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Cytochrome P450 Modified Polycrystalline Indium Tin Oxide Film as a Drug Metabolizing Electrochemical Biosensor with a Simple Configuration

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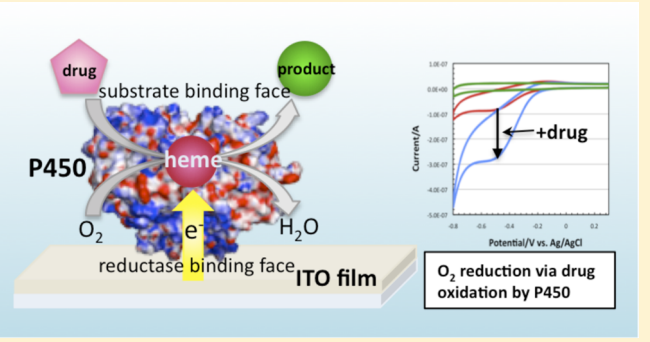
Supporting Information

ABSTRACT: The development of a biocatalytic electrode consisting of cytochrome P450 (CYP) proteins would be a key technology with which to establish simple drug metabolizing biosensors or screening devices for drug inhibitors. We have successfully detected the direct electron transfer (DET) from a human CYP layer or a CYP microsome adsorbed on a bare indium tin oxide (ITO) film electrode without any modification layers and applied it to drug metabolism evaluation. We compared the electrocatalytic properties of the two ITO films with different surface nanostructures (polycrystalline or amorphous). CYP on polycrystalline ITO film enhanced the electron transfer rate of oxygen reduction about fifteen times more than with amorphous film. The polycrystalline ITO film was a suitable electrode for the adsorption of CYP proteins while maintaining efficient DET and enzymatic activity, probably because of its larger surface area and negatively charged surface. The oxygen reduction current at the polycrystalline ITO film electrodes had increased 3- to 4-fold, specifically coupled with the oxidation of drugs (testosterone and quinidine) by the monooxygenase activity of CYP. In contrast, the oxygen reduction current completely disappeared in the presence of the CYP inhibitor (ketoconazole). Similar results could be obtained from the CYP microsome with sufficiently clear responses. These results indicate that the CYP modified polycrystalline ITO electrode offers the potential for electrochemically evaluating CYP activity for drug metabolism with a simple configuration.



Cytochromes P450 (CYPs) are members of a family of heme proteins, which are responsible for the metabolism of almost all drugs and chemicals, and 15 species of CYP have been found in human liver. Among the human CYP members, CYP3A4/5 is involved in the oxidation of almost half of the drugs that CYP members can metabolize.[1] In vivo, CYPs exist in the endoplasmic reticulum membrane of the hepatocyte and catalyze drugs via the NAD(P)H-dependent electron transfer pathway of cytochrome b_5 and P450 oxidoreductase. For several decades, many attempts had been made to develop drug metabolizing biosensors utilizing the superior electron transfer system between CYP and electron supply molecules combined with photometric detection.[2] However, these conventional drug metabolism assay methods had certain disadvantages arising from their complexity and high cost.

Recently, third generation CYP biosensors, which are NAD(P)H or mediator-free and designed to measure the direct electron transfer (DET) between CYP and an electrode, have been the main target, because of their simple and inexpensive sensing systems. Since a method for immobilizing CYP on an electrode surface has been one of the key technologies, various electrode materials and their morphologies have been introduced, including nanomaterials (nanocarbon, gold nanoparticles, silica, etc.) and various organic monolayers and polymers. Biochemical techniques such as genetically modified CYPs have also been developed.[3−5]

Gilardi's group performed a successful DET measurement of human CYP2E1 on a modified glassy carbon (GC) or gold electrode.[3] Using gene engineering technology, they constructed a chimera molecule of CYP and CYP-reductase to promote its electron transfer[4] and they introduced the point mutations into the CYP sequence for the efficiently oriented immobilization of CYP molecules onto the electrode.[5] Other groups embedded CYP molecules in the polycation film[6] or the membrane (Nanodisc)[7] to construct suitable soft interfaces on the electrode. Mie et al.[8] reported the DET measurement of CYP3A4 microsome on a naphthalene thiolate modified gold electrode, where the DET was promoted by the addition of its specific drug and diminished by its inhibitor. Recently, an electrode modified with complicated composite films consisting of graphene and gold nanoparticles[9] or copolymers and acetylene black[10] was used for CYP sensor. In almost all the

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Table 1. Comparison of Characteristics of Polycrystalline ITO and Amorphous ITO Film

	sheet resistance (Ω/sq)	thickness (nm)	rms roughness (nm)	C^0 (μF/cm^2)	atomic content of Sn (%)	contact angle (θ/2) (°)
polycrystalline (PC) ITO (n = 3−5)	18.1 ± 0.5	92.0 ± 10.4	3.7 ± 0.8	11.4 ± 0.3	2.9 ± 0.1	60.9 ± 2.0
amorphous (AM) ITO (n = 3−5)	168 ± 25	31.9 ± 1.7	1.6 ± 0.3	7.3 ± 0.4	1.4 ± 0.2	60.4 ± 2.9

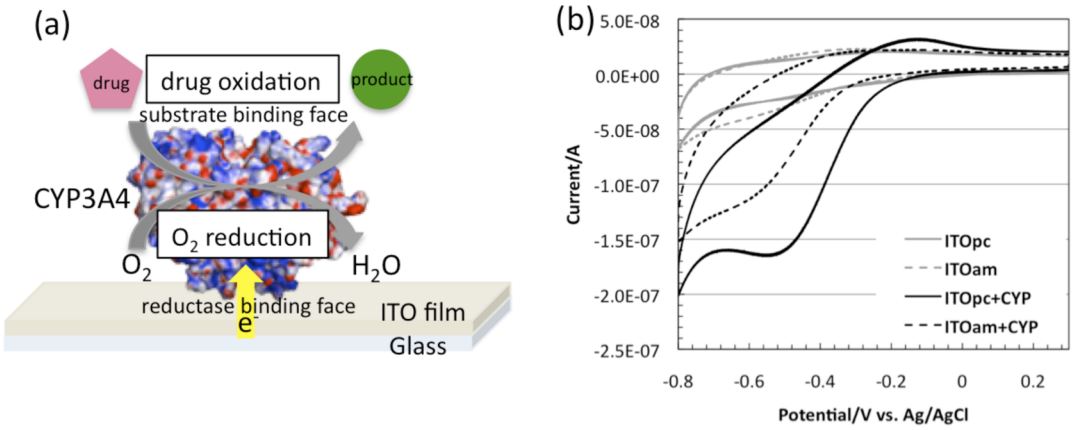

Figure 1. (a) Schematic representation of CYP450 redox reactions on an ITO film electrode. The CYP3A4 molecule (PDB ID: 1tqn) is colored according to surface electrostatic potential (red: negative; blue: positive). (b) DET via O_2 reduction by purified human CYP3A4 adsorbed on the bare ITO film electrode (pc: polycrystalline; am: amorphous). The measurements were performed in the 50 mM Tris-HCl buffer (pH 7.4) with a scanning rate of 0.02 V s^{-1}.

above studies, modified layers were used to improve the DET by controlling CYP orientation or forming the electron transfer path.

An alternative potential electrode material for cytochrome proteins is indium tin oxide (ITO) film. ITO is an n-type degenerate state semiconductor material with high electrical conductivity and high transparency, and it has been widely applied to display devices. According to the sputtering conditions, the internal nanostructures of ITO films can be varied (polycrystalline or amorphous). Yeh and Kuwana[11] first reported the DET of cytochrome c (cyt c) at an ITO film electrode as long as 35 years ago. Since then, many electrochemical studies[12−15] have reported on the DET of cyt c at a bare ITO electrode to understand its electron transfer mechanism. The surface of the ITO film is hydrophilic and negatively charged in a neutral condition, thus making it good for adsorption of cyt c, whose pI is around 10 and positively charged in a neutral buffer. With CYP modified ITO based electrodes, a nanocomposite modified GC electrode consisting of ITO nanoparticles, chitosan, and CYP microsome was recently developed and electrochemically driven drug metabolism was demonstrated.[16] However, the electrode configuration was very complicated, and there were no detailed studies about the effects of ITO structures on the DET properties of CYP.

Here, we studied the effects of a bare ITO film electrode surface structure on human CYP3A4 by using polycrystalline ITO and amorphous ITO film. We succeeded in detecting the DET from both pure and microsome CYP3A4 layers adsorbed on the bare polycrystalline ITO film electrode without any other modification and applied it to drug metabolism and inhibitor evaluation.

RESULTS AND DISCUSSION

We used two types of ITO films (polycrystalline and amorphous) and compared their electrochemical properties as a biocatalytic electrode. The basic characteristics of the ITO films are shown in Table 1. The polycrystalline ITO had lower resistivity (sheet resistance), and its apparent capacitance (C^0) was about 1.5 times larger than that of amorphous ITO despite both surfaces having similar contact angles. The rms roughness of polycrystalline ITO is about 2.3 times larger than that of amorphous ITO, indicating its larger surface area for electron transfer (ET). The cyclic voltammograms (CV) of 1 mM $Fe(CN)_6^{3-/4-}$ at the polycrystalline ITO electrode showed sharper redox peaks (data not shown). The ΔE_p values (0.1 V s^{-1}) were 72 and 524 mV, and the I_{pa} values were 7 and 2.8 μA for the polycrystalline and amorphous ITO film electrodes, respectively. When we take account of the sheet resistance and I_{pa} values, the much larger ΔE_p values at the amorphous ITO are not a result of the larger electrode resistance but of the lower apparent ET, which might be caused by the smaller surface area or chemical structure of ITO film. In fact, the atomic content of the Sn of the polycrystalline ITO was larger, which might indicate that its carrier density was larger than that of amorphous ITO.[17]

To develop a simple and high-throughput CYP drug metabolizing biosensor, many surface modification and enzyme immobilization techniques have been employed for stabilizing the CYP structure and its activity, because the detection of the electrocatalytic activity of CYP was very difficult with unmodified electrodes (e.g., GC or bare gold).[18] This is probably because a CYP molecule (ca. 7.8 × 10 × 13 nm; the size of the unit cell of a crystal) is larger than that of commonly studied redox enzymes such as cyt c (ca. 4.2 × 5.8 × 5.8 nm). In this paper, we describe our successful detection of DET from purified human CYP3A4 (without P450 oxidoreductase or cytochrome b_5) adsorbed onto a bare ITO film electrode as shown in Figure 1a. Figure 1b shows CV curves of DET resulting from O_2 reduction by CYP3A4 biocatalytic activity. A clear O_2 reduction peak appeared at −0.52 V (vs Ag/AgCl, for polycrystalline ITO, bold line). It is noteworthy that we have

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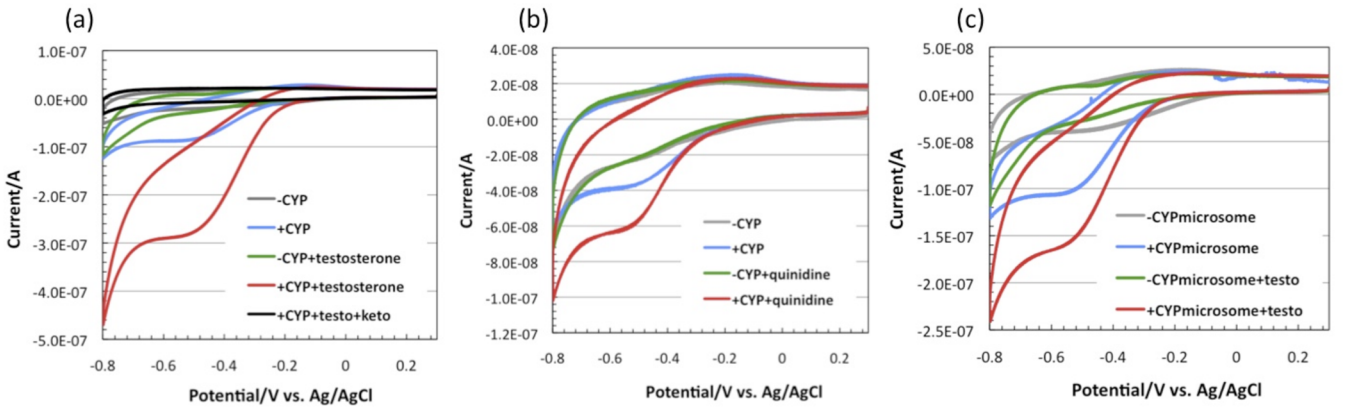

Figure 2. Electrochemical detection of DET via drug metabolism with purified human CYP3A4 (a, b) or CYP microsome (c) adsorbed on a bare polycrystalline ITO film electrode. Drugs (a, c: 100 μM testosterone; b: 100 μM quinidine) and the CYP3A4 inhibitor (500 μM ketoconazole) were added. The measurements were performed in 50 mM Tris-HCl buffer (pH 7.4) with a scanning rate of 0.02 V s^{-1}.

shown the first biocatalytic DET from CYP, only physically adsorbed on a bare electrode without any surface modification. On the basis of a recent review article,[18] the redox property of similar CYP on a bare electrode was reported by Fleming et al.[19] who also reported the electrochemical reaction of other CYP species of a bacterial source adsorbed directly onto a pyrolytic graphite electrode but showed no enzymatic reactions. An ITO film surface is hydrophilic and negatively charged in a neutral condition. CYP3A4 is known to have a patch of positively charged residues on the reductase binding face (proximal), opposite to the substrate binding face (distal)[20] (see scheme in Figure 1a). We hypothesized that these structural features helped to achieve efficient ET between the ITO electrode surface and CYP. The heme iron cofactor is buried deeply in the CYP molecule, and the distance from the electrode surface is estimated to be much larger than that of cyt c (about 8 Å).[21] Therefore, the orientation of CYP on an electrode must be very important.

An O_2 reduction peak also appeared with the amorphous ITO electrode at −0.59 V (Figure 1b, dotted line). From the difference between peak potentials (ΔE_p = 0.07 V), the ratio of the ET rates (k_{PC}/k_{AM}) was estimated to be about 15. The increased reduction currents were 140 and 80 nA for polycrystalline and amorphous ITO, respectively. The possible factors explaining the superior property of polycrystalline ITO over amorphous ITO were (1) the larger surface area caused by the rougher surface, which was assumed to be covered with more CYP molecules, and (2) the enzyme-sized nanostructured surface, which could have a shorter distance between the heme cofactor and the electrode. The hydrophilicities of both films were almost the same (see Table 1; the contact angles were about 60°). Other surface properties, such as the difference in surface electrical charge (not investigated), might be effective as regards the molecular adsorption and orientation of CYP molecules. Kato et al.[22] reported that amorphous ITO film is more negatively charged. Therefore, the difference between the surface roughnesses of polycrystalline and amorphous ITO film is a dominant factor with respect to the difference in the ET rates.

The DET from O_2 reduction caused by CYP3A4 adsorbed onto an unmodified GC electrode also increased (ΔI_{GC} = 70 nA at −0.44 V, CV data not shown), but the increased current was much smaller than that for polycrystalline ITO (ΔI_{PC} = 140 nA at −0.52 V). The capacitance of GC was much larger than that of ITO, which resulted in a large background current. Therefore, the reduction current increase was small compared with that of the background current. Other conventional electrodes including Au also exhibited a larger background current than an ITO film electrode. Indeed, a CYP-modified Au electrode exhibited a larger background current around the potential at which O_2 reduction did not occur.[8] These results clearly indicate the superiority of polycrystalline ITO film as a CYP biosensor electrode.

Next, we investigated electrochemically driven drug oxidation by purified CYP3A4 or CYP microsome on a polycrystalline ITO film electrode. Figure 2a shows a CV curve of O_2 reduction coupled with the oxidation of 100 μM testosterone by CYP3A4 (red line). The O_2 reduction current (at −0.52 V) had increased 3.7-fold by adding the drug. Without CYP3A4, no O_2 reduction current was observed (green line). This showed that testosterone was oxidized specifically by CYP3A4 enzymatic activity, not electrochemically at scanning potentials between 0.3 and −0.8 V (vs Ag/AgCl). Ketoconazole, a CYP3A4 inhibitor, which binds directly to the heme iron in the active center,[23] had completely blocked the O_2 reduction (bold black line), which also indicated a relationship with the drug oxidation by CYP3A4 enzymatic activity. Less than 2% volume of methanol in the assay solution had no effect on the CYP and CYP microsome catalytic activity.[8] We have also confirmed that the O_2 contained in the added drug solution (in methanol) had no effect by measuring with stock solutions without drugs. These results suggested that the CYP3A4 molecules retained their native structures on the bare polycrystalline ITO, which was not the case in previous studies.[18] Furthermore, the O_2 reduction current increase showed a clear dependence on the testosterone concentration (shown in the Supporting Information, Figure S1). CYP3A4 was able to oxidize another drug, namely, 100 μM of quinidine, and the O_2 reduction current also increased (Figure 2b). The O_2 reduction peak current (−60 nA at −0.52 V) was smaller than that for testosterone (−280 nA), which might reflect the difference in the specific activity of CYP3A4 for these drugs. Indeed, Agrawal et al. showed that the K_m values of CYP3A4 in a reconstituted mixture consisting of CYP3A4, CYP oxidoreductase, cyt b_5, lipid mix, and NADPH regeneration mix were 112 ± 5.78 and 327 ± 24.8 μM for testosterone and quinidine, respectively.[24]

When we consider the practical use of the CYP drug metabolizing sensor, liver microsomes constitute possible CYP

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specimens. Therefore, we also tried to detect DET from CYP3A4 microsome physically adsorbed onto a bare polycrystalline ITO (Figure 2c). The O_2 reduction current certainly increased with testosterone. The response obtained from CYP microsome was 1/2 with only a slight peak potential difference compared with that from the purified CYP despite the fact that the concentration of the applied CYP (1 μM in microsome) on the electrode surface was 1/20 smaller than that of the purified CYP. It could be explained that this relatively large current from CYP microsome might be caused by the CYP of more stable activities in its more native conditions. Moreover, the difference of surface concentrations between pure and microsome CYP are not as large as those in the applied solutions because CYP is preconcentrated on the ITO surface. Xu et al.[16] reported that the electrocatalytic response for 100 μM tolbutamide was about 15 nA on the other CYP microsome composite electrode (CYP2C9 microsome/ITO nanoparticles/chitosan/GC). Our ITO film electrode with a simple configuration (only microsome adsorbed onto ITO) was shown to perform the electrocatalytic DET more efficiently, because we assumed that the amount of immobilized CYP on our ITO film electrode was much smaller than that of the CYP microsome composite electrode. Moreover, many previous reports about the DET of CYP microsomes had employed modified electrodes with self-assembled monolayers or composites of various nanomaterials, demonstrating that hydrophobic surfaces were suitable for immobilizing CYP microsomes. In contrast, our ITO film was relatively hydrophilic and flat. These results indicated that polycrystalline ITO film had some superior properties (e.g., adequate interaction between ITO and CYP caused by the surface structure having similar dimensions with the enzymes or the surface charge) as a biocompatible material for a biocatalytic electrode rather than other electrode materials, irrespective of CYP situations (CYP alone or CYP microsome). This is highly advantageous in terms of constructing simple electrochemical biosensors for drug metabolites. For this, we must further evaluate the polycrystalline ITO electrode for its ability to detect signals from various types of CYPs and the related drugs/inhibitors.

CONCLUSION

In this study, we described the electrochemical detection of the drug metabolizing activities of CYP3A4 protein and CYP3A4 microsome at an ITO film electrode without any surface modification. We also revealed the superior properties of polycrystalline ITO film as a biocatalytic electrode with more efficient electron transfer activity than amorphous ITO film and other conventional electrodes. These results are technically informative with respect to the development of CYP drug metabolizing biosensors with a simple configuration and a high throughput.

ASSOCIATED CONTENT

Supporting Information

Experimental description and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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