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Analytical Biochemistry 332 (2004) 321–329
ANALYTICAL BIOCHEMISTRY
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Voltammetric determination of all DNA nucleotides

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Received 13 March 2004
Available online 21 July 2004

Abstract

The voltammetric oxidation of all deoxyribonucleic acid (DNA) monophosphate nucleotides is investigated for the first time over a wide pH range by differential pulse voltammetry with a glassy carbon electrode. Experimental conditions such as the electrode size, supporting electrolyte composition, and pH were optimized to obtain the best peak potential separation and higher currents. This enabled the simultaneous voltammetric determination of all four DNA bases in equimolar mixtures and detection limits in the nanomolar range at physiological pH. It was also possible to detect for the first time the oxidation of each of the purine and pyrimidine nucleotides free in solution or as monomers in single-stranded DNA.

Keywords: Guanine; Adenine; Thymine; Cytosine; Deoxynucleosides; Deoxynucleotides; DNA; Voltammetry; Anodic oxidation

The purine bases, guanine (G) and adenine (A), and the pyrimidine bases, thymine (T) and cytosine (C), are fundamental compounds in biological systems, participating in processes as distinct as energy transduction, metabolic cofactors and cell signaling, and are essential building blocks of nucleic acids [1]. Their sequence order in deoxyribonucleic acid (DNA) chains generates domains of information (genes) necessary to the living cell to build up proteins and self regulate its metabolism. Other noncoding and repetitive base sequence domains constitute structural motifs that appear to be important in DNA–protein interactions and recognition [1,2].

Chemical modification of each of the DNA bases causes molecular disturbance to the genetic machinery that leads to cell malfunction and death. For instance, oxidative DNA damage by free radicals and exposure to ionizing radiation generates several products within the double helix such as 8-oxoguanine, 2,8-oxoadenine, 5-formyluracil, 5-hydroxicytosine, etc., which are mutagenic [3]. It is well established that the oxidation of DNA is an important source of genomic instability since there is evidence that the oxidation products of DNA bases play important roles in mutagenesis, carcinogenesis, aging, and age-related disease [3–5]. Hence, great interest exists in the sensitive determination and full characterization of the mechanisms involved in oxidative damage of all DNA bases.

Electrochemical methods are very promising for the study of DNA oxidative damage and in the investigation of the mechanisms of interaction of drugs with DNA [6–8]. In particular, electrochemical DNA biosensors have proved to be excellent tools for investigating the effects of various endogenous and exogenous sources of hazard on the genomic material, allowing quick and low-cost determination of DNA damage. Most electrochemical DNA biosensors are based on the determination of purine oxidation peaks, principally the guanine peak, to monitor the degree of oxidative damage caused to DNA [6–9]. This is due to the fact that guanine has the lowest oxidation potential of all DNA bases [7,10] and that its principal oxidation product, 8-oxo-7,8-dihydroguanine (8-oxoGua),[1] is considered a useful biomar-

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[1] *Abbreviations used:* 8-oxoGua, 8-oxo-7,8-dihydroguanine; GCE, glassy carbon electrode; LOD, limit of detection.

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doi:10.1016/j.ab.2004.06.021

ker of DNA damage by oxidative stress [3–5] and can be easily quantified by voltammetry [11,12].

Several studies showed the importance of considering the influence on the chemical reactivity of guanine of the chemical environment surrounding guanine residues in DNA (which is determined by base sequence). For instance, it has been shown that cytosine hydrogen bond interaction with guanine affects the rate of guanine oxidation within the double helix of DNA [13]. This can influence the concentrations found for 8-oxoGua and the extent of DNA oxidative damage [3–5].

To increase the knowledge on the interactions and damage caused by chemical and physical sources on DNA, it is important to extend the investigation of electrochemical oxidation studies on DNA to simultaneously include information from all purine and pyrimidine DNA bases. This will result in a better understanding of the data already obtained based only on the guanine oxidation peak and will increase the overall understanding by electrochemical methods of the mechanisms of DNA oxidative damage.

Mechanistic studies on the electrochemical behavior of purine and pyrimidine derivatives have been performed in the past four decades. Most of this work is concerned with the electrochemical reduction of purine and pyrimidine derivatives on mercury electrodes and, although guanine, adenine, and cytosine could be reduced, no polarographic wave has been observed for thymine on the mercury electrode [6]. Oxidation of purine derivative compounds was extensively studied at solid electrodes, mainly carbon-based electrodes [6,7]. The electroactivity of pyrimidine derivative compounds at solid electrodes has been shown [14]. This led to the possibility of detecting voltammetrically the oxidation of all DNA bases at glassy carbon electrodes (GCEs) and confirmed that guanine and adenine are more easily detected than thymine and cytosine. This was done using sonovoltammetry, but the simultaneous sonovoltammetric detection of the four DNA bases was achieved only in alkaline electrolyte solutions containing pyrimidine bases with a concentration 10 times higher than the purine base concentration [14–16]. However, it is well known that in the DNA double helix the purine/pyrimidine ratio is equal to 1. Thus, it is a challenge to increase the sensitivity of pyrimidine voltammetric determination to allow the simultaneous electrochemical detection of all purine and pyrimidine DNA bases in real samples and at physiological pH.

Another major challenge is to extend the DNA voltammetric studies to nucleotides that will enable the simultaneous detection of all bases in single-stranded DNA (ssDNA) without the necessity of a previous hydrolysis step. Therefore, the main objective of the present work was to study equimolar mixtures of the four DNA purine and pyrimidine bases, their nucleotides, and ssDNA over a wide pH range using differential pulse voltammetry with glassy carbon electrodes of different sizes. Optimization of the experimental conditions was achieved and enabled low detection limits for the simultaneous determination of the four bases and nucleotides of DNA.

Materials and methods

Materials and reagents

Guanine, adenine, thymine, cytosine, guanosine 5-monophosphate (GMP), adenosine 5-monophosphate (AMP), thymidine 5-monophosphate (TMP), cytidine 5-monophosphate (CMP), lyophilized calf thymus single-stranded DNA, sodium salt poly(dT), and sodium salt poly(dC) were obtained from Sigma–Aldrich and used without further purification. Analytical-grade reagents and purified water from a Millipore Milli-Q system (conductivity $<0.1\,\mu S\,cm^{-1}$) were used for the preparation of phosphate, acetate, borate, and ammonium buffer electrolyte solutions.

Stock solutions (200 μM) of all bases were prepare in purified water. To guarantee complete dissolution of guanine, 10 μL of 9 M NaOH was added to the stock solution.

Nano- and microvolumes were measured using an EP-10 Plus and an EP-100 Plus motorized microliter pipette (Rainin Instrument, Woburn, MA, USA). The pH was measured with a Crison Model micropH 2001 pH meter with an Ingold combined-glass electrode. All experiments were done at room temperature (25 ± 1 °C).

Voltammetric cell and parameters

All voltammetric experiments were done using an μAutolab Type II with GPES version 4.9 software (Eco-Chemie, Utrecht, The Netherlands). A one-compartment electrochemical cell with a volumetric capacity of 100 μL was used containing a glassy carbon (GC) working electrode (Cypress, USA), a Pt wire counter electrode, and an Ag/AgCl reference electrode (3 M KCl saturated with AgCl). This cell was placed inside a Faraday cage at room temperature during all the measurements. In the present work glassy carbon disk electrodes with 3 mm, 1.5 mm, and 7 μm diameter were used.

The voltammetric parameters used, unless stated otherwise, were (a) differential pulse voltammetry, pulse amplitude 50 mV, pulse width 70 ms, scan rate $5\,mV\,s^{-1}$, equilibration time 10 s and (b) square wave voltammetry, pulse amplitude 25 mV, frequency 10 Hz, effective scan rate $20\,mV\,s^{-1}$. All potentials are referred to Ag/AgCl reference electrode.

Preconditioning of the glassy carbon electrodes

The GCEs were polished using alumina oxide (particle size 0.3 μm) before every electrochemical assay. After

polishing they were rinsed thoroughly with Milli-Q water for 30 s; the electrode was sonicated for 1 min in an ultrasound bath and again rinsed with water. After this mechanical treatment electrochemical preconditioning was carried out in three steps: first, the potential of the electrode was scanned in the electrolyte solution to check the cleanliness of the electrode surface between 0.30 and 1.50 V, at 5 mV s^{-1}; second, a 1.70-V potential was applied for 300 s; and third, the electrode was successively cycled between 0.30 and 1.30 V in a new supporting electrolyte solution, until a steady state baseline voltammogram was obtained. This procedure ensured very reproducible experimental results.

Acquisition and presentation of voltammetric data

All the experimental curves presented were background-subtracted and baseline-corrected using the moving average application with a step window of 5 mV included in GPES version 4.9 software. This mathematical treatment improves the visualization and identification of peaks over the baseline without introducing any artifact, although the peak intensity 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 and charge presented in all graphs were determined from the original untreated voltammograms.

Results and discussion

In previous work [14–16] the oxidation of all DNA bases was detected but in solutions containing pyrimidine bases with a concentration 10 times higher than the purine base concentration.

The differential pulse voltammogram obtained for a mixture of 20 μM guanine and adenine and 200 μM thymine and cytosine in pH 7.4, 0.1 M phosphate buffer supporting electrolyte solution using a 3-mm-diameter GCE shows four oxidation peaks of the same height magnitude (Fig. 1). The peak at 0.70 V is attributed to G oxidation, the easiest oxidizable of all DNA bases [6,7,10,14]. The following peaks are due to the oxidation of A at 0.96 V [14,17,18], of T at 1.16 V, and of C at 1.31 V [14]. Both the recorded voltammogram (dotted line) and that obtained after moving average baseline correction (solid line) are presented to show the improvement obtained with the baseline-correction procedure for the visualization and identification of peaks, allowing a better comparison of the relative peak currents of the four peaks.

In the voltammogram presented in Fig. 1, the ratio between purine and pyrimidine peak charge (area under the peak) [(G + A)/(T + C)] is equal to 1.3, but the concentrations of the pyrimidine bases are 10 times higher than those of the purine bases. This reflects the difficulty in detecting thymine and cytosine oxidation by voltammetry principally due to the fact that their oxidation occurs close to the oxidation of the supporting electrolyte [14]. This constitutes a drawback that needs to be overcome for the simultaneous detection of all four DNA bases when they are present in similar concentrations in a random sample.

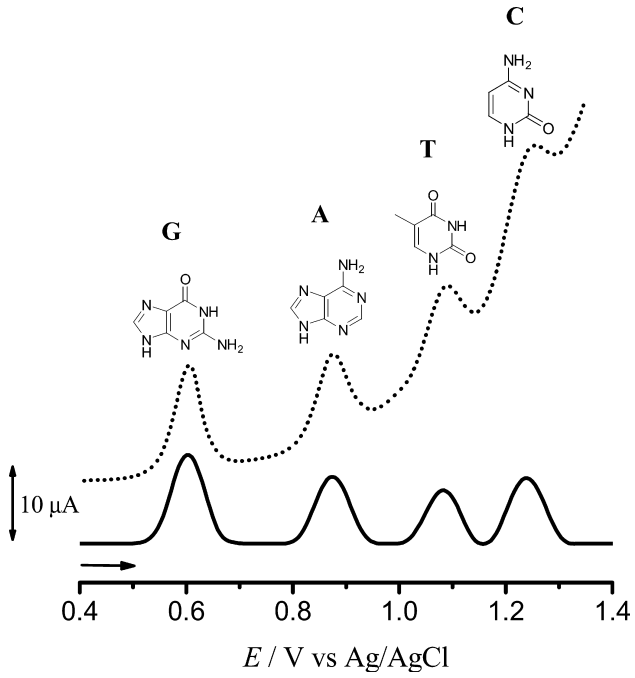

Fig. 1. Differential pulse voltammogram obtained with a 3-mm-diameter GCE electrode for the mixture of 2×10^{-5} M guanine (G) and adenine (A), 2×10^{-4} M thymine (T) and cytosine (C) in pH 7.4, 0.1 M phosphate buffer supporting electrolyte. (...) Recorded voltammogram; (—) baseline-corrected voltammogram. Pulse amplitude 50 mV; pulse width 70 ms; scan rate 5 mV s^{-1}.

Voltammetry of equimolar mixtures of DNA bases

In Fig. 2 are presented the differential pulse voltammograms obtained for a 20 μM equimolar mixture of the four bases in pH 7.4, 0.1 M phosphate buffer supporting electrolyte, using GCEs of diameter 1.5 mm (Fig. 2a) and 7 μm (Fig. 2b). Under these conditions it was possible to detect all four DNA bases in a 20 μM equimolar mixture. Using the 1.5-mm-diameter GCE, a ratio between purine and pyrimidine peak areas [(G + A)/(T + C)] equal to 10 (Fig. 2a) was obtained. This ratio was reduced five times when the 7-μm-diameter GCE was used (Fig. 2b). These results demonstrate that it is possible to detect simultaneously in a voltammetric scan all four DNA base present in solution in equal concentrations using a GC microelectrode. Nevertheless, the microelectrode has a smaller surface area and the disadvantage of also decreasing the

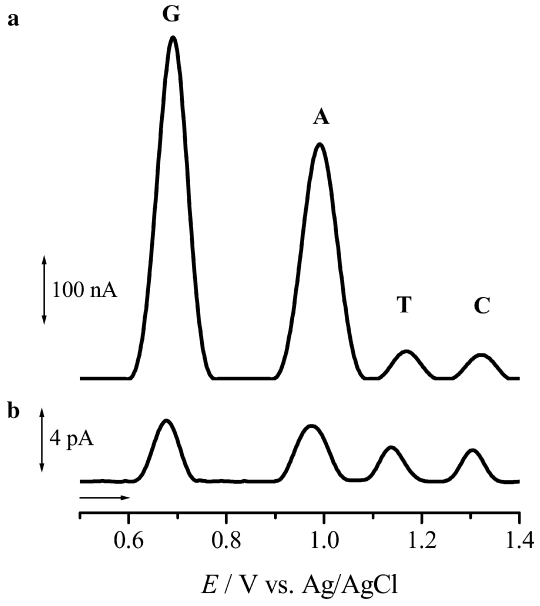

Fig. 2. Baseline-corrected differential pulse voltammograms obtained in a 20 μM equimolar mixture of guanine (G), adenine (A), thymine (T), and cytosine (C) in pH 7.4, 0.1 M phosphate buffer supporting electrolyte with: (a) 1.5-mm-diameter GCE and (b) 7-μm-diameter GCE. Pulse amplitude 50 mV; pulse width 70 ms; scan rate 5 mV s^{-1}.

current, which implies that it will be difficult to detect voltammetric signals for DNA base concentrations less than the micromolar range with this microelectrode. The compromise between an acceptable detection limit for practical applications and a reduced electrode area led to choosing the 1.5-mm-diameter GCE for the rest of this study. It was also found that the GCE preconditioning, described under Materials and methods, increased the current and reproducibility of peaks obtained for the equimolar mixture of the DNA bases.

The pH dependence of the differential pulse peak potential (E_p) and current (I_p) obtained for the 20 μM equimolar mixture of guanine, adenine, thymine, and cytosine was studied in the pH range between 3 and 12 (Fig. 3). To avoid interference of the supporting electrolyte composition on the voltammetric results, as will be considered below, phosphate salts supporting electrolyte solutions of ionic strength equal to 0.1 M were used over all the pH range studied.

A linear dependence over the whole pH range studied for guanine and adenine was found, with the slopes of the E_p–pH plots (dE_p/dpH) being equal to −60 and −58 mV, respectively (E_p/mV = 1.090–0.060 pH and E_p/mV = 1.375–0.058 pH). Thus, the numbers of protons and electrons involved in the oxidation of both purines were equal over the entire pH range [19].

For thymine and cytosine oxidation peaks a linear dependence was found within the pH interval 3 to 9, the values of dE_p/dpH being equal to −59 and −61 mV, respectively (E_p/mV = 1.567–0.059 pH and E_p/mV = 1.741–0.061 pH).

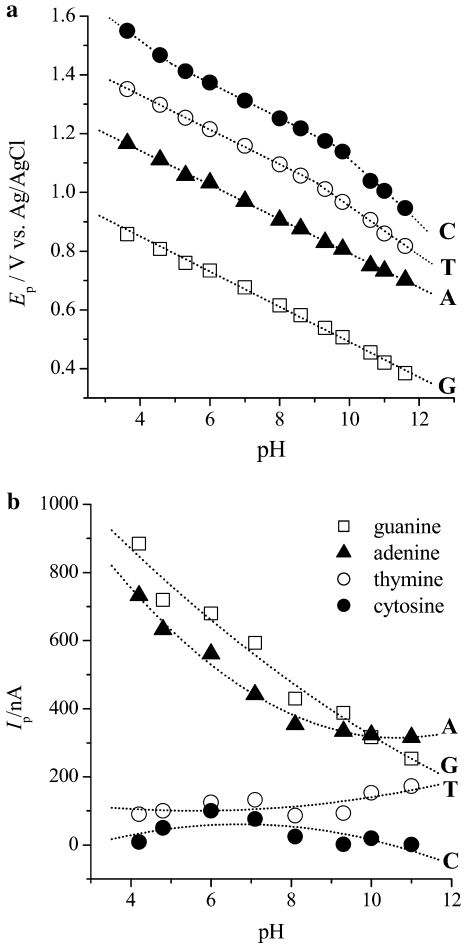

Fig. 3. Plots of (a) E_p vs pH and (b) I_p vs pH obtained from differential pulse voltammograms in a 20 μM equimolar mixture of guanine (G), adenine (A), thymine (T), and cytosine (C) in 0.1 M phosphate buffer supporting electrolyte using a preconditioned 1.5-mm-diameter GCE. Each symbol represents the arithmetic mean of $n = 3$ independent experiments.

For pH > 10, the slope increased for both pyrimidines. For thymine, a dE_p/dpH of −84 mV was found (E_p/mV = 1.788–0.084 pH), indicating that the ratio of the number of protons and electrons involved in the charge transfer changed from 1 to 1.5 for pH > 9.5, and this can be associated with the pK_a of thymine that is equal to 9.9 [1].

For cytosine, for pH < 4.5, a value of −88 mV for dE_p/dpH was obtained and this can be associated to the first pK_a of cytosine that is equal to 4.6 [1,20].

For cytosine, for pH > 10, a higher value of −106 mV per pH unit was obtained (E_p/mV = 2.175–0.106 pH). This indicates that the proton/electron ratio involved in the cytosine oxidation changed to ∼2, and the change in slope can be associated to the pK_a of cytosine that is equal to 10.2 [1,20].

The oxidation peak current (I_p) of all bases changes with pH as can be seen in Fig. 3b. It was found that the peak currents of guanine and adenine decreased al-

most linearly with pH. The current of thymine peak oxidation remained almost constant over the whole pH range studied, but a slight increase was observed for pH > 10, whereas a decrease in cytosine oxidation peak current was noticed for pH > 7.

This behavior in alkaline pH is shown in Fig. 4, where the differential pulse voltammograms obtained for 20 μM equimolar mixture of all DNA bases in pH 11.0, 0.1 M phosphate buffer supporting electrolyte solution with GCE preconditioned as described under Materials and methods are shown. The voltammogram obtained for the same mixture but without preconditioning of the GCE (dotted line) is also shown for comparison. However, while for guanine, adenine, and thymine the peak currents obtained are of the same order of magnitude, the cytosine oxidation peak is detectable only as a tiny peak. The identification of the cytosine and thymine oxidation peaks were confirmed by performing independent experiments in solutions containing only one of the bases and using the same voltammetric conditions. In addition to the decrease of the cytosine oxidation peak current for pH > 6 (Fig. 3b), the large decrease shown in Fig. 4 of the cytosine peak current is also due to progressive fouling of the electrode surface by the guanine and adenine oxidation products [18] as will be further discussed.

The electrolyte composition also influences the simultaneous detection of DNA bases. In fact, for pH > 8 higher peak currents were obtained for all bases when phosphate buffer supporting electrolyte was used, compared to the results obtained with borate or ammonia buffer solutions. For pH < 5, higher peak currents for purines and lower peak currents for pyrimidines were obtained when acetate buffer solutions were used instead of phosphate buffer supporting electrolyte solutions of equal pH.

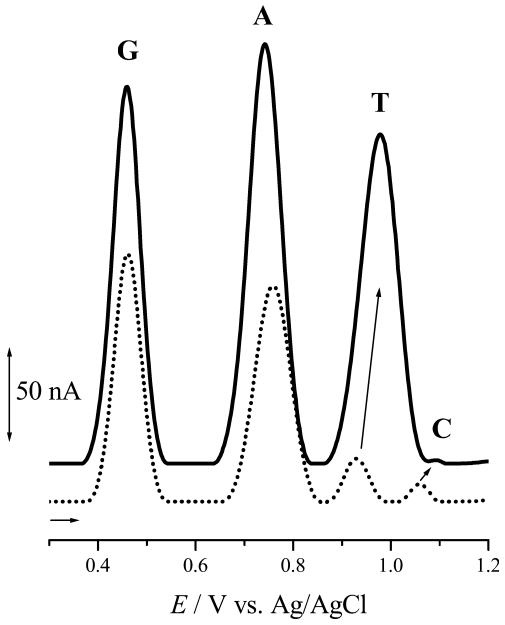

Fig. 4. Baseline-corrected differential pulse voltammograms obtained for a 20 μM equimolar mixture of guanine (G), adenine (A), thymine (T); and cytosine (C) in pH 11.0, 0.1 M phosphate buffer supporting electrolyte solution with a 1.5-mm-diameter GCE; (...) not conditioned; (—) preconditioned. The arrows indicate the changes observed in thymine and cytosine oxidation peak current after electrode preconditioning. Pulse amplitude 50 mV; pulse width 70 ms; scan rate 5 mV s^{-1}.

Analytical determinations of DNA bases in equimolar mixtures

The limit of detection (LOD) was for the first time determined for each DNA base in equimolar mixtures by differential pulse voltammetry in pH 7.4, 0.1 M phosphate buffer. This buffer was chosen as supporting electrolyte for several reasons. First, it is desirable to work close to the physiological pH. Second, a good compromise between peak separation and peak current was obtained at this pH as can be seen in Fig. 3. In fact, the lower the pH the more positive are the potentials at which all bases undergo oxidation, which is a drawback mainly for pyrimidine detection since their oxidation peaks begin to be masked by electrolyte decomposition. At high pH thymine and cytosine oxidation peak potentials became closer and the peaks tend to overlap; in addition the increase of thymine peak current with pH makes difficult the identification of the cytosine oxidation peak (Fig. 4).

The differential pulse voltammetric peak current obtained for each base in equimolar mixtures was determined for concentrations ranging from 0.2 to 50 μM in pH 7.4, 0.1 M phosphate buffer supporting electrolyte with the GCE electrochemically preconditioned (see Materials and methods). The plots of peak current (I_p) vs concentration for each base in the equimolar mixtures are given in Fig. 5. The data obtained by linear regression for each base over the concentration range, in which the peak current has a linear response with concentration, are presented in Table 1. Each point in the plots in Fig. 5 represents the average of values obtained in three experiments and each error bar represents the standard deviation.

The LODs attained for each DNA base in equimolar mixtures were found to be 0.05 μM for guanine, 0.07 μM for adenine, 0.89 μM for thymine, and 1.76 μM for cytosine, based in (3 × SD)/slope [21]. Good linearity was found between 0.2 and 10 μM for purines and between 1 and 20 μM for pyrimidines, always in equimolar mixtures. To our knowledge, this was the first time that this simultaneous quantification of all four DNA bases in equimolar mixtures has been achieved, together with a low LOD for each base in the mixed solution.

For solutions of only one purine base, lower LODs for guanine (7 nM [22]) and adenine (30 pM [23]) have been determined indirectly through the formation of

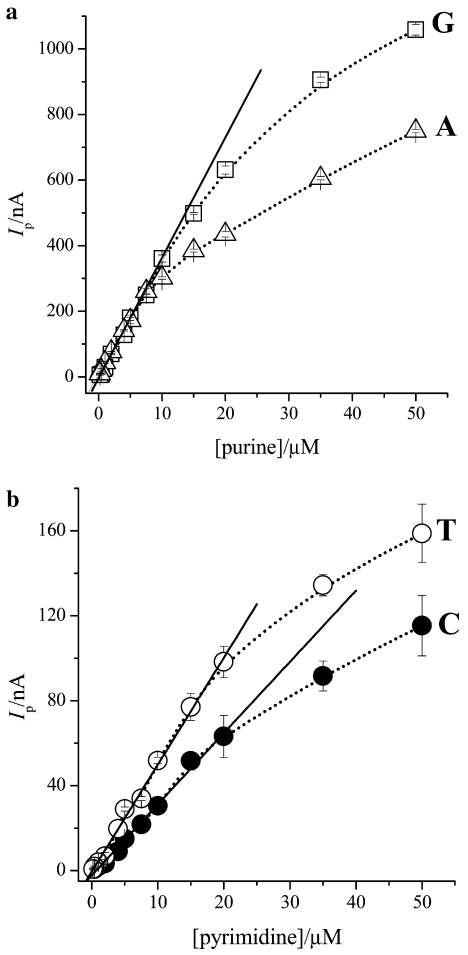

Fig. 5. Plots of I_p vs concentration: (a) guanine (G) and adenine (A); and (b) thymine (T) and cytosine (C) obtained for equimolar mixtures in pH 7.1, 0.1 M phosphate buffer supporting electrolyte solution by differential pulse voltammetry, using a preconditioned 1.5-mm-diameter GCE. Pulse amplitude 50 mV; pulse width 70 ms; scan rate 5 mV s^{-1}. Each error bar represents the standard deviation obtained for $n = 3$ experiments.

guanine– and adenine–copper complexes on mercury electrodes. Previous determination of the LODs for mixtures of thymine and cytosine by sonovoltammetry at GCE reported a value of 10 μM for both of these compounds [14].

The analysis of the I_p–concentration plots obtained for each of the DNA bases in equimolar mixtures by differential pulse voltammetry (Figs. 5a and b) gives information on the degree of interaction between each DNA base and the electrode surface and on lateral interactions between the DNA bases adsorbed on the surface.

The I_p–concentration plot for guanine (Fig. 5a) was found to fit well to Langmuir adsorption isotherm behavior [24,25] and it was already found that the formation of a submonolayer of adsorbed guanine at the electrode surface at low concentration occurred [18]. Contrarily, the I_p–concentration plot obtained for adenine (Fig. 5a) fits better a Temkin adsorption isotherm in which it is considered that the adsorption sites at the surface are not all equivalent due to surface inhomogeneities, the most favorable sites being first occupied by guanine oxidation products [18,26].

The I_p–concentration plot for thymine (Fig. 5b) fitted a Frumkin adsorption isotherm with positive interaction (positive g) [24,25], indicating an attractive interaction between the thymine and the electrode surface. This is interesting because it is expected that the electrode surface is already covered to some extent by guanine and adenine oxidation products [18,26] when the thymine oxidation begins to occur.

For cytosine (Fig. 5b), the I_p–concentration plot could be fitted by a Frumkin adsorption isotherm with negative interactions (negative g) which implies a weak or even repulsive interaction between the cytosine and the electrode surface and explains the difficulty in detecting the cytosine oxidation peak in an equimolar mixture solution.

Voltammetry of DNA nucleotides

In the structure of DNA each base is linked to a pentose–phosphate unit in the helix skeleton. Hence, the electrochemical oxidation of each deoxyribose-5-monophosphate was studied and compared with that of the corresponding free DNA base.

In Fig. 6 are presented the differential pulse voltammograms obtained in pH 7.4, 0.1 M phosphate buffer supporting electrolyte, with a GCE preconditioned as described, in solutions of a DNA nucleotide. It was possible to detect the oxidation peaks of GMP at 0.89 V [17,18] and AMP at 1.19 V [27]. For the first time the oxidation peaks of TMP at 1.41 V and CMP at +1.46 V were detected. In Fig. 6 is also shown the voltammogram obtained for the 20 μM equimolar mixture of all

Table 1
Linear regression analysis and limits of detection (LOD) using differential pulse voltammetry for the quantification of an equimolar mixture of DNA bases ($p < 0.0001$)

DNA base	LOD (μM)	Sensitivity (nA μM^{-1})	Linear range (μM)	Standard deviation (nA)	Regression coefficient	n
Guanine	0.06	34.46	0.2–10	0.64	0.9989	7
Adenine	0.07	33.67	0.2–10	0.79	0.9996	7
Thymine	0.89	5.04	1–20	1.51	0.9976	9
Cytosine	1.76	3.35	1–20	1.97	0.9967	9

Concentration range 2×10^{-9}–5×10^{-5} M each base. Experimental conditions as in Fig. 7.

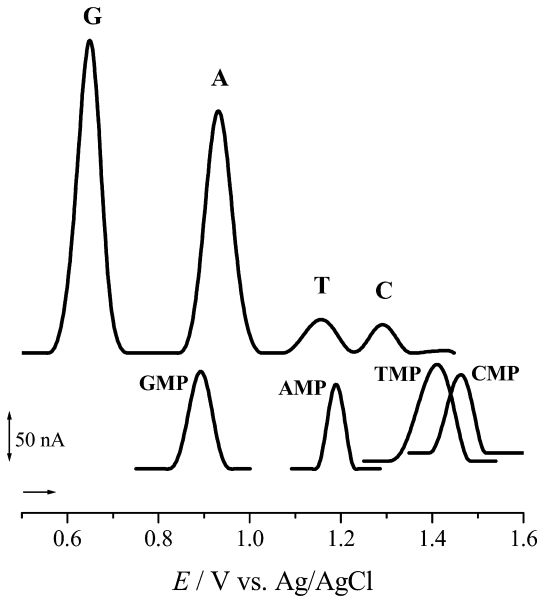

Fig. 6. Baseline-corrected differential pulse voltammograms obtained for a 20 μM equimolar mixture of guanine (G), adenine (A), thymine (T), and cytosine (C), 20 μM guanosine 5-monophosphate (GMP), 20 μM adenosine 5-monophosphate (AMP), 500 μM thymidine 5-monophosphate (TMP), and 500 μM cytidine 5-monophosphate (CMP) in pH 7.4, 0.1 M phosphate buffer supporting electrolyte with preconditioned 1.5-mm-diameter GCE. Pulse amplitude 50 mV; pulse width 70 ms; scan rate 5 mV s^{-1}.

DNA bases for a clear identification of the oxidation peak potential differences found between the bases and each respective 5-monophosphate nucleotide. The voltammograms were offset to facilitate peak identification and comparison.

Relative to the free DNA bases, the oxidation peaks of GMP, AMP, and TMP were always shifted by 250 mV to more positive potentials, whereas for CMP a difference of 170 mV was found. The oxidation of each corresponding nucleoside was observed to occur at the same potential of the corresponding 5-monophosphate nucleotide. These results are in agreement with previous work [14–18] and reflect the increased difficulty of oxidizing the deoxynucleosides and deoxynucleotides in comparison with the DNA bases. As the 2′-deoxyribose and the orthophosphate are not electroactive in the potential window used [6] and since the phosphate group appeared to have no influence in the oxidation peak potential, the observed shift in the oxidation peak of nucleosides and nucleotides relative to the corresponding base can be attributed to the inductive effect caused by the glycosidic bound on the π-system of purine and pyrimidine rings, making it more difficult to remove electrons from the bases [1,10].

In addition to the observed shift in the peak potential, the presence of the pentose–phosphate group causes a significative decrease in the oxidation peak current relative to the base, being even lower for the nucleotide than for the nucleoside. This diminution of the peak current can be explained by the lower diffusion coefficient of the nucleotide compared to that of the base and by the greater solvation energy caused by the polar sugar–phosphate group [1]. Moreover, the lower peak current obtained with nucleotides compared to that with nucleosides can be attributed to the steric effect caused by electrostatic interaction between the negatively charged phosphate group and the positively charged GCE surface during the potential scan, which orients the bases further from the electrode surface toward the solution bulk, thus increasing the energy necessary for reorganization of the nucleotide at the surface after adsorption and prior to charge transfer.

This diminution in peak current was observed to be more accentuated in the case of both pyrimidine nucleotides, and to obtain similar peak currents for all the nucleotides it was necessary to use solutions of pyrimidine nucleotides 25 times more concentrated than those used for purine nucleotides (Fig. 6).

The peak potential dependence on pH was studied for each nucleotide alone and was found to be similar to that observed for the corresponding base, noting the fact that for all pHs the oxidation peak potential was always shifted to more positive potentials as already described.

Detection of oxidation of all bases in ssDNA

A major challenge is the simultaneous detection of all bases in ssDNA in which the bases are linked to the sugar–phosphate backbone forming the biopolymer and are present in concentrations of the same order of magnitude.

The experimental results showed that the shift in the oxidation peak to more positive potentials observed for the nucleotides makes detection less easy. The smaller peak shift to more positive potentials observed for CMP (Fig. 6) implies that there will be an overlapping of TMP and CMP oxidation peaks when both are present in a sample and this makes the simultaneous determination of pyrimidine nucleotides directly in ssDNA even more difficult.

In Fig. 7 is shown the differential pulse voltammogram for a 40-μg/mL ssDNA solution in pH 7.4, 0.1 M phosphate buffer supporting electrolyte. The peaks denoted as G_r and A_r are attributed to the oxidation of guanine and adenine residues in the ssDNA according to present (see Fig. 6) and previous [7,17] results. The peak Py_r is attributed to the superposition of the peaks due to oxidation of the pyrimidine residues in the ssDNA. Experiments with poly(dT) and poly(dC) confirmed the pyrimidine nucleotides anodic voltammetric behavior (Fig. 7, inset). The higher peak current obtained for both purine residues compared to the peak current assigned to the pyrimidine residues oxidation is in agreement with the lower peak currents also ob-

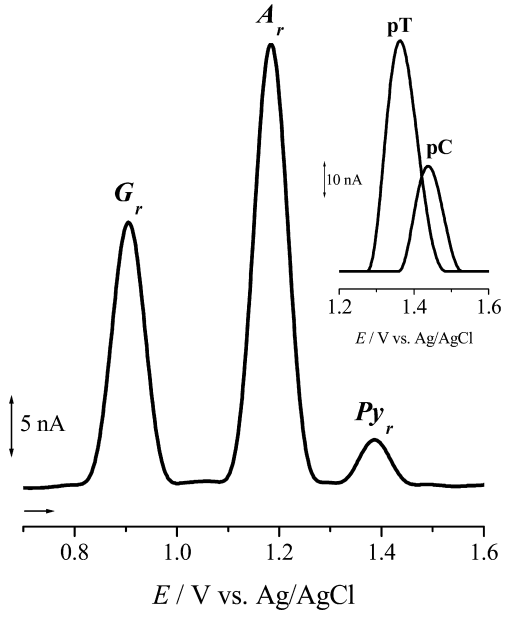

Fig. 7. Baseline-corrected differential pulse voltammogram obtained in a 40-μg/mL ssDNA solution pH 7.4, 0.1 M phosphate buffer supporting electrolyte with a preconditioned 1.5-mm-diameter GCE. G_r, guanine residue; A_r, adenine residue; Py_r, pyrimidine residue. (Inset) Baseline-corrected differential pulse voltammograms obtained in 100-μg/mL poly(dT) (pT) and poly(dC) (pC) solutions in pH 7.4, 0.1 M phosphate buffer supporting electrolyte. Pulse amplitude 50 mV; pulse width 70 ms; scan rate 5 mV s^{-1}.

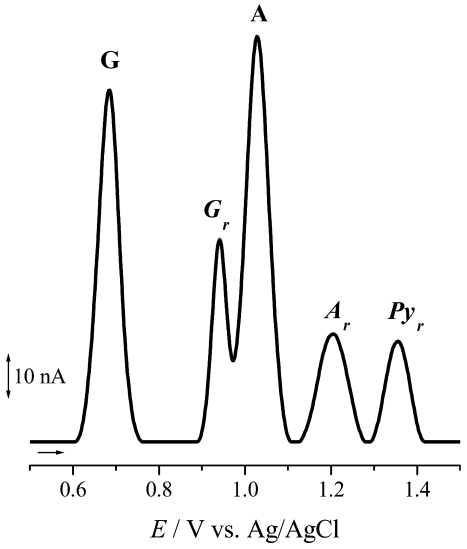

Fig. 8. Baseline-corrected differential pulse voltammogram obtained in a 40-μg/mL ssDNA solution pH 7.4, 0.1 M phosphate buffer supporting electrolyte with a preconditioned 1.5-mm-diameter GCE. The ssDNA was allowed to undergo mild acid depurination for 48 h prior to the experiment (see text for additional details). G, free guanine; A, free adenine; G_r, guanine residue; A_r, adenine residue; Py_r, pyrimidine residue in ssDNA. Pulse amplitude 50 mV; pulse width 70 ms; scan rate 5 mV s^{-1}.

tained for T and C nucleotide oxidation currents shown in Fig. 6. Nevertheless, it was possible to detect for the first time the oxidation peak currents corresponding to the pyrimidine residues in ssDNA.

In Fig. 8 is shown the differential pulse voltammogram obtained for a ssDNA solution allowed to undergo depurination by mild acid conditions (pH 3.6, 0.1 M acetate buffer) for 48 h [1,10]. In addition to the oxidation peaks due to the guanine (G_r), adenine (A_r), and pyrimidine (Py_r) residues in the ssDNA, two oxidation peaks attributed to free guanine (G) and adenine (A) are observed, indicating that depurination had occurred, causing, consequently, the diminution of both G_r and A_r oxidation peaks and the concomitant appearance of the free G and A oxidation peaks.

The difficulty in the voltammetric analysis of ssDNA occurs from oxidation peaks overlapping when the free bases and nucleosides or nucleotides are present in the same sample. As can be seen in Fig. 6, the oxidation peak of GMP will cause interference in the detection of the adenine oxidation peak and the AMP oxidation peak will overlap with the oxidation peak of thymine. This implies that for a proper characterization of an unknown DNA sample attention must be given to careful analysis of the voltammogram. The presence of shoulders or broader peaks in the voltammogram could simply mean that bases, nucleosides, and/or nucleotides, of DNA are present in a given sample due to DNA damage, as in the case shown in Fig. 8. Nevertheless, this apparent drawback becomes qualitatively advantageous when the main goal is to verify the integrity of a double or single strand of DNA. This shows that electrochemical methods are a good analytical tool that can be used to confirm the occurrence of any damage or enzymatic activity (e.g., glycosidic, nuclease) over DNA that may liberate free bases or disrupt the polymeric strand. In general, the detection of a guanine oxidation peak in a ssDNA sample is a good indication that enzymatic base excision or acid depurination has occurred to some extent.

Conclusion

The study of DNA oxidative damage is of great importance since it is well established that it constitutes an important source of genomic instability. Voltammetric methods are suitable to study DNA oxidation, but almost all previous studies focus only on the oxidation of purine derivative compounds.

The voltammetric results presented in this work extend previous voltammetric studies on DNA to include all four bases and corresponding nucleotides. For the first time equimolar mixtures of all DNA bases, nucleosides, and nucleotides have been quantified by differential pulse voltammetry.

An electrochemical preconditioning of the GCE enabled the achievement of a better peak separation and an enhancement of the current of the oxidation peaks

for all four DNA bases in pH 7.4 phosphate buffer supporting electrolyte, close to physiological pH. Detection limits in the nano- and micromolar ranges were obtained for purine and pyrimidine bases, respectively, together in solution.

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

Acknowledgments

Financial support from Fundação para a Ciência e Tecnologia (FCT), Ph.D. Grants PRAXIS XXI/BD/6134/2001 (J.A.P.P.) and PRAXIS SFRH/BD/877/2000 (V.C.D.), projects PRAXIS/P/AGR/12205/1998, POCTI (cofinanced by the European Community Fund FEDER), ICEMS (Research Unit 103), and European Projects QLK3-2000-01311 and HPRN-CT-2002-00186 are gratefully acknowledged.

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