Screening for Reactive Metabolites using Electro-optical Arrays Featuring Human Liver Cytosol and Microsomal Enzyme Sources and DNA

Linlin Zhao, John B. Schenkman and James F. Rusling

Supplementary Information

Experimental Details

Chemicals and Materials. The electrochemiluminescent ruthenium metallopolymer was synthesized and characterized following an established protocol.[1] Silica microspheres were from Polysciences Inc. (Warrington, PA. 500 nm (±10%) diameter, approximately 10% solids, d = 1.96g cm^{-3}). All other chemicals were from Sigma-Aldrich. Human liver microsomes (pooled, female) and human liver cytosol (single donor, female) were supplied by BD biosciences (Woburn, MA).

Array Film Characterization. Quartz crystal microbalance (QCM) analysis was employed to monitor the film growth and nominal thickness at each layer application step. Briefly, a droplet of RuPVP (2.5mg mL^{-1}), DNA (2 mg mL^{-1}), human liver cytosol or microsomes (20 mg protein mL^{-1}) was placed on the 9 MHz quartz resonator (0.16 cm^2, International Crystal, OK) for 15 min, except for 30 min for cytosol or microsomes. The resonator was then rinsed with water, dried under a gentle stream of nitrogen and placed into the QCM for frequency recording.

Film Fabrication. Array spots containing human liver cytosol and/or microsomes were assembled on a 1 x 1 in. pyrolytic graphite (PG) chip using established protocols.[2,3] The following solutions were used for film assembly: 2 mg mL^{-1} calf thymus DNA (10 mM, Tris pH 7.4, 50 mM NaCl), 2.5 mg mL^{-1} RuPVP 88 (88% H_2O/12% ethanol), 2.5 mg mL^{-1} RuPVP 50 (50% H_2O/50% ethanol), and 20 mg mL^{-1} total protein human liver cytosol (in 50 mM Tris pH 7.5) or microsomes (in 250 mM sucrose), with a final film construction of DNA/(RuPVP88/DNA)$_2$/(RuPVP50/DNA/cytosol)/(RuPVP88/DNA/cytosol)$_2$ or DNA/(RuPVP88/DNA)$_2$/(RuPVP50/DNA/cytosol)/(RuPVP88/DNA/cytosol)/RuPVP88/ DNA/microsomes. Analogous films were assembled on 500 nm-diameter silica beads following previous methods with modifications.[3] Briefly, 2 mL of poly(diallyldimethylammonium chloride) (PDDA) (2 mg mL^{-1}, 50 mM NaCl) was added to 1.8 mL (1.96g mL^{-1}) of silica beads and was allowed a 15-min assembly. The suspension was centrifuged for 1 min at 8000 rpm and rinsed with water to remove loosely bound polyions. The beads were then dispersed in 1.5 mL of ds-DNA solution (0.5 mg mL^{-1}) allowing a 15-min assembly, which was followed by rinsing. Similar steps were followed for cytosol or microsomes adsorption for 30 min. After fabrication, the films had a following architecture: PDDA/DNA/PDDA/cytosol or PDDA/DNA/PDDA/cytosol/microsomes. The modified beads were dispersed in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) to a final volume of 1.8 mL and stored near 0 ºC till use.

Metabolic Activation. ***Safety note****: ethylene dibromide and 2-amonifluorene are suspected carcinogens. All procedures were done while wearing gloves and under closed hoods.*

(1) ECL Array. Reaction was initiated by treating the array spots with the reaction solution and stopped by rinsing the solution off the arrays with water. For ethylene dibromide reaction, array spots were exposed to 50 mM MES buffer (pH 6.0) solution containing ethylene dibromide (80 μM) and 0.5 mM cofactor glutathione (GSH). Inhibition reactions were conducted at the same conditions, expect that spots were pretreated with 175 μM bilirubin at 37 ºC for 10 min. For cytosolic NAT and microsomal enzymes mediated 2-amonifluorene reaction, 50 mM MES buffer (pH 6.0) containing 100 μM 2-amonifluorene, 0.5 mM acetyl coenzyme A (AcCoA), 1.6 mM DTT, 0.5 mM EDTA and an NADPH generating system (10 mM glucose 6-phosphate, 4 units of glucose-6-phosphate dehydrogenase, 10 mM $MgCl_2$, 0.80 mM $NADP^+$) was incubated at 37 ºC for 10 min before exposing to array spots. Inhibition of NAT by luteolin was done by incubating spots for 10 min with

350 μM luteolin at 37 ºC before exposing to the same enzymatic activation solution. For cytosolic NAT mediated reaction, the NADPH regenerating system was neglected. The array chip is placed in an open-top electrochemical cell in a dark box with a CCD camera mounted on top to measure the ECL light emission and the ECL image was collected and processed following previously described methods.[2]

(2) Nanoreactors. Metabolic enzyme reactions were started by adding the substrate into dispersed silica beads (200 μL) with DNA/cytosol or DNA/cytosol/microsome films in the same reaction condition as in ECL array experiments. Reactions were allowed for 5 min and stopped by centrifugation. Silica beads with modified DNA were dispersed into 150 μL 10 mM potassium phosphate buffer (pH 7.0) followed by enzymatic digestion using previous protocol with modification.[4] Briefly, DNA coated silica beads were incubated with deoxyribonuclease I (400 unit/mg of DNA) for 12 h at 37 ºC, followed by incubation with phosphodiesterase I (0.2 unit/mg of DNA) and phosphatase, alkaline (1.2 unit/mg of DNA) for 5 h at 37 ºC. All solutions were filtered with 0.2 micron filter before injecting into LC.

CapLC-MS/MS Analysis. (1) CapLC. The capillary LC system (Waters, Milford, MA) was equipped with column switching, which allows the selective capture of nucleobase adducts in a trapping column, while the unmodified nucleobases were directed to waste. The trapping column (Atlantis dC18, 23.5 mm, 0.18 mm i.d., 5µm particle size) and analytical column (Atlantis dC18, 150 mm, 300 µm i.d., 5µm particle size) were from Waters (Milford, MA). A 10µL portion of the sample was loaded into the trapping column at a flow rate of 9.25 µL min^{-1} and flushed with ammonium acetate buffer at a flow rate of 9.25 µL min^{-1} for 1 min before switching to in-line position. Elution onto the analytical column was achieved at a flow rate of 9.25 µL min^{-1} with the following gradient: (A, 10 mM ammonium acetate buffer, pH 4.5; B, acetonitrile) 2min, 0% B; 13 min, 0-30% B; 2min 30% B; 8min, 30-0% B. A photodiode array detector was used to monitor the wavelength 210-300nm throughout the analysis.

(2) On-line CapLC-MS/MS: A Q-TRAP 4000 Applied Biosystems (Foster City, CA) mass spectrometer with Analyist 1.4 software was operated in the positive ion mode. Samples were analyzed in multiple reaction monitoring (MRM) mode at 4500eV ion spray voltage, 40 eV collision energy, 30 eV declustering potential and 0.2 s dwell time for each mass pair.

Results

Estimated amounts of cytosol, microsomes, DNA and RuPVP in the film were obtained from quartz crystal microbalance (QCM) studies.[4] The mass of each applied layer was estimated using Sauerbrey equation:

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

The nominal dry film thickness was determined using an empirically equation derived from high-resolution electron microscopy:

$$d\ (nm) \approx (-0.016 \pm 0.002)\ \Delta F\ (Hz) \qquad (2)$$

ΔF values and eq 1 were used to obtain weights of liver fractions and DNA. Equation 2 was used to estimate the average nominal thickness of the films, as summarized in Table 1.

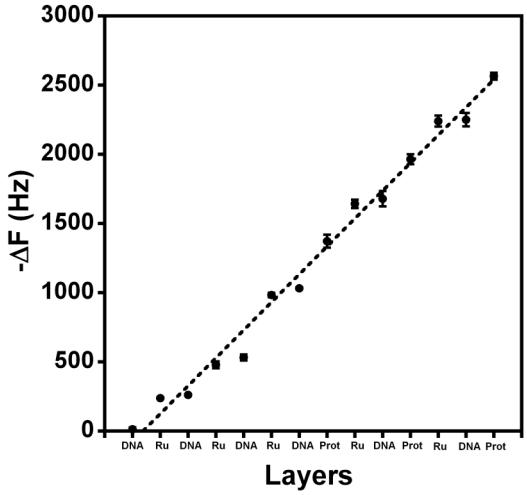

Figure S1. QCM frequency response upon application of each layer (Ru = RuPVP, DNA, and Prot = human liver cytosol) used in the construction of the ECL array films. The error bars shown is representative of 3 trials.

Table S1. QCM Characteristics of DNA/cytosol and DNA/cytosol/microsomes Films (data represent mean±SD from three trials).

Composition	Average Film Weight (μg cm^{-2})				Average Film Thickness (nm)
	RuPVP	DNA	Cytosol	Microsomes	
DNA/cytosol	7.86±0.32	1.02±0.42	5.13±0.35	———	41±1
DNA/cytosol/microsomes	7.35±0.40	1.23±0.28	3.44±0.38	0.92±0.26	39±1

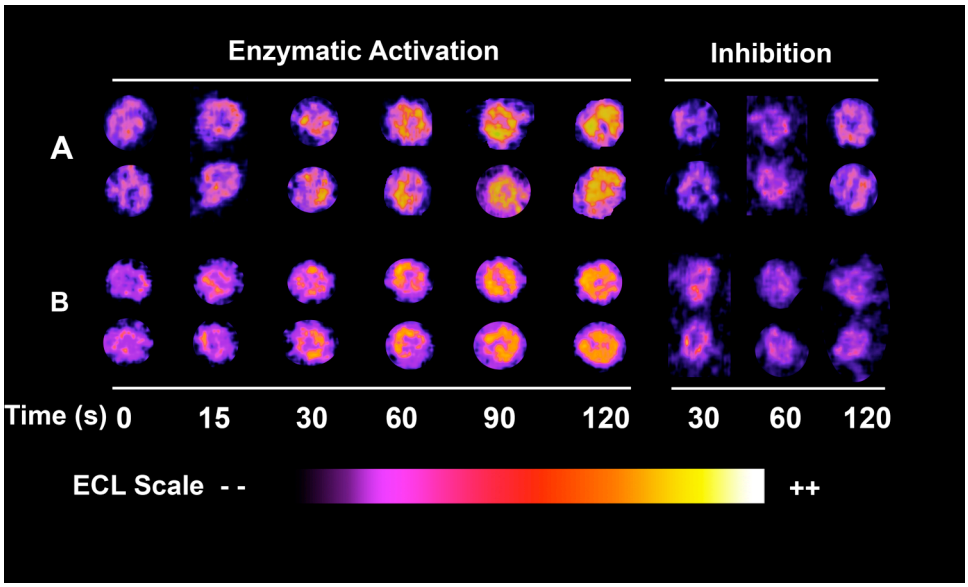

Figure S2. Reconstructed ECL array data from (A) spots of RuPVP/DNA/human liver cytosol exposed to 80 μM EDB, 0.5 mM glutathione in 50 mM MES buffer (pH 6.0) for denoted time. Inhibition reactions were conducted at the same conditions, expect that spots were pretreated with 175 μM bilirubin at 37 ºC for 10 min; (B) spots of RuPVP/DNA/human liver cytosol/human liver microsomes exposed to 100 μM 2-AF, 0.5 mM acetyl coenzyme A, 1.6 mM DTT, 0.5 mM EDTA and an NADPH regenerating system in a 50 mM MES buffer (pH 6.0). Inhibition reactions were conducted at the same conditions, expect that spots were pretreated with 350 μM luteolin at 37 ºC for 10 min.

References

1 L. Dennany, R. J. Forster and J. F. Rusling, *J. Am. Chem. Soc.*, 2003, **125**, 5213-5218.
2 E. G. Hvastkovs, M. So, S. Krishnan, B. Bajrami, M. Tarun, I. Jansson, J. B. Schenkman and J. F. Rusling, *Anal. Chem.* , 2007, **79**, 1897-1906.
3 S. Krishnan, E. G. Hvastkovs, B. Bajrami, D. Choudhary, J. B. Schenkman and J. F. Rusling, *Anal. Chem.* , 2008, **80**, 5279-5285.
4 L. Zhou, J. Yang, C. Estavillo, J. D. Stuart, J. B. Schenkman and J. F. Rusling, *J. Am. Chem. Soc.*, 2003, **125**, 1431-1436.