Detection and mechanistic investigation of halogenated benzoquinone induced DNA damage by photoelectrochemical DNA sensor

Suping Jia · Ben-Zhan Zhu · Liang-Hong Guo

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Abstract Halogenated phenols are widely used as biocides and are considered to be possibly carcinogenic to humans. In this report, a previously developed photoelectrochemical DNA sensor was employed to investigate DNA damage induced by tetra-halogenated quinones, the *in vivo* metabolites of halogenated phenols. The sensor surface was composed of a double-stranded DNA film assembled on a SnO₂ semiconductor electrode. A DNA intercalator, Ru(bpy)₂(dppz)²⁺, was allowed to bind to the DNA film and produce photocurrent upon light irradiation. After the DNA film was exposed to 300 μM tetrafluoro-1,4-benzoquinone (TFBQ), the photocurrent dropped by 20%. In a mixture of 300 μM TFBQ and 2 mM H₂O₂, the signal dropped by 40%. The signal reduction indicates less binding of Ru(bpy)₂(dppz)²⁺ due to structural damage of ds-DNA in the film. Similar results were obtained with tetra-1,4-chlorobenzoquinone (TCBQ), although the signal was not reduced as much as TFBQ. Fluorescence measurement showed that TFBQ/H₂O₂ generated more hydroxyl radicals than TCBQ/H₂O₂. Gel electrophoresis proved that the two benzoquinones produced DNA strand breaks together with H₂O₂, but not by themselves. Using the photoelectrochemical sensor, it was also found that TCBQ covalently bound with DNA did not produce additional oxidative damage in the presence of H₂O₂. The combined photoelectrochemistry, gel electrophoresis, and fluorescence data revealed distinctive differences between TFBQ and TCBQ in terms of DNA adduct formation and hydroxyl radical generation.

Keywords DNA damage · Photoelectrochemistry sensor · Benzoquinone

Introduction

Halogenated phenols are widely used in the pesticide industry and are ubiquitous in the environment due to their high stability and slow degradation [1, 2]. Among the halogenated phenols, pentachlorophenol (PCP), which is used for antimicrobial agents, detergents, and wood preservatives, has been listed by the US Environmental Protection Agency as a priority pollutant [3]. It is also classified as a Group 2B environmental carcinogen by the International Agency for Research on Cancer [4]. The metabolism and toxicology of PCP has been investigated extensively [5–7]. The carcinogenesis of PCP is believed to involve the *in vivo* enzyme-mediated biotransformation of PCP to its reactive metabolites such as tetrachlorohydroquinone (TCHQ) and tetrachloro-1,4-benzoquinone (TCBQ). Several modes of action have been investigated for DNA damage induced by these metabolites. DNA adducts were identified in calf thymus DNA reacted with TCBQ [8], in human HeLa S3 tumor cells treated with TCHQ [9], and in rats exposed to PCP [10]. Structure of a deoxyguanosine (dG)/TCBQ adduct was characterized carefully, and was found to be a dichlorobenzoquinone nucleoside [11]. The reaction was dependent on the type of nucleobases, as TCBQ reacted more readily with dG than with other bases. In another DNA adduct formation mechanism, PCP was found to be oxidized by horseradish peroxidase/H₂O₂ to the corresponding phenoxyl radicals, which then reacted with nucleobases to produce oxygen-bound C8-dG nucleoside adduct [12]. In addition to covalent adducts, oxidative DNA damage was also ob-

S. Jia · B.-Z. Zhu · L.-H. Guo (✉)
State Key Laboratory of Environmental Chemistry and
Ecotoxicology, Research Center for Eco-environmental Sciences,
Chinese Academy of Sciences,
18 Shuangqing Road, P.O. Box 2871, Beijing 100085, China
e-mail: LHGuo@rcees.ac.cn

served in calf thymus DNA, tumor cells and rats [8–10]. Reactive oxygen species were believed to be formed during the enzyme-catalyzed biotransformation of PCP. In the presence of Cu(II) and NADPH, low concentrations of TCBQ induced increased production of 8-hydroxydeoxyguanosine (8-HO-dG) and abasic sites [8].

More recently, by using salicylate hydroxylation assay and electron spin resonance method, it was found that halogenated quinones produced hydroxyl radicals ($\text{HO}\cdot$) in the presence of H_2O_2 in a mechanism independent of transition metal ions [13–15]. It was inferred that a nucleophilic reaction might take place between TCBQ and H_2O_2 , forming a trichlorohydroperoxyl-1,4-benzoquinone ($\text{TrCBQ-OHO}\cdot$) intermediate, which would decompose homolytically to produce $\text{HO}\cdot$. Metal-independent production of $\text{HO}\cdot$ from H_2O_2 was also observed with several other halogenated quinones including 2,5-dichloro-1,4-benzoquinone (2,5-DCBQ), 2-chloro-1,4-benzoquinone (2-CBQ), tetrafluoro-1,4-benzoquinone (TFBQ) and tetrabromo-1,4-benzoquinone (TBBQ).

Similar to PCP, the toxicity of other chlorinated phenols such as 2,4,6-trichlorophenol (2,4,6-TCP), 2,4,5-trichlorophenol (2,4,5-TCP), and 2,4-dichlorophenol (2,4-DCP) have also been well-documented. Reactivity of these chlorophenols toward dG and calf thymus DNA following activation by horseradish peroxidase/ H_2O_2 has been established [16–18], and the formation of 2,5-DCBQ-DNA and 2,6-DCBQ-DNA adducts was observed [19].

In the studies described above, several well-established techniques were employed in the identification and characterization of damaged DNA. 8-HO-dG is a commonly used marker for oxidative DNA damage, and is identified using HPLC separation followed by online electrochemical detection. DNA strand breaks are observed by gel electrophoresis. For DNA adduct identification and quantification, ^{32}P post-labeling is the method of choice, although the procedure is very time-consuming, including enzymatic digestion of DNA sample, purification, labeling with ^{32}P , and TLC or PAGE analysis [20]. Recently, we developed a photoelectrochemical DNA sensor for the rapid detection of structural DNA damage [21–23]. The sensor surface was composed of a double-stranded DNA (ds-DNA) film assembled on a SnO_2 semiconductor electrode. A DNA intercalator, $\text{Ru}(\text{bpy})_2(\text{dppz})^{2+}$, was allowed to bind to the DNA film and produce photocurrent upon light irradiation. After the DNA film was exposed to a DNA-damaging chemical, photocurrent would drop due to less binding of $\text{Ru}(\text{bpy})_2(\text{dppz})^{2+}$ to the damaged DNA film. It was shown that the sensor was able to detect both oxidative DNA damage induced by $\text{Fe}^{2+}/\text{H}_2\text{O}_2$, as well as DNA adducts with styrene oxide [21]. In the present work, the photoelectrochemical DNA sensor has been employed to detect DNA damage induced by tetra-halogenated benzoquinones.

We demonstrate that the sensor can detect very rapidly the DNA damage caused by the direct DNA adducts with the halogenated quinone alone, as well as by oxidative damage from their mixture with H_2O_2 . With its unique feature, some information about the damage reaction mechanism can also be obtained.

Experimental

Reagents

Tin-doped indium oxide (ITO) conductive glass was supplied by Weiguang Corp (Shenzhen, People's Republic of China). Fifteen percent tin (IV) oxide, as a colloidal dispersion of 15-nm particles, was obtained from Alfa Aesar (Ward Hill, MA), so was hydrogen peroxide. Poly-(diallyldimethyl ammonium chloride) (PDDA), coumarin-3-carboxylic acid (CCA), ethidium bromide, TCBQ and TFBQ were obtained from Sigma-Aldrich (St. Louis, MO). Double-stranded calf thymus DNA (ds-DNA, approximately 13K base pairs) was purchased from Merck (San Diego, CA). $\text{Ru}(\text{bpy})_2(\text{dppz})(\text{BF}_4)_2$ was synthesized according to the published procedure [24, 25]. All other chemicals and solvents were of analytical grade. Solutions were prepared in high-purity water from a Millipore Milli-Q (Biocel) water purification system.

Photoelectrochemical measurement

In the experiment, the preparation of SnO_2 nanoparticle electrodes and the fabrication of the DNA films on the SnO_2 electrode was the same as previously described [21]. The concentration of PDDA and ds-DNA solution for film deposition was 2 mg/mL and 0.5 mg/mL, respectively. The DNA-modified electrode was denoted as $\text{SnO}_2/\text{PDDA}/\text{DNA}$. TCBQ was dissolved in *N,N*-dimethylformamide (DMF) at a concentration of 150 mM and then diluted to 20 mM phosphate buffer (pH 7.3). TFBQ was dissolved in acetonitrile at a concentration of 150 mM and then diluted to 20 mM PB (pH 7.3). The DNA film on the electrode was damaged by exposing to halogenated quinone alone or a mixture of halogenated quinone and H_2O_2 in PB buffer at 37 °C with vortex (200 rpm) for 30 min. A DNA film electrode with either intact or damaged DNA was first rinsed with water for 3 min and then reacted with 50 μM $\text{Ru}(\text{bpy})_2(\text{dppz})^{2+}$ for 30 min in order for the signal reporter to bind to the DNA film. After the reaction, the unbound metal complex was washed off by water. Photocurrent was measured on a CHI 630A electrochemical analyzer (CH Instruments, Austin, TX) by placing the electrode in 30 mM oxalate buffer (pH 5.8), using a Pt counter electrode, a Ag/AgCl (3 M KCl) reference electrode, and a bias voltage of +0.1 V.

Gel electrophoresis

The damaged ds-DNA sample for gel electrophoresis was prepared by the incubation of 0.01 mg/mL ds-DNA with different damage reagent for 30 min at 37 °C. The incubated DNA sample was then electrophoresed on a 0.6% agarose gel in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0) and 0.5 µg/mL ethidium bromide for 35 min at 80 V.

Fluorescence measurement

Fluorescence intensity was measured on a Thermo Electron Varioskan Flash luminescence spectrometer (Waltham, MA), using 395 nm excitation, 450 nm emission, a 5-nm slit width, and a 430-nm emission cut-off filter. A well containing 1 mM CCA, 300 µM halogenated quinone, and 2 mM H₂O₂ in 20 mM phosphate buffer (pH 7.3) was shaken for 30 min at 37 °C before the measurement. The fluorescence intensity from wells containing a mixture of CCA and halogenated quinone (no H₂O₂) was used as the control, and the wells containing CCA only were used as the blank.

Results and discussion

As described in the “Introduction” section, halogenated quinones are electrophilic agents that have been proven experimentally to bind covalently to the nucleophilic sites in calf thymus DNA and poly-nucleosides to form DNA adducts, preferentially with dG [8–12]. In addition, halogenated quinones also produced highly reactive hydroxyl radical (HO·) in the presence of H₂O₂ [13–15]. A number of studies have shown that HO· can induce several classes of DNA oxidative damage, including single-strand break, double-strand break, abasic sites, and base oxidation [26]. It has been shown that the photoelectrochemical DNA sensor we developed previously can detect DNA damage caused either by DNA adducts with styrene oxide, or by HO· generated in Fe²⁺-mediated Fenton Reaction [21]. Therefore, the sensor can, in principle, detect DNA damage induced by halogenated quinones in the absence and presence of H₂O₂.

DNA damage by TFBQ was first investigated by the photoelectrochemical sensor. In the experiment, a DNA film was fabricated on a SnO₂ nanoparticle electrode using the layer-by-layer electrostatic self-assembly approach. After binding of the signal reporter Ru(bpy)₂(dppz)²⁺ to the DNA film, photoelectrochemical signal was measured in an oxalate solution. As shown in Fig. 1a, upon light illumination, a relatively stable photocurrent with a magnitude of approximately 730 nA was recorded. However, if

the sensor was first incubated in 300 µM TFBQ before binding with Ru(bpy)₂(dppz)²⁺, the photocurrent decreased to 586 nA. The reduction in photocurrent could be attributed to TFBQ/DNA adduct formation. Ru(bpy)₂(dppz)²⁺ is a DNA intercalator and binds selectively to the stacked base pairs in double-stranded DNA. DNA adducts with bulky molecules such as TFBQ introduce spatial hindrance to DNA base pairing and change its double-helical structure, thus resulting in less bound signal reporters. In our previous work, we have shown by fluorescence that styrene oxide/DNA adducts reduced the binding of Ru(bpy)₂(dppz)²⁺ with double-stranded calf thymus DNA [21]. Another possible reason for the reduction of photocurrent is that Ru(bpy)₂(dppz)²⁺ binding is not affected, but the electron transfer rate from Ru(bpy)₂(dppz)²⁺ to the electrode is slowed down by the adducts. This is unlikely in our system, because the DNA molecules are deposited on the electrode by electrostatic adsorption, and they should orientate mostly along the horizontal direction. Therefore, interfacial electron transfer does not go along the DNA chain, but rather through the underlying PDDA layer. Also shown in Fig. 1a is the

Fig. 1 **a** Photocurrent of Ru(bpy)₂(dppz)²⁺ bound to DNA electrode after it was exposed to (a) 20 mM PB buffer (pH 7.3) for 30 min, (b) 300 µM TFBQ for 30 min, (c) 300 µM TFBQ and 2 mM H₂O₂ for 30 min. Excitation light was switched on and off as indicated. **b** Photocurrent response of Ru(bpy)₂(dppz)²⁺ intercalated into the SnO₂/PDDA/DNA film as a function of the concentration of TFBQ in (a) TFBQ alone, (b) TFBQ and H₂O₂

photoelectrochemical response of the sensor after it was exposed to a mixed solution of 300 μM TFBQ and 2 mM H_2O_2 . The current dropped by 40% to 450 nA. The reason for the larger loss of signal is that, in addition to forming DNA adducts, TFBQ may also react with H_2O_2 and generate $\text{HO}\cdot$ that oxidizes DNA bases. Similar to DNA adducts, the oxidative damage also reduces the intercalation sites in DNA for the signal reporter.

Although photoelectrochemical sensors cannot identify the type of DNA damage (adduct or oxidative), they offer some unique advantages over the conventional techniques. One advantage is high sensitivity. As can be seen in Fig. 1b, DNA damage by 30–100 μM TFBQ was detected, whereas in ^{32}P post-labeling method the concentration was in the millimolar range [8]. Secondly, DNA usage in the sensor method is very low. It has been determined that the amount of DNA immobilized in a film is 3.2 ng mm^{-2} [27]. This is much less than the amount (micrograms) needed for ^{32}P post-labeling and gel electrophoresis experiments. Thirdly, once the DNA film is prepared on the sensor surface, DNA damage detection is simple and rapid. On the contrary, ^{32}P post-labeling method involves multiple steps and is very time-consuming.

In addition to TFBQ, TCBQ was also investigated. Figure 2 depicts the photocurrent signal of the DNA sensor exposed to TCBQ alone, and its mixture with H_2O_2 , respectively. The results show that, in the absence of H_2O_2 , TCBQ reduced the photocurrent, indicating covalent binding with DNA. The photocurrent follows the order $\text{TCBQ} < \text{TFBQ} < \text{blank}$, suggesting that TCBQ formed more DNA adducts than TFBQ. In the presence of H_2O_2 , the photocurrent of TCBQ is higher than TFBQ. It is also higher than TCBQ alone. Because it is possible for the quinones in the mixture to carry out DNA adduct reaction and oxidative damage at the same time, interpretation of the results is not straightforward, and will be discussed later after fluorescence and gel electrophoresis experiments.

Fig. 2 Photocurrent response of $\text{Ru}(\text{bpy})_2(\text{dppz})^{2+}$ bound to $\text{SnO}_2/\text{PDDA}/\text{ds-DNA}$ electrode after the DNA film was exposed to PB buffer, 300 μM TFBQ, 300 μM TCBQ without H_2O_2 (empty column) or with 2 mM H_2O_2 (filled column)

As mentioned above, TCBQ has been shown by ESR to produce $\text{HO}\cdot$ in its reaction with H_2O_2 by a metal-independent mechanism [15]. As we know, the most widely accepted mechanism for $\text{HO}\cdot$ production is through the metal ion mediated decomposition of hydrogen peroxide, the Fenton reaction [28]. It has been shown that hydroxyl radicals can bring about extensive damage to many biological macromolecules, including protein fragmentation, lipid peroxidation, carbohydrate degradation, and oxidative DNA damage. The newly proposed mechanism for $\text{HO}\cdot$ generation from reactions between halogenated quinones and H_2O_2 does not require the involvement of transition metal ions. It may partly contribute to the carcinogenesis of halogenated phenols such as hexachlorobenzene, chlorophenols, and Agent Orange, which can be metabolized in vivo to tetra-, di-, or monochlorinated quinones. To confirm the formation of $\text{HO}\cdot$ and compare the reactivity of the two halogenated quinones, fluorescence measurement was carried out. Coumarin-3-carboxylic acid (CCA) is a non-fluorescent molecule in aqueous solutions. It can react directly with $\text{HO}\cdot$ to produce the highly fluorescent compound 7-hydroxycoumarin-3-carboxylic acid. This reaction has been shown to be specific for the detection of $\text{HO}\cdot$ produced either by radiation or chemical reaction, and also quantitative, sensitive, and accurate [29, 30]. As Fig. 3 shows, when CCA was added into 300 μM halogenated quinone alone (c, e), the fluorescence intensity did not show any change compared with the blank (a, b). But the fluorescence intensity increased substantially after CCA was reacted with a mixture of 300 μM halogenated quinone and 2 mM H_2O_2 (d, f). The intensity in TFBQ/ H_2O_2 is about twice as much as that of TCBQ, indicating that TFBQ/ H_2O_2 produced more $\text{HO}\cdot$ than TCBQ/ H_2O_2 .

To further investigate the effect of quinone generated $\text{HO}\cdot$ on DNA, gel electrophoresis of DNA samples after reaction with quinone/ H_2O_2 was conducted. Because calf thymus DNA is a heterogeneous polymer of various chain

Fig. 3 Fluorescence of 1 mM CCA after reacting with (a) 20 mM PB buffer, (b) 20 mM PB and 2 mM H_2O_2 , (c) 300 μM TFBQ, (d) 300 μM TFBQ and 2 mM H_2O_2 , (e) 300 μM TCBQ, (f) 300 μM TCBQ and 2 mM H_2O_2

length, the intact DNA sample displayed a major band on the bottom of the image (length greater than 2,000 base pairs) and many less intense bands with shorter length (Fig. 4). Reaction with TFBQ alone did not change the pattern of DNA electrophoresis image, which suggests there is no DNA break in the reaction product. However, for the DNA samples reacted with 1 mM TFBQ / 2 mM H_2O_2 , the major band disappeared in the image. Instead, many new bands with shorter length emerged, representing DNA breaks. For TCBQ, much higher concentration (2 mM TCBQ/50 mM H_2O_2) was needed for the major band to lower its intensity as shown in Fig. 4b. The observation in gel electrophoresis experiments is consistent with fluorescence measurement in that TFBQ generated more $\text{HO}\cdot$ in fluorescence measurement than TCBQ, and also produced more DNA breaks in electrophoresis image. The trend is also consistent with the photoelectrochemical measurement results depicted in Fig. 2, where lower current reflects more DNA damage.

One unique feature of the photoelectrochemical DNA sensor is that the reaction is surface-based, and as such allows for easy separation between surface-bound and solution species. In our previous work, this surface reaction mode was exploited to investigate the role of DNA-associated Fe^{2+} ions in oxidative DNA damage [22]. It was demonstrated explicitly that Fe^{2+} ions indeed associated with the DNA molecules in the sensor film, and still engaged in Fenton reaction and DNA damage. The reactivity of covalently bound benzoquinones was also investigated in the current work by the sensor method. In the experiment, the DNA film electrode was assembled as usual, and then exposed to 300 μM TCBQ. After rinsing with water, the sensor was then reacted with 2 mM H_2O_2 . Finally, the electrode was allowed to bind with $\text{Ru}(\text{bpy})_2(\text{dppz})^{2+}$, and the photocurrent was measured. As shown in Fig. 5, the photocurrent was

Fig. 4 Agarose gel electrophoresis of different types of DNA, **a**: (1) DNA marker, (2) 0.01 mg/ml CT-DNA reacted with 1 mM TFBQ, (3) DNA reacted with 1 mM TFBQ/2 mM H_2O_2 , (4) DNA reacted with 1 mM TCBQ, (5) DNA reacted with 1 mM TCBQ/2 mM H_2O_2 , (6) intact DNA. **b** (1) DNA marker, (2) 0.01 mg/ml CT-DNA reacted with 2 mM TCBQ, (3) DNA reacted with 2 mM TCBQ/5 mM H_2O_2 , (4) DNA reacted with 2 mM TCBQ/50 mM H_2O_2 , (5) intact DNA

Fig. 5 Photocurrent of $\text{Ru}(\text{bpy})_2(\text{dppz})^{2+}$ bound to DNA electrode after the DNA film was exposed to (a) 20 mM PB for 1 h, (b) 300 μM TCBQ for 1 h, (c) 300 μM TCBQ/2 mM H_2O_2 for 1 h, (d) 300 μM TCBQ for 30 min, water rinse, then 2 mM H_2O_2 for 30 min

basically the same as the electrode that was reacted with TCBQ only (no H_2O_2). The result suggests that TCBQ covalently bound with DNA does not produce additional oxidative damage even in the presence of H_2O_2 .

Conclusions

In conclusion, DNA damage by halogenated phenol metabolites, TFBQ and TCBQ, was investigated by photoelectrochemical DNA sensor in combination with fluorescence measurement and gel electrophoresis. It was found that the two benzoquinones exhibited distinctively different reactivity in DNA damage. In the absence of H_2O_2 , TCBQ induced more DNA damage than TFBQ, presumably by forming covalent adducts. However, in the mixed solution with H_2O_2 , more severe DNA damage was observed with TFBQ. The $\text{HO}\cdot$ measured by a fluorescent probe and DNA breaks observed in gel electrophoresis are consistent with the photoelectrochemical results. The photoelectrochemical DNA sensor possesses advantages in sensitivity, sample consumption, and convenience, and offers a rapid and semi-quantitative tool for the detection of both oxidative DNA damage and covalent adduct formation, as well as for the study of reaction mechanisms.

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