Antileishmanial Drug Miltefosine-dsDNA Interaction in situ Evaluation with a DNA-Electrochemical Biosensor

W. B. S. Machini and A. M. Oliveira-Brett

Abstract: Leishmaniasis is one of the most important parasitic neglected disease. The electrochemical evaluation of the antileishmanial drug miltefosine-dsDNA interaction was investigated in incubated solutions and using dsDNA-electrochemical biosensors, following the changes in the oxidation peaks of guanosine and adenosine residues, and the occurrence of the free guanine residues, electrochemical signal. The electrochemical behaviour of miltefosine was also investigated, at a glassy carbon electrode, using cyclic, differential pulse and square wave voltammetry and no electrochemical redox processes were observed. The interaction mechanism of miltefosine-dsDNA occurs in two ways: independent of the dsDNA sequence, and leading to the condensation/aggregation of DNA strands, producing a rigid miltefosine-dsDNA complex structure, and a preferential interaction between the guanine hydrogen atoms in the C–G base pair and miltefosine, causing the release of guanine residues detected on the electrode surface. Miltefosine did not induce oxidative damage to DNA in the experimental conditions used.

Keywords: Antileishmanial drug · miltefosine · neglected tropical diseases · glassy carbon electrode · DNA interaction · DNA-electrochemical biosensor

1 Introduction

The progress with science and technology has brought improvements in quality of life and health, in addition to economic and social developments, but various diseases, namely the neglected tropical diseases, continue to affect the poor and marginalized people around the world [1].

According to the World Health Organization (WHO) [2], a disease that affects mainly the poor can be characterized as a disease of poverty, which is divided into two classes. The first class includes HIV/AIDS, malaria and tuberculosis, diseases which have received considerable attention from the community and investment in research and development in order to eradicate them. The other class consists in the neglected diseases, such as onchocerciasis (river blindness), Chagas disease, leprosy, tuberculosis, schistosomiasis (bilharzias), lymphatic filariasis, African trypanosomiasis (sleeping sickness), dengue and leishmaniasis.

The neglected diseases affect mostly the poor, with little visibility and low political voice, in developing countries, which thrive in poor housing and without high sanitation conditions, are most common in tropical climates, and therefore are often referred to as neglected tropical diseases. These diseases are practically neglected also by the research community, but can be prevented, controlled, and probably eliminated using adequate clinical solutions [3].

Leishmaniasis is one of the most important parasitic neglected diseases and refers to various clinical syndromes caused by protozoan kinetoplastid parasites found in Leishmania species [4], that are transmitted to mammalian by the bites of female sandflies from the Phlebotomus and Lutzomyia genera via anthroponotic or zoonotic cycles, and infect them [5].

In humans, the leishmanial infections may manifest in four most common forms, depending on the causative species, such as, cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), diffused cutaneous leishmaniasis (DCL), or visceral leishmaniasis (VL) [6].

It is estimated that about 350 million people worldwide are at risk of this parasitic disease [7].

Moreover, according to the WHO data [8], every year around 700,000 to one million new cases are recorded in the world, resulting in 20,000 to 30,000 deaths annually. In this sense, the recognition and treatment of leishmaniasis is currently of great public health concern in developing countries. However, the treatment of leishmaniasis remains a challenge due to problems, such as high cost of drugs, high drug-dosage, incidence and prevalence of drug resistance, side-effects, and lack of affordable new antileishmanial drugs [9].

One of the drugs used in the treatment of leishmaniasis is miltefosine (hexadecylphosphocholine, HePC), Scheme 1, an alkyllysophospholipid analogue. It was originally developed for the treatment of breast cancer and other solid tumours [10], without success due to the dose-limiting gastro-intestinal toxicity when used as an oral agent [11]. However, miltefosine presented an excellent antileishmanial activity, both in vitro and in experimental animals, making human trials possible, and

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The DNA-electrochemical biosensor detects specific binding processes of drugs with DNA [32], changes in the DNA oxidation peaks before and after the interaction [33], monitors the appearance of purine and pyrimidine bases, and the biomarkers of DNA purine bases oxidative damage: guanine (Gua) oxidation product, 8-oxoguanine (8-oxoGua) [34], and adenine (Ade) oxidation product, 2,8-dihydroxyadenine (2,8-oxoAde) [35].

The dsDNA-electrochemical biosensors have been successfully utilized to investigate the interaction of drugs with dsDNA [36–41] and compared with other methods showed great sensitivity towards detecting small perturbations of the double-helical structure and the detection of DNA oxidative damage, allowing the unravelling of detailed mechanistic interactions.

The antileishmanial mechanism of action of miltefosine is not clear and more than one site of action is suggested. In this paper the antileishmanial drug miltefosine-dsDNA interaction, for different time periods: (a) in incubated solutions, by DP voltammetry and UV-Vis spectrophotometry, and (b) in situ using the dsDNA-, poly[G]- or poly[A]-electrochemical biosensors, was investigated.

2 Experimental
2.1 Materials and Reagents
Miltefosine (hexadecylphosphocholine, HePC) (M5571, purity ≥ 98%), double stranded deoxyribonucleic acid sodium salt from calf thymus (dsDNA) (D1501), polyguanylic acid potassium salt (poly[G]) (P4404) and polyadenylic acid potassium salt (poly[A]) (P9403) and were obtained from Sigma-Aldrich, Spain, and used without further purification.

A stock solution of 1.0 mM miltefosine was prepared in deionised water and kept at 4°C. This procedure was in agreement with the long term storage Sigma-Aldrich Production Information for miltefosine storage and ensured that the drug activity was maintained. Solutions of different concentrations of miltefosine were prepared by dilution of the appropriate quantity in 0.1 M acetate buffer pH 4.5 supporting electrolyte. These solutions were never used for more than one day. Stock solutions of 300 µg mL⁻¹ of dsDNA, poly[G], and poly[A], were prepared in deionised water and diluted to the desired concentrations prior to use.

The supporting electrolyte used in all experiments was 0.1 M acetate buffer pH 4.5, and was prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity ≤ 0.1 µScm⁻¹) [42]. The pH measurements were carried out with a Crison micropH 2001 pH-meter with an Ingold combined glass electrode. Microvolumes were measured using a Pipetman® single chancel electronic pipette (Gilson Co, Inc., France). All experiments were performed at room temperature (25 ± 1°C).

2.2 Voltammetric Parameters and Electrochemical Cells
Voltammetric experiments were carried out using a µAutolab type III, running with GPES 4.9 software, Metrohm/Autolab, The Netherlands. The measurements were carried out using a three-electrode system in a 2 mL
one compartment electrochemical cell (eDAQ, Europe). Measurements were carried out using a glassy carbon electrode (GCE) (d=1.0 mm) as working electrode, a Pt wire as counter electrode, and an Ag/AgCl (3 M KCl) as reference electrode.

The experimental conditions for cyclic voltammetry (CV) were obtained using a potential increment of 2 mV at 25 mVs⁻¹ scan rate. For differential pulse (DP) voltammetry pulse amplitude 50 mV, pulse width 100 ms, step potential 2 mV, and scan rate 5 mVs⁻¹, were used. For square wave (SW) voltammetry, pulse amplitude 50 mV, frequency 13 Hz, and potential increment 2 mV corresponding to an effective scan rate of 25 mVs⁻¹, were used.

Prior to each measurement and preparation of each electrochemical biosensor, the GCE was washed with ethanol and Milli-Q water, and the surface was polished using diamond spray (particle size 3 µm) (Kemet, UK). After polishing, it was thoroughly rinsed with Milli-Q water. Following this mechanical pre-treatment, the GCE was placed in the appropriate buffer supporting electrolyte and DP voltammograms were recorded between the potential limits of $E_L$=0.0 V and $E_H$= +1.4 V, until a stable signal was obtained. This procedure was performed before each electrochemical experiment and ensured very reproducible experimental results.

### 2.3 Incubation Procedures

#### 2.3.1 Procedure 1 – Miltefosine-dsDNA Interaction in Incubated Solutions

Solutions of different miltefosine concentrations, 0.1 µM and 100 µM, and 50 µg/mL dsDNA, were incubated in 0.1 M acetate buffer solution pH 4.5, during different time periods. Control solutions of miltefosine, and 50 µg/mL dsDNA, were prepared in 0.1 M acetate buffer solution pH 4.5, and stored in similar conditions and during the same time periods. DP voltammograms were recorded in solution after different incubation times always using a clean GCE surface.

#### 2.3.2 Procedure 2 – Miltefosine-dsDNA-Electrochemical Biosensor Interaction

The dsDNA-electrochemical biosensors were prepared by covering successively the GCE surface with three drops each of 5 µL from a 50 µg/mL dsDNA solution diluted in 0.1 M acetate buffer pH 4.5. After placing each drop on the electrode surface the biosensor was dried under a constant flux of N₂(g). A similar procedure was used in order to obtain the poly[G]- and poly[A]-electrochemical biosensors.

This procedure ensures full coverage of the electrode surface by a multilayer dsDNA film [32], avoiding the formation of undesired thin and incomplete network film of co-adsorbed dsDNA-miltefosine, dsDNA, or miltefosine nonspecific binding to the electrode surface.

The dsDNA-, poly[G]- or poly[A]-electrochemical biosensors were immersed in solutions of 0.1 M miltefosine, in 0.1 M acetate buffer pH 4.5, and allowed to incubate during different periods of time as indicated in the text.

Afterwards, the dsDNA-, poly[G]- or poly[A]-electrochemical biosensors were removed from the solution, washed with deionised water in order to remove the unbounded miltefosine molecules and placed in the electrochemical cell, containing only the 0.1 M acetate buffer pH 4.5 supporting electrolyte, where the transduction was performed by DP voltammetry. For control experiments, the dsDNA-electrochemical biosensor was incubated in 0.1 M acetate buffer pH 4.5 supporting electrolyte during the same time periods and in similar conditions as the solutions with miltefosine.

### 2.4 UV-Vis Spectrophotometry

UV-Vis measurements were performed using a Spectrophotometer Hitachi U-2810 running with UV Solution Program (Digilab Hitachi, Tokyo, Japan). The experimental conditions for absorption spectra were: slit width 1.5 nm, sampling interval 0.5 nm and a scan speed of 100 nm/min⁻¹. All UV-vis spectra were measured from 230 nm to 400 nm, in a quartz glass cuvette with an optic path of 10.0 mm. UV-vis spectra for an incubated solution of 500 µM miltefosine and 50 µg/mL dsDNA, 0.1 M acetate buffer pH 4.5, was recorded. UV-vis spectra of control solutions, 50 µg/mL dsDNA, and 500 µM miltefosine, for the same time conditions, were also recorded.

### 2.5 Acquisition and Presentation of Data

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

All the UV-Vis spectra presented were shown without any further mathematical treatment and all values of $\lambda_{max}$ and absorbance were obtained from the original spectra.

### 3 Results and Discussion

#### 3.1 Voltammetric Behaviour of Miltefosine

The electrochemical behaviour of the antileishmanial drug miltefosine was also investigated, at a glassy carbon electrode, using cyclic, differential pulse and square wave voltammetry, in solutions of 100 µM miltefosine, in acetate
buffer pH 4.5. As expected no electrochemical redox processes were observed. This in agreement with the chemical structure of miltefosine, without electroactive centres which can be oxidized in aqueous solutions.

3.2 Voltammetric Behaviour of dsDNA, Poly[G] and Poly[A]
The oxidation behaviour of dsDNA, poly[G] and poly[A], in 0.1 M acetate buffer pH 4.5, was revisited using DP voltammetry, Figure 1, as a control, in order to enable the identification of the oxidation peaks that occurred after the in situ miltefosine-dsDNA, miltefosine-poly[G] and miltefosine-poly[A] interactions.

![Fig. 1. Control DP voltammograms baseline-corrected, at GCE, in 0.1 M acetate buffer pH 4.5 supporting electrolyte, for 50 \( \mu \text{g mL}^{-1} \) dsDNA, 50 \( \mu \text{g mL}^{-1} \) poly[G] and 50 \( \mu \text{g mL}^{-1} \) poly[A], \( v = 5 \text{ mV s}^{-1} \).](image)

The DP voltammograms for 50 \( \mu \text{g mL}^{-1} \) dsDNA, in 0.1 M acetate buffer pH 4.5, showed two small peaks corresponding to the oxidation of desoxyguanosine (dGuo), at \( E_{\text{pa}} = +0.98 \text{ V} \), and desoxyadenosine (dAdo), at \( E_{\text{pa}} = +1.25 \text{ V} \), Figure 1. The small current from the dGuo and dAdo peaks is due to greater difficulty of the purine bases electron transfer within the DNA the double strand to the CGE surface [32].

The DP voltammograms of poly[G] and poly[A] obtained in the same conditions showed, as expected, only one oxidation peak each, Figure 1. The poly[G] homopolymer contains only guanine (Gua) residues and the oxidation occurred at the dGuo residue, at \( E_{\text{pa}} = +0.98 \text{ V} \), while poly[A] homopolymer contains only adenine (Ade) residues and the oxidation occurred at the dAdo residues, at \( E_{\text{pa}} = +1.25 \text{ V} \).

3.3 Miltefosine-dsDNA Interaction in Incubated Solutions
3.3.1 Electrochemistry
The miltefosine-dsDNA interaction, in incubated solutions using DP voltammetry, to evaluate the possibility of miltefosine to cause conformational changes in the DNA double helix, hydrogen bonding cleavage, and/or dsDNA base oxidative damage, was investigated.

The effect of the miltefosine-dsDNA interaction was electrochemically followed comparing the changes on the dsDNA oxidation peaks, dGuo and dAdo, in the absence and presence of miltefosine, and monitoring the occurrence of free Gua oxidation and free Ade oxidation peaks, and the purine biomarkers: 8-oxoGua, Gua oxidation product, and 2,8-oxoAde, Ade oxidation product.

Solutions of different miltefosine concentrations, 0.1 \( \mu \text{M} \) and 100 \( \mu \text{M} \), and 50 \( \mu \text{g mL}^{-1} \) dsDNA, were incubated in 0.1 M acetate buffer solution pH 4.5, during different time periods.

After each measurement, the GCE surface was cleaned following the cleaning and conditioning procedure described in Section 2.2, in order to remove all oxidation products adsorbed at the electrode surface.

The miltefosine-dsDNA interaction, in concentrated solutions, containing 100 \( \mu \text{M} \) miltefosine incubated with 50 \( \mu \text{g mL}^{-1} \) dsDNA, in 0.1 M acetate buffer pH 4.5, using a GCE, Figure 2, was investigated.

The DP voltammogram obtained immediately after the addition of miltefosine to the dsDNA solution showed immediately a large decrease in both anodic peak currents, corresponding to the oxidation of dGuo and dAdo, when compared with the control dsDNA solution.

This is due to a strong condensation and/or aggregation of DNA double helix, due to a strong interaction between miltefosine and dsDNA.

In order to better understand the effect of miltefosine-dsDNA interaction over time, different incubation time periods, in diluted solutions, containing 0.1 \( \mu \text{M} \) miltefosine incubated with 50 \( \mu \text{g mL}^{-1} \) dsDNA, in 0.1 M acetate buffer pH 4.5, using a GCE, by DP voltammetry, Figure 3, were investigated.

The DP voltammogram obtained immediately after the addition of 0.1 \( \mu \text{M} \) miltefosine to the solution containing 50 \( \mu \text{g mL}^{-1} \) dsDNA, Figure 3, showed the two dsDNA oxidation peaks, dGuo, at \( E_{\text{pa}} = +0.98 \text{ V} \) and dAdo, at \( E_{\text{pa}} = +1.25 \text{ V} \), with a small decrease in the peak currents, when compared with the control dsDNA solution.

After 15 minutes incubation time the dsDNA oxidation peak currents continued to decrease, Figure 3, which clearly demonstrated that a conformational change in the dsDNA structure occurred after the miltefosine-dsDNA interaction [18,43,44].
was more difficult, and the dGuo and dAdo oxidation peak currents decreased [18,43–45].

Increasing the incubation time, to 30 and 60 minutes, both dGuo and dAdo oxidation peak currents continue to decrease, which is in agreement with a strong condensation/aggregation of the dsDNA caused by the miltefosine-dsDNA interaction.

The DP voltammogram obtained after 60 minutes dsDNA solution incubation, in the diluted 0.1 μM miltefosine solution, Figure 3, showed a big decrease of the dsDNA oxidation peak currents, similar to the incubation in the concentrated 100 μM miltefosine solution, Figure 2, which confirms a strong condensation/aggregation of dsDNA caused by the interaction between miltefosine and DNA.

In all DP voltammograms obtained in miltefosine-dsDNA incubated solutions, Figures 2 and 3, no oxidation peak related to the presence of 8-oxoGua or 2,8-oxoAde, at $E_{pa} = +0.45$ V, the DNA oxidative damage biomarkers [34,35], was detected.

### 3.3.2 UV-Vis Spectrophotometry

The UV-Vis evaluation of miltefosine-dsDNA interaction was also investigated. The differences in the absorption spectra of dsDNA were monitored in the absence and presence of miltefosine in order to determine the influence of miltefosine interaction on the dsDNA conformation and morphology.

Absorption spectra in the UV-Vis region for control solutions of 500 μM miltefosine, 50 μg mL⁻¹ dsDNA, and immediately after the addition of 500 μM miltefosine to the 50 μg mL⁻¹ dsDNA solution, in 0.1 M acetate buffer pH 4.5, Figure 4, were recorded.

The control spectra obtained in the range from 400 to 230 nm, Figure 4, for 50 μg mL⁻¹ dsDNA, in 0.1 M acetate buffer pH 4.5, for (—, red) control 50 μg mL⁻¹ dsDNA, and (—) after incubation of 0.1 μM miltefosine with 50 μg mL⁻¹ dsDNA, during different time periods: 0, 15, 30 and 60 minutes, ν = 5 mVs⁻¹.

The DNA purine bases were embedded within the condensed more compact dsDNA structure and less exposed to the GCE surface, the purine bases oxidation was more difficult, and the dGuo and dAdo oxidation peak currents decreased [18,43–45].

Increasing the incubation time, to 30 and 60 minutes, both dGuo and dAdo oxidation peak currents continue to decrease, which is in agreement with a strong condensation/aggregation of the dsDNA caused by the miltefosine-dsDNA interaction.

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buffer pH 4.5, showed one absorption band, at $\lambda_{\text{max}} = 260$ nm, attributed to the $\pi - \pi^*$ transitions from the heterocyclic rings of the nucleotides. The control spectra for 500 $\mu$M miltefosine did not show any absorption bands.

The spectra obtained immediately after the addition of 500 $\mu$M miltefosine to the 50 $\mu$g mL$^{-1}$ dsDNA solution showed the absorption band, at $\lambda_{\text{max}} = 260$ nm, with a great decrease in the intensity of absorption, which corroborates the fact that miltefosine promotes a strong condensation and/or aggregation of dsDNA.

3.4 *In situ* Interaction of Miltefosine with the dsDNA-Electrochemical Biosensors

During the electrochemical experiments of the miltefosine-dsDNA interaction in incubated solutions, an incomplete network film of co-adsorbed free molecules of dsDNA and miltefosine, and the miltefosine-dsDNA complexes, were deposited on the GCE surface [46].

Thus, a complete coverage of the electrode surface is important to avoid undesired nonspecific adsorption of these molecules and complexes to the electrode surface. The dsDNA electrochemical biosensor was prepared after the immobilization of DNA multilayers to the GCE surface [32], the procedure is described in Section 2.3.

The use of the dsDNA-electrochemical biosensor enabled observing, *in situ* and in real time, the changes that occurred to the dsDNA immobilized on the electrode surface during the miltefosine-dsDNA interaction, detected by the changes in the purine bases oxidation peak currents of the dsDNA-electrochemical biosensor, the appearance of free purine base oxidation peaks, and oxidation products peaks, recorded after different incubation times, and compared with the results, obtained in the same experimental conditions, using a control dsDNA-electrochemical biosensor.

The DP voltammogram for the control dsDNA-electrochemical biosensor showed, as expected, only both dGuo, at $E_{pa} = +0.99$ V, and dAdo, at $E_{pa} = +1.26$ V, oxidation peaks, Figure 5 (—, red).

A new dsDNA-electrochemical biosensor was always prepared for each experiment and incubated during different time periods, 15, 30 and 60 minutes, in diluted solutions, containing 0.1 $\mu$M miltefosine, in 0.1 M acetate buffer pH 4.5.

After each interaction time the multilayer dsDNA-electrochemical biosensor was washed with deionised water, in order to remove the unbound miltefosine, dried under $N_2(g)$ atmosphere, and transferred to the supporting electrolyte solution, 0.1 M acetate buffer pH 4.5, where the DP voltammograms were obtained, Figure 5.

The DP voltammogram after 15 min incubation, in diluted 0.1 $\mu$M miltefosine solutions, Figure 5, showed a decrease of the dGuo and dAdo purine bases residues oxidation peak currents in the polynucleotide chain, due to the miltefosine-dsDNA interaction, and the appearance of one new oxidation peak, at $E_{pa} = +0.76$ V, when compared with the results obtained with the control dsDNA-electrochemical biosensor.

Fig. 5. DP voltammograms baseline-corrected, in 0.1 M acetate buffer pH 4.5, of dsDNA-electrochemical biosensor (—, red) control and (—) after incubation with 0.1 $\mu$M miltefosine during different time periods: 15, 30 and 60 minutes, $v = 5$ mV s$^{-1}$.

Considering that the electrode surface is completely covered by dsDNA molecules and miltefosine has no electroactive centers which can be oxidized, this new peak is due to the release of free Gua from the DNA helix, creating an abasic site in the dsDNA structure. The occurrence of the free Gua peak is explained considering that miltefosine induced cleavage of the bond between Gua and the sugar moiety, in the dsDNA guanosine residues, leading to the release of free Gua, which is oxidized at a lower potential, at $E_{pa} = +0.76$ V [46–48].

The dsDNA-electrochemical biosensors, for long incubation times, 30 min and 60 min, in diluted 0.1 $\mu$M miltefosine solutions, were also investigated, Figure 5. The free Gua oxidation peak current increased with increasing incubation time. The progressive decrease with time, of the dGuo and dAdo oxidation peak currents observed, is in agreement with the results obtained with the miltefosine-dsDNA interaction in incubated solutions, Figure 3.

The specific interaction between the dGuo residues and miltefosine was evidenced based on the occurrence of the free Gua oxidation peak, at $E_{pa} = +0.76$ V, due to the oxidation of the deoxyribose Cl’ carbon [36,44,48] by miltefosine that caused the liberation of Gua from the dsDNA backbone.

The results clearly showed that miltefosine interacted with dsDNA, under the experimental conditions investigated, leading to strong conformational changes in the DNA double-helical structure, and the release of free Gua.

Although the free Gua oxidation peak was observed, the appearance of the Gua (8-oxoGua) or/and Ade (2,8-oxoAde) oxidation product peaks, at $E_{pa} = +0.45$ V
[34,35], which would correspond to oxidative damage caused to DNA, was not electrochemically detected. This means that miltefosine did not induce oxidative damage to dsDNA in these experimental conditions.

3.5 In situ Interaction of Miltefosine with Poly[G]- and Poly[A]-Electrochemical Biosensors

The homopolynucleotides, poly[G] and poly[A], were used to prepare poly[G]- and poly[A]-electrochemical biosensors, Section 2.3, in order to clarify with which purine base, Gua or Ade, a more specific preferential miltefosine-dsDNA interaction occurred, Figure 6.

The DP voltammogram of the poly[G]-electrochemical biosensor, in 0.1 M acetate buffer pH 4.5, showed one peak, at $E_{pa} = +1.00$ V, corresponding to dGuo oxidation, Figure 6A. A new poly[G]-electrochemical biosensor was incubated in 0.1 mM miltefosine during 15 min, dried under N$_2$ atmosphere, and afterwards transferred to 0.1 M acetate buffer pH 4.5 where DP voltammetry was performed. The DP voltammogram registered using the poly[G]-electrochemical biosensor, Figure 6A, showed the oxidation of dGuo at the same potential as the control, $E_{pa} = +1.00$ V, with a decreased in the peak current. Also, a small new oxidation peak appeared, at $E_{pa} = +0.74$ V, indicating, and confirming the results, obtained using the dsDNA-electrochemical biosensors, concerning the release of free Gua bases. After longer incubation times, 30 and 60 minutes, the DP voltammograms showed a progressive decrease with time of the dGuo oxidation peak, and an increase with incubation time of the free Gua oxidation peak.

In all DP voltammograms the poly[G]-electrochemical biosensors incubated in 0.1 mM miltefosine, Figure 6A, no oxidation peak related to the presence of the DNA oxidative damage biomarker 8-oxoGua, at $E_{pa} = +0.45$ V [34], was observed, indicating that poly[G] oxidative damage did not occur, in these experimental conditions.

The DP voltammogram of the poly[A]-electrochemical biosensor, in 0.1 M acetate buffer pH 4.5, Figure 6B, showed one peak, at $E_{pa} = +1.25$ V, corresponding to dAdo oxidation. Using new poly[A]-electrochemical biosensors, incubations in 0.1 mM miltefosine during 15, 30 and 60 minutes, were carried out. Afterwards the poly[A]-electrochemical biosensors were dried under N$_2$ atmosphere, and then transferred to an electrochemical cell with 0.1 M acetate buffer pH 4.5, where the DP voltammograms were recorded.

Only a progressive decrease of the dAdo oxidation peak current occurred, Figure 6B, due to conformational changes of the poly[A] structure, with less exposure of the Ade bases for oxidation at the GCE surface. No peak corresponding to the DNA oxidative damage biomarker 2,8-oxoAde, at $E_{pa} = +0.45$ V [35], was observed, indicating that no poly[A] oxidative damage occurred.

The experiments using poly[G]- and poly[A]-electrochemical biosensors confirmed the results obtained with the ds DNA-electrochemical biosensors, and revealed new important information in order to understand the molecular mechanism involved in the miltefosine-dsDNA interaction.

The miltefosine-dsDNA interaction mechanism occurs in two ways: independent of the dsDNA sequence, and leading to the condensation/aggregation of DNA strands, producing a rigid miltefosine-dsDNA complex structure [18,48], detected in incubated solutions, and a preferential interaction between the guanine hydrogen atoms in the C–G base pair and miltefosine, causing the release of
guanine residues that are oxidised at the GCE surface [48], detected using dsDNA-electrochemical biosensors.

4 Conclusions

The miltefosine-dsDNA interaction was investigated in incubated solutions and using dsDNA-electrochemical biosensors, and caused modifications in the DNA morphological structure, which were also confirmed using polyhomonucleotides of guanosine and adenosine, the poly [G]- and poly[A]-electrochemical biosensors.

The interaction mechanism miltefosine-dsDNA causes condensation/aggregation of DNA strands, and the occurrence of a guanine abasic site in the dsDNA structure. Nevertheless, in the experimental conditions used, miltefosine did not induce oxidative damage to DNA.

The discovery of new drugs for the leishmaniasis treatment, a most important parasitic neglected disease, with limited therapeutic options and emerging resistance to the available drugs, remains very important.

The use of DNA-electrochemical biosensors bring new perspectives for a better understanding of their mechanisms of action, as well as the development and the improvement in searching for new antileishmanial agents.

5 Glossary

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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
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<td>FTIR</td>
<td>Fourier Transform Infrared</td>
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<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<td>UV-Visible</td>
<td>Ultraviolet-Visible</td>
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<td>CV</td>
<td>Cyclic voltammetry</td>
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<td>DP</td>
<td>Differential pulse</td>
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<td>SW</td>
<td>Square wave</td>
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<td>GCE</td>
<td>Glassy carbon electrode</td>
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<td>HePC</td>
<td>Hexadecylphosphocholine</td>
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<td>dsDNA</td>
<td>Double-strand deoxyribonucleic acid</td>
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