Past, Present and Future Challenges of Biosensors and Bioanalytical Tools in Analytical Chemistry: A Tribute to Professor Marco Mascini

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CHAPTER NINE

Applications of DNA-Electrochemical Biosensors in Cancer Research

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1. INTRODUCTION

Despite the unremitting efforts in prevention, early detection, and treatment, cancer remains one of the four leading threats to human health.
and development. According to the *International Agency for Research on Cancer* (IARC), in 2012 there were 14.1 million new cancer cases and 8.2 million cancer deaths worldwide, and it is estimated that there will be 21.7 million new cases of cancer each year by 2030.

Nanotechnology and biosensor technology have the potential to impact cancer treatment, detection, and prevention, through the development of biotechnological devices, for screening new therapeutic agents that interact with specific targets, detection of cancer biomarkers, and analysis of new environmental hazard compounds. Electrochemical biosensors received particular attention, due to their robustness, easy miniaturization, excellent detection limits, use of small analyte volumes, and ability to be used in turbid biofluids, which make them exceptional tools for rapid and simple on-field detection.

The DNA molecule can be seen from different viewpoints in molecular pharmacology. DNA represents the primary element of the genetic machinery, it is involved in complex interactions with different proteins and protein complexes, and it is the primary target for many drugs, including antibiotics and antineoplastic drugs. DNA, its components, and derivatives can act as drugs by themselves (e.g., azidothymidine used in AIDS and cancer treatment), and DNA is the basis for the development of DNA molecular diagnostics (e.g. polymerase chain reaction assay) for numerous diseases [1]. Due to its important chemical and biophysical characteristics, and its high specificity for recognition and binding to other molecules, nanotechnology and biosensor technology consider DNA for the construction of DNA-based sensor devices.

The DNA-electrochemical biosensor represents a good model for simulating nucleic acid interactions with cell membranes, specific DNA sequences, proteins, drugs, or harmful carcinogenic compounds [2–8], and present nowadays well-established applications in medical, environmental, and food control areas.

A DNA-electrochemical biosensor is a sensing device composed of an electrode, the electrochemical transducer, interfaced with a DNA immobilized layer on the electrode surface that acts as a biological recognition element, to detect target analytes that interact with DNA at nanoscale, inducing morphological, structural, and electrochemical changes in the DNA layer, which are further translated into an electrochemical signal, Scheme 1 [2,3,5–7,9–11]. Several electrochemical strategies are used in DNA-electrochemical biosensors applications: (i) the direct label-free detection of DNA bases’ electrochemical signals, (ii) the detection of redox reactions of
In this chapter, the advances on the DNA-electrochemical biosensors design and its applications in detection of cancer treatment drugs, protein cancer biomarkers, and carcinogens will be briefly discussed. Special attention is given to the label-free DNA-electrochemical biosensor devices that directly monitor the changes in the oxidation and/or reduction peaks of the DNA after the interaction with the analyte [2–8]. This type of DNA-electrochemical biosensor is highly sensitive, up to femtomoles of analyte, and allows the use of different electrode substrates, which makes it suitable for inexpensive miniaturization for clinical diagnosis and on-site environmental monitoring.

2. DNA-ELECTROCHEMICAL BIOSENSOR DEVELOPMENT

The development and miniaturization of DNA-electrochemical biosensors requires both (i) the correct assessment of the DNA immobilization on the electrode surface, Scheme 2A-middle and (ii) a good understanding of the electrochemical response of the immobilized DNA, Scheme 2A-right. The DNA adsorption and stability on the surface of the electrode is a complicated process and requires special attention, since it influences the DNA characteristics, the chemical compounds accessibility to the DNA, the sensor response and its performance. Several internal (DNA base sequence, length, concentration) and external parameters (the electrode
Scheme 2 DNA-electrochemical biosensor and DNA oxidative damage detection, showing DNA immobilization, and surface and voltammetric characterization. (A) Development and (B) applications.
characteristics and electrode pretreatment conditions, DNA adsorption procedure, pH, ionic strength, applied potential, ion concentration, time) directly influence the immobilized DNA structural conformation and biophysical properties.

DNA is composed of nucleotides, each containing a phosphate group, a sugar group, and a nitrogen base, the purines adenine (A) and guanine (G) and the pyrimidines thymine (T) and cytosine (C), Scheme 3A. The main structural conformation for natural DNA is the double-stranded DNA (dsDNA), Scheme 3D-left, in Watson-Crick base-pairs, Scheme 3B, the cellular DNA being almost exclusively in this form [1]. However, DNA can be found in a variety of other conformations, such as double helixes with different types of loops (bulge, internal, hairpin, junction, knotted loops, etc.), single helixes, triplex helixes, or four-stranded secondary structures, such as \textit{i-motifs} formed by DNA sequences rich in C or G-quadruplex (GQ) structures, formed by DNA sequences rich in G that occur in the telomeric regions at the ends of the chromosomes, Scheme 3C and D [1,8,12]. Many electrochemical studies were devoted to the investigation of the DNA and DNA constituents’ redox behaviour, and to understand, dependent on DNA conformation, the nature of the DNA-electrode surface interactions [4,11,13–20].

The free A and C bases can be reduced at mercury electrodes, in aqueous media at acidic pH, in a process involving the transfer of four electrons for A and three electrons for C [21,22]. The reduction behaviour of A did not change when A is incorporated into nucleosides or nucleotides [21,22]. At negative potentials, close to the electrolyte discharge, G free base is reduced to an unstable, oxidizable product [21,22]. In single-stranded DNA (ssDNA), the A and C residues are also reducible at mercury electrodes, and G residues are reduced at very negative potentials of $\sim -1.80$ V in neutral pH, the G residues reduction product, with a lower reduction potential, being more easily studied [21,22]. The T residues reduction in DNA has not been observed [21,22].

All free DNA bases present pH-dependent oxidation at carbon electrodes, displaying anodic peaks at $\sim +0.70$ V for G, +0.96 V for A, +1.16 V for T, and +1.31 V for C, in neutral pH $\sim 7.0$, Fig. 1A [23,24]. The G oxidation product is 8-oxoguanine (7,8-dihydro-8-oxoguanine or 8-oxoG) [25], and the A oxidation product is 2,8-oxoadenine (8-oxoA or 2,8-DHA) [26], which are oxidized at a lower potential, of $\sim 0.35$ V at glassy carbon electrode (GCE), Fig. 1B [25,26]. The DNA nucleosides and nucleotides oxidation at GCE occurs at potentials $\sim 0.20$ V more
Scheme 3 (A) Chemical structure of DNA nucleotides, nucleosides, and bases; (B) Watson-Crick base-pairing; (C) Homo-ODNs base-pairing and (D) Schematic representation of the DNA double strand, G-quadruplex, and i-motif configurations. Adapted from V.C. Diculescu, A.-M. Chiorcea-Paquim, A.M. Oliveira-Brett, Applications of a DNA-electrochemical
positive than the corresponding free bases [24], due to steric effects imposed by the glycosidic bond on the π-system of purine and pyrimidine rings that are now further away from the GCE surface, and more difficult to be oxidized.

The dsDNA oxidation in solution, at GCE, is pH dependent, showing two small anodic peaks, corresponding to the G residues oxidation (G₉), and A residues oxidation (Aᵣ) in the double-helix DNA, Fig. 1D [3,5,8,9,27]. The T and C residues oxidation is more difficult to detect, since it occurs with a very low current, at very high positive potentials, near to the potential of oxygen evolution. Differential pulse (DP) voltammograms of ssDNA in solution, at GCE, also show the G and A residues oxidation peaks, Fig. 1D, but the ssDNA oxidation peak currents are much higher than the currents of dsDNA, due to easier transfer of electrons from the inside of the single helix that presents the base residues in closer proximity to the electrode surface.

The advances in the electrochemical and atomic force microscopy (AFM) characterization of short- and long-chain DNA sequences have determined in detail the key factors controlling the distribution, size, and shape of highly ordered two- and three-dimensional DNA nanostructures on the electrode surface [4,11,13—20].

DNA adsorption was initially investigated on mercury [3,22,28], and later on carbon electrodes [3,5,9]. At both electrodes, dsDNA presents smaller adsorption than ssDNA, since it is less flexible, and has the hydrophobic base residues more protected inside the double helix. Electrochemical characterization of the DNA adsorption process onto electrode surfaces was combined with other analytical methods, such as ellipsometry and surface enhanced Raman spectroscopy [3], and now with AFM [4,11,13—20].

Long-chain dsDNA and ssDNA adsorb spontaneously at the surface of carbon electrodes, forming thin two-dimensional network films, whose characteristics are directly dependent on the DNA concentration and pH, Fig. 2A, B, E, and F. Applying a positive potential of ~ +0.30 V, versus AgQRE to the carbon electrode surface, which is not sufficiently high to oxidize the G and A residues on the dsDNA and ssDNA, more robust and stable DNA films are formed, Fig. 2C, D, G, and H.

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Figure 1 DP voltammograms baseline corrected at GCE, in pH 7.0 solutions: (A, ▴) 20 μM equimolar mixture of guanine (G), adenine (A), thymine (T) and cytosine (C), and (A, ▿) 20 μM uracil (U), (B) 0.5 μM 8-oxoG and 2.8-DHA, (C) 40 μg/mL poly(dG) and poly(dA), 100 μg/mL poly(dT) and poly(dC), 250 μg/mL poly(dU), and (D) 60 μg/mL (——) dsDNA and (——) ssDNA. Adapted from A.M. Oliveira-Brett, J.A.P. Piedade, L.A. Silva, V.C. Diculescu, Voltammetric determination of all DNA nucleotides, Anal. Biochem. 332 (2004) 321–329, http://dx.doi.org/10.1016/j.ab.2004.06.021; S.C.B. Oliveira, A.M. Oliveira-Brett, DNA-electrochemical biosensors: AFM surface characterisation and application to detection of in situ oxidative damage to DNA, Comb. Chem. High Throughput Screen. 13 (2010) 628–640 with permission.
Figure 2 AFM images of an HOPG electrode modified by: (1) (A–D) 60 µg/mL dsDNA (A and B) free adsorption in (A) pH 5.3 and (B) pH 7.0, (C and D) at applied potential of +0.30 V (vs. AgQRE) in (C) pH 5.3 and (D) pH 7.0; (2) (E–F) 5 µg/mL ssDNA (E and F) free adsorption in (E) pH 5.3 and (F) pH 7.0, (G and H) at applied potential of +0.30 V (vs. AgQRE) in (G) pH 5.3 and (H) pH 7.0. Adapted from A.M. Oliveira Brett, A.-M. Chiorcea, Effect of pH and applied potential on the adsorption of DNA on highly oriented pyrolytic graphite electrodes. Atomic force microscopy surface characterisation, Electrochem. Commun. 5 (2003) 178–183 with permission.
Thin dsDNA adsorbed films that form network structures with holes exposing the electrode surface define different active surface areas on the DNA-electrochemical biosensor, Fig. 3A. The uncovered electrode surface may act as an array of microelectrodes with nanometer or micrometer dimensions [29]. In a thin network film of DNA-electrochemical biosensor, the incompletely covered electrode surface will allow the diffusion and nonspecific adsorption of the analyte molecules from the bulk solution to the electrode surface, Fig. 3A-right, blue arrows. This can lead to two contributions to the electrochemical signal, one from the simple adsorbed analyte, and other from the damage caused by the analyte to the immobilized dsDNA, being difficult to distinguish between the two signals.

The DNA-electrochemical biosensors built with a thick multilayer immobilized DNA film have the advantage, due to a complete surface coverage, that the undesired nonspecific adsorption of the analyte molecules to the electrode surface is impossible, Fig. 3B [29], the sensor response will be only determined by the interaction of the compound with the immobilized dsDNA-electrochemical biosensor in pH 4.5, and schematic representation of their interaction with the analyte. Adapted from A.-M. Chiorcea, A.M. Oliveira Brett, Atomic force microscopy characterization of an electrochemical DNA-biosensor, Bioelectrochemistry 63 (2004) 229–932, http://dx.doi.org/10.1016/j.bioelechem.2003.09.029 with permission.
dsDNA on the electrode surface, without any electrochemical contribution from the analyte.

Short-chain oligodeoxyribonucleotides (ODNs) are used as recognition elements in DNA-electrochemical biosensor devices. Adaptation of immobilization techniques to miniaturization allows the manufacture of DNA microarrays with several hundred thousand sequences per square centimetre. The sensor response is particularly sensitive to ODN’s small structural variations, since base residues can easily associate in a variety of arrangements, Scheme 3C, different from the Watson–Crick base-pairs, Scheme 3B.

The adsorption morphology and the redox behaviour of the short homo-ODNs containing only one type of base, d(A)_{10}, d(C)_{10}, and d(T)_{10}, were studied using AFM at highly oriented pyrolytic graphite (HOPG) and DP voltammetry at GCE, concerning their ability to form complex secondary structures and higher-order nanostructures, dependent on the incubation time, solution pH, and ODN concentration [12]. The short homo-ODNs, d(A)_{10}, d(C)_{10}, and d(T)_{10}, are single stranded under physiological pH, and the flexible single helixes aggregate and coil, adsorbing as network films with knobby appearance. In mild acid pH solutions, d(A)_{10} double-helical conformations were observed by AFM as network films with lower surface coverage, and detected by DP voltammetry by the occurrence of the A residues oxidation peak current decrease, whereas d(C)_{10} forms i-motifs, and were observed by AFM as spherical aggregates [12].

G-rich ODNs have great medical and nanotechnological potential, because they can self-assemble into GQs, Scheme 3D-middle, and higher-order nanostructures. They are also considered important cancer-specific molecular targets for anticancer drugs, since the GQ stabilization by small organic molecules can lead to telomerase inhibition and telomere dysfunction in cancer cells.

The electrochemical behaviour of DNA sequences to self-assemble into GQ configurations has been studied [30–33]. The first report on the electrochemical oxidation of GQs concerned the investigation of two different length thrombin-binding aptamer (TBA) sequences, d(G_2T_2G_2TGTG_2T_2G_2) and d(G_3T_2G_3TGT_3T_2G_3) [30]. The different adsorption patterns and degree of surface coverage observed by AFM were correlated with the sequence base composition, presence/absence of K^{+}, and voltammetric behaviour observed by DP voltammetry. In the absence of K^{+}, in Na^{+} containing solutions, the formation of GQs is slow, and the oxidation of both TBA sequences showed one anodic peak, corresponding to the G residues oxidation in the TBA single strands. In
the presence of K\(^+\), both TBA sequences fold into GQs, and only a few single-stranded sequences are observed on the HOPG surface, since no adsorption of the stable and rigid GQs occurs. DP voltammetry showed the decrease of the G residues oxidation peak and the occurrence of a new GQ peak at a higher potential, corresponding to the G residues oxidation in the GQs. The GQ higher oxidation potential is due to the greater difficulty of electron transfer from the inside of the GQ structure to the electrode surface than the electron transfer from the G residues in the more flexible single strands.

The redox behaviour and adsorption of the \(d(G)_{10}\), \(d(TG_9)\), and \(d(TG_8T)\) ODN sequences that fold into parallel tetramolecular GQ structures, Scheme 3D-middle, were studied by AFM and DP voltammetry at carbon electrodes [32–34]. The results demonstrated that GQ formation is directly influenced by the ODN sequence and concentration, pH, and presence of monovalent cations (Na\(^+\) vs. K\(^+\)). DP voltammetry allowed the detection of the ODN single strands folding into GQs and G-based nanostructures, in freshly prepared solutions, at concentrations 10 times lower than usually detected using other techniques currently employed to study the formation of GQs. Single-stranded ODNs, in Na\(^+\) containing solutions and for short incubation times, were detected using AFM as network films and polymeric structures and using DP voltammetry by the occurrence of only the G residues oxidation peak (G\(_r\)), Fig. 4. GQ structures, in Na\(^+\) containing solutions and for long incubation times or in K\(^+\) containing solutions, were detected using AFM as spherical aggregates and using DP voltammetry by the decrease of the G residues oxidation peak and the GQ oxidation peak occurrence, increase, and shift to more positive potentials, in a time-dependent manner, Fig. 4 [12,34]. Concerning the self-assembling into higher-order nanostructures, the homo-ODN sequence \(d(G)_{10}\) was the only sequence forming G-nanowires observed using AFM, Fig. 4C, \(d(TG_9)\) forms short G-based super structures that adsorbed as rod-shaped aggregates, and \(d(TG_8T)\) forms no nanostructures, due to the presence of T residues at both 5' and 3' ends [34].

The \(d(TG_4T)\) telomeric repeat sequence, of the free-living ciliate protozoa *Tetrahymena*, forms tetramolecular GQ structures that are considered simpler models of biologically relevant human quadruplexes, being used to obtain high resolution data on drug-DNA interactions [35]. The well-known conformation of the \(d(TG_4T)\) GQs, and their extraordinary stiffness, enabled the \(d(TG_4T)\) molecules to be considered good building block candidates for the development of novel devices, with medical and nanotechnology
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Figure 4 (A and B) DP voltammograms baseline corrected in 3.0 μM d(G)₁₀, in pH 7.0: (A) Incubation time dependence — in the absence of K⁺ ions (■■■) 0, (●●●) 24, (■■) 48 h and (•) 14 days incubation, and in the presence of 1 mM K⁺ ions (●●●) 0 and (—) 24 h incubation; (B) K⁺ ions concentration dependence, 0 h incubation (●●●) in the absence and in the presence of (left, ▴) 100 μM, (left, ■■■) 5 mM, (—), 100 mM, (right, ■■■) 200 mM, (right, ●●●) 500 mM and (right, ▴) 1 M K⁺ ions; (C) AFM images of 0.3 μM d(G)₁₀, onto HOPG in the absence and in the presence of 100 mM and 200 mM K⁺ ions, for different incubation times. Adapted from A.-M. Chiorcea-Paquim, P.V. Santos, R. Eritja, A.M. Oliveira-Brett, Self-assembled G-quadruplex nanostructures: AFM and voltammetric characterization, Phys. Chem. Chem. Phys. 15 (2013) 9117—9124, http://dx.doi.org/10.1039/c3cp50866h with permission.

applications. The transformation of the d(TG₄T) sequence from single strand into GQ configuration, influenced by the Na⁺ and K⁺ ions concentration, was successfully detected using AFM on HOPG and DP voltammetry at GCE [35]. The d(TG₄T) GQs self-assembled very fast in K⁺ ions solutions, and slowly in Na⁺ ions solutions, revealing a time and a K⁺ ions concentration-dependent adsorption process and redox behaviour. The optimum K⁺ ions concentration for the formation of d(TG₄T) GQs was similar to the
healthy cells’ intracellular K\(^+\) ions concentration. The d(TG\(_4\)T) higher-order nanostructures self-assembled slowly in Na\(^+\) ion solutions and were detected by AFM as short nanowires and nanostructured films. The absence of higher-order nanostructures in K\(^+\) ion solutions showed that the rapid formation of stable GQ structures induced by the K\(^+\) ions is relevant for the good function of cells.

Long-chain polynucleotides poly(dG) and poly(G) are widely prevalent in the human and other genomes at both DNA and RNA levels and are used in DNA-electrochemical biosensor, as models to determine the preferential interaction of drugs with G-rich segments of DNA. AFM at HOPG and DP voltammetric studies at GCE, showed that the poly(G) single strands self-assemble into short GQ regions for short incubation times, while large poly(G)-GQ aggregates with low adsorption are formed after long incubation times, in the presence of monovalent Na\(^+\) or K\(^+\) ions, Fig. 5A–C [36]. DP voltammograms in freshly prepared poly(G) solutions showed only the G residues oxidation peak (G\(_r\)), Fig. 5D, due to the G residues oxidation in the poly(G) single strand. Increasing the incubation time, the G residues oxidation peak decreases and disappears, and a GQ oxidation peak in the poly(G)-GQ morphology appears, at a higher oxidation potential, dependent on the incubation time, presenting a maximum after 10 days incubation, and reaching a steady value after \(~\sim\) 17 days incubation, Fig. 5D.

The bottom-up immobilization of DNA sequences that self-assemble in either double- and single-stranded form, or in unusual but biologically relevant configurations, such as GQs and \(i\)-motifs, and large nanostructures allows different applications. Long chains dsDNA, ssDNA, and the purine homo-polynucleotide poly(A) and poly(G), as well as short-chain ODNs in single-stranded, double-stranded and GQ configurations, were successfully used for screening cancer therapeutic agents, cancer biomarkers, and hazard compounds.

In DNA-electrochemical biosensors applications, the target analyte interacts with the DNA recognition layer immobilized at the electrode surface, Scheme 2B-left, inducing morphological changes in the dsDNA helical structure, Scheme 2B-middle. The sensing strategy of label-free DNA-electrochemical biosensors consists in the electrochemical detection of the difference between the intact dsDNA, Scheme 2A-right, and the DNA damaged structure, after the interaction with the analyte, Scheme 2B-right.

When the DNA interaction with the chemical compound in the cell induce oxidation of G and A residues in the dsDNA, the formation of
8-oxoG or 2,8-DHA causes important transversion mutagenic lesions, and can be the starting point for cellular dysfunction, which in turn could lead to a state of illness [25,26]. Therefore, the DP voltammetric detection of 8-oxoG and 2,8-DHA biomarkers of DNA bases oxidative stress using DNA-electrochemical biosensor, Scheme 2B-right, allows the direct detection of DNA oxidative damage after interaction with the analyte.
3. DNA-ELECTROCHEMICAL BIOSENSOR IN EVALUATION OF CANCER TREATMENT DRUGS

A variety of new biopharmaceutical drugs have been developed in the last years, to bind and interact strongly with dsDNA, providing new therapeutic opportunities for cancer and many inherited diseases. The understanding of the mechanisms involved in the drug-dsDNA specific interaction is essential to comprehend the toxicity, as well as chemotherapeutic effects, of those compounds [37,38]. DNA-electrochemical biosensors are important tools for the detection and evaluation of pharmaceutical compounds’ specific binding to nucleic acids, due to their high sensitivity for the detection of small perturbations of DNA morphology. The DNA–electrochemical biosensor use in the analysis of drugs, medicines, and other analytes of interest in the pharmaceutical area have received increased attention.

3.1 Monoclonal antibodies

Immunotherapy is a treatment that uses the patient body’s own immune system to help fight cancer, being considered one of the most promising strategies for the treatment of specific types of cancer. Monoclonal antibodies (mAb) recognize and attach to specific proteins produced by cells. Several different mAbs are successfully used in clinical treatments, and some newer types are in clinical trials. In the light of the mAbs’ advances, the study of the interaction between mAbs and dsDNA is of utmost importance to predict its action mechanism and to understand its toxicity.

Rituximab (RTX) is a human/murine chimeric mAb that specifically targets the transmembrane protein CD20 of B-cells. The oxidation mechanism of native and denatured RTX was investigated by DP voltammetry at GCE. Native RTX presented only one oxidation peak of tyrosine and tryptophan residues, whereas in denatured RTX were detected three peaks corresponding to the oxidation of tyrosine, tryptophan, and histidine residues [39]. The dsDNA-RTX interaction was investigated by DP voltammetry in incubated samples and using a multilayer dsDNA-electrochemical biosensor, and by gel electrophoresis [40]. The voltammetric study showed a strong condensation of the DNA double-helical structure promoted by the dsDNA-RTX interaction, detected by the decrease and disappearance of the A residues oxidation peak current, the decrease of the G residues oxidation peak current, and the occurrence of free G and A bases oxidation peaks, but no DNA base oxidative damage was detected.
Bevacizumab (BEVA) is a mAb used in clinical oncology to treat several types of cancer, such as colon, lung, kidney, ovarian, and brain cancers. The electrochemical oxidation of native and denatured BEVA was investigated in solution over a wide pH range and using BEVA-thin film voltammetry on a GCE [41]. For native BEVA, only one pH-dependent oxidation peak, corresponding to tyrosine and tryptophan amino acid residues oxidation, was observed. The unfolding of BEVA-thin film on GCE surface after denaturation with chemical agents enabled the exposure of more electroactive amino acid residues to the electrode surface, and additional peaks, due to cysteine and histidine amino acid residues oxidation were observed [41]. The interaction of BEVA with dsDNA was investigated by DP voltammetry and gel electrophoresis in incubated solutions and using the dsDNA-electrochemical biosensor [42]. The voltammetric results showed the decrease/disappearance of the G and A residues oxidation peaks after incubation, Fig. 6, indicating that the BEVA–dsDNA interaction leads to the formation of a complex adduct that prevents the interaction of DNA purine bases with the electrode surface. For long incubation times the occurrence of the free G base oxidation peak was observed, but no oxidative damage DNA was electrochemically detected [42].

3.2 Nucleoside analogues

Nucleoside analogues represent a major class of chemotherapeutic agents for the treatment of cancer, especially leukaemia. Because of their structural similarity with the natural nucleosides, these analogues are taken up by cells, turned into their triphosphate species, and incorporated into DNA or RNA.

The redox behaviour of the adenosine analogues clofarabine (CLF) [43] and cladribine (CLD) [44] was investigated at GCE using cyclic, DP, and square wave (SW) voltammetry, in different pH supporting electrolytes. The CLF and CLD oxidation are irreversible and pH-dependent processes, which occur with the transfer of two protons and two electrons, following diffusion-controlled mechanisms. The in situ evaluation of the DNA interaction with CLF, Fig. 7A [43], and CLD [44], using a dsDNA-electrochemical biosensor, was investigated. The interaction with both purine nucleoside analogues caused dsDNA structural modifications in a time-dependent manner, but no DNA oxidative damage was observed. The results were confirmed using poly(G)- and poly(A)-electrochemical biosensors.

The electrochemical behaviour of the cytosine nucleoside analogue gemcitabine (GEM) was investigated and no electrochemical process was observed [45]. The interaction of GEM with DNA was investigated
Figure 6 DP voltammograms baseline corrected at GCE, in 100 µg/mL dsDNA, in pH 7.0: (▬) before and (A) after incubation with 100 µg/mL BEVA during (▼) 0 and (●●●) 48 h, and (B and C) after incubation with (●●●) 10 µg/mL and (▼) 500 µg/mL BEVA during (B) 0 and (C) 48 h. Reproduced from L.I.N. Tomé, N.V. Marques, V.C. Diculescu, A.M. Oliveira-Brett, In situ dsDNA-bevacizumab anticancer monoclonal antibody interaction electrochemical evaluation, Anal. Chim. Acta. 898 (2015) 28–33, http://dx.doi.org/10.1016/j.aca.2015.09.049 with permission.
Figure 7 DP voltammograms baseline corrected, at a dsDNA-electrochemical biosensor, in pH 4.5 (●●●) before and after incubation with: (A) 100 μM CLF, during (●) 5 and (—) 15 min, and (B) 10 μM GEM, during (●) 15 min and (—) 4 h. Adapted from H.E. Satana, A.D.R. Pontinha, V.C. Diculescu, A.M. Oliveira-Brett, Nucleoside analogue electrochemical behaviour and in situ evaluation of DNA—clofarabine interaction, Bioelectrochemistry 87 (2012) 3–8, http://dx.doi.org/10.1016/j.bioelechem.2011.07.004; R.M. Buoro, I.C. Lopes, V.C. Diculescu, S.H.P. Serrano, L. Lemos, A.M. Oliveira-Brett, In situ evaluation of gemcitabine—DNA interaction using a DNA-electrochemical biosensor, Bioelectrochemistry 99 (2014) 40–45, http://dx.doi.org/10.1016/j.bioelechem.2014.05.005 with permission.
in incubated solutions and at a DNA-electrochemical biosensor, and showed modifications of the DNA morphological structure, but no oxidative damage to DNA was observed, Fig. 7B. The interaction mechanism occurred in two consecutive steps. The initial process was independent of the DNA sequence and led to the condensation/aggregation of dsDNA. The formation of a GEM-DNA rigid structure promoted a second step, favouring the interaction between the G residues and the fluorine atoms in the GEM ribose moiety and provoking the release of G bases detected at the electrode surface.

3.3 Kinase inhibitors

Small molecules able to target and inhibit protein kinases, enzymes responsible for phosphorylation processes, have been recognized to be efficient in anticancer therapy. In vitro studies demonstrated that kinase inhibitors and/or their metabolites can increase the amount of DNA damage, and showed that they act through intercalation into DNA or formation of alkali-labile sites and/or DNA strand breaks [46]. The interaction between different kinases inhibitors and dsDNA, using label-free electrochemical biosensors, has been investigated [46–48].

Imatinib is a relatively small molecule with activity against the protein tyrosine kinase, a protein expressed by all patients with chronic myelogenous leukaemia, which is currently undergoing extensive evaluation of its activity against other types of tumour [49,50]. The interaction of DNA with imatinib in bulk solution and at a dsDNA-electrochemical biosensor was investigated [46]. The imatinib-dsDNA interaction led to modifications in the dsDNA structure, detected by changes of the G and A residues oxidation peaks, Fig. 8A, and the imatinib redox product, the electrochemical generated metabolite, caused DNA oxidative damage. Using poly(A)- and poly(G)-electrochemical biosensors, it was proved that the interaction between imatinib and DNA takes place preferentially at the A-rich segments, and an imatinib-dsDNA interaction mechanism was proposed. The electrochemical generation of imatinib oxidation product inside the DNA double helix leads to A residues oxidation and the electrochemical detection of 2,8-DHA, marker of dsDNA oxidative damage [46].

Danusertib is an Aurora kinases inhibitor, which also binds and inhibits with high affinity other tyrosine kinases, which makes danusertib an effective drug for the treatment of multiple tumours. Danusertib interaction with dsDNA was studied in solution and using a dsDNA-electrochemical
Figure 8 DP voltammograms baseline corrected, in pH 4.5, at a dsDNA-electrochemical biosensor, after incubation with: (A) 5 μM imatinib, during 2 min, (▬) without and (▬) with +0.90 V conditioning potential during 2 min, and (B) 10 μM danusertib, during 60 min, (▬) without and (▬) with +0.85 V conditioning potential during 30 min. Adapted from V.C. Diculescu, M. Vivan, A.M.O. Brett, Voltammetric behavior of anti-leukemia drug glivec. Part III: in situ DNA oxidative damage by the glivec electrochemical metabolite, Electroanalysis 18 (2006) 1963–1970, http://dx.doi.org/10.1002/elan.200603602; V.C. Diculescu, A.M. Oliveira-Brett, In situ electrochemical evaluation of dsDNA interaction with the anticancer drug danusertib nitrenium radical product using the DNA-electrochemical biosensor, Bioelectrochemistry 107 (2016) 50–57, http://dx.doi.org/10.1016/j.bioelechem.2015.10.004 with permission.
biosensor, Fig. 8B [47]. The dsDNA-danusertib interaction occurs in two sequential steps: first, the danusertib’s positively charged piperazine moiety is bound electrostatically to the dsDNA phosphate backbone; then, in a second step, a more stable danusertib–DNA complex is formed, that involves the danusertib pyrrolo–pyrazole moiety, which causes small morphological modifications in the DNA double helix, detected by DP voltammetry by changes of the G and A residues oxidation peaks and confirmed by electrophoretic and spectrophotometric measurements [47].

Controlling the applied potential to the dsDNA-electrochemical biosensor surface, the danusertib amino group oxidation enabled the in situ electrochemical generation of nitrenium cation radical, and the study of its interaction with dsDNA, Fig. 8B [47]. The decrease of G residues oxidation peak was in agreement with the covalent attachment of the danusertib nitrenium cation radical redox metabolite, to the C8 position of the G residues, protecting them and preventing their oxidation. Using homo-polynucleotides of known sequence it was confirmed that the danusertib–dsDNA interaction takes place preferentially at G-rich segments, an interaction mechanism was proposed, and the formation of danusertib redox metabolite–G adduct formation was explained [47].

Lapatinib (LPT) is a dual kinase inhibitor used for breast cancer and other solid tumours. The electrochemical oxidation mechanism of LPT was studied in detail by cyclic, DP, and SW voltammetry, in the pH range 0.30–10.00 [48]. LPT exhibits three anodic peaks, corresponding to the transfer of two electrons for each peak. The oxidation mechanism of the first anodic peak was associated with the (2-methylsulfonylethylamino)methyl oxidation to alcohol, and the second anodic peak with the 4-methoxyaniline oxidation to quinone imine. The dsDNA-LPT interaction was studied using a DNA-electrochemical biosensor, and by spectroscopic techniques [48]. Based on electrochemical and spectroscopic methods, it was confirmed that LPT intercalates into the DNA double helix.

3.4 Antimetabolites

Antimetabolites are a group of drugs that became important for cancer chemotherapy, being highly effective in targeting and inhibiting the enzymes involved in malignant cells lines. Methotrexate (MTX) is an antimetabolite of folic acid that targets the enzyme dehydrofolate reductase, which plays a supporting but essential role for the synthesis of thymine nucleotide. MTX is used in the treatment of many neoplasms, such as acute leukaemia, head and neck cancer, and micrometastases of osteosarcoma,
being also used as alternative treatment for psoriasis and rheumatoid arthritis resistant to conventional therapies. The MTX-dsDNA interaction was studied by DP voltammetry at GCE and AFM at HOPG, Fig. 9 [51], and the mechanism of interaction in incubated solutions was explained taking into consideration the correlation between the redox behaviour and morphological modifications. AFM images showed reorganization of the immobilized DNA on the surface of HOPG after incubation with MTX, and the formation of a more densely packed and thicker MTX-DNA lattice. In agreement with the AFM, the voltammetric data showed that structural dsDNA modifications occur in a time-dependent manner. The decrease of dsDNA oxidation peaks observed at short incubation times was consistent with a mechanism that involves condensation and formation of more rigid structures due to the intercalation of MTX into DNA strands. The dsDNA-electrochemical biosensor enabled in situ monitoring of both

![Figure 9](image-url)
DNA and MTX oxidation peaks, showing that the dsDNA bending process imposes kinking of the strands, which facilitates the intercalation of MTX, causing unwinding of the dsDNA structure and the purinic bases’ exposure to the GCE surface. The preferred affinity of MTX to A-rich segments, explained by the weakness of the H-bonds between A—T, was confirmed using single-stranded poly(A)- and poly(G)-electrochemical biosensors [51].

3.5 G-quadruplex ligands

Another class of anticancer drugs is represented by small ligand molecules that are specifically designed to bind to G-rich DNA sequences, at the ends of chromosomes. Their ability to induce and stabilize the DNA folding into four-stranded GQ configurations at the level of telomeres prevent the telomeric DNA from unwinding and opening to telomerase, thus indirectly targeting the telomerase and inhibiting its catalytic activity, which further leads to the senescence and apoptosis of tumour cells. Remarkable progress has been made in the development of selective GQ ligands; some entered in clinical trials for cancer therapy, presenting significant telomerase inhibition or suppression of the transcription activity of oncogenes. The trisubstituted acridine compound BRACO-19 has been developed as a ligand for stabilizing GQ structures that produce short- and long-term growth arrest in cancer cell lines. However, BRACO-19 revealed to be relatively non GQ-selective, having also significant binding affinity for duplex DNA. Therefore, a series of new triazole-linked acridine ligands, for example, GL15 and GL7, with enhanced selectivity for human telomeric GQ binding versus duplex DNA binding has been designed, synthesized, and evaluated. BRACO-19, GL15, and GL7 present a complex, pH-dependent, and adsorption-controlled irreversible oxidation mechanism, at a GCE [52,53]. The interaction between DNA and the acridine ligands GL15 and GL7 was investigated in incubated solutions and using dsDNA-, poly(G)-, and poly(A)-electrochemical biosensors [53]. Both GL15 and GL7 interact with dsDNA in a time-dependent manner, causing condensation of the dsDNA morphological structure, with preferential affinity for the G-rich segments, but do not cause DNA oxidative damage.

The interactions of the GQ-targeting triazole-linked acridine ligand GL15 with the short-chain length *Tetrahymena* telomeric DNA repeat sequence d(TG₄T) and with the long poly(G) sequence have been reported at the single-molecule level [54]. In the presence of GL15, GQ formation was detected by AFM via the adsorption of GL15-d(TG₄T)-GQ and GL15-poly(G)-GQ small spherical aggregates, and large GL15-poly(G)-GQ assemblies, and by
DP voltammetry via GL15 and Gᵣ oxidation peak current decrease and disappearance, and the occurrence of a GQ oxidation peak, Fig. 10. The AFM and voltammetric results showed that the GL15 molecule interacts with both sequences in a time-dependent manner. An excellent correlation was observed between the d(TG₄T) and poly(G) structural changes and redox behaviour, before and after interaction with GL15, and was directly influenced by the presence of monovalent Na⁺ or K⁺ ions in solution. These results are consistent with the interaction of triazole-linked acridine derivatives with terminal G–quartets in an individual GQ, Fig. 10A–right. The

(A) In the presence of Na⁺ ions

(B) In the presence of K⁺ ions

Figure 10  DP voltammograms baseline corrected at GCE, in solutions of 3.0 μM d(TG₄T) incubated with 4.0 μM GL15, in pH 7.0, for a range of incubation times, in the presence: (A) Na⁺ and (B) K⁺ ions. Adapted from A.-M. Chiorcea-Paquim, A.D.R. Pontinha, R. Eritja, G. Lucarelli, S. Sparapani, S. Neidle, et al., Atomic force microscopy and voltammetric investigation of quadruplex formation between a triazole-acridine conjugate and guanine-containing repeat DNA sequences, Anal. Chem. 87 (2015) 6141–6149. http://dx.doi.org/10.1021/acs.analchem.5b00743 with permission.
binding of GL15 to d(TG₄T) and poly(G) both strongly stabilized the GQs and accelerated GQ formation, in both Na⁺ and K⁺ ions solutions, although only the K⁺-containing solution promoted the formation of perfectly aligned tetramolecular GQs [54].

### 3.6 Antibiotics

Anthracycline antibiotics are a class of anticancer drugs, originally derived from *Streptomyces* bacterium, which are active against a wide variety of solid tumours and haematological malignancies [55]. However, their use is considerably limited due to tumour resistance and their toxicity to healthy tissue, especially cardiotoxicity [56]. Daunorubicin was the first anthracycline compound to be characterized. Doxorubicin, also known by the trade name Adriamycin, is a hydroxyl derivative of daunorubicin, being one of the most widely used chemotherapeutic agents, generally prescribed in combination with other drugs. It is approved for a long list of cancer types, being one of the most effective drugs for solid tumour treatment (breast cancer, small cell lung and ovarian cancer) [56–58]. Doxorubicin intercalation and in situ interaction with dsDNA were investigated using a DNA-electrochemical biosensor [56,57]. Interaction of doxorubicin with DNA is dependent on the applied potential. The reduction of DNA intercalated doxorubicin leads to the formation of a doxorubicin radical that oxidatively damages DNA and the formation of biomarker 8-oxoG. A mechanism for doxorubicin reduction and oxidation in situ, when intercalated into the double helix, was presented and the formation of the mutagenic 8-oxoG explained [56,57].

Idarubicin (IDA) is a synthetic analogue of daunorubicin, presenting an improved activity for different types of leukaemia. IDA oxidation showed an irreversible, pH-dependent process that occurs with the transfer of one electron and one proton, by cyclic and DP voltammetry, at GCE [59]. The mechanism of interaction of IDA with dsDNA was determined in incubated solutions and using multilayer dsDNA and poly(A) and poly(G)-electrochemical biosensors. Intercalation of IDA in the DNA double helix was detected by the decrease of the G and A residues oxidation peak currents with increasing incubation time. However, no oxidation peaks of the purine base oxidation products, 8-oxoG and 2,8-DHA, were observed.

### 3.7 Thalidomide

Thalidomide (TD) is an immunomodulatory, antineoplastic, and leprostatic agent. Initially used as a sedative and an anti-emetic drug to combat morning
sickness, it was banned from clinical use in the 1960s due to its severe teratogenic effects. TD came back to be used initially to treat erythema nodosum leprosum, and then various cancers, particularly multiple myeloma. Voltammetric, AFM, UV-vis and electrophoresis studies showed that TD interacts specifically with the dsDNA by intercalation, inducing dsDNA structural changes in a time-dependent manner [60]. TD has affinity for both G and A residues, and the interaction between TD and dsDNA induced major changes in the oxidation peaks of both TD, and G and A residues, directly dependent on the dsDNA and TD concentrations and incubation time. Oxidative damage caused to DNA by TD was detected electrochemically by the occurrence of the 8-oxoGua and/or 2,8-DHA oxidation peak. Multiple time- and concentration-dependent effects on the dsDNA structure are induced by TD. Initially, the aggregation and disappearance of the G and A residues oxidation peaks is related to the TD induced DNA condensation. Afterwards, the formation of thinner TD-DNA films, and the increase of the G and A residues oxidation peaks, with increasing the incubation time, is related to the TD intercalation into the DNA, followed by the double helix unwinding.

### 3.8 Metal complexes

The discovery of the Pt(II) complex cisplatin was considered a significant achievement in cancer therapy [61], cisplatin being widely used to treat a variety of tumours including ovarian, cervical, head and neck, and testicular cancers. However, its clinical use decreased over time, due to its toxicity and acquired resistance, which triggered the interest in the development of more efficient Pt(II)- and other metal-containing complexes [61].

As alternatives to first-generation agents such as cisplatin, the development of new chemotherapeutic agents led to the synthesis of polynuclear metal complexes, a new class of third-generation anticancer agents with specific chemical and biological properties. In particular, Pd(II) complexes aimed to achieve lower toxicity, enhanced solubility, and tumour selectivity, when compared with the Pt(II) ones. The interaction with dsDNA of two polynuclear Pd(II) chelates with the biogenic polyamines spermidine (Spd) and spermine (Spm) was studied at room temperature, using AFM, DP voltammetry, and gel electrophoresis [62]. After the interaction with Pd(II)-Spd and Pd(II)-Spm, Fig. 11, a reorganization of the DNA self-assembled network on the surface of the HOPG and a decrease in G and A residues oxidation peaks at GCE were observed, consistent with the model mechanism of interaction of Pd(II) and Pt(II) complexes with dsDNA.
that leads to the formation of Pd(II)-DNA adducts and/or aggregates. The Pd(II)-Spd complex presents a stronger interaction with dsDNA, owing to its molecular structure comprising three Pd(II) centres and two Spd molecules, with six possible coordination sites to dsDNA, as compared to Pd(II)-Spm that contains only two Pd(II) ions and one Spm ligand, yielding only four possible coordination sites. Furthermore, the voltammetric results showed that the interaction with either of the Pd(II) polyamine complexes caused no oxidative damage to dsDNA. Due to their polycationic chains, the polyamines Spd and Spm interact specifically with the negatively charged phosphate groups of the DNA double helix by electrostatic forces, thus stabilizing its structure. The voltammetric results showed that the interaction is observed even at polyamines’ low concentration, but causing no oxidative damage to DNA [62].

![Figure 11](image_url)  
**Figure 11** (A and B) AFM images at HOPG modified by: (A) 10 μg/mL dsDNA, and (B) 10 mg/mL dsDNA incubated with 50 mM Pd(II)-Spm, during 24 h (C) DP voltammograms baseline corrected at GCE modified by: (●) 10 μg/mL dsDNA and 10 μg/mL dsDNA incubated with 100 μM Pd(II)-Spm, in pH 4.5, during (■■■) 10 and (●●●) 30 min. Adapted from O. Corduneanu, A.-M. Chiorcea-Paquim, V. Diculescu, S.M. Fiuza, M.P.M. Marques, A.M. Oliveira-Brett, DNA interaction with palladium chelates of biogenic polyamines using atomic force microscopy and voltammetric characterization, Anal. Chem. 82 (2010) 1245–1252, http://dx.doi.org/10.1021/ac902127d with permission.
The lipoic acid-palladium (LAPd) complex was originally designed as a non-toxic chemotherapeutic agent, in a prescription version called DNA Reductase, and consists of a palladium bonded to both end-groups of a lipoic acid (LA). The interaction of dsDNA with LAPd and Poly-MVA, a dietary supplement containing LAPd polymer that exists as trimers of LAPd complex joined to thiamine was studied at room temperature, using AFM and DP voltammetry [63]. The DP voltammetric results demonstrated the interaction of both LAPd and Poly-MVA with dsDNA, but no oxidative damage caused to dsDNA was detected. The interaction of dsDNA with low concentrations of LAPd complex leads to less knotted and bended, and more extended, dsDNA molecules, when compared with control dsDNA adsorbed on the HOPG surface. The LAPd molecules interact and adsorb strongly on HOPG, in comparison with LA, probably due to the incorporation of palladium into the ligand structure. The stability of both the LAPd complex and the LAPd-containing Poly-MVA solution, after applying high negative or high positive potentials, was tested. The DP voltammetry and AFM showed that, while in the case of LAPd complex palladium removal is still possible, in the Poly-MVA solution the same was not achieved [63].

3.9 Natural compounds

Naphthoquinones are secondary metabolites of some fungi, plants, and bacteria, with antibiotic, antiviral, antifungal, antiparasitic, anti-inflammatory, antiproliferative, and cytotoxic activity. Biflorin is an ortho-naphthoquinone isolated from Capraria biflora, a perennial shrub distributed in countries of tropical America, that is used as cytotoxic and antioxidant agent. Biflorin showed a cytotoxic activity against different tumour cell lines, demonstrating a good antitumour therapeutic potential, especially for skin, breast, and colon cancer cells. The pharmacokinetics of biflorin was evaluated by electrochemistry and spectrophotometry [64], showing that biflorin interacts with dsDNA by intercalation, and also with ssDNA, which may lead to DNA damage. The effects of biflorin–dsDNA interaction were addressed through a molecular cytogenetic approach, using the comet assay and the chromosome aberration induction evaluation. Biflorin, compared to the negative control, presented approximately four- and six-fold increase in DNA damage. Also, like other naphthoquinones such as β-lapachone and lapachol, biflorin presents antitumour and cytotoxic activities, but lacks mutagenic potential. The absence of either clastogenic or aneuploidogenic activity of the compound reinforced its safety.
Naphthoquinone-based complexes with metal ions may present higher cytotoxic properties in comparison with naphthoquinone itself or with metal ions. Lawsone is a derivate of 1,4-napthoquinone with significant cytotoxic properties, demonstrated on different cell lines, due to its ability to induce production of reactive oxygen species (ROS). Therefore, ternary lawsone-copper(II) complexes are a very interesting group of compounds in cancer research [65]. The voltammetric behaviour of seven ternary copper(II) complexes of lawsone with additional O-donor (water) and N-donor ligands (pyridine, 2-, 3-, and 4-aminopyridine, 3-hydroxypyridine, and 3,5-dimethylpyrazole) was studied by cyclic and DP voltammetry [65]. Their interaction with DNA was investigated using a DNA-electrochemical biosensor prepared on carbon paste electrodes. All lawsone complexes showed ability to interact with dsDNA. The most simple complex is \( \text{Cu(lawsone)}_2(\text{H}_2\text{O})_2 \cdot 0.5\text{H}_2\text{O} \), with significant prooxidant properties, which may contribute to its cytotoxicity.

Flavonoids are naturally occurring polyphenolic compounds with antioxidant activity that, depending on the concentration, can also act as prooxidants, to produce free radicals and cause DNA damage and mutagenesis. The prooxidant activity is generally catalyzed by metals, such as Fe and Cu, present in biological systems. Knowledge about the malignant tissue-specific anticancer effects of flavonoids can be applied in chemoprevention, as well as in cancer treatment [66].

Quercetin exhibits several anticancer properties, such as cell signalling, pro-apoptotic, antiproliferative, and antioxidant effects, and growth suppression. On the other hand, synergistic effects of chemotherapeutic agents when combined with quercetin were described [67], quercetin acting both as chemosensitization and chemoprotective agent in anticancer treatment. Quercetin interaction with dsDNA was investigated electrochemically using two types of DNA-electrochemical biosensors at GCE in order to evaluate the occurrence of DNA damage caused by oxidized quercetin [68]. The results showed that quercetin binds to dsDNA where it can undergo oxidation; the radicals formed during quercetin oxidation cause breaks of the hydrogen bonds in the dsDNA finally giving rise to 8-oxoG, biomarker of DNA oxidative damage. A mechanism for oxidized quercetin-induced damage to dsDNA immobilized onto a GCE surface was proposed. In bulk solution, a very weak interaction between quercetin and DNA was found to take place [69]. In addition, extensive quercetin-induced DNA damage via reaction with Cu(II) has been reported. An electrochemical study of the DNA–Cu(II)–quercetin system in solution showed a time-dependent
damage on the dsDNA structure, recognized via the G and A residues oxidation peaks increase, and confirmed spectrophotometrically via the 260 nm adsorption band increase [69].

The antioxidant and prooxidant properties of the semi-synthetic flavonolignan 7-O-galloylsilybin (7-GSB) have been studied [70]. The presence of a galloyl moiety enhances the antioxidant capacity of 7-GSB, when compared to that of silybin (SB). These results were supported by electrochemistry, DPPH $\cdot$ (2,2-diphenyl-1-picrylhydrazyl radical) scavenging activity, total antioxidant capacity (CL-TAC), and DFT (density functional theory) calculations. A three-step oxidation mechanism of 7-GSB was proposed at pH 7.4 and confirmed by molecular orbital analysis. The complex formed by 7-GSB with Cu(II) was also studied and the prooxidant effects of the metal complexes were tested according to their capacity to induce DNA oxidative modification and cleavage. The results showed that 7-O-galloyl substitution to SB concomitantly enhances the antioxidant capacity and decreases the prooxidant effect/DNA damage after copper complexation.

### 3.10 Free radicals

ROS species, such as superoxide radical, hydrogen peroxide, singlet oxygen, and the highly reactive hydroxyl radical, are very reactive radicals, ions, or molecules that have a single unpaired electron in their outermost shell of electrons [71]. ROS species are known to be cytotoxic and have been associated with the occurrence of several diseases, including cancer, heart disease, and aging [72]. Most ROS are generated as by-products during mitochondrial electron transport; also, various carcinogens may act by generating ROS species during their metabolism. Oxidative damage to DNA by ROS has been established as cause of cancer since the 80’s [72,73], and the majority of mutations involve modification of G residues, causing G $\rightarrow$ T transversions, and accumulation of 8-oxoG [72]. Compared with normal cells, cancer cells present higher levels of endogenous oxidative stress in culture and in vivo, resulting in very high levels of ROS. Therefore, studies suggested that these excessive levels of ROS in cancer cells can be exploited for selectively eliminating the tumour cells by further pharmacological ROS attacks [74].

Oxygen radicals can be electrochemically generated in situ, from redox reactions, at the electrode surface. Immobilizing DNA at the electrode surface allows the potential control of the oxygen radicals’ formation (the DNA-damaging species) and subsequent electrochemical detection of the
damage they cause to the surface-attached DNA. The DNA damage causing strand brakes formation was detected using an electrochemical biosensor, based on a covalently closed supercoiled DNA-modified mercury electrode [75]. The hydroxyl radicals generated in electrochemically controlled iron/EDTA-mediated Fenton reaction, as well as radical intermediates of oxygen electroreduction, cleaved the DNA anchored at the mercury electrode surface. The extent of DNA damage increased with the shift of the electrode potential to negative values, displaying a sharp inflection point matching the potential of $[\text{Fe(EDTA)}]^{2-}/[\text{Fe(EDTA)}]^{1-}$ redox pair [75].

A DNA-electrochemical biosensor, based on native DNA adsorbed on hybrid coating consisting of nanoparticles of silver decorated with a macrocyclic receptor with catechol groups and covalently attached to a neutral red conductive polymer, was used for the estimation of DNA damage, in a model system based on the Fenton reagent [76]. Oxidative damage of DNA caused by the Fenton reagent resulted in synchronous increase of the electron transfer resistance and capacitance measured by EIS. The DNA-electrochemical biosensor was tested for the evaluation of antioxidant capacity of green tea infusions [76].

The hydroxyl radicals electrochemically generated in situ on a boron-doped diamond electrode (BDDE) have been investigated, in different electrolyte media, over a wide pH range. An oxidation peak at high positive potentials, corresponding to the transfer of one electron and one proton in pH < 9 electrolytes, associated with the electrochemical generation of hydroxyl radicals from the water discharge process, was observed [77]. The effect of BDDE surface termination (causing oxygen or hydrogen terminations), immediately after cathodic or anodic electrochemical pretreatment, and the influence of the pretreatment on the electrochemical oxidation of dsDNA, poly(G) and poly(A) homopolynucleotides, GMP and AMP nucleotides, DNA bases, and biomarker 8-oxoG, were investigated [78,79]. The interaction and adsorption of DNA and its components on the BDDE surface pretreated cathodically was facilitated due to the BDDE higher conductivity. On the other hand, after anodic pretreatment a wider potential window of BDDE was obtained enabling the detection of the pyrimidine bases. The hydroxyl radicals produced on BDDE surface at high positive potentials were highly reactive, and consequently the BDDE surface was not completely inert.

The dsDNA-BDDE-electrochemical biosensor, prepared by immobilization of dsDNA on an oxidized BDDE surface, was successfully used to detect the dsDNA oxidative damage caused by in situ electrogenerated
hydroxyl radicals \[80\]. Controlling the time of BDDE applied potential, different hydroxyl radical concentrations were electrochemically generated in situ, enabling the hydroxyl radicals pre-concentration onto the thick multilayer immobilized dsDNA. Monitoring the G and A residues oxidation peak currents modification, Fig. 12, it was concluded that the hydroxyl radicals oxidatively damage the immobilized dsDNA, leading to the dsDNA structure modifications, demonstrated by the occurrence of the 8-oxoG oxidation peak, a biomarker of DNA oxidative damage, and confirmed by electrophoresis\[80\].

3.11 Ionizing radiation

The application of ionizing radiation in cancer treatment was recognized soon after the discovery of X-rays and has been used for several decades in curative and palliative treatments of cancerous solid tumours \[81,82\]. Ionizing radiation can change the structure of biomolecules, creating potentially harmful effects. Although radiation has the potential to damage a multitude of biomolecules, inside a cell, the structure of most concern is DNA, to which different types of damage can be induced: single- and
double-strand breaks, modifications of the base residues, destruction of the sugar, cross-links, and formation of dimers.

In particular, γ-radiation has been used in radiotherapy for several decades, and it is known that exposure gives rise to genomic instability leading to mutagenesis, carcinogenesis, and cell death. The γ-radiation can be absorbed directly by DNA, leading to ionization of both the bases and sugar, in a mechanism described as the direct effect of generating single and tandem DNA damage.

However, approximately 65% of the DNA damage is not caused directly by radiation, but is an indirect effect of free radicals, such as hydroxyl radicals, that are formed from the radiolysis of surrounding water molecules that attack DNA [82]. The characterization of different kinds of DNA damage caused by γ-radiation exposure was studied by DP voltammetry at GCE, using dsDNA, ssDNA, poly(dG), poly(dA), poly(dT), and poly(dC) aqueous solutions previously exposed to γ-radiation doses between 2 and 35 Gy [82]. The generation of 8-oxoG, 2,8-DHA, 5-formyluracil, base-free sites, and single- and double-stranded breaks, in the γ-irradiated DNA samples, was detected electrochemically, the damage depending on the γ-irradiation time. It was found that the peak currents for 8-oxoG increase linearly with the γ-radiation dose applied to the different nucleic acid samples, and values between 8 and 446 8-oxoG molecules per 10^6 G molecules per applied Gy were obtained. The electrochemical observations were confirmed by the electrophoretic migration profile, obtained for the same ssDNA and dsDNA γ-irradiated samples, by non-denaturing agarose gel electrophoresis [82].

4. DNA-ELECTROCHEMICAL BIOSENSOR IN DETECTION OF CANCER BIOMARKER PROTEINS

The NCI defines a biomarker as a biological molecule found in blood, body fluids (e.g., stool, urine, sputum, or nipple discharge), or tissues, whose presence represents a sign of an abnormal process, condition or disease [83]. Cancer biomarkers include proteins, nucleic acids, antibodies, peptides, etc., and can be used to non-invasively differentiate a healthy person from a patient with a specific cancer disease [84]. Among cancer biomarkers, proteins are particularly important, since more than 1261 proteins are believed to be differentially expressed in human cancer [85]. Cancer biomarker protein candidates include proteins involved in a variety of processes, such as oncogenesis, angiogenesis, development, differentiation, proliferation,
apoptosis, hematopoiesis, immune and hormonal responses, cell signalling, nucleotide function, hydrolysis, cellular homing, cell cycle and structure, the acute phase response, and hormonal control [85].

The identification and quantification of proteins is essential in many medical and biomedical applications related to cancer early detection and treatment. Protein detection has been investigated by different techniques, such as AFM, molecular affinity scanning force microscopy, ellipsometry, fluorescence spectroscopy, acoustic plate mode sensors, surface plasmon resonance, etc.

DNA-electrochemical biosensors have started to be used in the direct detection of different proteins–DNA interaction. DNA-electrochemical biosensors, designed to specifically detect emerging cancer biomarker proteins, can open the perspective of many potential applications in oncology, including risk assessment, differential diagnosis, determination of prognosis, prediction of response to treatment, and monitoring of disease progression.

Cancer risk can be determined by various genetic, metabolic, and lifestyle factors that alter the metabolism. Cytochrome P450 1A2 (CYP1A2) is responsible for the metabolism of oestrogens, and many exogenous compounds, including caffeine, and its activity can be correlated with hormones, blood lipids, and lifestyle factors associated with breast cancer [86]. The direct, label-free detection of the human cytochrome CYP1A2 interaction with dsDNA was investigated by DP voltammetry, in incubated solutions and at the surface of a dsDNA-electrochemical biosensor, and by UV-Vis spectrophotometry. CYP1A2 interacted with dsDNA causing its condensation and DNA oxidative damage, Fig. 13. A preferential interaction between the dsDNA G residues and CYP1A2 was found, as free G and 8-oxoG oxidation peaks were detected. This was confirmed using poly(G)- and poly(A)-electrochemical biosensors [87].

Pur proteins are sequence-specific single-stranded nucleic acid-binding proteins, which in humans are critical for myeloid cell, muscle and brain development. Purα and Purβ members have been implicated in several types of diseases, as diverse as cancer (e.g., acute myelogenous leukaemia), premature aging, and fragile-X mental retardation syndrome [88].

The interaction of the protein Purβ of mouse (mPurβ) with ssDNA, and of Escherichia coli protein MutH with dsDNA, was studied at a DNA-electrochemical biosensor [89]. Impedance spectroscopy and DP voltammetry distinguished their interaction specificity and recognition, the mPurβ for single strands and the MutH for double strands. DP voltammograms recorded for the DNA before and after the interaction with proteins,
confirmed the DNA residues oxidation peak modifications after binding to the proteins. The DNA-electrochemical response depended on the proteins concentration, and the DNA-proteins complexes’ formation constants were estimated based on the DP voltammetric results [89].

Aptamers are small single-stranded nucleic acids molecules that present specific three-dimensional structure, synthesized by chemical or enzymatic procedures, to specifically bind to target molecules and inhibit their biological functions [90,91]. Due to their high affinity for a series of proteins, they are largely used in biosensor development applied to protein quantification assays. Aptamers can fold into different three-dimensional configurations, such as GQ, stem-and-loop, hairpin, and three-stem helix junctions [92].

Particular attention received the short aptamers that adopt GQ configurations, which can bind to a wide variety of molecular targets, mainly proteins (such as thrombin, nucleolin, signal transducer and activator of transcription STAT3, human RNase H1, protein tyrosine phosphatase Shp2, VEGF, HIV-1 integrase, HIV-1 reverse transcriptase, HIV-1 reverse transcriptase, HIV-1 nucleocapsid protein, M. tuberculosis polyphosphate kinase 2, sclerostin, insulin, etc.), but also to some other targets (hematoporphyrin IX, hemin, ochratoxin, potassium ions, ATP) [93–96].

Figure 13 DP voltammograms baseline corrected, in pH 7.0: (—) control dsDNA-electrochemical biosensor, and (▬) dsDNA-electrochemical biosensor incubated with 0.5 mg/mL CYP1A2, during 10 min, 1, 3, and 5 h. Adapted from I.C. Lopes, A.M. Oliveira-Bret, Human cytochrome P450 (CYP1A2)-dsDNA interaction in situ evaluation using a dsDNA-electrochemical biosensor, Electroanalysis (2017) (in press) with permission.
Therefore, the development of electrochemical biosensors using aptamers as recognition units in cancer diagnosis early stages become a promising strategy [97].

Thrombin is a serine protease and a coagulation protein in the bloodstream that controls the coagulation mechanism. Cancer patients are at increased risk of deep vein thrombosis and pulmonary embolism, therefore the need for monitoring the thrombin generation during cancer treatments [98]. In addition, thrombin induces invasion and metastasis in various cancers, although the mechanisms by which it promotes tumorigenesis are not well understood [99]. For this reason, the detection of thrombin using TBAs has been extensively studied. In fact, the TBA-thrombin model has been used for the development of electrochemical aptasensors with different architectures [33,100].

A systematic study of the interaction between the serine protease thrombin and two different TBA sequences, d(G2T2G2TGTG2T2G2) and d(G3T2G3TGT3T2G3), was carried out on two different types of carbon electrode, GCE using DP voltammetry and HOPG using AFM [31,34]. The thrombin interaction with the TBA’s primary and secondary structures, were detected through changes in G and A residues oxidation peaks in the TBA sequences. The complex between thrombin and single-stranded TBA sequences involved coiling of the single strands around thrombin, leading to the formation of a robust TBA-thrombin complex that maintains the symmetry and conformation of thrombin. The thrombin oxidation peaks occurred at more positive potentials after single-stranded TBA-thrombin complex formation. In the presence of K⁺ ions, the aptamers folded into GQ structures that facilitate the interaction with thrombin. The TBA-thrombin complex adsorbs with the aptamer GQ always in preferential contact with the electrode surface and the thrombin molecules on top of the aptamer GQ structure.

5. DNA-ELECTROCHEMICAL BIOSENSOR IN RISK ASSESSMENT OF HAZARDOUS COMPOUNDS

Identification of unknown carcinogenic compounds and screening the presence of these hazardous substances in the environment are crucial strategies for cancer risk assessment and prevention.

Carcinogens are hazardous compounds that increase the risk of cancer, by increasing the opportunities for DNA changes to occur. NCI’s list of possible cancer-causing substances include aflatoxins, aristolochic acids, arsenic, asbestos, aspartame, benzene, cadmium, ethylene oxide, formaldehyde,
hexavalent chromium compounds, nickel compounds, strong inorganic acid mists containing sulphuric acid, radon, vinyl chloride, and several compounds found in cosmetics, antiperspirants, or hair days beauty salon treatments [101]. However, new harmful compounds are evaluated and identified every day. Furthermore, the toxicity of many well-known carcinogens (e.g., the previous described metal complexes, naphthoquinones, cyanotoxins, free radicals, and ionizing radiation) is now exploited as anticancer drugs, for selectively eliminating the tumour cells. In this context, DNA-electrochemical biosensors can be effectively used from a cancer prevention perspective, for rapid preliminary screening of chemical mutagens and carcinogens in environmental and food samples.

5.1 Metal ions

Epidemiological studies have shown that occupational and environmental exposure to specific metals is associated with an increased risk of different cancers and adverse health effects. Transition and post-transition metal ions can interact specifically with DNA inducing changes of the DNA double helix. In particular, arsenic and arsenic compounds, beryllium and beryllium compounds, cadmium and cadmium compounds, hexavalent chromium compounds, lead, cobalt, and nickel compounds are proven carcinogens in laboratory animals [102].

DP voltammetry was used to study the in situ dsDNA interaction mechanism with different chromium species, Cr(III), Cr(IV), Cr(V), and Cr(VI) [103]. The Cr(III) reactive intermediates oxidation by the O₂ dissolved in solution cause DNA oxidative damage, and the Cr(VI) reactive intermediates oxidation leads to conformational modifications of the dsDNA double helix. The Cr(IV) and Cr(V) reactive intermediates are produced, in situ, in Cr(III) oxidation by O₂ dissolved in solution, and, in vivo, in Cr(VI) reduction by ascorbate and glutathione. The DNA-electrochemical biosensor enabled the separate investigation of the interaction and toxicity of each chromium species with DNA and a better understanding of the molecular mechanisms involved in chromium species-induced neoplasia. Using poly-nucleotides of known sequences, it was confirmed that chromium reactive intermediates preferentially interact with dsDNA at G-rich segments, leading to oxidative damage and formation of 8-oxoG [103].

DNA damage by Cr(V) and/or Cr(IV) intermediates of Cr(VI) electrochemical reduction was also evaluated using a supercoiled DNA-modified mercury electrode [104]. DNA at the mercury electrode surface is cleaved in the presence of the chromium species in an electrode
potential-dependent manner. The AC voltammetric signal sensitive to the formation of DNA strand breaks increased after incubation of the DNA-modified electrode in solutions of Cr(VI), at potentials sufficiently negative for the Cr(VI) reduction. These processes lead to DNA damage, and may involve reactive intermediates of Cr(VI) reduction and/or ROS [104].

Heavy metal ions, lead, cadmium and nickel, are well-known carcinogens with different natural origins [105,106]. A dsDNA-electrochemical biosensor was used for the in situ evaluation of Pb(II), Cd(II), and Ni(II) interaction with dsDNA [105], showing that the interaction leads to different modifications in the dsDNA structure, electrochemically recognized as changes in the G and A residues oxidation peaks. Using poly(G)- and poly(A)-electrochemical biosensors, it has been confirmed that the interaction between Pb(II) and DNA causes oxidative damage and takes place preferentially at A-rich segments, with the formation of 2,8-DHA, the A residues oxidation product, biomarker of DNA oxidative damage. The Pb(II) bound to dsDNA can still undergo oxidation. The interaction of Cd(II) and Ni(II) causes conformational changes, destabilizing the double helix, which can facilitate the action of other oxidative agents on DNA [105].

AFM and DP voltammetry at spontaneously adsorbed DNA–metal complexes film also showed that Pb(II), Ni(II), Cd(II), and Pd(II) interact specifically with the dsDNA, due to a high affinity to form covalent bonds with nitrogenous bases, inducing conformational modifications in the B-DNA structure [106]. An increase of the electrode surface coverage by the Pb–DNA, Cd–DNA and Ni–DNA complexes, when compared with control dsDNA, was observed. The local denaturation of the double helix, due to the metal–DNA interaction, facilitates the hydrophobic interactions between the DNA bases and the hydrophobic carbon electrode surface. The Pd(II) cations induced the greatest morphological changes in the DNA adsorption pattern, leading to the formation of Pd–DNA complex thicker aggregates, caused by DNA condensation. The compact Pd–DNA structures observed by AFM and the DP voltammetry suggest that the Pd(II)–DNA interaction is very strong and stable [106].

5.2 Pollutants

Pollutants are chemical substances present in greater concentration than normal, and introduced in the environment (air, water, soil, and biota), as a result of agricultural and industrial activities, and presenting detrimental effect on human health [107]. Many pollutants have been found to cause
cancer in animal species or humans. DNA-electrochemical biosensors can be used to further predict and understand the pollutant effects on organisms, as well as to develop analytical methodologies for their sensitive detection.

Acrylamide (AA) is an \(\alpha,\beta\)-unsaturated carbonyl compound, used as a building block in making polyacrylamide and acrylamide copolymers, widely used in industrial processes, such as the production of paper, dyes, and plastics, and in the treatment of drinking water and wastewater. Acrylamide can also be found in certain foods when heated above 120°C. IARC considers acrylamide to be a probable human carcinogen, based on its neurotoxicity, genotoxicity, and reproductive toxicity found in rodent models [108]. A DNA-electrochemical biosensor based on graphene-ionic liquid-Nafion/(horseradish peroxidase/DNA)\(_3\) modified pyrolytic graphite electrode was prepared by the layer-by-layer method, which provided an effective strategy for mimicking and detecting DNA damage induced by AA in vivo [108]. The results indicated that under the coexistence of horseradish peroxidase (HRP), \(\text{H}_2\text{O}_2\), and AA, HRP was activated by \(\text{H}_2\text{O}_2\) and catalyzed the transformation of AA into glycidamide. The formation of DNA adducts with AA and glycidamide disturbed the double helix of DNA, which exposed the G residues inside the dsDNA. Based on the changes of the G residues oxidation peak, DNA damage was directly detected. In addition, glycidamide induced more serious DNA damage than AA, which provided further evidence for the mainly carcinogenic activity of glycidamide.

Hydroquinone is a chemical compound used for decades as a skin lightening agent, banned initially from cosmetics due to mid-term effects such as leucoderma/vitiligo or exogenous ochronosis. However, more studies suggest that carcinogenesis may be expected as well, due to the hydroquinone metabolites formed in the liver, such as hydroquinone’s conjugates \(p\)-benzoquinone and glutathione [109]. The electrochemical behaviour of hydroquinone and its DNA-damaging mechanisms were investigated using a DNA-electrochemical biosensor constructed with chitosan (CTS) and polyaniline (PANI) [110]. The results showed that the redox peak current was remarkably increased after GCE modification by PANI/CTS. The electrochemical oxidation of hydroquinone on the dsDNA/PANI/CTS/GCE electrode was an adsorption-controlled irreversible and a two-electron two-proton transfer process. The dsDNA damage by hydroquinone was concentration dependent, increasing along with the hydroquinone oxidation peak current increase, and the dsDNA G residues oxidation peak current decrease [110].
Polycyclic aromatic hydrocarbons (PAHs) are a group of different chemicals, formed during the incomplete burning of coal, oil and gas, garbage, or other organic substances like tobacco or charbroiled meat, being currently classified as human carcinogens [111]. Benzo(a)pyrene (BaP) is one of the most studied PAHs, classified by IARC as probable human carcinogen. BaP and its metabolite were detected at a DNA-electrochemical biosensor, built by layer-by-layer assembling of HRP and dsDNA at nafion-solubilized single-wall carbon nanotubes-ionic liquid (SWCNTs-NA-IL) composite film [112]. The biosensor was characterized by cyclic and DP voltammetry, EIS, SEM, and computational methods. UV-vis spectrophotometry was also used to investigate DNA damage induced by BaP and its products in solution. The DNA-electrochemical biosensor was investigated separately in BaP, H$_2$O$_2$, and in their mixture. The results showed that the interaction with BaP caused unwinding of DNA double helix and exposure of the bases.

The cyanobacterial blooms in aquatic environments are of increased concern in many lakes, rivers, and brackish waters worldwide and more than 40 types of cyanobacteria are known to produce a variety of harmful compounds as secondary metabolites called cyanotoxins. Microcystin-LR (MC-LR) and nodularin (NOD) are among the most commonly reported toxins produced by cyanobacteria, with strong hepatotoxic, genotoxic, and carcinogenic potential, which have been associated with the induction of DNA damage in vitro and in vivo [113,114]. Apart from the environmental, hazardous, tumour promoting activity of MC-LR and NOD, especially liver cancer, based on the strong MC-LR cytotoxicity in vitro, it was proposed that MC-LR could be held as structural basis for the development of novel targeted compounds against pancreatic cancer [115]. From the pharmacological viewpoint, cyanotoxins represent a rich source of natural cytotoxic compounds with a potential to target specific cancers [116].

The MC-LR electrochemical behaviour at GCE was investigated by cyclic, DP, and SW voltammetry [113], showing that MC-LR oxidation is a diffusion-controlled irreversible and pH-independent process that occurs with the transfer of one electron and does not involve the formation of any electroactive oxidation product. Upon incubation during 24 h, in different pH electrolytes, homogeneous chemical degradation of MC-LR in solution occurred. The chemically degraded MC-LR was electrochemically detected by the occurrence of a new oxidation peak, at a lower oxidation potential, corresponding to an irreversible, pH-dependent process, and involving the formation of two new redox products that undergo reversible oxidation.
The chemical degradation of MC-LR in solution was confirmed by high-performance liquid chromatography with UV detection at room temperature.

The interaction of MC-LR and NOD with dsDNA was investigated in incubated solutions and at DNA-electrochemical biosensors [114]. It was shown that MC-LR, NOD, and their chemical degradation products interact with dsDNA, causing the aggregation of DNA strands, detected by the time-dependent decrease of the G and A residues oxidation peaks, Fig. 14 [114]. In incubated solutions, where dsDNA strands are allowed to move freely, the interaction leads to the release of free A bases. The interaction between DNA and MC-LR or NOD also causes a basic damage, upon cleavage of the bond between the DNA phosphate sugar backbone that, if left unrepaired, can lead to mutations during the replication process.

6. CONCLUSIONS

This chapter presents advances on the design and applications of label-free DNA-electrochemical biosensors, in a cancer research perspective.
Screening new antineoplastic drugs with increased specificity, early diagnosis of cancer biomarkers, identification of unknown carcinogenic compounds, and detection of the presence of hazardous substances in the environment are crucial strategies for evaluation of new effective cancer treatment drugs, risk assessment of hazardous compounds, and prevention.

The development of DNA-electrochemical biosensors, built by bottom-up immobilization of DNA sequences that self-assemble in either double- and single-stranded form, or in unusual but biologically relevant configurations, such as GQs and i-motifs, is revised. The influence of the DNA immobilization parameters and the analytical characterization, using combined electrochemical and surface characterization techniques is crucial and emerges as a necessary step for the development of new, more sensitive DNA-electrochemical biosensors. The applications of DNA-electrochemical biosensors, for label-free detection of anticancer pharmaceutical compounds, protein cancer biomarkers, and carcinogens, can now provide new information very useful for planning therapeutic intervention, in a wide variety of human cancers.

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