Published in final edited form as:

Biosens Bioelectron. 2007 November 30; 23(4): 492–498.

Electrochemiluminescent/Voltammetric Toxicity Screening Sensor using Enzyme-Generated DNA Damage

Minjeong So^a, Eli G. Hvastkovs^a, John B. Schenkman^b, and James F. Rusling^{a,b}

^a Department of Chemistry, University of Connecticut, Storrs, Connecticut 06269-3060

^b Department of Pharmacology, University of Connecticut Health Center, Farmington, Connecticut 06032

Abstract

Simultaneous optical and voltammetric detection of bioactivated genotoxicity is reported for the first time employing ultrathin films of DNA, model metabolic enzymes, and electrochemiluminescence (ECL) generating metallopolymer [Ru(bpy)₂PVP₁₀]²⁺ on pyrolytic graphite (PG) electrodes. Cytochrome P450_{cam} and myoglobin were used as model monooxygenase enzymes to mimic *in vivo* processes. Sensor film growth and component amounts were monitored using a quartz crystal microbalance (QCM). Subsequent to the enzyme reaction, DNA damage in the sensor films was measured simultaneously using a simple apparatus combining a standard voltammetry cell coupled with an optical fiber and photomultiplier tube. The model enzyme reaction converted styrene to styrene oxide, which reacts with DNA nucleobases. ECL and SWV signals increased with enzyme reaction time on the scale of several min, and provided relative enzyme turnover rates for DNA damage suitable for toxicity screening applications. Within 1 min, the sensor detects ~3 damaged bases per 10,000 DNA bases using this simultaneous detection.

Keywords

Biosensor; Genotoxicity; Enzyme bioactivation; DNA damage; Electrochemiluminescence; Square wave voltammetry

1. Introduction

Genetic damage from metabolites of lipophilic drugs and pollutants generated by human liver enzymes represents a major toxicity pathway. (Singer and Grunberger, 1983; Pauwels et al., 1996) Many genotoxic, carcinogenic chemicals are bioactivated by so called Phase I enzymes to electrophilic forms that can react with DNA. Bioactivation often involves oxidation catalyzed by the cyt P450 family of enzymes (Schenkman and Greim, 1993; Ortiz de Montellano, 2005; Guengerich and Shimada, 1991). Covalent DNA adducts of these bioactivated molecules with nucleobases are important biomarkers of human cancer risk (Tarun and Rusling, 2005 (1)).

Drug toxicity remains a major cause of drug candidate failure, driving up overall costs of necessary medicines (Caldwell and Yan, 2006). Existing genotoxicity tests are designed to detect compounds that induce genetic damage *in vitro* and *in vivo*. Examples include bacterial

Correspondence to: James F. Rusling.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

mutagenesis tests and cytogenetics assays (Snyder, 2003). In general, assessment of new drug and other chemical toxicity is often hampered by cost and time issues. Therefore, rapid, inexpensive means of genotoxicity screening that could be used in very early stages of discovery and development could prove quite value.

We have described electrochemical genotoxicity biosensors based on enzyme-DNA films that detect relative DNA damage rates resulting from enzyme bioactivation of chemicals (Zhou et al., 2003; Wang et al., 2003; Rusling, 2004) and direct damage of DNA (Zhou and Rusling, 2001). A layer by layer technique was employed to immobilize DNA and enzymes on the sensor surfaces, and film construction was verified by measuring frequency decrease using a quartz crystal microbalance (QCM) (Zhou et al., 2003; Lvov et al., 1998). DNA damage was detected using catalytic square wave voltammetry (SWV) with soluble $\text{Ru}(\text{bpy})_3^{2+}$ ($\text{bpy} = 2,2'$ -bipyridine), which catalyzes oxidation of guanines (Thorp, 1998; Johnston et al., 1995). Sensor response vs. enzyme reaction time provided relative DNA damage rates that were related to indices of animal toxicity and were validated by directly measured rates of nucleobase adduct formation in DNA films using LC-MS/MS (Tarun and Rusling, 2005 (1,2)); Yang et al., 2005).

We previously reported that the ruthenium metallopolymer catalyst, $[\text{Ru}(\text{bpy})_2(\text{PVP})_{10}]^{2+}$ (RuPVP) with six N-Ru bonds can be used to detect DNA damage by direct electrochemiluminescence (ECL) (Dennany et al., 2003). Similar to soluble $\text{Ru}(\text{bpy})_3^{2+}$, RuPVP oxidizes guanines in DNA films to give ECL in the following proposed pathway:

Ru^{II} -PVP is oxidized by the electrode (eq 1) at 1.2 V vs SCE and in turn oxidizes guanines in DNA (eq 2). The guanine radical formed can be further oxidized by Ru^{III} -PVP to give the photoexcited $\text{Ru}^{\text{II}*}$ -PVP (eq 3) which emits light at 610 nm upon electronic relaxation (eq 4). Alternatively, $\text{Ru}^{\text{II}*}$ -PVP can be generated by interaction from Ru^{I} -PVP and Ru^{III} -PVP to produce the excited $\text{Ru}^{\text{II}*}$ state. (Dennany et al., 2003)

Detection of ECL can discriminate against common electrochemical and optical interferences due to near zero background and lack of source radiation. Many ECL systems employ $\text{Ru}(\text{bpy})_3^{2+}$ with tri-n-propylamine (TPA) as a sacrificial reductant (Xu and Bard, 1995; Miao and Bard, 2003; Miao and Bard, 2004). This approach is widely used for detection of DNA hybridization. However, the approach illustrated by Scheme 1 utilizes guanines in the DNA as the sacrificial reductant (Dennany et al., 2003). As nucleobase adducts form with metabolites during the enzyme reaction step, dsDNA is partly unraveled within the films, providing closer access of the catalytic sites to the guanines during the detection step, and correspondingly larger ECL and voltammetric signals (Zhou et al., 2003; Dennany et al., 2003).

In this paper, we evaluate for the first time a genotoxicity biosensor format utilizing enzyme-DNA films containing RuPVP utilizing dual ECL and electrochemical detection of DNA damage. The sensor detects DNA damage from enzyme metabolites after enzyme-catalyzed bioactivation of a procarcinogenic chemical. Compared to our previous report demonstrating direct DNA damage *without* bioactivation (Dennany et al., 2003), we show here that complex enzymatic reactions can be incorporated into this detection format to monitor individual genotoxic metabolic processes. We employed procarcinogenic test molecule styrene in this study. Oxidation of styrene by cytochrome (cyt) P450_{cam} or myoglobin or (Mb) generates reactive styrene oxide that damages DNA in the films (Zhou et al., 2003). After the enzyme reaction, the sensor is placed into the dual electrochemical-ECL cell for detection (Scheme 2). Using RuPVP as a catalyst allowed measurement of relative DNA damage rates within 1 min. by ECL and voltammetry.

2. Experimental

2.1. Chemicals and Materials

The ECL metallopolymer, $[\text{Ru}(\text{bpy})_2(\text{PVP})_{10}](\text{ClO}_4)$ (denoted as RuPVP) (bpy = 2,2'-bipyridyl; PVP = poly(4-vinylpyridine)), was prepared (Sullivan et al., 1978; Hogan and Forster, 1999) and characterized (Sprintschnik et al., 1977; Durhan et al., 1980; Clear et al., 1981) by previously published methods as described in the Supplementary Information file. Calf thymus DNA, Type XV, 13 000 avg. base pairs, 41.9% G/C, and salmon testes DNA (ST-DNA), 2000 avg base pairs, 41.2% G/C were from Sigma. Horse heart myoglobin (Mb) was from Sigma (MW 17, 400) dissolved in pH 4.5 acetate buffer and filtered through an Amicon YM30 membrane (30,000 MW cutoff). *Pseudomonas putida* cyt P450_{cam}(101) (MW 46 500) was expressed in *E. coli* DH5R containing P450_{cam} cDNA and purified following a previously reported procedure (O'Keefe et al., 1978). Water was purified with a Hydro Nanopure system to a specific resistance of >16 M Ω . All other chemicals were reagent grade.

2.2. Electrochemical and ECL Measurements

Square wave voltammetry (SWV) was measured using CH Instruments 660A and CH 430 electrochemical analyzers with 95% ohmic drop compensated using 4 mV step height, 25 mV pulse height, and 5 Hz frequency. The electrolyte was 10 mM pH 5.5 sodium acetate buffer and 50–150 mM NaCl. This pH provides optimal DNA reaction rates with styrene oxide (Zhou and Rusling, 2001).

Simultaneous SWV/ECL measurements were made in an electrochemical cell employing a saturated calomel reference electrode (SCE), a Pt wire counter electrode, and a film-coated PG disk working electrode. The electrochemical cell was constructed to position the sensor surface close to the cell bottom in a reproducible manner (Scheme 2).

ECL was collected through an optical fiber connected to a Photon Counting Module (MP933RS, Perkin-Elmer) containing a Channel Photomultiplier, a high voltage power supply, a discrimination amplifier and a pulse shaper. The optical fiber was positioned directly underneath the sensor outside the cell with the terminal end coupled to the photon counter. The detection module communicated directly to a personal computer with an integrated RS232 interface. SWV and ECL signals were measured with the cell in light-tight box.

2.3. Sensor Film Preparation

Metallopolymer-DNA-Enzyme films were constructed by the layer-by-layer electrostatic assembly method (Lvov, 2000) following an optimized protocol as follows. 4 mm diameter disk electrodes made from ordinary basal plane pyrolytic graphite (PG, Advanced Ceramics) were polished with 400 grit SiC paper and sonicated in ethanol and pure water successively for 1 min, then rinsed with water and dried with nitrogen. Each layer was formed by placing a drop of each adsorbate solution on PG electrode in a closed and dark environment to control evaporation for 10–20 min, the time required to achieve steady state adsorption (Lvov, 2001; Rusling and Zhang, 2003). Cyt P450_{cam} was applied at 4 °C to maintain enzyme stability. Each layer was rinsed with water after deposition to remove weakly adsorbed molecules, then dried with nitrogen between adsorption steps. Adsorption times and drop volumes for each solution were as follows: (1) 10–15 min, 30 μL drop of 2.5 mg mL^{-1} RuPVP in 50% ethanol/water; (2) 15 min, 30 μL drop of 2 mg mL^{-1} DNA in 5 mM pH 7.1 TRIS buffer and 0.50 M NaCl; (3) 20 min, 30 μL drop of 3 mg mL^{-1} Mb in 10 mM pH 4.5 sodium acetate buffer; and (4) 20 min, 20 μL of 0.75 mg mL^{-1} cyt P450_{cam} in pH 5.5 sodium acetate buffer. Final film architectures are denoted by the order of layer deposition as (RuPVP/DNA)₂/(Mb/DNA)₂, (RuPVP/DNA)₃/(Mb/DNA)₂, and (RuPVP/DNA)₃/(cyt P450_{cam}/DNA)₂. The assembled films were kept at 4 °C in the dark overnight before use.

2.4. Characterization of Sensor Films

A quartz crystal microbalance (QCM, USI Japan) was used to monitor film assembly at each step of formation using gold-coated QCM resonators (9 MHz, AT-cut, International Crystal Mfg. Co.). The resonators were cleaned and treated with 3-mercapto-1-propanol and 3-mercaptopropionic acid to form an initial negative layer on the gold surface as previously reported (Lvov, 2000; Lvov, 2001; Rusling and Zhang, 2003). Films were assembled on resonators as described above. The frequency change (ΔF) was measured at ambient temperature after layer deposition and drying in a stream of nitrogen.

2.5 Incubation of Films with Styrene

Safety Note

Styrene is suspected human carcinogens and somewhat volatile. Procedures should be done in a closed hood while wearing gloves, using sealed cells: Incubation of metallopolymer/DNA/enzyme films was done in a thermostatted vessel at 37 °C containing 2% styrene and 0.2–1 mM hydrogen peroxide in 10 mL of 10 mM pH 5.5 buffer containing 50 mM NaCl. After incubation in the stirred solution, electrodes were rinsed with water and transferred to the cell containing 10 mM pH 5.5 buffer containing 150 mM NaCl for SWV/ECL measurements.

3. Results

3.1 Quartz Crystal Microbalance (QCM) and Characterization of Sensor Films

Sensor films of architecture $(\text{RuPVP/DNA})_n/(\text{enzyme/DNA})_n$ were constructed, where n is the number of bilayers. Films were initially characterized by making them on QCM resonators, and measuring frequency change for dry films at every step of the fabrication. The Sauerbrey equation gives the relation between adsorbed mass and frequency change (ΔF , Hz) of the quartz resonator. For 9 MHz quartz resonators, the dry film mass per unit area M/A (gcm^{-2}) is (Lvov, 2001)

$$M/A (\text{gcm}^{-2}) = -\Delta F (\text{Hz}) / (1.83 \times 10^8) \quad (5)$$

The nominal thickness (d) of dry films was estimated from (Lvov et al, 1995; Lvov et al, 1998)

$$d (\text{nm}) \approx -0.016 \Delta F (\text{Hz}) \quad (6)$$

Linear increases in $-\Delta F$ indicated linear film mass increases as each layer was added (See Supporting Information), denoting reproducible film formation. Amounts of each component in the film and average nominal thicknesses were estimated from eqs 5 and 6 (Table 1). Results show that layer-by-layer electrostatic assembly adsorbing alternately charged layers of RuPVP, DNA and enzymes provided stable ultrathin films of nanometer scale thickness. Additionally, the total amount of RuPVP per layer averaged 1.5 nmol cm^{-2} , the total amount of Mb in films was $0.17 \text{ nmol cm}^{-2}$ while the total amount of cytP450_{cam} was $0.04 \text{ nmol cm}^{-2}$. The smaller amount of cytP450_{cam} is consistent with its larger size compared to Mb (Zhou et al., 2003). Final film architectures of $(\text{RuPVP/DNA})_2/(\text{Mb/DNA})_2$ or $(\text{RuPVP/DNA})_3/(\text{cyt P450}_{\text{cam}}/\text{DNA})_2$ are referred to as RuPVP/DNA/Mb or RuPVP/DNA/P450_{cam} for the duration of the paper.

To optimize the composition of RuPVP/DNA/enzyme films for sensing, we first employed the more readily available Mb as a model enzyme for epoxidation of styrene. DNA damage from the metabolite styrene oxide in $(\text{RuPVP/DNA})_2/(\text{Mb/DNA})_2$ films was monitored by SWV. (See Supporting Information) A preliminary study showed that films of architecture (RuPVP/

DNA)₃/(enzyme/DNA)₂, i.e. with 3 layers of RuPVP, improved the ECL signal/noise, and so this film architecture was used for all sensor studies described herein. One RuPVP/DNA bilayer on average contributed 20 μ A SWV peak current with 15–20% increase in peak current with additional bilayers. Therefore, the background signal from RuPVP bilayers does not increase so significantly as to decrease sensitivity of the voltammetric detection. In addition, while one RuPVP/DNA bilayer is sufficient for electrochemical DNA damage detection, we found that ECL from only one layer of RuPVP was not detected using the current photomultiplier tube (PMT) configuration. Therefore, it was necessary to increase the number of RuPVP/DNA bilayers to facilitate ECL emission (Hvastkovs et al., 2007).

The concentration of hydrogen peroxide for the activation of enzymes for substrate conversion while minimizing DNA damage was optimized. Concentrations of 0.2–1 mM hydrogen peroxide had no measurable influence on SWV of DNA films alone, implying no DNA damage. Hydrogen peroxide at 0.5 mM was found to be optimum for the sensor incubation steps. (See Supporting Information)

3.2. Simultaneous ECL and SWV Detection of Metabolite-generated DNA damage

Cyt P450_{cam} was employed as a representative cyt P450 Phase I metabolic enzyme model. Cyt P450_{cam} is a bacterial P450 form that encompasses many of the same metabolic functions as human cyt forms (Hvastkovs et al., 2007). Compared to human isoforms, it is expressed and purified in higher yields and is soluble (i.e. not membrane bound, which imparts stability); therefore it represents a convenient P450 form with which to work.

Figure 1 shows simultaneously acquired ECL and SWV data. The ECL is represented as a bar graph showing 2-second integrated photomultiplier tube acquisitions upon scanning from 0.4 to 1.35 V vs. SCE using SWV. Figure 1a demonstrates the typical ECL response before (0 min) and after reaction (4 min) of RuPVP/DNA/P450_{cam} films with styrene/hydrogen peroxide to produce styrene oxide. ECL detected at approximately +1.15 V vs. SCE after incubation with styrene/H₂O₂ is significantly larger than for the control. This suggests increased DNA damage resulting in increased rate of catalytic oxidation of DNA and corresponding increase in ECL at this potential. Figure 1b shows SWVs obtained at the same time as the ECL acquisition, in which peak current increased after the 4 min. enzyme reaction.

Figure 2 shows ECL and SWV sensor response vs. enzyme reaction time from experiments similar to those in Figure 1. Voltammetric data are expressed as final/initial SWV peak heights over a range of enzyme reaction times. The ECL signal used for the ratios was the sum of photon counts from +1.0 to +1.35 V vs. SCE. The ECL and SWV sensor ratios vs. time measure the rate of change from DNA damage in the film, which reflects relative genotoxicity (Rusling, 2004). Such ratio plots also help to compensate for inter-electrode variability. As the sensor measures DNA damage and this may occur as a result of the enzyme reaction, it is of necessity a single use sensor. Sensors constructed using the layer-by-layer technique typically exhibited $\pm 10\%$ reproducibility based on acquired catalytic SWV oxidation signals. This can be due to variance in the polished electrode surface area for each subsequent experiment, the layering process, or a combination. Figure 2 clearly shows a linear increase in ECL and SWV peak response vs. enzyme reaction time for the cytP450_{cam}-based sensors. The voltammetric increase mirrors the ECL increase. The lack of trends of ECL or SWV signals for control incubations with H₂O₂ alone demonstrates that at this concentration, the peroxide does not influence the DNA in the film, as reported previously (Zhou, et. al., 2003).

Similar ECL and SWV peak current increases were seen using Mb as the model enzyme in the sensor films (see Supplementary Information). Similar to Figure 1, larger ECL and SWV peaks were observed with incubation times of several min compared to initial ECL and SWV peaks. Figure 3 shows final/initial ECL and SWV ratios for RuPVP/DNA/Mb films incubated with

styrene/H₂O₂. ECL and SWV increased in similar fashion with incubation time. No significant changes in ECL and SWV peaks were found for control incubations with only styrene or only H₂O₂. In ECL/SWV detection, films containing Mb gave larger signals than those of cytP450_{cam} for the first 3 min. At longer incubation of more than 7 min, both Mb films and cytP450_{cam} films had similar peak ratios in both SWV and ECL measurements. Error bars in Figures 2 and 3 represent standard deviations of ~10% (n = 4 to 8). These repeatability estimates are consistent with the QCM results that reflect construction of the active layers, and which show ±10–20% overall inter-experimental difference (cf. Figure S1, Table 1). Reporting the initial/final signal ratios as in Figures 2 and 3 improves variability compared to reporting the absolute signal (Zhou et al., 2003).

As with purely voltammetric toxicity sensors, comparisons with direct measurement of nucleobase damage rates from LC-MS (Tarun and Rusling, 2005 (1); Tarun and Rusling, 2005 (2); Yang et al., 2005) suggests that the ECL and SWV ratio plots estimate relative rates of DNA damage. The relative turnover rates were obtained by measuring the initial slope of the ECL and SWV ratio plots and dividing by the amount of enzyme present in the films (Γ) as estimated by QCM (cf. Table 1). While the initial slope of the damage ratio vs. reaction time (Table 2) for Mb is higher than that for cyt P450_{cam}, the relative turnover rate for Mb is smaller due to the larger amount of the smaller Mb in the films. The average relative turnover rate of cyt P450_{cam} resulting in DNA damage was 2.2 (min⁻¹nmol⁻¹cm²) and that of Mb was 1.0 (min⁻¹nmol⁻¹cm²). Thus, cyt P450_{cam} was almost twice as active in the film in converting styrene to styrene oxide compared to Mb.

4. Discussion

Results above show that sensors based on enzyme/DNA films with simultaneous ECL/voltammetry detection can provide relative rates for DNA damage as slopes of response vs. enzyme reaction time (Figures 2 and 3). Simultaneous measurement of ECL and SWV is relatively easy, and provides an internal 2-output check on the relative turnover rates obtained. The relative rate depends on the rate of reaction of the enzyme to form metabolites, and the rate of reaction of the metabolite with DNA. In this case, styrene oxide is the only product of the enzyme reactions in the films (Lvov et al., 1995), and the slopes of the response curves in Figures 2 and 3 directly reflect the rate of styrene oxide production in the film. Based on relative turnover rates, Cyt P450_{cam} was >2-fold more active for styrene conversion than Mb by both ECL and SWV (Table 2). This result is expected since cyt P450 is a true enzyme and oxidation catalysis is not a standard biological function for Mb. This is also consistent with previous results with voltammetric genotoxicity sensors using cyt P450_{cam} and Mb to detect DNA damage from metabolites of styrene (Zhou et al., 2003) and benzo[a]pyrene (Wang et al., 2005; Hvastkovs et al., 2007).

Results also lend confidence that different DNA damage rates will reflect the activities of different enzymes in the films, a feature that is important for toxicity screening. Expression of the different cyt P450 enzyme isoforms in different individuals can vary dramatically, resulting in different exposures to chemical or drug toxicity (Guengerich, 2001; Guengerich et al., 1999). Thus, identification of the specific cyt P450s associated with genotoxicity of a given drug is an important application of these sensors.

Intimate mixing of layers in protein-polyion films made by the electrostatic layer-by-layer approach used in our sensors was demonstrated by neutron reflectivity (Lvov, 2000). Although the films are made one layer at a time, the final film structure features considerable interlayer mixing and close contact between RuPVP, DNA and enzyme molecules, consistent with the current view of significant disordered nature of polyion multicomposite layered films (Decher, 1997; Lvov, 2000 & 2001). These intermixed films represent a 50 nm by 0.2 cm² reaction

volume with high concentrations of enzyme, DNA, and RuPVP. Film disorder is a significant advantage, since intermixing allows the formation of metabolites in close proximity to high concentrations of DNA to enhance reaction rates. Also, damaged DNA is in close proximity to high concentrations of RuPVP catalyst to improve efficiency of the detection step (cf. Scheme 1). In addition, the layer-by-layer assembled film of metallopolymer, enzymes and DNA is very stable (Wang et al, 2003) and typically show <20% loss in activity over 2 month storage at 4 °C (Lvov et al, 1998).

Incorporating RuPVP in these reagentless sensors provides slightly more sensitive SWV detection of DNA damage rates compared to soluble Ru(bpy)₃²⁺. The SWV ratios reported here (Figure 2 and 3) are about 10% larger than previously reported using soluble Ru(bpy)₃²⁺ under otherwise similar conditions. (Zhou et al., 2003) The initial slope of 0.072 min⁻¹ in the damage ratio plots for cytP450_{cam} (Table 2) is ~20% larger compared to the previous results using soluble Ru(bpy)₃²⁺ which was 0.059 min⁻¹ for cytP450_{cam}. Assuming that the rates of bioactivation of styrene by cytP450_{cam} in the two systems are similar, the increase in initial slope reflects a larger average rate of reaction between DNA and catalyst RuPVP in the film compared to the soluble catalyst. Furthermore, surface confinement of RuPVP has been reported to provide greater efficiency and higher ECL intensity than conventional solution phase ECL (Dennany et al., 2006). Table 2 also shows that ECL detection offers slightly better sensitivity than SWV, but reason are not fully understood.

The most important function of the sensors is the ability to rapidly measure relative DNA damage rates, which is related to relative genotoxicity (Tarun and Rusling, 2005 (2)). Detection limits were estimated by correlating the increase in ECL or SWV ratio with previous LC-MS measurement of styrene oxide-guanine and other adducts from damaged DNA (Tarun and Rusling, 2005 (1); Yang et al., 2005; Tarun et al., 2006). That is, in similar films, we directly measured the rate of styrene oxide-guanine formation by LC-MS, so we know how many nucleobases are damaged per unit time under similar as that used in the sensors. The LC-MS analysis showed that the amount of N7-guanine-styrene oxide formed by the in-situ-generated styrene oxide in DNA/Mb films increased linearly with incubation time in accord with sensor response, and at a rate of 0.3 pmol of guanine min⁻¹ (Tarun et al., 2006). In the present sensors, based on QCM data (Table 1), the amount of DNA guanine in our sensor films is 0.9 nmol. A sensor response 3-fold larger than the noise for both ECL and SWV occurs in <1 min enzyme reaction (Figure 2 and 3). Taking the amount of styrene oxide-guanine found in 1 min from the LC-MS studies, we estimate a conservative detection limit of ~0.03% of total guanines or 3 damaged bases in 10,000. Thus, these sensors have the ability to assess the genotoxicity of procarcinogenic compounds in several min. of enzyme reaction and can detect low DNA damage levels.

5. Conclusion

In summary, the coupling of ECL, SWV and metabolite generation with enzymes in a single DNA-based sensor allows sensitive simultaneous detection of DNA damage rates that can be used for toxicity screening. The multiple transduction platform allows for a thorough monitoring of toxicity mechanisms using DNA damage as the endpoint and ensures that signal increases are indeed due to DNA damage and not background processes. Significant information about relative enzyme kinetics for genotoxic metabolite generation can be acquired with two outputs simultaneously using a simple apparatus. The sensor does not require soluble catalyst or sacrificial reductant. The individual dual output sensors will be an excellent complement to ECL sensor arrays (Hvastkovs et al., 2007). Not only are similar reaction rate trends seen using cyt P450_{cam} vs. Mb in these electrodes compared to the arrays, but these individual sensors will allow for more thorough monitoring of bioactivation processes. Due to the individual addressability of the dual output sensors described here, one can envision initial

high throughput toxicity monitoring using an ECL array platform, followed optimization of damage protocols and conditions and a more directed study of individual enzymes using the simultaneous detection apparatus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the National Institute Environmental Health Services (NIEHS) of the NIH via US PHS grant No. ES03154. The authors thank Dr. Byoungchul Lee and Sumi Shima for experimental assistance.

References

- Caldwell GW, Yan L. *Curr Opin Drug Disc & Devel* 2006;9:47–50.
- Clear JM, Kelly JM, O'Connell CM, Vos JG. *J Chem Res Synopses* 1981;9:260–1.
- Decher G. *Science* 1997;277:1232–1237.
- Dennany L, Forster RJ, Rusling JF. *J Am Chem Soc* 2003;125:5213–5218. [PubMed: 12708874]
- Dennany L, Hogan CF, Keyes TE, Forster RJ. *Anal Chem* 2006;78:1412–1417. [PubMed: 16503588]
- Durhan B, Wilson SR, Hodgson DJ, Meyer TJ. *J Am Chem Soc* 1980;102:600–607.
- Guengerich FP, Parikh A, Turesky RJ, Josephry PD. *Mutation Res* 1999;428:115–124. [PubMed: 10517985]
- Guengerich FP, Shimada T. *Chem Res Toxicol* 1991;4:391–407. [PubMed: 1912325]
- Guengerich FP. *Chem Res Toxicol* 2001;14:611–650. [PubMed: 11409933]
- Hogan CF, Forster RJ. *Anal Chim Acta* 1999;396:13–21.
- Hvastkovs EG, So' M, Krishnan S, Bajrami B, Tarun M, Jansson I, Schenkman JB, Rusling JF. *Anal Chem* 2007;79:1897–1906. [PubMed: 17261025]
- Johnston DH, Glasgow KC, Thorp HH. *J Am Chem Soc* 1995;117:8933–8938.
- Lvov YM, Lu Z, Schenkman JB, Zu X, Rusling JF. *J Am Chem Soc* 1998;120:4073–4080.
- Lvov, Y. *Protein Architecture: Interfacing Molecular Assemblies and Immobilization Biotechnology*. Lvov, Y.; Möhwald, H., editors. Marcel Dekker; New York: 2000. p. 125-167.
- Lvov, Y. *Handbook Of Surfaces And Interfaces Of Materials*. In: Nalwa, RW., editor. Nanostructured Materials, Micelles and Colloids. 3. Academic Press; San Diego: 2001. p. 170-189.
- Lvov Y, Ariga K, Ichinose I, Kunitake T. *J Am Chem Soc* 1995;117:6117–6123.
- Miao W, Bard AJ. *Anal Chem* 2003;75:5825–5834. [PubMed: 14588023]
- Miao W, Bard AJ. *Anal Chem* 2004;76:5379–5386. [PubMed: 15362895]
- Munge B, Estavillo C, Schenkman JB, Rusling JF. *ChemBiochem* 2002;4:101–108.
- O'Keefe DH, Ebel RE, Petersen JA. *Methods Enzymol* 1978;52:151–157. [PubMed: 672625]
- Ortiz de Montellano, PR., editor. *Cytochrome P450*. Kluwer/Plenum; New York: 2005.
- Pauwels W, Vodiceka P, Severi M, Plna K, Veulemans H, Hemminki K. *Carcinogenesis* 1996;17:2673–2680. [PubMed: 9006105]
- Rusling JF. *Biosensors & Bioelectronics* 2004;20:1022–1028. [PubMed: 15530799]
- Rusling, JF.; Zhang, Z. *Biomolecular Films*. Rusling, JF., editor. Marcel Dekker; New York: 2003. p. 1-64.
- Schenkman, JB.; Greim, H., editors. *Cytochrome P450*. Springer-Verlag; Berlin: 1993.
- Singer, B.; Grunberger, D. *Molecular Biology of Mutagens and Carcinogens*. Plenum; New York: 1983.
- Snyder RD. *Business Briefing: Future Drug Discovery* 2003:68–71.
- Sprintschnik G, Sprintschnik HW, Kirsch PP, Whitten DG. *J Am Chem Soc* 1977;90:4947.
- Sullivan BP, Salmon DJ, Meyer TJ. *Inorg Chem* 1978;17:3334–3341.
- Tarun M, Bajrami B, Rusling JF. *Anal Chem* 2006;78:624–627. [PubMed: 16408950]
- Tarun M, Rusling JF. (1) *Anal Chem* 2005;77:2056–2062.

- Tarun M, Rusling JF. (2) Crit Rev Eukaryotic Gene Expr 2005;15:295–315.
- Thorp HH. Trends Biotechnol 1998;16:117–121.
- Wang B, Jansson I, Schenkman JB, Rusling JF. Anal Chem 2005;77:1361–1367. [PubMed: 15732919]
- Wang B, Rusling JF. Anal Chem 2003;75:4229–4235. [PubMed: 14632140]
- Xu XH, Bard AJ. J Am Chem Soc 1995;117:2627–2631.
- Yang J, Wang B, Rusling JF. Molecular Biosystems 2005;1:251–259. [PubMed: 16880989]
- Yang J, Zhang Z, Rusling JF. Electroanalysis 2002;14:1494–1500.
- Zhou L, Rusling JF. Anal Chem 2001;73:4780–4786. [PubMed: 11681451]
- Zhou L, Yang J, Estavillo C, Stuart JD, Schenkman JB, Rusling JF. J Am Chem Soc 2003;125:1431–1436. [PubMed: 12553846]

Figure 1.

Simultaneous ECL and SWV from a sensor featuring $(\text{RuPVP/DNA})_3/(\text{cytP450}_{\text{cam}}/\text{DNA})_2$ films on PG electrodes in pH 5.5 acetate buffer before (0 min) and after (4 min) incubations at 37°C with 2% styrene and 0.5 mM H_2O_2 at pH 5.5: (a) integrated ECL intensity (counts/s) vs. potential profiles. (b) SWV amplitude, 25 mV; frequency, 5 Hz; step, 4 mV.

Figure 2. Influence of incubation time on the final/initial ECL (●) and SWV (▲) ratios for sensors featuring $(\text{RuPVP}/\text{DNA})_3/(\text{cytP450}_{\text{cam}}/\text{DNA})_2$ films incubated at 37 °C with 2% styrene + 0.5 mM H_2O_2 , and controls using 0.5 mM H_2O_2 alone for ECL (○) and SWV (Δ).

Figure 3.

Influence of incubation time on the final/initial ECL (●) and SWV (▲) ratios for sensors featuring $(\text{RuPVP}/\text{DNA})_3/(\text{Mb}/\text{DNA})_2$ films reacted at 37°C with saturated styrene and 0.5 mM H_2O_2 , controls using 0.5 mM H_2O_2 alone for ECL (○) and SWV (△), and controls using styrene alone for ECL (□) and SWV (▽).

Scheme 1.

Scheme 2.

Conceptual drawing of sensor system for simultaneous ECL and voltammetry detection. The SCE reference electrode (a), PG working electrode (b) and Pt counter electrode (c) are located in a glass cell with an extended cylindrical glass base. A fiber optic cable (d) is located on the outside of the cell directly underneath the sensor surface. RuPVP, DNA (brown), and enzyme (blue) layers forming the active sensor film are shown to the right. The structure of RuPVP is shown at the top. For clarity, only one layer of RuPVP is shown on the sensor, although several can be used to enhance S/N.

Table 1

Average Characteristics of metallopolymer/DNA/enzyme films from QCM Results.

Film architecture	Total thickness (nm)	Total mass RuPVP ($\mu\text{g cm}^{-2}$)	Total mass DNA ($\mu\text{g cm}^{-2}$)	Total mass enzyme ($\mu\text{g cm}^{-2}$)
(RuPVP/ST-DNA) ₂ /(Mb/ST-DNA) ₂	47±6	4.6±0.3	8.9±0.3	3.0±0.2
(RuPVP/DNA) ₃ /(Cyt P450 _{cam} /DNA) ₂	49±6	7.8±0.5	7.2±0.5	1.9±0.5

Table 2

Relative rate of DNA damage from bioactivation of styrene by ECL and SWV

Enzyme	Γ (nmol cm ⁻²) ^a	Initial slope (min ⁻¹)		Rel. turnover rate (min ⁻¹ nmol ⁻¹ cm ²)	
		ECL	SWV	ECL	SWV
Cyt P450 _{cam} Mb	0.04±0.01	0.103	0.072	2.58±0.69	1.79±0.48
	0.17±0.01	0.204	0.124	1.20±0.07	0.73±0.04

^afrom QCM