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Gold sputtered electrode surfaces enhance direct electron transfer reactions of human cytochrome P450s

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ABSTRACT

We immobilized human cytochrome P450 (CYP), a membrane-bound enzyme, onto both smooth and nanostructured surfaces of gold electrodes via a naphthalene thiolate monolayer film. Rapid electron transfer of CYP with an electrode as a redox partner took place when the enzyme was immobilized onto an electrode surface with nanostructures. This structure was easily prepared by conventional sputtering techniques. A well-defined pair of peaks was observed at −0.175 V (vs. SHE) with the largest heterogeneous electron transfer rate constant of 340 s^{-1} for human CYP. The positive redox potential shift of 45 mV upon drug (testosterone) binding was clearly detected, which corresponded to a change in the spin states of heme iron in CYP. The present study showed that gold sputtered surfaces are very useful for direct electron transfer reactions of human CYP isoforms.

1. Introduction

There has been increasing interest in achieving direct electron transfer between redox proteins and an electrode for applications in bioassays, biosensors, and bioreactors. This system can be used to activate the enzyme at an electrode by controlling the electrode potential instead of using oxidizing and reducing reagents [1,2], which enables the construction of reagent-less and cost-effective systems for the applications described above.

CYPs are heme proteins that form a large family involved in drug metabolism and the biosynthesis of steroids, lipids, vitamins, and natural products. Since human CYP plays a crucial role in drug metabolism, measuring its activity is very important to develop drug research strategies [3]. NADPH and NADPH-regenerating enzyme systems are generally used to supply electrons to CYP, and their activity is measured by the product formation (or substrate depletion) rates using HPLC after separating the reaction components. Achieving a fast electron supply from an electrode is a promising approach to develop rapid screening assays for CYPs [4]. Since plain electrode surfaces are usually not effective for direct electrochemistry of redox proteins, special types of modified electrodes were developed [2,5]. However, very limited studies on the direct electrochemistry of human CYPs have been reported [6–8], and some limitations were discussed [8]. Recently, we found that hydrophobic thin films of aromatic thiolates on an electrode are useful for electrochemically-driven reactions of CYP microsomes [9]. In the previous report, gold electrodes roughened by oxidation–reduction cycles (ORC) were used to observe direct electron transfer. In the present study, to investigate the relationship between electrode surface structures and electron transfer reactions of CYPs, we prepared gold electrodes with several surface structures and immobilized purified CYP3A4 isoform (the most important isoform which catalyzes more than half of current drugs) via a monolayer film of naphthalene thiolate (Np-S) to compare their electrochemical responses. We report herein our findings that 1) a gold sputtered electrode, which can be easily prepared by usual sputtering techniques, was as useful as an ORC-treated electrode for direct electron transfers of human CYPs; and 2) nanostructures created by gold particles, where each particle contacts other particles, were crucial for the rapid electron transfer reaction.

2. Experimental

2.1. Materials

Human CYP isoforms were obtained from Invitrogen (the purity was more than 85% and 10 amino acids at N-terminal were deleted for bacterial expression). Testosterone (TST) and erythromycin (from Wako) were used as substrate drugs. All other reagents used in this study were of analytical grade.

2.2. Preparation of the nanostructured electrode surface

Gold sputtered electrodes were prepared by sputtering gold onto a gold disk electrode (ø = 3 mm) using a JEOL sputter system at ambient temperature and 0.7 Torr under Ar atmosphere. ORC-treated gold electrodes were prepared according to a method previously

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reported [10]. Potentials of 0.28 V vs. Ag|AgCl|sat. KCl and 1.22 V were applied for 30 s in a 0.1 M HCl solution and the cycle was repeated 20 times. Glassy carbon electrode (GC) with sparsely deposited gold nanoparticles was prepared according to the reported method [11]. A GC electrode (ø = 3 mm) was immersed into 0.5 M H_2SO_4 containing 1 mM Na[$AuCl_4$] and 0.1 M iodide ions. A potential step from 1.1 to 0 V was utilized to perform the electrodeposition of the Au nanoparticles for 60 s.

2.3. Preparation of the CYP immobilized electrode

Surfaces of gold electrodes were modified with Np-S by overnight dip treatments in ethanol solutions containing ca. 0.1 mM Np-S. After modification, a 2 μL solution of CYP (20 μM) was placed on the electrode surface for 30 min at 25 °C. The electrode was washed with a 0.1 M phosphate buffer containing 20% glycerol. The modification was confirmed by FTIR reflection adsorption spectroscopy with clear amide I and II bands at 1672 and 1543 cm^{-1}, respectively.

2.4. Electrochemical measurements

Voltammetry was performed with an electrochemical analyzer (CH Instruments Inc., USA) with a normal three-electrode configuration consisting of an Ag|AgCl|sat.KCl reference electrode, a Pt auxiliary electrode, and a CYP immobilized working electrode.

3. Results and discussion

3.1. Rapid electron transfer of human CYP on nanostructured surfaces

Fig. 1a shows cyclic voltammograms of the ORC-treated gold electrode where CYP3A4 was immobilized on a monolayer film of Np-S. A pair of peaks was clearly observed at a formal potential ($E^{0\prime}$) of −0.175 V vs. SHE in a buffer solution (pH 7.4), whereas the CYP-unmodified Np-S monolayer on gold showed no voltammetric peaks (not shown). Peaks in the voltammogram of the CYP3A4 modified electrode were attributed to the oxidation and reduction of the heme iron ($Fe^{III/II}$). The obtained $E^{0\prime}$ value was close to the reported value (−0.220 V) of lipid bound CYP3A4 measured by the titration method [12]. Interestingly, no faradaic currents were observed in the voltammogram (Fig. 1b) when CYPs were immobilized onto a Np-S monolayer film on the mirror-like gold surface (without ORC treatment). Since the surface area of the ORC-treated surface estimated by calculating the charge consumed during the formation of the surface oxide monolayer in H_2SO_4 solution [11] was only 2.4 times larger than the non-treated smoother surface, the difference in the electrode surface area alone cannot explain the above results. Fig. 2a and b shows the surface morphologies of the gold electrode surfaces with and without ORC treatment. The gold surface without ORC treatment was relatively plain with a mirror-like surface, whereas the aggregation of nanoparticles of 5–20 nm was observed on some parts of the ORC-treated roughened surface. The result is consistent with recent trends that have shown direct electron transfers of several redox enzymes using nanomaterials such as nanotubes and nanoparticles [13–19].

Metal sputtered films are known to have nanostructured surfaces of densely-packed grains (Fig. 3a), which are similar to the aforementioned ORC-treated surfaces. However, there have been no clear reports on the usefulness of metal sputtered surfaces for direct electron transfer reactions of redox enzymes. Therefore, we examined whether the sputtered electrode would facilitate the direct electron transfers of CYPs using two types of gold sputtered electrode surfaces. One type was the as-sputtered surface, and its AFM image showed the presence of 20 nm grains; each grain contacted to another (Fig. 3a). The other surface was prepared by sputtering, followed by heat treatment (300 °C for 1 h) to smooth the as-sputtered surface [20], and a much smoother surface was obtained as shown in Fig. 3b. After Np-S-coating and CYP3A4 immobilization of these two surfaces, the electrochemical measurements were performed. Well-defined peak currents of CYP were observed in the voltammogram from the nanostructured (as-sputtered) surface, but no obvious faradaic currents were detected from the CYP immobilized onto the smoother surface. These results are consistent with those obtained in the ORC-treated electrode experiments. The voltammetric responses of the CYPs on as-sputtered surface were as strong as on the ORC-treated surface and quite similar at various potential sweep rates, suggesting that the interfacial electron transfer kinetics were similar. The sputtering method can easily provide a reproducible nanostructured surface, and sputtered films are also commercially available. Therefore, gold sputtered surfaces will be useful electrodes for the observation of direct electron transfer reactions of CYPs.

To validate the utility of the sputtered film electrode, we characterized the electrochemical properties of CYP3A4 immobilized on a gold sputtered surface via naphthalene thiolate. Since a larger reduction current than oxidation one was observed at a slower scan rate (<ca. 1 V s^{-1}), which corresponded to the catalytic reduction of dioxygen by CYP on the electrode surface [7,9], we used relatively high scan rates (5 to 100 V s^{-1}) to obtain non-turnover voltammograms. Both cathodic and anodic peak currents increased linearly with scan rate, indicating the electrode reaction of surface bound species. The apparent rate constant for the heterogeneous electron transfer

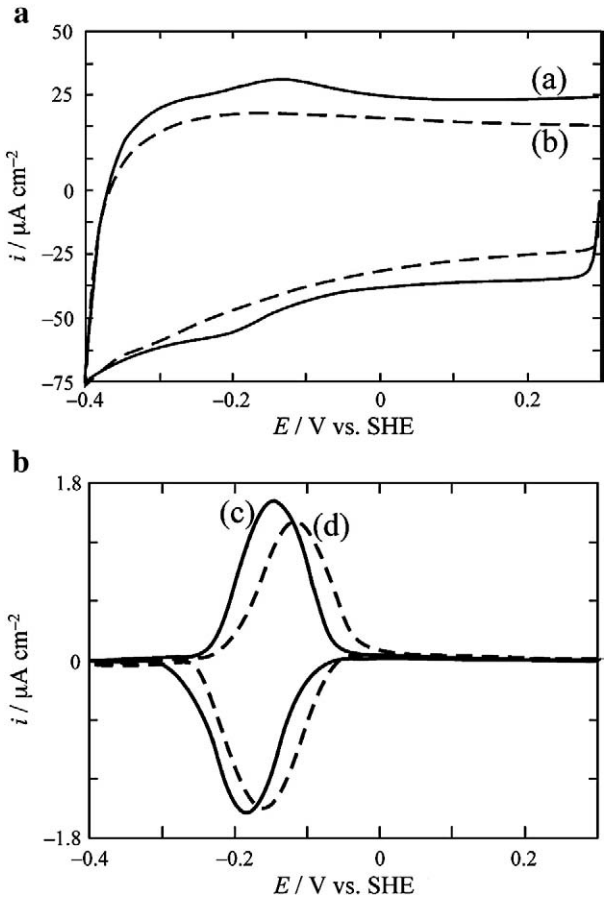

Fig. 1. (a) Cyclic voltammograms of CYP3A4 immobilized electrodes in a 0.1 M phosphate buffer (pH 7.4) at a scan rate of 20 V s^{-1}. CYP3A4 was immobilized onto Np-S monolayer films constructed on gold disk electrodes with (a; solid line) and without (b; dashed line) ORC treatment. (b) Baseline corrected cyclic voltammograms of CYP3A4 immobilized electrodes in a 0.1 M phosphate buffer (pH 7.4) at a scan rate of 5 V s^{-1} in the absence (c; solid line) and presence (d; dashed line) of 200 μM testosterone. CYP3A4 was immobilized onto Np-S monolayer films constructed on gold as-sputtered electrodes.