

Toxicity Screening by Electrochemical Detection of DNA Damage by Metabolites Generated In Situ in Ultrathin DNA–Enzyme Films

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Abstract: Rapid detection of DNA damage could serve as a basis for in vitro genotoxicity screening for new organic compounds. Ultrathin films (20–40 nm) containing myoglobin or cytochrome P450_{cam} and DNA grown layer-by-layer on electrodes were activated by hydrogen peroxide, and the enzyme in the film generated metabolite styrene oxide from styrene. This styrene oxide reacted with double stranded (ds)-DNA in the same film, mimicking metabolism and DNA damage in human liver. DNA damage was detected by square wave voltammetry (SWV) by using catalytic oxidation with Ru(bpy)₃²⁺ (bpy = 2,2'-bipyridine) and by monitoring the binding of Co(bpy)₃³⁺. Damaged DNA reacts more rapidly than intact ds-DNA with Ru(bpy)₃³⁺, giving SWV peaks at ~1 V versus SCE that grow larger with reaction time. Co(bpy)₃³⁺ binds more strongly to intact ds-DNA, and its SWV peaks at 0.04 V decreased as DNA was damaged. Little change in SWV signals was found for incubations of DNA/enzyme films with unreactive organic controls or hydrogen peroxide. Capillary electrophoresis and HPLC–MS suggested the formation of styrene oxide adducts of DNA bases under similar reaction conditions in thin films and in solution. The catalytic SWV method was more sensitive than the Co(bpy)₃³⁺ binding assay, providing multiple measurements over a 5 min reaction time.

Introduction

Damage of genetic material by cytochrome P450-derived metabolites^{1,2} of lipophilic pollutants and drugs in mammalian liver is a major toxicity pathway.^{3–6} Reactions between these metabolites and DNA lead to structural changes which can serve as markers for genetic disease.^{7,8} Methods for detecting such DNA damage could serve as effective in vitro screens for the toxicity of new organic chemicals at an early point in their commercial development.

Electrochemical methods offer a rapid, relatively inexpensive approach to detecting DNA damage and hybridization.^{9–13}

Adenine and guanine bases in DNA undergo electrochemical oxidations which give much larger signals when DNA is single stranded or chemically damaged. In the double helix form, nucleic acid bases are protected and minimal oxidation occurs.

We recently demonstrated that ultrathin films of double stranded (ds)-DNA and polycations on electrodes could be used for the detection of DNA damage from known damage agent styrene oxide, the liver metabolite of styrene. Styrene oxide forms up to 11 covalent adducts with guanine and adenine moieties^{4–8} that disrupt the double helix. We employed square wave voltammetry (SWV) coupled with a soluble¹⁴ or polymeric electrode-bound¹⁵ ruthenium complex catalyst or with a soluble metal complex probe that binds more strongly to ds-DNA than to damaged DNA.¹⁶ These approaches allowed detection of damaged DNA in the films within 5–10 min of incubation with the metabolite.

We also demonstrated that myoglobin or cyt P450s in thin films can be used to produce liver metabolites accurately and efficiently.¹⁷ In this paper, we report ultrathin films that combine enzyme-catalyzed generation of toxic metabolite with subse-

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quent detection of DNA damage. To this end, we constructed films by alternating adsorbed layers of ds-DNA with myoglobin or cyt P450_{cam} to generate styrene oxide from styrene. The resulting damage to DNA from reaction with styrene oxide was detected by SWV in the same film using electrochemical catalysis or probe binding protocols.

Experimental Section

Chemicals and Materials. DNA was calf thymus (CT) ds-DNA (Sigma, type XV, 13 000 av base pairs, 41.9% G/C) and salmon testes (ST) ds-DNA (Sigma, ~2000 av base pairs, 41.2% G/C). Horse heart myoglobin (Mb) was from Sigma (MW 17 400) dissolved in pH 5.5 buffer and filtered¹⁸ through an Amicon YM30 membrane (30 000 MW cutoff). *Pseudomonas putida* cyt P450_{cam} (MW 46 500) was expressed in *E. coli* DH5 α containing P450_{cam} cDNA and purified as described previously.¹⁹ Poly(diallyldimethylammonium chloride) (PDDA), toluene, and benzaldehyde were from Aldrich. Deoxyribonuclease I (type IV, from Bovine Pancreas), phosphodiesterase I, alkaline phosphatase, styrene, and styrene oxide were from Sigma. Sodium dodecyl sulfate (SDS) was from Acros. Water was treated with a Hydro Nanopure system to a specific resistivity > 16 m Ω cm. All other chemicals were reagent grade.

Voltammetry. CH Instruments 660A and CH 430 electrochemical analyzers were used for square wave voltammetry (SWV), with an ~95% ohmic drop compensated. The thermostated cell employed a saturated calomel reference electrode (SCE), a Pt wire counter electrode, and a film-coated working electrode disk ($A = 0.2 \text{ cm}^2$) 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 and 20 or 50 mM NaCl, pH 5.5. Solutions were purged with purified nitrogen, and a nitrogen atmosphere was maintained for voltammetry.

Film Construction. Films were grown on PG disks that had been abraded on 400 grit SiC paper and on coarse Emery paper (3M Crystal Bay) and then ultrasonicated in water for 30 s. Layers of ds-DNA and protein were adsorbed^{20,21} alternately for 15 min onto the rough PG electrodes and washed with water between adsorption steps. Optimal conditions were derived from previous work.^{14,16,17} Adsorbate solutions were as follows: (a) 2 mg mL⁻¹ DNA in 5 mM pH 7.1 TRIS buffer and 0.50 M NaCl; (b) 2 mg mL⁻¹ PDDA in 50 mM NaCl; (c) 3 mg mL⁻¹ Mb in 10 mM pH 5.5 acetate buffer; and (d) 1 mg mL⁻¹ cyt P450_{cam} in the pH 5.5 buffer. Films of architecture denoted PDDA/DNA/(Mb/DNA)₂ or PDDA/DNA/(cyt P450_{cam}/DNA)₂ were employed.

Film assembly was monitored at each step with a quartz crystal microbalance (QCM, USI Japan) using 9 MHz QCM resonators (AT-cut, International Crystal Mfg.). To mimic the carbon electrode surface used for voltammetry, a partly negative monolayer was made by treating gold-coated ($0.16 \pm 0.01 \text{ cm}^2$) resonators with 0.7 mM 3-mercaptopropanol and 0.3 mM 3-mercaptopropionic acid in ethanol.¹⁴ Films were assembled as for PG electrodes. Resonators were dried in a stream

of nitrogen before measuring the frequency change (ΔF). Adsorbed mass was estimated with the Sauerbrey equation.²¹ For 9 MHz quartz resonators, the dry film mass per unit area M/A is

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

The nominal thickness (d) of dry films was estimated with an expression confirmed by high-resolution electron microscopy:²²

$$d (\text{nm}) \approx (-0.016 \pm 0.002) \Delta F (\text{Hz}) \quad (2)$$

Incubation of Films with Styrene. Incubation of DNA/protein films for metabolite generation and reaction with DNA was done in a thermostated vessel at 37 °C containing 1–4% styrene (by vol). To activate the enzymes for styrene oxide formation, 0.2–2 mM H₂O₂ was added¹⁷ to 10 mL of pH 5.5 buffer containing 50 mM NaCl. After incubation, electrodes were rinsed with water and transferred to pH 5.5 buffer containing 50 μM Ru(bpy)₃²⁺ for analysis by catalytic SWV.¹⁴ For the probe binding method,¹⁶ washed electrodes were transferred to pH 5.5 buffer containing 20 μM Co(bpy)₃³⁺.

Safety Note: Styrene and styrene oxide are suspected human carcinogens and somewhat volatile. Procedures should be done in a closed hood while wearing gloves, using sealed cells.

Capillary Electrophoretic Analysis. A Beckman PACE 5000 with a UV detector at 254 nm was used for capillary electrophoresis (CE) to analyze hydrolyzed DNA that had been reacted with styrene oxide. ST ds-DNA (1 mg mL⁻¹) in 50 mL of buffer or (PDDA/DNA)₂ films on $3 \times 10 \text{ cm}$ carbon cloth in solution were reacted in 50 mL of buffer and 0.87 mmol of styrene oxide at 37 °C with stirring. Reacted DNA in solution or in films was hydrolyzed by incubating with deoxyribonuclease I (0.1 mg/mg of DNA) for 20 h at 37 °C, followed by incubation with phosphodiesterase I (0.01 unit/mg of DNA) and phosphatase, alkaline (0.6 unit/mg of DNA) for 5 h at 37 °C.²³ The resulting samples, consisting mainly of individual nucleosides, were analyzed using CE with 20 mM pH 7 phosphate buffer and 100 mM SDS. The capillary was 75 $\mu\text{m} \times 50 \text{ cm}$, with a polyimide coating. To make standard styrene oxide adducts, guanosine or adenosine (0.5 mg mL⁻¹) were reacted with styrene oxide.

Liquid Chromatography–Mass Spectrometry (LC–MS). A Perkin-Elmer LC with diode-array (255 and 280 nm) and mass spectrometer (Micromass, Quattro II) detection was used. Full-scan spectra were taken at low cone voltage (15 V) in the positive ion mode (ESI). The HPLC column was Restek Ultra C-18 reversed phase (i.d. 2.1 mm, length 10 cm, particle size 5 μm). Solvents were (A) 5% acetonitrile/95% water with 0.05% TFA and (B) 100% acetonitrile with 0.05% TFA. The gradient at 300 $\mu\text{L}/\text{min}$ was 100% A for 5 min, ramped to 100% B in 10 min, and was then 100% B for 10 min. Multiple reaction monitoring (MRM) MS/MS mode employed argon at $1.7 \times 10^{-3} \text{ mmHg}$, cone voltage 15 V, and collision energy 15 eV.

Results

QCM Monitoring of Film Assembly. Films of architecture DNA/(protein/DNA)₂ (see table of contents graphic) were used to strike a balance between high enzyme loading favorable for the catalytic conversion and mass transport limitations that occur with thicker films.²⁴ QCM frequency shifts measured during growth of the films were nearly linear (Figure 1), suggesting regular film growth with reproducible layers of DNA and proteins.

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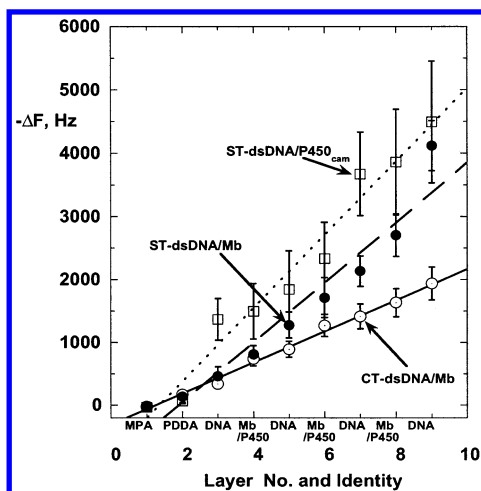


Figure 1. QCM frequency shifts for cycles of alternate Mb/ds-DNA and cyt P450_{cam}/ST ds-DNA adsorption on gold resonators coated with mixed monolayers of mercaptopropionic acid/mercaptopropanol as first layer and PDDA as second layer (av values for nine [Mb/CT ds-DNA] (○), five [Mb/ST ds-DNA] (●), and four [cyt P450_{cam}/ST ds-DNA] (□) replicate films).

Table 1. Average Characteristics of Protein/DNA Films from QCM Results

film	thickness (nm)	wt DNA ($\mu\text{g cm}^{-2}$)	wt protein ($\mu\text{g cm}^{-2}$)
DNA(Mb/CT ds-DNA) ₂	20	3.1	3.6
DNA(Mb/ST ds-DNA) ₂	30	5.8	4.9
DNA(cyt P450 _{cam} /ST ds-DNA) ₂	40	15	2.9

ΔF values and eq 1 were used to obtain weights of protein and DNA. Equation 2 was used to estimate the average nominal thickness of the films (Table 1).

PDDA/DNA/(cyt P450_{cam}/DNA)₂ films were the thickest consistent with the larger size of cyt P450_{cam} compared to Mb. Cyt P450_{cam} layers also adsorbed the most DNA, even though the isoelectric pH of cyt P450_{cam} is 4.6 and the protein is slightly negative under our adsorption conditions. This behavior is consistent with previously observed binding properties of this enzyme, which utilizes localized surface patches of both negative and positive charge.²⁵ PDDA/DNA/(Mb/ST ds-DNA)₂ films contained more protein and DNA and were 50% thicker than PDDA/DNA/(Mb/CT ds-DNA)₂ films (Table 1).

Voltammetric Response to Metabolite Generation. When myoglobin or cyt P450_{cam} in polyion films are activated by hydrogen peroxide, styrene is converted to styrene oxide. The iron heme in the protein is oxidized by hydrogen peroxide to an oxyferryl intermediate, which transfers an oxygen atom to the olefinic double bond of styrene. This reaction proceeds when DNA is the polyion.^{24,26} Even though styrene oxide reacts with DNA, it can be detected in significant amounts after 1 h of reaction. In the present work, we added hydrogen peroxide to solutions containing styrene so that the protein in the film would catalyze the production of styrene oxide to react with DNA. Reactions were done at pH 5.5, since this pH was the previously established optimum for the reaction of styrene oxide with DNA²⁷ and also gives adequate turnover for the epoxidation.²⁴

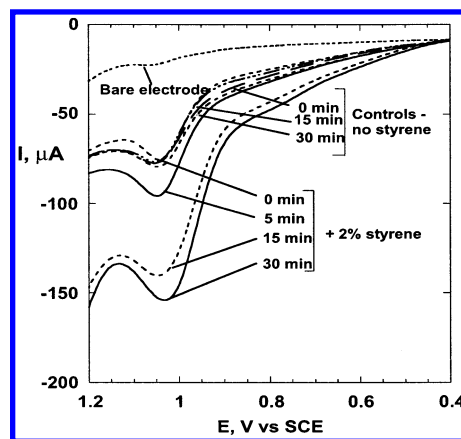
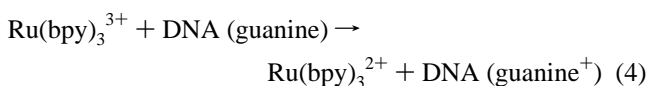
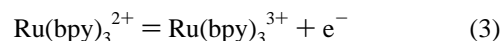


Figure 2. Square wave voltammetry of PDDA/ds-DNA/(Mb/ds-DNA)₂ films on rough PG in pH 5.5 buffer containing 50 μM Ru(bpy)₃²⁺ before and after incubations at 37 $^{\circ}\text{C}$ with 2% styrene (no styrene in controls) and 0.2 mM H₂O₂ in aerobic buffer solution (SWV amplitude, 25 mV; frequency, 15 Hz; step, 4 mV).

In the first DNA analysis method, we used square wave voltammetry with a small amount of ruthenium tris(2,2'-bipyridyl) [Ru(bpy)₃²⁺], which increases sensitivity by catalytically oxidizing guanines in DNA (eqs 3 and 4).²⁸ When ds-DNA in the film is damaged by styrene oxide, guanines released from the protection of the double helix as well as adducts of adenine become easily oxidized and the catalytic current (eqs 3 and 4) increases.^{14,15}



After reaction in 0.2 mM hydrogen peroxide/2% styrene, Mb/DNA electrodes were washed and then placed into a solution of 50 μ M Ru(bpy)₃²⁺. Catalytic SWV oxidation peaks were observed at about 1 V versus SCE, and the peak height (negative peaks, Figure 2) increased with the reaction time of the film. Control electrodes which were incubated in hydrogen peroxide without styrene showed catalytic oxidation peaks of similar heights to freshly prepared films. This confirms that this low concentration of peroxide has a minimal influence on the intact ds-DNA, which still reacts at a finite rate with Ru(bpy)₃²⁺. The peaks for electrodes that had been activated by hydrogen peroxide with styrene present increased with reaction time, presumably because of the disruption of the double helix by the formation of adducts of DNA bases with styrene oxide. The heights of these peaks did not depend on styrene concentrations between 1 and 4%. Concentrations of hydrogen peroxide of 2 and 10 mM gave larger peaks but smaller increases with time and larger control peaks. A hydrogen peroxide concentration of 0.2 mM gave the largest differences between reacted samples and controls.

Average SWV catalytic peak currents for ds-DNA/Mb films increased with reaction time at relatively larger rates for the

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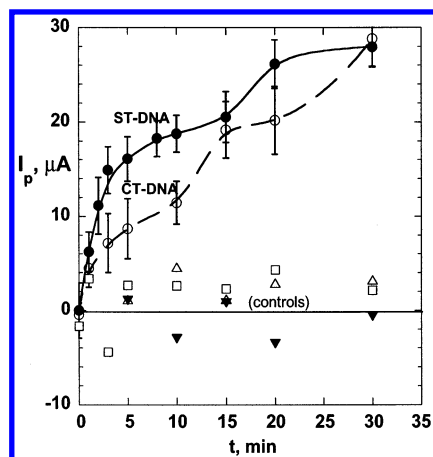


Figure 3. Influence of reaction time with 2% styrene + 0.2 mM H_2O_2 on catalytic peak current in 50 μM $\text{Ru}(\text{bpy})_3^{2+}$ for PDDA/ds-DNA/(Mb/ds-DNA)₂ films using CT DNA (○) and ST DNA (●) (5–15 trials per data point). Also shown are controls representing the incubation of ST DNA/Mb films with 2% styrene but no H_2O_2 (□), 2% toluene + 0.2 mM H_2O_2 (▼), and 0.2 mM benzaldehyde + 0.2 mM H_2O_2 (△) (av peak current for unreacted DNA/Mb electrodes was subtracted).

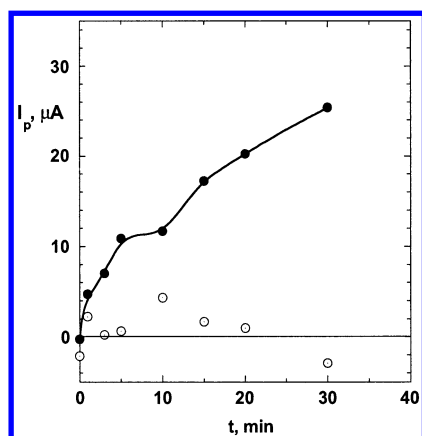


Figure 4. Influence of reaction time with 2% styrene + 0.2 mM H_2O_2 on the average catalytic peak current for PDDA/ST ds-DNA/(cyt P450_{cam}/ST ds-DNA)₂ films (●) in 50 μM $\text{Ru}(\text{bpy})_3^{2+}$ solution. Control (○) is for incubation with 0.2 mM H_2O_2 .

first 5 min then at lower rates at longer times (Figure 3). Larger initial rates of peak current increase were found with Mb/ST ds-DNA films than with Mb/CT ds-DNA films. Error bars in Figure 3 are mainly the result of film-to-film variability. No significant increases in peak current or trends with incubation time were found when films were incubated with toluene and hydrogen peroxide, styrene alone, benzaldehyde, or hydrogen peroxide alone (Figure 3, controls).

Figure 4 shows that similar results were obtained when DNA-(cyt P450_{cam}/ST ds-DNA)₂ films were incubated with styrene and hydrogen peroxide. Again, a rapid increase in SWV peak current for the first 5 min was followed by a slower increase from 5 to 30 min. However, the rate of increase was about 30% less than that with Mb/ST DNA films (cf. Figure 3). Controls with hydrogen peroxide alone gave no significant increases in peak current.

Our second DNA-damage detection method employed tris-(2,2'-bipyridyl)cobalt (III) [$\text{Co}(\text{bpy})_3^{3+}$] as an electroactive probe, which binds more strongly to intact ds-DNA in films compared to DNA damaged by styrene oxide.¹⁶ In these studies, we found that the SWV peak decreases after reaction with 0.2

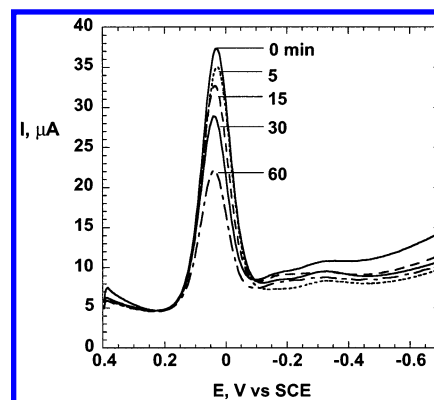


Figure 5. SWV of PDPA/ST ds-DNA/(Mb/ST ds-DNA)₂ films on rough PG reacted at 37 °C with 4% styrene and 2 mM H_2O_2 at pH 5.5 and then transferred to 20 μM $\text{Co}(\text{bpy})_3^{3+}$ in pH 5.5 buffer (SWV amplitude, 25 mV; frequency, 15 Hz; step, 4 mV). One electrode was used for each assay.

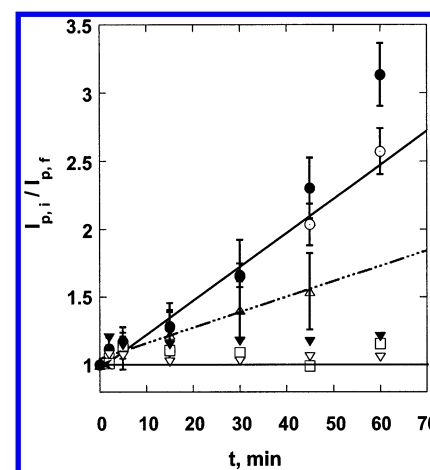


Figure 6. Influence of reaction time with 4% styrene + 2 mM H_2O_2 for PDPA/ds-DNA/(Mb/ds-DNA)₂ films using CT DNA (●) and ST DNA (○) and for PDPA/CT ds-DNA/(cyt P450_{cam}/CT ds-DNA)₂ (△) on peak current in 20 μM $\text{Co}(\text{bpy})_3^{3+}$. Also shown are controls representing incubations of ST DNA/Mb films with 4% styrene but no hydrogen peroxide (□), 4% toluene + 2 mM hydrogen peroxide (▼), and 50 μM benzaldehyde with no hydrogen peroxide (▽). Error bars represent standard deviations for five replicates.

mM hydrogen peroxide and 2% styrene were not large enough to be useful. The optimum concentrations were 2 mM hydrogen peroxide and 4% styrene. After incubation of films in this medium for a given time, electrodes were washed and placed into 20 μM $\text{Co}(\text{bpy})_3^{3+}$. Figure 5 shows that the largest peaks for the reduction of $\text{Co}(\text{bpy})_3^{3+}$ at 0.04 V versus SCE were found when the ds-DNA in the film was intact, because the probe binds in the largest amounts to these films. The peak height decreased with incubation time, suggesting the gradual loss of the film's ability to bind the probe. The small peak at ca. -0.3 V versus SCE represents the $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ reduction of the protein.

The time course of the reaction can readily be monitored via the ratio of initial SWV peak current for a given film ($I_{p,i}$) to that after reaction ($I_{p,f}$). With Mb in the films, similar linear increases of the $I_{p,i}/I_{p,f}$ ratio with time were observed over 45 min (Figure 6). In this case, ST and CT ds-DNA showed similar slopes of peak ratio versus reaction time (solid line) with Mb in the film. Films containing cyt P450_{cam} gave smaller slopes of peak ratio versus time (dashed line), perhaps because of smaller molar amounts of active enzyme in these films compared

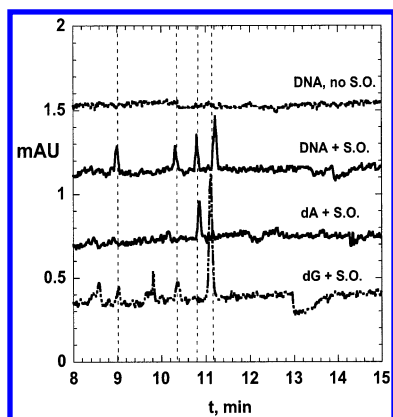


Figure 7. Partial capillary electropherograms showing the possible peaks of DNA–styrene oxide adducts of enzyme hydrolyzed (PDDA/ST ds-DNA)₂ films after incubation with styrene oxide (S.O.) for 48 h at 37 °C, in comparison to samples of guanosine (dG) and adenosine (dA) after reaction with styrene oxide. Dashed lines identify peaks common to reacted samples of DNA and dG or dA. The much larger peaks of unreacted nucleosides occur at $t_R < 8$ min (not shown).

to Mb films. Control experiments involving the incubation of films in toluene and hydrogen peroxide, benzaldehyde, or hydrogen peroxide alone gave no significant increases or trends in peak current ratios with incubation time. (Figure 6).

Confirmation of DNA–Styrene Oxide Adducts. We previously showed that styrene oxide is formed from styrene using Mb and cyt P450_{cam} films.^{17,24} However, available capillary electrophoresis and HPLC–MS/MS methods were not sensitive enough to detect and identify damaged DNA directly from the protein–DNA films. Thus, we used capillary electrophoresis and HPLC–MS to confirm that the reaction of styrene oxide with DNA in (PDDA/DNA)₂ films and in solution under our conditions gave the reported DNA–styrene oxide adducts. After incubation of ds-DNA with saturated styrene oxide, DNA was hydrolyzed enzymatically to the individual nucleosides. Figure 7 demonstrates capillary electrophoretic analyses of ST ds-DNA in a (PDDA/DNA)₂ film on carbon cloth reacted with styrene oxide. When the capillary electropherograms of undamaged hydrolyzed ST ds-DNA that showed only the four unreacted DNA nucleosides are compared, there were new peaks evident at retention times (t_R) between 8 and 12 min.

Peaks for the damaged hydrolyzed DNA at $t_R = 9.0$, 10.4, and 11.2 min also appeared in a deoxyguanosine sample that had been reacted with styrene oxide. The peak at $t_R = 10.8$ min corresponds to a peak in a deoxyadenosine sample that had been reacted with styrene oxide. Similar results were obtained when DNA was in solution or in these films.

Confirmatory results were obtained by HPLC–MS/MS. UV–HPLC data of damaged DNA showed the suspected dG and dA adducts as a series of peaks at $t_R = 15$ –21 min, with the major peak in this group at 18 min. Electrospray ionization mass spectral detection with a display of mass at 388 M/e, corresponding to dG–styrene oxide adducts (see Supporting Information), showed a major peak at $t_R = 18$ min, and the 372 M/e display, corresponding to dA–styrene oxide adducts, gave a major peak at about 17.8 min. In the multiple reaction monitoring (MRM) mode, the most significant daughter ions for both of these peaks corresponded to the loss of a sugar group ($m/z = 116$) from the parent ions. These experiments showed at least four styrene oxide adducts each for dA and dG (see

Supporting Information). These results confirm the formation of dA–styrene oxide and dG–styrene oxide adducts under reaction conditions similar to those used for the films. However, the sensitivity limitations of these methods required reaction times much longer than those for the SWV methods.

Discussion

We showed previously that the activation of layered polyanion films of cyt P450_{cam} and Mb with H₂O₂ produces nanomole quantities of styrene oxide and benzaldehyde per hour from styrene.^{17,24} The enzyme-catalyzed product is styrene oxide, resulting from oxygen transfer from an oxyferryl enzyme intermediate presumably formed by reaction of the ferric enzyme with peroxide.²⁹ Benzaldehyde formation from styrene and hydrogen peroxide is not enzyme catalyzed. Results in Figures 2–6 suggest that styrene oxide formed within PDDA/ds-DNA (enzyme/ds-DNA)₂ films damages DNA, and the resulting DNA damage can be detected by catalytic or probe binding voltammetric assays. Our view is that formation of styrene oxide within the nanometer thickness films in high local concentrations very close to the DNA facilitates reactions with DNA bases before the epoxide can exit the film.

Control studies show (Figures 2, 3, 4, and 6) that the changes in the SWV peaks with time do not occur from the effect of hydrogen peroxide alone, benzaldehyde, or toluene and hydrogen peroxide. This suggests that SWV signal changes after reaction in styrene/peroxide solutions result from styrene oxide production. We previously reported that SWV signals changed in similar ways when DNA–polyion and DNA–protein films were reacted with the metabolite styrene oxide.^{14–16} In that work, the catalytic SWV signals were shown to be due to the oxidation of guanines and adenine–styrene oxide adducts in the damaged, partly unfolded DNA.¹⁴ Further, electrophoretic and HPLC–MS/MS analyses confirmed that styrene oxide adducts with adenine and guanine bases in DNA form under our reaction conditions. Thus, it is very likely that the increased catalytic SWV peaks (Figures 2–4) and peak ratios for Co(bpy)₃³⁺ in the probe method (Figures 5 and 6) result from the damage of DNA by styrene oxide.

The catalytic SWV method develops signals at lower hydrogen peroxide concentrations and at shorter incubation times than the probe method, as seen by comparing Figures 3 and 4 with Figure 6. In general, the catalytic approach may prove to be the most sensitive for toxicity screening. In addition, results in Figure 3 showing faster signal development with ST DNA films than with CT DNA suggest that the method may allow discrimination between different source types of DNA. However, the probe method has the advantage of a very low redox potential (0.04 V vs SCE), where few interferences would be oxidized or reduced. Thus, the probe method may be essential where such interferences occur, for example, a DNA base adduct that is electroactive. The probe method seems to be relatively insensitive to the type of DNA, but the damage signals take longer to develop.

For both methods, films containing Mb show a slightly more rapid change in signal versus time (Figures 3, 4, and 6). We showed previously that at 4 °C cyt P450_{cam} and Mb at pH 5.5–6

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have similar turnover rates for hydrogen peroxide mediated styrene oxidation.²⁴ However, from the enzyme weights (Table 1) we estimate that the ST ds-DNA/(Mb/ST ds-DNA)₂ films contain 0.06 nmol of protein, while ST ds-DNA/(cyt P450_{cam}/ST ds-DNA)₂ films contain only 0.012 nmol of this larger enzyme. These results suggest that the less expensive more readily obtained Mb may be a good choice for some screening applications as a substitute for the more metabolically correct cyt P450s. However, interindividual variability in cyt P450s are important in toxicity studies.³⁰ Thus, we are currently attempting to incorporate larger amounts of these enzymes into DNA films to facilitate comparative in vitro kinetic studies.

In summary, our results show that ultrathin films of DNA and proteins that catalyze toxic metabolite formation can be used for voltammetric detection of metabolite-generated DNA damage. SWV using soluble Ru(bpy)₃²⁺ as catalyst was the most sensitive, but a method employing binding of Co(bpy)₃³⁺ may be complementary in situations where oxidizable interferences are encountered. From the QCM results, the films used in this

work contained 0.6–3 μg of DNA and 0.6–1 μg of enzyme on 0.2 cm² of electrodes. These amounts might be decreased ca. 20 000-fold by using ultramicroelectrodes of a 10 nm diameter. As described herein, the method would not be used for biological samples but for pure organic compounds. It remains to validate the method with many more organic compounds producing genotoxic and nontoxic metabolites and to fine-tune the sensitivity. However, we have shown in preliminary studies that our general approach is also applicable to DNA methylating agents. Thus, we feel that the methodology presented shows promise for the in vitro screening of toxicity of organic metabolites and their parent compounds.

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Supporting Information Available: Three additional figures presenting HPLC–MS results on DNA samples reacted with styrene oxide and then hydrolyzed. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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(30) For general discussion^{30a,b} and results on styrene metabolism,^{30c,d} see: (a) Geungerich, F. R. *Trends Pharmacol. Sci.* **1989**, *10*, 107–109. (b) Gonzalez, F. J. *Trends Pharmacol. Sci.* **1992**, *13*, 346–352. (c) Nkajima, T.; Elovaara, E.; Gonzalez, F. J.; Gelboin, H. V.; Raunio, H.; Pelkonen, O.; Vaino, H.; Aoyama, T. *Chem. Res. Toxicol.* **1994**, *7*, 891–896. (d) Wenker, M. A. M.; Kezic, S.; Monster, A. C.; De Wolff, F. A. *Xenobiotica* **2001**, *31*, 61–72.