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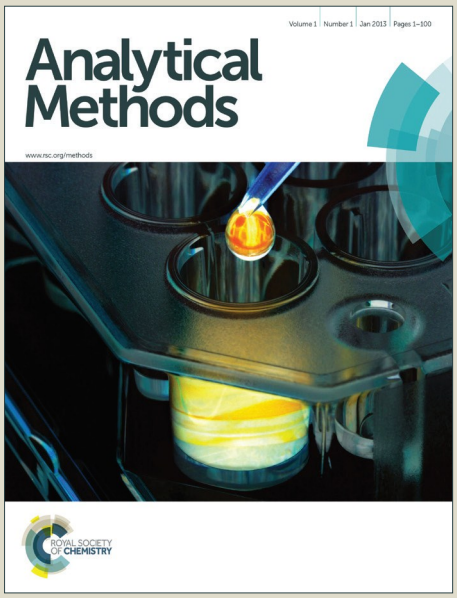

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Carbon materials for the electrooxidation of nucleobases, nucleosides and nucleotides toward cytosine methylation detection- A review

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Improved analytical methods for the determination of the methylation degree of DNA is of vital relevance as it may allow the detection of certain diseases such as carcinomas and infertility among others, in the early stages of development. Amongst the analytical methods for the detection and quantification of epigenetic modifications in DNA, electroanalytical platforms are emerging as potential and feasible tools for clinical purposes. This review describes the basic fundamentals of the electrochemical response of nucleobases, nucleosides, nucleotides and DNA in general; from the pioneer studies at mercury electrodes to the most recent ones during the last two decades. Concerning these latter studies, we will exclusively focus on carbonaceous electrodes such as carbon, graphite, glassy carbon, boron doped diamond, carbon nanofibers, carbon nanotubes and graphene. This review will also provide a vision about the feasibilities of the electrochemical sensors development for the simultaneous determination and quantification of naturally occurring DNA bases and nucleotides as well as the cytosine methylation in DNA using carbon materials.

Introduction

Nucleotides are organic molecules formed by covalent attachment of a five-carbon monosaccharide (pentose), a nitrogenous base and a phosphate group. Nucleotides play different roles in life processes, but the most important one refers to the formation of the structural units of the ribonucleic acid (RNA) and the deoxyribonucleic acid (DNA).[1, 2] The nitrogenous bases are cyclic organic compounds that include two or more nitrogen atoms and their sequence in the genes gives each living organism their identity itself.[3] The main bases found in DNA are Guanine (G), Adenine (A), Thymine (T) and Cytosine (C), in which they are classified into two groups, such as purines (A and G) and pyrimidines (C and T). Changes in DNA can either have no effects on the life of an organism or otherwise cause important consequences on it. If changes in genomic sequence occur, we talk about "mutations", whereas if we refer particularly to a change of a single nucleotide, we talk about a single nucleotide polymorphism (SNP). However, when covalent modifications at the DNA strand take place without any change in the gene sequence, this is called "epigenetic modifications".[4] Unfortunately, these epigenetic changes are not fully understood yet. One of the most relevant epigenetic modifications is that related to the methylation of cytosine, which consists in the methylation of the C5 carbon atom of the cytosine moiety. Methylation usually occurs in areas enriched in guanine and cytosine called CpG islands, which are often located in the gene promoters Promoter sequences are DNA sequences, which are typically located directly upstream or at the 5' end of the transcription initiation site, define where transcription of a gene by RNA polymerase begins).[5] DNA hypermethylation may cause silencing of tumor-suppressor genes, provoking many types of human tumors,[6] such as carcinomas,[5, 7] lung,[8] thyroid,[9] leukemia,[10] prostate,[11] and pancreas[12] tumors. Furthermore, it has been observed that when methylation occurs, other diseases such as infertility,[13-17] Beckwith-Wiedemann syndrome[18] (overgrowth syndrome)[19] and Angelman syndrome[20] (neurogenetic disorder)[21], among others, can be caused. Overall, it is well-established that the knowledge of the epigenetic changes that occur in human diseases would allow us to detect these diseases in their early stages and hence it could be vital to address their treatment in the future. The potentially reversible state of this process is an ideal target to create therapeutic strategies, which would imply the re-activation, or silencing, of specific genes.

Methylation detection and quantification is found in the literature through the use of several conventional techniques, such as bisulfite sequencing,[22, 23] PCR (Polymerase Chain Reaction),[24] MSP (Methylation specific PCR),[25] immuno-based detection,[26] liquid,[26-29] gas[26, 30, 31], and capillary[26, 32, 33] chromatography and microarrays.[34] Despite the sensitivity of the above mentioned techniques, they are not introduced into the routine of the clinical analysis laboratories because they are expensive and time consuming. In this regard, in recent years, the development of electrochemical sensors has been proposed as a promising alternative. Sensors are devices that convert physical or chemical information into a useful signal that can be processed, thereby quickly providing information of interest and without complex analysis.[35] Electrochemical sensors enjoy of a prominent position in the market for analytical instrumentation, and when compared with other types of chemical sensors they are simpler devices without need of very sophisticated measuring equipment. In summary, electrochemical sensors have certain advantages: firstly, the electrical signal is translated and easily processed into a quantitative value, such as analyte concentration; secondly, electrochemical sensors are miniaturized devices, allowing the analyst to work with small sample volumes; thirdly, the limits of detection are reduced, so low concentrations of the analyte to be determined is provided; and finally, electrochemical sensors

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are often mass produced, which grants them to be marketed as disposable devices due to their low cost.[36] Those characteristics gives rise us the examination of mutations and epigenetics modifications in a simpler, cheaper and faster way, in order to implement them in clinical analysis laboratories as diagnostic applications, hopefully in the near future.

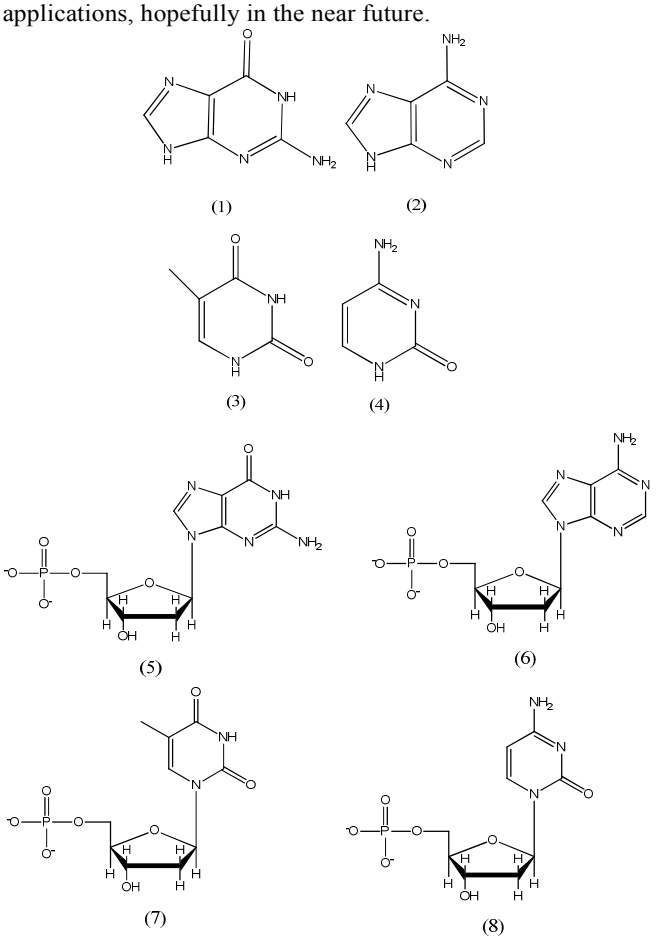

Figure 1. Structure of purine and pyrimidine nucleobases and their nucleotides derivatives. (1) Guanine, (2) Adenine, (3) Thymine, (4) Cytosine, (5-8) correspond to nucleotides (a nucleobase attached to a 5-carbon sugar, namely deoxyribose, with one phosphate group); (5) 2´-deoxyguanosine-5´-phosphate (GMP), (6) 2´-deoxyadenosine-5´-phosphate (AMP), (7) 2´-deoxythymidine-5´-phosphate (TMP), (8) 2´-deoxycytidine-5´-phosphate (CMP).

For the development of electrochemical sensors to be then applied to the detection and quantification of epigenetic modifications, it is of crucial importance to be aware of the electrochemical response of the different nucleobases (see Fig. 1) as well as those coming from more complex molecules, such as nucleotides, single strand DNA (ssDNA) and double strand DNA (dsDNA). In particular, their electrochemical reduction and oxidation responses at different electrode materials have been the subject of innumerable contributions. The literature in this field is vast, and the purpose of this article is to review, from our point of view, the most significant achievements concerning the electrochemistry of nucleobases, nucleosides, nucleotides and oligonucleotides using carbonaceous materials, which can prompt the development of electrochemical sensors over the years for the detection and determination of methylation in DNA and other emerging epigenetic modifications.

Direct reduction of DNA bases: The mercury legacy

The first data concerning the electroactivity of nucleobases was published in 1946.[37] Earliest works in 1960s were performed using polarographic techniques and showed that adenine and cytosine were reduced in aqueous medium at pH 4.2 with a half wave potential of -1.33 V and -1.44 V, respectively, versus a saturated calomel (SCE) reference electrode at a mercury electrode.[38-41] In the case of adenine, its reduction involved the transfer of four electrons while only three in the case of cytosine. Using polarography, no changes were observed between nucleobase, nucleoside or nucleotide, but it was possible to distinguish ssDNA of different lengths.[38, 42-44] It was also observed that cytidine and nucleotide poly(C) showed a slightly more positive reduction potential peak (Ep) than the corresponding free base. When the pH values rose to values higher than 4, a shift of the reduction towards more negative potentials was observed, suggesting an important role of the protonation.[41] On the other hand, guanine could be only reduced at very negative potentials. In fact, the potentials required for its reduction were close to the electrolyte discharge and unstable products were formed. Nevertheless, the subsequent oxidation of the reduction products were, through cyclic or anodic stripping voltammetry, better observed than the nucleobase reduction itself.[45] However, no reductive signals were observed in aqueous medium for uracil and thymine[38, 39] using mercury electrodes, as they overlapped with the water reduction process. Therefore, non-aqueous solvents such as dimethylsulfoxide or acetonitrile, with wider electrochemical potential windows were employed.[46]

In general, the reduction of the different nucleobases is complicated, requiring very negative reduction potentials and involving the formation of many radical species.[46, 47] Recently, for example, ionic liquids have been used on platinum electrodes for studying guanine reduction obtaining a reduction peak at about -2.2 V vs a Ag pseudo reference electrode.[48]

As far as the DNA electrochemical response is concerned, Palecek´s group used a mercury electrode to study the reduction and oxidation of DNA and, using oscillographic polarography, they were able to correlate the concentration of captured DNA with the redox process thereof.[49] Currently, it is known that nucleic acids are electroactive, but their reduction and oxidation at electrodes are irreversible, producing signals at highly negative or positive potentials.[50-52] In further works, a methodology to discriminate between single and double stranded DNA through direct DNA reduction was also developed.[53] Thus, it was demonstrated that the polarographic method could be used as a technique to study the denaturation or melting (when DNA double helix collapse and the DNA strands separate), premelting[54] and renaturation or hybridation (when the denaturation is reverted and the separated strands reform their double helical structure).[55]

One of the main problems that had to be addressed was the volume of sample needed, which had to be necessarily reduced. By introducing adsorptive transfer stripping voltammetry (AdTSV), the sensitivity of the determination of DNA improved by several orders of magnitude[56-58] and allowed working with comparable volumes to those used in gel electrophoresis of nucleic acids. In this electrochemical technique, DNA is adsorbed on the mercury electrode, resisting a subsequent washing, and it was observed that

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the voltammetric response of immobilised DNA did not differ substantially from that obtained by conventional voltammetry with the electrode immersed in the solution containing the DNA. Thus, it was demonstrated that both ssDNA and dsDNA are irreversibly adsorbed on mercury electrodes.[59] Also, Miller et al. studied the surface activity of the nucleic acids adsorption upon mercury electrodes, showing that the adsorption took place between 0 and -1.1 V and the desorption at 1.2 V[60-62] and ssDNA desorbs at more negative potentials.[63]

Direct oxidation of DNA bases. Oxidation mechanisms

Although early works studied the reduction of DNA, in recent years there has been a boom in the study of oxidation due to its higher sensitivity and reproducibility in nucleobase, nucleoside and nucleotide detection. For this reason we will focus on the oxidation of DNA and its components in the following section. First of all, the different mechanisms of oxidation of nucleobases will be presented. Later, in the following sections their oxidations on different carbonaceous electrodes will be summarized and some relevant contributions discussed.

Purines (adenine and guanine) are oxidized at low potentials (being guanine the easiest to be oxidised). In addition, both bases give oxidation peaks in a wide range of pH (0-12.5).[64, 65] Guanine oxidation is an irreversible process that occurs in two steps. The first step is the oxidation of guanine to 8-oxo-7,8-dihydroguanina (8-oxoGua), which is an irreversible process and requires two protons and two electrons, following the path shown in Fig. 2. This product is considered a biomarker of DNA damage by oxidative stress[66, 67] and can be easily quantified by differential pulse voltammetry.[68] The second step is the oxidation of 8-oxoGua to 8-oxoGua$_{ox}$, involving another two protons and two electrons through a reversible reaction. The whole mechanism of oxidation of guanine follows a two-step mechanism involving four electrons and four protons.

In the case of adenine, its electrooxidation occurs in three steps, through a mechanism involving six electrons and six protons[69] yielding 8-oxo-adenine (Fig. 3). During this process, adenine molecules adsorb very strongly on the electrode surface (e.g. glassy carbon) in comparison with the oxidation products.[70]

The mechanisms for the oxidation of thymine and cytosine are illustrated in Fig. 4 and 5, respectively. The similarities for both mechanisms include both the nucleophilic attack of water to the radical cation 3a and 4a (yielding 3b and 4b and 4c, respectively) and the deprotonation pathway of the radical cation to form the radical of the thymine and cytosine bases, leading to the radical species 3c and 3d for the oxidation of thymine and 4d for the oxidation of cytosine.[71] A subsequent oxidation of oxidative thymine or cytosine will lead to radical reactions with oxygenated species or even molecular oxygen to provide stable products or further decomposition reactions.

Even more interesting, together with cytosine oxidation, is the oxidation of methylcytosine towards the formation of methyl oxidation products (Fig. 6). For instance, the deprotonation of the methyl group of 5-methylcytosine (9) occurs to form 5-methyl-(2′-deoxycytidylyl) radical (9d), which can be easily attacked by free oxygen to give rise to peroxyl radical (9g) and hydroperoxide (9j). Subsequently, those compounds can be decomposed to 5-hydroxymethylcytosine (9i) and 5-formilcytosine (9h).[71]

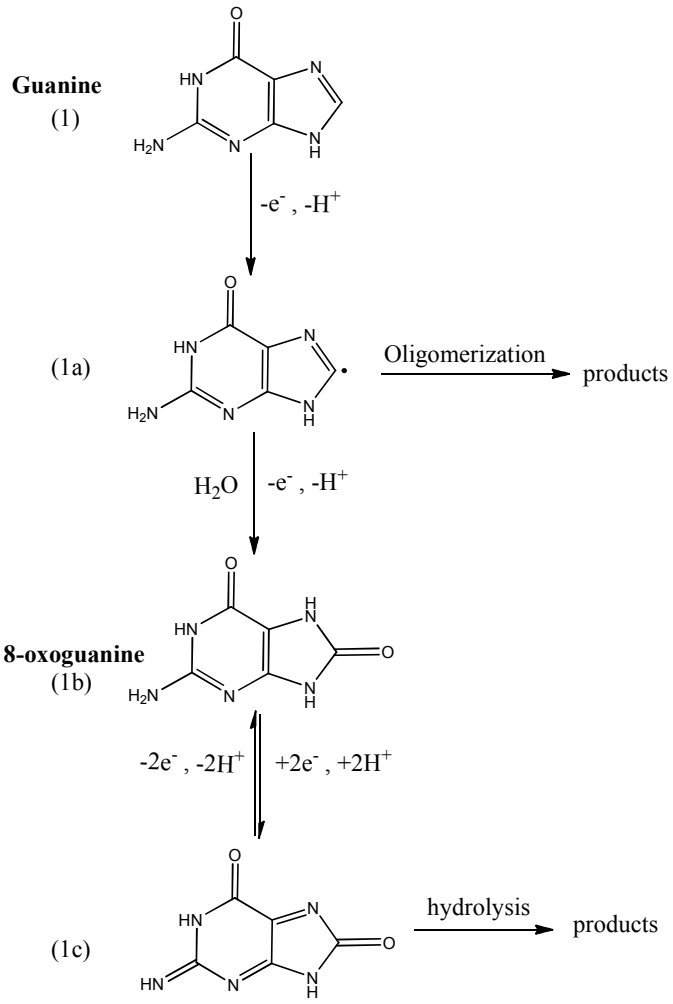

Figure 2. Mechanism for the electrochemical oxidation of Guanine in aqueous solution. (1) Guanine; (1a) guanine radical; (1b) 8-oxoGua; (1c) 8-oxoGua$_{ox}$

Carbon materials for the direct oxidation of DNA bases

Although direct DNA oxidation has been studied with different materials such as silver,[72, 73] platinum,[48] copper,[74] gold[75] and mercury,[76, 77] among others, the most widely used electrodes for studying the direct oxidation of nucleobases, nucleosides and nucleotides have been carbon based electrodes. Namely, these electrodes are graphite, glassy carbon, boron doped diamond, graphene, nanocarbon films and carbon nanotubes due to their unique properties in terms of structure and electric resistivity. The following sections will address the state of the art for most significant achievements in the electrooxidation of nucleobases, nucleosides, nucleotides and oligonucleotides with these different carbon materials.

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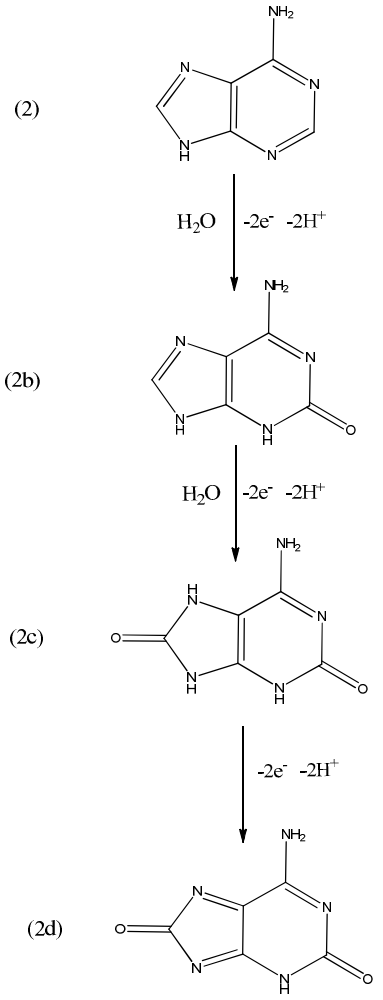

Figure 3. Mechanism for the electrochemical oxidation of adenine in aqueous solution. (2) Adenine; (2b) 8-oxo-adenine; (2c) 2,8-oxo-adenine; (2d) 2,8-oxo-adenine$_{ox}$

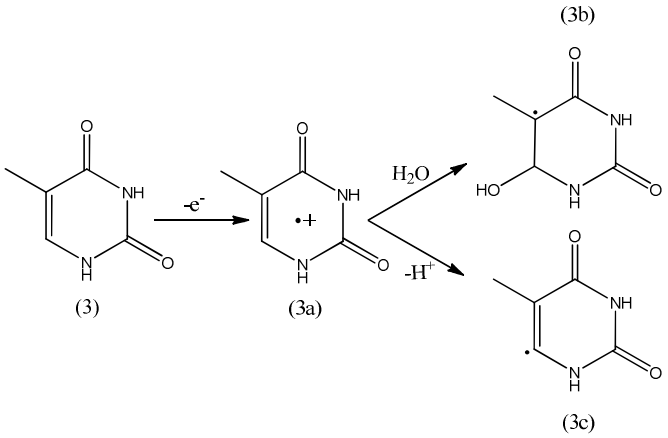

Figure 4. Proposed electrochemical mechanism for the oxidation of thymine in aqueous solution. (3) Thymine; (3a) radical cation of thymine; (3b) (3c) and (3d) radicals of thymine.

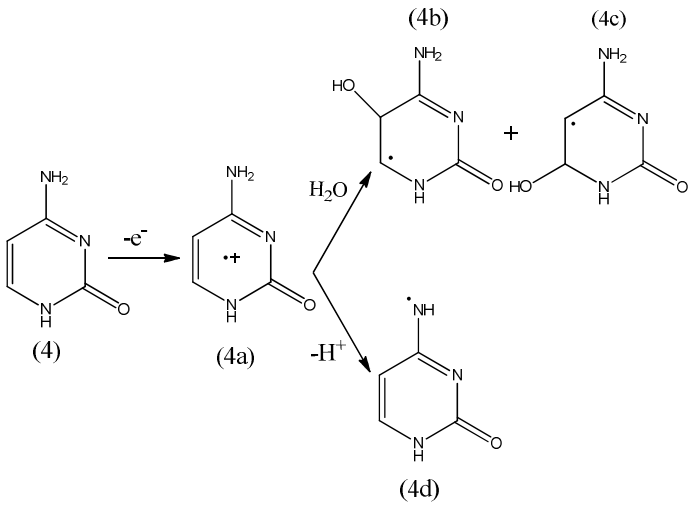

Figure 5. Proposed electrochemical mechanism for the oxidation of cytosine in aqueous solution. (3) Cytosine; (4a) radical cation of cytosine; (4b), (43c) and (4d) radicals of cytosine.

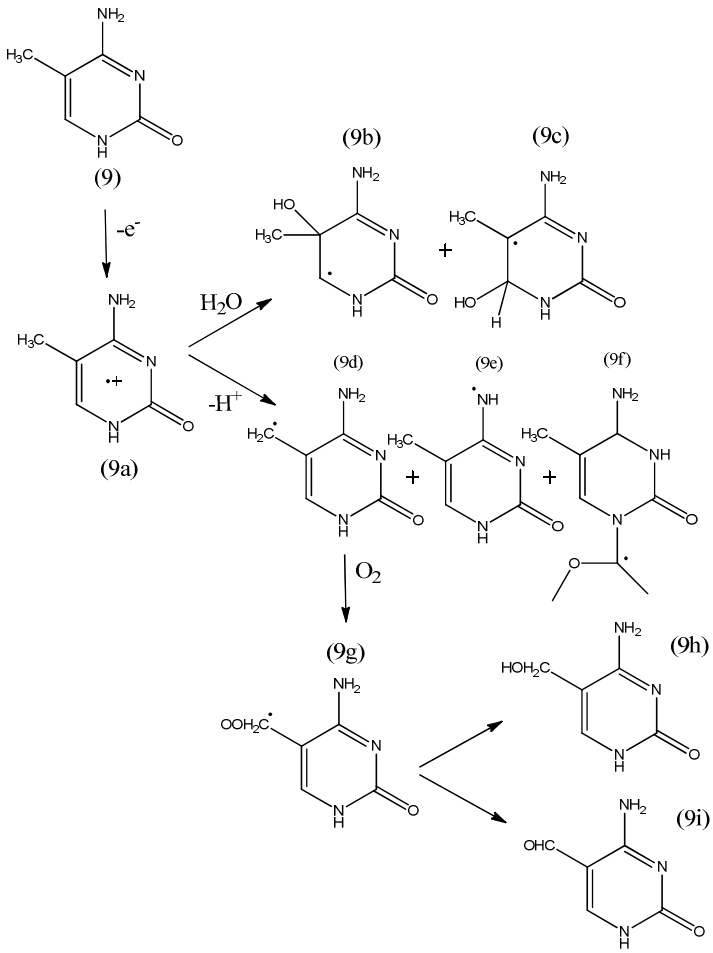

Figure 6. Proposed electrochemical mechanism for the oxidation of methylcytosine in aqueous solution. (9) Methylcytosine; (9a) radical cation of methylcytosine; (9b) (9c) and (9e) methylcytosine radicals; (9f) methylcytosine with sugar moiety; (9d) 5-methyl-(2′-deoxycytidylyl) radical. (9g) Methylcytosine peroxyl radical (9j) hydroperoxide; (9h) 5-formilcytosine and (9i) 5-hydroxymethylcytosine.

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Graphite

Smith and Elving[42] were the first who observed the oxidation of guanine on graphite electrodes in 1962. Initially, with pyrimidines, it was assumed that there was no electrochemical activity, not only at graphite electrodes,[40, 43, 78] but also at carbon electrodes in general.[79] This assumption was based on the fact that the detection of pyrimidine oxidation is complex with carbon electrodes, mainly due to interferences from background currents which hinder the detection of oxidation peaks. It was not until the incorporation and application of the square wave voltammetry (SWV), when carbonaceous electrodes started competing with electrochemical reduction processes at mercury electrodes in terms of sensitivity, thus allowing oligonucleotide detection in samples with sub-micromolar concentrations.[80, 81] Moreover, the wider electrochemical potential window of the carbon electrodes even garanteed the electrochemical detection of the methylated cytosine, which occurs at more positive potentials.[82]

Dryhurst[83] found that in the presence of guanosine, adsorbed guanine was displaced from the pyrolytic graphite electrode surface, resulting in a decrease in the guanine voltammetric peak. Other authors found that with carbon electrodes, it was common to encounter stability problems due to strong adsorption events suffered by both purine and pyrimidine bases.[84, 85] Conversely, opposite results were reported by Gilmmin and Hart, who observed no adsorption effect neither on glassy carbon nor on carbon paste electrodes.[86]

Brabec and Dryhurst[83] introduced graphite electrodes for the investigation of polynucleotides.[87] For example, single strand poly(A), was adsorbed on graphite, giving rise to relatively good signals because of its flexible structure, which conforms to a large extent to the contours of the rough electrode surface and hence allowing many adenine residues to be accessible to the electrode. However, double strand polynucleotide(A.A) has a more rigid structure and consequently it cannot conform so readily to the contours of the electrode surface, thus giving lower voltammetric peak currents since less adenine residues are accessible to the electrode surface.[87]

From a more pragmatic point of view, disposable screen printed graphite electrodes (SPGEs) can offer rapid, facile and economical evaluation of the electrochemical response of nucleobases,[86, 88, 89] including not only their detection and quantification but also the determination of the methylation degree of DNA,[90] as well as nucleosides and nucleotides.[90-92] These electrochemical devices are cheap and easy to produce upon a large scale, allowing them to be disposable and thus, having the potential to be used in protocols for clinical analysis laboratories in the future. Unfortunately, we have to bear in mind that with these electrochemical platforms we can find variability in the results about the electrooxidative response of DNA components, both in peak intensity and peak potential, due to likely differences between carbon paste formulations.[90, 92, 93] In this regard, Stempkowska and co-workers studied the oxidation of the nucleosides monophosphate with carbon paste electrode. They reported that the main peaks for their oxidations were centered at +1.00 V for the GMP (5), 1.28 V for AMP (6), 1.46 for TMP (7) and 1.53 V for CMP (8).[92] Moroever, peak potentials of G, A and T shifted linearly with pH with slopes close to the Nernstian theoretical value of 57 mV pH^{-1} at 25 ºC, while for C and mC the slope values were slightly higher (63 mV pH^{-1} and 70 mV pH^{-1}). The same group also observed that T base gave an electrochemical signal in oligonucleotides only when its amount reached almost 50 % of all bases. In addition, its adsorption competes with that of C, so the latter gives rise to a well-defined response either when T is absent or when the amount of C significantly exceeds that of T in an oligonucleotide.[92]

Brotons *et al.* recently obtained the oxidation peaks of the nitrogenous bases at +0.58 V for G, 0.88-0.98 V for A, 1.02 for T. 1.21 V for C, 1.12V for mC and 0.84 V for mG versus a pseudo reference electrode of Ag/AgCl paste using SPGEs with planes like-edge characteristics.[90, 94] Also, we determined the anodic peak potentials of several 6-mer oligonucleotides with peak potential values of +0.82, +1.07, +1.1 and +1.19 V regarding poly (G) (5´-GGGGGG-3´), poly (A) (5´-AAAAAA-3´), poly (T) (5´-TTTTTT-3´) and poly (C) (5´-CCCCCC-3´), respectively.[90] Among these oligonucleotides, polynucleotide (T) gave the poorest electrochemical response in comparison with the others polynucleotides, showing only a shoulder related to its electrooxidation. It is important to highlight the generally lower currents observed in peak intensity when working with nucleotides instead of the free nucleobases. This decrease is mainly due to the inductive effect caused by the glycosidic bond in the *pi*-system of purine and pyrimidine rings, thus making it more difficult to withdraw electrons from the bases.[90] The same authors also used screen printed graphite electrodes to explore the electrochemical response of several 6-mer oligonucleotides with different base sequences (e.g 5'-TTTCGC-3, 5´-AAACGC-3, 5´-TTACGC-3 and 5´-TAACGC-3) in 0.1 M acetate buffer pH 5. The oxidation of G, A and presumably T took place at around +1.0 V making almost undistinguishable the simultaneous identification of these bases. However, the oxidation of C was reported at around +1.3 V. In addition, it was also possible to qualitatively distinguish between non-methylated and methylated oligonucleotides (e.g. 5´-CGCGCG-3´ and 5´-mCGmCGmCG-3´) as can be observed in Fig. 7 and 8.[90]

Glassy Carbon (GC)

Several research groups have conducted an extensive study about the oxidation of nucleobases, nucleosides and nucleotides at GC electrodes. This electrode displays the oxidation peaks of the nitrogenous bases at +0.7 V for the guanine ,+0.96 V for adenine, +1.16V for thymine and +1.31 V for cytosine versus a reference electrode of AgCl/Ag in phosphate buffer pH 7.4.[88] As stated before, purines (G and A) are more easily oxidized than pyrimidines (T and C), which require higher potentials for their oxidation.[88, 95, 96] Generally, in the case of purines well defined signals are obtained during cyclic voltammetry, while in the case of cytosine, its oxidation overlaps with the electrooxidation of water/electrolyte.[96] On the other hand, thymine shows a well-defined oxidation wave with a half-wave potential of +1.27 V versus a AgCl/Ag reference electrode.[96]

When a pentose and a phosphate group are attached to the nucleic base moiety (i.e. a mononucleotide) the oxidation peaks are centred at +0.89 V, for GMP (5)[88, 97, 98], [7, 96, 97] at +1.19 V, for AMP (6), at +1.41 V for TMP (7)[88] and at 1.46 V for CMP (8)[88] versus a reference electrode of AgCl/Ag using differential pulse voltammetry (DPV) in 0.1 M phosphate buffer solution pH 7.4. In comparison with the aforementioned peak potentials of the nucleobases, it is clear that all peaks are shifted to more positive potentials (differences range from 0.15 to 0.25 V) as it can be observed in Fig. 9. This shift makes detection more difficult because of the previously mentioned hindering effect.[90] Additionally, there is a decrease in the peak current after adding the pentose and the phosphate group, which can be explained by lower diffusion coefficient of the nucleotide than the free

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base and by the higher solvation energy caused by the polar sugar–phosphate group[99]. Moreover, the peak current becomes even lower when working with nucleotides instead of nucleosides. This decrease is attributable to the steric effect caused by the electrostatic interaction between the negatively charged phosphate group and the electrode surface that is positively charged during the potential scanning, orienting the base moiety far away from the electrode surface. Thus, an increase in the energy required for the reorganization of the nucleotide on the surface after adsorption and before the charge transfer takes place.[88]

Interestingly, a correlation has been found between the peak potentials of the nucleobases and the electrolyte pH. In fact, a linear trend is observed when plotting E_p vs pH. In the case of the purines for the whole pH range studied (3.5-11.5) the slope of this line is -60 and -58 mV pH^{-1} for guanine and adenine, respectively.[40, 88] These values suggest that the number of protons and electrons involved in the oxidation mechanism is equal, as previously discussed in Fig. 2 and 3. For pyrimidines, similar slope values were reported in the range of pH 3-9, being equal to -59 and -61 mV for thymine and cytosine, respectively. Nevertheless, for pHs higher than 10, a slope of -84 and -106 mV was found for thymine and cytosine, respectively, indicating that the ratio of the number of protons and electrons involved in the charge transfer changed from 1 to 1.5 and to ~2, respectively. In addition, in the case of cytosine, for pH<4.5 a value of -88 mV was obtained, which was related to the first pK_a of cytosine, which is equal to 4.6.[88]

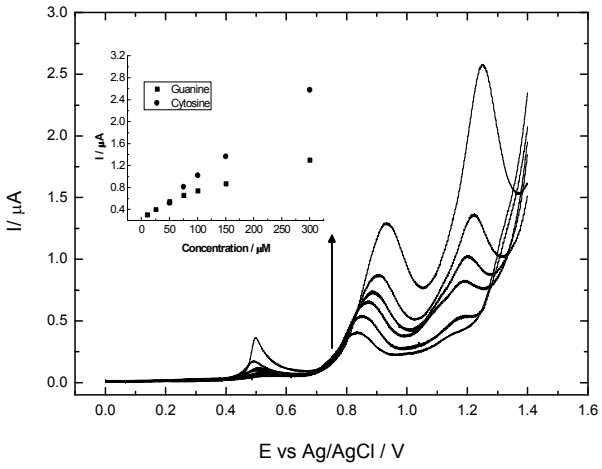

Figure 7. SWVs of 5′-CGCGCG-3′ as a function of concentration in 0.1 M acetate buffer pH 5.0. Inset figure: plots of anodic peak intensity of G and C obtained from the SWV response of 5′-CGCGCG-3′ against oligonucleotide concentration. SWV parameters: modulation amplitude, 50 mV; modulation frequency, 10 Hz; modulation step, 2 mV. Starting potential at 0 V. Oligonucleotide concentrations: 25, 50, 75, 100, 150 and 300 µM. Reproduced from Ref. [90] with permission from The Royal Society of Chemistry.

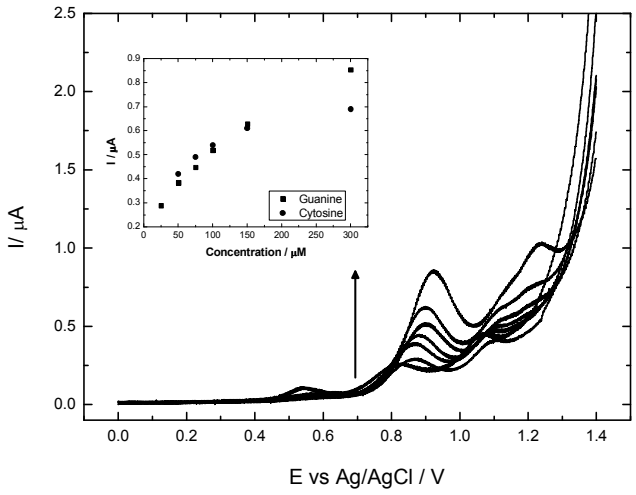

Figure 8. SWVs of 5′-mCGmCGmCG-3′ as a function of concentration in 0.1 M acetate buffer pH 5.0. Inset figure: Plots of anodic peak intensity of G and mC obtained from the SWV response of 5′-mCGmCGmCG-3′ versus oligonucleotide concentration. SWV parameters: modulation amplitude, 50 mV; modulation frequency, 10 Hz; modulation step, 2 mV. Starting potential at 0 V. Oligonucleotide concentrations: 10, 25, 50, 75, 100, 150 and 300 µM**.** Reproduced from Ref. [90] with permission from The Royal Society of Chemistry.

Moreover, a relationship between the peak current and the concentration was found for all bases, although each base fitted with a unique isotherm. Guanine fitted with the Langmuir adsorption isotherm,[88, 100] adenine with the Temkim isotherm[70, 88, 98] and thymine[88, 100] and cytosine[88] fitted with Frumkin isotherm (T with positive, attractive, and C with negative, weak or repulsive, interactions). This explains that a guanine submonolayer adsorbed on the electrode is formed already at low concentrations. The most favourable sites are occupied by guanine oxidation products, due to the fact that the surface is not as uniform and hinders the adenine adsorption. Unexpectedly, despite the previous adsorption of adenine and guanine, thymine has a high attraction on the electrode surface, and cytosine has a weak or even repulsive interaction with the electrode surface which explains the difficulty in detecting it. [88] In addition, there is generally a signal decrease during successive scans when using cyclic voltammetry of both purine [101] and pyrimidine[96] nucleobases. This effect is mainly ascribed to the adsorption of oxidation products on the electrode surface. Finally, it is worth mentioning that there still exists some controversy regarding the role of adsorption in the electrochemical determination of the components of DNA with glassy carbon electrodes. While some groups have reported an adsorption effect for this electrode[89, 102-104], opposite results have been published by other groups. For example, Gilmartin and Hart observed no effect of adsorption on glassy carbon electrodes.[86]

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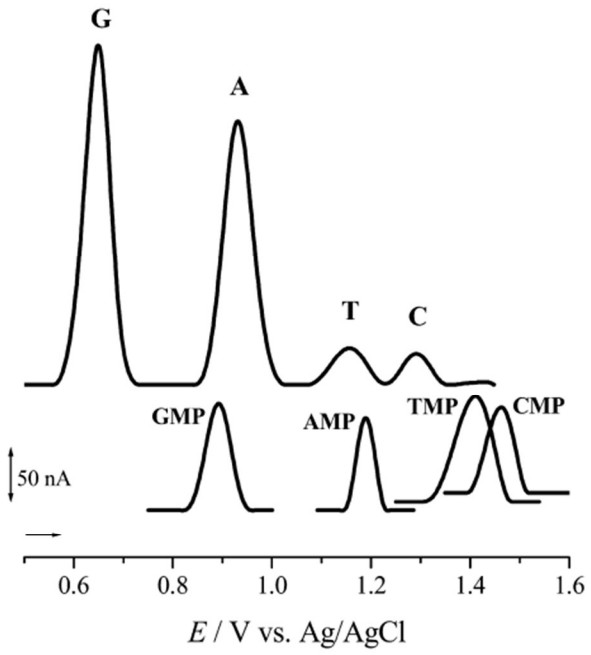

Figure 9. Baseline-corrected differential pulse voltammograms obtained for a 20 μM equimolar mixture of G A T and C and for 20 μM GMP, 20 μM AMP) 500 μM TMP, and 500 μM CMP in pH 7.4, 0.1 M phosphate buffer supporting electrolyte with preconditioned 1.5-mm-diameter GC. Pulse amplitude 50 mV; pulse width 70 ms; scan rate 5m V s^{-1}. Reprinted from Ref. [88]. Copyright (2004), with permission from Elsevier.

Finally, by studying more complex systems, such as ssDNA at GC, three different peaks attributed to the oxidation of guanine, adenine and pyrimidines were observed, going from low to high potential, respectively[97]. Long DNA chains make relatively little contact with the electrode surface, because the strand does not fit at the electrode surface, while free bases can do it[97]. When dsDNA is analysed, the bases are located inside the double helix, so it is even more difficult for them to reach the electrode surface and thus, oxidation signals are more difficult to be detected at the voltammogram[82].

Boron-doped diamond electrodes (BDD)

BDD electrodes are very advantageous to detect nucleobases due to some of their properties, including high reproducibility and stability, and robustness under extreme conditions where conventional electrode materials may suffer erosion and fouling. In addition, BDD shows a wide potential window in aqueous solutions so that those substances that oxidize at very positive potentials can be electrochemically detected with only small background currents.

There is literature about the oxidation of nitrogenous bases both with anodically oxidized BDD (AO-BDD) and as-deposited or pristine BDD (AD-BDD) electrodes with slightly different results. For example, for the AD-BDD electrode, the oxidation peaks of the nitrogenous bases are centered at +1.13 V for guanine, +1.54 V for adenine, and 1.60 V for cytosine, whereas it is not clear for thymine being buried with the water oxidation current (100 mM ammonium buffer pH 4.25).[105] On the other hand, with the AO-BDD electrode the oxidation peaks for the same nucleobases are centred at 1.18 V, 1.56 V, 1.96 V and 1.77 V for guanine, adenine, thymine and cytosine, respectively (100 mM ammonium buffer solution pH 4.25). In addition, in the case of AO-BDD, peaks are better defined both for purines and pyrimidines, while with AD-BDD, difficulties are found in order to observe the oxidation peak of thymine, since at high potentials (> +1.5 V) BDD surface is oxidized and the measurement is unstable[105], as shown in Fig 10. Note that, to the best of our knowledge, this is the only publication we have found where the oxidation of the cytosine occurs at less positive potential than the oxidation of the thymine, what seems to contradict results upon other carbonaceous materials.

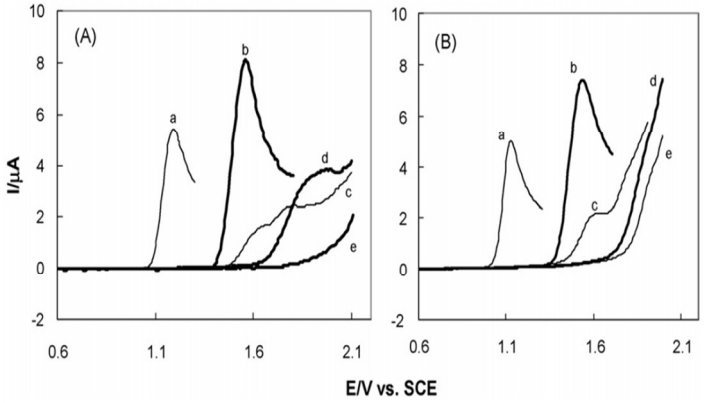

Figure 10. Linear sweep voltammograms of 100 μM (a) guanine, (b) adenine, (c) cytosine and (d) thymine in 100 mM ammonium acetate buffer solution (pH 4.25) together with (e) the corresponding background voltammogram at (A) AO and (B) AD-BDD electrodes. Scan rate was 100 mV/s. Reprinted from Ref. [105]. Copyright (2007), with permission from Elsevier.

In addition, among these two BDD types of electrodes, the oxidation of positively charged molecules is easier on AO-BDD, while, conversely, for negatively charged molecules is on AD-BDD electrodes. [106] Nevertheless, in the case of nucleobases, only slight differences in peak potentials were found between AD-BDD and AO-BDD electrodes. This fact is explained due to the fact that in acid medium, these bases behave as neutral or slightly positive charged molecules -as a result of hydrogen bonding with water molecules- so the effect of attraction / repulsion with interactions with the electrode surface is minimized.[105] With regard to the strong adsorption of molecules on the electrode surface, it is interesting to highlight that while that can provide high sensitivity, at the same time that can result in the formation of insulating layers on the surface, causing electrode fouling and non-lineal calibration curves.[107]

On the other hand, it can be stated that the oxidative processes of the nucleobases are under diffusion control, being the peak currents directly proportional to the square root of the scan rate ($v^{1/2}$) in the scan rate range from 25 to 300 mVs^{-1},[105] as well as for dGMP (deoxyguanosine monophosphate), tRNA (transfer RNA), ssDNA and dsDNA.[108]

As previously mentioned, the theoretical value of -60 mV pH^{-1} involves the mobilization of an equal number of electrons and

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protons. In the case of adenine and guanine this value is slightly higher in absolute value (-80 mV and -75 mV pH^{-1}, respectively) when using an AO-BDD electrode. In this regard, Ivandini et al. explained that the more positive potential required for purine oxidation can lead to other oxidation routes that could follow an oxidation process involving three protons and two electrons (i.e. -88 mV pH^{-1}).[105] However, at more alkaline pHs, the oxidation potential was kept constant which suggests a pH-independent process. Furthermore, thymine oxidation suggested a pH dependence with a slope of -60 mV pH^{-1}, which indicates the equal number of protons and electrons involved in the oxidation mechanism.[105]

Concerning the electrochemical response of mononucleotides, cathodically pretreated BDD electrodes revealed that the oxidation peaks of GMP, AMP, TMP and CMP are centred at 1.18 V, 1.52 V, 1.69 V and 1.85 V, respectively.[109] Nevertheless, those values shifted to 1.09 V, 1.46 V, 1.71 V and 1.89 V for GMP, AMP, TMP and CMP, respectively, if an anodically pretreated BDD electrode was employed using square wave voltammetry (SWV) in 0.1 M Britton-Robinson buffer solution (pH 7.0)[109]. However, irrespectively of the pretreated BDD surface, TMP and CMP responses required a deconvolution and/or background-substraction procedures [88, 109].

If a step forward is taken, more complex systems, such as ssDNA, were also studied. In this case, for GC, three different peaks were observed, which were ascribed, from lower to higher potential, to the oxidation of guanine, adenine and with pyrimidines. By comparing GC with BBD electrodes, it was first concluded that the surface pretreatment of the BDD electrode played an important role and was vital for the improvement of the electrochemical signal. For instance, AD-BDD gave almost a two-fold increase in the current in comparison with AO-BDD. Nevertheless, the signal of the ssDNA oxidation in BDD decreased during the second cycle due to electropassivation or fouling of the electrode. The reason for this is, on the one hand, the adsorption of the ssDNA molecule at the electrode surface, and, on the other hand, the oxidation of the electrode surface because generally voltammetric studies are conducted using very high, positive potential (0-1.5 V vs saturated calomel electrode (SCE)).[109]

Nanocarbon films

Kato and co-workers used electron cyclotron resonance (ECR) nanocarbon film electrodes[110] to develop an electrochemical analysis technique to detect short sequences of DNA[111] and methylation in DNA.[112, 113] With these electrodes, better responses were obtained compared to those observed with GC and BDD when analysing nucleotide monophosphates. The excellent chemical and electrochemical properties of the ECR films were ascribed to their homogeneous and stable structure that consists solely of nanocrystalline sp^2 and sp^3 carbon, which induces high electrode activity with aromatic DNA bases as a result of *pi–pi* interactions.[111, 113, 114] This fact provides excellent electrochemical characteristics, including a low background current, a wide potential window and a negligible surface fouling by analytes after oxidation.

When the free bases are examined by square wave voltammetry, the oxidation peaks are roughly at the same potentials than those obtained for the mononucleotides being +1.1 V for G and GMP, +1.39 V for A and AMP, +1.69 V for T and TMP and +1.72 V for C and CMP measured in 50 mM acetate buffer pH 5.0.[111, 113, 115]. Kato and co-workers [114] were also able to differentiate nucleobases in short chains of ssDNA. However, in this case, a remarkable diminution in sensitivity was observed in comparison with short nucleotides, because of the higher average distance between each electroactive base and the surface of the electrode, giving rise to conformational impediments together with a diminution of the diffusion coefficient because of the molecular size.[114] To solve this problem, Goto et al. digested enzymatically the ssDNA strand thereby recovering peak intensity of the different nucleotides and as it can be observed in Fig. 11 they were capable of determine the methylation rate of CpG60s-mers after digestion.[112] The same group achieved SNPs detection in combination with different concentrations of two oligonucleotide samples by using ECR nanocarbon films throughout SWV in acetate buffer (50 mM, pH 3.3) containing 2 M sodium nitrate.[111, 113] Additionally, ECR electrode also proved the feasibility to differentiate between both methylcytosine and cytosine free bases present in short oligonucleotides.[111, 113] Finally, these electrodes have also been used in combination with HPLC to study the 8-hydroxy-2'-deoxyguanosine in urine samples, giving better results than GC.[116]

Graphenes

Graphene is a fashion material which has attracted much attention during the last years in a wide number of scientific fields.[117, 118] Graphene is the first example of a strictly two-dimensional material (2-D), with a single sheet of one carbon atom thickness. Graphene displays fascinating physico-chemical properties[117-120] because of its flexibility and hardness,[119] together with a high electrical conductivity at room temperature.[120] Moreover, from graphene moiety, other carbon materials can be constructed such as fullerenes (0-D), carbon nanotubes (1-D) and graphite (3-D).[120]

Zhou et al.[95] developed an electrochemical sensor based on a chemically reduced graphene oxide coated onto a glassy carbon substrate /GC. With this sensor, they were able to simultaneously obtain well defined signals of all four nucleobases. The better applicability of this graphene-based electrode compared, for example, with GC and graphite electrodes, is attributed to its single sheet nature, higher conductivity, larger surface area, antifouling properties and higher electron transfer kinetics for nucleobase oxidation. This gives rise to a high density of edgelike-plane defect sites and oxygen containing functional groups on the CR-GO film, thus providing many active sites which are beneficial for speeding up electron transfer between the electrode and species in solution.[121-123] Zhou and co-workers also studied different natural mutations such us the changes from 5′-CAT-GAA-CCG-3′ to 5′-CAT-GAA-CCA-3′ -with a transition from G to A- and from 5′-CAT-GAA-CCG-3′ to 5′-CAT-GAA-CTG-3′ implying a transition from C to T[95] with very attractive results as shown in Fig. 12. Furthermore it has been found that chemically reduced graphene based electrodes, are able to provide separate signals for the four bases both in ssDNA and dsDNA without the need for DNA hydrolysis or labeling. [95, 124]

To reduce the graphenes, it is taking force the electrochemical method, instead of the chemical one, because it is easy, fast, efficient and clean technique compared with the chemical method. Electrochemical sensors based on reduced graphene have shown electrochemical catalytic activity towards a variety of products[125, 126]. Zheng et al. studied the simultaneous oxidation of 5-mC and C on electrochemically reduced graphene-modified GC electrodes which

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enhanced the signal 11.9 and 3.3 times with respect to those obtained with bare GC. They also performed quantitative measurements of 5-mC and C in CpG islands with varying degrees of methylation without prior hydrolysis or hybridization. [127]

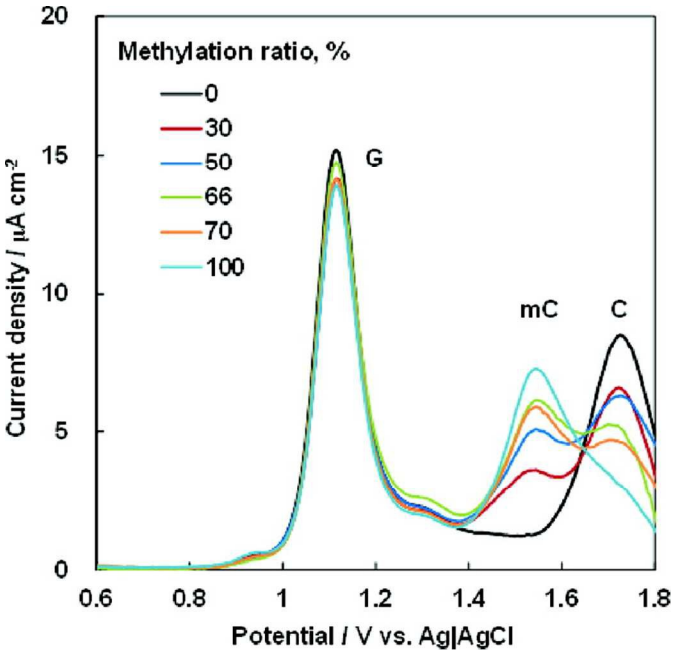

Figure 11. Background subtracted SWVs of 2 μM of digested CpG60s-mers with different methylation ratios at the nanocarbon film electrode in 50 mM, pH 4.4 acetate buffer containing 2 M $NaNO_3$. Amplitude =25 mV; ΔE = 5 mV; frequency = 10 Hz. Reprinted with permission from Ref. [112]. Copyright (2011) American Chemical Society.

Moreover, electrochemically anodized epitaxial graphene, consisting of oxygen-related defects, was found to be a good platform for the detection of nucleic acids, and other molecules.[128] In fact, mixtures of nucleic acids (A, T, C, G) could be resolved as individual peaks using differential pulse voltammetry. Furthermore, the simultaneous detection of all four bases in dsDNA without pre-hydrolysis step was achieved, and was also capable of distinguishing between ssDNA and dsDNA.[128]

In order to increase the number of flat edge sites, which give unique electrochemical and chemical characteristics Ambrosi et al.[129] published an elegant work involving nanofiber-stacked graphene (SGNFs) which consisted in nanofiber graphene sheets located in perpendicular orientation relative to the long axis of the fiber. This configuration gives unique properties since only the edge planes are exposed,[129-132] compared to usual graphite or CNTs. Concerning their electrochemical properties, it is worth mentioning that it is known that the "basal planes" of the graphene are substantially electrochemically less active than the flat edge sites of graphene sheets. This difference in activity of the graphene sites explains the higher electrochemical activity of SGNFs compared with other carbon materials. Graphene electrodes were also used for studying ssDNA (5′-GAACAAAGGTGTAACGGCAG-3′, a specific oligonucleotide for the human influenza A (H1N1) virus). A greater electrochemical response was obtained compared with other electrode carbon materials such as GC, multiwalled carbon nanotubes (MWCNT), edge plane pyrolytic graphite (EPPG) and GmP (graphite microparticles). However, it was also observed a lower current for the mononucleotide CMP than for free cytosine.[129]

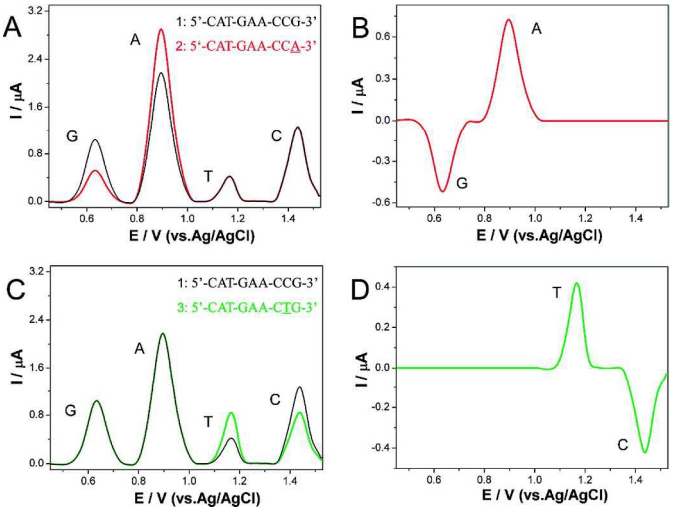

Figure 12. Detection of SNPs of oligonucleotides including the sequence from codon 248 of the p53 gene at the CR-GO/GC electrode. (A) DPVs of wild-type oligonucleotide **1** and its single-base mismatch **2** (G→A mutation). (B) Subtraction of the DPVs of **1** and **2**. (C) DPVs of wild-type **1** and its single-base mismatch **3** (C→T mutation). (D) Subtraction of the DPVs of **1** and **3**. Concentrations for different oligonucleotides (A-D): **1** (1 μM), **2** (1 μM) and **3** (1 μM). Electrolyte: 0.1 M pH 7.0 PBS. Reprinted with permission from Ref. [95]. Copyright (2009) American Chemical Society.

Huang et al. used the carboxylic acid functionalized graphene (CAFG) as a platform for the simultaneous determination of A and G as free bases or in thermally denatured ssDNA. The results showed improved current signals both for adenine and guanine in comparison with GC. They attributed these changes to the electrochemical and electrostatic adsorption on CAFG of the two positively charged bases.[133]

Although DNA is better detected at acid pH [175], based-on-graphene electrodes also allow DNA detection at neutral pH, probably due to the surface stacking effects between graphene and the molecule that exhibit high electron transfer rate[134].

Single and multi-walled carbon nanotubes

The versatility of the carbon-carbon bond provides the opportunity for attaching different functional groups at the ends of carbon nanotubes (CNT) and allows the use of new materials for sensing applications. The electrocatalytic reactivity of the multi-walled carbon nanotubes (MWCNT) is mainly due to edge plane defects existing at the ends of the nanotubes and around the tube wall where the concentric tubes end.[122, 123, 135] Furthermore it has been found that the stability and distribution of nanotubes depend on the structure and properties of the substrates,[136] so various modifications of the nanotubes can be found.

Wang's group obtained improved voltammetric signals when studying the direct electrochemical oxidation of DNA in a MWCNT-modified GC electrode in comparison with a naked one. In addition,

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and although guanine oxidation occurs at slightly high potentials, the MWCNT-modified electrodes promote an enhanced detection due to the interfacial accumulation of CNTs rather than an electron transfer effect.[137] The resulting improved catalytic activity of choline film was attributed to the positive charge of-$NH^{+}(CH_3)_3$ groups, which increased the density of edge-plane-like active sites of GC for effective promotion of electron transfer between the electrode and the solution.[89] The same group was also able to determine the degree of methylation using a subtraction method based on the complementarity of bases, thereby being able to distinguish between 5-mC and T, which show oxidation signals at the same potential, using overoxidized polypyrrole directed MWCNT film modified GC (PPyox / MWCNT / GC).[138]

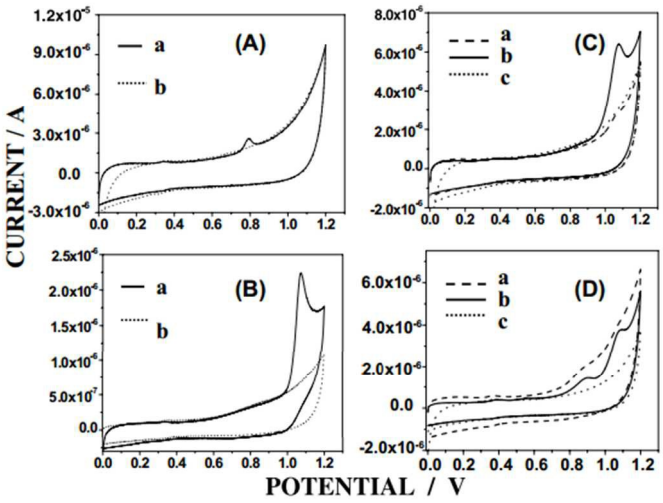

Figure 13. (A, a; B, a) Cyclic voltammograms obtained at pretreated CNT paste electrodes (CNTPE) (by applying 1.3 V for 20 s in a 0.200 M acetate buffer solution pH 5.00) for $2.13x10^{-6}$ M guanine solution (A) and $1.25x10^{-6}$ M adenine solution (B). (A, b; B, b) Cyclic voltammograms obtained at pretreated CNTPE in a 0.200 M acetate buffer solution pH 5.00. (C, a; D, a) Cyclic voltammograms obtained at pretreated CNTPE (by applying 1.3 V for 20 s in a 0.200 M acetate buffer solution pH 5.00) for 1.0 mg/l oligo$(dG)_{21}$ solution (C) and 10.0 mg/l dsDNA solution (D) with no accumulation. (C, b; D, b) Cyclic voltammograms obtained at pretreated CNTPE in a 0.200 M acetate buffer solution pH 5.00 after accumulation in a 0.200 M acetate buffer solution pH 5.00 containing 1.0 mg/l oligo$(dG)_{21}$ (C) (for 5 min) or 10.0 mg/l dsDNA solution (D) (for 10 min). (C, c; D, c) Cyclic voltammograms obtained at pretreated CNTPE in a 0.200 M acetate buffer solution pH 5.00. Scan rate: 0.100 V/ Ag/AgCl. Reprinted from Ref. [139]. Copyright (2004), with permission from Elsevier.

Pedano et al. prepared a carbon paste electrode enriched with MWCNT and studied the adsorption and electrochemical oxidation of nucleic acids, obtaining a large enhancement of the guanine oxidation signal, compared to that obtained at its analogue carbon (graphite) paste electrode, both in polynucleotides and short oligonucleotides as observed in Fig. 13.[139] The same group also demonstrated the importance of the surface for further adsorption and electrooxidation of nucleic acids, being necessary a pretreatment to improve the performance of the CNT paste. On the other hand, the results indicate that the interaction of the nucleic acids with the CNT paste presents mainly a hydrophobic character.[139]

Moreover, Deng et al. used a glassy carbon electrode modified with boron-doped carbon nanotubes as electrode to detect the different nucleobases, providing another potential platform for direct DNA oxidation.[140]Furthermore, Gooding and co-workers reported better results when using glassy carbon electrodes modified with *bamboo* type carbon nanotubes than when modified with SWCNTs or than those on bare GC electrodes for the oxidation of DNA bases. The observed superior electrochemical performance (larger currents and smaller peak separation between oxidation and reduction waves) was ascribed to the presence of edge planes of graphene at regular intervals along the walls of the bamboo nanotubes.[141]

The redox behavior of A, G and T has been also improved with nanocomposites of α or ß-cyclodextrin (CD) and MWCNT deposited on GC electrodes[142, 143]. α-CD/MWCNT considerably improved the sensitivity towards T,[143] while ß-CD/MWCNT allowed a simultaneous determination of G, A and T being the corresponding peaks well-separated.[142] Similarly, hidroxypropyl-ß-cyclodextrin on a film of MWCNT coated with gold nanoparticles and deposited on GC, Au, and on indium tin oxide were used to simultaneously detect tyrosine, G, A and T.[144] In addition, these electrodes showed high reproducibility and long-term stability.

Finally, Ye and Ju used screen printed electrodes modified with MWCNT for the detection of ssDNA and RNA in a fast and sensitive way from the electrochemical oxidation of guanine and adenine.[145]

Modified electrodes

Traditional solid electrodes often suffer from fouling effects due to the accumulation of oxidation products at the electrode surface, resulting in a decreased sensitivity and reproducibility.[146] Metal particles are being used increasingly for the modification of electrodes due to their catalytic properties.[147] TiO_2, in its various forms (nanoparticles, nanotubes, nanoneedles) has features that make it attractive for the modification of electrodes, such as its good biocompatibility, high conductivity and low cost.[148, 149] Fan et al. showed that the electrocatalytic activity of adenine and guanine increased in glassy carbon electrodes modified with TiO_2-graphene composites.[150]

Zeolite modifications have also been widely studied[151, 152]. Zeolites are microporous aluminosilicate minerals characterized by their ability to be hydrated and dehydrated reversibly. Physical, chemical and structural characteristics make them good candidates as electrode modifiers. The mixture of zeolites and graphite has been documented[152] and publications can already be found, for example for carbon electrodes modified with TiO_2 nanoparticles-magnesium (II) doped natrolite zeolite for detecting free bases having a powerful electrooxidation behaviour, showing oxidation peaks well separated of G, A and T[93], but it has not been shown effective with C and mC yet.

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Working electrode	Reference electrode	Buffer solution	Peak potential / V G	A	T	C	Ref
GC	AgCl/Ag	Acetate 0.1 M pH 7.0	0.70	0.96	1.16	1.30	88
SPGE	AgCl/Ag	Phosphate 0.1 M pH 5.0	0.58	0.90	1.02	1.21	90
BDD	Cl-(sat.)/Hg2Cl2/Hg	Amonium acetate 0.1 M pH 4.5	1.13	1.54	--	1.60	105
Ox-BDD	Cl-(sat.)/Hg2Cl2/Hg	Amonium acetate 0.1 M pH 4.5	1.18	1.56	1.77	1.96	105
ECR	AgCl/Ag	Amonium acetate 0.1 M pH 4.5	1.10	1.39	1.69	1.72	115
CR-GO/GC	AgCl/Ag	Phosphate 0.2 M pH 7.0	0.60	0.90	1.25	1.10	95

Table 1. Peak potentials obtained from the electrooxidation of free DNA bases upon different carbon materials.

Working electrode	Reference electrode	Buffer solution	Peak potential / V GMP	AMP	TMP	CMP	Ref
GC	AgCl/Ag	Phosphate 0.1M pH 7.4	0.89	1.19	1.41	1.46	88
SPGE	AgCl/Ag	Acetate 0.1M pH5.0	1.00	1.28	1.46	1.53	92
Red-BDD	AgCl/Ag	Britton-Robinson 0.1 M pH 7.0	1.18	1.52	1.69	1.85	109
Ox-BDD	AgCl/Ag	Britton Robinson 0.1 M pH 7.0	1.09	1.46	1.71	1.89	109

Table 2. Peak potentials obtained from the electrooxidation of nucleotides upon different carbon materials.

Concluding remarks and future perspectives

Different carbon materials have been already examined for the electrochemical oxidation of nucleobases and their nucleoside and nucleotide derivatives. In this regard, Tables 1 and 2 summarise the peak potentials obtained from the electrooxidation of free DNA bases and nucleotides upon different carbon materials. The potentials of the different electrooxidation peaks have evidenced that, irrespectively of the carbon material, the pyrimidine moieties are more difficult to be oxidized and consequently the sensitivity decreases. Thus, cytosine and thymine identification and quantification are not easily achieved with conventional carbon materials such as carbon, graphite or glassy carbon materials. The use of BDD electrodes opened considerably the anodic electrochemical window to tackle more sensitively the electrooxidation of pyrimidine bases as well as their nucleosides and nucleotides. Moreover, BDD surface prevented remarkably the electrode fouling making it a real advantage for its viability in electrochemical sensing applications. However, the appearance of carbon films combining sp2 and sp3 hybridization and the use of single and multiwalled carbon nanotubes and both graphene oxides and reduced graphene oxides led to sensitivity increases, fouling reduction and much better peak resolution.

Despite simultaneous determination of all nucleobases has been demonstrated by a wide number of carbon materials, the simultaneous identification and quantification of cytosine methylation in DNA is still found in an incipient phase. Thus, even although the electrooxidation of methylcytosine and cytosine as well as their nucleotides derivatives can really be distinguishable by several electrochemical techniques, the situation becomes more complicated when managing more complex molecules such oligonucleotides and ssDNA. In this regard, the need for lytic digestion of oligonucleotides, genes or ssDNA to single nucleotides, separation and purification of the lysis reaction crude and the total interference elimination are vital issues to be addressed. Furthermore, thymine electrooxidation occurs very close to that of methylcytosine. Therefore, either novel electrochemical procedures or novel carbon materials have to be tested for a distinguishable and unambiguous determination. The lack of studies for the validation of electrochemical sensors at determining methylation at cytosine residues is still a matter that research groups have to pay greater attention to assess the viability in clinical application. This review may provide the appropriate background for the development of

novel analytical tools for the determination of cytosine methylation in DNA. Finally, miniaturization of electrochemical devices will allow researchers to solve problems associated with the sample amount, cost, rapidness and robustness. In this way, electrochemical sensors may be integrated into conventional analytical procedures as a complement tool with options in multichannel devices.

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