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Comparison of DNA-Reactive Metabolites from Nitrosamine and Styrene Using Voltammetric DNA/Microsomes Sensors

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Abstract

Voltammetric sensors made with films of polyions, double-stranded DNA and liver microsomes adsorbed layer-by-layer onto pyrolytic graphite electrodes were evaluated for reactive metabolite screening. This approach features simple, inexpensive screening without enzyme purification for applications in drug or environmental chemical development. Cytochrome P450 enzymes (CYPs) in the liver microsomes were activated by an NADPH regenerating system or by electrolysis to metabolize model carcinogenic compounds nitrosamine and styrene. Reactive metabolites formed in the films were trapped as adducts with nucleobases on DNA. The DNA damage was detected by square-wave voltammetry (SWV) using Ru(bpy)₃²⁺ as a DNA-oxidation catalyst. These sensors showed a larger rate of increase in signal vs. reaction time for a highly toxic nitrosamine than for the moderately toxic styrene due to more rapid reactive metabolite-DNA adduct formation. Results were consistent with reported in vivo TD₅₀ data for the formation of liver tumors in rats. Analogous polyion/ liver microsome films prepared on 500 nm silica nanoparticles (nanoreactors) and reacted with nitrosamine or styrene, provided LC-MS or GC analyses of metabolite formation rates that correlated well with sensor response.

Keywords

Reactive metabolites; Sensors; DNA damage; Liver microsomes; Toxicity screening

1. Introduction

Toxicity caused by reactions of metabolites with DNA and proteins is a major issue in the development of drugs and environmental chemicals [1 – 5]. Bioactivation of xenobiotic compounds by liver cytochrome P450 (CYP) enzymes often leads to the generation of reactive metabolites [6 – 9]. These reactive metabolites damage DNA by forming adducts which eventually can become carcinogenic if DNA repair mechanisms fail. Hence, DNA damage caused by reactive metabolites, resulting in so called genotoxicity, can be used for testing drugs under development [10, 11]. While useful, conventional methods to assess genotoxicity employing microbiology or animal models are limited by expense, throughput and prediction of human toxicity [12, 13]. However, novel rapid and inexpensive screening methods based on simple biochemical approaches can complement existing assays [11].

Metabolite mediated liver toxicity is a major toxicity pathway [6 – 8, 14]. We recently developed electrochemical and electro-optical sensors capable of detecting reactive metabolites [15 – 20] by employing purified CYPs and DNA films prepared by a layer-by-layer (LbL) technique for high-throughput reactive metabolite screening. In these sensors, the enzyme reaction produces metabolites and the resulting DNA damage is detected by catalytic voltammetry using soluble $\text{Ru}(\text{bpy})_3^{2+}$ catalyst [15, 16], single electrode electrochemiluminescence (ECL) /voltammetry sensor [17, 18], or ECL arrays [19, 20]. We demonstrated correlations of the rate of sensor signal increase vs. enzyme reacting time with DNA adduct formation rates detected by LC-MS/MS using enzyme/DNA films on solid surfaces [21, 22].

The bottleneck in the development of these sensors, especially when several enzymes are required, is the isolation and purification of CYP enzymes. On the other hand, liver microsomes containing major drug metabolizing CYP enzymes and their reductases are cheap and commercially available. Liver microsomes are extensively used in the drug industry for in vitro metabolism studies, utilizing in vivo metabolic pathway of NADPH-CYP reductases [23, 24].

We recently showed that rat liver microsome/DNA films in an electro-optical electrochemiluminescence (ECL) array format could easily distinguish between highly toxic *N*-nitrosamines and less toxic styrene [25]. In the current study, we employ rat liver microsomes (RLM) and DNA/polyion films assembled using layer-by-layer electrostatic adsorption on pyrolytic graphite electrodes in a single sensor format, and test this sensor against model toxic compounds *N*-nitrosopyrrolidine (NPYR) and styrene. In addition, we present layer-by-layer characterization of the sensor films by microbalance and microscopic techniques. Activation of RLMs in the layer by electrolysis or NADPH was compared to bioactivate the model compounds. The reactive metabolites thus formed were then trapped as nucleobase adducts in the DNA film, and the DNA damage was detected by catalytic electrochemical oxidation utilizing soluble $\text{Ru}(\text{bpy})_3^{2+}$ [15, 26]. While this format is not as sophisticated or as high throughput as the ECL electro-optical arrays, the sensors are easily and rapidly prepared, and only electrochemical equipment is required.

The bioactivation of NPYR by CYPs occurs via ahydroxylation forming α -hydroxy tetrahydrofuran (2-OH-THF) as the major metabolite and several other reactive electrophilic intermediates that form DNA adducts mainly with guanines [27, 28]. Styrene is metabolized to styrene oxide, which forms up to 11 covalent adducts with guanine and adenine bases of DNA that disrupt the double helix [29 – 31]. Pathways of DNA adduct formation by bioactivation of NPYR and styrene are illustrated in Scheme 2, and were confirmed in this work by LC-MS using polyion/microsomes nanoreactors.

In brief, this study addresses utilization of electrode surfaces with films of liver microsomes containing drug metabolizing enzymes and dsDNA for the study of reactive metabolite formation by a simple electrochemical readout utilizing $\text{Ru}(\text{bpy})_3^{2+}$ catalyst.

2. Experimental

2.1. Materials and Methods

Liver microsomes from rats (RLM, F344, male rats, total protein 20 mg mL⁻¹) containing CYP enzymes, CYP reductases (CPR), and cytochrome b₅ were from BD-Biosciences (Woburn, MA, USA). Calf thymus double stranded DNA (dsDNA, Type-I, 41.9% G/C), *N*-nitrosopyrrolidine (NPYR), styrene, 2-hydroxy tetrahydrofuran (2-OH-THF), poly(diallyldimethylammonium chloride) (polycation, PDDA), and tris(2,2'-

bipyridyl)dichloro ruth-enium(II)hexahydrate $[\text{Ru}(\text{bpy})_3^{2+}]$ were from Sigma. Glucose-6-phosphate sodium salt, nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate dehydrogenase were also from Sigma. Hydroxylated SiO_2 nanoparticles [500 nm ($\pm 10\%$) diameter] were from Polysciences Inc.(Warrington, PA). Water was treated with a Hydro Nanopure system to a specific resistivity 18 m Ω cm. All other chemicals were reagent grade.

2.2. Voltammetry

A CHI 660A electrochemical analyzer was used for square-wave voltammetry (SWV) in a cell employed with a saturated calomel reference electrode (SCE), a Pt-wire counter electrode, and 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. Electrolyte was 50 mM phosphate buffer plus 0.1 M KCl, pH 7.0 containing 50 μM $\text{Ru}(\text{bpy})_3^{2+}$.

2.3. Layer-by-layer Film Assembly

The (PDDA/DNA/PDDA/RLM)₂ films (denoted as PDDA/DNA/RLM) on PG electrodes were constructed by the layer-by-layer electrostatic assembly method as previously reported [15 – 20, 32]. In brief, basal plane PG electrodes were polished on 400 grit SiC paper (3 M Crystal Bay), and then ultrasonicated in ethanol for 30 s followed by water for 30 s and dried in nitrogen. Layers of PDDA, DNA and RLM were then adsorbed one layer at a time in the sequence (PDDA/DNA/PDDA/RLM)₂ on the rough PG electrodes. PDDA and DNA layers were adsorbed at room temperature for 20 min. RLM and subsequent layers were adsorbed at 48°C for 30 min. The electrodes were washed with water between adsorption steps to remove weakly adsorbed molecules. These conditions were optimized using quartz crystal microbalance measurements [15] as discussed below. The following solutions were used to make the voltammetry and QCM sensors: (a) 2 mg mL⁻¹ double stranded DNA in 10 mM Tris buffer, pH 7.1 plus 50 mM NaCl; (b) 2 mg mL⁻¹ PDDA in water plus 50 mM NaCl; and (c) RLM as supplied.

2.4. Quartz Crystal Microbalance (QCM)

Assembly of PDDA, DNA and RLM sensor films as for PG electrodes was monitored using quartz crystal microbalance (QCM, USI Japan) with 9 MHz QCM resonators (AT-cut, International Crystal Mfg.) as reported previously [15, 16]. To mimic the PG electrode surface used for voltammetry sensors, a partly negative monolayer was made by treating gold-coated ($0.16 \pm 0.01 \text{ cm}^2$) resonators with 5 mM 3-mercaptopropionic acid in ethanol [15, 16]. Films were assembled as for PG electrodes on the negatively charged gold resonators with PDDA adsorbed as first layer, DNA as second layer and so on in the order (PDDA/DNA/PDDA/RLM)₂. Resonators were rinsed in water between adsorption steps and dried in a stream of nitrogen before measuring the frequency change (ΔF) for each adsorbed layer. The total preparation time for the (PDDA/DNA/PDDA/RLM)₂ assembly by QCM sensor is about 4 h.

2.5. Transmission Electron Microscope (TEM)

For the TEM (Philips EM300, USA) imaging of rat liver microsomes, about 4 μL of microsomal solution was adsorbed on a carbon coated copper grid for 1 min. For this study, the supplied RLM was diluted 40 times in 50 mM phosphate buffer, pH 7.0 and used (final total protein concentration was 0.5 mg mL⁻¹). Following the adsorption, the copper grid was rinsed with water to remove salts and loosely bound microsomes. Then 5 μL of 0.5%

phosphotungstic acid was placed on the microsome adsorbed copper grid for 1 min, then rinsed with water, dried and used for TEM imaging.

2.6. Atomic Force Microscope (AFM)

A Nanoscope IV multimode atomic force microscope (Digital Instruments, CA, USA) was used in tapping mode in air to monitor the nominal thickness and surface topography of PDDA/DNA/PDDA/RLM film at each layer adsorption on mica surface. The conditions of the adsorption were the same as that used for PG electrodes discussed above. After each adsorption step, the mica chip was rinsed in water to remove salts and loosely bound molecules. The following solutions were used for the AFM study: (a) PDDA (0.5 mg mL^{-1}) in water; (b) DNA (10 mg mL^{-1}) in water; (c) RLM (1 mg mL^{-1}) in 50 mM phosphate buffer, pH 7.0.

2.7. Activation of RLM and Voltammetric Detection

Film assemblies of PDDA/DNA/RLM on PG electrodes were incubated in 1 mM of test compound (NPYR or styrene) and NADPH generating system (10 mM MgCl_2 , 0.8 mM NADP, 10 mM glucose-6-phosphate, and 1 unit mL^{-1} of glucose-6-phosphate dehydrogenase) in 50 mM phosphate buffer, pH 7.0 at 37°C for different times up to 30 min. Here, the Fe(III) heme of microsomal CYP accepts an electron from NADPH via CYP reductase, followed by dioxygen binding and a second electron transfer (from cyt b_5 or CYP reductase), resulting in an active oxidant CYP form that subsequently oxidizes bound substrates [9, 10, 24]. This metabolic pathway can activate pollutants and drugs to reactive intermediates that damage DNA by forming covalent adducts with DNA bases mainly guanines due to its high nucleophilicity.

2.8. Metabolite Identification and Quantitation

For the identification and structural confirmation of reactive metabolite formation in the sensors, analogous PDDA ($2 \text{ mg mL}^{-1} + 50 \text{ mM NaCl}$ in water) and RLM (used as supplied) films were assembled on silica nanoparticles (aliquots of 0.2 mL suspension in 50 mM phosphate buffer, pH 7.0) by the layer-by-layer technique in the order (PDDA/RLM)₂ as reported recently [33]. After each layer adsorption at 4°C for 30 min, the nanoparticles were separated from the dispersion by centrifugation (2 min 8000 rpm) followed by resuspension in water and centrifugation for three times to remove weakly adsorbed molecules.

These particles containing the (PDDA/RLM)₂ films (nanoreactors) were incubated in 1 mM NPYR or 1 mM styrene and NADPH generating system in 50 mM phosphate buffer, pH 7.0 at 37°C for different times up to 30 min same as that used for the sensors. The reaction was stopped by rapidly centrifuging the reaction solution to settle the nanoparticles and the supernatant was collected and analyzed by capillary LC-MS to identify the major NPYR metabolite, 2-hydroxy tetrahydrofuran (2-OH-THF) [33]. Similarly, for the styrene reaction with the (PDDA/RLM)₂ films, gas chromatography (GC) was used to detect styrene oxide [22, 34], the styrene metabolite. Calibration plots were made using commercially available 2-OH-THF and styrene oxide standards to quantify the metabolites formed from the nanoreactors.

2.9. Chromatography and Mass Spectrometry

For the analysis of 2-hydroxy tetrahydrofuran obtained from the supernatant of nanoreactors reaction with NPYR, a trapping column (Atlantis, dC18, 23.5 mm, 0.18 mm, ID 5 mm particle size) and the analytical column (Atlantis, dC18, 150 mm, 300 mm, ID 5 mm particle size) were used (Waters, Milford, MA) similar to that reported recently [33]. The capillary

LC was equipped with a photo diode array detector (scanning wavelengths from 200–450 nm). About 10 μL of sample injection for each analysis was loaded into the trapping column at a flow rate of 4.25 $\mu\text{L min}^{-1}$ and washed with water for 1 min at 10 $\mu\text{L min}^{-1}$.

The trapped metabolite and substrate were then back-flushed from the trapping column onto the analytical column at 4.25 $\mu\text{L min}^{-1}$ using the following elution gradient: 5 min 10% B, 5 min 10% to 30% B, 30 min 30% B, 5 min 30% to 10% B, 5 min 10% B (A, 10 mM acetate buffer, pH 5.5; B, methanol). Electrospray ionization mass spectrometry (ESI-MS) employed a Micromass Quattro II (Beverly, MA) operated in the positive ion mode (ESI^+). The following conditions were used: cone voltage = 15 V, collision energy = 20 eV and collision gas (Ar) pressure = 5×10^{-3} mbar. The metabolite was analyzed by total ion chromatogram (TIC). Average retention times were 6.3 min for 2-hydroxy tetrahydrofuran and 12 min for NPYR.

For the analysis of styrene oxide from the styrene reaction with $(\text{PDDA}/\text{RLM})_2$ nanoreactors, the reaction mixture from different reaction times were extracted with hexane after settling the particles by centrifugation. The obtained styrene oxide in hexane was analyzed by gas chromatography (GC, HP 6890, USA) employing a flame ionization detector with a temperature program of 50°C initially for 0.5 min, then 7.5°C/min to 100°C, then 50°C/min to the final T of 250°C as described elsewhere[34]. Average retention times were 4.2 min for styrene and 6.4 min for styrene oxide.

3. Results and Discussion

3.1. Film Characterization

QCM measurements during layer-by-layer assembly of $(\text{PDDA}/\text{DNA}/\text{PDDA}/\text{RLM})_2$ film used in the voltammetric sensors are shown in Figure 1. Plot of change in frequency, $-\Delta F$ (Hz) vs. sequential layers identified by type for the $(\text{PDDA}/\text{DNA}/\text{PDDA}/\text{RLM})_2$ film on gold resonators is linear suggesting regular and reproducible film growth. Here PDDA is adsorbed as first layer on the 3-mercaptopropionic acid coated negatively charged gold resonators followed by DNA and so on to attain the $(\text{PDDA}/\text{DNA}/\text{PDDA}/\text{RLM})_2$ film assembly. The frequency decrease observed for each layer adsorption was used to obtain thickness and mass of the adsorbed layer [15]. The larger frequency decrease for the microsomal layer than polyions can be attributed to the larger size of the RLM compared to the polyion layer thickness (DNA or PDDA) of about 1 to 2 nm [35, 36].

Table 1 shows the nominal thickness and amounts of PDDA, DNA and RLM films in the sensor assembly. The average thicknesses for the first and second microsomes layers were 4.0 ± 0.6 and 6.1 ± 0.2 nm respectively, but it is likely that the microsomes are flattened and interspersed with polymer strands in a rather disordered structure to give the 4 – 6 nm nominal layer thicknesses, analogous to nano-particles in polyion films fabricated by this method [37, 38].

In order to characterize the morphology and size of microsomes adsorbed on a surface, TEM images of the RLM were obtained. The TEM image of RLM film adsorbed on a copper grid fixed by phosphotungstic acid (Fig. 2) shows disc-like features of microsomes consisting of phospholipids, vesicles, and membrane bound metabolic enzymes of various sizes ranging from 40 – 200 nm.

To obtain the topography and feature sizes of the layer-by-layer assembly of PDDA, DNA, and RLM films on the sensor, AFM characterization was performed. Figure 3 represents the AFM images of PDDA, PDDA/DNA, and PDDA/DNA/PDDA/RLM films adsorbed on mica surface. The PDDA films gave the smoothest surface (Fig. 3a), adsorption of DNA

forming PDDA/DNA layer adds more structure (Fig.3b), and when microsomes are adsorbed hollow circular wells can be seen in Fig. 3c (e.g. see arrow). These characteristic well features were seen only after microsomes is adsorbed as the fourth layer to the PDDA/DNA/PDDA film.

The average horizontal diameter of these circular wells (Fig. 3c) was 251 ± 36 nm and vertical height (Fig. 3d) was 19 ± 7 nm. The most likely explanation for these features is flattened microsomes, consistent with the microsomal layer thickness found by QCM. Thus, characteristic changes in surface topography from AFM images can monitor the stepwise layer-by-layer assembly of the PDDA/DNA/PDDA/RLM film. The microscopic studies correlate well with the higher mass and nominal thicknesses found for microsomes layers in QCM studies.

3.2. Electrolysis of RLM and SWV Sensor

Electrolysis of microsomes enriched with CYPs in thin polyelectrolyte films at negative potentials in the presence of oxygen has been shown to produce metabolic products from oxidation of styrene [39]. Voltammetric studies suggested that electrons enter the microsomal films via CYP reductases and are then transferred to CYPs to initiate the metabolic pathway [39, 40]. Here we evaluated the possibility that the reactive metabolites generated in this system can be trapped by DNA as adducts in the sensor films, and are sufficient to allow electrochemical detection.

For this purpose, PDDA/DNA/RLM sensors were prepared and electrolyzed at -0.7 V vs. SCE, slightly negative of the -0.5 V reduction potential of the reductase, in 1 mM NPYR for 1 h at 37°C and analyzed by SWV (Fig. 4). As a control experiment, sensors were electrolyzed only in buffer or not electrolyzed. A catalytic peak for DNA oxidation was observed at 1.01 V vs. SCE. The non-catalytic peak at ca. 0.65 V occurred from RLM alone which was confirmed from the SWV of (PDDA/RLM)₃ films (data not shown).

3.3. Enzymatic Activation of RLM and SWV Sensor

Figure 5. shows square-wave voltammograms of PDDA/DNA/RLM sensors with increasing times of reaction up to 30 min with 1 mM NPYR and NADPH system (10 mM MgCl_2 , 0.8 mM NADP, 10 mM glucose-6-phosphate, and 1 unit mL^{-1} of glucose-6-phosphate dehydrogenase) in 50 mM phosphate buffer, pH 7.0 at 37°C . The sensor electrodes after incubation in NPYR or styrene (1 mM) and NADPH generating system for the specified times were rinsed in water to stop the reaction, and then SWV was acquired in a cell containing 50 mM phosphate buffer + 0.1 M KCl, pH 7.0 and $50 \mu\text{M}$ $\text{Ru}(\text{bpy})_3^{2+}$ catalyst. Here, the oxidized Ru(III) at the electrode surface further electrochemically oxidizes the reactive metabolites adducted DNA having more exposed guanines compared to the intact double stranded DNA which results in increased peak current (Fig. 5) in the SWV [15 – 20, 26]. The established pathway for electrocatalytic DNA oxidation is shown below [where Gua = Guanine]. Adducted DNA is more disordered than ds-DNA, and thus guanines are more accessible to the catalyst, resulting in a larger catalytic current [15 – 20] (Scheme 1). In this way, adducts formed from reactive metabolites provide larger signals in the sensor.

The peak current at 1.01 V vs. SCE increased with increasing reaction time up to 30 min (Fig. 5), in accordance with our previous sensor reports for the purified CYP metabolizing styrene [15] and benzo[a]pyrene [16] to reactive metabolites detected as DNA adducts [15, 16]. Controls (in Fig. 5) are the sensor electrodes incubated only in NPYR with no NADPH system. After the reaction, the electrodes were rinsed in water, and SWVs were acquired in pH 7.0 buffer containing $50 \mu\text{M}$ $\text{Ru}(\text{bpy})_3^{2+}$. Similarly sensors were evaluated for styrene

metabolism under the same conditions where no increase in peak current was observed until 30 min (data not shown) at which a slight increase in peak current was seen (Fig. 6A).

3.4. Correlation of Sensor Response with Metabolite Production

Figure 6A. shows the increase in sensor signal as SWV peak current ratio after and before the reaction ($I_{p,f}/I_{p,i}$) for PDDA/DNA/RLM sensor electrodes incubated in NPYR (a) or styrene (b) plus NADPH system as electron donor with increasing times of reaction (in min) up to 30 min at 37°C in 50 mM phosphate buffer, pH 7.0. The $I_{p,i}$ is the peak current for sensors not exposed to NPYR or styrene, and $I_{p,f}$ is the peak current after exposure as described above for increasing time. Similarly, the peak current ratios for control PDDA/DNA/RLM film electrodes exposed to only NPYR or NADPH system alone for the same time and conditions (10 mM $MgCl_2$, 0.8 mM NADP, 10 mM glucose-6-phosphate, and 1 unit mL^{-1} of glucose-6-phosphate dehydrogenase) are also shown in Figure 6A (c and d).

The formation of the major 2-hydroxy tetrahydrofuran (2-OH-THF) metabolite from NPYR, and styrene oxide from styrene with reaction time (in pmol vs. reaction time) using (PDDA/RLM)₂ nanoreactors are shown in Figure 6B (see Sec.2.8 for details).

Representative capillary LC-MS chromatogram in total ion current (TIC) mode (Fig. 7a) was obtained by analysis of the NPYR reaction mixture after 10 min incubation with (PDDA/RLM)₂ nanoreactors in the presence of NADPH system. The MS spectra were acquired by monitoring the elution of compounds with selected m/z of 89 Da corresponding to the mass of 2-OH-THF metabolite from NPYR (Scheme 2). Calibration standards for the metabolite 2-OH-THF were used to identify and quantify the amount formed in the sensor film with reaction time (Fig. 6B, 2-OH-THF).

The GC chromatogram in Figure 7b corresponds to the product of styrene reaction with (PDDA/RLM)₂ nano-reactors in NADPH generating system forming a detectable amount of styrene oxide only after 30 min reaction time (Fig. 6B, styrene oxide).

Dividing the initial slope of sensor ratio plot (Fig. 6A) or metabolite amount with time (Fig. 6B) by the amount of enzyme present in the film provides relative turnover rates of the sensor and nanoreactors as shown in Table 2. The relative turnover rates of the voltammetry sensors and metabolites generation from silica nanoreactors were compared with the TD_{50} values [chronic dose in mg/kg body weight/day inducing mixed liver tumors in half of test rat population at end of standard life span] of NPYR and styrene in rat liver (Table 2). A smaller TD_{50} value corresponds to higher toxicity of a compound and hence should show a higher sensor ratio increase with reaction time than a less toxic compound.

The results presented above show that electrochemical toxicity sensors featuring microsome/DNA films provide responses that correlate well with rates of metabolite production estimated by LC-MS and TD_{50} values (Table 2) confirming that these sensors are useful for in vitro toxicity screening. The advantages of the modified single electrode voltammetry sensor are simplicity and ease of fabrication [15, 16]. The disadvantage is that the time of analysis is longer than our reported electrochemiluminescence (ECL) arrays, and throughput is limited [19, 25]. Nevertheless, the approach is useful for some applications in which a quick answer is needed to a very specific question, and avoids arrays spotting and optical detection needed for ECL arrays. While either chemical or electrochemical activation can be used to drive the enzyme reaction, the NADPH regenerating system as electron source via microsomal CYP reductase to CYPs seems to be faster and more efficient than electrolysis, although qualitatively sensor results are similar (cf. Figs. 4 and 5).

When the CYPs in RLMs are reduced by electrons supplied from the electrolysis process via CYP reductases [39], or through NADPH cycle involving the two electron transfers from CYP reductases to CYPs [24], metabolic activation of NPYR to reactive intermediates occurs resulting in DNA adducts in the film. This causes partial unwinding of DNA and exposure of guanine residues which upon SWV scan in buffer containing 50 μM $\text{Ru}(\text{bpy})_3^{2+}$ gives more peak current than the unreacted film, as documented previously [15, 16]. The slope of the increase in $I_{p,f}/I_{p,i}$ ratio with reaction time correlates with the extent of DNA adduct formation [41] and hence monitors the amount of accumulated reactive metabolites in the film with time (Fig. 6A). The negligible increases in peak current ratios with reaction time for the control films suggest that treatment of either NPYR or NADPH alone does not produce any signal. Therefore, the observed sensor responses are indeed due to the metabolic bioactivation of NPYR by microsomes following NADPH-CYP reductase pathway producing DNA reactive metabolites in the films. Thus the sensor results are consistent with the fact that nitrosamines indeed require metabolic activation to exert their carcinogenicity [27]. Styrene as the test compound under similar conditions as the NPYR sensor gave a much smaller increase in the sensor signal with time until 30 min of incubation (Fig. 6A) and hence smaller slope of $I_{p,f}/I_{p,i}$ with time was observed. This is consistent with styrene being bioactivated to a lesser extent than NPYR by rat liver microsomes [25].

Relative sensor response slopes for NPYR and styrene correlated well with the amount of metabolites formed from each compound in nanoreactors measured by LC-MS (Fig. 6B). The amount of major NPYR metabolite formation, 2-hydroxy tetrahydrofuran (2-OH-THF, Scheme 2) increased linearly to a few hundred pmol within 30 min reaction time (Fig. 6B, and Fig. 7a for LC-MS chromatogram) whereas styrene under similar reaction conditions formed only 3.7 pmol styrene oxide in 30 min (Fig. 6B, and Fig. 7b for gas chromatogram). At lower incubation times (<20 min), negligible styrene oxide was detected by GC from the nanoreactors reacted with styrene and NADPH system, the same trend as found for the sensor with styrene (Fig. 6A).

Thus, the higher turnover rate observed for NPYR compared to styrene for the voltammetry sensors and capillary LC-MS analysis is consistent with the higher toxicity of this nitroso compound over styrene. These observations are in accordance with the lower toxicity parameter TD_{50} , the dose of compound at which 50% of test rats developed liver tumors, for NPYR compared to styrene (Table 2) [42]. The observed turnover rates of voltammetry sensors also agree well with our recently reported electro-chemiluminescent (ECL) array data for nitrosamine and styrene [25].

4. Conclusions

We have shown here that simple liver microsomes/DNA film voltammetry sensors made layer-by-layer on pyrolytic graphite electrodes are capable of detecting DNA-reactive metabolites from model toxic compounds and can provide in vivo toxicity screening. Sensor surfaces having immobilized drug metabolizing enzymes and DNA have potential biological applications for simple drug reactive metabolite screening with an electrochemical output by using $\text{Ru}(\text{II})$ catalyst. Further, the sensor response and metabolite formation rates correlated well with the reported in vivo TD_{50} values for rat liver validating this approach for in vitro toxicity screening of new drugs and other potentially toxic chemicals in development.

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Fig. 1. QCM frequency shifts for cycles of alternate PDDA/DNA and PDDA/RLM adsorption on gold resonators coated first with a monolayer of 3-mercaptopropionic acid rendering a negatively charged resonator surface. The final film assembly used in the voltammetric sensors is (PDDA/DNA/PDDA/RLM)₂ (average values $\pm$ *SD* for 3 replicate resonators).

Fig. 2.
TEM characterization of morphology of RLM adsorbed on carbon coated copper grid.

Fig. 3. Characterization of topography and feature sizes of layer-by-layer assembly of PDDA, DNA, and RLM by AFM: Tapping mode atomic force microscopy (AFM) images of a) PDDA film; b) PDDA/DNA film; c) PDDA/DNA/PDDA/microsomes film on mica surface, and d) is the height image of (c).

Fig. 4.

Square-wave voltammograms of PDDA/DNA/RLM films on rough PG electrodes. The CYP enzymes in RLMs were reduced by electrons supplied via CYP reductase from electrolysis at -0.7 V vs. SCE for 1 h in the presence of O_2 in 50 mM phosphate buffer plus 50 mM KCl, pH 7.0 at 37°C in a) 1 mM NPYR; b) buffer having no NPYR and c) PDDA/DNA/RLM film not subjected to electrolysis. After electrolysis, the electrodes were rinsed in water and SWVs were acquired in 50 mM phosphate buffer plus 0.1 M KCl, pH 7.0 containing $50\text{ }\mu\text{M Ru(bpy)}_3^{2+}$.

Scheme 1.

Fig. 5.

Square-wave voltammograms of PDDA/DNA/RLM films incubated in 1 mM NPYR with NADPH generating system (10 mM MgCl_2 , 0.8 mM NADP, 10 mM glucose-6-phosphate, and 1 unit mL^{-1} of glucose-6-phosphate dehydrogenase) at 37°C in 50 mM phosphate buffer + 50 mM KCl, pH 7.0, for increasing times of reaction (in min) as labeled on the curves. Here the generated NADPH supplies electrons to CYPs via CYP reductase present in microsomes to metabolize the substrates under study [24] (see Sec. 2.7 for details). Also shown are control PDDA/DNA/RLM films incubated only in NPYR (no NADPH) under the same conditions. The SWVs were acquired in 50 mM phosphate buffer plus 0.1 M KCl, pH 7.0 containing $50\ \mu\text{M}\ \text{Ru}(\text{bpy})_3^{2+}$.

Fig. 6.

A) Influence of a) 1 mM NPYR + NADPH system (•); b) 1 mM styrene + NADPH system (◆); c) only NADPH system (△); and d) only NPYR (□) reaction time on sensor peak ratio after: before enzyme reaction ($I_{p,f}/I_{p,i}$) with PDDA/DNA/RLM films on PG electrodes at 37°C in 50 mM phosphate buffer, pH 7.0. B) The amount of 2-hydroxy tetrahydrofuran (2-OH-THF) metabolite formed from NPYR or styrene metabolites measured by LC-MS or GC (in pmol) with reaction time when (PDDA/RLM)₂ films on silica nanoparticles (nanoreactors) were reacted with 1 mM NPYR or 1 mM styrene plus NADPH generating system in 50 mM phosphate buffer, pH 7.0 at 37°C.

Scheme 2.
Metabolic pathways of test compounds.

Fig. 7.

a) Representative LC-MS chromatogram in total ion current (TIC) mode for the (PDDA/RLM)₂ nanoreactor film reacted with 1 mM NPYR + NADPH generating system in 50 mM phosphate buffer, pH 7.0 at 37°C for 10 min; b) Gas chromatogram of (PDDA/RLM)₂ film reacted with 1 mM styrene + NADPH generating system under the same conditions as in (a) for 30 min at which detectable styrene oxide was seen.

Table 1

Average characteristics of PDDA/DNA/RLM assembly from QCM.

Assembly	Thickness (nm)	PDDA ($\mu\text{g cm}^{-2}$)	DNA ($\mu\text{g cm}^{-2}$)	RLM ($\mu\text{g cm}^{-2}$)
(PDDA/DNA/PDDA/RLM) ₂	15.1 $\pm$ 0.5	1.0 $\pm$ 0.3	0.7 $\pm$ 0.1	3.5 $\pm$ 0.3

Table 2

Comparison of sensors response and nanoreactors metabolite production vs. enzyme reaction time to in vivo toxicity data for rats.

Compound	Sensor turnover rate ^[a] (min ⁻¹ (mg protein) ⁻¹)	Nanoreactors turnover rate ^[b] (pmol min ⁻¹ (mg protein) ⁻¹)	TD50 values in rat liver ^[c]
<i>N</i> -Nitrosopyrrolidine	17 ± 2.3	59 ± 6	1.5 ^[d]
Styrene	1.4 ± 0.2	0.3 ± 0.04	23 ^[e]

^[a] Obtained by dividing the initial slope of SWV sensor ratio (Fig. 6A) by the amount of RLM present in the assembly from QCM (Table 1)

^[b] obtained by dividing the initial slope of metabolite production vs. time (Fig. 6B) by the amount of RLM present in the nanoreactors (determined by Bradford protein assay)

^[c] from L. S. Gold [42], The Carcinogenic Potency Database, <http://potency.berkeley.edu>, 2007

^[d] ad libitum in drinking water

^[e] gavage administration.