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Voltammetric investigation of DNA damage induced by nitrofurazone and short-lived nitro-radicals with the use of an electrochemical DNA biosensor

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ABSTRACT

Electrochemical behavior of nitrofurazone (NFZ) was investigated with the use of cyclic voltammetry (CV) and differential pulse voltammetry (DPV) methods. The pH-dependence of NFZ was studied at a glassy carbon electrode (GCE) in ethanol/Britton–Robinson buffer (30:70), and short-lived nitro-radicals were generated by the reduction of NFZ at high pHs (>7.0). In the presence of DNA, the DPV peak current of NFZ decreased and the peak potential shifted negatively, which indicated that there was an electrostatic interaction between NFZ and DNA. An electrochemical dsDNA/GCE biosensor was prepared to study the DNA damage produced in the presence NFZ; this process was followed with the use of the $Co(phen)_3^{2+}$ electroactive probe. Also, the oxidation peaks of guanosine (750 mV) and adenosine (980 mV) indicated that DNA damage was related directly to the nitro-radicals. Experiments demonstrated that DNA damage occurred via two different steps while NFZ was metabolized and nitro-radicals were produced. Novel work with AFM on the NFZ/DNA interaction supported the suggestion that *in vivo*, the nitro-radicals were more cytotoxic than the NFZ molecules. A linear DPV calibration plot was obtained for NFZ analysis at a modified dsDNA/GCE (concentration range: 2.50×10^{-6}–3.75×10^{-5} mol L^{-1}; limit of detection: 8.0×10^{-7} mol L^{-1}), and NFZ was determined successfully in pharmaceutical samples.

1. Introduction

In general, complementary and supplementary electrochemical techniques are often useful to study small molecule/biopolymer (proteins or DNA) interactions in different aqueous media. Such reactions commonly involve redox processes, which often damage the host bio-molecule. This study is focused on the interaction of the nitrofurazone (NFZ) and its potential to damage DNA; cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were the analytical techniques of choice. NFZ is a synthetic nitrofuran derivative, and on the one hand, it is an effective fungicide, amoebicide and skin sensitizer used against various allergies and infections animals and humans (de Bengoa Vallejo et al., 2011; Trossini et al., 2010; Escobosa et al., 2009) as well as in aquaculture (Tittlemier et al., 2007), but on the other hand, in recent years, this antimicrobial drug has been linked with carcinogenic effects, particularly, in large doses (Ito et al., 2005; Takahashi et al., 2000; Takegawa et al., 2000; Bartel et al., 2009). At present, the International Agency for Research on Cancer (IARC) categorizes NFZ as a Group 3 drug i.e. it is not deemed to be carcinogenic to humans (International Agents for Research on Cancer (IARC), 2011). Consequently, studies of the interaction of NFZ with DNA and the mechanism involved are of interest and importance.

In this context, Hiraku et al. (2004) reported that NFZ could be metabolized by cytochrome P450 to produce short-lived nitro-radicals; their electron spin resonance spectroscopy investigations showed that radicals were generated from NFZ, and found that NFZ metabolites (nitro-radicals) were involved in tumor initiation by facilitating oxidation of DNA; also, NFZ itself participated in tumor promotion and progression through enhancement of cell proliferation.

Ona et al. (2009) reported that NFZ was reduced by cellular nitroreductases to form N^2-deoxyguanine adducts that were associated with mutagenesis and toxicity; they also found that nucleotide excision repair was a primary mechanism for processing damage induced by NFZ. Zhou et al. (2011) found that ciliates, treated with NFZ, produced damaged DNA. These conclusions were made on the basis of results of a random amplified polymorphic DNA (RAPD) assay. Thus, these studies indicated that NFZ itself and its metabolites could be cytotoxic in cellular systems. Apart from the RAPD method, the PCR bioanalysis (Ito et al., 2005) and the high

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performance liquid chromatography (HPLC; electrochemical detector; Hiraku et al., 2004) have been used with similar indicative results. However, in general, these techniques are time-consuming, and involve costly equipment and maintenance. Electrochemical methods of analysis, especially those involving electrochemical biosensors, offer simple, relatively cheap equipment with rapid detection as alternative applications (Zhang et al., 2010b; Xu et al., 2011; Xiong et al., 2011). For dsDNA, the two strands of nucleotides are twisted into a double helix, held together by hydrogen bonds between the adenine(A)–thymine(T) and guanine(G)–cytosine(C) bases of each strand. Thus, it is very difficult to detect the electrochemical signals of these four bases. However, when DNA is damaged by free-radicals, the double helix of the DNA will be broken and this will facilitate the oxidation of the bases (especially guanine) or generate DNA adducts. Such drastic changes to the DNA structure would affect the subsequent replication and transcription of DNA, and this may lead to cancer. However, DNA damage is difficult to detect directly by applying electrochemical analysis particularly with guanosine and adenosine because the bases are shielded by the dsDNA, which interferes with the electron transfer to the electrode's surface. Thus, an indirect method with the use of redox electroactive probes (Zhang et al., 2010a; Liao et al., 2010), has been used in this work—specifically the $Co(phen)_3^{2+}$ complex.

DNA biosensors have been previously used to study DNA damage induced by short-lived nitro-radicals (Abreu et al., 2002; Vyskočil et al., 2010), and the reduction of nitro-aromatic compounds to generate nitro-radicals was achieved as follows: 1. by successive CV cathodic scanning, and 2. reduction at a fixed potential. The first method showed that if the nitro-aromatic reduction was facilitated by successive cathodic cycling, the DNA damage was more severe when compared to that produced by reduction at a fixed potential. It appeared that the damage increased when short-lived nitro-radicals were freshly generated during sequential reduction of the NFZ during the CV cycling. However, interestingly, in these investigations, the production of nitro-radicals was assumed and no direct evidence of their existence was presented. In contrast, voltammetric analysis showed that ethyl-*m*-nitrobenzoate disproportionated in water/DMF, and a nitro-radical formed (Carbajo et al., 2000). Similar studies of the NZF molecule in protic, mixed and aprotic media indicated that reduction of the nitro-radical anion was present in all three media (Bollo et al., 2005). In addition, kinetics research of the related reactions further supported the formation of the nitro-radicals (Fotouhi and Faramarzl, 2004). These studies provided the basis to further explore the potential role of the nitro-radical anion, derived from the NFZ, in induction of carcinogenesis.

Recently, the investigation of the toxicity of NFZ has focused on its presence as a food residue, and many techniques have been used for its analysis e.g. HPLC (Lin and Jeng, 2001), cathodic stripping voltammetric (Khodari et al., 1999) and others (Wang and Zhang, 2006; Thongsrisomboon et al., 2010; Tubino et al., 2009). Chen et al. (2010) synthesized a new fluorescence sensor to analyze NFZ.

Thus, the aims of this work were: 1. to investigate the chemistry of the nitro-radicals generated by the reduction of the NFZ in protic media with the use of electrochemical techniques; 2. to use a DNA biosensor to investigate the interaction of DNA with NFZ in the same media, and to establish if the DNA was damaged by the nitro-radical and/or the NFZ molecule itself and 3. to develop a method for quantitative analysis of NFZ by the DNA biosensor.

2. Experimental

2.1. Chemicals

Double-stranded calf thymus DNA (type I, No. D1501; Sigma-Aldrich Co., Shanghai) and nitrofurazone (NFZ; Sigma-Aldrich Co., Shanghai) were used as received. DNA solution (1.0 mg mL^{-1}) was prepared by dissolving 50.0 mg of the solid in 50 mL 50 mmol L^{-1} sodium chloride solution. NFZ stock solution (5×10^{-3} mol L^{-1}) was prepared by dissolving 0.1 g crystals in 100 mL dimethylformamide (DMF). $[Co(phen)_3](ClO_4)_2 \cdot 2H_2O$ solution (5.0 mmol L^{-1}) was prepared by dissolving the crystals (0.4670 g) in 100 mL 10 mmol L^{-1} phosphate buffer (pH=7.00). Ethanol/Britton–Robinson buffer (30:70, v/v), a protic medium, was the electrolyte. Sample solutions were purged with pure nitrogen for 5 min prior to any measurements. All chemicals were Analytical Grade reageants and double-distilled water was used throughout.

2.2. Instrumentation

CV and DPV experiments were performed with the use of a CHI 660 A electrochemical workstation (CH Instruments, Shanghai, China). A conventional three-electrode system was used for the measurements, and it was equipped with a glassy carbon electrode (GCE, 3 mm diameter) or a modified dsDNA/GCE as the working electrode, a platinum auxiliary and an Ag/AgCl reference electrodes. Atomic force microscope (AFM) was the AJ-III model (Shanghai Aijian Nanotechnology) in the tapping mode. Standard silicon cantilevers (spring constant, 0.6–6 N/m) were used at their typical resonance frequencies e.g. 60–150 kHZ. Mica sheets were from the General Research Institute (Shanghai). Buffer pH was monitored with an Orion SA720 meter (US). All experiments were carried out at 25 ± 0.5 °C.

2.3. Preparation of the dsDNA/GCE

The GCE was polished sequentially with 1.0, 0.3 and 0.05 μm alumina slurry on a polishing felt, and then, the electrode was sonicated in 0.5 mol L^{-1} dilute nitric acid, ethanol and deionized water, sequentially, for 5 min at a time. After gentle mechanical cleaning, the GCE was further cleaned by repeated potential scanning between −1.0 and 1.0 V in 10 mL 0.25 mol L^{-1} sulfuric acid until a reproducible cyclic voltammogram was observed. This procedure activated the electrode surface so as to assist the immobilization of the dsDNA (Pang et al., 1996). The electrode surface was then washed with double-distilled water and left to dry. Subsequently, 30.0 μL 1.0 mg mL^{-1} dsDNA stock solution was deposited on the surface of the GCE and dried for 6 h in air at room temperature. The obtained dsDNA/GCE was washed with deionized water and immersed in a protic medium (pH=9.10) for 2 min to remove any loosely adsorbed DNA. The prepared dsDNA/GCE electrode was then submitted to CV analysis in the presence of 5.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ (dissolved in 0.1 mol L^{-1} KCl) as an electroactive probe; the potential was scanned from −0.1 to 0.6 V with a scan rate of 100 mV s^{-1}.

2.4. Immobilization of dsDNA or NFZ–dsDNA on the mica sheet

Mica sheets were freshly cleaved with adhesive tape prior to each experiment, then an AFM image was obtained and sample cleanliness was checked. A thick dsDNA or NFZ–dsDNA film was prepared by covering the mica sheets with 30 μL 1.0 mg mL^{-1} DNA solution (containing 5×10^{-3} mol L^{-1} Mg^{2+}) or a mixed solution (30 μL 1.0 mg mL^{-1} DNA, 5×10^{-3} mol L^{-1} Mg^{2+} and 5×10^{-3} mol L^{-1} NFZ); these films were allowed to stand in a sterile environment to dry overnight before further processing. The thin dsDNA and NFZ–dsDNA films were also prepared by covering the mica sheet with the above solution diluted 100 times.