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# Thin film voltammetry of metabolic enzymes in rat liver microsomes

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**Abstract**

We report herein thin film voltammetry and kinetics of electron transfer for redox proteins in rat liver microsomes for the first time. Films were made layer-by-layer from liver microsomes and polycations on pyrolytic graphite electrodes. Cyclic voltammograms were chemically reversible with a midpoint potential of −0.48 V vs SCE at 0.1 V $s^{-1}$ in pH 7.0 phosphate buffer. Reduction peak potentials shifted negative at higher scan rates, and oxidation–reduction peak current ratios were ~1 consistent with non-ideal quasireversible thin film voltammetry. Analysis of oxidation–reduction peak separations gave an average apparent surface electron transfer rate constant of 30 $s^{-1}$. Absence of significant electrocatalytic reduction of $O_2$ or $H_2O_2$ and lack of shift in midpoint potential when CO is added that indicates lack of an iron heme cofactor suggest that peaks can be attributed to oxidoreductases present in the microsomes rather than cytochrome P450 enzymes.


*Keywords:* Liver microsomes; Thin film voltammetry; Kinetics; Cytochrome P450s; Oxidoreductases

## 1. Introduction

Liver microsomes play an extensive role in drug discovery as a source of enzymes for *in vitro* metabolism and inhibition studies. Cytochrome P450 (CYP) enzymes are the major ''Phase I'' enzymes in liver microsomes that metabolize lipophilic drugs and pollutant molecules [1–3]. Most CYP enzyme reactions involve oxidation, although other reactions are known [4]. For new drug approval, details about which of the many liver CYPs are involved in metabolism is a requirement in some countries [4]. In the metabolic pathway, the iron heme of CYP accepts an electron from NADPH via NADPH-reductase with electrons flowing from NADPH via reductase prosthetic group flavin adenine dinucleotide (FAD) to flavin mononucleotide (FMN) to the CYP, followed by dioxygen binding and a second electron transfer, resulting in an active oxidant CYP form that subsequently oxidizes bound substrates [1,4].

We recently reported direct electrochemistry of so called *Supersomes*, or microsomes genetically enriched in CYP1A2 or CYP3A4 [5]. The voltammetric peaks found were attributed to oxidoreductases such as CYP NADPH-reductase, not CYPs. These oxidoreductases include NADPH-cytochrome P450 reductase, the natural electron donor for all microsomal CYP-catalyzed oxidations [1,3]. Electrochemical reduction of these oxidoreductases in the supersome films was able to convert styrene to styrene oxide in low yield, presumably via electron transfer to oxidoreductases, and then to the CYPs [5].

We have used pure CYP enzymes in genotoxicity screening sensors and arrays in which DNA damage for enzyme-generated metabolites is monitored [6–9]. Human CYP enzymes are not commercially available in pure form, and their isolation and purification is labor intensive. On the other hand, rat liver microsomes are cheap, commercially available sources of CYPs and other metabolic enzymes. In the place of pure enzymes, microsomes have the potential to eliminate the bottleneck of multiple enzyme purification, and broaden toxicity biosensor array applications with respect to drug discovery and development. In this communication, we describe a first successful step

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toward such a goal. We report for the first time construction and characterization of stable thin films on electrodes from rat liver microsomes (RLM) and polyions of opposite charge, and voltammetry and electron transfer kinetics of the oxidoreductases in these films.

## 2. Experimental

### 2.1. Chemicals and materials

Liver microsomes from rat (F344, male) containing cytochrome P450 (CYP) enzymes and oxidoreductases were from BD-Biosciences (Woburn, MA, USA). The activities of enzymes as supplied by the manufacturer are: oxidoreductases 330 nmol $mg^{-1}$ $min^{-1}$, CYP 3A, 2C, 2E1,1A and 4A were 3.3, 5.1, 1.1, 0.18 and 1.2 nmol $mg^{-1}$ $min^{-1}$, respectively against their specific substrates. Total CYP and cyt $b_5$ were given as 590 and 760 pmol/mg respectively. Horse heart myoglobin (Mb, MW 17400), poly (diallyldimethylammonium chloride) (PDDA), poly(sodium 4-styrene sulfonate) (PSS), and hydrogen peroxide were from Sigma. Water was treated with a Hydro nanopure system to a specific resistivity $\geqslant 18$ m$\Omega$ cm. Amicon YM30 membrane filter (30 000 MW cutoff) was used to filter the Mb solution prepared in 10 mM acetate buffer, pH 4.5 [10]. All other chemicals were reagent grade.

### 2.2. Instrumentation

A CH instrument 660A electrochemical analyzer was used for cyclic voltammetry (CV). The cell employed a saturated calomel reference electrode (SCE), Pt-wire counter electrode and film-coated working electrode disk ($A = 0.2$ $cm^2$) of ordinary basal plane pyrolytic graphite (PG, Advanced Ceramics). Ohmic drop was compensated ~95%. Buffer solutions were purged with purified nitrogen for 20 min. before acquiring voltammograms.

To monitor the layer-by-layer assembly of films, we used a quartz crystal microbalance (QCM, USI Japan) with 9 MHz QCM resonators (AT-cut, International Crystal Mfg.). A partly negative monolayer was made on gold-coated ($0.16 \pm 0.01$ $cm^2$) resonators (9 MHz frequency) with 0.5 mM 3-mercaptopropionic acid in ethanol [6]. Layers were adsorbed onto this negatively charged gold resonator surface. The conditions of layer adsorption and duration to form stable PDDA and microsomes assemblies were optimized via a QCM monitoring of film growth. Resonators were dried in a stream of nitrogen before measuring the frequency change ($\Delta F$) for each layer, from which adsorbed mass and nominal thickness were estimated [11].

### 2.3. Film construction

PDDA/microsome films were constructed on PG electrode by using the layer-by-layer alternate electrostatic assembly [11]. PDDA polycations were chosen to have opposite charge to the microsomes. PG electrodes were abraded with 400 grit SiC paper, then ultrasonicated for 30 s in ethanol followed by 30 s in water, and dried in nitrogen. Films on PG of architecture $(PDDA/microsomes)_3$ and $(PSS/Mb)_3$ were used for CV studies.

Layers of PDDA (1 mg $mL^{-1}$ in 0.05 M NaCl) and negatively charged microsomes were adsorbed in alternative steps on rough PG surfaces for 20 and 30 min. respectively at 4 °C. The electrodes were rinsed in water between adsorption steps. Similarly, Mb films as controls were made with three bilayers of PSS (3 mg $mL^{-1}$ in 0.05 M NaCl) and positively charged Mb (p$I$ 6.9, 3 mg $mL^{-1}$ acetate buffer, pH 4.5) [12].

## 3. Results

Film growth was monitored by QCM measurements during the assembly of $(PDDA/microsome)_3$ films on gold resonators, and showed a decrease in frequency ($-\Delta F$) for each sequentially adsorbed layer suggesting regular and reproducible film growth (Fig. 1). The decreases in frequency for microsomes layers were relatively large, because the microsomes themselves are much larger than PDDA molecules. Using the appropriate equations [11], $\Delta F$ values were used to find a nominal film thickness of $10.8 \pm 0.2$ nm. Microsome content was $3.2 \pm 0.1$ μg $cm^{-2}$ and PDDA was $0.5 \pm 0.1$ μg $cm^{-2}$. Thicknesses of individual layers were ~3 nm for microsomes and 0.5 nm for PDDA. In experiments to be reported elsewhere, we have shown that these microsome films are catalytically active for substrate oxidation [13].

Fig. 2a represents cyclic voltammograms (CV) of microsomes in the $(PDDA/microsome)_3$ films with increasing scan rates. A reversible pair of reduction–oxidation peaks with a midpoint potential of −0.48 V vs SCE at 0.1 V $s^{-1}$ in phosphate buffer, pH 7.0 was observed. Peak current vs scan rate was linear up to 1000 mV $s^{-1}$. Reduction peak potentials shifted more negative with increasing scan rate

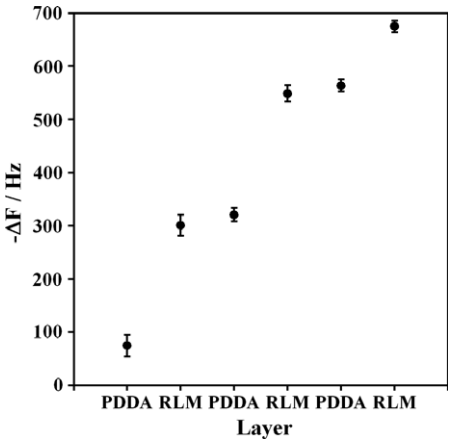


Fig. 1. QCM frequency changes for adsorption steps during $(PDDA/microsome)_3$ film growth on gold resonators coated with a monolayer of mercaptopropionic acid as first layer. (Error bars reflect SD for 3 resonators.)