Electrochemical biosensor featuring a two-enzyme pathway and DNA for screening toxic reactive metabolites of arylamines†

Minjeong So,[a] John B. Schenkman[b] and James F. Rusling*[ab]

Received (in Cambridge, UK) 1st April 2008, Accepted 10th June 2008
First published as an Advance Article on the web 18th July 2008
DOI: 10.1039/b805447a

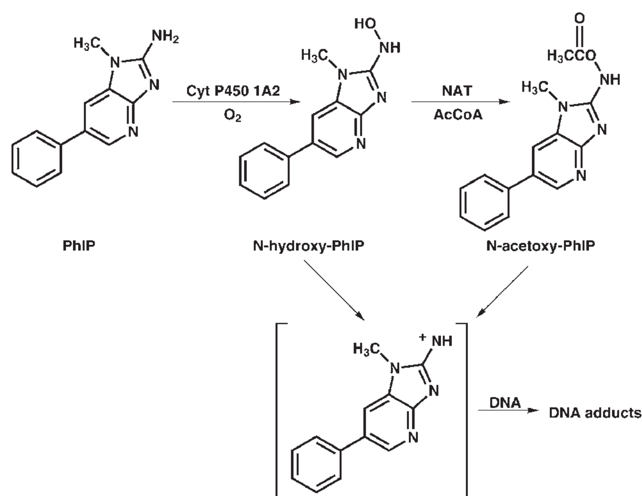
We demonstrate for the first time a biosensor featuring a sequential two-enzyme pathway suitable to screen potentially toxic reactive metabolites generated during metabolism.

Conventional biosensors, *e.g.* those for blood glucose, typically utilize a single enzyme. Layer-by-layer (LbL) electrostatic adsorption is amenable to incorporation of multiple biomolecules into thin films of nanometre thickness, and has been used to make multi-enzyme bioreactors.[1] Recently, sulfite oxidase was layered on top of an electron transfer protein to make an electrochemical sensor for sulfite.[2] We used LbL methods to make films of DNA and metabolic enzymes as biosensors to detect reactive metabolites from environmental pollutants and drugs.[3] Enzymes convert the chemical to metabolites that may react in the films to form DNA adducts.[4] The resulting DNA damage is detected by catalytic voltammetry using $Ru(bpy)_3^{2+}$ (bpy = 2,2′-bipyridine) or a ruthenium metallopolymer. We recently fabricated these sensors in array formats.[5]

Enzymes metabolize xenobiotic molecules by oxidation and bioconjugation. Most oxidations are catalyzed by cytochrome P450 (cyt P450 or CYP) enzymes. Bioactivation to reactive metabolites by cyt P450s is a major source of toxicity.[6] However, bioconjugation enzymes account for ~25% of marketed drug metabolism and can mediate the bioactivation of chemicals in sequential reactions with cyt P450s. In this communication, we report the first combination of a cyt P450 and a bioconjugation enzyme in LbL films with DNA to create a two-enzyme metabolic pathway that generates DNA-reactive products. The films were configured in a biosensor, and results herein demonstrate their applicability for toxicity screening.

For proof of concept, we chose a sequential metabolic pathway characteristic of arylamines involving oxidation catalyzed by cytochrome P450 1A2 (CYP1A2) followed by acylation by conjugation enzyme acetyl coenzyme A (AcCoA) dependent *N*-acetyltransferase (NAT).[7] We made films of DNA, CYP1A2 and NAT on pyrolytic graphite (PG) disks to make the two-enzyme sensors (see ESI†). Quartz crystal microbalance weighings of the layers during film formation were used to obtain the amounts of enzymes and DNA in the films (Fig. S1, Table S1, ESI†).

[a] *Department of Chemistry, University of Connecticut USA. E-mail: james.rusling@uconn.edu*
[b] *Department of Cell Biology, University of Connecticut USA*
† Electronic supplementary information (ESI) available: Experimental details, Fig. S1–S3. See DOI: 10.1039/b805447a



Scheme 1 Metabolism of PhIP in the two-enzyme sequence.

PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) was chosen as the test substrate because it is a potent heterocyclic amine mutagen bioactivated by NAT in series with CYP1A2.[8] PhIP metabolites form covalent nucleobase adducts (Scheme 1).[9] Metabolic bioactivation of PhIP occurs primarily in the liver by CYP1A2-mediated N-oxidation of the exocyclic amine to form *N*-hydroxy-PhIP (*N*-OH-PhIP).[10] Acylation of *N*-OH-PhIP by NAT generates *N*-acetoxy-PhIP. Both derivatives decompose to a DNA-reactive nitrenium cation that forms PhIP–DNA adducts, most commonly at the C8 position of guanines.[11,12]

First, we monitored DNA damage arising from N-hydroxylation of PhIP by CYP1A2 alone. CYP1A2/DNA sensors were exposed to solutions of PhIP and H_2O_2 in pH 7.5 buffer. Fig. 1(a) shows square wave voltammetric (SWV) curves obtained by using soluble $Ru(bpy)_3^{2+}$ as the catalyst. The mechanism for detecting DNA damage in the films is similar to that elucidated by Thorp for DNA in solution.[13] The electrode oxidizes the catalyst to $Ru(bpy)_3^{3+}$ which oxidizes intact guanines on the DNA. The role of guanine reactions in signal development was demonstrated in previous studies with films of polynucleotides having only one base type, and only polyG reacted with the catalyst.[14] The faster the reaction of $Ru(bpy)_3^{3+}$ with DNA, the larger the sensor response. Damaged DNA gives larger SWV peaks than intact ds-DNA because when adducts form on the bases ds-DNA is partly disrupted and guanines are more closely approached by catalyst, increasing the reaction rate and the catalytic peak.[4]

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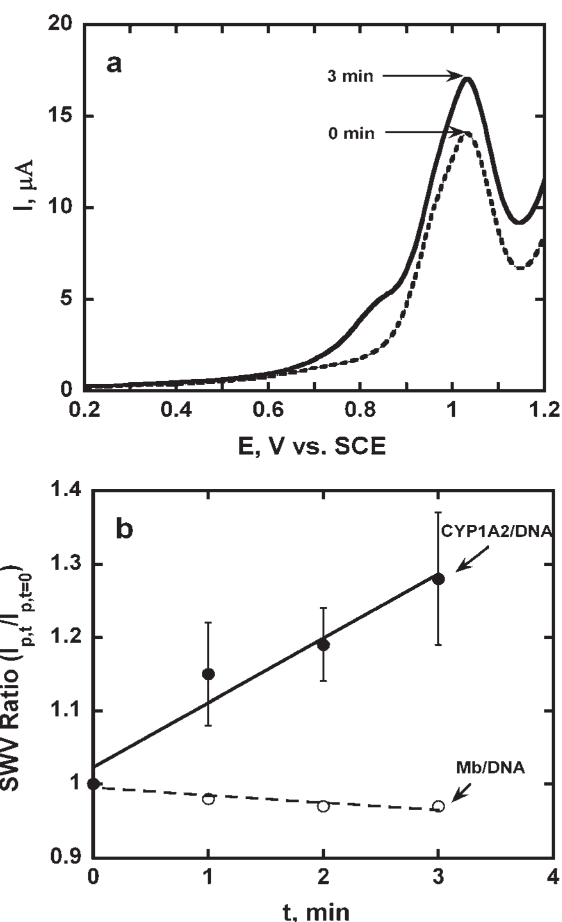

Fig. 1 (a) SWVs for sensors featuring PDDA/DNA/(CYP1A2/DNA)$_2$ films (denoted CYP1A2/DNA) in pH 5.5 buffer containing 50 μM $Ru(bpy)_3^{2+}$ before (0 min) and after (3 min) incubations at 37 °C with 50 μM PhIP and 1 mM H_2O_2 (for enzyme activation)[14] at pH 7.5. (SWV ampl. 25 mV; freq. 15 Hz; 4 mV step). (b) Influence of incubation time with 50 μM PhIP and 1 mM H_2O_2 on CYP1A2/DNA sensor final/initial peak ratio (●) (error bars are sd); and on PDDA/DNA/(Mb/DNA)$_2$ control (denoted Mb/DNA) (○) (relative sd in controls from 3–5 trials: ±8%).

Thus, DNA damage is the reason for the increase in peak current in Fig. 1(a) at 1.02 V *vs.* SCE after reaction of the sensor with PhIP.

Initial slopes of single enzyme/DNA sensor response *vs.* enzyme reaction time for a range of substrates correlated with rates of nucleobase adduct formation measured by LC-MS after DNA hydrolysis,[4] showing that sensor slopes monitor relative DNA damage rates. Fig. 1(b) shows that the ratio of final (after incubation) to initial sensor peaks increased with PhIP reaction time, suggesting a significant rate of DNA damage. Control films of myoglobin Mb/DNA gave no signal increase under our conditions. This suggests that DNA damage occurred from activation of PhIP by CYP1A2, but not by Mb, which does not hydroxylate heterocyclic amines.[15]

The influence of NAT alone on DNA damage was investigated by using sensors with PDDA/DNA/(NAT/DNA)$_2$ (denoted NAT/DNA) films. These sensors were incubated with PhIP–AcCoA medium in pH 7.5 buffer.[16] SWV peak ratios for NAT/DNA films gave negligible increases in peak current with increasing enzyme reaction time (Fig. S3, ESI†). This result suggests that PhIP does not undergo N-acetylation under these conditions, consistent with the known low activity of NAT for N-acetylation.[8b]

Films containing CYP1A2, NAT and DNA were used to mimic the sequential bioactivation of PhIP (Scheme 1). Sensors were fabricated with the catalytic polyvinylpyridine (PVP) metallopolymer ($[Ru(bpy)_2(PVP)_{10}]^{2+}$ denoted RuPVP) in the films to improve sensitivity of detection and provide reagentless detection.[4,5a] Fig. 2(a) shows responses before and after exposure of CYP1A2/NAT/DNA sensors to the PhIP–H_2O_2–AcCoA reaction cocktail. The increase in peak current at 1.15 V suggests metabolite generated DNA damage after

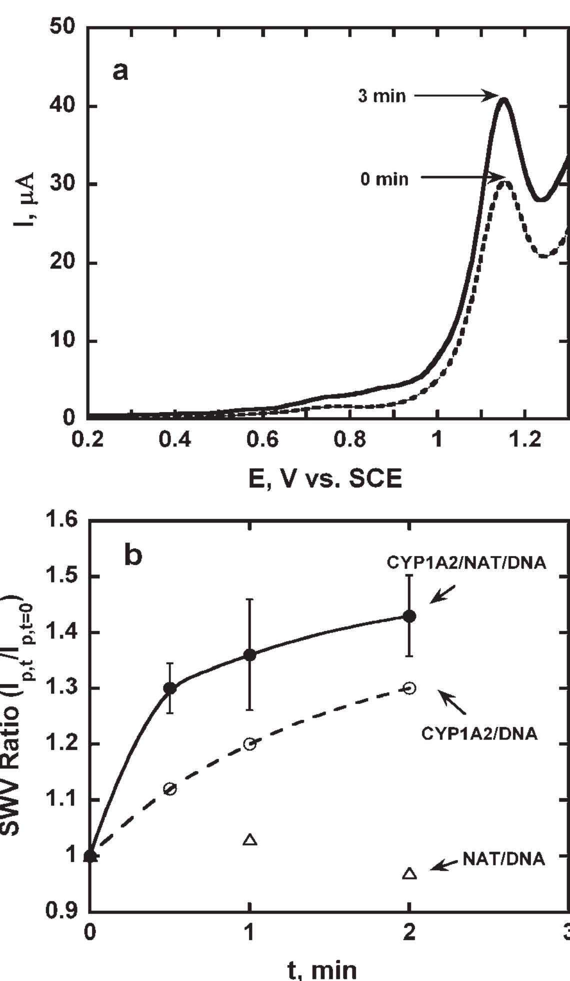

Fig. 2 (a) SWVs of DNA/RuPVP/DNA/(CYP1A2/DNA)$_2$/(NAT/DNA)$_2$ 2-enzyme sensors in pH 5.5 buffer before (0 min) and after (3 min) incubations at 37 °C in pH 7.5 buffer containing 50 μM PhIP, 1 mM H_2O_2, 0.5 mM AcCoA, 1 mM DTT and 1 mM EDTA (SWV ampl. 25 mV; freq. 15 Hz; step 4 mV). (b) Influence of enzyme incubation time on final/initial peak ratio for DNA/RuPVP/DNA/(CYP1A2/DNA)$_2$/(NAT/DNA)$_2$ 2-enzyme sensors (●), control 1 (○) DNA/RuPVP/DNA/(CYP1A2/DNA)$_2$ sensor with 50 μM PhIP and 1 mM H_2O_2, and control 2 (△) DNA/RuPVP /DNA/(NAT/DNA)$_2$ sensor with 50 μM PhIP, 0.5 mM AcCoA, 1 mM DTT, 1 mM EDTA (relative sd in controls from 3–5 trials was ±8%).

the enzyme reactions. Fig. 2(b) shows that peak ratios for CYP1A2/NAT/DNA sensors increased much faster with incubation time compared to CYP1A2/DNA films that only catalyze N-hydroxylation. Initial slopes of the damage ratio plots were 0.60 min^{-1} for CYP1A2/NAT/DNA and 0.20 min^{-1} for CYP1A2/DNA, indicating a faster rate of DNA damage for the 2-enzyme sensor. No significant increase was found in the ratio plot for control NAT/DNA sensors, consistent with results using soluble $Ru(bpy)_3^{2+}$.

Fig. 1 and 2 suggest that PhIP can be bioactivated directly by CYP1A2 in films to produce a reactive intermediate that damages DNA. Protonation of *N*-hydroxy-PhIP followed by elimination of water has been suggested to form an electrophilic nitrenium ion.[9,11] Can this be the only source of DNA damage in both one- and two-enzyme sensors? To answer this, we used results from QCM (Table S1, ESI†) to reveal relative concentrations of enzyme in each film. The 60 nm thick CYP1A2/NAT/DNA films provide 0.16 μmol cm^{-3} DNA intermixed with 20 μmol cm^{-3} cyt P450 1A2 in a 9×10^5 μm^3 film reaction volume. CYP1A2/DNA sensors with 44 nm thickness provide 0.15 μmol cm^{-3} DNA and 28 μmol cm^{-3} cyt P450 1A2 in a 7×10^5 μm^3 reaction volume. While concentrations of DNA are similar in the two films, the amount of CYP1A2 is larger in a smaller reaction volume in the single-enzyme film, providing a larger CYP1A2 concentration than in the 2-enzyme films. Thus, it is unlikely that larger peaks for CYP1A2/NAT/DNA sensors are caused by CYP1A2 oxidations alone. Further, control NAT/DNA sensors gave no signal increases. Thus, the larger peak increase for CYP1A2/NAT/DNA sensors compared to CYP1A2/DNA in Fig. 2(b) must result from the two-enzyme pathway in Scheme 1, *i.e.* formation of *N*-OH-PhIP by CYP1A2 followed by NAT mediated O-acetylation to form highly reactive *N*-acetoxyl esters. This facilitates more rapid formation of the nitrenium ion (Scheme 1) in the films that presumably reacts with guanines on DNA to provide the sensor signals.

In summary, results above show that two metabolic enzymes, CYP1A2 and NAT, can be incorporated into a film with DNA by LbL techniques to mimic a sequential metabolic reaction. The reactive product of the metabolic pathway in these sensors is detected *via* DNA damage, appropriate for *in vitro* toxicity screening for reactive metabolites. This work suggests that multi-enzyme films catalyzing sequential reactions are suitable for incorporation into toxicity screening arrays[5] together with a range of other metabolic enzymes.

The authors gratefully acknowledge financial support from the National Institute of Environmental Health Sciences (NIEHS), NIH (US PHS grant No. ES03154) and thank Dr Eli Hvastkovs and Dr Ingela Jansson for experimental assistance in expression and purification of CYP 1A2.

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