Genotoxicity screening for *N*-nitroso compounds. Electrochemical and electrochemiluminescent detection of human enzyme-generated DNA damage from *N*-nitrosopyrrolidine†

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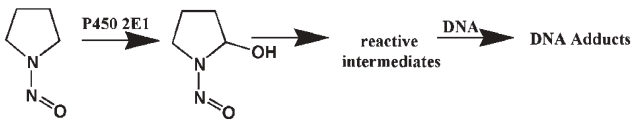
We report for the first time voltammetric/electrochemiluminescent sensors applied to predict genotoxicity of *N*-nitroso compounds bioactivated by human cytochrome P450 enzymes.

Metabolic activation of lipophilic pollutants and drugs in mammalian liver by cytochrome (cyt) P450 enzymes can cause DNA damage in a major genotoxicity pathway.[1] Detection of DNA damage caused by metabolites can be used in toxicity screening and molecular genotoxicity studies.[2,3] We recently described electrochemical sensors for genotoxicity screening that combine metabolic enzymes and DNA in thin films.[2] The enzyme reaction may produce reactive metabolites, and relative rates of reaction with DNA are detected by catalytic voltammetry[2a,3] or mass spectrometry (MS).[2b] By comparison with nucleobase formation rates measured by MS, these sensors have been validated so far for hydrocarbons such as styrene, benzo[*a*]pyrene, and simple methylating agents, and can detect ~0.05% DNA damage.[2a,3] However, to be widely applicable, the sensors need to predict genotoxicity for other classes of compounds such as nitrogen heterocycles, which appear often in drugs and toxic chemicals. Herein, we report the first application of *in vitro* sensors with voltammetric and electrochemiluminescent outputs to predict genotoxicity caused by metabolites of *N*-nitrosamines, formed in the sensors by reactions catalyzed by cyt P450 2E1.

Nitrosamines constitute a large class of compounds that are reported as carcinogenic in more than 30 species.[4] Cytochrome P450s play a key role in bioactivating *N*-nitrosamines for genotoxicity, mainly by α-hydroxylation of carbon adjacent to the heterocyclic nitrogen. *N*-Nitrosopyrrolidine (NPYR) is a suspected human carcinogen with well established rodent carcinogenicity.[5] Exogenous and endogenous sources of *N*-nitrosopyrrolidine include diet, tobacco smoke and nitrosation of pyrrolidine.[6,7] *N*-Nitrosopyrrolidine requires metabolic bioactivation to exert carcinogenic effects,[8] and cyt P450 2E1 is a major enzyme that bioactivates *N*-nitrosopyrrolidine (Scheme 1).[9] Adduct formation occurs between the metabolites of *N*-nitrosopyrrolidine and the deoxyguanosines of DNA (see Fig. S1).

Hecht *et al.* detailed the mechanism of DNA adduct formation from the reaction of α-acetoxy *N*-nitrosopyrrolidine, a stable precursor to α-hydroxy *N*-nitrosopyrrolidine, and deoxyguanosine (dG) using LC/MS and UV–vis spectra.[10] They identified 13 possible adducts between α-acetoxy *N*-nitrosopyrrolidine and dG, demonstrating the complex nature of this reaction. In the present work, we used a similar reaction scheme in thin films to develop *in vitro* toxicity screening sensors for such molecules. However, to date, *in vitro* genotoxicity testing for nitrosamines has only included microsomal and reconstituted enzymatic systems. Therefore, alternative screening methods addressing desirable analytical aspects (*i.e.* cost, throughput, rapid sample handling) are needed.



Scheme 1

Electrochemical methods offer simple, rapid, inexpensive approaches to detect DNA damage.[2a,11] A particularly sensitive approach is to use ruthenium tris(2,2′-bipyridyl) $[Ru(bpy)_3^{2+}]$ to electrocatalytically oxidize guanines in DNA.[11c] We previously showed that electrode-immobilized ruthenium bipyridyl polymer $[Ru(bpy)_2PVP_{10}]^{2+}$, denoted as RuPVP, can also be used to catalytically oxidize guanine in DNA in a similar fashion, according to Scheme 2, where G = guanine.[12]

Damage to the nucleobases causes DNA to partly unwind, and allows better access of the catalyst to guanines. This causes faster cycling of $Ru^{III/II}$ in eqn 2 and yields an enhanced catalytic oxidation current detected by square wave voltammetry (SWV).[3] Electrochemiluminescence (ECL) at 610 nm is generated from RuPVP by further oxidation of guanine (eqns 3 and 4), or by reaction of reduced Ru^{I} and Ru^{III} to produce excited Ru^{II}*.[12] Detected simultaneously, SWV and ECL signals are proportional to the relative amount of chemically damaged DNA bases in the sensor films.[12] Immobilized RuPVP metallopolymer in the sensor film removes the need for soluble catalyst, or for sacrificial reductant as the guanines serve the latter purpose.

$$Ru^{II}\text{-PVP} \leftrightarrow Ru^{III}\text{-PVP} + e^- \text{ (at electrode)} \quad (1)$$
$$Ru^{III}\text{-PVP} + G \rightarrow Ru^{II}\text{-PVP} + G^{\bullet} + H^+ \quad (2)$$
$$G^{\bullet} + Ru^{III}\text{-PVP} \rightarrow G_{2ox} + Ru^{II*}\text{-PVP} \quad (3)$$
$$Ru^{II*}\text{-PVP} \rightarrow Ru^{II}\text{-PVP} + h\nu\,(610\text{ nm}) \quad (4)$$

Scheme 2

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In the present work, DNA–RuPVP–enzyme films were constructed as active components of genotoxicity sensors. Layers of RuPVP, DNA and enzymes were adsorbed sequentially on basal plane pyrolytic graphite disks (PG, $A = 0.2$ cm^2)[2,3,12] and rinsed with water between adsorption steps (see Supplemental Information). Quartz crystal microbalance (QCM) studies confirmed regular and reproducible film formation (ESI Fig. S2). Film architectures expressed as order of layer deposition for SWV were DNA/RuPVP/DNA/cyt P450 2E1/DNA or DNA/RuPVP/DNA/myoglobin/DNA. For ECL, additional bilayers of DNA/RuPVP were necessary to improve signal/noise.

Cyt P450 2E1 immobilized in similar thin films demonstrated proper electrochemical behavior characteristic of the native enzyme (ESI Figs. S3–S4). Peroxide activates cyt P450 enzymes to give the same metabolites as *in vivo*, where activation occurs *via* NADPH and cyt P450 reductase.[13a,b] To drive the enzyme reaction, sensors were exposed to 150 μM *N*-nitrosopyrrolidine (NPYR, Sigma) + 1 mM H_2O_2 in acetate buffer pH 5.5 at 37 °C. A water rinse of the sensor stopped the reaction. Fig. 1a shows SWVs obtained for sensors for increasing time of the enzyme reaction.

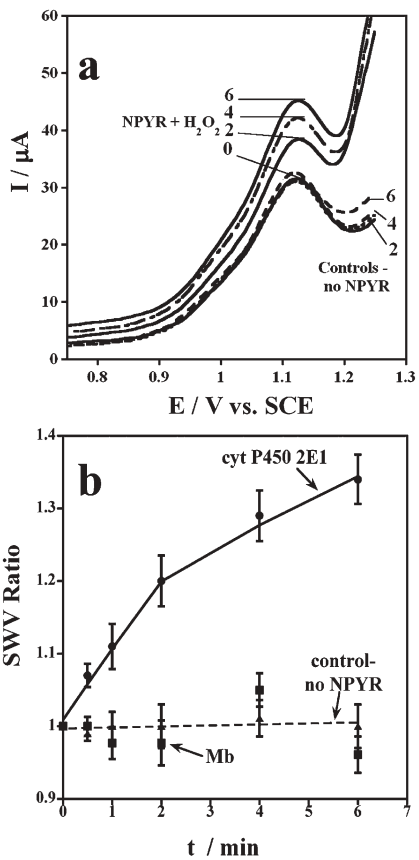

Fig. 1 Voltammetric sensor data after exposure to NPYR: (a) SWV of DNA/RuPVP/DNA/cyt P450 2E1/DNA films before and after incubations at 37 °C with 150 μM NPYR and 1 mM H_2O_2 in 10 mM acetate buffer + 50 mM NaCl, pH 5.5. Controls show incubations with only 1 mM H_2O_2 (labeled times in min) (SWV ampl. 25 mV; freq. 15 Hz; step 4 mV). Damage exposure peaks are offset for clarity; controls are not. (b) Influence of NPYR/H_2O_2 incubation time on SWV peak current ratio for DNA/RuPVP/DNA/cyt P450 2E1/DNA (solid line) and DNA/RuPVP/DNA/Mb/DNA films. Controls are incubation of DNA/cyt P450 2E1 films with only 1 mM H_2O_2 (dashed line). Error bars are sd for $n = 3$ trials.

The peak potential was 1.13 V *vs.* SCE and peak currents increased with incubation time. Fig. 1b shows the increase in signal as peak current ratio ($I_{p,f}/I_{p,i}$), where $I_{p,i}$ is the peak for sensors not exposed to NPYR and peroxide, and $I_{p,f}$ is the peak after the enzyme reaction. This ratio minimizes deviations due to inter-electrode variability. NPYR is bioactivated by cyt P450 2E1 to reactive metabolites. The $I_{p,f}/I_{p,i}$ increase in Fig. 1 is due to guanine–NPYR metabolite adduct formation, which increases the catalytic oxidation rate as mentioned above. The signal increase reflects DNA damage similar to that reported previously from styrene and benzo[*a*]pyrene metabolites.[2a,3,12] Fig. 1b also shows control peak ratios for sensors exposed to H_2O_2 only, and for RuPVP/DNA/Mb sensors exposed to NPYR and H_2O_2. The H_2O_2-only control shown in Fig. 1a and plotted in Fig. 1b demonstrates that peroxide does not damage DNA or contribute to the oxidative current under these conditions. The negligible signal increase when using Mb as a model oxygenase[13c] in the films suggests Mb does not bioactivate NPYR under these conditions. ESI Figs. S6 to S8 demonstrate that voltammetric peaks are due to RuPVP and increases due to catalytic DNA oxidation.

Figs. 2a and 2b are digitally reconstructed images from ECL arrays[12b] that demonstrate the ECL response when the DNA/cyt P450 2E1 and DNA/Mb films are exposed to increasing times of damage solution (see Supplementary Information for description of ECL arrays[12b]). Each spot in the array contains enzyme, DNA and RuPVP. The increase in light intensity as the cyt P450 2E1 reaction proceeds results from increasing damage to the DNA. Results for RuPVP/DNA/Mb confirm that Mb does not bioactivate NPYR. Fig. 2c shows an ECL ratio plot comparing the ECL from different enzyme reaction times to that at $t = 0$,

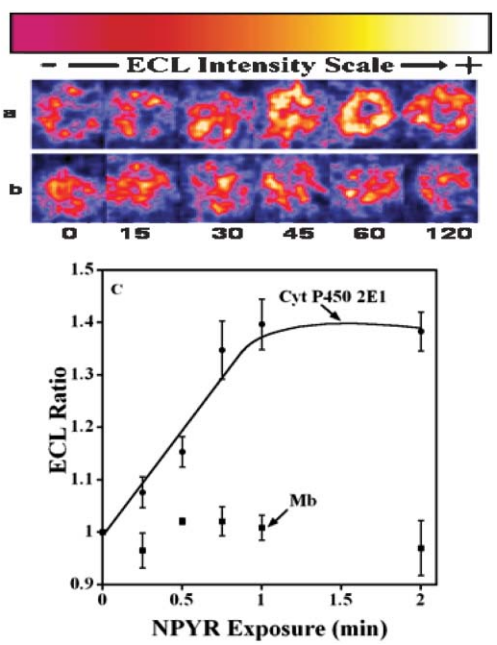

Fig. 2 ECL array data after exposure to NPYR: (a) Digitally reconstructed image demonstrating CCD captured ECL emitted from RuPVP/DNA/cyt P450 2E1 array and (b) RuPVP/DNA/Mb array exposed to 150 μM NPYR + 1 mM H_2O_2 for the denoted amounts of time (s). (c) Ratio plot demonstrating the ECL signal increase from films containing cyt P450 2E1 or Mb.

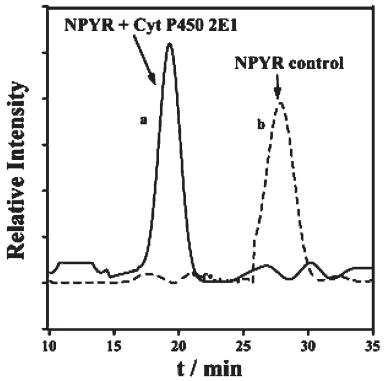

Fig. 3 Single ion recording capLC-MS chromatogram detecting *m*/*z* 70–100 Da for: (a) 150 μM NPYR + 1 mM H_2O_2 reacted 10 min with CYP 2E1 immobilized on silica microspheres; (b) 150 μM NPYR + 1 mM H_2O_2 reacted with control microspheres with no enzyme.

similar to the SWV ratio plots. The ECL ratio increased with reaction time for the RuPVP/DNA/cyt P450 2E1 films, denoting increasing amounts of DNA damage from *N*-nitrosopyrrolidine metabolites.

Increased ECL signals (Fig. 2c) at smaller reaction times compared to SWV (*cf.* Fig. 1b) are attributable to differences in film composition and detection format. Larger amounts of RuPVP and DNA in the ECL films force more ruthenium centers in close proximity to DNA resulting in more ECL. The higher amount of RuPVP in ECL films was necessary to generate sufficient light to be imaged by the CCD camera.[12b] These additional RuPVP layers were unnecessary for voltammetric analysis, and only one layer of RuPVP was used to generate a satisfactory signal to background ratio as seen in Fig. 1a. The rapidly achieved plateau in Fig. 2c is likely due to increased amounts of RuPVP and prolonged oxidation conditions compared to SWV, resulting in detection of the majority of damaged guanines formed during NPYR/H_2O_2 exposure. We also observe plateau responses for electrochemical studies at lengthy NPYR exposure times (data not shown), as well as for direct DNA damage studies,[12a] lending causal evidence.

Verification of active NPYR metabolite produced by cyt P450 2E1 was accomplished by forming analogous films on hydroxylated 0.5 μm silica microspheres, running the enzyme reaction, and analyzing reaction solutions by CapLC-MS (see Supplemental Information for protocols). MS was acquired by monitoring the elution of compounds with selected *m*/*z* of 70–100 Da range (single ion recording, SIR). Fig. 3a shows the chromatogram for a SIR-MS of NPYR reaction solution extract in 10 mM acetate buffer plus 50 mM NaCl, pH 5.5 (150 μM NPYR + 1 mM H_2O_2) after exposure for 10 min to microspheres with immobilized cyt P450 2E1. This shows the $[M + H]^+$ product peak in the chromatogram at 19 min (solid line). The observed $[M + H]^+$ MS peak at *m*/*z* 89 Da for this peak (Fig. S5) is consistent with the major *N*-nitrosopyrrolidine α-hydroxylation metabolite 2-hydroxytetrahydrofuran (2-OH-THF).[14] Controls not exposed to cyt P450 2E1 showed an SIR chromatogram peak at 27 min (dashed line, Fig. 3b). The MS for the 27 min peak showed a peak at *m*/*z* 101 Da associated with $[M + H]^+$ of *N*-nitrosopyrrolidine.

Overall, the genotoxicity sensors detected DNA damage from the NPYR metabolites from cyt P450 2E1 by SWV and ECL. Bioactivation of NPYR to reactive metabolites in our sensor films was effected by cyt P450 2E1 but not Mb, indicating the specificity of the sensor to enzyme identity. This result agrees with previous reports that cyt P450 2E1 is a major human liver enzyme involved in metabolizing NPYR.[9]

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