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In situ evaluation of heavy metal–DNA interactions using an electrochemical DNA biosensor

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Abstract

Heavy metal ions, lead, cadmium and nickel, are well known carcinogens with natural different origins and their direct mode of action is still not fully understood. A dsDNA-electrochemical biosensor, employing differential pulse voltammetry, was used for the *in situ* evaluation of Pb^{2+}, Cd^{2+} and Ni^{2+} interaction with dsDNA. The results confirm that Pb^{2+}, Cd^{2+} and Ni^{2+} bind to dsDNA, and that this interaction leads to different modifications in the dsDNA structure. These modifications were electrochemically recognized as changes in the oxidation peaks of guanosine and adenosine bases. Using homopolynucleotides of guanine and adenine it has been proved that the interaction between Pb^{2+} and DNA causes oxidative damage and preferentially takes place at adenine-containing segments, with the formation of 2,8-dihydroxyadenine, the oxidation product of adenine residues and a biomarker of DNA oxidative damage. The Pb^{2+} bound to dsDNA can still undergo oxidation. The interaction of Cd^{2+} and Ni^{2+} causes conformational changes, destabilizing the double helix, which can enable the action of other oxidative agents on DNA.

Keywords: dsDNA-electrochemical biosensor; Heavy metal ions; Differential pulse voltammetry; Oxidative damage; 2,8-dihydroxyadenine

1. Introduction

Metal ion–DNA interactions are important in nature, often changing the genetic material's structure and function. The interaction of DNA with heavy metals, such as Pb, Cd and Ni, has been extensively investigated since they are involved in processes leading to DNA damage. The ions of Pb, Cd and Ni have been found in different sources, foods, beverages, soil, plants, natural waters, etc. The International Agency for Research on Cancer (IARC) lists Pb and Cd compounds as possible human carcinogens, based on experimental animal trials, while the carcinogenic properties of Ni are related to tumour promotion [1].

The DNA has four different potential sites for binding of metal ions, the negatively charged phosphate oxygen atoms, the ribose hydroxyls, the base ring nitrogens, and the exocyclic base keto groups [2]. Most transition metal ions interact with more than two different sites and their interactions with DNA are more complicated. They frequently bind indirectly to the phosphate groups and directly to the bases with the N7 atom of purines or N3 of pyrimidines [3].

Various methods with great sensitivity and specificity have helped to characterize the nature of Pb^{2+}, Cd^{2+} and Ni^{2+} interactions with DNA, such as UV–VIS, IR and Raman spectroscopy [4–6], HPLC [5], electrochemistry [7–12], among others [13–18]. However, the direct mechanism of Pb, Cd and Ni metal ions induced tumour formation is poorly understood and conflicting results have been reported [15,19,20].

Electrochemical techniques, such as pulse techniques, are suitable for studies of biological systems, for instance DNA–heavy metal interactions [8–10,12], since they are fast, low cost and have high sensitivity. One advantage of the use of pulse techniques, is that they bring a great improvement in signal-to-noise ratio compared to steady state techniques and in many cases greater selectivity [21]. The DNA-electrochemical biosensor, using differential pulse voltammetry, has been successfully utilized to investigate the interaction of small molecules with DNA, and comparing with other methods shows great sensitivity towards detecting small perturbations of the double-helical structure.

An electrochemical DNA biosensor is an integrated receptor-transducer device that uses DNA as a biomolecular recognition

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element to measure specific binding processes with DNA, through electrochemical transduction. The most important factor for the construction of efficient DNA-based electrochemical biosensors is the immobilization of the DNA probe on the electrode surface [22–25].

Among the electrochemical transducers, carbon electrodes demonstrate several unique properties. The extensive potential window in the positive direction allows sensitive electrochemical detection of the oxidative damage caused to DNA by monitoring the appearance of the oxidation peaks of the DNA bases. Different adsorption immobilization procedures, electrostatic adsorption or evaporation, of a monolayer or multilayer DNA films have been used. The multilayer dsDNA-electrochemical biosensor consists of an electrode with dsDNA deposited on the transducer surface by successive monolayer coverage. Mac Mode AFM images have shown that the multilayer immobilization method leads to total coverage of the carbon electrode surface, thus the adsorption of undesired substances on the transducer surface does not occur, and the biosensor response is due only to the interaction of the substance with dsDNA [24,25].

Additionally, the electrochemical oxidation signal of the components of DNA, such as, nucleotides, nucleosides, purine and pyrimidine bases, can be employed as biological recognition elements for the determination of a more specific interaction. Different DNA-electrochemical biosensors can be prepared from known selected sequences of the DNA components, as in homopolynucleotides and heteropolynucleotides. Thus, multilayer dsDNA-electrochemical biosensors can be a powerful tool in elucidating the nature of DNA–metal ion binding and detecting the conformational changes or oxidative damage resulting from these interactions.

Indicators of DNA oxidative damage are 8-oxo-7,8-dihydroguanine (8-oxoGua) and 2,8-dihydroxyadenine (2,8-oxoAde), which are the oxidation products of guanine and adenine, respectively [24,25]. These oxidation products can form relatively stable base pairs with cytosine or thymine during DNA replication, being considered one of the natural causes of mutagenesis. The direct oxidation of DNA by hydroxy radicals has been reported to generate a substantial amount of 8-oxoGua, but only a small quantity of 2,8-oxoAde. In contrast, 2,8-oxoAde is exclusively generated by the oxidation of dATP in the nucleotide pool. The difference in the origins of each oxidized base suggests that 8-oxoGua and 2,8-oxoAde cause mutagenesis in different manners [26], hence the importance of screening for the occurrence of each base oxidation products.

The current study is aimed at giving a better understanding of the nature of the mechanism of interaction of Pb^{2+}, Cd^{2+} and Ni^{2+} with and binding to dsDNA in aqueous solution, using a multilayer dsDNA-electrochemical biosensor and differential pulse voltammetry detection.

2. Experimental

2.1. Materials and reagents

Lead (II) and cadmium (II) nitrate, and nickel (II) chloride analytical grade were from Merck. Calf thymus dsDNA, polyguanylic (poly[G]) and polyadenylic (poly[A]) acid were from Sigma and used without further purification. Stock solutions of 4 mM Pb^{2+}, Cd^{2+} and Ni^{2+}, and 300 μg mL^{-1} dsDNA, poly[G] and poly[A] were prepared in deionised water and diluted to the desired concentration. All solutions were prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity ≤ 0.1 μS cm^{-1}). Nafion® solution, 5 wt.% in alcohol and water, was from Aldrich. Nafion® films were prepared according to a previously described procedure [27], applying 5 μL of a 0.25% solution of Nafion® in ethanol over the electrode surface, followed immediately by 3 μM of *N*, *N*′-dimethylformamide casting solvent. The solvents were evaporated in a warm air stream.

Microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pippettes (Rainin Instrument Co. Inc., Woburn, USA). The pH measurements were carried out using a Crison micropH 2001 pH-meter with an Ingold combined glass electrode. All experiments were done at room temperature (25 ± 1 °C).

2.2. Voltammetric parameters and electrochemical cells

Voltammetric experiments were carried out using a μAutolab running with GPES 4.9 software, Eco-Chemie, Utrecht, The Netherlands. The experimental conditions for differential pulse voltammetry (DPV) were: pulse amplitude 50 mV, pulse width 70 ms, scan rate 5 mV s^{-1}. Measurements were carried out using a glassy carbon electrode (GCE) (d = 1.5 mm), with a Pt wire counter electrode, and a Ag/AgCl (3 M KCl) electrode as reference, in a 0.5 mL one-compartment electrochemical cell.

The GCE was polished using diamond spray (particle size 6 μm) before every electrochemical assay. After polishing, the electrode was rinsed thoroughly with Milli-Q water for 30 s; then it was sonicated for 1 min in an ultrasound bath and again rinsed with water. After this mechanical treatment, the GCE was placed in buffer supporting electrolyte and various DP

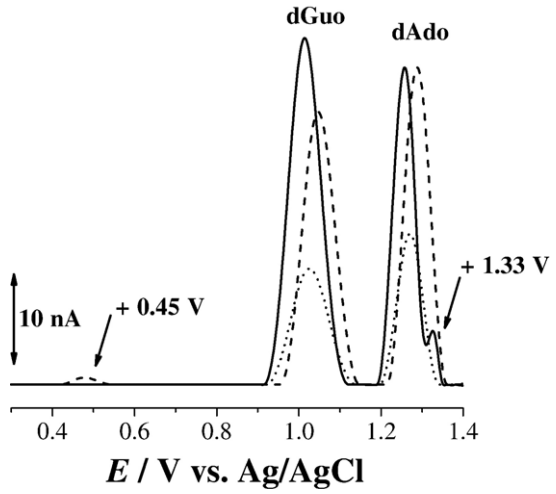

Fig. 1. Background-subtracted DP voltammograms in pH 4.5 0.1 M acetate buffer with a multilayer dsDNA-electrochemical biosensor: (●●●) before and (—) after incubation during 1 h with Pb^{2+} utilizing Procedure 1, and (▪▪▪) after incubation during 48 h with Pb^{2+} following Procedure 2.

voltammograms were recorded until a steady state baseline voltammogram was obtained. This procedure ensured very reproducible experimental results.

2.3. Acquisition and presentation of voltammetric data

All the voltammograms presented were background-subtracted and baseline-corrected using the moving average with a step window of 5 mV included in GPES version 4.9 software. This mathematical treatment improves the visualization and identification of peaks over the baseline without introducing any artifact, although the peak height is in some cases reduced (<10%) relative to that of the untreated curve. Nevertheless, this mathematical treatment of the original voltammograms was used in the presentation of all experimental voltammograms for a better and clearer identification of the peaks. The values for peak current presented in all graphs were determined from the original untreated voltammograms after subtraction of the baseline.

2.4. DNA-biosensor preparation and incubation procedure

The multilayer dsDNA biosensors were prepared by successive covering of the GCE surface with three drops of 5 μL each of 60 μg mL^{-1} dsDNA solution. After placing each drop on the electrode surface the biosensor was dried under a constant flux of N_2. The poly[G] and poly[A] biosensors were prepared in the same way.

Two different procedures for the incubation with metals ions were used:

Procedure 1: The incubation was carried out by covering the DNA-biosensor surface with two drops of 5 μL each of a 2 mM metal ion solution and allowing the surface to dry in normal atmosphere, for different periods of time.

Procedure 2: The incubation with the metal ions was carried out by immersing the biosensor in a 2 mM metal ion solution, for different periods of time.

3. Results and discussion

3.1. In situ evaluation of Pb^{2+} metal ion interaction with DNA

The multilayer dsDNA-electrochemical biosensor was used to evaluate the possible interaction involving metal ions and DNA. The effects of metal ions on dsDNA were followed by DP voltammetry, comparing the changes of the dsDNA-biosensor signals before and after the interaction. For each experiment a new multilayer dsDNA biosensor was prepared.

The dsDNA biosensor was always prepared as described in Section 2.4 and characterized in pH 4.5 0.1 M acetate buffer by DP voltammetry. The results showed well-defined small oxidation peaks due to the oxidation of desoxyguanosine (dGuo) at E_{pa}=+1.03 V, and desoxyadenosine (dAdo) at E_{pa}=+1.28 V, Fig. 1. The DP voltammogram of the dsDNA biosensor shows small oxidation peaks due to the difficulty of the electron transfer from the inside of the double-stranded rigid form of DNA to the electrode surface [24,25].

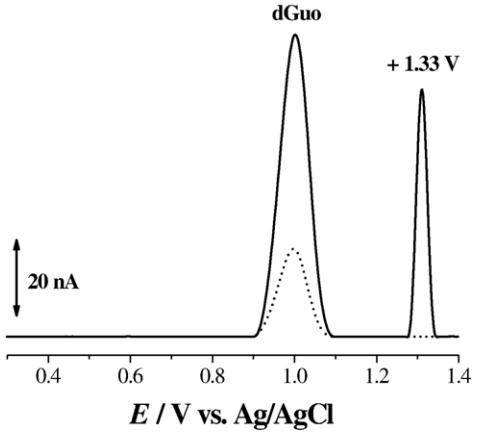

Fig. 2. Background-subtracted DP voltammograms in pH 4.5 0.1 M acetate buffer with a multilayer poly[G]-electrochemical biosensor (●●●) before and (—) after incubation during 15 h in Pb^{2+} utilizing Procedure 1.

A newly prepared dsDNA biosensor was incubated during 1 h in Pb^{2+} solution, following Procedure 1, then washed with deionised water in order to remove the unbound ions and transferred to pH 4.5 0.1 M acetate buffer where the DP voltammogram was recorded. The voltammogram shows an increase of the height of the oxidation peaks of dGuo and dAdo, and a new oxidation peak at E_{pa}=+1.33 V, Fig. 1, corresponding to the oxidation of Pb^{2+} to Pb^{4+}. The results indicate that Procedure 1 leads to a pre-concentration of Pb^{2+} ions in the film, which then interact with the dsDNA. After incubation following Procedure 1, the dsDNA film was removed from electrode surface and a DP voltammogram was recorded in buffer. No peak corresponding to the oxidation of Pb^{2+} to Pb^{4+} was observed, proving the total coverage of the electrode surface after modification with dsDNA.

A control experiment was performed, to enable the clarification of the occurrence of the peak at E_{pa}=+1.33 V, using a GCE modified by a Nafion® film, instead of the dsDNA film on the GCE. The GCE modified by a Nafion® film was prepared and Procedure 1 was repeated and in the DP voltammogram the peak at E_{pa}=+1.33 V still appeared, confirming that it was not a result of Pb^{2+} interaction with DNA, but corresponded to the oxidation of Pb^{2+} to Pb^{4+}. This experiment also showed that adsorption of Pb^{2+} takes place within the DNA film, and that besides the interaction with DNA, as shown by the increase of the oxidation peaks of dGuo and dAdo, Fig. 2, the free Pb^{2+} is pre-concentrated in the DNA film and can undergo oxidation. DNA is a biopolymer and deposited on the electrode surface behaves as a matrix like any polymer modified electrode surface enabling pre-concentration, in this case of the free Pb^{2+}, in the DNA biopolymer film on the electrode.

In another experiment, a new dsDNA biosensor was incubated for 48 h in the Pb^{2+} solution, following Procedure 2. The results also show an increase of the oxidation peak curents of dGuo and dAdo, while a new peak at E_{pa}=+0.45 V appeared, but no peak at E_{pa}=+1.33 V occurred, Fig. 1.

Other experiments with different concentrations of Pb^{2+} and incubation times were carried out following Procedure 2. An increase in current of the peak at E_{pa}=+0.45 V and of the

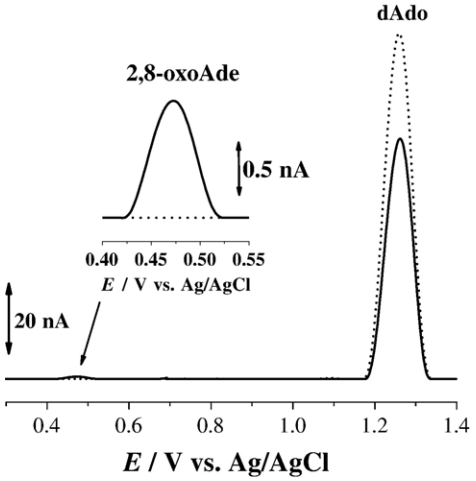

Fig. 3. Background-subtracted DP voltammograms in pH 4.5 0.1 M acetate buffer with a multilayer poly[A]-electrochemical biosensor (●●●) before and (—) after incubation during 3 h in Pb^{2+} utilizing Procedure 1.

oxidation peaks of dGuo and dAdo, proportional to the increase of Pb^{2+} concentration and incubation time, was noted.

The data obtained suggest that Pb^{2+} interacts with dsDNA immobilized on the GCE surface leading to modifications in the double-helical structure in a time-dependent manner. As the double helix unwinds, the DNA bases are more exposed to the electrode surface thus facilitating their oxidation, which can explain the occurrence of higher peak currents for dGuo and dAdo with increasing incubation times. Thus, the results of this study already confirm the toxic effects of Pb^{2+} on dsDNA and the dsDNA-electrochemical biosensor enables a fast and easy DNA damage detection procedure.

Moreover, the oxidative damage caused to DNA by Pb^{2+} can also to be detected electrochemically by monitoring the appearance of the oxidation product peak, which will be identified below as 2,8-oxoadenine, at E_{pa}=+0.45 V, in pH 4.5 0.1 M acetate buffer [28–30].

3.2. In situ evaluation of Pb^{2+} ion interaction with homopolynucleotides

In order to obtain more information about the origin of the peak at E_{pa}=+0.45 V several experiments were performed, using a GCE modified with homopolynucleotides of chosen bases, namely poly[G] and poly[A]. The poly[A] and poly[G] modified electrodes were only used in order to clarify with which base, adenine (A) or guanine (G), occurs a more specific interaction.

The GCE surface was modified as described in Section 2.4 with poly[G], the homopolynucleotide that contains only guanine residues. The DP voltammogram in pH 4.5 0.1 M acetate buffer shows a peak at E_{pa}=+1.02 V, corresponding to dGuo oxidation, Fig. 2. In a subsequent experiment, the poly [G]-electrochemical biosensor was incubated during 15 h, folowing Procedure 1, and then transferred to acetate buffer where DPV was performed. Compared with the previous results, there is an increase in the height of the peaks corresponding to dGuo oxidation and of Pb^{2+} to Pb^{4+}, E_{pa}=+1.33 V, Fig. 2. However, no peak at E_{pa}=+0.45 V was observed, indicating that no guanine oxidative damage had occurred. The dGuo peak current increase can be explained considering conformational changes, by the unwinding of the quadruple and/or double helix structure of poly[G] in acid media, pH 4.5 [2,31,32], thus exposing the guanine bases for oxidation, but not leading to the formation of oxidation products.

The above experiment was repeated modifying the GCE with poly[A], the homopolynucleotide that contains only adenine residues. The DP voltammogram of the poly[A] biosensor, recorded in pH 4.5 0.1 M acetate buffer, showed a peak at E_{pa}=+1.26 V corresponding to dAdo oxidation, Fig. 3. The poly[A] biosensor was incubated for 3 h with Pb^{2+}, also following Procedure 1, and then transferred to acetate buffer where DPV was performed. The results showed a decrease of the signals corresponding to dAdo oxidation, and the appearance of a peak at E_{pa}=+0.45 V, corresponding to oxidation of 2,8-oxoAde [30], indicating that poly[A] oxidative damage had occurred. No peak at E_{pa}=+1.33 V was observed. The dAdo peak current decrease can be explained considering either that the poly[A] underwent oxidative damage through the oxidation of the adenine residues in a process that led to the formation of 2,8-oxoAde, and/or the formation of a metal complex between adenine residues and Pb^{2+} [33], no free Pb^{2+} remaining, as confirmed by the absence of the lead oxidation peak, E_{pa}=+1.33 V.

The experiments using poly[G] and poly[A] multilayer biosensors added new information to the understanding of the molecular mechanism involved in Pb^{2+}–DNA interaction, clearly showing that the peak at E_{pa}=+0.45 V is directly associated with the oxidation of the adenine residues of the multilayer dsDNA biosensor, since the oxidation potential corresponds to the oxidation of 2,8-oxoAde.

3.3. In situ evaluation of Cd^{2+} and Ni^{2+} ions interaction with DNA

The ions of the metals cadmium and nickel are known carcinogens to humans and to animals. The interaction of these

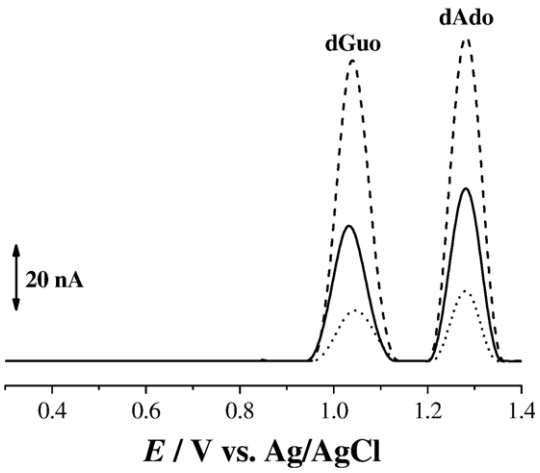

Fig. 4. Background-subtracted DP voltammograms in pH 4.5 0.1 M acetate buffer of a multilayer dsDNA-electrochemical biosensor: (●●●) before and (—) after incubation during 60 h in Cd^{2+} utilizing Procedure 2, and (▪▪▪) after incubation during 48 h in Ni^{2+} utilizing Procedure 2.

metals with DNA is frequently discussed and the possible occurrence of DNA oxidative damage. Thus, the above experiments were repeated with Cd^{2+} and Ni^{2+} in order to investigate their potential in inducing hydrogen bonding cleavage and/or oxidative damage to DNA using the multilayer dsDNA biosensor.

A DP voltammogram was obtained after incubating a multilayer dsDNA biosensor during 60 h in a Cd^{2+} solution, following Procedure 2, Fig. 4. The results showed only a large increase in current of the oxidation peaks corresponding to dGuo and dAdo residues, while no other oxidation peaks were recorded.

In another experiment, a multilayer dsDNA biosensor was incubated during 48 h in Ni^{2+} solution, following Procedure 2. While the data shows similar results, Fig. 4, as in the case of Cd^{2+}–DNA interaction, it can be noted that higher peak currents were obtained after the incubation of the dsDNA biosensor with Ni^{2+} than with Cd^{2+}. Consequently, these experiments clearly demonstrate that both Cd^{2+} and Ni^{2+} interact by binding to dsDNA strands causing conformational changes, but in addition suggest that the Ni^{2+} ions have a greater affinity for dsDNA than the Cd^{2+} ions. Nevertheless, in both cases no evidence of DNA oxidative damage caused by Cd^{2+} and Ni^{2+} was observed, Fig. 4.

Other experiments with different concentrations of Cd^{2+} and Ni^{2+} and incubation times were carried out following Procedure 2. In all cases only a current increase of the oxidation peaks of dGuo and dAdo was seen, which was proportional to the increase of Cd^{2+} and Ni^{2+} concentration and incubation time (not shown).

In both cases, the peak current increase was due to the loosening of the double helix structure of dsDNA, breaking of the hydrogen bonds and unlocking of the bases for oxidation on the GCE surface, but the interaction did not lead to the base oxidation products. However, this conformation effect leaves the DNA bases more exposed to the action of other oxidative agents.

4. Conclusions

The study of the interaction of DNA with the heavy metal ions of Pb, Cd and Ni, using a multilayer dsDNA biosensor, helped clarify the mechanism by which these metals bind to dsDNA and their possible mutagenic properties. The different interaction of Pb^{2+} with dsDNA, leading to oxidative damage, compared with Ni^{2+} and Cd^{2+}, which only caused conformation structural changes, was shown.

Using modified electrodes with polynucleotides of known sequences, it was confirmed that Pb^{2+} interacts with dsDNA preferentially at adenine-containing segments, leading to oxidative damage and formation of the 2,8-oxoAde oxidation product. These results enable a better understanding of the molecular mechanism involved in Pb^{2+} induced neoplasia.

While no oxidative damage to dsDNA by Cd^{2+} and Ni^{2+} was observed, the results indicate that these heavy metal ions also affect the double helix structure of dsDNA, causing conformational changes and opening the way to initiation of the action of other oxidative agents.

The sensitivity of the multilayer dsDNA-electrochemical biosensor also offers the possibility to follow the interaction of metal ions with DNA under different conditions. However, it is evident that a correlation between multilayer dsDNA-electrochemical biosensor results and data obtained by means of other methods, especially *in vivo* methods, is necessary.

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