Published in final edited form as:

Analyst. 2016 October 21; 141(20): 5722–5729. doi:10.1039/c6an01237j.

Microfluidic Array for Simultaneous Detection of DNA Oxidation and DNA-Adduct Damage

Boya Song^a, Min Shen^a, Di Jiang^a, Spundana Malla^a, Islam M. Mosa^a, Dharamainder Choudhary^b, and James F. Rusling^{a,b,c,d}

^aDepartment of Chemistry, University of Connecticut, Storrs, CT, USA 06269

^bDepartment of Surgery and Neag Cancer Center, University of Connecticut Health Center, Farmington, CT, USA 06032

^cInstitute of Materials Science, University of Connecticut, Storrs, CT, USA 06269

^dSchool of Chemistry, National University of Ireland, Galway

Abstract

Exposure to chemical pollutants and pharmaceuticals may cause health issues caused by metabolite-related toxicity. This paper reports a new microfluidic electrochemical sensor array with the ability to simultaneously detect common types of DNA damage including oxidation and nucleobase adduct formation. Sensors in the 8-electrode screen-printed carbon array were coated with thin films of metallopolymer osmium or ruthenium bipyridyl-poly(vinylpyridine) chloride (OsPVP, RuPVP) along with DNA and metabolic enzymes by layer-by-layer electrostatic assembly. After a reaction step in which test chemicals and other necessary reagents flow over the array, OsPVP selectively detects oxidized guanines on the DNA strands, and RuPVP detects DNA adduction by metabolites on nucleobases. We demonstrate array performance for test chemicals including 17 β -estradiol (E₂), its metabolites 4-hydroxyestradiol (4-OHE₂), 2-hydroxyestradiol (2-OHE₂), catechol, 2-nitrosotoluene (2-NO-T), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and 2-acetylaminofluorene (2-AAF). Results revealed DNA-adduct and oxidation damage in a single run to provide a metabolic-genotoxic chemistry screen. The array measures damage directly in unhydrolyzed DNA, and is less expensive, faster, and simpler than conventional methods to detect DNA damage. The detection limit for oxidation is 672 8-oxodG per 10⁶ bases. Each sensor requires only 22 ng of DNA, so the mass detection limit is 15 pg (~10 pmol) 8-oxodG.

Graphical Abstract

A microfluidic electrochemical sensor array detects DNA oxidation and adduct formation for a fast genotoxicity chemistry screen for test compounds.

[†]Electronic Supplementary Information (ESI) available: Details about experimental section and additional figures and tables. See DOI: 10.1039/x0xx00000x

1. Introduction

There is a pressing need to improve in vitro assessments of possible toxicity risks for humans from emerging environmental pollutants and pharmaceutical candidates. Toxic effects related to the damage of genetic material (i.e. DNA) are referred to by the term *genotoxicity*. DNA damage gives rise to mutations, and can be a key event in carcinogenesis.¹ There are two major categories of DNA damage: (1) oxidative damage, in which the nucleobases are oxidized by reactive oxygen species (ROS), which can be formed by redox cycling involving certain pollutant or drug metabolites,² and (2) damage in which metabolites or parent compounds react chemically with nucleobases to form so called DNA adducts.^{3,4} These adducts with nucleobases can also induce depurination and strand breakage. While LC-MS and other approaches can measure DNA adducts^{5–7} and oxidized DNA (as 8-oxo-deoxyguanosine, 8-oxodG),^{8–14} protocols most often require hydrolysis of the damaged DNA, and may be too time consuming and expensive for screening large numbers of new chemicals.

We previously developed individual microfluidic electrochemical and electrochemiluminescent (ECL) sensor arrays to measure metabolite-related DNA damage in films from adduct formation^{15–17} and for separate measurements of 8-oxodG in intact DNA in solution.^{18,19} Herein we report a new array that employs thin films of metabolic enzymes, DNA and catalytic Ru and Os metallopolymers to measure metabolite-related DNA damage from both adduct formation and oxidation simultaneously.

Reactive metabolites of chemicals can react with DNA nucleobases to form covalently linked nucleobase adducts that may be key players in carcinogenesis, and are important biomarkers of genotoxicity.^{20,21} Oxidative DNA damage²² can result from oxidative stress, which reflects an imbalance between ROS levels and the capacity of antioxidant defenses. Oxidative stress can occur in cellular metabolism.²³ In the presence of NADPH and metal ions, metabolites can be involved in formation of ROS that oxidize DNA bases.²⁴ An important biomarker for oxidative stress is the predominant product of DNA oxidation, 8-oxo-7-hydro-2'-deoxyguanosine (8-oxodG).²⁵

This paper describes the first microfluidic array that detects both DNA adduction and DNA oxidation simultaneously by using sensors coated with thin films containing DNA, metabolic enzymes and metallopolymers $[\text{Me}(\text{bpy})_2(\text{PVP})_{10}]^{2+}$ (bpy = 2,2'-bipyridine, PVP = polyvinylpyridine; Me = Ru or Os). Sensors coated with RuPVP/DNA/enzyme films detect covalent DNA damage from adduct formation by providing catalytic voltammetric peaks proportional to the amount of exposed deoxyguanosine (dG) due to the disorder of DNA double strands after damage.¹⁵ Sensors in the array with OsPVP/DNA/enzyme films oxidize

8-oxodG in the presence of intact nucleobases to provide catalytic voltammetric peaks proportional to the amount of 8-oxodG.¹⁸ RuPVP has the higher oxidation potential near 1.2 V vs Ag/AgCl (0.05 M NaCl) and selectively oxidizes guanines.⁴ OsPVP with oxidation potential of 0.3 V vs Ag/AgCl (0.05 M NaCl) selectively oxidizes 8-oxodG but not guanines.¹⁸ We evaluated array performance using chemicals and metabolites known to cause DNA damage including 17 β -estradiol (E₂), its metabolites 4-hydroxyestradiol (4-OHE₂), 2-hydroxyestradiol (2-OHE₂), catechol, 2-nitrosotoluene (2-NO-T), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 2-acetylaminofluorene (2-AAF) and tobacco smoke. Results were consistent with literature of DNA damage by these species, and show that the array can simultaneously reveal DNA-adduct based damage and DNA oxidation.

2. Experimental

Chemicals and materials

Screen-printed carbon arrays were from Kanichi, UK. Synthesis and characterization of metallopolymer [Os(bpy)₂(PVP)₁₀Cl]⁺ (OsPVP) were by previously described procedures.^{26,27} Full experimental details and material sources are in the ESI.

Sensor array fabrication

The screen-printed carbon array contains eight electrodes to allow eight measurements simultaneously. Human liver microsomes (HLM) were used in films as human metabolic enzyme sources and contain up to a dozen active cyt P450 isoforms, and significant amounts of a few bioconjugation enzymes.^{3,20,21} Cationic polymer poly(diallyldimethylammonium chloride) (PDDA) was used as an interlayer in the films to hold together layers of negatively charged DNA and microsomes. To detect DNA oxidation, four of the sensors in the array were coated with OsPVP and salmon testes double stranded DNA (ds-DNA) with and without human liver microsomes (HLM) by layer-by-layer electrostatic film self-assembly³ based on the application. To detect DNA-adduct damage, similar films in which RuPVP replaced OsPVP were used. These films are denoted in subsequent text as PDDA/PSS/(metallopolymer/DNA)₃ or PDDA/PSS/(metallopolymer/DNA)₃/PDDA/HLM/PDDA/DNA. Films were characterized by quartz crystal microbalance (QCM) measurements (see ESI). The microfluidic array device features a two-channel syringe pump and a switch valve connected in series to the inlet of the 8-sensor array (ESI, Scheme S1).¹⁸

Voltammetric analysis of damaged DNA

Safety note: 4-hydroxyestradiol (4-OHE₂), 2-hydroxyestradiol (2-OHE₂), 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 2-acetylaminofluorene (2-AAF) and 2-nitrosotoluene (2-NO-T) are carcinogens. Proper protections should be taken.

General procedures for using the array involved: (i) pumping a reactive solution across the sensors in the detection chamber for desired times to effect possible DNA damage, then pumping in an electrolyte wash solution, and (ii) running square wave voltammetry (SWV) with the electrolyte solution flow stopped to measure relative catalytic currents for oxidation and DNA-adduct formation. For DNA oxidation, the reaction solution containing the test

compound, 0.5 mM Cu^{2+} and NADPH regenerating system (1 U/mL G6PDH enzyme, 2.5 mM G6P, 0.5 mM NADP^+ , 1 mM Mg^{2+}) in 50 mM pH 7.0 Tris buffer with 50 mM NaCl (Tris buffer) was pumped into the microfluidic sensor at constant flow rate ($50 \mu\text{L min}^{-1}$) for different reaction intervals. Then the microfluidic channel was flushed with 10 mM pH 5.5 acetate buffer + 50 mM NaCl for 2 min. Flow was stopped and SWV recorded on all 8 electrodes by using an eight-channel CHI 1040b electrochemical workstation. SWV were done at 4 mV step, 25 mV pulse, and 15 Hz frequency.

Constant potential of $-0.65 \text{ V vs. Ag/AgCl}$ was applied to sensors coated with enzyme/DNA film while flowing reactant solution to activate metabolic enzymes. Reactant solutions for adduct formation were: (1) 150 μM NNK in 50 mM pH 7.0 Tris buffer with 50 mM NaCl (Tris buffer) + 0.5 mM Cu^{2+} + NADPH regenerating system; (2) 250 μM 2-AAF in Tris buffer + 0.5 mM Cu^{2+} + NADPH regenerating system. (In some cases, this enzyme-activation step was also used for DNA oxidation.) After electrolysis, the array was flushed with buffer for 2 min, flow stopped and SWV recorded.

UPLC-MS/MS measurement of 8-oxodG in oxidized DNA

Thin films of DNA were grown by the LbL approach on 1 μm carboxylate-functionalized magnetic beads (Invitrogen) to make $(\text{PDDA}/\text{DNA})_3$ films. The DNA-coated magnetic beads were incubated with catechol, 2-NO-T, 4-OHE₂, 2-OHE₂ or estradiol metabolite extracts with 0.5 mM Cu^{2+} and NADPH regenerating system to oxidize DNA. Beads were then washed twice with pH 7 Tris buffer and subjected to enzymatic hydrolysis.¹⁸ Hydrolyzed sample solutions were vacuum filtered using OmegaTM membrane 96-well filtration plate (3KDa MW cutoff). (Scheme 1) 10-fold diluted nucleoside samples were analyzed using a Dionex Ultimate 3000 UHPLC interfaced to an Applied Biosystems 4000 QTRAP triple quadrupole linear ion trap mass spectrometer (AB Sciex) in positive ion mode (details in ESI).

3. Results

Metabolite-induced DNA oxidation

Thin films containing DNA and metallopolymer, with and without HLM were assembled on the sensors using layer-by-layer (LbL) alternate electrostatic film deposition (see experimental).³ Film construction was characterized by QCM using 9 MHz gold-quartz resonators to measure mass and nominal film thickness. (See ESI.) Linear relationships of $-F$ versus layer number demonstrated stable and reproducible film depositions, with average nominal thickness of $\sim 40 \text{ nm}$. (Figures S1 and S2). Average amount of DNA per film is 22 ng.

Test compounds with known metabolic chemistry leading to DNA damage were used to evaluate the arrays. We focused first on sensors for oxidation of DNA in the sensor films, since we have earlier addressed DNA-adduct related measurements in detail and here simply included them in the array using earlier protocols.^{3,4} Metabolite-related DNA oxidation was demonstrated by using 17 β -estradiol (E_2), and its major metabolites, 4-OHE₂, 2-OHE₂,

known to cause DNA oxidation.²⁸ 2-Nitrosotoluene (2-NO-T) is the major metabolite of o-toluidine, a common pollutant.²⁹

Tumor development initiated by metabolic conversion of the estrogen 17 β -estradiol (E₂) to 4-OHE₂ has been observed in rodent models.²⁸ These metabolites, 2-OHE₂ and 4-OHE₂ containing quinone moieties are considered major contributors to estrogen-related carcinogenesis (Scheme 2). In the assays, test compound solution containing Cu²⁺ and NADPH regenerating system flows over the thin films of DNA on the sensors. ROS are formed (Scheme 2 to 4) in the microfluidic channel and oxidize nucleobases in the DNA films. OsPVP on the sensors selectively catalyzes deoxyguanosine (dG) oxidation in the DNA strands to 8-oxodG,¹⁸ giving catalytic SWV peaks.

Figure 1a, 1c show the Os and Ru SWV curves resulting from exposing DNA films on the sensor arrays to 4-OHE₂ or 2-OHE₂ in the Cu²⁺ + NADPH reaction cocktail. Os SWV peaks at ~0.3 V vs Ag/AgCl in the array increased with reaction time, suggesting oxidation of dG to the 8-oxodG biomarker on the DNA strands (Scheme 2). Longer reaction times increased Os SWV peaks (Figure 1b, 1d), suggested increasing DNA oxidation with time. However, the Ru SWV peaks were not influenced significantly by the oxidation reaction, suggesting that except for oxidation, very little additional DNA damage occurs in these systems. The Ru³⁺ form of RuBPY does not catalytically reduce 8-oxodG because all of the 8-oxodG has been reduced directly on the electrode (oxidation potential ~0.6 V vs Ag/AgCl^{30,31}) by the time the applied potential reaches the more positive Ru²⁺ oxidation peak. Controls represent the effect of reactant solutions without test compounds on sensor elements coated with the same films. SWV peaks of OsPVP and RuPVP sensors remained unchanged after control reactions.

As above, chemicals with catechol-like quinone functionality can generate ROS to oxidize DNA when undergoing redox cycling as in Scheme 3. DNA oxidation by catechol with Cu²⁺ and NADPH was measured as a positive control by our array, and gave results qualitatively similar to those in Figure 1, consistent with this view. (Figure S3), as reported earlier for soluble DNA.¹⁸ The array responses for Os peaks (Figure 1) show that both 2- and 4-OHE₂ facilitate DNA oxidation. Specifically, 2-OHE₂ produces less (~50 %) 8-oxodG than 4-OHE₂ under equivalent condition (Figures 1b and 1d). This observation is consistent with reports that 2-OHE₂ is less toxic than 4-OHE₂ in vivo.³² In animal models, E₂ induces tumors in tissues in which E₂ is predominantly converted to the 4-OHE₂, whereas tumors fail to develop in organs in which 2-OHE₂ is the predominant metabolite.³³

2-NO-T is an example of a ROS-promoting metabolite without a quinone moiety. This test compound with Cu²⁺ + NADPH also gave and increased Os peaks, but no change in Ru peaks (Figure S4), even though the damage mechanism (Scheme 4) is different than for quinoid compounds. This shows that the array responds to DNA oxidation independent of the chemical pathway.

Detection of DNA adducts formation on the array

NNK and 2-AAF were chosen as model test compounds for DNA adduct formation, since both of them are known carcinogens that undergo metabolism to produce DNA-reactive

metabolites which form DNA adducts (Scheme 5).⁴ NNK is one of the most prevalent carcinogens in tobacco smoke.³⁴ 2-AAF is a discontinued insecticide and suspected human carcinogen.³⁵ Cyt P450s are the major metabolic enzymes responsible for the bioactivation. Human liver microsomes (HLM) were used as the source of cyt P450s in our array films to provide a representative source of metabolic enzymes. The sensor films used were HLM/Ru or OsPVP/DNA.

For arrays utilizing enzyme conversion of the test compounds to metabolites, reactant solution was pumped over the eight enzyme/DNA film-coated electrodes at constant flow rate to generate metabolites and react with DNA within the films. A potential of -0.65V vs. Ag/AgCl was applied to activate cyt P450 enzymes in the microsomal films as reported previously.¹⁵ Os and Ru SWV responses and peak ratios vs. enzyme reaction time are shown in Figure 2 (NNK) and Figure S5 (2-AAF). Controls feature reactant solution without test compounds. Ru SWV peak ratios for NNK and 2-AAF increased with reaction time, but no significant response was found for controls (Figures 2 and S5). Results suggest the extent of adduct-based DNA damage, mainly resulting from the formation of covalent DNA adducts which disorder the double helix, resulting in dG exposure to Ru sites in the polymer, faster catalytic oxidation and larger peak currents.^{3,4} On the other hand, the Os SWV peaks (Figure 2) did not change for reaction times up to 20 min, the time scale for DNA oxidation in Figure 1. We conclude that NNK and 2-AAF metabolites do not induce DNA oxidation with Cu^{2+} and NADPH. OsPVP cannot oxidize dG since its oxidation potential is too low.³⁸

Detection of DNA damage in mixtures

To detect the two types of DNA damage simultaneously, the reactant solutions containing NNK and catechol was used in the array. These two representative compounds were chosen because they have been shown to induce DNA adduct formation and DNA oxidation, respectively, and we don't have an example of one compound that causes both DNA oxidation and adduct formation. Additionally, these compounds are main components in cigarette smoke, which is a common environmental pollutant.^{39, 40}

Plots of SWV peak current ratio vs. reaction time are shown for OsPVP (Figure 3a) and RuPVP (Figure 3b). The solid curves represents SWV ratio vs. reaction time obtained for the mixture of both compounds, and the dashed curves indicates the SWV ratios vs. reaction time from detecting one compound by the same sensor. The slopes for both Ru and Os curves are not significantly different according to t-tests at 95% confidence interval when using a mixture, or the one compound that actually produces the signal, i.e. catechol for OsPVP and NNK for RuPVP.

Detection of DNA damage from cigarette smoke

As a practical application, the sensor was used to analyze the capacity of cigarette smoke to damage DNA using thin films of DNA, HLM and the metallopolymer. The cigarette smoke was trapped by an artificial inhalation device, and then extracted into THF¹⁸ (see ESI). Figure 4 shows the Os and Ru SWV of the sensors after treatment with a reaction cocktail including the smoke extract. Both Os and Ru SWV peaks show significant increases after reaction. These results are consistent with our previous observations that cigarette smoke

extract with Cu^{2+} , and NADPH oxidizes DNA in solution.¹⁸ The trend of Os SWV peak current ratio vs. reaction time was converted into number of 8-oxodG per 10^6 nucleobases from a calibration curve developed by using oxidized DNA samples whose 8-oxodG concentrations were measured by LC-MS/MS (Figure S9) as previously.¹⁸ The trend was similar with that of catechol induced DNA oxidation detected by the array (Figure S3), consistent with catechol being a major components of cigarette smoke. On the other hand, the trend of Ru SWV peak current ratio vs. reaction time was similar to that of NNK induced DNA damage detected by the same sensor array (Figure 2). NNK is also abundant in cigarette smoke, and a major contribution to DNA adduct formation when exposing DNA to cigarette smoke. Comparing these data to Figure 2, we estimated that the DNA adduct-based damage from the cigarette smoke in this experiment is equivalent to that induced by $140\ \mu\text{M}$ NNK.⁴¹

Detection of DNA damage by 17β -estradiol

17β -estradiol (E_2) is an endogenous steroid hormone that may be associated with breast cancer incidence. Epidemiological data shows a strong positive correlation between estrogen levels and lifetime risk of breast cancer development in women.⁴² E_2 metabolites 2-OHE₂ and 4-OHE₂ also correlate with estrogen-related carcinogenesis (Scheme 2).⁴³ E_2 also promotes breast cancer in rodents.⁴⁴

Using HLM or rat liver microsomes (RLM) on the array with NADPH, Cu^{2+} and E_2 as reactant in experiments similar to those in Figure 3 gave no detectable DNA oxidation. Thus we verified DNA oxidation using an offline metabolic conversion. E_2 was reacted with RLM enzymes to form the metabolites in solution. RLM was used since it more effectively converts E_2 to metabolites than HLM. Products were extracted, concentrated and reconstituted in DMSO (see ESI for details). Figure 5 shows Os and Ru SWV of array sensors coated with DNA and metallopolymers after reacting with the E_2 metabolite mixture with Cu^{2+} and NADPH. The Os peaks increased (Figure 5a) with reaction time, indicating the formation of 8-oxodG. Development of Os SWV peak ratio ($I_{p,t}/I_{p,i}$) vs. reaction time (Figure 5b) and the lack of response for RuPVP were similar to that of the pure metabolites (Figure 1). This illustrates that offline metabolite generation for a test compound can also be used to evaluation possible DNA oxidation.

UPLC–MS/MS measurement of E_2 metabolites

The formation of 4-OHE₂ and 2-OHE₂ as reactive metabolites after E_2 metabolism by metabolic enzyme RLM was confirmed by LC MS/MS (see ESI details). Figure 6a shows the multiple-reaction monitoring (MRM) chromatogram of metabolites after incubating E_2 with RLM and extracting as above. The MRM peak for transition 287-147 (blue peak) clearly shows hydroxylated metabolites 4-OHE₂ and 2-OHE₂ are formed after the incubating E_2 with metabolic enzyme, as confirmed with standards.

Figure 6b, 6c are tandem mass spectra of peaks in Figure 6a which correspond to the 4-OHE₂ and E_2 standards. Additionally, the MS pattern of Figure 6b and 6c are also consistent with the MS/MS database METLIN (Scripps Center, CA).⁴⁵

UPLC–MS/MS measurement of DNA oxidative damage

Amounts of 8-oxodG in DNA oxidized by catechol, 2-NO-T, 4-OHE₂, 2-OHE₂ and E₂ metabolites were measured using UPLC–MS/MS to validate 8-oxodG formation in each reaction for specific time to complement sensor results.

Magnetic beads (1 μ m) decorated with (PDDA/DNA)₃ films were used as bioreactors to produce DNA oxidative products induced by test compounds, followed by enzymatically hydrolysis, filtration and UPLC-MS/MS detection of 8-oxodG. Multiple reaction monitoring (MRM) mode was used to measure amounts of dG and 8-oxodG in these samples. Figure S6 shows the MRM chromatogram of transition m/z 268 to 152 at 6.2 min corresponding to peaks of dG and transition m/z 284 to 168 at 7.3 min for 8-oxodG. Insert in Figure S6 show an MRM chromatogram with mass transition m/z 284 to 168 indicating the formation of 8-oxodG. Relative amounts of 8-oxodG (number of 8-oxodG per 10⁶ nucleobases) for each oxidation time of each test compounds were obtained from these results. Figure S7 and S8 are the trends of relative amounts of 8-oxodG found in the UPLC–MS/MS assay for each test compound. The LC–MS/MS limit of detection of 8-oxodG was 10 fmol 8-oxodG per 2.2 pmol of DNA (one 8-oxodG per 10⁶ nucleobases).

4. DISCUSSION

Results above demonstrate the successful development of an electrochemical screening platform that simultaneously detects DNA damage from oxidation and nucleobase adduct formation. To achieve this task, sensors in the array were equipped with thin films containing metabolic enzymes, DNA, and either OsPVP or RuPVP to electrocatalyze DNA damage products to detect their reactions with DNA. By monitoring responses of OsPVP and RuPVP sensors with metabolic enzymes in the films, a metabolite-related genotoxic chemistry profile can be developed. We illustrated performance of the array by evaluating metabolite-related DNA oxidative damage for estrogens and important pollutants (Figures 1 and 2), and demonstrated simultaneous detection of oxidation and adduct formation (Figure 3 and 4). Observation of DNA oxidation or adduct formation is consistent with literature reports on these compounds. UPLC-MS/MS was used to measure the relative amount of 8-oxodG in DNA samples exposed to 4-OHE₂, 2-OHE₂, catechol and 2-NO-T (Figure S7). These 8-oxodG curves mirror trends in OsPVP ratios vs. reaction time in the sensor arrays (Figure 1, 4 and 5, S3 and S4), lending additional confidence that the sensors are measuring 8-oxodG in the DNA strands. DNA adducts formed by NNK and 2-AAF via HLM bioactivation was previously confirmed using LC-MS/MS.³

Ease of use, fast detection (5 min/assay) and versatility are important analytical features of the array. The potential genotoxicity estimates for test compounds requires minimal sample workup, unlike competing methods (e.g. LC-ECD, LC-MS). The limit of detection (LOD) of the array for DNA oxidation is 672 8-oxodG per 10⁶ bases. Each sensor holds 22 ng of DNA, so the mass detection limit is 15 pg (~10 pmol) 8-oxodG. This is better by three orders of magnitude than that of our voltammetric array that measures oxidized DNA in solution.¹⁸ For DNA adduct-based damage, we showed previously that our electrochemical approaches can detect ~3 damaged bases in 10⁴ intact bases in DNA, and this is adequate to reliably measure relative rates of DNA damage that correlate with other toxicity metrics.²¹

An important feature of the present system is the low DNA sample size that provides good potential for clinical and research applications, especially where amounts of DNA extracted from tissue is a major limitation.^{46, 47}

The assay using cigarette smoke presents a practical example measuring DNA oxidation and adduct formation from a common environment pollutant. Cigarette smoke is a mixture of >5000 compounds including nearly 100 known carcinogens, mutagens, and tumor promoters.⁴⁸ It is a major cause of lung cancer, which is a leading cause of cancer deaths in the USA⁴⁹ and worldwide.⁵⁰ Cigarette smoke contains 73 chemicals classified as carcinogenic by the International Agency for Research on Cancer (IARC) and there is evidence that >20 induce lung tumors.^{51,52} Among these, there is strong evidence that NNK is a cause of lung cancer by forming DNA adducts through metabolic bioactivation.⁵³ Cigarette smoke also contains catechol and contributes to cigarette smoke-mediated ROS generation (Scheme 3) and carcinogenicity.⁴⁸ Figure 4 clearly shows the ability of the array to measure relative rates of DNA oxidation and adduct formation in the same experiment.

E₂ was examined as a risk factor for breast cancer, and because its major metabolites 4-OHE₂ and 2-OHE₂ are also available for study.⁵⁴ These electrophilic quinone metabolites undergo redox cycling with Cu²⁺ and NADPH to form ROS to oxidize DNA (Scheme 2).^{55, 56} Cyt P450 1B1 favors 4-OHE₂ metabolite formation by catalyzing estrogen 4-hydroxylation, whereas the 2-OHE₂ metabolite depends mainly on Cyt P450s 1A1 and 1A2 for conversion.^{57,58} High levels of estradiol and Cyt P450 1B1 in breast cancer tissues have been suggested as a possible cause of breast cancer.⁵⁹

Array results with Cu²⁺ and NADPH show that at equal concentrations 4-OHE₂ induces more than twice the level of DNA oxidation compared to 2-OHE₂ (Figure 1). This suggests that 4-OHE₂ induces more ROS than 2-OHE₂, leading to enhanced DNA oxidation. Our findings are also consistent with epidemiological data in human⁴² and animal studies^{60,33} in which 4-OHE₂ was more carcinogenic than 2-OHE₂.⁶¹

For investigations above showing both responses, the DNA-adduct signal develops much faster than the the DNA oxidation signal (Figures 3 and 4). This is a limitation of the array, and we have not yet found a system which can produce metabolites in the device in the presence of Cu²⁺ and NADPH that showed measurable DNA oxidation. With E₂, we demonstrated DNA oxidation with 0.5 mM of the metabolites (Figure 1), but could not observe oxidation with E₂ when HLM or RLM were present in sensor films. This is despite the fact that microsomal cyt P450s did convert E₂ to expected metabolites (Figure 6). We speculate that this is because the concentrations of metabolites produced by the microsomal enzyme films are too small (probably μM or less) to generate sufficient ROS to oxidize DNA in the films. We are currently investigating conditions and modified arrays that might accomplish this task. However, in the meantime, we demonstrated using E₂ that metabolites could be generated off line and introduced to the array to investigate their DNA oxidation capacity.

In summary, this paper describes development and evaluation of a microfluidic voltammetric sensor array to simultaneously detect DNA oxidation and DNA-adduct-based damage. The

array is cheaper and faster than existing separate analyses, does not require DNA hydrolysis, and uses only 22 ng DNA per sensor. This approach can be used to screen test compounds for possible DNA damage, then the damaging compounds' genotoxicity pathways can be investigated in molecular detail using LC-MS/MS.⁴

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the National Institute of Environmental Health Sciences (NIEHS), NIH, USA, Grant No. ES03154 for generous financial support of this research.

References

1. Kryston TB, Georgiev AB, Pissis P, Georgakilas AG. *Mutat Res Fund Mol Mech Mut.* 2011; 711:193–201.
2. Cooke MS, Evans MD, Dizdaroglu M, Lunec J. *The FASEB Journal.* 2003; 17:1195–1214. [PubMed: 12832285]
3. Rusling JF, Wasalathanthri DP, Schenkman JB. *Soft Matter.* 2014; 10:8145–8156. [PubMed: 25209428]
4. Hvastkovs EG, Rusling JF. *Anal Chem.* 2016; 88:4584–4599. [PubMed: 27043322]
5. Park BK, Boobis A, Clarke S, Goldring CEP. *Nature Rev Drug Discov.* 2011; 10:292–307. [PubMed: 21455238]
6. Guengerich FP. *Chem Res Toxicol.* 2001; 14:611–650. [PubMed: 11409933]
7. Tarun M, Rusling JF. *Anal Chem.* 2005; 77:2056–2062. [PubMed: 15801738]
8. Cadet J, Douki T, Ravanat JL. *Mutat Res.* 2011; 711:3–12. [PubMed: 21329709]
9. Helbock HJ, Beckman KB, Shigenaga MK, Walter PB, Woodall AA, Yeo HC, Ames BN. *Proc Natl Acad Sci U S A.* 1998; 95:288–293. [PubMed: 9419368]
10. Lagadu S, Pottier I, Sichel F, Laurent C, Lefaix JL, Prevost V. *Biomarkers.* 2010; 15:707–714. [PubMed: 20868227]
11. Kasai H. *J Radiat Res.* 2003; 44:185–189. [PubMed: 13678349]
12. Serrano J, Palmeira CM, Wallace KB, Kuehl DW. *Rapid Commun Mass Spectrom.* 1996; 10:1789–1791. [PubMed: 8953779]
13. Taghizadeh K, McFaline JL, Pang B, Sullivan M, Dong M, Plummer E, Dedon PC. *Nat Protoc.* 2008; 3:1287–1298. [PubMed: 18714297]
14. Hu C-W, Wu M-T, Chao M-R, Pan C-H, Wang C-J, Swenberg JA, Wu K-Y. *Rapid Commun Mass Spectrom.* 2004; 18:505–510. [PubMed: 14966860]
15. Wasalathanthri DP, Faria RC, Malla S, Joshi AA, Schenkman JB, Rusling JF. *Analyst.* 2013; 138:171–178. [PubMed: 23095952]
16. Wasalathanthri DP, Malla S, Bist I, Tang CK, Faria RC, Rusling JF. *Lab Chip.* 2013; 13:4554–4562. [PubMed: 24113555]
17. Wasalathanthri DP, Li D, Song D, Zheng Z, Choudhary D, Jansson I, Lu X, Schenkman JB, Rusling JF. *Chemical Science.* 2015; 6:2457–2468. [PubMed: 25798217]
18. Song B, Pan S, Tang C, Li D, Rusling JF. *Anal Chem.* 2013; 85:11061–11067. [PubMed: 24164630]
19. Bist I, Song B, Mosa I, Keyes T, Martin A, Forster RJ, Rusling JF. *ACS Sensors.* 2016; 1:272–278. [PubMed: 27135053]
20. Rusling, JF.; Hvastkovs, EG.; Schenkman, JB. *Drug Metabolism Handbook.* Nassar, A.; Hollenburg, PF.; Scatina, J., editors. J. Wiley; N. J: 2009. p. 307-340.
21. Hvastkovs EG, Schenkman JB, Rusling JF. *Annu Rev Anal Chem.* 2012; 5:79–105.

22. Salim S. *Curr Neuropsychopharmacol*. 2014; 12:140–147. [PubMed: 24669208]
23. Deavall DG, Martin EA, Horner JM, Roberts R. *J Toxicol*. 2012; 2012:13.
24. Nathan C, Cunningham-Bussell A. *Nat Rev Immunol*. 2013; 13:349–361. [PubMed: 23618831]
25. Valavanidis A, Vlachogianni T, Fiotakis C. *J Environ Sci Health, Part C: Environ Carcinog Ecotoxicol Rev*. 2009; 27:120–139.
26. Forster RJ, Vos JG. *Macromolecules*. 1990; 23:4372–4377.
27. Dennany L, Forster RJ, Rusling JF. *J Am Chem Soc*. 2003; 125:5213–5218. [PubMed: 12708874]
28. Cavalieri E, Chakravarti D, Guttenplan J, Hart E, Ingle J, Jankowiak R, Muti P, Rogan E, Russo J, Santen R, Sutter T. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*. 2006; 1766:63–78. [PubMed: 16675129]
29. Ohkuma Y, Hiraku Y, Oikawa S, Yamashita N, Murata M, Kawanishi S. *Arch Biochem Biophys*. 1999; 372:97–106. [PubMed: 10562421]
30. Matter B, Malejka-Giganti D, Csallany AS, Tretyakova N. *Nucleic Acids Res*. 2006; 34:5449–5460. [PubMed: 17020926]
31. Prat F, Houk KN, Foote CS. *J Am Chem Soc*. 1998; 120:845–846.
32. Cavalieri EL, Rogan EG. *Future Oncol*. 2009; 6:75–91. [PubMed: 20021210]
33. Liehr JG, Ricci MJ. *Proc Natl Acad Sci*. 1996; 93:3294–3296. [PubMed: 8622931]
34. Bajrami B, Krishnan S, Rusling JF. *Drug Metab Lett*. 2008; 2:158–162. [PubMed: 19356087]
35. Heflich RH, Neft RE. *Mutat Res*. 1994; 318:73–174. [PubMed: 7521935]
36. Oikawa S, Hirose I, Hirakawa K, Kawanishi S. *Carcinogenesis*. 2001; 22:1239–1245. [PubMed: 11470755]
37. Gayathri SB, Kamaraj P. *Int J Pharm Sci Health Care*. 2014; 4:24–39.
38. Dennany L, Forster RJ, White B, Smyth M, Rusling JF. *J Am Chem Soc*. 2004; 126:8835–8841. [PubMed: 15250737]
39. Hoffmann D, Hoffmann I, El-Bayoumy K. *Chem Res Toxicol*. 2001; 14:767–790. [PubMed: 11453723]
40. Vadhanam MV, Thaiparambil J, Gairola CG, Gupta RC. *Chem Res Toxicol*. 2012; 25:2499–2504. [PubMed: 22994544]
41. Mani V, Kadimisetty K, Malla S, Joshi AA, Rusling JF. *Environ Sci Technol*. 2013; 47:1937–1944. [PubMed: 23331021]
42. MacMahon B. *Int J Cancer*. 2006; 118:2373–2378. [PubMed: 16358260]
43. Fernandez SV, Russo IH, Russo J. *Int J Cancer*. 2006; 118:1862–1868. [PubMed: 16287077]
44. Nandi S, Guzman RC, Yang J. *Proc Natl Acad Sci*. 1995; 92:3650–3657. [PubMed: 7731959]
45. Tautenhahn R, Cho K, Uritboonthai W, Zhu Z, Patti GJ, Siuzdak G. *Nat Biotech*. 2012; 30:826–828.
46. El Bali L, Diman A, Bernard A, Roosens NHC, De Keersmaecker SCJ. *J Biomol Tech*. 2014; 25:96–110. [PubMed: 25365790]
47. Tan SC, Yip BC. *J Biomed Biotechnol*. 2009; 2009:574398. [PubMed: 20011662]
48. Vadhanam MV, Thaiparambil J, Gairola CG, Gupta RC. *Chem Res Toxicol*. 2012; 25:2499–2504. [PubMed: 22994544]
49. Siegel RL, Miller KD, Jemal A. *CA: A Cancer Journal for Clinicians*. 2015; 65:5–29. [PubMed: 25559415]
50. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. *Int J Cancer*. 2015; 136:E359–386. [PubMed: 25220842]
51. Hecht SS. *Int J Cancer*. 2012; 131:2724–2732. [PubMed: 22945513]
52. Hecht SS. *Nicotine Tob Res*. 2012; 14:18–28. [PubMed: 21324834]
53. Hecht SS. *Chem Res Toxicol*. 1998; 11:559–603. [PubMed: 9625726]
54. Santen RJ, Yue W, Wang J-P. *Steroids*. 2015; 99(Part A):61–66. [PubMed: 25168343]
55. Bolton JL, Trush MA, Penning TM, Dryhurst G, Monks TJ. *Chem Res Toxicol*. 2000; 13:135–160. [PubMed: 10725110]

56. Cavalieri E, Chakravarti D, Guttenplan J, Hart E, Ingle J, Jankowiak R, Muti P, Rogan E, Russo J, Santen R, Sutter T. *Biochim Biophys Acta (BBA) - Reviews on Cancer*. 2006; 1766:63–78. [PubMed: 16675129]
57. Jansson I, Stoilov I, Sarfarazi M, Schenkman JB. *Pharmacogenetics*. 2001; 11:793–801. [PubMed: 11740343]
58. Choudhary D, Jansson I, Sarfarazi M, Schenkman JB. *Pharmacogenetics genom*. 2008; 18:665–676.
59. Parl FF, Dawling S, Roodi N, Crooke PS. *Ann N Y Acad Sci*. 2009; 1155:68–75. [PubMed: 19250193]
60. Zhao Z, Kosinska W, Khmelnitsky M, Cavalieri EL, Rogan EG, Chakravarti D, Sacks PG, Guttenplan JB. *Chem Res Toxicol*. 2006; 19:475–479. [PubMed: 16544955]
61. Cavalieri EL, Rogan EG. *Future Oncol*. 2009; 6:75–91. [PubMed: 20021210]

Figure 1. Difference SWVs in pH 5.5 buffer after incubating with 0.5 mM (a) 4-OHE₂ and (c) 2-OHE₂, 0.5 mM CuCl₂, NADPH regenerating system for microfluidic array sensors featuring films of PDDA/PSS/(OsPVP/DNA)₃ and PDDA/PSS/(RuPVP/DNA)₃ (SWV ampl., 25 mV; freq., 15 Hz; step, 4 mV). (b, d) Influence of reaction time on SWV peak current ratio ($I_{p,f}/I_{p,i}$) of PDDA/PSS/(OsPVP/DNA)₃ and PDDA/PSS/(RuPVP/DNA)₃ films for the same reaction with 4-OHE₂ and 2-OHE₂. Controls are array incubated in the same mixture absence of test compounds. (Error bars represent SD for n= 4.)

Figure 2.

Difference SWVs in pH 5.5 buffer after incubating with 150 μ M NNK, 0.5 mM CuCl_2 and NADPH regenerating system while applying potential of -0.65V vs. Ag/AgCl for films of (a) PDDA/PSS/(RuPVP/DNA) $_3$ /PDDA/HLM/PDDA/DNA and PDDA/PSS/(OsPVP/DNA) $_3$ /PDDA/HLM/PDDA/DNA (SWV ampl., 25 mV; freq., 15 Hz; step, 4 mV). (b) Influence of substrate incubation time on SWV peak current ratio ($I_{p,f}/I_{p,i}$) for the same sensors reacted with NNK. Controls are incubations in the absence of NNK. (Error bars represent SD for $n=4$.)

Figure 3.

Influence of incubation time on (a) Os and (b) Ru SWV peak current ratio ($I_{p,f}/I_{p,i}$) for sensors with DNA/HLM/metallopolymers films reacted with a mixture of catechol and NNK (solid lines); and individual (dashed lines) for catechol (a) or NNK while applying potential of $-0.65\text{ V vs. Ag/AgCl}$ (b). Controls are incubations with the same reactant solution with no test compounds. (Error bars represent SD for $n = 4$.)

Figure 4.

Difference SWVs in pH 5.5 buffer after incubating with 100-fold diluted cigarette smoke extract, 0.5 mM CuCl₂, and NADPH regenerating system for sensors featuring films of (a) PDDA/PSS/(RuPVP/DNA)₃/PDDA/HLM/PDDA/DNA and PDDA/PSS/(OsPVP/DNA)₃/PDDA/HLM/PDDA/DNA (SWV ampl., 25 mV; freq., 15 Hz; step, 4 mV). Influence of incubation time on (b) ratio of 8-oxodG found with OsPVP, and (c) Ru SWV peak current ratio ($I_{p,f}/I_{p,i}$) using the same sensors reacted with cigarette smoke extracts. Controls are array incubated in the same reactant solution in the absence of cigarette smoke extract. (Error bars represent standard deviations for n= 4.)

Figure 5.

Difference SWVs in pH 5.5 buffer after incubating with E_2 metabolite extracts, 0.5 mM CuCl_2 , NADPH regenerating system on sensors with films (a) PDDA/PSS/(RuPVP/DNA)₃ and PDDA/PSS/(OsPVP/DNA)₃ (SWV ampl., 25 mV; freq., 15 Hz; step, 4 mV). Influence of reaction time on (b) ratio of 8-oxodG found with OsPVP, and (c) Ru SWV peak ratio ($I_{p,f}/I_{p,i}$) for sensors reacted with E_2 metabolites. Controls are arrays incubated with the same reactant solution with no E_2 metabolites. (Error bars are SD for $n=4$.)

Figure 6.

MRM chromatogram of the E₂ metabolites (a) measuring m/z transitions 271-145 (red, E₂) and m/z 287-147 (blue, hydroxylestradiol) and tandem mass spectra of (b) E₂ and (c) hydroxylestradiol from the resulting peaks in (a) at collision energy 55 eV.

Scheme 1.

UPLC-MS analyses of DNA oxidation: (A) coating of beads with DNA for reaction with generated ROS; (B) Reaction of DNA on beads and isolation of hydrolysis products for UPLC-MS detection of 8-oxodG.

Scheme 2.

Possible mechanism of DNA oxidation induced by E_2 metabolism.²⁸

Scheme 3.
Possible mechanism of DNA oxidation induced by catechol.³⁶

Scheme 4.

Possible mechanism of DNA oxidation induced by o-toluidine and 2-nitrosotoluene.³⁷