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Microfluidic Electrochemical Array for Detection of Reactive Metabolites Formed by Cytochrome P450 Enzymes

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Abstract

A novel, simple, rapid microfluidic array using bioelectronically driven cytochrome P450 enzyme catalysis for reactive metabolite screening is reported for the first time. The device incorporates an 8-electrode screen printed carbon array coated with thin films of DNA, [Ru(bpy)₂(PVP)₁₀](ClO₄) {RuPVP}, and rat liver microsomes (RLM) as enzyme sources. Catalysis features electron donation to cyt P450 reductase in the RLMs and subsequent cyt P450 reduction while flowing an oxygenated substrate solution past sensor electrodes. Metabolites react with DNA in the film if they are able, and damaged DNA is detected by catalytic square wave voltammetry (SWV) utilizing the RuPVP polymer. The microfluidic device was tested for a set of common pollutants known to form DNA-reactive metabolites. Logarithmic turnover rates based on SWV responses gave excellent correlation with the rodent liver TD₅₀ toxicity metric, supporting the utility of the device for toxicity screening. The microfluidic array gave much better S/N and reproducibility than single electrode sensors based on similar principles.

Cytochrome P450 enzymes (cyt P450s) comprise a family of iron heme NADPH-dependent monooxygenases involved in the majority of human metabolic reactions.^{1–4} Oxidative metabolism of xenobiotic molecules by cyt P450s is an evolutionary adaptation aimed at facilitating excretion by producing water-soluble metabolites. However, *reactive* metabolites are often formed that induce toxic effects via reactions with DNA, proteins, and other biomolecules.^{4,5} Predicting the formation of these reactive metabolites is important for development of safe drugs and environmental chemicals.^{6,7} Cytotoxicity bioassays, animal testing and *in silico* methods are used widely to provide predictions of drug and chemical toxicity^{5–7,8} Unfortunately, even when using a sophisticated battery of such tests, about 30% of drugs manifest toxicity issues only after reaching human clinical trials, and a few even after marketing.^{5–7}

Oxidative catalysis by cyt P450s is facilitated by donation of electrons from NADPH via cyt P450 reductase (CPR) to the enzyme's iron heme center (Scheme S1, Supporting information (SI)).^{4,5,9} Replacing NADPH by an electrode is a cost-effective and simplifying approach for devices utilizing these enzymes in thin films. For example, cyt P450s in layer-by-layer (LbL) films with polyions^{10,11} or insoluble surfactant films¹² have been utilized to facilitate direct electron exchange between the electrode and enzymes. However, direct electron donation to cyt P450s in aerobic media leads to activation of the enzyme by hydrogen peroxide generated in a complex process involving reduced cyt P450s.⁹ This differs from the natural pathway, and may be less efficient in some cases.^{13–16} Catalytic activity is improved in genetically engineered fusion proteins of cyt P450s and the reductase

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domain of CPR or flavodoxin.^{17,18} In this approach, electrons are injected into the redox protein fused to the cyt P450, then transferred to the enzyme.

We recently reported a third approach that accurately reproduces the natural cyt P450 catalytic cycle on electrodes by using LbL films that combine microsomal CPR with excess human liver cyt P450s. Electrons are transferred from electrode to CPR to cyt P450s in these films.^{9,19} Substrate conversion rates suggested bioelectronic catalysis with these films was as fast or faster than the conventional NADPH-driven process.

Over a decade ago, we began developing new devices and methodologies for molecular-based chemical and drug toxicity screening.^{7,20,21} These techniques are based on 20–50 nm-thick LbL films that combine metabolic enzymes with DNA. The enzyme reactions produce metabolites that react with DNA bases.²² The rate of DNA damage is measured as an indicator of genotoxicity. Early devices utilized hydrogen peroxide to drive cyt P450 reactions on voltammetric sensors^{23,24} and electrochemiluminescent (ECL) arrays utilizing the redox polymer $\text{Ru}(\text{bpy})_2^{2+}$ (polyvinylpyridine).^{25,26} These devices evolved to include liver microsomes and bicistronic single cyt P450 preparations in the films as sources of cyt P450 enzymes and CPR, and the reactions were driven by NADPH.^{27,28} Similar enzyme/DNA films on nanoparticles were developed as “bioreactors” for identifying structures and measuring formation rates of DNA-metabolite adducts using liquid chromatography-mass spectrometry (LC-MS).^{29–32}

Up to now, these toxicity screening arrays have utilized manual wet chemistry operations. However, microfluidic approaches are attractive to improve hydrodynamic control of reaction chemistry and introduce partial automation.^{33–36} A microfluidic device featuring immobilized cyt P450 fusion proteins on gold electrodes for drug metabolite profiling was reported recently.³⁷

The present paper describes the first integration of bioelectronically actuated natural cyt P450 catalysis using rat-liver microsome/DNA films into a microfluidic device designed to detect reactive metabolites. As with our previous toxicity screening arrays,⁷ reactive metabolites are formed and subsequently trapped by reacting with DNA in the films, and the rate of DNA damage is monitored. However, this new device employs electrochemical actuation of the cyt P450 enzymes instead of using hydrogen peroxide or NADPH. Thus, enzyme bioactivation accurately mimics the natural pathway of cyt P450s without using the expensive NADPH as electron source.

The microfluidic system used was adapted from a recently developed protein assay device.³⁸ A 63 μL polydimethylsiloxane (PDMS) channel fabricated by molding is held in a poly(methylmethacrylate) (PMMA) housing with inlet connected to a syringe pump and sampling valve (Scheme 1). Long Ag/AgCl reference and Pt counter wire electrodes and a replaceable 8-sensor array are symmetrically located along the channel to minimize cross talk. Each electrode is coated with redox polymer $[\text{Ru}(\text{bpy})_2(\text{PVP})_{10}](\text{ClO}_4)$ {RuPVP (bpy = 2,2'-bipyridyl; PVP = poly(4-vinylpyridine)}, DNA, and a microsomal enzyme source. The enzyme reaction is run first, followed by square wave voltammetry (SWV) detection of the resulting DNA damage.³⁹

The microfluidic device showed distinct analytical advantages compared to stand-alone toxicity sensors based on similar films when tested on a series of organic pollutants that form DNA-reactive metabolites. Reproducibility and analysis time compared to stand-alone electrodes was improved, and all sensors gave good correlation with the rodent TD₅₀ liver toxicity metric.

EXPERIMENTAL SECTION

Chemicals and materials

Ruthenium metallopolymer [Ru(bpy)₂(PVP)₁₀](ClO₄) (RuPVP (bpy = 2,2-bipyridyl; PVP = poly(4-vinylpyridine)) was synthesized and characterized by established protocols.³⁹ 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, M_w= 207.23) was from Toronto Research Chemicals (Ontario, Canada). Benzo[a]pyrene (B[a]P, M_w= 252.31), *N*-nitrosopyrrolidine (NPYR, M_w= 100.12), styrene(M_w= 104.15), *N*-(9*H*-fluoren-2-yl)acetamide (2-AAF, M_w= 223.27), tetrahydrofuran (THF, M_w= 72.11), poly(diallyldimethylammonium chloride) (PDDA, average M_w= 100,000–200,000), poly(sodium 4-styrenesulfonate) (PSS, average M_w= 70000), calf thymus DNA (Type I) and all other chemicals were from Sigma. Pooled rat liver microsomes (RLM-F344, 20 mg mL⁻¹ in 250 mM sucrose) were from BD Gentest (Woburn, MA) and contained (a) 20 mg mL⁻¹ total protein content, (b) total cyt P450 content of 590 pmole mg⁻¹ of protein using the method of Omura and Sato,⁴⁰ (c) CPR activity of 330 nmol cyt c reduced (mg of total protein × min)⁻¹, (d) 760 pmol mg⁻¹ cyt b₅, Specific cyt P450 enzyme activities in picomol product per mg of total protein per minute were: (a) cyt P450 3A – 3300 from testosterone 6β-hydroxylase assay, (b) cyt P450 2C – 5100 from testosterone 16α-hydroxylase assay, (c) cyt P450 2E1 – 1100 from p-nitrophenol hydroxylase assay, (d) cyt P450 1A – 180 from 7-ethoxyresorufin O-diethylase assay, and (e) cyt P450 4A – 1200 from [¹⁴C] lauric acid-hydroxylase assay. The bicinchoninic acid (BCA) protein assay kit (μBCA assay kit, model number = 23235) was from Thermoscientific (IL, USA). Screen printed carbon 8-electrode arrays were from Kanichi, UK.

Microfluidic system

This has been adapted from a previously reported protein detection device.³⁸ Full details are in the Supporting Information file.

Film construction

LbL films of architecture PDDA/PSS/(RuPVP/DNA)₂/PDDA/RLM/PDDA/DNA were deposited a layer at a time from 1 μL droplets placed on each carbon electrode on an array using optimized solution compositions reported previously,^{26–28} (a) PDDA, 2 mg mL⁻¹ in 0.05 M NaCl; (b) PSS, 3 mg mL⁻¹ in 0.5 M NaCl; (c) RuPVP, 3 mg mL⁻¹ 50% V/V ethanol; (d) Calf thymus DNA, 2 mg mL⁻¹ in 10 mM TRIS + 0.5 M NaCl, pH 7.4; (e) RLM, 20 mg mL⁻¹ in potassium phosphate buffer, pH 7.4. To achieve steady state adsorption, 1 μL of each adsorbate solution was incubated on electrodes for 20 min. at 4 °C, except RLM and DNA where 30 minute incubations were used.¹⁰ Similar films were fabricated on basal plane pyrolytic graphite (PG) electrodes, after first polishing with 400 grit SiC paper and sonicating in ethanol and pure water successively for 1 min, then rinsing with water and dried.

Electrochemical and surface characterization

Full details are in Supporting Information file.

Metabolite generation and measurements

Safety note: Styrene, NNK, NPYR, 2-AAF and Benzo[a]pyrene and their metabolites are known carcinogens. All manipulations were done under a closed hood while wearing gloves.

Metabolites were generated within the microfluidic device by flowing oxygenated reactant solutions over the 8 enzyme/DNA film-coated electrodes in the microfluidic device at 50 μL min⁻¹ with constant potential electrolysis at -0.65 V vs. Ag/AgCl (0.14 M KCl) using an

eight-electrode CHI 1030 electrochemical workstation at ambient temperature $22 (\pm 2) ^\circ\text{C}$ (see Figure S2, SI). The following oxygenated buffer solution compositions were used, (a) 2 mM styrene in 50 mM phosphate + 0.1 M NaCl of pH 7.4, (b) 0.2 mM NPYR in 50 mM phosphate + 0.1 M NaCl of pH 7.4, (c) 0.2 mM NNK in 50 mM phosphate + 0.1 M NaCl of pH 7.4, (d) 0.05 mM 2-AAF 1% dimethyl sulfoxide (DMSO) in 10 mM acetate + 0.1 M NaCl of pH 4.5, and (e) 0.025 mM B[a]P 1% DMSO in 50 mM phosphate + 0.1 M NaCl, pH 7.4. 2 mM THF in 50 mM phosphate + 0.1 M NaCl of pH 7.4 was used as a negative control. After washing with anaerobic 50 mM phosphate + 0.1 M NaCl buffer of pH 7.4 for 5 minutes at $50 \mu\text{L min}^{-1}$, square wave voltammograms (SWV) were recorded. (ampl. 25 mV; freq. 15 Hz; step 4 mV). This procedure was used to obtain SWV signals at different electrolysis times.

Metabolite generation for single PG electrode sensors was accomplished by constant potential electrolysis at -0.65 V vs. SCE in 1 mL oxygenated solutions of substrates for a desired time interval while the electrode was rotated at 1000 rpm. SWV was recorded in anaerobic 50 mM phosphate plus 0.1 M NaCl buffer, pH 7.4.

RESULTS

Film construction and characterization

Films containing DNA, RLM, and RuPVP were made by alternate electrostatic layer-by-layer (LbL) film deposition in which each new layer had a charge opposite of the preceding one.^{10,11} Optimized film compositions giving the best SWV signals for toxicity screening were developed for the array and the PG electrodes. Film fabrication was monitored using a quartz crystal microbalance (QCM). For this purpose, films were grown on gold-coated QCM crystals, and dried between each layer deposition. Stable and reproducible film growth was confirmed by QCM results (Figure S3, SI). Amounts of film components and thicknesses are shown in Table 1. Assay data used to obtain the amount of protein in the films are also included in Table 1 (also, see Figure S4, SI).

Atomic force microscopy (AFM) was used to obtain topographic and phase contrast images of PDDA/PSS/(RuPVP/DNA)₂/PDDA/RLM films on smooth mica (Figure 1). Figure 1a shows a single layer of PDDA with nominal thickness of $1.7 \pm 0.3 \text{ nm}$ (from QCM), where a relatively smooth film of polycation is apparent. After the deposition of PDDA/PSS/(RuPVP/DNA)₂ layers (Figure 1b), a less-uniform film with occasional pebble-like features was observed. Surface roughness increased 26-fold compared to the PDDA film (Table 2). This morphology may be caused by the non-uniform adsorption of RuPVP on the surface followed by strong electrostatic interaction with the oppositely charged DNA. Figure 1c is an image of the film after deposition of PDDA followed by RLM. A dense coverage with a distribution of circular well-like structures of variable size was found, with a 2-fold increase in surface roughness over the underlying layer. Microsomes create these well-like vesicular features by partially flattening on surfaces, as concluded from previous electron microscopy and AFM studies.^{41,42} Horizontal diameter of the circular structures was $141 \pm 35 \text{ nm}$ and vertical height was $12 \pm 5 \text{ nm}$. Electron microscopy on RLMs revealed similar circular features with variable diameters of 80–400 nm.⁴¹

Phase contrast AFM provides information on surface composition and viscoelasticity,⁴³ and phase boundaries can sometimes be resolved. The phase contrast image in Figure 1(d) reveals variable size distribution of the well-like structures associated with RLMs. A drastic decrease in QCM frequency (Figure S3, SI) upon adsorption of RLMs suggested a surface layer with nominal thickness $34 \pm 4 \text{ nm}$. This is confirmed in Figures 1(c) and (d), where morphology changes from Figure 1b are consistent with coverage by the relatively large RLMs..

RLM LbL films on electrodes gave quasireversible cyclic voltammetry (CV) at a midpoint potential of -0.48 V vs. SCE characteristic of cyt P450 reductase (CPR).⁴⁴ A quasi reversible CV (Figure S5, SI) was also found for RLM in the films used in the present work with oxidation-reduction midpoint potential of -0.48 V vs. SCE. As with related microsomal CPR films,^{9,19,44} this is consistent with electrons being donated from the electrode to CPR and not to cyt P450s, which have more positive redox potentials.¹⁹ Reasons for the unique electron flow pathway involve complexes between cyt P450s and CPR, and have been reported previously.^{9,19}

Toxicity screening

In the presence of substrate and oxygen, electrons delivered to CPR can be transferred to cyt P450s to drive the enzyme reactions electrochemically.⁹ Using this approach, electrode-driven activation of cyt P450 enzymes in the microsomal films was evaluated for genotoxicity screening while monitoring DNA damage by SWV.^{7,21} Microfluidic arrays were compared with stand-alone PG electrode sensors using similar DNA/RLM/RuPVP films optimized for each measurement configuration. Metabolites generated by cyt P450s react with DNA in the film to form DNA-metabolite adducts, and the SWV peak at 1.15 V vs. Ag/AgCl (0.14 M NaCl) reflects the extent of DNA damage.^{21,23,39}

We tested our approach using pollutant molecules known to produce reactive metabolites via cyt P450 oxidations. These included styrene,³² 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK),⁴⁵ *N*-nitrosopyrrolidine (NPYR),⁴⁶ *N*-(9*H*-fluoren-2-yl)acetamide (2-AAF),³² and benzo[*a*]pyrene (B[*a*]P).²⁵ Metabolite-DNA adduct chemistries are well understood for these compounds, which undergo metabolic oxidations including hydroxylation and epoxidation (Scheme 2.).

All 8 electrodes in the microfluidic array were equipped with DNA/RLM films and were exposed to oxygenated substrate solutions at $50 \mu\text{L min}^{-1}$ for desired times while applying a constant potential of -0.65 V vs. Ag/AgCl to drive the catalytic process.¹⁹ After subsequent washing with buffer, SWVs were recorded with the flow stopped in anaerobic pH 7.4 buffer. SWV peak current increased with enzyme reaction time for all pollutants (Figure 2). SWV peak current density in the microfluidic array was significantly larger than that of the single PG electrode (Figure S6, SI). Eight replicate SWVs were obtained per measurement in the microfluidic system, which gave about 2-fold better reproducibility than the single electrode sensors in most cases (Figure S8, SI). The increase in SWV peak current with enzyme reaction time is related to the extent of DNA damage caused by cyt P450 metabolites.^{20,23,24}

Toxicity screening data are presented as the SWV peak ratio ($I_{p,f}/I_{p,i}$) (Figure 3), i.e., the ratio of SWV difference peak current after enzyme catalysis ($I_{p,f}$) to that of the control with no enzyme reaction ($I_{p,i}$). The initial slopes of these lines are proportional to the relative rate of DNA damage.^{20,24,26} The negative control 2 mM THF did not demonstrate significant DNA damage under our assay conditions (Figure 3f). This is consistent with its relatively low genotoxicity, and suggests a negligible influence on the SWV signal from a non-reactive organic component in the sample.

SWV peak ratio slope per μg of protein per mM of substrate provides relative turnover rates for the formation of metabolites that damage DNA, and as such should be related to liver genotoxicity. These turnover rates show very good correlations with a previously reported ECL-based toxicity array²⁸ (Figure 4a) as well as with the rodent liver toxicity metric TD₅₀ (Figure 4b). The ECL based toxicity array²⁸ utilizes similar thin films of RLM, DNA and RuPVP as in the microfluidic array to measure relative rates of DNA damage. Figure 4 (a) illustrates excellent agreement of the log of enzyme turnover rates obtained by the two array designs.

TD₅₀ is the chronic dose (mg/kg body weight per day) of a substance that causes mixed liver tumors in half of a test male rodent population by end of the standard life span.^{28,48,49} The log of enzyme turnover rates from the sensor array show good correlation with reciprocal of the rodent liver TD₅₀, (Figure 4(b)). We express this toxicity metric as 1/TD₅₀, (reciprocal of TD₅₀), which is proportional to liver toxicity and correlates directly with the log of turnover rate. The lack of response for THF (Figure 3f) is consistent with its TD₅₀ value⁴⁸ of 407 (1/TD₅₀ = 0.0025).

DISCUSSION

Results above support the utility of a purely electrochemical microfluidic array featuring DNA, RuPVP and microsomes to screen for DNA-reactive metabolites using bioelectronically driven cyt P450 enzyme reactions. As with the NADPH-driven enzyme reactions in similar films,^{20,21,26–28} catalytic sensor response increases with the enzyme reaction time for pollutant conversion (Figure 2).

As depicted in Figure 3, there are no appreciable SWV ratio changes for controls, and negligible responses for THF, a compound with very low genotoxicity. Given previous correlations with increases in amounts of specific nucleobase adducts with enzyme reaction time in similar films measured by LC-MS,^{20,21,26–32} the data in Figures 2 and 3 provide clear evidence of DNA damage from metabolites formed by cyt P450 enzymes. Further, turnover rates derived from assay results show excellent correlation with rodent 1/TD₅₀ values of the compound set (Figure 4b), as well as with recently reported results from ECL toxicity arrays (Figure 4a).²⁸

The microfluidic platform provides a number of advantages over single sensors and non-microfluidic arrays.^{20,21} Clearly, smaller size and multiplexity facilitates use of smaller amounts of expensive enzyme sources and low reagent consumption leading to faster, more cost effective assays. Fluid dynamics under laminar flow is known to enhance sensitivity of detection.³³ This is reflective in the present study by SWV turnover rates with dramatically larger values than for the single electrode mode, leading to better signal/noise and reproducibility of microfluidic assisted platform (Figure S8, SI). A constant maintenance of substrate concentration in the electrode array under laminar flow as well as more efficient utilization of enzymes are logical causes for enhanced enzyme kinetics that provided improved sensitivity over the single electrode platform. Comparison between these two sensor designs reveals <10% average relative standard deviation (RSD) in microfluidic assisted toxicity array compared ~15% for single electrode sensors (Figure S8, SI). Moreover, RSD of NNK, NPYR and 2-AAF was significantly lower for the microfluidic array compared to the single sensor. This suggests that enzyme kinetics for these 3 substrates are governed by the rate of mass transport of substrate, as their metabolic chemistries feature initial hydroxylation by cyt P450 enzymes (Scheme 2). Average RSD of B[a]P gave similar values for both sensor platforms, consistent with a prominent radical bioactivation pathway in B[a]P metabolism (Scheme 2(e)), where the effect from mass transport limitations on reaction kinetics may be smaller due to very high reactivity of the radical cations.

Previous studies on RuPVP, enzyme and DNA assemblies showed very good stability and retention of ~80% activity after two months of storage at 4 °C.^{10,23,49} RLM is a common source of cyt P450 enzymes and other cofactors required for bioelectronically actuated catalysis. The amount of protein in the film can be measured with a BCA assay.⁵⁰ Furthermore, virtually any source of metabolic enzymes can be used in the LbL films including single cyt P450 *supersomes*, human liver microsomes, cytosol, liver S9 fraction, or pure enzymes.^{7, 20,21,28}

Styrene had lowest turnover rate in the selected compound set consistent with *in vivo* and *in vitro* studies.^{27,28} NNK and NPYR demonstrated larger SWV turnover rates that are similar to each other, basically due to similar reaction chemistry of the two compounds.^{45,46} *In vitro* ECL genotoxicity assays confirmed the same result (Figure 4a).^{27,28} Lower reactivity of NNK and NPYR metabolites compared to that of 2AAF and B[a]P may be due to the instability of active diazonium ions in water.^{28,51}

2-AAF and B[a]P gave the largest turnover rates in the compound set for the microfluidic array (Figure 4) and the single electrode sensor (Figure S7, SI). B[a]P bioactivation occurs via two major pathways (Scheme 2e), namely (1) conjugation, where a diol-epoxide intermediate acts as the reactive species, and (2) a radical pathway, where a cation radical is reactive.²⁵ Previous studies suggest prominence of the radical pathway over the diol-epoxide pathway *in vivo*, despite confirmed formation of diol-epoxide.^{25,52}

2-AAF bioactivation occurs via N-hydroxylation by cyt P450s in RLMs, (mainly Cyt P450 1A2) to form N-hydroxy-2-acetylaminofluorene (N-hydroxy-2-AAF, Scheme 3, compound 2), which is a very reactive metabolite. However, removal of the acetyl group from N-hydroxy-2-AAF by deacylase enzymes in RLM generates N-hydroxyaminofluorene (N-hydroxy-AF, Scheme 3, 3), which then undergoes dehydroxylation in acidic media to yield highly reactive arylnitrenium ion (Scheme 3, 5). The pH 4.5 buffer was used in this protocol to provide mildly acidic conditions to generate arylnitrenium ion. This acid-catalyzed pathway was reported to be a major cause of bladder cancer in mammals, where lower pHs in the bladder facilitate generation of reactive metabolites from arylamines.⁵³

In summary, the present study shows for the first time that electrochemically-driven cyt P450 catalysis following the natural pathway^{9,19} can be used for *in vitro* screening for reactive metabolites using 8-electrode arrays on a microfluidic platform. Excellent correlation of the results with reported TD₅₀ values suggests the value of this array as a genotoxicity prediction tool. This new approach simplifies the technology compared to previous ECL arrays, since the same 8-electrode system can be used for enzyme reactions and detection and provides automation of the reaction chemistry. Electrode driven methodology removes the requirement of expensive NADPH and does not require detection of light as in ECL arrays. Nevertheless, the ECL systems have the current advantage in multiplexing, since they can be configured with up to 50 individual spots, and do not require individually addressable sensor electrodes.²¹ Future progress in electrochemical microfluidic arrays for toxicity screening will require higher throughput and inclusion of metabolic bioconjugation enzymes. These goals are currently being pursued in our laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Images of topographical features of films on flat mica obtained by tapping mode atomic force microscopy (AFM) for LbL films of: (a) PDDA (b) PDDA/PSS/(RuPVP/DNA)₂ (c) PDDA/PSS/(RuPVP/DNA)₂/PDDA/RLM, and (d) phase contrast AFM image of (c).

Figure 2.

Representative difference SWVs at different enzyme reaction times in anaerobic pH = 7.4 phosphate buffer for microfluidic array sensors featuring optimized films of PDDA/PSS/(RuPVP/DNA)₂/PDDA/RLM/PDDA/DNA, (SWV ampl. 25 mV; freq. 15 Hz; step 4 mV) (a) 2 mM Styrene, (b) 0.2 mM NPYR, (c) 0.2 mM NNK, (d) 0.05 mM 2-AAF, (e) 0.025 mM B[a]P, (f) 2 mM THF.

Figure 3.

Influence of substrate incubation time on SWV peak current ratio ($I_{p,f}/I_{p,i}$) for microfluidic toxicity sensor array using PDDA/PSS/(RuPVP/DNA)₂/PDDA/RLM/PDDA/DNA, films: (a) 2 mM styrene, (b) 0.2 mM NPYR, (c) 0.2 mM NNK, (d) 0.05 mM 2-AAF, (e) 0.025 mM B[a]P, (f) 2 mM THF. Controls are incubations without substrate or exposure to the substrate without electrolysis, which gave equivalent results. Error bars represent standard errors for n=4–8.

Figure 4. Correlation of the logarithm of turnover rates ($\{\mu\text{g protein}^{-1}\} \text{ s}^{-1} \text{ mM}^{-1}$) measured by the microfluidic array (a) with previously reported ECL array turnover rates²⁸ and (b) with reciprocal of reported TD₅₀ values.²⁸

Scheme 1.

Strategy for detection of reactive metabolites formed by cytochrome P450 enzyme catalysis using a microfluidic 8-electrode device

Scheme 2.

DNA nucleobase adduct formation from metabolic cyt P450 bioactivation of (a) styrene, (b) NPYR, (c) NNK, (d) 2-AAF and (e) B[a]P.

Scheme 3. Bioactivation of 2-AAF by RLM enzymes in acidic medium.⁵³

Table 1

Film characteristics from QCM and protein assays

Total mass of RuPVP/ $\mu\text{g cm}^{-2}$	Total mass of DNA/ $\mu\text{g cm}^{-2}$	Total mass of RLM/ $\mu\text{g cm}^{-2}$	Mass of total protein/ μg		Total thickness/nm
			On PG electrodes	On array electrodes	
5.6 (± 0.3)	11 (± 1)	11.3 (± 0.3)	8 (± 3) $\times 10$	1.5 (± 0.1) $\times 10$	89

^a From BCA total protein assay (Supporting Information), 35.6 (± 0.1) μg of protein per mg of RLM, electrode area (PG = 0.16 cm^2 , array electrode = $3.85 \times 10^{-3} \text{ cm}^2$)

Table 2Mean surface roughness of films from AFM^a

Surface layer architecture	Mean surface roughness/nm
PDDA	0.10 ± 0.03
PDDA/PSS/(RuPVP/DNA) ₂	2.6 ± 0.1
PDDA/PSS/(RuPVP/DNA) ₂ /PDDA/RLM	4.4 ± 0.1

^a Surface roughness was estimated using Nanoscope IV Version 5.30r3.Sr3 software (Veeco).