. The KOH-1.0 mol/L treated Ti (e) is more sharp and the diameter is shorter than the KOH-15 mol/L treated Ti.

3.2. Biocatalysis of fabricated bioelectrode

Fig. 2 shows the apparent Tafel plots for the pure Ti-, HRP/Ti-, and HRP/nTOFs/Ti-electrodes. HRP/nTOFs/Ti-electrodes were fabricated by three different surfaces which are treated by various concentrations of KOH aqueous solution (1.0, 15 and 25 mol/L). A 1000 U/mL HRP solution was used. This plot shows the point of electron transfer in the electrode, called the onset potential ($E^{0/}$). $E^{0/}$ for HRP/nTOFs/Ti-electrodes was different from those for pure Ti- and HRP/Ti-electrodes, and $E^{0/}$for HRP/nTOFs/Ti-electrodes is similar to the reported $E^{0/}$ of HRP at around −0.33 to −0.40 V (Elena and Elena, 2002). It is noted that the DET reaction can occur only when utilizing HRP/nTOFs/Ti-electrodes; this reaction is then attributed to the nanofiliform surface. These results contribute to an enhancement DET as follows: from comparison between pure Ti-electrodes and HRP/nTOFs/Ti-electrodes, the electron transfer occurred between HRP and nTOFs, suggesting HRP was successfully immobilized on the nTOFs, and its electrochemical activity was well retained. Moreover, a comparison of HRP/Ti-electrodes and HRP/nTOFs/Ti-electrodes showed that nTOFs are a good matrix for immobilizing HRP and they have a suitable geometry for DET.

3.3. DET of fabricated bioelectrode

Fig. 3 shows the cyclic voltammograms (CVs) of nTOFs/Ti- and HRP/nTOFs/Ti-electrodes (treated by 1000 U/mL HRP solution) in pH 7.0 phosphate buffered saline (PBS) at 100 mV/s. The electrode with 35 and 200 nm in the AFPs diameter were used for this. For the nTOFs/Ti-electrode, no peak is observed at the electrochemical window. However, reduction peaks appeared at around −0.270 to 0.290 V for the HRP/nTOFs/Ti-electrode with < 70 nm reaction site electrode, attributable to the DET reaction between HRP and the underlying electrode. Once again, we defined that AFPs are the reaction sites of DET in this paper. The electrochemical result indicates that HRP was successfully immobilized on the nTOFs and its electrochemical activity was well retained. The results are very similar to those of the $E^{0/}$ derived from the Tafel plot; thus, DET occurred between HRP and nTOFs in fabricated HRP/nTOFs/Ti-electrodes. However, in case of the above 400 nm reaction site electrodes in which the nanotubular structure disappeared, the DET reaction could not be clearly observed. It is very interesting to see that the DET reaction is related to morphology. It was previously reported that the DET reaction occurred when the diameter of the reaction site of the electrode materials was < 50 nm (Xiao et al., 2003). For inducing DET, it is a key point to have specific shape AFP to reach the redox center, which is why we focus on the shape. We comfirmed the diameter and shape of APFs by SEM analysis; when the diameter of AFPs is longer, the shape becomes accordingly smoother and almost flat on the surface. From this information, DET occurred in this system when the diameter of the AFPs was < 100 nm. The CV results were enough to show the influence. Based on the results of the diameter of the AFPs, it was thus suggested that the electrodes