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Electrochemistry for Probing DNA Damage

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1. OVERVIEW

In a health perspective the need for the analysis of gene sequences, oxidative damage to DNA and the understanding of DNA interactions with molecules or ions led to the development of DNA-based biosensors. The DNA-based biosensor is a device that incorporates immobilized DNA as molecular recognition element in the biological active layer on the surface, and measures specific binding processes with DNA mainly using electrochemical, optical and piezoelectric transducers. The fact that the DNA sequences are unique to each organism means that any self-replicating biological organism can be discriminated.

The DNA-based biosensor is also a complementary tool for the study of biomolecular interaction mechanisms of compounds with DNA, enabling the screening and evaluation of the effect caused to DNA by health hazardous compounds and oxidizing substances. There are hundreds of compounds which bind and interact with DNA. Exposure to toxic chemicals is the cause of many human cancers; these carcinogens act by chemically damaging the DNA. Thus it is very important to explain the factors that determine affinity and selectivity in binding molecules to DNA, identify these chemicals and ascertain their potency so that human exposure to them can be minimized.

The reactions with chemicals cause changes in the structure of DNA and the base sequence leading to perturbations in DNA replication. A quantitative understanding of the reasons that determine selection of DNA reaction sites is useful in designing sequence-specific DNA binding molecules for application in chemotherapy and in explaining the mechanism of action of neoplasic drugs.

Electrochemical techniques have the advantage in DNA-biosensor design of having a rapid response time, being quantitative, sensitive, suitable for automation, cost effective, disposable, enabling *in situ* generation of reactive intermediates and detection of DNA damage and solving analytical problems in a wide range of contexts in order to be commercially viable. Comprehensive descriptions of research on DNA and DNA sensing [1–10] show the great possibilities of using electrochemical transduction in DNA diagnostics.

2. DNA-ELECTROCHEMICAL BIOSENSORS

Electrochemical research on DNA is of great relevance to explain many biological mechanisms. The DNA-electrochemical biosensor is a very good model for simulating nucleic acid interaction with cell membranes, potential environmental carcinogenic compounds and for clarifying the mechanisms of action of drugs used as chemotherapeutic agents. An electrochemical sensor for detecting DNA damage consists of an electrode with DNA on its surface. DNA-electrochemical biosensors enable the study of the interaction of DNA immobilized on the electrode surface with analytes in solution, the DNA acting as a promoter between the electrode and the biological molecule under study. Interactions of the surface-confined DNA with a DNA damaging agent are converted, via changes in electrochemical properties of the DNA recognition layer, into measurable electrical signals [11]. The interaction of a number of substances with DNA has been successfully studied using such a kind of biosensor and the interpretation of results have contributed to the elucidation of the mechanisms by which DNA is damaged by hazardous compounds [12–16].

When compared with optical, piezoelectric or other transducers the electrochemical transduction is dynamic in that the electrode is itself a tuneable charged reagent as well as a detector of all surface phenomena, which greatly enlarges the electrochemical DNA biosensing capabilities. However,

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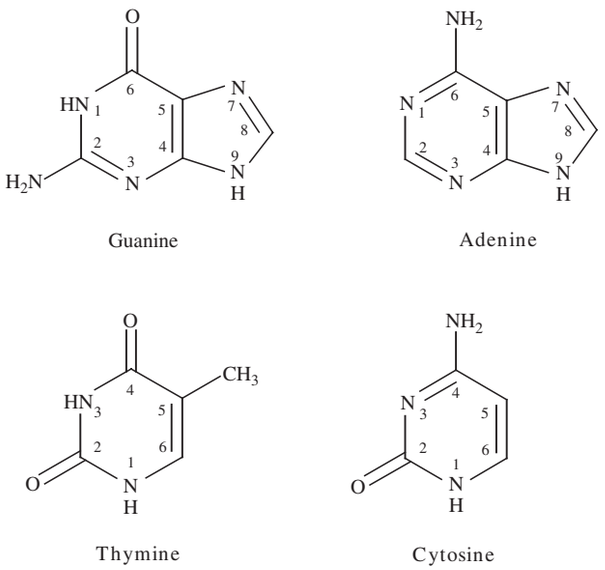
it is necessary that the analyte is electroactive, i.e., capable of undergoing heterogeneous electron transfer reactions, in order to use an electrochemical transducer. To design DNA-based biosensors, it is essential to understand the surface structures of the modified surfaces and so it is important to know which DNA groups are electroactive.

The double helical structure of DNA consists of two polynucleotide chains running in opposite directions and made up of a large number of deoxyribonucleotides, each composed of a base, a sugar, and a phosphate group. The four different bases, Scheme 1, the purines, adenine (A) and guanine (G), and pyrimidines, thymine (T) and cytosine (C) which are all electroactive [6, 17, 18] are on the inside of the double helix and their distance and accessibility to the electrode surface is determinant for nucleic acids' electrochemical behavior. The electrochemical behavior of DNA and adsorption at different types of electrodes has been investigated for a number of years first using a dropping mercury electrode and more recently solid electrodes [6, 7].

The electrochemical behavior with double strand DNA (dsDNA) and single strand DNA (ssDNA) illustrates the greater difficulty for the transition of electrons from the inside of the double-stranded rigid form of DNA to the electrode surface, than from the flexible single-stranded form of DNA where the guanine and adenine residues are in close proximity to the electrode surface. The roughness of a solid electrode surface means that dsDNA has some difficulty in following the surface contours whereas unwound ssDNA molecules fit more easily into the grooves on the electrode surface, because of their greater flexibility. Differential pulse voltammograms obtained with a bare glassy carbon electrode (GCE) did not show any oxidation peak for dsDNA in the first voltammogram, Fig. 1. The very small peaks that appeared after forty scans were caused by changes in conformation of the double helix due to the positive applied potential that enabled some guanine and adenine residues



Scheme 1. Chemical structures of guanine (G), adenine (A), thymine (T), and cytosine (C).

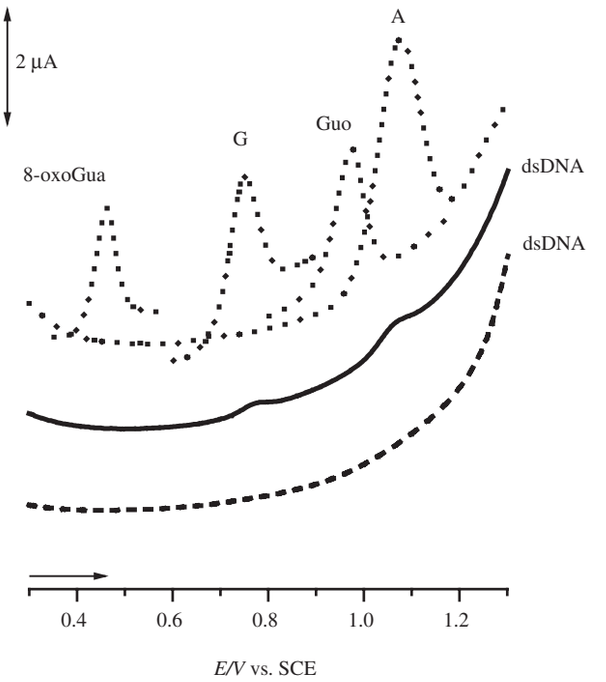


Figure 1. Differential pulse voltammograms obtained with a bare GCE in pH 4.5 0.1 M acetate buffer of: (•••) 15 μM 8-oxoguanine (8-oxoGua); (•••) 15 μM guanine (G); (•••) 15 μM guanosine (Guo); (•••) 100 μM adenine (A); and 60 μ g/ml dsDNA (---) 1st and (—) 40th voltammogram. Pulse amplitude 50 mV, pulse width 70 ms, scan rate 5 mV s^{-1}. Reprinted with permission from [14], A. M. Oliveira Brett et al., *Talanta* 56, 969 (2002). © 2002, Elsevier.

to be more accessible to the electrode surface [14]. The differential pulse voltammogram for the oxidation of dsDNA using a small diameter GCE ($d = 1.5$ mm) in acetate buffer showed better the two tiny signals corresponding to the oxidation of guanosine and adenosine residues in the polynucleotide chain [115].

Electrochemical oxidation on carbon electrodes [17–24] showed that all bases–guanine (G), adenine (A), cytosine (C) and thymine (T)–can be oxidized, Fig. 2, following a pH dependent mechanism. The voltammetric studies on DNA shown in this figure include all four bases—for the first time equimolar mixtures of all DNA bases, nucleosides and nucleotides have been quantified by differential pulse voltammetry [18]. Electrochemical pre-conditioning of the glassy carbon electrode enabled a better peak separation and an enhancement of the current of the oxidation peaks for all four DNA bases in pH 7.4 phosphate buffer supporting electrolyte, close to physiological pH. Detection limits in the nano- and micromolar range were obtained for purine and pyrimidine bases, respectively, together in solution.

The results showed for the first time that the pyrimidine nucleosides and nucleotides are electroactive on glassy carbon electrodes and that, besides the easy detection of the purines, it was also possible to detect simultaneously the oxidation of pyrimidine residues in ssDNA [18].

Electrochemical reduction of natural and biosynthetic nucleic acids at a dropping mercury electrode (DME) [1, 6, 12, 13] showed that adenine and cytosine residues

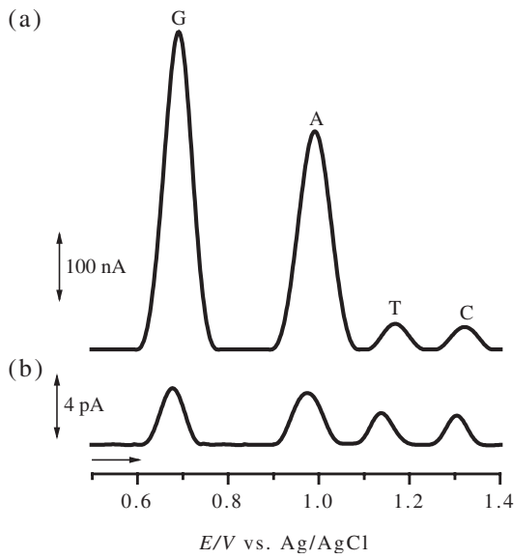


Figure 2. Base line corrected differential pulse voltammograms obtained in a 20 mM equimolar mixture of guanine (G), adenine (A), thymine (T), and cytosine (C) in pH 7.4 0.1 M phosphate buffer supporting electrolyte with: (a) 1.5 mm, (b) 7 μm diameter GCE. Pulse amplitude 50 mV, pulse width 70 ms, scan rate 5 mV s^{-1}. Reprinted with permission from [18], A. M. Oliveira-Brett et al., *Anal. Biochem.* (2004). In press. © 2004.

as well as guanine residues in a polynucleotide chain are reducible, Fig. 3. The cyclic voltammogram of ssDNA at a hanging mercury drop electrode (HMDE) showed a cathodic peak due to irreversible reduction of cytosine (C) and adenine (A). The reduction of guanine (G) occurs at very negative potentials but a peak due to the oxidation of the reduction product of (G) could be detected in the reverse scan [6].

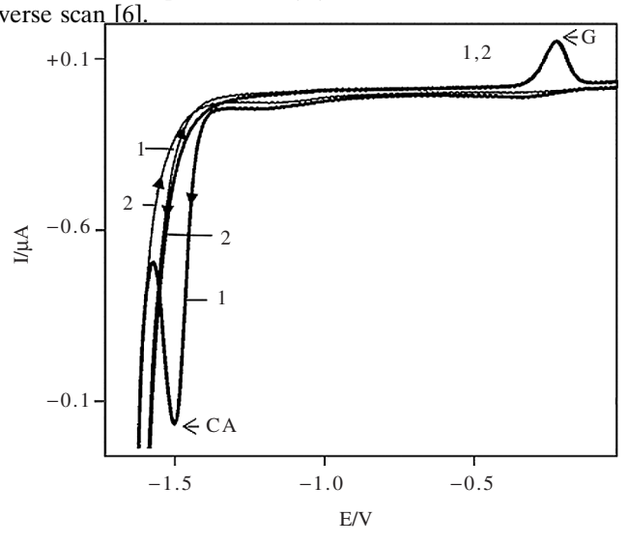


Figure 3. Cyclic voltammogram of ssDNA obtained upon repeated cycling. (CA) peak due to reduction of C and A, (G) peak due to oxidation of reduction product of G. Reprinted with permission from [6], E. Palecek et al., in "The Encyclopedia of Electrochemistry" (A. J. Bard and M. Stratmann, Eds.) in "Bioelectrochemistry" (George Wilson, Ed.), Vol. 9, Ch. 12, p. 365 and references therein. Wiley-VCH Verlag, Weinheim, FRG, 2002. © 2002, Wiley.

A critical issue in the development of a DNA-electrochemical biosensor is the sensor material and the degree of surface coverage that influences directly the sensor response, so electrode surface characteristics represent an important aspect in the construction of sensitive DNA-electrochemical biosensors for rapid detection of DNA interaction and damage.

The study of the adsorption of DNA at electrode surfaces is of fundamental interest, since the interaction of DNA with charged surfaces can be expected in biological systems. In fact, a number of studies of DNA adsorption was conducted at mercury electrodes [25–33] and carbon electrodes [34, 35] and in general a weaker adsorption was observed with dsDNA than with ssDNA.

Atomic Force Microscopy (AFM) has been proved to be a powerful tool for obtaining high-resolution images of adsorbed DNA. Images of DNA conformations, unusual structures and DNA-protein complexes have been obtained. However, the DNA molecules do not bind strongly enough to conducting substrates and the AFM tip tends to sweep away the adsorbed macromolecules. Magnetic AC mode AFM (MAC Mode AFM) permits the visualization of the molecules weakly bound to the substrate material and can be very helpful in the investigation of molecules loosely attached to the conducting surface of electrochemical transducers. MAC Mode AFM in an electrochemically-controlled environment is capable of resolving the surface morphological structure of nucleic acid adsorbates and contributes to the understanding of the mechanism of adsorption and the nature of DNA-electrode surface interactions.

Using *ex situ* MAC mode AFM [36–40] it was possible to visualize directly the surface characteristics of dsDNA films prepared on a highly oriented pyrolytic graphite (HOPG) electrode. It was found that different immobilization methodologies lead to structural changes on the DNA biosensor surface and consequently different sensor response. The HOPG surface is extremely smooth, which enables the identification of the topography changes when the surface is modified with dsDNA [39]. In Fig. 4 are AFM images of a HOPG substrate modified by a thick and a thin layer of dsDNA. The two different immobilization procedures of dsDNA at the surface of a HOPG electrode have been evaluated, the thin dsDNA adsorbed film forming a network structure, with holes not covered by the molecular film exposing the electrode surface, and the thick dsDNA film completely covering the electrode surface with a uniform multilayer film, presenting a much rougher structure.

The dsDNA networks formed at the HOPG electrode during the formation of thin dsDNA layers define different active surface areas of the DNA electrochemical biosensor. The uncovered regions may act as a system of microelectrodes with nanometer or micrometer dimensions, Fig. 5. The two dimensional dsDNA networks form a biomaterial matrix to attach and study other molecules.

The major problem encountered with electrodes modified by a thin film of dsDNA is the fact that the electrode is not completely covered allowing the diffusion of molecules from bulk solution to the surface and their non-specific adsorption. The thin film DNA-electrochemical biosensor in a solution containing an electroactive hazard compound will give an electrochemical signal with two contributions. One

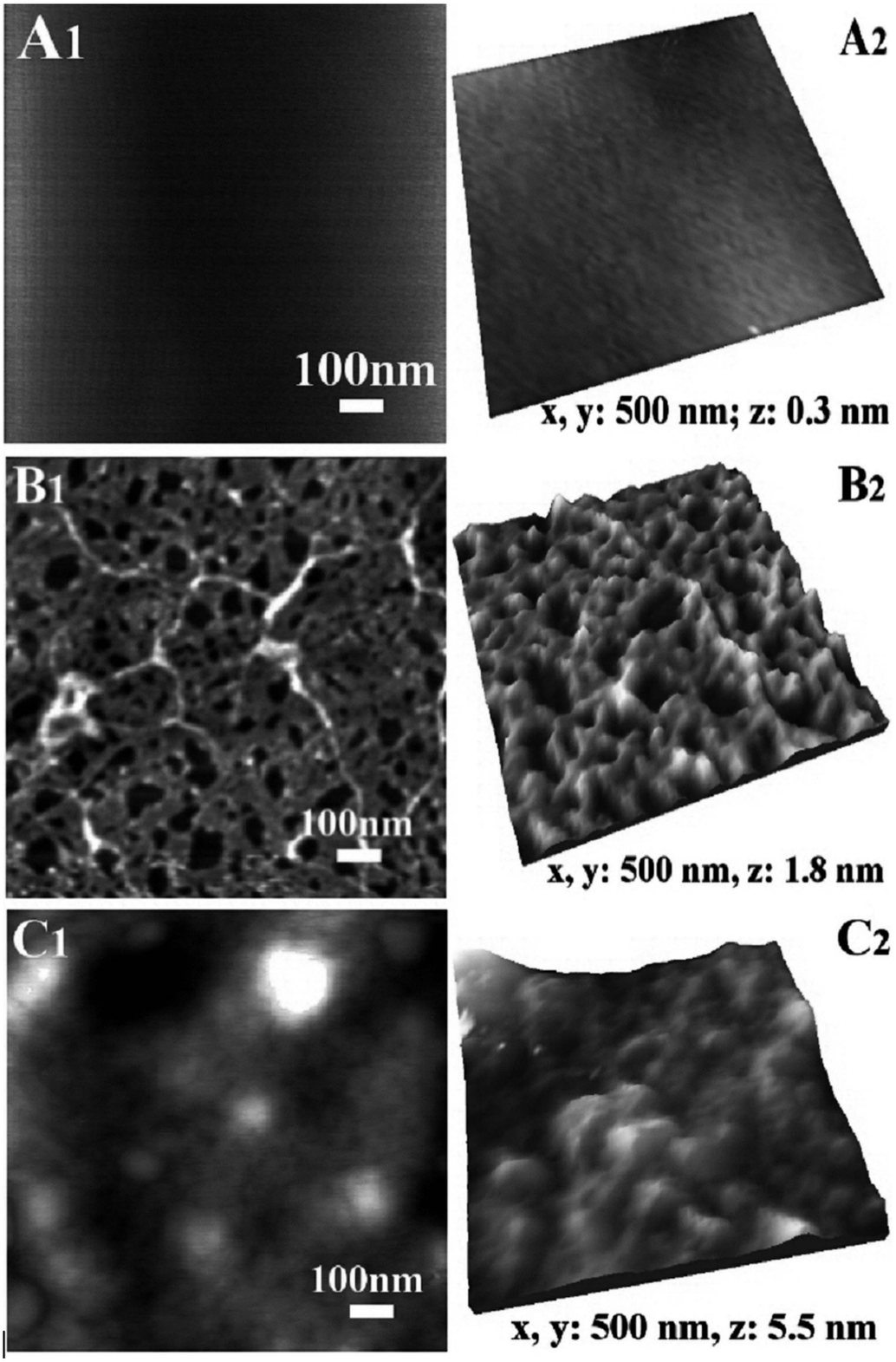


Figure 4. MAC mode AFM topographical images in air of: (A1 and A2) clean HOPG electrode surface; (B1 and B2) thin film dsDNA-biosensor surface, prepared onto HOPG by 3 min free adsorption from a solution of 60 μg/ml dsDNA in pH 4.5 0.1 M acetate buffer electrolyte; (C1 and C2) thick film dsDNA-biosensor surface, prepared onto HOPG by evaporation from solution of 37.5 mg/ml dsDNA in pH 4.5 0.1 M acetate buffer electrolyte; (A1, B1, and C1) two-dimensional view 1 μm $\times$ 1 μm scan size and (A2, B2, and C2) three-dimensional view 500 nm $\times$ 500 nm scan size. Reprinted with permission from [39], A. M. Oliveira Brett and A.-M. Chiorcea, *Bioelectrochemistry* 63, 229 (2004). © 2004, Elsevier.

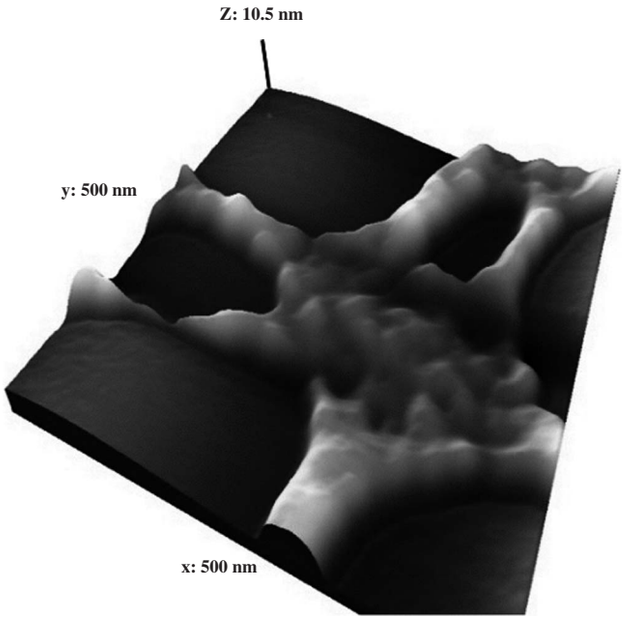


Figure 5. MAC mode AFM topographical image in air of thin dsDNA-biosensor surface, prepared by applying an adsorption potential of +300 mV (vs. Ag wire) to the HOPG electrode immersed into a 60 μ g/mL dsDNA pH 7.0, 0.1 M phosphate buffer electrolyte solution; three-dimensional view 500 nm×500 nm scan size.

of these is from the electron transfer reaction of electroactive hazard compound simply adsorbed on the uncovered areas, Figs. 4, 5 and (7)C. The other is from the damage caused by the hazard compound to the immobilized dsDNA on the electrode surface, Figs. 4 and (8)C, and it is difficult to distinguish between the two signals [14].

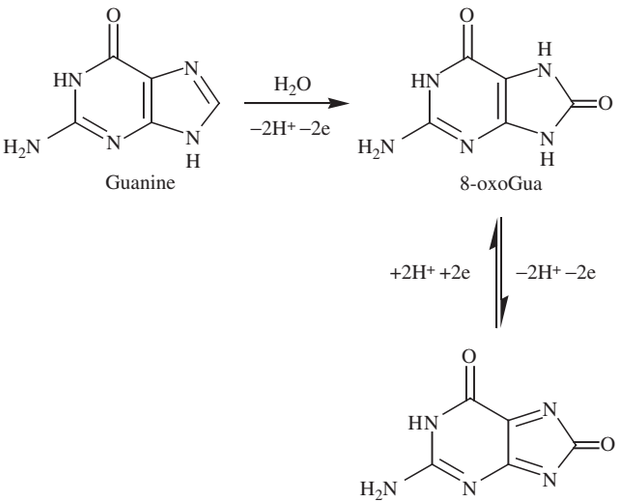
The big advantage of the thick film of dsDNA is that the electrode surface is completely covered by dsDNA so that undesired binding of molecules to the electrode surface is impossible, Figs. 4 and (8)C. The DNA biosensor response is thus only determined by the interaction of the compound with the dsDNA in the film, without any contribution from the electrochemical reaction of the compound at the substrate surface.

Other forms of carbon besides HOPG electrodes, such as glassy carbon electrodes (GCE) or carbon paste electrodes (CPE), are usually used as the sensor material in a DNA-electrochemical biosensor and substrate for DNA immobilization but it is considered that the interactions, the adsorption and the degree of surface coverage, between DNA and the different carbon surfaces are very similar.

3. ELECTROCHEMICAL DETECTION OF DAMAGE TO DNA BASES

Oxidative DNA damage caused by oxygen-free radicals leads to multiple modifications in DNA, including base-free sites and oxidized bases. The damage caused to DNA bases is potentially mutagenic [41–47].

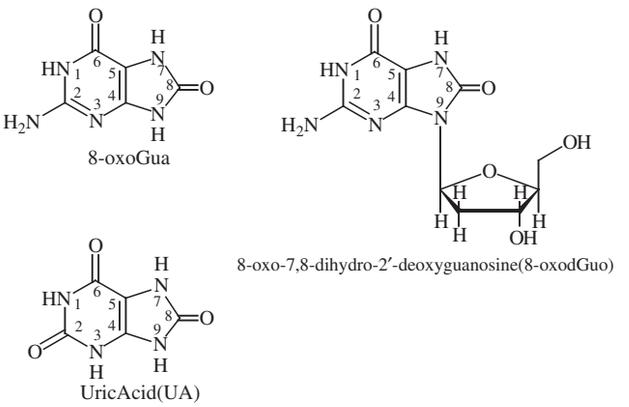
The major product of DNA oxidative damage is 8-oxo-7,8-dihydroguanine (8-oxoGua), Fig. 1, an electroactive compound which is the product of oxidation of



Scheme 2. Oxidation mechanism of guanine.

guanine, Scheme 2, the most easily oxidized base in DNA [7], Figs. 1 and 2. This modified base is highly mutagenic [48, 49] causing G → T transversion and loss of base-pairing specificity [50–52]. Since it was first reported two decades ago [53], 8-oxoGua, namely its deoxynucleoside 8-oxo-7,8-dihydro-2′deoxyguanosine (8-oxodGuo), Scheme 3, has been the subject of intensive investigation and has become widely accepted as a biomarker of oxidative DNA damage and cellular oxidative stress [52–56]. Also 8-oxoGua and 8-oxodGuo are major products of DNA oxidative damage that can be enzymatically repaired, being excreted into urine.

Elevated levels of 8-oxodGuo were found in the urine and lung tissues of smokers [57, 58] as well as in body fluids and DNA from human tissues of patients with disorders such as cancer, atherosclerosis, chronic hepatitis, cystic fibrosis, diabetes, acquired immunodeficiency syndrome, neurodegenerative and age-related diseases [56, 59–62]. One approach to assess this oxidative DNA damage is the measurement of 8-oxoGua or 8-oxodGuo content in DNA



Scheme 3. Chemical structures of 8-oxo-7,8-dihydroguanine (8-oxoGua), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) and uric acid (UA).

isolated from tissues that would represent steady-state levels arising from the balance between oxidative damage and enzymatic repair [55].

Endogenous production of 8-oxoGua due to aerobic cellular metabolism leads to basal levels of one 8-oxoGua/10^6 DNA bases, being excreted into urine at basal concentrations below 100 nM [63]. Due to their high sensitivity, voltammetric techniques enable the detection of such low concentrations of 8-oxoGua [64]. In urine, purines such as hypoxanthine and uric acid (UA) are co-excreted in concentrations 10^4-fold higher than 8-oxoGua. UA is the major end product of purine metabolism in humans and its levels in urine are also indicative of various diseases. It has a structure very similar to 8-oxoGua, Scheme 3, and is thus a strong interferent. Assessment of urinary levels of both 8-oxoGua and 8-oxodGuo may provide a non-invasive approach to evaluate the DNA repair capability in individuals and be used as biomarkers of cellular oxidative stress.

At present, HPLC with electrochemical detection (HPLC-ECD) is the most commonly used technique to assess urinary 8-oxodGuo, but HPLC with tandem mass spectrometry (HPLC-MS/MS) is also being used, showing high sensitivity and a better specificity. However, HPLC-ECD is easier to use and less demanding of resources. There is a significant lack of HPLC-ECD based methods for 8-oxoGua detection in human urine, despite 8-oxoGua being also electrochemically detectable [64] at a lower potential than 8-oxodGuo [65]. A selective method based on HPLC-ECD was developed to enable simultaneous detection of 8-oxoGua and 8-oxodGuo, products of DNA oxidative damage, after UA elimination by uricase, consisting of HPLC isocratic elution with amperometric detection, enabling a detection limit for 8-oxoGua and 8-oxodGuo lower than 1 nM in standard mixtures [66, 67]. Regardless of the complexity and inter-individual variability of urine samples, the method was tested with urine samples from children (3–8 years old) with metabolic disorders [66], Fig. 5, and it was confirmed that at the applied working potential, hypoxanthine does not give any electrochemical signal [68] and does not interfere at all in 8-oxoGua signal.

The oxidation of the other DNA bases, Fig. 2, is much more difficult due to their high oxidation potentials. Chemical modification of each of the DNA bases causes molecular disturbance to the genetic machinery that leads to cell malfunction and death. For instance, oxidative DNA damage by free radicals and exposition to ionizing radiation generates several products within the double helix besides 8-oxoguanine, such as 2,8-oxoadenine, 5-formyluracil, 5-hydroxicytosine, etc. which are mutagenic [41]. It is well established that the oxidation of DNA bases is an important source of genomic instability since there is evidence that the oxidation products of DNA bases play an important role in mutagenesis, carcinogenesis, ageing and age-related disease [41, 47, 69].

Hence, great interest exists in the sensitive determination and full characterization of the mechanisms involved in oxidative damage of all DNA bases. Electrochemical methods are very promising for the study of DNA oxidative damage as well as in the investigation of the mechanisms of interaction of drugs with DNA [6–10]. In particular, electrochemical DNA-biosensors have proved to be excellent tools for investigating the effect of various endogenous and exogenous sources of hazard on genomic material, allowing quick and low cost determination of DNA damage.

4. ELECTROCHEMICAL DETECTION OF DNA DAMAGE

As indicated above, many compounds bind and interact with DNA. Toxic chemicals damage DNA and can cause human cancers. The changes in the structure of DNA and the base sequence can lead to perturbation in DNA replication. The products of oxidation of DNA bases lead to substitutions, deletions and insertions in the base sequence of DNA, the process called mutagenesis [70]. The clarification of the mechanisms that determine affinity and selectivity in binding molecules to DNA, enabling a quantitative understanding of the reasons that determine selection of DNA reaction sites, is useful in designing sequence-specific DNA binding molecules for application in chemotherapy and in explaining the action of neoplasic drugs [71].

The DNA interacting drugs prevent cell growth, but not only cancer cell growth; the cytotoxic effect also blocks the growth of normal cells. The lack of selectivity of cancer drugs is one of the main problems in cancer chemotherapy and DNA-biosensors are an important tool for the investigation of the chemical and biological mechanism of drugs which are active against cancer cells.

Electrochemical methods [11] have shown great potential in studying the adsorption and reactions of biological molecules at electrified interfaces. They have been used by a number of researchers who have been studying metal ion-DNA interactions [72, 73]. Using DNA-electrochemical biosensors, electrochemical methods will probe DNA interactions and will continue to exploit the remarkable specificity of biomolecular recognition to provide analytical tools that can measure the presence of a single molecular species in a complex mixture, pre-screen hazard compounds that cause damage to DNA and help to explain DNA-protein interactions.

The possible relation between oxidative damage and metal ion concentrations is not completely clear. Metal ions such as Na^+, K^+, Mg^{2+}, and Ca^{2+} exist in the body in high concentrations and the nucleic acids and nucleotides occur as complexes coordinated with these ions. A metal ion has four different potential coordination sites for binding with DNA: the negatively charged phosphate oxygen atoms, the ribose hydroxyls, the base ring nitrogens and the exocyclic base keto groups.

There are many forms that a metal ion can take but it is the free metal ion that is most toxic and trace levels of free toxic metals, such as copper, cadmium, lead and zinc, can be determined using electrochemical methods [73–75]. The heavy (or transition) metal elements, cadmium, chromium and nickel, are carcinogenic and the most toxic metals known. They catalyse the formation of highly reactive species causing DNA damage, and its site specificity is determined by the chemical property of the reactive species formed. Thus, metal-catalyzed ROS generation may play a critical role in carcinogenesis induced by chemicals. It is accepted that DNA adduct formation participates in

tumor initiation [76], while oxidative DNA damage would be involved in tumor promotion [77].

Nevertheless, the mechanism of metal carcinogenesis is not clear due to the very complex nature of metal interactions in biological systems. Metal ions react with superoxide anion (O_2^-) and H_2O_2 to produce highly reactive species such as hydroxyl free radicals (OH) and metal-oxygen complexes in biological systems, resulting in metal-mediated oxidative DNA damage.

Studies of metallointercalation agents [78–80] in the presence of dsDNA have been done with solutions containing the redox complexes $Co(phen)_3^{3+/2+}$, $Fe(phen)_3^{2+/3+}$, $Co(bpy)_3^{3+/2+}$, $Fe(bpy)_3^{2+/3+}$, and $Os(bpy)_3^{2+/3+}$ (where phen = 1,10-phenanthroline and bpy = 2,2′-bipyridyl). Binding was interpreted in terms of the interplay of electrostatic interactions of the metal coordination complexes with the charged sugar-phosphate and the intercalative, hydrophobic, interactions within the DNA helix, i.e., the stacked base pairs. Chronopotentiometric detection of the $Co(bpy)_3^{3+}$ indicator was used [81] to monitor the hybridization onto a screen-printed carbon electrode of short DNA sequences from *E. coli* pathogen in environmental water samples. The redox couple $Co(bpy)_3^{3+/2+}$ was also used to evaluate the adsorption of DNA on gold electrodes [81–83].

Studies of redox-active metallointercalation agents in the presence of dsDNA have been done with solutions containing the redox complexes of cobalt, iron and osmium [5, 6, 10, 13, 84]. Osmium tetroxide complexes with tertiary amines (Os, L) have been used as a chemical probe of DNA structure. The simultaneous determination of (Os, L)-DNA adducts and free (Os, L) using a pyrolitic graphite electrode was possible due to their peak separation on the potential scale being sufficiently large.

Layer-by-layer electrostatic assembly of DNA, enzymes, polyions, and catalytic redox polyions of nanometer thickness on electrodes, were designed to detect DNA damage as they can provide active elements for sensors for screening the toxicity of chemicals and their metabolites, and for oxidative stress [85, 86].

Metallopolyion films catalyze DNA oxidation and were incorporated into DNA/enzyme films enabling the detection of structural damage to DNA as a basis for toxicity screening and leading to "reagentless" sensors. Films of the osmium polymer $[Os(bpy)_2(PVP)_{10}Cl]^+$[PVP=poly(vinylpyridine)] were used to monitor DNA oxidation selectively; including the analogous ruthenium metallopolymer in the sensor provides a monitor for oxidation of other nucleobases [85, 86].

The binding of ligand and ligand substituents in complexes of ruthenium(II) [87] has been investigated in a systematic fashion and the binding parameters for the series compared in order to determine the different ligand functionalities and sizes in the binding with DNA, i.e., intercalation and surface binding. It was found that if one compares the various factors that contribute to stabilizing the metal complexes of ruthenium on the DNA helix it appears that the most significant factor is that of molecular shape. The complexes that fit most closely to the DNA helical structure, those in which the van der Waals interactions between complex and DNA are maximized, display highest binding affinity.

A doubly metallated 15 base-pair double helix containing ruthenium and rhodium at each end of the strands [87] showed the efficiency of DNA for coupling electron donors and acceptors over a very long range, greater then 4 nm. The dsDNA was found to behave like a piece of molecular wire with fast electron transfer rates ($\geq 10^{10}$ s^{-1}) for the photoinduced electron transfer between the metallointercalators [88–90] and semiempirical Hartree-Fock calculations of H_{AB} for DNA mediated electron transfer [91] were described.

Oxidative damage to DNA was demonstrated to depend upon oxidation potential [92] and be promoted from a remote site as a result of an electron hole migration through the DNA π-stack, the hole migrates down the double helix to damage guanine, a site sensitive to oxidative nucleic acid damage within the cell.

During the transfer of genetic information, the interactions between DNA and the divalent ions Mg^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Cr^{2+}, Cd^{2+}, and Pb^{2+} play an essential role in promoting and maintaining the nucleic acid functionalities. Some are recognized for their carcinogenicity as they damage DNA molecules and alter the fidelity of DNA synthesis [93, 94]. Nickel, chromium, and cadmium, have been recognized as the most effective carcinogens. Many inorganic nickel compounds have been tested and their effect on a cell or tissue has been established as an interaction with the base donor systems, especially within unwound parts of nucleic acids [95–97]. Damage to DNA structure has also been described due to the effects of Zn [98], Cd [98], and Pb [99]. The interaction modes of metalloporphyrins with DNA [100] have a different electrochemical behavior depending on the metal ions: Cu, Ni, Zn, or Cd, and the porphyrin ligands.

The adsorptive and voltammetric characteristics of Cu(II) complexes with guanine, guanosine and adenosine were exploited [101] in order to detect these bases after separation by capillary zone electrophoresis and the enzyme-mimic catalytic activity of a DNA-Cu^{2+} complex [102] was used to develop an amperometric quinacrine sensor using an oxygen electrode covered by the complex entrapped in polyacrylamide gel.

The cytostatic activity of various platinum drugs has shown that platinum coordination complexes cause irreversible inhibition of DNA synthesis due to covalent binding with DNA [100]. This often causes the treatment to be accompanied by adverse reactions. Differential pulse voltammetry with the static mercury drop electrode has been used to investigate the interactions of a group of eight anticancer-active Pt(II) and Pt(IV) complexes in solution with DNA, as well as the conformational alterations induced by the binding of the drugs [104–108]. The interactions of carboplatin in solution with DNA were investigated using differential pulse voltammetry at a glassy carbon modified electrode [97, 98]. It was found that the binding of platinum drugs causes local distortions in the DNA molecule with the formation of interstrand cross-links. Research on metal ion nucleic acid complexes was advanced when antitumour activities of platinum (II) compounds were discovered.

Similarly, the detection of DNA damage involving strand breaks was observed [109] using a hanging mercury drop electrode (HMDE). Extensive cleavage of electrode-confined DNA by reactive oxygen species (ROS) was

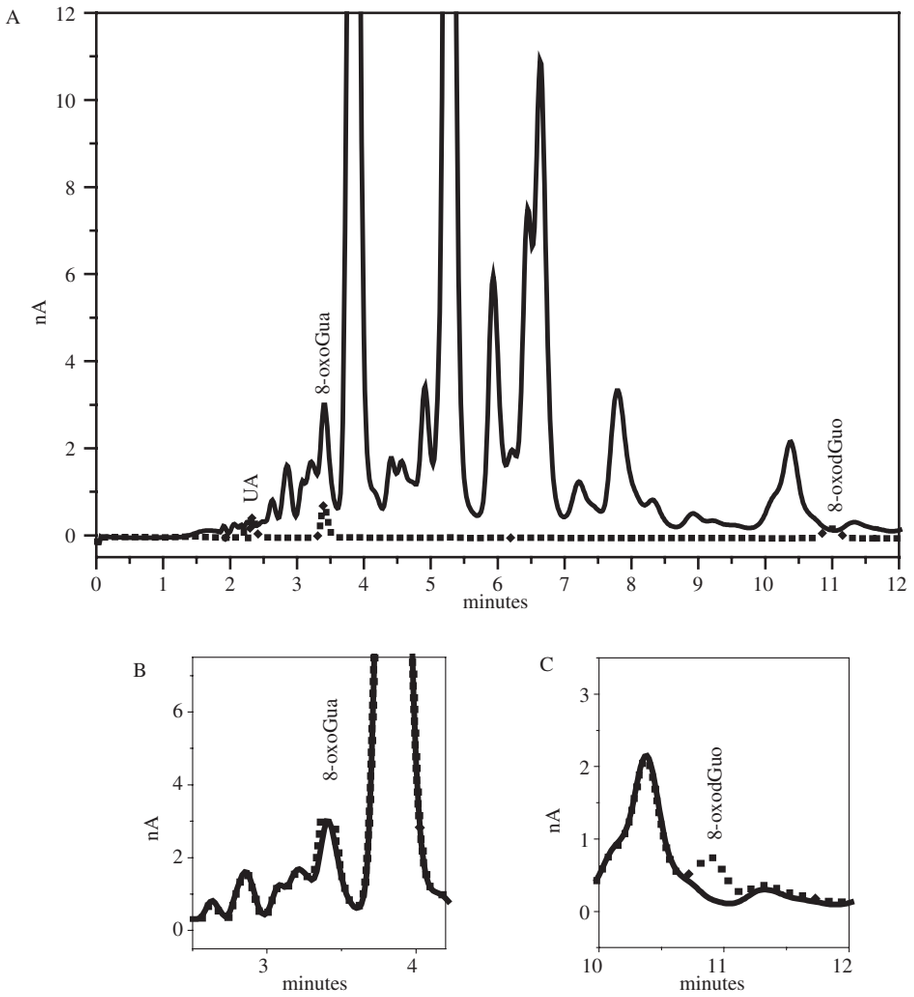


Figure 6. (A) Chromatograms obtained from (•••) 10 μL injection of a 100 nM standard mixture of UA, 8-oxoGua and 8-oxodGuo and (—) 10 μL injection of an urine sample from a child with metabolic disorders, after being processed by SPE. Same urine sample (—) before and (•••) after being spiked with 250 nM standard solutions of (B) 8-oxoGua and (C) 8-oxodGuo. Mobile phase: 50 mM phosphate buffer + 6% MeOH+2 mM KCl, final pH 6.2. Reprinted with permission from [66], I. A. Rebelo et al., *Talanta* 63, 323 (2004). © 2004, Elsevier.

obtained in the absence of chemical reductants when redox cycling of the metal (iron/DNA complex) was controlled. Not only the cleaving agents were detected but also the DNA cleavage was modulated, by generating the DNA-damaging species electrochemically.

Metals are considered to act not only as carcinogens but also to activate carcinogenic chemicals. A number of aromatic compounds induce oxidative DNA damage through metal catalyzed reactive oxygen species (ROS) generation. There is a deep interest in identifying free radical scavengers or antioxidants that inhibit oxidative DNA damage. Owing to their polyphenolic nature, flavonoids, compounds found in rich abundance in all land plants, often exhibit strong antioxidant properties [110–112]. Initially, flavonoids were investigated as potential chemopreventive agents against certain carcinogens. Previous intake of a large quantity of flavonoid inhibited the incidence of ROS produced damage to DNA. In sharp contrast with the commonly accepted role, there is also considerable evidence that flavonoids themselves are mutagenic and have DNA damaging ability [111, 112].

One example is quercetin, which under certain circumstances acts as a prooxidant and has mutagenic activity [113, 114]. There is experimental evidence that the formation of quercetin radicals via autooxidation leads to the generation of superoxide radicals. Also, it is proposed that quercetin can directly reduce transition metals, thus providing all the elements necessary to generate the highly oxidizing radical OH. Therefore, quercetin can promote oxidative damage to DNA through the generation of these highly reactive oxygen species. Extensive quercetin-induced DNA damage via reaction with Cu(II) was reported. In an electrochemical study of the DNA-Cu(II)-quercetin interactions, several situations were studied using a bare GCE in a solution containing dsDNA incubated with quercetin or quercetin-Cu(II) complex and using a DNA-electrochemical biosensor, which gave strong evidence that it was the radicals formed during oxidation of the catechol moiety in the

quercetin molecule, via reaction with Cu(II) or electrochemically, that damaged the DNA [115, 116].

The anticancer activity and host toxicity of compounds with a quinone group was also investigated. There is considerable death of tumour cells, together with the induction of breaks in DNA single and double strands, although a low binding value for the semiubiquinone with DNA was found [117].

The antitumour action of the natural antibiotic bleomycin is thought to involve the aerobic degradation of DNA by the Fe^{2+}-bleomycin complex. In order to probe the mechanism of antitumor action of bleomycin, the 4-ethylamido[5,(2′-thienyl)-2-thiophene] imidazole iron(II) complex was synthesized [118]. It was studied in non-aqueous solution using cyclic voltammetry and showed antitumour activity *in vitro*, its action causing cleavage of the double helical DNA.

Spectroscopic methods have been complemented by electrochemical methods in investigating the binding strength and specificity of antineoplasic drugs with DNA at the molecular level, as well as in explaining their toxic action. Some tumour cells, but not normal cells, have nucleic acids associated with the cell surface which can suppress many cell immunological reactions.

The detection of chemicals that cause irreversible damage to DNA is very important, as this may lead to hereditary or carcinogenic diseases. A short-time screening test for carcinogens based on ac voltammetric measurements was developed to study *in vitro* damage to DNA caused by the action of alkylating mutagens [119]. Nitroimidazoles are among the most important nitroheterocyclic drugs of interest in cancer chemiotherapy. It was observed that adenine and guanine interact with intermediates generated during nitroimidazole reduction [120], causing irreversible damage to DNA and suggesting mutagenic properties of these compounds.

The mechanism of reduction of a group of nitroimidazoles: metronidazole, secnidazole and tinidazole was investigated using the DNA-biosensor [121–124] in a new way by pre-concentrating the analyte on the electrode surface containing DNA and studying either their reduction or the oxidation of reduction products retained on the electrode surface. It was possible to follow the reduction, and the reversible reoxidation of the hydroxylamine derivative (RNHOH) to the corresponding nitroso derivative (RNO), as well as the condensation reaction between the hydroxylamine and nitroso derivatives to form the azoxycompound (RNO:NR) and the interaction with triple helix nucleic acid (H-DNA). The DNA-biosensor contained a small amount of H-DNA segments which were detected by the hydroxylamine, a well known chemical probe for single strands in H-DNA [125].

DNA-modified electrodes have also been used for trace measurements of toxic amine compounds [126] and for trace measurements of phenothiazine compounds with neuroleptic and antidepressive action [127] as well as for detection of radiation-induced DNA damage [128].

Conventional cancer chemotherapy is as toxic as it is beneficial. The DNA interacting drugs prevent cell growth, but not only cancer cell growth and the cytotoxic effect blocks also the growth of normal cells. The lack of selectivity of cancer drugs is one of the main problems in cancer chemotherapy [129, 130] and the chemical and biological mechanism of drugs active against cancer cells must be investigated deeply.

Antisense and triplex-forming oligonucleotides are currently being developed as therapeutic agents for cancers and viral infections [131]. They are highly effective and safe. The recognition of the potential biological roles of H-DNA and the interest in the triple-helical nucleic acid research also includes genetic applications.

The potent antineoplasic antibiotic, also an antiviral and antibacterial agent, the basic oligopeptide netropsin, is known to increase the stiffening of double helical DNA segments to which it is bound. In agreement with this, voltammetric oxidation peaks at graphite electrodes of a DNA solution containing netropsin were smaller for the peak corresponding to the oxidation of the adenine residues than in solutions without netropsin [132]. These results show the effect of the A.T-specific drug netropsin. Electrochemistry has the advantage of permitting direct observation of changes in the A.T and G.C peaks in double stranded DNA solutions with and without netropsin. This is a particularly good example which demonstrates the relevance of electrochemistry for research into specific binding of biologically active ligands to DNA.

The binding affinities of a group of cancerostatic anthracycline antibiotics to DNA were evaluated by differential pulse and other voltammetric techniques using mercury electrodes [133] and the formation of complexes with DNA in relation to the sugar residue was measured. It was found that binding was higher for derivatives with two basic sugars and lower for derivatives with one neutral sugar. Neverthless the cytostatic properties of these anthracycline antibiotics also depend on the influence of rates of dissociation, splitting and transport to the DNA of the tumor.

Electrochemical voltammetric *in situ* detection of dsDNA oxidative damage caused by reduced adriamycin, an antibiotic of the family of anthracyclines, intercalated into DNA, was carried out using a DNA-electrochemical biosensor. Oxidation and reduction of adriamycin molecules intercalated in dsDNA were investigated [14] in order to understand the *in vivo* mechanism of action of DNA with this anti-neoplasic drug. However, it is not possible to detect adriamycin-DNA damage by monitoring only changes in the adriamycin oxidation peak, $E_p = +0.50$ V, due to adriamycin's strong adsorption [134]. The damage to immobilized dsDNA causes the appearance of oxidation peaks from DNA bases and this should always be measured and taken into account, Figs. (7)B and (8)B.

Using a thin film of dsDNA, Fig. 7, the electrode is not completely covered allowing non-specific adsorption of adriamycin molecules diffusing from the bulk solution to the surface, giving an electrochemical signal with two contributions: from adsorbed adriamycin and from the adriamycin-dsDNA interaction, Fig. 7(B), [14]. Using the thick film of dsDNA, Fig. 8, the electrode surface is completely covered by dsDNA so the DNA-biosensor response corresponds to the adriamycin-dsDNA interaction, Fig. (8)B [14].

The results showed that the interaction of adriamycin with dsDNA is potential-dependent. The reduction of adriamycin, at $E_p = -0.60$ V, originates radicals that damage dsDNA causing the contact between DNA guanine, $E_p = +0.78$ V, and adenine $E_p = +1.08$ V, bases and

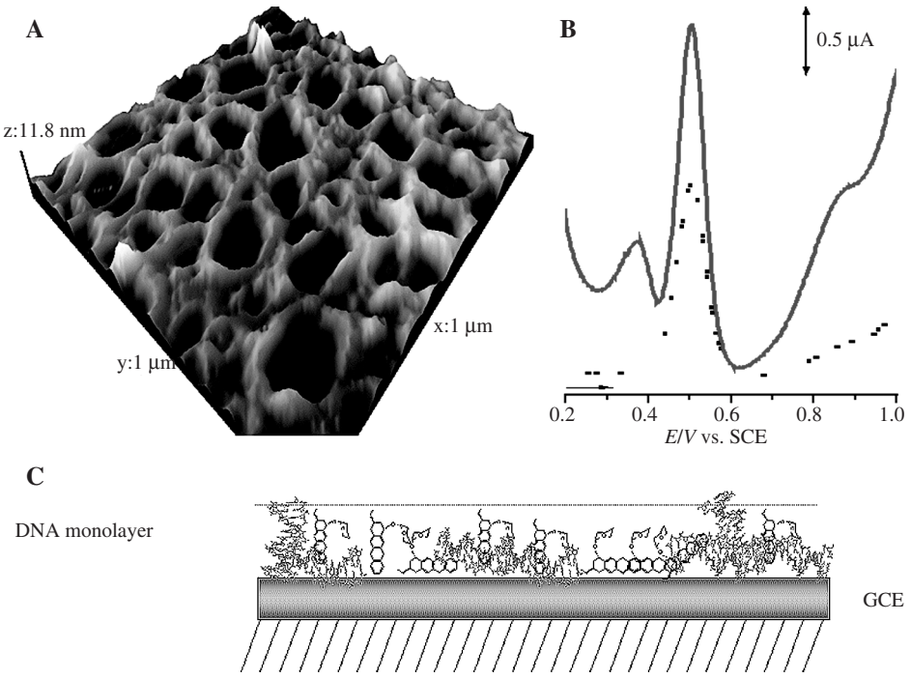


Figure 7. (A) MAC mode AFM topographical image in air of thin film dsDNA-biosensor surface, prepared onto HOPG by 3 min free adsorption from a solution of 60 μg/ml dsDNA in pH 4.5 0.1 M acetate buffer electrolyte; three-dimensional view 1 μm × 1 μm scan size. (B) Background-subtracted differential pulse voltammograms in pH 4.5 0.1 M acetate buffer obtained with a thin layer dsDNA-modified GCE after being immersed during 10 min in a 1 μM adriamycin solution and rinsed with water before the experiment in buffer: (•••) without applied potential; (—) subsequent scan after applying a potential of −0.6 V during 120 s. Pulse amplitude 50 mV, pulse width 70 ms, scan rate 5 mV s^{-1}. Reprinted with permission from [14], A. M. Oliveira Brett et al., *Talanta* 56, 969 (2002). © 2002. (C) Scheme of DNA monolayer modified GCE with adsorbed adriamycin.

the electrode surface such that their oxidation is easily detected. A mechanism for adriamycin reduction and oxidation *in situ* when intercalated in dsDNA immobilized onto the glassy carbon electrode surface was proposed. This mechanism leads to the formation of the mutagenic 8-oxoguanine, $E_p = +0.38$ V, whose redox behavior was studied [64, 66, 67].

The DNA-electrochemical biosensor can provide very relevant information because the mechanisms of interaction of DNA-drug at charged interfaces mimics better the *in vivo* DNA-drug complex situation, where it is expected that DNA will be in close contact with charged phospholipid membranes and proteins, rather than when the interaction is in solution. Complexes between short oligodeoxynucleotides (ODN) with a variable dG_xdC_y base composition and liposomes composed of the cationic lipid DOTAP (ODN lipoplexes), which can be important for the understanding and development of gene therapy vectors based on ODN lipoplexes, were studied by differential pulse voltammetry at a glassy carbon electrode [135, 136]. It was found that the ODN base sequence influences the physicochemical properties of the lipoplexes. This means that DNA sequences are not only essential, as they code for proteins and are relevant for DNA/protein interaction and genetic regulation, but that there is also a sequence dependence interaction between DNA and the lipids in the cellular membrane.

DNA-biosensors have been used for trace measurements of toxic amine compounds, phenothiazine compounds with neuroleptic and antidepressive action as well as detection of radiation-induced DNA damage [130]. Screening tests for carcinogens based on voltammetric measurements were developed to study *in vitro* damage to DNA caused by the action of pollutants [137], pathogens [4] and detection of DNA-adduct formation that start the carcinogenic process, such as benzo[*a*]pyrene-DNA adducts [138]. The application of DNA-electrochemical biosensors to detection in food of bacterial and viral pathogens responsible for disease, due to their unique nucleic acid sequences, is also attractive.

The development of electrochemical DNA-biosensors opened a wide perspective using a particularly sensitive and selective method for the detection of specific interactions. The possibility of foreseeing the damage that these compounds cause to DNA integrity arises from the possibility of pre-concentration of either the starting materials or the redox reaction products on the DNA-biosensor surface, thus permitting the electrochemical probing of the presence of short-lived intermediates and of their damage to DNA [14, 115, 116, 123–126].

Effectively, the DNA-electrochemical biosensor enables pre-concentration of the drug under investigation onto the electrode sensor surface and *in situ* electrochemical generation of radicals, which cause damage to the DNA immobilized on the glassy carbon electrode surface and can be detected electrochemically. The whole detection procedure occurs in minutes.

However, non-uniform coverage of the electrode surface by DNA and adsorption of drug on the bare GCE, Fig. 7(C), may lead to contributions from both simple adsorbed analyte and from the products of the damage caused to dsDNA immobilized on the electrode surface,

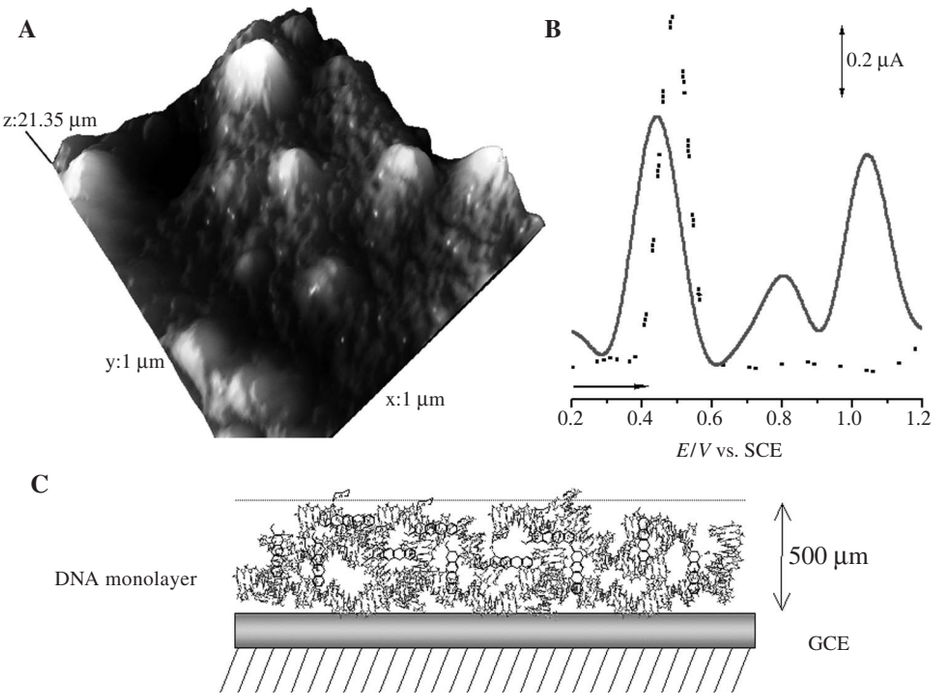


Figure 8. (A) MAC mode AFM topographical image in air of thick film dsDNA-biosensor surface, prepared onto HOPG by evaporation from solution of 37.5 mg/ml dsDNA in pH 4.5 0.1 M acetate buffer electrolyte, three-dimensional view 1 μm × 1 μm nm scan size. (B) Differential pulse voltammograms in pH 4.5 0.1 M acetate buffer solution, obtained with a GCE modified by a DNA multilayer, after being immersed during 10 min in a 1 μM adriamycin solution and washed with water before the scan in acetate buffer (•••) without applied potential; (—) after application of −0.6 V during 120 s. $v = 5\,\text{mV s}^{-1}$. Reprinted with permission from [14], A. M. Oliveira Brett, M. Vivian, I. R. Fernandes, and J. A. P. Piedade, *Talanta* 56, 969 (2002). © 2002. (C) Scheme of DNA multilayer modified GCE with adsorbed adriamycin.

which need to be carefully distinguished. The damage to immobilized DNA always leads to the appearance of oxidation peaks from DNA easily oxidized bases, guanine and adenine, or even 8-oxoguanine [64, 66, 67], which should be monitored and taken into account.

The understanding of the mechanism of action of drugs that interact with DNA will explain the differences in reactivity between similar compounds. This knowledge can be used as an important parameter for quantitative structure-activity relationships (QSAR) and/or molecular modeling studies, as a contribution to the design of new structure-specific DNA-binding drugs, and for the possibility of prescreening the damage they may cause to DNA integrity.

5. CONCLUSIONS

Electrochemical research on DNA is of great relevance to explain many biological mechanisms. The DNA-modified electrode is a very good model for simulating the nucleic acid interaction with cell membranes, potential environmental carcinogenic compounds and to clarify the mechanisms of action of drugs used as chemotherapeutic agents.

The use of DNA-electrochemical biosensors for the understanding of DNA interactions with molecules or ions exploits the use of voltammetric techniques for *in situ* generation of reactive intermediates and is a complementary tool for the study of biomolecular interaction mechanisms. Voltammetric methods are an inexpensive and fast detection procedure. Additionally, the interpretation of electrochemical data can contribute to elucidation of the mechanism by which DNA is oxidatively damaged by such substances, in an approach to the real action scenario that occurs in the living cell.

The development of the DNA-electrochemical biosensor has opened wide perspectives using a particularly sensitive and selective method for the detection of specific interactions. The possibility of foreseeing the damage that these compounds cause to DNA integrity arises from the preconcentration of either the starting materials or the redox reaction products on the DNA-biosensor surface, thus permitting the electrochemical probing of the presence of short-lived intermediates and of their damage to DNA.

GLOSSARY

Adenine a purine base that pairs with thymine in the DNA double helix.

Adenosine the nucleoside containing adenine as its base.

AFM atomic force microscopy.

AMP adenosine monophosphate.

AP sites apurinic or apyrimidinic site resulting from the loss of a purine or pyrimidine residue from DNA

Biosensor a device incorporating a biological sensing element connected to a transducer.

Base pair two nucleotides inn DNA that are paired by hydrogen bonds—for example, G with C and A with T.

Bond energy strength of the chemical linkage between two atoms, measured by the energy needed to break it.

Carcinogen agent, such as a chemical or a form of radiation, that causes cancer.

Chromatography separation technique in which a mixture of substances is separated by charge, size, or some other property by allowing it to partition, between a moving phase and a stationary phase.

Complementary nucleotide sequence two nucleic acid sequences are said to be complementary if they can form a perfect base-paired double helix with each other.

Cytidine the nucleoside having cytosine as its base.

Cytosine a pyrimidine base that pairs with guanine.

Denaturation change in conformation of a nucleic acid caused by heating or by exposure to chemicals and usually resulting in loss of biological function.

Deoxyribonucleic acid (DNA) polynucleotide formed from covalently linked deoxyribonucleic units that serve as the carrier of genetic information.

Diffusion movement of molecules in the direction of lower concentration due to random thermal movements.

Double helix the structure of DNA first proposed by Watson and Crick, with two interlocking helices joined by hydrogen bonds between paired bases.

Functional group group of covalently linked atoms, such as hydroxyl group ($-$OH) or amino group ($-NH_2$), the chemical behavior of which is well characterized.

GCE glassy carbon electrode.

CPE carbon paste electrode.

Guanine A purine base that pairs with cytosine.

Guanosine The nucleoside having guanine as its base.

HMDE hanging mercury drop electrode.

HPLC high pressure liquid chromatography.

HPLC-ECD HPLC with electrochemical detection.

HPLC-MS/MS HPLC with tandem mass spectrometry.

H-DNA triple helix nucleic acid.

Hydrogen bond A weak bond in which an atom shares an electron with a hydrogen atom; hydrogen bonds are important in the specificity of base pairing in nucleic acids.

In situ in place.

In vitro in an experimental situation outside the organism.

In vivo in a living cell or organism.

Lesion a damaged area in a gene (a mutant), or a chromosome.

MAC mode AFM magnetic AC mode atomic force microscopy.

Macromolecule A large polymer such as DNA.

Melting denaturation of DNA.

Mutagen an agent that is capable of increasing the mutation rate.

Mutant an organism or cell carrying a mutation.

Mutant site the damaged or altered area within a mutated gene.

Nanometer (nm) unit of length used to measure molecules, 1 nm $= 10^{-3}$ μm $= 10^{-9}$ m $= 10$ Å (angstroms).

Nitrogen bases types of molecules that form important parts of nucleic acids, composed of nitrogen-containing ring structures; hydrogen bonds between bases link the two strands of a DNA double helix.

Nucleoside a nitrogen base bound to a sugar molecule.

Nucleotide a molecule composed of a nitrogen base, a sugar, and a phosphate group; the basic building block of nucleic acids.

Nucleotide pair a pair of nucleotides (one in each strand of DNA) that are joined by hydrogen bonds.

Nucleotide-pair substitution the replacement of a specific nucleotide pair by a different pair, often mutagenic.

8-oxodGuo 8-oxo-7,8-dihydro-2′deoxyguanosine.

8-oxoGua 8-oxo-7,8-dihydroguanine.

Phosphodiester bond a bond between a sugar group and a phosphate group, such bonds form the sugar-phosphate backbone of DNA.

Purine bases nitrogen-containing ring compounds found in DNA called adenine and guanine.

Pyrimidine bases nitrogen-containing ring compounds found in DNA called thymine and cytosine.

ROS reactive oxygen species.

QSAR quantitative structure-activity relationships.

Thymidine the nucleoside having thymine as its base.

Thymine a pyrimidine base that pairs with adenine.

UA uric acid.

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