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Square Wave Voltammetric Detection of Chemical DNA Damage with Catalytic Poly(4-Vinylpyridine)−$Ru(bpy)_2^{2+}$ Films

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Reversible, catalytic films of poly(4-vinylpyridine)−Ru(bpy)$_2^{2+}$ [PVP−Ru(bpy)$_2^{2+}$, bpy = 2,2′-bipyridine] on pyrolytic graphite (PG) electrodes were evaluated for the detection of damage to double-stranded (ds) DNA by using square wave voltammetry (SWV). Damage of both calf thymus and salmon testes ds-DNA in solution was induced by incubation of DNA at 37 °C with styrene oxide, the liver metabolite of styrene, and a suspected carcinogen. Both types of ds-DNA incubated in solution with saturated styrene oxide gave a linear increase in catalytic peak current up to 30 min, and an estimate of two damaged DNA bases in one thousand could be detected. The increase in catalytic current is attributed to better access of the catalyst redox sites to oxidizable bases in the damaged, partly unwound DNA. A self-contained "toxicity sensor" was also evaluated, which consisted of films of [PVP−Ru(bpy)$_2^{2+}$] on PG electrodes coated with films of ds-DNA and polydiallyldimethylammonium polycations assembled layer-by-layer. These films also gave an increase in catalytic peak current upon incubation in saturated styrene oxide, and an estimate of 1 damaged base in 1000 could be detected. Control films or solutions of ds-DNA treated in buffer or buffer containing unreactive toluene resulted in no significant changes in the catalytic peak current with incubation time.

Human liver cytochrome P450 enzymes convert lipophilic pollutants and drugs to metabolites that can often form covalent adducts with DNA, constituting a major mechanism for chemical toxicity.[1−3] Adducts formed by metabolites with DNA serve as important markers of human exposure to environmental and occupational mutagens and carcinogens.[4−7] With the advent of combinatorial chemistry, extremely large numbers of new organic chemicals are synthesized annually. Rapid in vitro methods to detect chemical DNA damage would be very useful for toxicological screening of these new chemicals and their metabolites.

Voltammetry is an attractive approach for rapid, inexpensive assays of damaged DNA and has been explored for DNA in solution using mainly mercury electrodes.[8,9] The problem of sensing chemical DNA damage is similar to sensing DNA hybridization, for which a variety of electroanalytical schemes have been devised.[10−17] The key in detecting hybridization is to distinguish intact double stranded (ds) DNA from single stranded DNA, which is related to distinguishing ds-DNA from chemically damaged DNA that has partly unwound. We reported using derivative square wave voltammetry to detect DNA damage in films by reaction of DNA with styrene oxide.[18] More recently, we improved sensitivity by using catalytic SWV[19] with $Ru(bpy)_3^{2+}$ or direct SWV using $Co(bpy)_3^{3+}$ as a binding probe.[20] These methods employed soluble metal complexes and were estimated to be capable of detecting 0.05−0.1% damaged bases in 6-nm-thick layered polyion/ds-DNA films.

In a preliminary report, we showed that films of the electrochemically reversible metallopolyion poly(4-vinylpyridine)−$Ru(bpy)_2^{2+}$ [PVP−$Ru(bpy)_2^{2+}$] adsorbed to ordinary pyrolytic graphite electrodes could be used to distinguish between double- and single-stranded (ss) DNA.[21] The catalytic oxidation pathway is similar to that elucidated by Thorp and co-workers,

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who showed that $Ru(bpy)_3^{3+}$ specifically oxidizes guanine in DNA and oligonucleotides[22,23] in the following electrochemical catalytic pathway:

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

$$Ru(bpy)_3^{3+} + DNA(guanine) \rightarrow Ru(bpy)_3^{2+} + DNA(guanine^+) \quad (2)$$

Cycling $Ru(bpy)_3^{3+}$ back to $Ru(bpy)_3^{2+}$ by the fast chemical step in eq 2 yields a kinetically dependent catalytic current in voltammetry that is enhanced over that of $Ru(bpy)_3^{2+}$ or DNA alone. Better accessibility of the guanine bases in ss-DNA provides larger catalytic peak currents by voltammetry than for ds-DNA, in which the guanines are partly protected in the double helix.

In this paper, we report the SWV detection of chemically induced damage of ds-DNA in solution by using poly(4-vinylpyridine)−$Ru(bpy)_2^{2+}$ films on pyrolytic graphite (PG) electrodes. DNA was damaged by incubation with known damage agent styrene oxide, the cytochrome P450 derived metabolite of styrene. In addition, films assembled layer-by-layer from DNA and polydiallyldimethylammonium ions on top of the metallopolymer on electrodes were used to detect DNA damage in a self-contained catalytic electrode not requiring a soluble catalyst or probe.

EXPERIMENTAL SECTION

Chemicals and Apparatus. Double-stranded calf thymus (CT) DNA (type XV, ∼10 K base pairs, 41.9% G/C), salmon testes (st) ds-DNA (∼2 K base pairs, 41.2% G/C) were from Sigma. Ruthenium chloride (99.9%) was from Fluka. Poly(diallyldimethylammonium chloride) (PDDA) and poly(4-vinylpyridine) (PVP, av M_w 60 000) were from Aldrich. Styrene oxide was from Sigma. Water was purified with a Hydro Nanopure system to specific resistance >15 mΩ-cm. Synthesis, purification and characterization of *cis*-dichloro-bis(2,2′-bipyridine)ruthenium followed a procedure in the literature.[24] All other chemicals were reagent grade.

A CH instruments 660A electrochemical analyzer was used for square wave voltammetry (SWV). A three-electrode thermostated cell employed a saturated calomel electrode (SCE), pyrolytic graphite (PG) working electrode and Pt wire counter electrode. SWV was performed at 25 °C, 4 mV step height, 25 mV pulse height, and 5 Hz frequency, with ohmic drop 98% compensated. The buffer for most analytical work was 20 mM acetate pH 5.5 + 50 mM NaCl. All solutions were purged at least 5 min with purified nitrogen before SWV.

Film Assembly. PG disk electrodes (Advanced Ceramics, $A = 0.16$ cm^2) were abraded manually on 400 grit SiC paper, ultrasonicated in ethanol for 30 s and in water for 1 min, then rinsed with water. Electrodes were then immersed in 5% poly(4-vinylpyridine) [PVP] in methanol for 5 min, washed with methanol and water, and dried.[21] The electrode was then immersed for 15 min in 20 mM *cis*-dichloro-bis(2,2′-bipyridine)ruthenium [$Ru(bpy)_2^{2+}$] in the dark, then washed with water and dried.

Films of PDDA and ds-DNA were constructed layer-by-layer on top of the [PVP−$Ru(bpy)_2^{2+}$] layer by alternate electrostatic adsorption.[19,20,25−27] Layers were constructed by alternately dipping electrodes into solutions 0.5 mg mL^{-1} ds-DNA and 2 mg ml^{-1} PDDA for 15 min to allow adsorption saturation, washing with water between adsorption steps.

Incubation of ds-DNA with Styrene Oxide. ***Safety note:*** *Styrene oxide is a suspected human carcinogen and somewhat volatile. Gloves were worn, and all manipulations were performed under a closed hood. All reactions were performed in closed vessels in closed hoods.*

To solutions of ds-DNA (0.2 mg/mL, 9 mL) pH 5.5, 120 μL of neat styrene oxide was added, and the mixture was incubated at 37 °C with stirring. Some experiments were also performed using 50 μL of styrene in 2 mL of buffer with similar results. All of these mixtures are emulsions. After 30 min, unreacted styrene oxide was extracted with 3 × 200 μL ethyl ether. A [PVP−$Ru(bpy)_2^{2+}$]-coated PG electrode was used to analyze the remaining clear aqueous solution containing DNA. In control experiments, ds-DNA was incubated with pure buffer and 100 μL of toluene in 9 mL of buffer. Films of $[Ru(bpy)_2-PVP]^{2+}$ coated with $(DNA/PDDA)_3$/DNA were incubated in stirred 5 mL of pH 5.5 buffer + 75 μL styrene oxide at 37 °C. Before SWV, incubated films were rinsed with water and dried in a stream of nitrogen, then analyzed in fresh buffer without styrene oxide.

RESULTS

Analysis of Damaged DNA in Solution. Styrene oxide is the major enzyme-generated liver metabolite of styrene. It forms covalent adducts with ds-DNA in vitro and in vivo.[28] Addition to guanine at positions N7, N2, and O6 account for 95% of all adducts[4−7,18,29,30] A small fraction of styrene oxide adducts of DNA also form with adenine.[31] Under our incubation conditions (37 °C), the optimal rate of DNA damage by styrene oxide occurs at pH 5.5. We recently found evidence for at least 8 different adducts after incubation of DNA with styrene oxide by capillary electrophoresis of hydrolyzed samples.[18,20]

As reported previously, [PVP−$Ru(bpy)_2^{2+}$] films on PG electrodes consistently give reversible forward and reverse SWV and CV peaks at potentials centered at about 0.74−0.78 vs SCE at pH 5.5.[21] Using our preparation methods, the films contained ∼0.075 nmol cm^{-2} electroactive centers. Figure 1a shows difference SWVs

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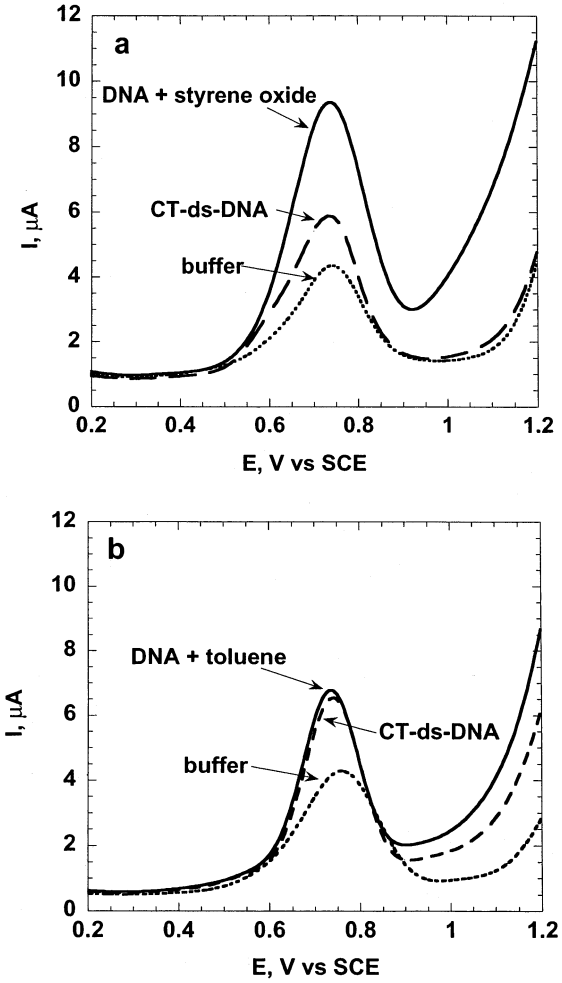

Figure 1. Difference square wave voltammograms at 5 Hz of [PVP−$Ru(bpy)_2^{2+}$] films in pH 5.5 buffers and in buffers containing (a) 0.2 mg mL^{-1} CT ds-DNA and the same solution after incubation for 30 min at 37 °C with saturated styrene oxide (120 μL in 9 mL); (b) 0.2 mg mL^{-1} CT ds-DNA and the same solution after incubation for 30 min at 37 °C with saturated toluene.

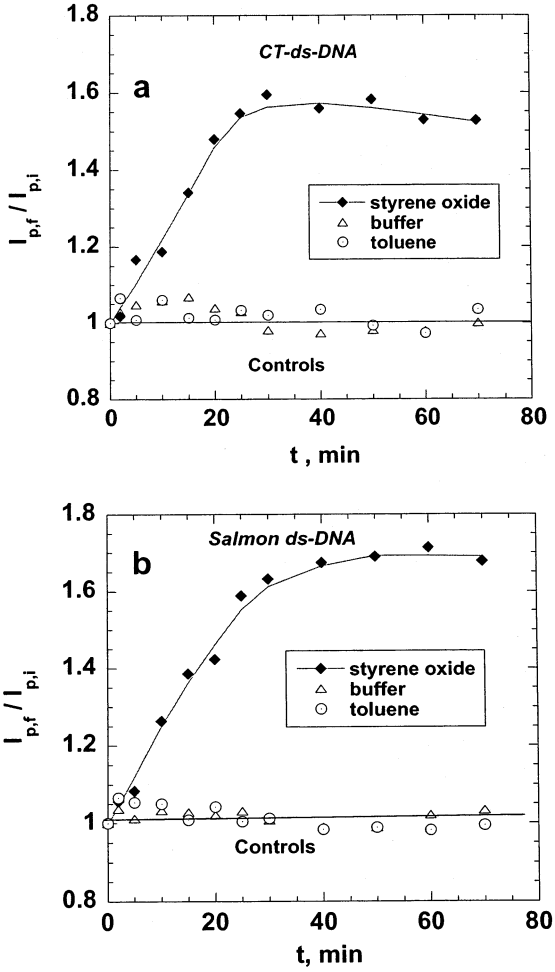

Figure 2. Influence of time on the ratios of final SWV peak current (5 Hz) of [PVP−$Ru(bpy)_2^{2+}$] films for solutions of two types of ds-DNA after incubations of DNA in saturated solutions of styrene oxide, toluene, and in pure buffers to the initial peak current in the same solution before incubation.

of such films in pure buffer, buffer containing intact CT ds-DNA, and CT ds-DNA that was incubated for 30 min with styrene oxide. An increase in current for the Ru^{2+}/Ru^{3+} oxidation peak in the film was observed in solutions of intact ds-DNA, consistent with a catalytic process. The peak current increased after ds-DNA was incubated with styrene oxide. There were no significant changes in the peak potential for incubated and nonincubated films.

Washing the films with water after SWV in damaged and undamaged DNA solutions resulted in the original voltammogram being reproduced within ±5%, as reported previously for ss- and ds-DNA solutions.[21] One coated electrode can be used for 20 or more analyses. Catalytic peak currents obtained as a result of ds-DNA incubation with toluene were within experimental error of those in intact ds-DNA solutions (Figure 1b). Toluene does not form adducts with DNA[19,20] and serves as a control. In addition, we did not observe direct oxidation peaks in DNA solutions between 0.2 and 1.1 V vs SCE using [PVP−$Ru(bpy)_2^{2+}$] electrodes.

SWV catalytic peak currents increased with time of incubation with styrene oxide for CT ds-DNA and for salmon testes DNA solutions and reached limiting plateaus after 30 min. These data are expressed in Figure 2 as plots of the ratio of final peak current $I_{p,f}$ at any incubation time to the initial peak current $I_{p,i}$ in the intact ds-DNA solution, which helps correct for electrode-to-electrode variations. For the linear portions of these plots at $t<30$ min, CT DNA gave a slope of 0.021 min^{-1} with a correlation coefficient of 0.974, and salmon testes DNA gave a slope of 0.022 min^{-1} with a correlation coefficient of 0.981. SWV of DNA solutions incubated in buffer alone and in buffer with toluene gave nearly constant peak current ratios, suggesting that ds-DNA is not significantly damaged under these conditions, even after 60 min (Figure 2).

We reported earlier that the rate of CT ds-DNA damage as detected by derivative SWV was optimum at pH 5.5.[25] However, the formal potentials of the metallopolymer films used for analysis here were also pH-dependent (Figure 3). This pH dependence had a slope of 43 mV pH^{-1} below pH 5, and was less dependent on pH above pH 5. The break point of this dependence corresponds[32] to an apparent p*K* of 5, which is about one pH unit above the p*K* of dissolved PVP. It is likely that this pH dependence arises from protonation of the polymer on the surface of the electrode.

The practical significance of this pH dependence of the metallopolymer's formal potential is that the rate of the catalytic

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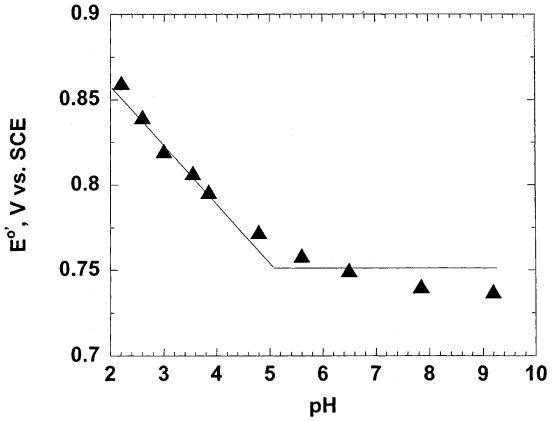

Figure 3. Influence of pH on the formal potential of [PVP−Ru(bpy)$_2^{2+}$] films from SWV at 5 Hz.

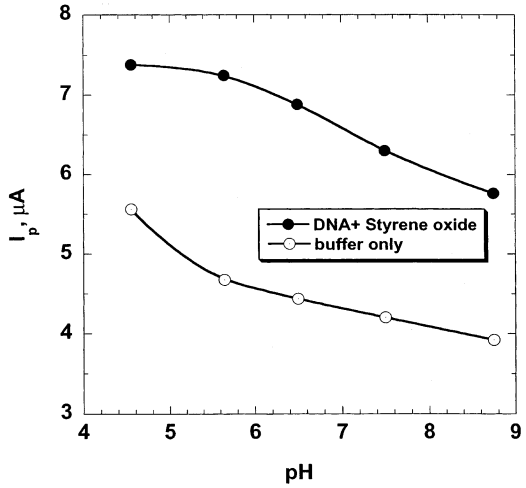

Figure 4. Influence of pH on the peak current of [PVP−Ru(bpy)$_2^{2+}$] films from SWV at 5 Hz in buffers and in 0.2 mg mL^{-1} CT ds-DNA incubated for 30 min with saturated styrene oxide.

reactions between [PVP−Ru(bpy)$_2^{3+}$] and the guanines in DNA should increase as the formal potential of polymer becomes more positive.[33] Since catalyst formal potential could influence the magnitude of the peak current, we investigated pH effects on the SWV current for damaged DNA solutions at different pH values. We found an increase in peak current as pH decreased from 9 to ~5.6 (Figure 4), with a relatively constant value between pH 5.6 and 4.5. Since low pH values could lead to styrene oxide hydrolysis and might adversely influence DNA structure, we used pH 5.5 for the majority of damage studies.

Analysis of Damaged ds-DNA Films. The versatile method of layer-by-layer electrostatic adsorption of oppositely charged polyions[19,26,27] was used to construct films of cationic PDDA and anionic ds-DNA on top of the [PVP−Ru(bpy)$_2^{2+}$] layer on PG electrodes. Figure 5 represents the architecture of the films denoted [PVP−Ru(bpy)$_2^{3+}$]/(ds-DNA/PDDA)$_3$/ds-DNA that were used. These films were stable for several weeks when stored at 5 °C. From our previous quartz crystal microbalance studies of CT-ds-DNA/PDDA multilayer films,[19] we estimate that the (ds-DNA/PDDA)$_3$/ds-DNA coatings are roughly 12 nm thick.

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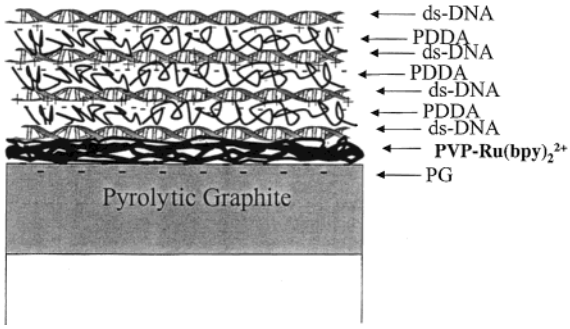

Figure 5. Conceptual cartoon representing PVP−Ru(bpy)$^{2+}$/(CT ds-DNA/PDDA)$_3$/CT ds-DNA films.

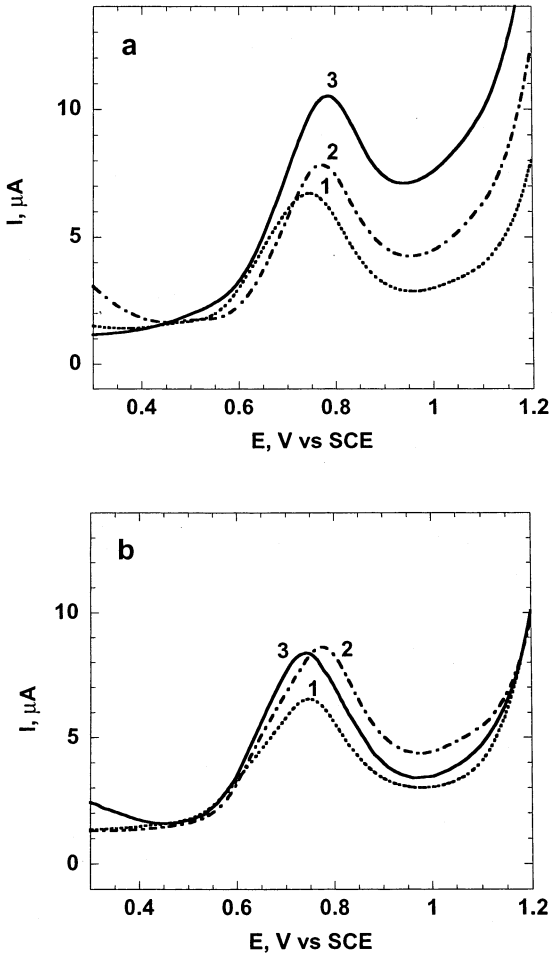

Figure 6. Difference SWV at 5 Hz of films at pH 5.5. Both curves 1 are for PVP−Ru(bpy)$^{2+}$ films in buffer, and (a) PVP−Ru(bpy)$^{2+}$/(CT-dsDNA/PDDA)$_3$/CT-ds-DNA film before (curve 2) and after incubation (curve 3) for 30 min in saturated styrene oxide at 37 °C (b) PVP−Ru(bpy)$^{2+}$/(CT-dsDNA/PDDA)$_3$/CT-ds-DNA film before (curve 2) and after incubation (curve 3) for 30 min in saturated toluene at 37 °C.

Figure 6 compares the SWV peak currents of PVP−Ru(bpy)$^{2+}$ and PVP−Ru(bpy)$^{2+}$/(CT ds-DNA/PDDA)$_3$/CT ds-DNA in pH 5.5 buffer. The current was a bit larger for the films with DNA overlayers, consistent with catalytic oxidation of the guanines in the double helix structure via eqs 1 and 2. Catalytic current of the PVP−Ru(bpy)$^{2+}$/(CT ds-DNA/PDDA)$_3$/CT ds-DNA films decreased gradually upon repetitive scanning because of the diminishing number of guanines available to be oxidized on the electrode.

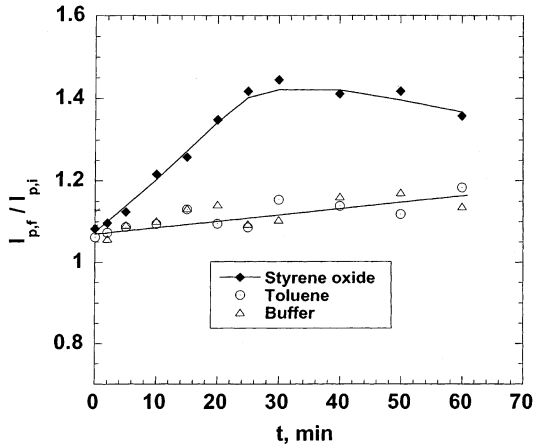

Figure 7. Influence of time on the ratio of final SWV peak current (5 Hz) after incubation of films of PVP−Ru(bpy)$^{2+}$/(CT ds-DNA/PDDA)$_3$/CT ds-DNA in saturated solutions of styrene oxide, toluene, and in pure buffers to the initial peak current of PVP−Ru(bpy)$^{2+}$ (no DNA) in pH 5.5 buffer.

Figure 6a shows that the catalytic peak current increased after PVP−Ru(bpy)$^{2+}$/(CT ds-DNA/PDDA)$_3$/CT ds-DNA films were incubated with styrene oxide. The peak current after 30 min incubation was almost twice the initial peak current of the metallopolymer film alone. Control experiments involving incubating the film with toluene did not yield any catalytic signal enhancement (Figure 6b), but small peak shifts were found.

To account for electrode-to-electrode reproducibility (roughly ±10%, judging from peak currents), we compared the ratio of SWV peak currents after incubations of PVP−Ru(bpy)$^{2+}$/(CT ds-DNA/PDDA)$_3$/CT ds-DNA films to those of the underlying PVP−Ru(bpy)$^{2+}$ layer alone. The ratio of final current to initial metallopolymer current (∼± 4%) for given films was plotted vs time of incubation (Figure 7). This current ratio increased linearly with time of styrene oxide incubation up to about 25 min, with a slope of 0.013 min^{-1} and a correlation coefficient of 0.995. There was a slight decrease in the catalytic peak current for incubations longer than 30 min. Films incubated in buffer or in buffer containing toluene showed negligible changes in catalytic peak currents.

DISCUSSION

Results in Figures 1, 2, 6, and 7 clearly show that PVP−Ru(bpy)$^{2+}$ films on PG electrodes give increased catalytic SWV responses to DNA treated with the known damage agent styrene oxide, but no current increases when treated with unreactive toluene in control incubations. We can correlate the increased current for DNA incubated with styrene oxide with capillary electrophoresis results, showing that under our conditions, 1.2% damage/h occurs (see below) for the first several hours of the reaction.[20] Thus, results demonstrate that SWV using reversible catalytic PVP−Ru(bpy)$^{2+}$ films on PG electrodes is a viable method for detecting damage of DNA from reactive metabolites both in solutions and in films of DNA. Furthermore, PVP−Ru(bpy)$^{2+}$ film electrodes can be repeatedly used in solution studies[21] and can be stored for up to 3 months at 5 °C without significant degradation of catalytic activity.

In the solution experiments, after 10 min of incubation with styrene oxide, the SWV peak current ratio $I_{p,f}/I_{p,i}$ for CT and salmon testes ds-DNA samples was 3-fold greater than the average peak current ratio for controls (Figure 2). Comparisons with the capillary electrophoresis results discussed above shows that this 10-min incubation time corresponds to a practical detection limit of the SWV method of ∼0.2% damage or 2 damaged bases/1000.

DNA damage after styrene oxide incubation was also detected in films of PVP−Ru(bpy)$^{2+}$/(CT ds-DNA/PDDA)$_3$/CT ds-DNA grown layer-by-layer, with a linear plots of $I_{p,f}/I_{p,i}$ vs incubation time up to ∼25 min. For these films, the SWV $I_{p,f}/I_{p,i}$ ratio for films incubated with styrene oxide was 3-fold greater than controls after ∼5 min, corresponding to a practical detection limit of 0.1% damage or 1 damaged base/1000. These films also have the advantage of containing the immobilized catalyst in the film along with the DNA, and they are stable under storage for several weeks at 5 °C. However, these films are limited to a single use, since the styrene oxide and catalytic oxidation reactions use up significant DNA.

The slope of $I_{p,f}/I_{p,i}$ vs time for PVP−Ru(bpy)$^{2+}$/(CT ds-DNA/PDDA)$_3$/CT ds-DNA films incubated with styrene oxide was 0.013 min^{-1}, as opposed to ∼0.022 min^{-1} in the solution studies. This is consistent with slightly less reactivity of the surface-bound ds-DNA that would be partly protected by having a fraction of its reactive sites bound to the underlying PDDA layer. Good linearity of SWV peaks over the first 25−30 min of the styrene oxide incubation suggests that the PVP−Ru(bpy)$^{2+}$ films on PG electrodes would be useful for estimating relative rates of DNA damage in solution. This result is consistent with previous findings for PDDA/DNA films that suggested that significant DNA damage by styrene oxide occurred only for the outer layer of DNA.[20]

The mechanism of the increase in catalytic current for chemically damaged DNA is likely to be related to a larger average rate of reaction between PVP−Ru(bpy)$^{3+}$ and chemically damaged DNA, as compared to the reaction with intact ds-DNA (eq 2). Thorp and co-workers found that reactions of soluble Ru(bpy)$_3^{3+}$ with ss-DNA are faster than for ds-DNA, which we confirmed previously for the PVP−Ru(bpy)$^{2+}$ films.[21] The explanation here is that the DNA bases in the double-helix DNA structure are less accessible to external oxidizing agents, and the bases in ss-DNA are more accessible. A smaller distance of closest approach of the Ru(bpy)$_2^{3+}$ active sites in the polymer to the oxidizable bases in ss-DNA leads to faster reaction rates and larger catalytic peaks. As mentioned above, styrene oxide forms covalent adducts with guanines and adenines in ds-DNA. Adduct formation disrupts the double helix and allows closer contact between oxidizable moieties on the DNA and the active oxidizing agent, thus increasing the catalytic peak current.

In principle, optimization of pH could increase the sensitivity of the catalytic SWV oxidation method because of the pH dependence of the PVP−Ru(bpy)$^{2+}$ formal potential (Figure 3). The main oxidized species in DNA is guanine, oxidized directly at ∼1.1 V vs SCE under our conditions.[18] According to theory, as the potential difference between the catalyst formal potential and the reactant reduction potential decreases, the rate of reaction and consequently the catalytic current should increase.[33] Data in Figure 4 show that peak current increased with decreasing pH, but the increase is not very large, and the peak current is nearly constant between pH 5.6 and 4.5. Wishing to avoid very low pH values because they are nonphysiological, we used pH 5.5 for

analytical studies, where the rate of reaction of styrene oxide with DNA is also optimum.[25]

In summary, results herein demonstrate that easily prepared PVP−$Ru(bpy)_2^{2+}$ films on PG electrodes present a promising approach for detecting chemical DNA damage from metabolites. Proof of concept has been provided for ds-DNA in solution and in DNA/polycation films grown layer-by-layer on top of the catalyst film. Detection limits are comparable to those for SWV employing soluble $Ru(bpy)_3^{2+}$ as a catalyst,[19] with the advantage here that the electrode itself contains the immobilized catalyst. We are currently evaluating layered films containing DNA, PVP−$Ru(bpy)_2^{2+}$ catalyst, and enzymes with the aim of generating a variety of toxic metabolites in situ and assaying the resulting DNA damage using the same electrode.

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