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

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Experimental Details

Chemicals and Materials

Calf thymus DNA (Type I, 41.9 % G/C), horse heart myoglobin (Mb, MW 17400), N-Nitrosopyrrolidine (NPYR), hydrogen peroxide, poly(diallyldimethylammonium chloride) (PDDA) and poly (sodium 4-styrene sulfonate) (PSS) were from Sigma. Hydroxylated SiO_2 microspheres (0.5 μm) were from Polysciences Inc. Water was treated with a Hydro Nanopure system to a specific resistivity > 18 mΩ cm. Amicon YM30 membrane filter (30 000 MW cutoff) was used to filter the Mb solution. Human Cyt P450 2E1 (MW 50 kDa) was expressed in *E.coli* containing the proper cDNA and was purified as described previously.[1] All other chemicals were reagent grade.

The electrochemiluminescent polymer $[Ru(bpy)_2PVP_{10}](ClO_4)_2$ was prepared following established literature protocols.[2] Briefly, cis-$Ru(bpy)_2Cl_2$, was refluxed in water for one hour before adding a 5 fold molar excess of $LiClO_4$ and a 10 fold excess of poly(vinyl pyridine) (PVP, MW 280,000) (i.e. 1 ruthenium/10 pyridine units) along with absolute ethanol for a final solution concentration of 80:20 ethanol:water. This mixture was refluxed for 24 hours with monitoring by UV-vis spectroscopy and voltammetry. A wavelength maximum of 465 nm was observed for the product. The final product was dissolved in ethanol and precipitated with ether.

Safety note*: N-Nitrosopyrrolidine is carcinogenic in experimental animals.* All procedures were done while wearing gloves, and under closed hoods.

Instrumentation and procedures

Voltammetry. A CH instrument 660A electrochemical analyzer was used for cyclic and square wave voltammetry (SWV). The cell employed a saturated calomel reference electrode (SCE), a Pt-wire counter electrode and a film-coated working electrode disk (A = 0.2 cm^2) of ordinary basal plane pyrolytic graphite (PG, Advanced Ceramics). SWV conditions were 4 mV step height, 25 mV pulse height, and 15 Hz frequency. The electrolyte was 10 mM sodium acetate buffer containing 50 mM NaCl, pH 5.5 (see Figure S1).

Electrochemiluminescence (ECL). For ECL detection, the block electrode was placed in a 150 ml Pyrex beaker filled to 60 ml with 10 mM sodium acetate buffer + 0.15 M NaCl pH 5.5. The counter electrode was a platinum ring wire placed directly above the electrode with an Ag/AgCl reference electrode placed to the right of the electrode block as described previously.[3] The beaker was placed in a the gel documentation dark box (SynGene Chemigenius Bio-Imaging System, Fredrick, MD) for ECL data acquisition by a CCD camera. A potential of +1.25 V was applied to the block for 20s using a CH Instruments (Austin, TX) model 1232 Electrochemical Analyzer Data analysis and quantification was done using GeneSnap and Gene Tools software provided by SynGene.

Quartz Crystal Microbalance (QCM). To monitor the layer-by-layer assembly of DNA, RuPVP and cyt P450 2E1 or Mb on the sensors, we used quartz crystal microbalance (QCM, USI Japan) with 9 MHz QCM resonators (AT-cut, International Crystal Mfg.). Layers were adsorbed on a gold resonator (0.16 ± 0.01 cm^2) that was pre-adsorbed with a monolayer of 0.5 mM 3-mercaptopropionic acid. Resonators were dried in a stream of nitrogen before measuring the

frequency change (ΔF) for each layer. Adsorbed mass was estimated with the Sauerbrey equation.[4,5] For 9 MHz quartz resonator, the dry film mass per unit area M/A is

$$M/A\ (g\ cm^{-2}) = -\Delta F\ (Hz)/\ (1.83 \times 10^{8}) \qquad (1)$$

The nominal thickness (d) of each dried layer was estimated with an expression confirmed by high-resolution electron microscopy:[6]

$$d\ (nm) \approx (-0.016 \pm 0.002)\ \Delta F\ (Hz) \qquad (2)$$

Capillary LC-MS/MS. A trapping column (Atlantis, dC18, 23.5 mm, 0.18mm, i.d., 5μm particle size) and the analytical column (Atlantis dC18, 150 mm, 300 μm I.D., 5 μm particle size) were from Waters (Milford, MA). The column switching system in the capillary LC was used to trap and concentrate the products from the reaction. A 10 μL portion of the sample was loaded into the trapping column at a flow rate of 4.25 μL min^{-1}, the mobile phase was ammonium acetate buffer (10 mM, pH 5.0) and methanol. Elution at the analytical column was achieved at a flow rate of 4.25 μL min^{-1} using a gradient method.

Electrospray ionization mass spectrometry (ESI-MS) was used, employing a Micromass Quattro II (Beverly, MA) with electrospray source and operated in the positive ion mode (ES^{+}). The procedure used to identify the product was SIR (single ion recording).

Sensor Film Assemblies. DNA-metallopolymer-protein films were constructed one layer at a time by the layer-by-layer electrostatic assembly method.[4] Basal plane PG electrodes were polished on 400 grit SiC paper (3M Crystal Bay) and then ultrasonicated in ethanol for 30s followed by in water for 30s. Layers of DNA, RuPVP layers (in dark) were adsorbed for 15 min and protein layers for 30 min (protein and subsequent DNA layers were adsorbed at 4°C) onto the rough PG electrodes to achieve saturated adsorption and washed with water between adsorption steps.[4,5]

Adsorbate solutions for SWV sensors were as follows: (a) 2 mg mL^{-1} DNA in 10 mM Tris buffer, pH 7.1 plus 50 mM NaCl; (b) 1 mg mL^{-1} Cyt P450 2E1 in 50 mM potassium phosphate buffer, pH 7.0; (c) 0.3 mg mL^{-1} Ru-PVP metallopolymer in 1:1 ethanol/water (v/v) mixture; and (d) 3 mg mL^{-1} Mb in 10 mM pH 4.5 acetate buffer. Under these conditions, DNA is negatively charged and the metallopolymer Ru-PVP is positively charged. For proteins, the pH ranges were determined to optimize the positive charge on the enzyme based on pI values and also for stability reasons regarding the human cyt P450 enzymes. Films of architecture DNA/Ru-PVP/DNA/Cyt P450 2E1/DNA (denoted as DNA/cyt P450 2E1) were employed for SWV sensors.

For ECL experiments, the basal plane pyrolytic graphite (PG) electrode block (Advanced Ceramics, 1 in^2) was attached to a copper plate connector using silver epoxide and insulated using a standard acrylic polymer so that only the face of the electrode was exposed.[3] The block was first polished on 600 grit SiC paper (Buehler) and sonicated in pure water for 1 minute, followed by rinsing with pure water, absolute ethanol and drying under a stream of nitrogen. Films were grown following previously established protocols[4,5] with some modifications that were experimentally determined in order to produce strong ECL signal and produce distinct, approximately uniform spots on the electrode surface:

1) 2-μl drops of DNA (2 mg ml^{-1}, 10 mM Tris pH 7.1 + 50 mM NaCl) were manually applied by micropipette to the electrode at the demarcated locations. After 15 minutes, spots were rinsed with pure water and dried briefly with a stream of nitrogen.

2) 1-μl RuPVP (1 mg ml^{-1}, 88% H_2O, 12% ethanol) drops were then applied at the same locations in the dark for 15 minutes followed by water rinsing and nitrogen drying.

3) This DNA/RuPVP sequence was repeated once more so that two bi-layers of DNA/RuPVP were in each spot on the electrode surface.

4) After the second RuPVP application, DNA was applied again, allowed to adsorb for 15 minutes, rinsed with pure water, and dried with nitrogen. At this juncture, 150-µl RuPVP (1 mg ml^{-1}, 50% H_2O, 50% ethanol) was applied over the entirety of the surface (i.e. – not just in the spot locations) for the third and fourth bi-layer applications.

5) After the fourth bi-layer, DNA was applied again for 15 minutes followed by two cycles of enzyme and DNA applications. 1-µl enzyme applications were applied from solutions of the following concentrations: myoglobin (3 mg ml^{-1}, 10 mM sodium acetate, pH 4.5), cyt P450 2E1 (1 mg ml^{-1}, 50 mM potassium phosphate, pH 7.0). The adsorption conditions were same as that used for SWV studies.

The final films had the following makeup: $(DNA/RuPVP)_4/(DNA/enz)_2(DNA)$, where enz is the myoglobin or cyt P450 enzyme used. After the last DNA layer was rinsed, the electrode was allowed to stand protected from light at 4°C for at least 12 hours.

Film preparation for CapLC-MS/MS study. 0.2 mL of SiO_2 microspheres (Polysciences, Inc., Warrington PA; used as received) was added to 1.0 mL of PDDA solution (2 mg/mL in water + 50 mM NaCl) and allowed to stand for 20 min to assemble the polymer layer into the SiO_2 microspheres. Then the suspension was centrifuged for 2 min at 8000 rpm to remove the supernatant and the beads were washed with water 3 times to remove weakly bound PDDA. The SiO_2 beads were then redispersed in 1.0 mL PSS solution (2 mg/mL in water + 0.5 M NaCl), allowed to stand for 20 min, centrifuged and washed in water as before. These SiO_2-PDDA/PSS films were then incubated in 0.2 mL of CYP450-2E1 solution (0.5 mg/mL in 50 mM phosphate buffer, pH 7.0) for 30 minutes, then centrifuged and washed 5 times with water to remove loosely bound enzyme.

Incubation: SiO_2/PDDA/PSS/CYP450-2E1 beads were incubated in stirred solutions at 37°C containing 150 µM NPYR and 1 mM H_2O_2 in pH 5.5 acetate buffer. After the incubation, the solution was centrifuged, the supernatant solution was collected and filtered through centricon filters with cut-off mass of 3000 Da (Amicon, Beverly, MA) and analyzed by CapLC-MS/MS analysis as described above.

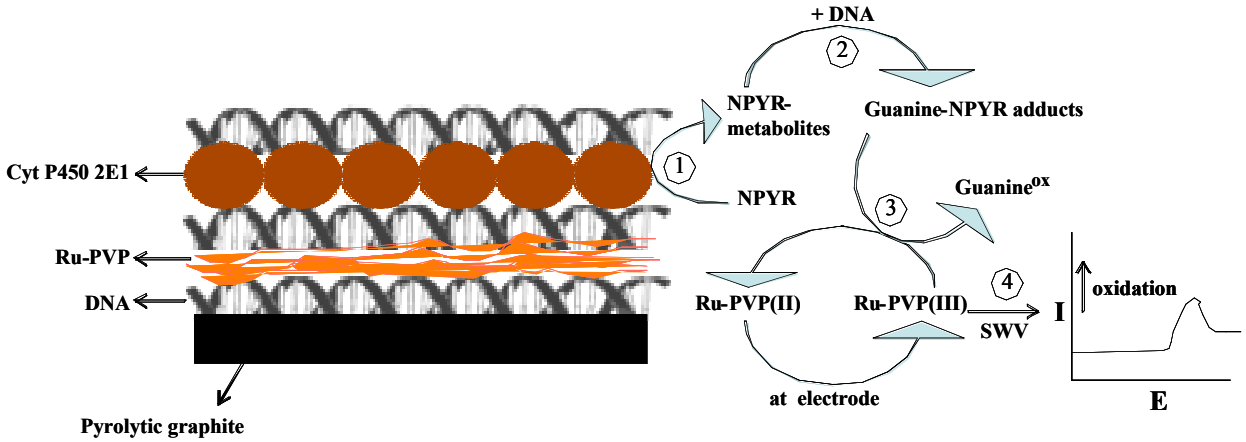

Figure S1. DNA/RuPVP/DNA/2E1/DNA sensor representation showing pathway for DNA damage detection caused by the metabolites produced from NPYR by Cyt P450 2E1.

Results

Figure S2 represents the QCM frequency shifts measured after the alternate adsorptions of DNA, RuPVP and cyt P450 2E1 or Mb layers on gold resonators pre-adsorbed with 3-mercaptopropionic acid as a first layer. From the frequency decrease at each adsorption step, one can understand the formation of DNA/RuPVP/protein layer assemblies used in this study. From the obtained ΔF values and by using eq 1 and eq 2, the amount of protein, DNA and RuPVP in the assemblies and their corresponding thicknesses were determined (Table 1).

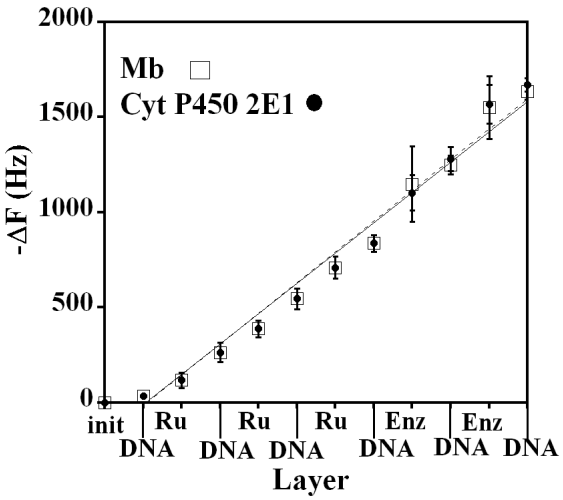

Figure S2. QCM frequency shifts for cycles of alternate DNA/RuPVP/enzyme (cyt P450 2E1 or Mb) adsorptions on gold resonators pre-adsorbed with 3-mercaptopropionic acid as first layer (Ru denotes for RuPVP and Enz denotes for either cyt P450 2E1 or Mb as shown in the legend).

Table 1. Average characteristics of DNA/RuPVP/protein layer assemblies.

Sensor Film	thickness (nm)	wt DNA ($\mu g\ cm^{-2}$)	wt protein ($\mu g\ cm^{-2}$)	wt RuPVP ($\mu g\ cm^{-2}$)
$DNA/(RuPVP/DNA)_4/(cyt\ P450\ 2E1/DNA)_2$	33.5	4.90±0.88	3.60±0.36	2.96±0.52
$DNA/(RuPVP/DNA)_4/(Mb/DNA)_2$	32.3	4.69±1.15	3.40±1.3	2.96±0.52
DNA/RuPVP/DNA/cyt P450 2E1/DNA	13.5	1.41±0.25	1.78±0.18	0.74±0.13
DNA/RuPVP/DNA/Mb/DNA	13.0	1.34±0.33	1.70±0.65	0.74±0.13

Table 1 shows the nominal films thickness and the amounts of DNA, RuPVP and protein for the assemblies used for sensors, as obtained from QCM.

Voltammetry of Cyt P450 2E1. Figure S3 shows the cyclic voltammogram of Cyt P450 2E1 in the film assembly PSS/(cyt P450 2E1/PSS)$_2$ on PG electrode acquired in 50 mM phosphate, pH 7.4 plus 0.1 M KCl buffer purged with nitrogen at a scan rate of 0.1 Vs^{-1}. A reversible peak pair for the Fe(III)/Fe(II) redox couple in cyt P450 2E1 with peak potential centered at -0.38 V is seen. The charge (Q) obtained by integration of forward-scan voltammogram for one-electron reduction of the Fe(III) proteins is related to the amount of electroactive protein per unit electrode area (Γ) by the equation Q = nFAΓ, where n is the number of electrons involved in the redox reaction, F is Faraday's constant and A is electrode area. Γ of 0.02 nmol/cm^2 was obtained for the bilayer of Cyt P450 2E1 used in the film. These reversible peaks occur in a potential range consistent with the behavior of native cyt P450s.[7]

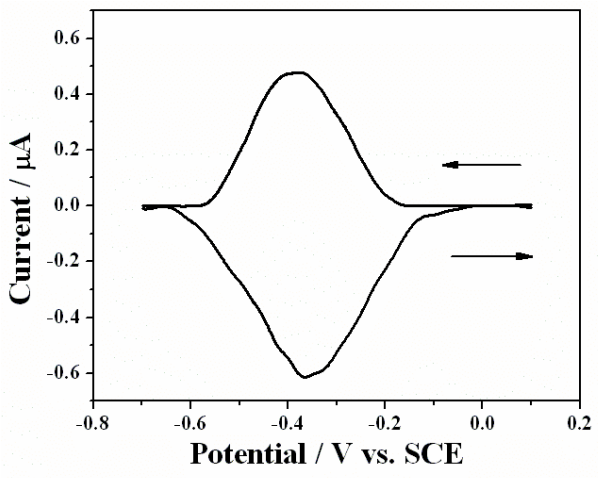

Figure S3. Background subtracted reversible cyclic voltammogram at a scan rate of 0.1 Vs^{-1} for PSS/(cyt P450 2E1/PSS)$_2$ film on rough PG electrode in 50 mM phosphate, pH 7.4 buffer + 0.1 M KCl purged with nitrogen.

Electrocatalytic oxygen reduction by cyt P450 2E1. Electrocatalytic oxygen reduction is a key property of heme enzymes like cyt P450s.[5b,7] In presence of oxygen, Fe(II) formed after the reduction of Fe(III) heme forms a Fe(II)-O_2 complex. Electrochemical reduction of this complex produces hydrogen peroxide at the Fe(III)/Fe(II) redox potential. This catalytic reduction can be detected by cyclic voltammetry as an increase in reduction peak current in the presence of oxygen and the disappearance of the oxidation peak for Fe(II), consistent with its fast reaction with oxygen.[7] Figure S4 shows the cyclic voltammogram of cyt P450 2E1 in the film assembly PSS/(cyt P450 2E1/PSS)$_2$ acquired in 50 mM phosphate, pH 7.4 plus 0.1 M KCl buffer saturated with oxygen at a scan rate of 0.1 Vs^{-1}.

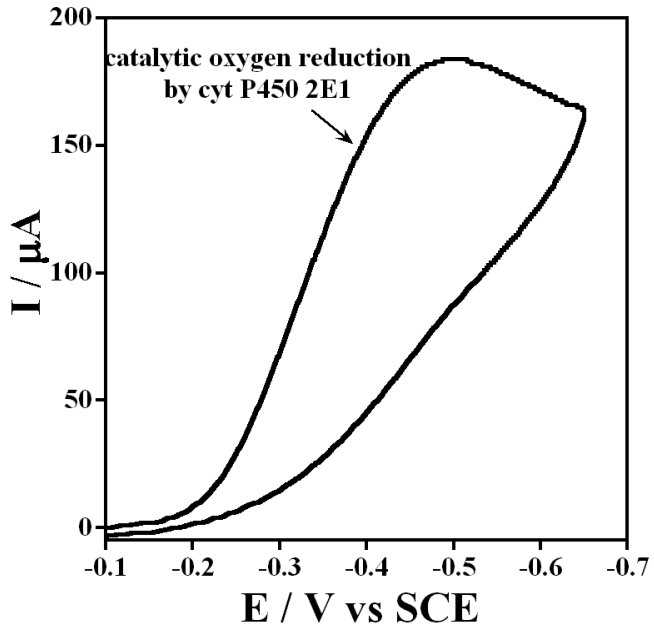

Figure S4. Cyclic voltammogram of PSS/(cyt P450 2E1/PSS)$_2$ film on rough PG electrode in 50 mM phosphate, pH 7.4 plus 0.1 M KCl buffer saturated with oxygen at a scan rate of 0.1 Vs^{-1}.

Figure S5 shows the MS $[M+H]^+$ SIR spectrum of the major metabolite 2-Hydroxy tetrahydrofuran (M+H 89Da) formed from the metabolic activation of N-Nitrosopyrrolidine by cyt P450 2E1 immobilized on silica microspheres in the presence of 1 mM H_2O_2.

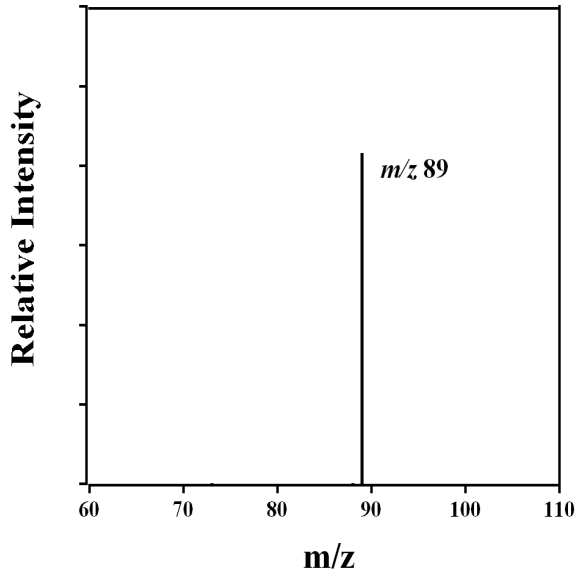

Figure S5. MS $[M+H]^+$ of 150 μM N-Nitrosopyrrolidine+1 mM H_2O_2 solution in 10 mM acetate buffer (pH 5.5) exposed to cyt P450 2E1 immobilized on fused silica microspheres. The M+H m/z peak at 89 Da corresponds to 2-hydroxy tetrahydrofuran, the major metabolite formed from the α-hydroxylation of N-Nitrosopyrrolidine by cyt P450 2E1.

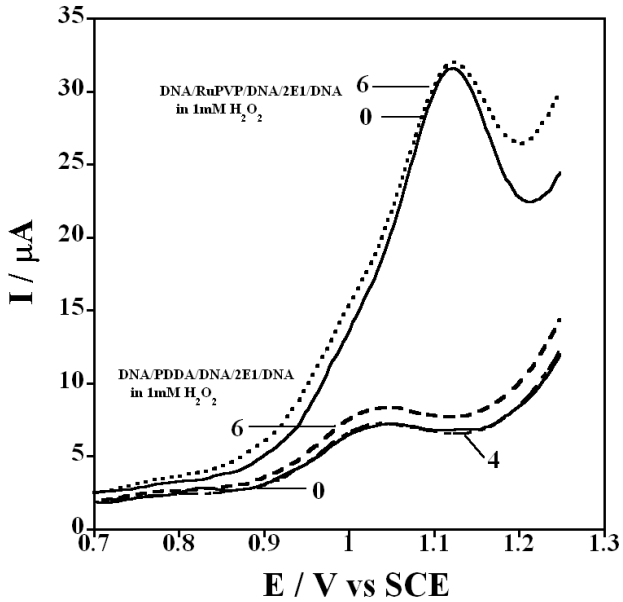

Figure S6. Effect of H_2O_2 on the SWV peak current of DNA/polyion/enzyme layers with or without RuPVP layer with the time of incubation in 10mM sodium acetate buffer plus 50mM NaCl, pH 5.5 containing 1mM H2O2.After incubation, the electrodes were rinsed in water and SWV was acquired in 10mM sodium acetate buffer plus 50mM NaCl, pH 5.5.

From figure S6, catalytic oxidation of DNA by RuPVP polymer can be seen compared to when RuPVP is absent. Also, H_2O_2 does not produce any significant increases in signal by oxidizing RuPVP or cyt P450 2E1. Hence the observed increase in peak current represented in fig.1a is due to cyt P450 formed reactive NPYR metabolites causing DNA damage.[14]

Figures S7 and S8 demonstrate the electrochemical properties of the RuPVP polymer as proof that the peaks shown in Fig. 1 are due to the oxidation of this polymer on the electrode surface.

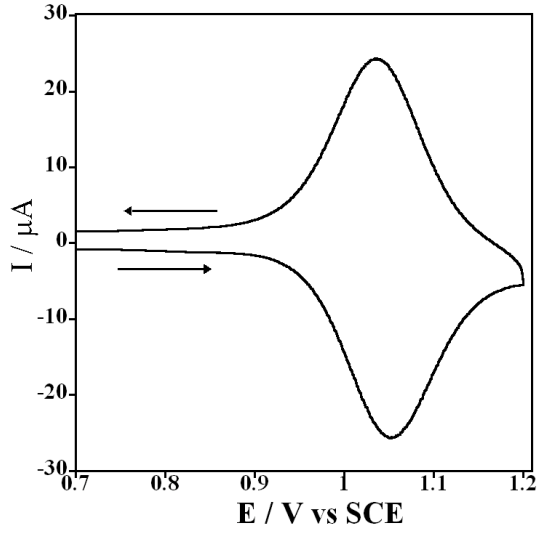

Figure S7. Reversible cyclic voltammogram for the redox reaction of RuPVP layer (0.3 mg/mL in ethanol) dried on PG-electrode acquired in 0.5M HCl at a scan rate of 0.1 V/s.

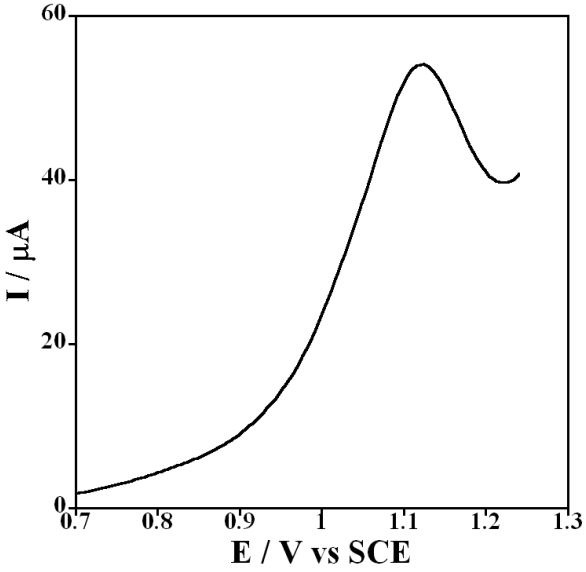

Figure S8. Square wave voltammogram of RuPVP layer (0.3 mg/mL in ethanol) dried on PG-electrode acquired in 10 mM acetate buffer plus 50mM NaCl, pH 5.5.

Approximate Limits of Detection (LOD). Detection of small amounts of DNA with the sensors damage are important only with respect to the length time it takes to measure rates of the sensor response vs enzyme reaction time.. Better LOD means that these rates can be measured faster. Estimates of LOD can be based on previous studies correlating the sensor signal obtained with capLC/MS-MS validity experiments using the same films as in the electrochemical DNA damage sensors.[8] Those reports previously demonstrated that approx. 0.57 nM min^{-1} guanine damage occurs in these films upon xenobiotic exposure. Based S/N of 3:1 the LOD for SWV occurred at approx. 1 minute and for ECL it occurred at approx. 30s resulting in a rough detection of 72 and 36 pmol over those time limits, respectively. This corresponds to about 0.05% of damaged nucleobases.

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