# Electrochemical biosensor featuring a two enzyme pathway and DNA for screening toxic reactive metabolites of aryl amines

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## Electronic Supplementary Information

## Experimental Details

**Chemicals and Materials.** Cytochrome P450 1A2 (CYP1A2, MW 52,000) was expressed from DH5$\alpha$ *Escherichia coli* containing the proper cDNA and isolated and purified as described previously.[1] Myoglobin (Sigma, MW 17,400, horse heart) was dissolved in 10 mM pH 4.5 sodium acetate buffer and filtered through an Amicon YM30 membrane (MW 30,000 cutoff). Arylamine acetyltransferase from pigeon liver (NAT, EC 2.3.1.5, 10 % protein, 0.9 U/mg protein, pH 8.0, 25 ℃, one unit acetylates 1.0 nmol p-nitroaniline/min, Mw 33,000[2]), acetyl coenzyme A sodium salt (AcCoA), DL-dithiothreitol (DTT), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), Calf thymus DNA (Type XV, 13000 avg. base pairs, 41.9 % G/C), tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate and poly(diallydimethylammonium chloride) (PDDA) were from Sigma-Aldrich. 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) was purchased from Toronto Research Chemicals (Toronto, ON). The ECL metallopolymer, [Ru(bpy)$_2$(PVP)$_{10}$](ClO$_4$) (denoted as RuPVP) (bpy = 2,2'-bipyridyl; PVP = poly(4-vinylpyridine)), was prepared and characterized by previously published methods.[3] Water was purified with a Hydro Nanopure system to specific resistance >16 M$\Omega$. All other chemicals were reagent grade.

**Voltammetry**. Square Wave Voltammetry (SWV) was done using CH Instruments 660A and 430 electrochemical analyzers. The 3-electrode electrochemical cell was protected from light and employed a saturated calomel reference electrode (SCE), Pt wire counter, and a film-coated pyrolytic graphite (PG) disk as the sensor electrode. SWV conditions were 4 mV step height, 25 mV pulse height, and 5 Hz frequency with 95 % ohmic drop compensated.

**Sensor Construction.** DNA-enzyme films were prepared by the layer-by-layer electrostatic assembly method[4] following an optimized protocol. Disks (A= 0.15 cm$^2$) of ordinary basal plane PG (Advanced Ceramics) were abraded with 400 grit SiC paper and sonicated in ethanol and pure water successively for 1 min, then rinsed with water and dried. Layers were formed by placing a μL-sized drop of each adsorbate solution on the PG electrode for 15-20 min. Cyt P450 1A2 and NAT enzymes were adsorbed at 0 $^o$C to maintain enzyme stability, and the electrode was then rinsed with water to remove weakly adsorbed enzyme and dried in a stream of nitrogen between adsorption steps. The times for steady state adsorption,[4] volume and concentration of each adsorbate solution were: (1) 15 min, 30 μL of 2 mg mL$^{-1}$ PDDA in 50 mM NaCl; (2) 15 min, 30 μL of 2 mg mL$^{-1}$ DNA in 5 mM pH 7.1 Tris buffer and 0.50 M NaCl; (3) 20 min, 20 μL of 5 mg mL$^{-1}$ NAT in 50 mM pH 7.2 Tris buffer; and (4) 20 min, 20 μL of 0.6 mg mL$^{-1}$ (11 μM) Cyt P450 1A2 in 50 mM pH 7.4 phosphate buffer; (5) 10-15 min, 30 μL drop of 2.5 mg mL$^{-1}$ RuPVP in 50 % ethanol/water. Final film architecture was denoted as the order of layer adsorption, i.e. (PDDA/DNA)$_2$, PDDA/DNA/(CYP1A2/DNA)$_2$, PDDA/DNA/(NAT/DNA)$_2$, and
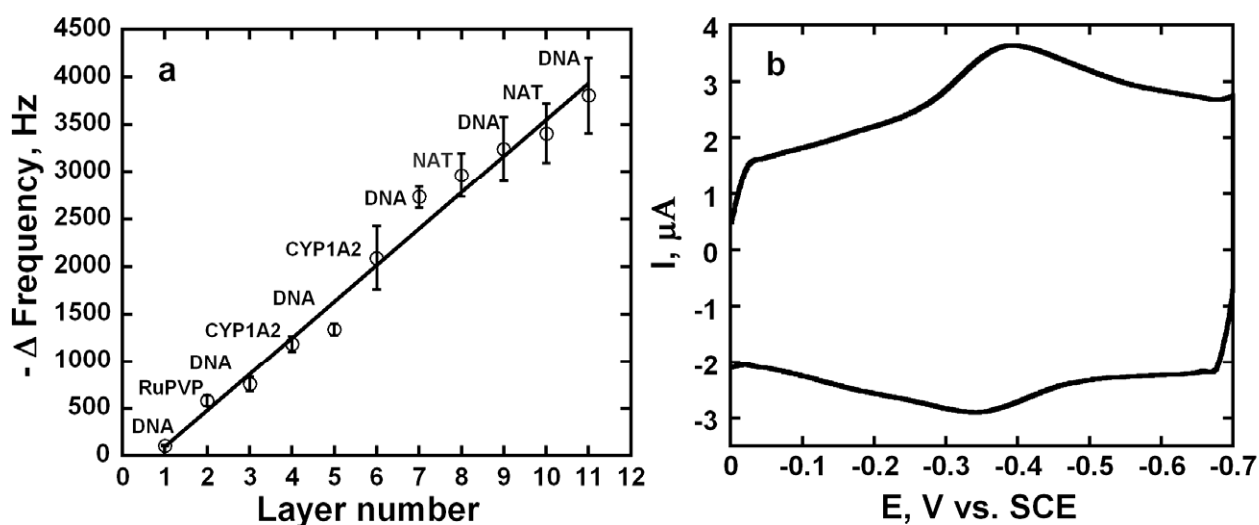
DNA/RuPVP/DNA/(CYP1A2/DNA)$_2$/(NAT/DNA)$_2$. We refer to these as DNA, CYP1A2/DNA, NAT/DNA and CYP1A2/NAT/DNA films, respectively. Quartz crystal microbalance (QCM, USI Japan) was used to monitor film assembly at each step using gold coated QCM resonators (9 MHz, AT-cut, International Crystal Mfg. Co.). The gold-coated resonator were cleaned and derivatized with 0.7 mM 3-mercapto-1-propanol and 0.3 mM 3-mercaptopropionic acid to mimic the negatively charged graphite surface. Films were assembled on resonators as described above and dried in a stream of nitrogen before QCM measurements. The mass per unit area and nominal thickness of dry films were estimated from QCM frequency as previously described. [4,5]

**Enzyme reactions in films. Safety Note***: 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine is suspected human carcinogens. Procedures should be done in a closed hood while wearing gloves, using sealed cells.* Stock solutions of PhIP were prepared in acetonitrile/DMSO (50:50). Stock solutions of 20 mM DTT, EDTA and AcCoA were prepared in water and kept at -20 °C. Incubation of films were done in a thermostatted vessel at 37 °C for the range of 0.5 - 5 min. Incubation of cyt P450 1A2/DNA films was done in 50 μM PhIP and 1 mM hydrogen peroxide in 5 mL of pH 5.8 or 7.5 buffer. Incubation of NAT/DNA films was done in 0.4 mL of pH 7.5 phosphate buffer containing 50 μM PhIP, 1 mM DTT, 1 mM EDTA and 0.5 mM AcCoA in 1.5 mL microcentrifuge tube. After the reaction, the electrode was rinsed with water and transferred to an electrochemical cell containing Ru(bpy)$_3$$^{2+}$ for SWV analysis. Incubation of RuPVP/CYP1A2/NAT films was done in 0.4 mL of pH 7.5 phosphate buffer containing 50 μM PhIP, 1 mM hydrogen peroxide, 1 mM DTT, 1 mM EDTA and 0.5 mM AcCoA in 1.5 mL microcentrifuge tube and then transferred to the cell containing 10 Mm pH 5.5 buffer containing 50 mM NaCl for SWV measurements.

Preliminary results on film sequence was that CYP 1A2/NAT films gave better performance than NAT/CYP 1A2 films. In CYP 1A2/NAT films, PhIP would diffuse into the inner film layers containing cyt P450 1A2, form N-OH-PhIP, then be conjugated with AcCoA to form N-acetoxy-PhIP as they diffuse out past outer NAT layers. Accessibility of the coenzyme AcCoA (relatively high mol wt) in outer NAT layer is apparently better than for inner NAT layers.

## Additional Results
**Sensor Film Characterization.** Desired film composition and reproducible construction was investigated using a quartz crystal microbalance (QCM) to measure film weight after each layer adsorption step. Figure 1 (a) shows the average change in QCM frequency (-ΔF), proportional to mass/area (μg cm$^{-2}$),[4] upon sequential adsorption of individual RuPVP, DNA, CYP1A2, and NAT layers onto a gold-quartz QCM resonator to make DNA/RuPVP/DNA/(CYP1A2/DNA)$_2$/(NAT/DNA)$_2$ (denoted CYP1A2/NAT/DNA) films similar to those used in the sensors. The QCM values of -Δ*F* increased linearly with layer number, indicating regular and reproducible film growth. Table S1 summarizes the surface coverage of each component and nominal film thickness obtained from QCM. Cyclic voltammograms in Figure S1 (b) demonstrated that the CYP1A2 enzyme in the film are electrochemically active on a pyrolytic graphite (PG) electrode and gives reversible Fe$^{III}$/Fe$^{II}$ peaks characteristic of the intact enzyme.[6]

**Figure S1.** Sensor Film Characterization: (a) Quartz Crystal Microbalance frequency shifts for cycles of alternate adsorption on gold quartz resonators during preparation of DNA/RuPVP/DNA/(CYP 1A2/DNA)$_2$/(NAT/DNA)$_2$ films. (b) Cyclic voltammogram of PDDA/DNA/(CYP1A2/DNA)2 films on PG electrode at 0.5 V/s in 0.1 M KCl pH 7.5 phosphate buffer.

**Table S1. Average Characteristics of sensor films from QCM Results.**

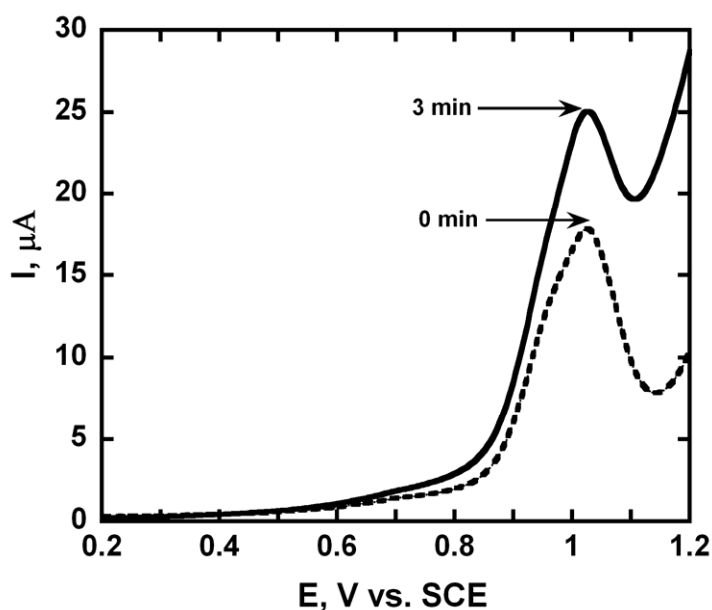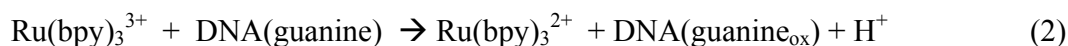| Film | Thickness[a] | Total mass [b] ($\mu$g cm$^{-2}$) | | | |
|---|---|---|---|---|---|
| | (nm) | RuPVP | CYP1A2 | NAT | DNA |
| CYP1A2/NAT/DNA | 60±6 | 2.6±0.3 | 6.5±0.5 | 2.2±0.3 | 8.3±0.5 |
| CYP1A2/DNA[c] | 44±6 | 2.6±0.3 | 6.5±0.5 | - | 5.5±0.5 |

[a] from $d = (-0.016 \pm 0.002)\ \Delta F$ (nm), [b] from $M/A$ (gcm$^{-2}$) $= -\Delta F$ (Hz)/(1.83 x 10$^8$), see ref. 4 for details.
[c] DNA/RuPVP/DNA/(CYP1A2/DNA)$_2$

Hydroperoxides activate cyt P450 enzymes to give the same metabolites as in vivo, where activation occurs via NADPH and cyt P450 reductase.[7] Thus, hydroxylations catalyzed by CYP1A2 were initiated by the oxidation of the iron heme in the enzymes by hydroperoxides to an oxyferryl intermediate.[8] Hydrogen peroxide is known to be an efficient initiator of CYP1A2 -catalyzed metabolic activation of heterocyclic amines,[9] and can be used without the necessity for an electron donor and cyt P450 reductase.[10] The sensors also employed hydrogen peroxide driven oxidations by cyt P450 enzymes. The activity of cyt P450 1A2 immobilized on PG

electrode as the sensor film PDDA/DNA/(CYP1A2/DNA)$_2$ was estimated using voltammetric detection. The well-known hydrogen peroxide-driven oxidation of styrene by CYP1A2 was used as the test reaction. We previously demonstrated hydrogen peroxide mediated oxidation of styrene to styrene oxide with films of CYP1A2.[6b] The product, styrene oxide, was trapped by DNA in CYP1A2/DNA films and induced DNA damage, was detected by square wave voltammetry (SWV) using catalytic oxidation with Ru(bpy)$_3^{2+}$.[11] (Scheme S1) Figure S2 demonstrates typical SWV response before (0 min) and after reaction (3 min) of CYP1A2/DNA films with styrene/ H$_2$O$_2$, A 20 % increase in SWV peak current after reaction for 3-5 min reflects the formation of styrene oxide by CYP1A2 in the film, and the resulting DNA damage. Additionally, our previous control experiments showed that hydrogen driven oxidation of styrene and DNA damage does not occur in the absence of oxidoreductase enzymes like CYP1A2.[3,11] Activity measurements of AcCoA-dependent NAT enzyme in LbL films were described previously.[12]

**Scheme S1**

$$Ru(bpy)_3^{2+} = Ru(bpy)_3^{3+} + e^- \qquad \text{(at electrode)} \tag{1}$$

$$Ru(bpy)_3^{3+} + DNA(guanine) \rightarrow Ru(bpy)_3^{2+} + DNA(guanine_{ox}) + H^+ \tag{2}$$



**Figure S2.** SWVs of PDDA/DNA/(CYP1A2 /DNA)$_2$ films in pH 5.5 acetate buffer containing 50 µM Ru(bpy)$_3^{2+}$ before (0 min) and after (3 min) incubations at 37 $^o$C with 2% styrene and 1 mM H$_2$O$_2$ in pH 7.5 phosphate buffer. This experiment confirms enzyme activity of CYP1A2 for the well-known conversion of styrene to styrene oxide, which then damages DNA (see ref. 4).

## Bioactivation of PhIP by NAT
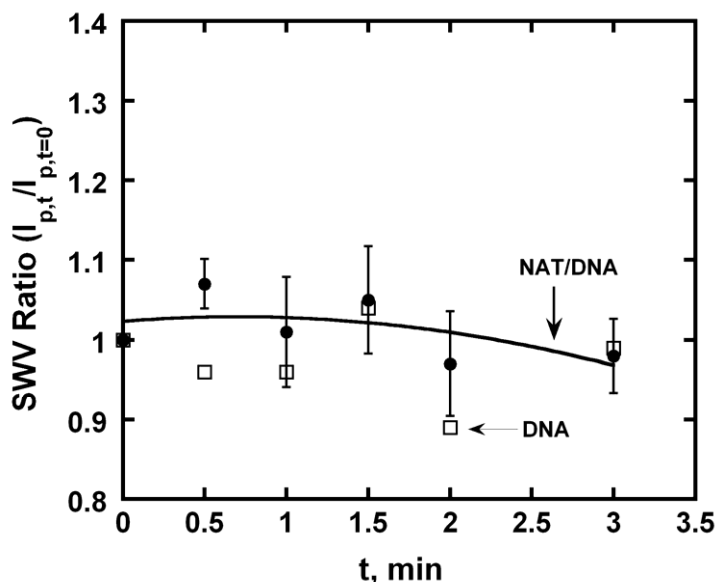


**Figure S3.** Influence of incubation time on the catalytic peak currents for PDDA/DNA/(NAT/DNA)$_2$ reacted at 37 $^{\circ}$C in pH 7.5 containing 50 μM PhIP, 0.5 mM AcCoA, 1 mM DTT, 1 mM EDTA. Control (□) : Incubation of (PDDA/DNA)$_2$ film with 50 μM PhIP. This experiment shows that NAT does not convert substrate to reactive metabolites under these conditions.

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