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Photoelectrochemical Sensor for the Rapid Detection of *in Situ* DNA Damage Induced by Enzyme-Catalyzed Fenton Reaction

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Photoelectrochemical sensors were developed for the rapid detection of oxidative DNA damage induced by Fe^{2+} and H_2O_2 generated *in situ* by the enzyme glucose oxidase. The sensor is a multilayer film prepared on a tin oxide nanoparticle electrode by layer-by-layer self-assembly and is composed of separate layers of a photoelectrochemical indicator, DNA, and glucose oxidase. The enzyme catalyzes the formation of H_2O_2 in the presence of glucose, which then reacts with Fe^{2+} and generates hydroxyl radicals by the Fenton reaction. The radicals attack DNA in the sensor film, mimicking metal toxicity pathways *in vivo*. The DNA damage is detected by monitoring the change of photocurrent of the indicator. In one sensor configuration, a DNA intercalator, $Ru(bpy)_2(dppz)^{2+}$ (bpy = 2,2′-bipyridine, dppz = dipyrido[3,2-a:2′,3′-c]phenazine), was employed as the photoelectrochemical indicator. The damaged DNA on the sensor bound less $Ru(bpy)_2(dppz)^{2+}$ than the intact DNA, resulting in a drop in photocurrent. In another configuration, ruthenium tris(bipyridine) was used as the indicator and was immobilized on the electrode underneath the DNA layer. After oxidative damage, the DNA bases became more accessible to photoelectrochemical oxidation than the intact DNA, producing a rise in photocurrent. Both sensors displayed substantial photocurrent change after incubation in Fe^{2+}/glucose in a time-dependent manner. And the detection limit of the first sensor was less than 50 μM. The results were verified independently by fluorescence and gel electrophoresis experiments. When fully integrated with cell-mimicking components, the photoelectrochemical DNA sensor has the potential to become a rapid, high-throughput, and inexpensive screening tool for chemical genotoxicity.

Introduction

Of the millions of man-made chemicals, around 100 000 are still on the market. In addition, about 4000 new chemicals are produced each year. Unfortunately, the vast majority of these chemicals do not have sufficient safety and health data, thus posing a great danger to human health and the ecosystem (*1, 2*). Many chemicals have been found to possess carcinogenic toxicity. Some of these carcinogens assert their toxic effect by causing damage to DNA, leading to gene mutation. In general, DNA damage is produced by one of the two major chemical routes, DNA oxidation by reactive oxygen species (ROS) and DNA adduct formation with exogenous chemicals and their *in vivo* metabolites (*3, 4*). According to the International Agency for Research on Cancer, Cr(VI), Ni(II), Be, Cd(II), and As(III) compounds have been confirmed to be human carcinogens, and Co(II) and Fe(II)-nitrilotriacetate are possibly carcinogenic to humans (*5*). A number of studies have shown that metals induce their toxic effects primarily through their ability to produce ROS. Iron is the most abundant transition metal in biological systems. Fe^{2+} has been found to react with H_2O_2 to produce the extremely reactive hydroxyl radical, the so-called Fenton reaction. The radical can induce several classes of DNA damage, including single-strand breakage, double-strand breakage, abasic sites, and base oxidation (*6*).

Currently, it is recommended that the genetic toxicity screening test should include three cell-based assays for gene mutation in bacteria, gene mutation in mammalian cells, and chromosomal aberration in mammalian cells, respectively (*7*). While these assays are powerful for toxicity screening, the most obvious drawbacks include low throughput, long duration, and high demand for manpower. DNA damage products have also been identified and quantified by a wide range of analytic techniques (*8*), such as gas chromatography/mass spectrometry, high-performance liquid chromatography, ^{32}P-postlabeling, gel electrophoresis, and immunoassays. Recently, electrochemical and electrochemiluminescent sensors and sensor arrays have been developed for the rapid detection of DNA damage (*9–13*). In particular, to mimic the *in vivo* toxicity pathway, cytochrome P450 has been incorporated into the sensor film to convert, *in situ*, styrene into the DNA-reactive form, styrene oxide (*14*). However, the approach has not been employed in the detection of metal-induced DNA damage.

We have been developing photoelectrochemical sensors for the detection of DNA damage as a rapid screening tool for the genetic toxicity of metals and organic compounds (*15, 16*). The photoelectrochemical technique is attractive because of two major aspects. First of all, due to the total separation of the excitation source (light) and the detection signal (current), the method is potentially very sensitive. Second, the use of electronic detection makes the photoelectrochemical instrument simple and low-cost, particularly in an array format. It compares favorably with the optical detection methods such as fluorescence, chemiluminescence, and electrochemiluminescence, which have to use complex and expensive optical imaging devices and sophisticated image-recognition software. Over the years, photoelectrochemistry-based analytical methods have been employed in the quantitation of DNA hybridization (*17–19*), ligand/receptor binding (*20*), and immunoassays (*21*).

In this paper, the enzyme glucose oxidase was incorporated into the multicomponent photoelectrochemical sensor film to generate H_2O_2 *in situ* in the presence of glucose. When the test sample contained Fe^{2+}, a Fenton reaction between the metal ion and H_2O_2 produced hydroxyl radicals and caused severe damage to the DNA molecules in the film, which was manifested by a substantial change in the photocurrent signal. The incorporation of glucose oxidase into the sensor mimics the metal-mediated ROS generation pathway *in vivo* and also eliminates the use of the unstable

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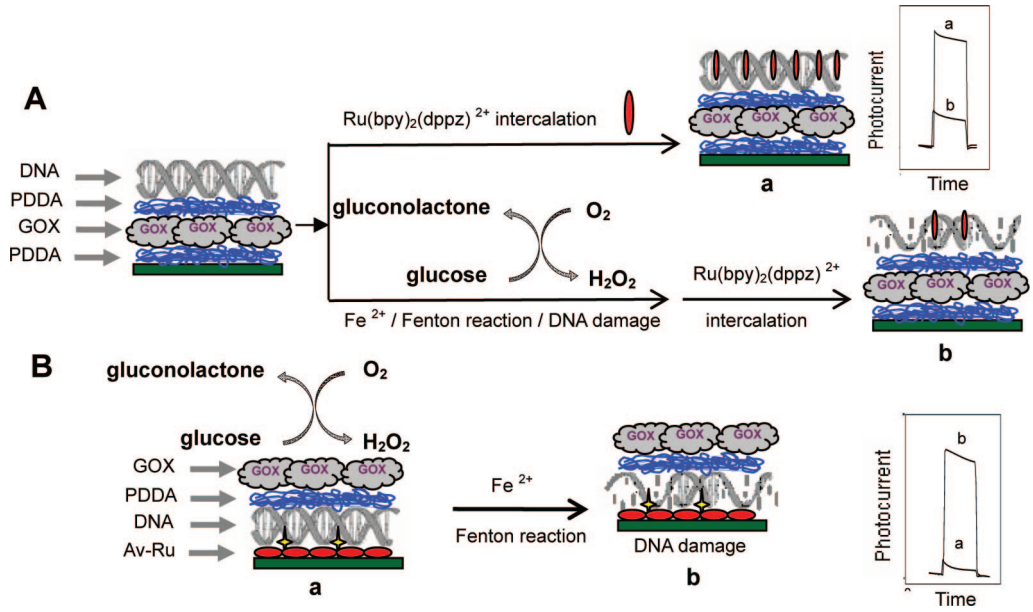
SCHEME 1. Schematic Diagram Illustrating the DNA Damage Detection Method Based on (A) Photoelectrochemical Indicator and (B) Photoelectrochemically Catalyzed Base Oxidation



H_2O_2 as a reagent. To our knowledge, this is the first report of a sensor for metal-induced, enzyme-catalyzed DNA damage.

Experimental Section

Reagents. Double- and single-stranded calf thymus DNA (ds-DNA and ss-DNA), avidin, thiazole orange (TO), glucose oxidase (GOx), poly(dimethyldiallylammonium chloride) (PDDA), and bis(2,2-bipyridine)(4-methyl-4-carboxybipyridine)-ruthenium *N*-succinimidyl ester bis(hexafluorophosphate) (RuNHS) were purchased from Sigma Aldrich. $Ru(bpy)_2(dppz)Cl_2$ was synthesized according to a published procedure (*22*). RuNHS-labeled avidin (avidin-Ru) was prepared as described previously (*15*). The ratio of Ru to avidin was 3.8:1. The ultrasonicated ds-DNA was obtained by ultrasonicating ds-DNA for 30 min.

Film Assembly. SnO_2 nanoparticle electrodes were prepared by following the previous method (*15, 16*). Two types of sensor films were constructed on the prepared SnO_2 electrodes (Scheme 1). The base oxidation sensor film was constructed by sequentially covering the SnO_2 electrode with avidin-Ru/avidin (3 μg/mL avidin-Ru/0.25 mg/mL avidin), ds-DNA (0.5 mg/mL), PDDA (2 mg/mL), and GOx (10μM) for 1 h. The final film was denoted as SnO_2/avidin-Ru/ds-DNA/PDDA/GOx. In the photoelectrochemical indicator method, a SnO_2 electrode was sequentially coated with PDDA, GOx, PDDA, and ds-DNA, denoted as SnO_2/PDDA/GOX/PDDA/ds-DNA. The same procedure was used to obtain films containing ss-DNA and ultrasonicated ds-DNA.

DNA Damage Reaction. The film-covered electrodes described above were incubated in an aqueous solution of $FeSO_4$/glucose (in H_2O, pH 3.0) at 37 °C with vortexing (200 rpm). After the required time, the electrode was taken out and rinsed with water.

Photoelectrochemical Measurement. The photocurrent was measured as described previously (*16*). For SnO_2/avidin-Ru/ds-DNA/PDDA/GOx sensor, the measurement was carried out in a phosphate buffer. For SnO_2/PDDA/GOX/PDDA/ds-DNA sensor, after DNA damage reaction and washing, the electrode was further reacted with 50 μM $Ru(bpy)_2(dppz)^{2+}$ for 30 min for the intercalation to take place. After washing, the photocurrent was measured in oxalate solution.

Fluorescence Measurement. Fluorescence was measured as described previously (*16*). A 96-well microplate containing 10 μg/mL of nucleic acids and 9 μM TO in a pH 7.3 phosphate buffer was shaken for 2 min before measurement. The damaged nucleic acid sample was obtained by reacting 0.1 mg/mL DNA with 50 mM glucose, 2 μM GOx, and 100 μM $FeSO_4$ in a pH 3.0 aqueous solution at 37 °C for the required time.

Gel Electrophoresis. The damaged ds-DNA sample for gel electrophoresis was prepared by the incubation of 0.1 mg/mL ds-DNA, 50 mM glucose, 2 μM GOx, and 100 μM $FeSO_4$ for 1 h at 37 °C. The incubated DNA sample was then electrophoresed on a 1.2% agarose gel in 0.5 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0) and 0.5 μg/mL ethidium bromide for 25 min at 7.5 V/cm.

Results and Discussion

Previously, we reported two types of photoelectrochemistry-based DNA sensors for the detection of DNA damage caused by styrene oxide and Fe^{2+}/H_2O_2 (*16*). However, both sensors require the presence of H_2O_2 in the case of transition metals for the Fenton reaction to occur and can only detect reactive organic chemicals which form covalent adducts with DNA directly. Their utility as a screening tool for genetic toxicity is therefore limited. In the current work, glucose oxidase was incorporated as a single layer into the multilayer sensor film to catalyze the generation of H_2O_2. The film was assembled by the same layer-by-layer electrostatic adsorption technique as described in previous work (*16, 23*). Because GOx is negatively charged, a cationic polymer, PDDA, was deposited on the semiconductor surface before and after GOx adsorption to obtain the SnO_2/PDDA/GOx/PDDA/ds-DNA film depicted in Scheme 1A. The amount of the enzyme deposited on the PDDA-modified surface was determined to be 179.7 ng/cm^2 by a quartz crystal microbalance.

To conduct the photocurrent measurement, a high-affinity DNA intercalator, $Ru(bpy)_2(dppz)^{2+}$, was employed as the signal reporter. After binding of $Ru(bpy)_2(dppz)^{2+}$ with DNA, photocurrent was measured in an oxalate solution, which served as an electron donor to recycle the signal reporter and amplify the signal. The intact sensor film SnO_2/PDDA/GOx/PDDA/ds-DNA produced a photocurrent in the range

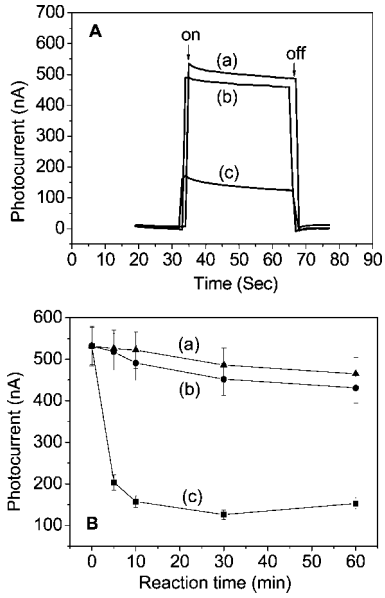


FIGURE 1. (A) Photocurrent response of $Ru(bpy)_2(dppz)^{2+}$ bound to the SnO_2/PDDA/GOx/PDDA/ds-DNA film after reaction for 30 min in (a) 50 mM glucose, (b) 1 mM $FeSO_4$, and (c) 1 mM $FeSO_4$/50 mM glucose. (B) Photocurrent as a function of the reaction time in (a) 50 mM glucose, (b) 1 mM $FeSO_4$, and (c) 1 mM $FeSO_4$/50 mM glucose. The excitation light was switched on and off as indicated.

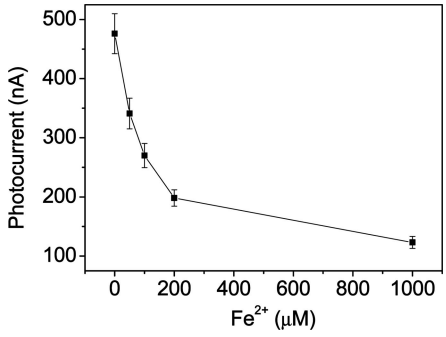


FIGURE 2. Photocurrent of $Ru(bpy)_2(dppz)^{2+}$ bound to the SnO_2/PDDA/GOX/PDDA/ds-DNA film as a function of Fe^{2+} concentration after reaction for 30 min in Fe^{2+}/50 mM glucose.

of 500 nA or so, as shown in Figure 1A. However, after incubation of the sensor in 1 mM Fe^{2+}/50 mM glucose (pH 3.0) at 37 °C for half an hour, the current dropped to about 150 nA. Incubation in either Fe^{2+} or glucose alone only caused a slight decrease in photocurrent. Presumably, ROS are generated in the enzyme-containing sensor film in the presence of both Fe^{2+} and glucose, inducing oxidative damage to DNA in the film. Since $Ru(bpy)_2(dppz)^{2+}$ is a double-strand specific DNA binder (*24*), it binds to the damaged DNA much less than the intact DNA, resulting in a drop in the photocurrent signal. Figure 1B shows the photocurrent responses as a function of the damage reaction time. As can be seen, the current dropped rapidly at the early stages of the reaction and then became steady after 30 min. The final current was only about 20% of the original value. Apparently, the damage proceeded at a very rapid rate, which is consistent with the known reaction kinetics of the highly reactive ROS species. Figure 2 is the Fe^{2+}-concentration–response curve, which shows that the photocurrent decreased as the Fe^{2+} concentration was increased from 0 to 1 mM, revealing a dependence of the damage reaction on the metal concentration. The detection limit for the metal ion is less than 50 μM, which is environmentally more relevant than the

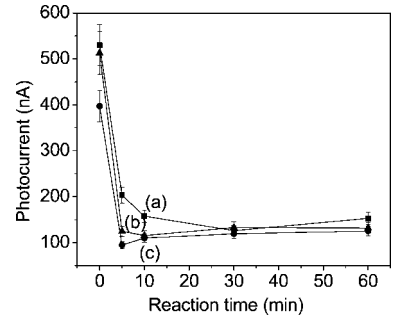


FIGURE 3. Photocurrent of $Ru(bpy)_2(dppz)^{2+}$ bound to the SnO_2/PDDA/GOx/PDDA/(nucleic acid) film as a function of the reaction time in 1 mM $FeSO_4$/50 mM glucose. The nucleic acid film is (a) ds-DNA, (b) ultrasonicated ds-DNA, and (c) ss-DNA.

millimolar concentration offered by the enzyme-free sensor in our previous work.

During the experiment, it was found that incubation of the DNA sensor in a 1 mM Fe^{2+} solution alone (without any glucose) reduced the photocurrent greatly when the solution pH was higher than 3.5. Since the photocurrent was measured in an oxalate buffer after rinsing, the effect must come from the metal ions adsorbed on the sensor surface during the incubation step. It has been reported that metal ions having suitable redox potentials, such as Fe^{3+} and Cu^{2+}, alter the electron transfer pathways of the photoexcited electrons of some fluorescent dyes (*25*). The same process may take place with the Ru complex by the surface-adsorbed Fe ions. The detrimental effect was eliminated almost completely when the pH of the Fe^{2+} solution was lowered to 3.0 or less. Considering that glucose oxidase has low activity in acidic solutions (*26*) and Fe^{2+} suppresses the photocurrent response at high pH values, a value of pH 3.0 was selected for the Fe^{2+}/glucose solution in the DNA damage experiments. As shown later in the work, even at pH 3.0, the activity of glucose oxidase is high enough to generate sufficient H_2O_2 for the DNA damage reaction.

In addition to ds-DNA (average length 13 000 base pair), the damage reaction of two other types of nucleic acids, namely, ultrasonicated ds-DNA (1000 base pair as determined by gel electrophoresis) and ss-DNA (average length 700 nucleotides), was also investigated by the photoelectrochemical sensor configuration. Figure 3 shows the measured photocurrent as a function of the reaction time in 1 mM Fe^{2+}/50 mM glucose. It is obvious from the graph that the damage process of the ultrasonicated ds-DNA and ss-DNA proceeded at an even faster rate than that of the intact ds-DNA and was completed in 5 min. The faster reaction rate is expected, as the more open structure of both shortened ds-DNA and ss-DNA allows for easier access to the DNA bases by ROS. It is also evident from the figure that, after the damage was completed, the photocurrent dropped to almost the same level for all three types of nucleic acids. The data suggest that the three nucleic acids in the multilayer film were damaged to the same extent by the hydroxyl radicals.

The DNA damage process was verified independently by fluorescence measurement, using TO as a double-strand specific DNA binder. TO displays negligible fluorescence when free in solution but becomes intensely fluorescent after binding with ds-DNA (*27*). The interaction of TO with DNA is strongly dependent on the structure of the nucleic acid. This character offers a convenient method for the study of DNA damage process. For the fluorescence measurement, damaged nucleic acid samples were obtained by reacting with 50 mM glucose, 2 μM GOx, and 100 μM $FeSO_4$ in solution for the required time. The control sample was obtained by reacting DNA with glucose and Fe^{2+} only (no GOx). After the reaction, 10 μg/mL of the nucleic acid sample was taken out

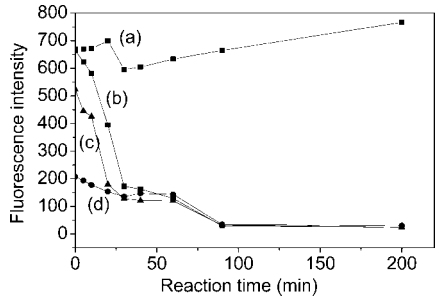


FIGURE 4. Fluorescence intensity of 9μM TO in the presence of different types of nucleic acids (10 μg/mL) as a function of their reaction time with 100 μM $FeSO_4$, 50 mM glucose, and 2 μM GOx in solution. (a) Unreacted ds-DNA, (b) ds-DNA, (c) ultrasonicated ds-DNA, and (d) ss-DNA.

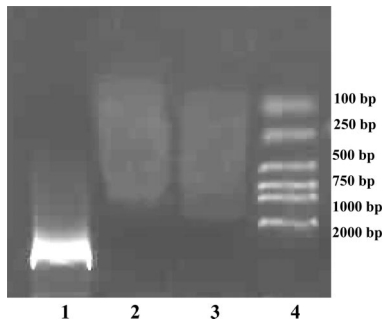


FIGURE 5. Agarose gel electrophoresis of different types of DNA. Lane 1: 0.1 mg/mL ds-DNA reacted with 100 μM $FeSO_4$/50 mM glucose, pH 3.0 (no GOx). Lane 2: ds-DNA reacted with $FeSO_4$/glucose/GOx (2 μM), pH 3.0. Lane 3: ds-DNA reacted with $FeSO_4$/glucose/GOx, pH 7.0. Lane 4: DNA markers.

and mixed with 9 μM TO for the fluorescence experiment. Figure 4 shows the change of fluorescence intensity as a function of the reaction time for the intact ds-DNA and ultrasonicated ds-DNA and ss-DNA. In general, the trend illustrated in the fluorescence measurement is consistent with that obtained in the photoelectrochemical experiment. It displays a fast decay in the fluorescent signal with the reaction time, indicating rapid kinetics of the damage reaction. The control sample did not show substantial change in fluorescence, which proves that the DNA damage requires the action of the enzyme. Because the binding specificity of TO toward ds-DNA is higher than that of $Ru(bpy)_2(dppz)^{2+}$, its initial fluorescence with ss-DNA is substantially lower than that of ds-DNA.

In the DNA damage experiment on the photoelectrochemical sensor, pH 3.0 was used to minimize the scavenging effect by Fe^{2+}. However, this low pH is not normally used for GOx. To illustrate the activity of glucose oxidase at pH 3, and to investigate the extent of DNA damage, a gel electrophoresis experiment was performed. In the experiment, 0.1 mg/mL of ds-DNA was reacted with 50 mM glucose, 2 μM GOx, and 100 μM $FeSO_4$ (either pH 3.0 or 7.0). After the reaction, the DNA sample was run on an agarose gel, together with the markers and a control DNA which was obtained by reacting with 50 mM glucose/100 μM $FeSO_4$ (no GOx). In Figure 5, there is a major band (length greater than 2000 base pairs) for the control that is absent in the images for the other two DNA samples. Instead, many weak bands representing different DNA lengths appeared for the latter, illustrating extensive DNA breakage. The difference between the control and sample indicates that GOx was active at pH 3.0 and induced DNA damage in the presence of glucose and Fe^{2+}. The similarity between the two DNA samples reacted at pH 3.0 and 7.0 suggests that the activity of the enzyme at the acidic pH was high enough and produced sufficient H_2O_2 to

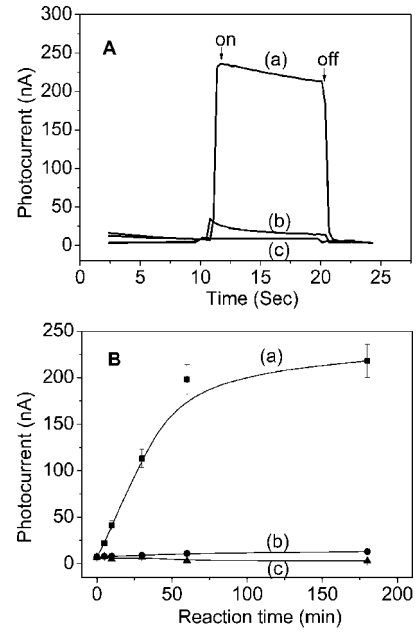


FIGURE 6. (A) Photocurrent response of the SnO_2/avidin-Ru/ds-DNA/PDDA/GOx film after reaction for 3 h in (a) 1 mM $FeSO_4$/50 mM glucose, (b) 1 mM $FeSO_4$, and (c) 50 mM glucose. (B) Photocurrent as a function of the reaction time in (a) 1 mM $FeSO_4$/50 mM glucose, (b) 1 mM $FeSO_4$, and (c) 50 mM glucose. The excitation light was switched on and off as indicated.

cause the same degree of damage as the neutral solution. It should be noted here that the above experiment was not intended to simulate in the test tube the H_2O_2 concentration generated on the sensor or within living cells. In addition, although there is a substantial amount of evidence in the literature that suggests ROS including hydroxyl radicals induce protein damage as well (*28*), our photoelectrochemical and gel electrophoresis data indicate that the enzyme in the film and in solution has enough activity in the presence of hydroxyl radicals to carry on the DNA damage reaction.

A different sensor configuration employed in our previous work is the deposition of $Ru(bpy)_3^{2+}$ on the electrode directly. And DNA damage in the sensor film is monitored by the photocurrent, which is related to the oxidation of the DNA bases by the photogenerated $Ru(bpy)_3^{3+}$oxidant. This configuration eliminates the addition of the DNA binder, $Ru(bpy)_2(dppz)^{2+}$, and oxalate used in the first configuration and represents more closely a reagent-less sensor. For this reason, the second configuration was also employed for the sensor that incorporated GOx, namely, SnO_2/avidin-Ru/ds-DNA/PDDA/GOx, as depicted in Scheme 1B. Without the damage reaction, the film electrode produced a very low background photocurrent of about 5 nA when measured in a phosphate buffer. In contrast, after 1 h of incubation in a solution of 1 mM Fe^{2+}/50 mM glucose, pH 3.0, the current increased to almost 200 nA (Figure 6A). The photocurrent was found to be strongly dependent on the glucose concentration and reached the maximum at 50 mM glucose (shown in Figure S1 in the Supporting Information). Control experiments with Fe^{2+} or glucose alone under the same conditions did not produce any change in the photoelectrochemical signal. The substantial rise in photocurrent in the presence of both Fe^{2+} and glucose can be attributed to the disruption of the rigid DNA helical structure by ROS generated in the Fenton reaction, and consequently the increased accessibility of the DNA bases for photoelectrochemical oxidation, as proposed and confirmed in our previous work (*15, 16*). Figure 6B shows the photocurrent change as a function of the incubation time. The photocurrent increased with the incubation time up to 1 h and then leveled

off afterward. A direct comparison can be made between Figure 6 in this manuscript and Figure 5 in our previous publication (*16*). After 1 h of incubation, the enzyme-incorporated sensor produced 4 times higher photocurrent than the enzyme-free sensor. In addition, the rising portion of the curve in Figure 6 is steeper than in the previous work, indicating the new sensor responded faster to the damaging agent than the old one. The high signal on the enzyme sensor is tentatively rationalized by the close proximity of the enzyme to the DNA layer in the sensor film and, therefore, the elevated local concentration of ROS in the vicinity of the DNA.

In summary, the work presented here has demonstrated that the GOx-incorporated multilayer film photoelectrochemical sensor can be used for the detection of *in situ* oxidative DNA damage by the metal-induced Fenton reaction. As shown above, the results are generally consistent with the gel electrophoresis and fluorescence measurement, but the photoelectrochemical method is more rapid and simpler to implement. Other enzymes can also be incorporated into the multilayer sensor film to simulate alternative chemical toxicity mechanisms so as to detect DNA damages induced by chemicals other than transition metals. This direction is currently pursued in our laboratory, and the preliminary results are very encouraging. However, it should be kept in mind that genetic toxicity is not merely about direct damage to DNA by a chemical. It also involves cell membrane permeability, transport, metabolism, interference with the DNA repair system, and so forth. The current sensor can only detect direct damage to DNA. An ideal sensor for genetic toxicity needs to incorporate all of the essential elements of a cell-based assay to carry out all of the above functions, yet with faster speed and better reproducibility than the cell assays. Enzymes are one of the essential elements, and their incorporation is a necessary and important step toward the final goal of an integrated, cell-mimicking sensor. When fully integrated, the DNA sensor has the potential to become a powerful tool for the rapid, low-cost, and large-scale screening of chemical genotoxicity.

Acknowledgments

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Supporting Information Available

Details of experiments in determining the glucose concentration for the DNA damage reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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