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Improved electrocatalytic metabolite production and drug biosensing by human liver microsomes immobilized on amine-functionalized magnetic nanoparticles

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ABSTRACT

A biocatalytic system constructed by electrostatically immobilizing drug metabolizing human liver microsomes (HLM, negatively charged due to phospholipids) onto positively charged amine-functionalized magnetic nanoparticles (MNP_{amine}, 100 nm hydrodynamic diameter) for biosensing of cytochrome P450 (CYP)-specific drug candidates and electrocatalyzing drug conversion into metabolites is reported. The MNP-adsorbed HLM biomaterial (denoted as MNP_{amine}/HLM) was magnetically separated from the bulk suspension of HLM, washed twice in phosphate buffer (pH 7.0), and adsorbed onto polished edge-plane pyrolytic graphite (EPG) electrodes. Direct electrochemical properties and oxygen reduction currents were obtained by cyclic voltammetry (CV). Direct electron transfer kinetics from the microsomal redox proteins present in the adsorbed film of MNP_{amine}/HLM on EPG surface was accomplished using square wave voltammetry due to its smaller background charging currents and improved faradaic peak intensity than CV. The MNP_{amine}/HLM bioelectrodes exhibited a formal potential of −0.46 V vs Ag/AgCl reference (1 M KCl), which was in agreement with the FAD/FMN cofactor containing CYP-reductase (CPR) as the electron receiver from the electrode. This conclusion was drawn based on the formal potentials of only CPR (~-0.47 V vs Ag/AgCl) containing bactosomal films adsorbed on EPG electrodes (bactosomes are bacterial membrane expressed enzymes). Whereas, bactosomal CYP 2C9 film showed a much more positive formal potential (−0.34 V vs Ag/AgCl). A heterogeneous electron transfer rate constant of $19 \pm 5\ s^{-1}$ was exhibited by the MNP_{amine}/HLM film. Oxygen reduction currents and electrocatalytic diclofenac hydroxylation characteristics of the CYP enzymes present in the HLM film indicated the electron mediation by the CYP-reductase from the electrode to CYP-heme centers to initiate the catalytic cycle. Amperometric i-t curves with diclofenac drug concentration yielded the apparent Michaelis-Menten affinity constants (K_M^{app}) of MNP_{amine}/HLM and only HLM films as 302 ± 33 and $365 \pm 36\ \mu M$, respectively. This is the first electrocatalysis report of bioelectrodes featuring HLM-bound MNP_{amine} for applications in drug activity assays, enhanced metabolite production, and sensitive biosensing of CYP-specific drugs and environmental pollutants. Eliminations of tedious isolation and purification of membrane-containing CYPs, and the need for expensive NADPH cofactor (as electrode donates the reducing equivalents in an inexpensive and sustainable manner) are added advantages. Our future work aims to gain quantitative metabolite estimations upon scalability of such bioelectrode designs useful for pharmacological and toxicological evaluations of new drugs in development.

1. Introduction

Metallic and semiconductor nanoparticles are attractive materials for designing efficient electrocatalytic and electroanalytical systems [1,2]. In particular, magnetic nanoparticles (MNPs) have received significant attention in enzyme electrocatalysis and biosensors [3–10]. This is due to the unique properties of MNPs such as high surface area, ease of functionalization with desired surface chemical groups, intrinsic peroxidase-like activity [11], and ease of isolating MNP-bound proteins by simple application of

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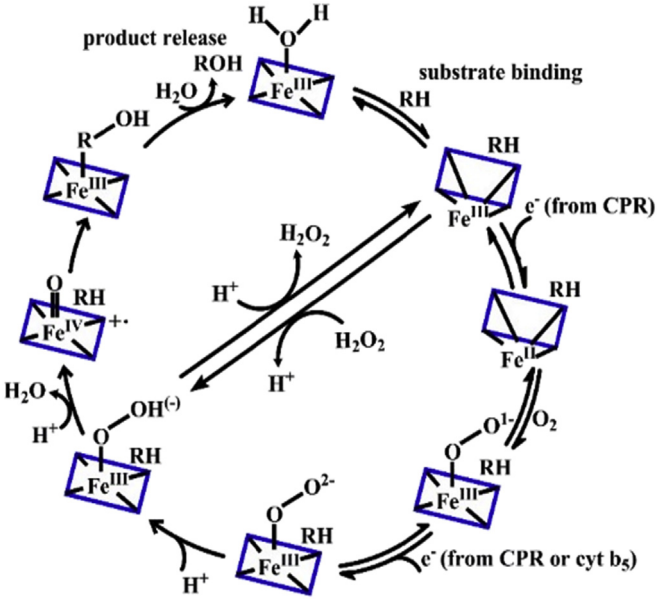
magnetic field using a tiny magnet [12,13].

Most of the reported studies were based on immobilizing purified redox proteins with MNPs to carry out electrocatalysis and develop electrochemical biosensors. In contrast, the understanding of the influence of MNPs on the membrane-containing cytochrome P450 (CYP) electrochemistry present in liver microsomes is unexplored. In this study, we demonstrate that human liver microsomes (HLM) immobilized electrostatically onto amine-functionalized MNPs (MNP_{amine}) produce enhanced metabolites and display highly sensitive drug biosensing (typical of the microsomal CYP enzymes) than those in the absence of MNP_{amine} attachment. The findings suggest that similar approach is broadly applicable for bioactive immobilization of any membrane-bound CYP isoforms.

HLM are subcellular fractions extracted from homogenized liver. HLM are a convenient *in vitro* system to study CYP-driven drug metabolism and drug-drug interactions. CYP enzymes play a major role in xenobiotic metabolism and are responsible for nearly 75% of phase-I oxidative metabolism of drugs occurring in liver [14–16]. The *in vivo* catalytic pathway of CYP involves electron transfer from NADPH to CYP-reductase (CPR) and then to the CYP heme centers to initiate CYP-catalyzed drug monooxygenation in the presence of molecular oxygen (Scheme 1) [17].

Successful attempts have been made for electrochemically driving CYP catalysis either using purified isoforms of CYPs [20–23], CYP-fused with a CPR domain [24], and by microsomal films immobilized on electrodes [25–29]. Recently, our group demonstrated that surface roughness and hydrophilic nature of graphite electrodes (e.g., EPG and high purity graphite electrodes) offered higher electrocatalytic currents and biofilm stability for a directly adsorbed film of HLM [30]. In addition, we recently demonstrated the crucial role of CYP-reductase in CYP bactosomal films (bacterial expressed CYP 2C9 or 3A4 with its reductase was used), adsorbed onto gold-cysteamine monolayer modified electrodes, in offering several fold greater turnover rates than in the absence of reductase [31]. We have also shown that HLM adsorbed onto multiwalled carbon nanotubes (MWNT) modified EPG electrodes can provide several fold enhanced electrocatalytic drug metabolite product yields with better biofilm stability and reusability features than the unmodified electrodes [32].



Scheme 1. Catalytic cycle of human CYP enzymes [18,19].

In this article, our objective is to expand our understanding on the favorable roles of magnetic nanomaterials on the voltage-driven biocatalysis of membrane-containing CYP liver enzymes. Such understanding is important in establishing novel nano-bioanalytical approaches for low-cost, volume-efficient stereo-selective biocatalytic and biosensing applications useful in drug development and environmental pollutant screening.

2. Experimental

2.1. Materials

Human liver microsomes (HLM, total protein: 20 mg mL^{-1}, total amount of CYP enzymes: 0.579 nmol mg^{-1} protein, NADPH-cytochrome c reductase activity: 171 nmol mg^{-1} protein min^{-1}, cytochrome b_5: 0.439 nmol mg^{-1} protein), either CYP 2C9 or CPR expressed bactosomes (bacterial membrane expression systems), and control bactosomes with no expressed CYP or CPR (CB) were purchased from XenoTech (Lenexa, KS, USA). The CYP 2C9 concentration in the purchased CYP bactosomes was 2.8 nmol mL^{-1} with a total protein concentration of 13 mg mL^{-1} bactosomes. The CPR concentration in the only CPR containing bactosomes was 9.7 nmol mL^{-1}. Amine-functionalized magnetic nanoparticles (MNP_{amine}, aminosilane matrix with a magnetite core, 100 nm hydrodynamic diameter, 1 g contains ~ 1.8×10^{15} particles) were purchased from Chemicell GmbH Inc. (Eresburgstrasse, Berlin, Germany). EPG electrode discs (geometric area: 0.2 cm^2) were prepared from pyrolytic graphite blocks (1" × 1" × 0.5", Momentive Performance Ltd., Strongsville, OH). Diclofenac was purchased from Sigma-Aldrich. All other chemicals were analytical grade.

2.2. Instrumentation

The standard 3-electrode electrochemical cell consists of an Ag/AgCl reference electrode (1 M KCl, CH instruments Inc., Austin, TX, USA), a Pt-wire counter electrode, and MNP_{amine}/HLM (or HLM or only MNP_{amine} or only CYP 2C9 or CPR bactosomes) adsorbed EPG working electrode was used. A CH instrument (Model: CHI 1040) was used to perform cyclic voltammetry and square wave voltammetry experiments in argon saturated phosphate buffer, pH 7.0. Rotating disc catalytic oxygen reduction experiments were performed at 300 rpm electrode rotation rate in saturated oxygen buffer using a rotator connected to a rotation controller unit (Eco-Chemie Autolab, Metrohm Inc., Riverview, FL, USA). Amperometric i-t curves for drug biosensing was monitored at an applied constant potential of −0.6 V vs Ag/AgCl, constant air, 25 °C, pH 7.0, phosphate buffer.

The hydrodynamic size and surface charges of MNP_{amine} and MNP_{amine}/HLM were characterized by ZetaPALS potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA). A Varian Cary 100 Bio UV–vis spectrophotometer was used to confirm the MNP_{amine} surface immobilization with HLM based on the notable difference in the HLM solution absorbance before and after adsorbing onto the MNP_{amine} particles. Metabolite formed from the electrocatalysis of diclofenac was identified using high performance liquid chromatography-mass spectrometry technique (LC-MS, premier column C_{18}, length 10 cm, Shimadzu LCMS 2010EV).

2.3. Construction of MNP_{amine}/HLM modified EPG electrode

Ten microliters of HLM was mixed with 6 μL of MNP_{amine} (25 mg mL^{-1}), and kept at 4 °C for 30 min to allow the negatively charged HLM electrostatically adsorb onto the positively charged amine functionalized MNP. The MNP_{amine}-bound HLM was magnetically separated, and resuspended in 10 μL of phosphate