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DNA Adsorption on Carbonaceous Materials

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Abstract The immobilization of DNA on different solid supports has become an important issue in different fields ranging from medicine to analytical chemistry and, more recently, molecular electronics. Among the different immobilization procedures, adsorption is the simplest and the easiest to automate, avoiding the use of procedures based on previous activation/modification of the substrate and subsequent immobilization, which are tedious, expensive and time-consuming. Carbon-based materials are widely used for this task due to their electrochemical, physical and mechanical properties, their commercial availability, and their compatibility with modern microchip fabrication technology.

Moreover, carbonaceous materials are widely used as transducers for electrochemical sensors. The knowledge of the adsorbed DNA morphology on carbon surfaces can be used to develop stable and functional DNA layers for their use in DNA analytical devices with improved properties.

Presented here is a concise description of surface immobilization of DNA, oligonucleotides, and DNA derivatives by adsorption onto carbonaceous materials, and the properties of the DNA layer adsorbed on carbonaceous solid phase.

Keywords DNA · Adsorption · Materials · Graphite · Carbon · Composite · Nanotube · Electrochemical sensing

Abbreviations

A	Adenine
ABS	Acetate buffer solution
AFM	Atomic force microscopy
BDD	Boron-doped diamond
BLM	Bilayer lipid membrane
C	Cytosine
CNT	Carbon nanotube
CNTP	Carbon nanotube paste
CP	Carbon paste
dsDNA	Double-stranded DNA or native DNA
G	Guanine
GC	Glassy carbon
$GC_{(ox)}$	Anodized glassy carbon
GEC	Graphite epoxy composite
HOPG	Highly ordered pyrolytic graphite
MWCNT	Multi-wall carbon nanotube
ODN	Oligodeoxynucleotide
PBS	Phosphate buffer solution
PG	Pyrolytic graphite
SCE	Saturated calomel electrode
ssDNA	Single-stranded DNA or denatured DNA
SWCNT	Single-wall carbon nanotube
T	Thymine

1 Introduction

The growing demand for genetic information in an increasingly broad range of disciplines has led to research into the development of new techniques for genetic analysis. The Human Genome Project (HGP) [1] has stimulated the development of analytical methods that yield genetic information quickly and reliably. Examples of this development are the DNA chips [2–4] and lab-on-a-chips based on micro fluidic techniques [5]. Additionally, the knowledge

obtained from the HGP has expanded the market that requires genetic devices, hence generating new applications. However, this expanding market would obviously benefit from simple, cheap and easy to use analytical devices, especially for industrial applications.

Therefore, the development of new methodologies possessing the convenience of solid-phase reaction, along with advantages of rapid response, sensitivity and ease of multiplexing is now a challenge in the development of new biochemical diagnostic tools. Electrochemical biosensors and chips can meet these demands, offering considerable promise for obtaining sequence-specific information in a faster, simpler and cheaper manner than traditional hybridization assays. Such devices possess great potential for numerous applications, ranging from decentralized clinical testing, to environmental monitoring, food safety and forensic investigations.

The use of nucleic acids recognition layers is a new and exciting area in analytical chemistry which requires extensive research.

To prepare electroanalytical devices based on DNA, the immobilization of the biological species must be carefully considered. The most successful immobilization techniques for DNA appear to be those involving multi-site attachment (either electrochemical or physical adsorption) or single-point attachment (mainly covalent immobilization or strept(avidin)/biotin linkage) [6]. Single-point attachment is beneficial to hybridization kinetics, especially if a spacer arm is used. However, among the different DNA immobilization procedures reported, multi-site adsorption is the simplest and most easily automated technique, avoiding the use of pre-treatment procedures based on previous activation/modification of the surface transducer and subsequent DNA immobilization. Such pre-treatment steps are known to be tedious, expensive and time-consuming. Furthermore, the adsorption properties of DNA on various supports (e.g., nylon, nitrocellulose) have been known for a long time [7].

Electrochemical detection of successful DNA hybridization events should be also considered. Although it is based mostly on external electrochemical markers, such as electroactive indicators or enzymes, the exploitation of the intrinsic DNA oxidation signal requires a multi-site attachment such as adsorption as the immobilization technique.

The direct electrochemical detection of DNA was initially proposed by Paleček [8, 9], who recognized the capability of both DNA and RNA to yield reduction and oxidation signals after being adsorbed. The DNA oxidation was shown to be strongly dependent on the DNA adsorption on the substrate; it requires meticulous control of the DNA-adsorbed layer.

While immobilization and detection are important features, the choice of a suitable electrochemical substrate is also of great significance in determining the overall performance of the analytical electrochemical-based device, especially regarding the immobilization efficiency of DNA.

The development of new transducing materials for DNA analysis is a key issue in the current research efforts in electrochemical-based DNA analytical devices. The use of platinum, gold, indium–tin oxide, copper solid amalgam, mercury and other continuous conducting metal substrates has been reported [6]. However, this chapter is focused on carbon-based materials and their properties for immobilizing DNA by simple adsorption procedures.

2 Carbonaceous Materials

The extraordinary ability of carbon to combine with itself and other chemical elements in different ways is the basis of organic chemistry. As a consequence, there is a rich diversity of structural forms of solid carbon because it can exist as any of several allotropes. It is found abundantly in nature as coal, as natural graphite and also in much less abundant form as diamond.

Engineered carbons [10] are the product of the carbonization process of a carbon-containing material, conducted in an oxygen-free atmosphere. Depending on the starting precursor material (hydrocarbon gases, petroleum-derived products, coals, polymers, biomass), the product of a carbonization process will have different properties, including the adsorption capability. Traditional engineered carbons can take many forms, such as coke, graphite, carbon and graphite fiber, carbon monoliths, glassy carbon (GC), carbon black, carbon film, and diamond-like film [10]. More recently, a promising new carbon-based material—carbon nanotubes—has been developed using the vapor deposition technique.

Engineered carbons have found intensive use as adsorbents because of their porous and highly developed internal surface areas as well as their complex chemical structures.

As with the majority of organic molecules, DNA can be easily adsorbed on carbon-based material. Adsorption processes can be driven in both liquid and gaseous media by physical forces. The porous structure and the chemical nature of the carbon surface are significantly related to its crystalline constitution. The crystal structure of graphite consists of parallel layers of condensed, regular hexagonal rings. The in-plane C – C distance is intermediate between the Csp^3–Csp^3 and the Csp^2 = Csp^2 bond lengths (Fig. 1).

The pore structure and surface area of carbon-based materials determine their physical characteristics, while the surface chemical structure affects interactions with polar and nonpolar molecules due to the presence of chemically reactive functional groups. Active sites—edges, dislocations, and discontinuities—determine the reactivity of the carbon surface. As shown in Fig. 1, graphitic materials have at least two distinct types of surface sites, namely, the basal-plane and edge-plane sites [11]. It is generally considered

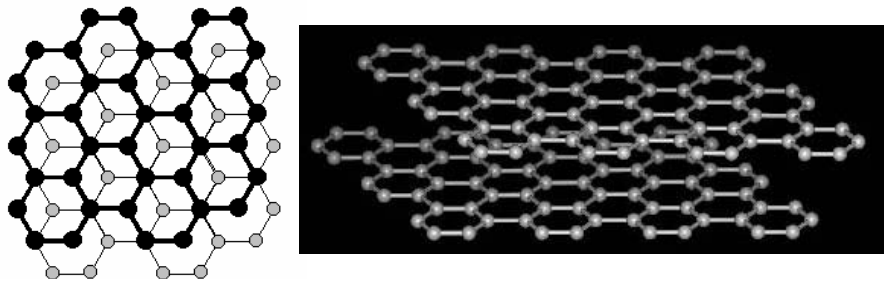

Fig. 1 Positional relationship between two identical graphene planes. Graphite structure can be described as an alternate succession of these basal planes. The *right panel* was taken from the image gallery of Prof. R. Smalley (to be found at http://smalley.rice.edu/ and reprinted with his kind permission

that the active sites for electrochemical reactions are associated with the edge-plane sites, while the basal plane is mostly inactive.

Heteroatoms (usually oxygen) play an important role in the chemical nature of the carbon "active" surface [10]. The adsorption process is thus strongly dependent on the type, quantity, and bonding of these functional groups in the structure. Heteroatoms distributed randomly in the core of the carbon matrix may be non-reactive due to their inaccessibility. However, the heteroatoms can be also concentrated at the exposed surface of carbons or presented as an "active" dislocation of the microcrystalline structure. Much of the research being carried out is focused on the identification and characterization of oxygen-containing functional groups in oxidized carbon surfaces, such as carboxyl, phenolic, quinonic, and lactones, but also in the changes that take place in the carbon surface under different oxidation treatments.

The electrochemical oxidation pretreatment was found to improve the electrochemical behavior by introducing more active edge sites on the treated carbon surface. The effect of oxidation on the chemical composition is related to the increased concentration of strong and weak acidic groups found upon electrochemical oxidation of the graphite surface [12]. The acidity of carboxylic groups on the oxidized carbon surface could be stronger than that of a carboxylic resin. The weight increase after electrochemical pretreatment was attributed to the formation of the oxidized graphite and the intercalation of solvent molecules and anions into graphitic material. A model of a fragment of oxidized carbon surface illustrating the general chemical character of the oxidized carbon surface is shown in Fig. 2.

Among the different carbonaceous materials, GC and pyrolytic graphite (PG) and the graphite-powder-based composites such as carbon paste (CP) are the most popular choices as electrochemical transducer materials.

GC is made by heating a high molecular weight carbonaceous polymer to 600–800 °C. Most of the non-carbon elements are volatilized, but the backbone is not degraded. Regions of hexagonal sp^2 carbon are formed during

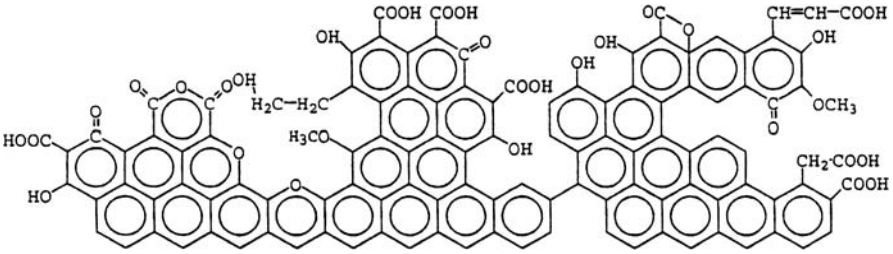

Fig. 2 Hypothetical fragment of an oxidized carbon surface. The figure was taken from [10] with kind permission from Prof. M. Streat

this treatment, but they are unable to form extensive graphitic domains without breaking the original polymer chain. GC is impermeable to liquid, so porosity is not an issue [13]. Pretreated GC has been obtained by (1) polishing and/or ultrasonication, (2) chemical oxidation or (3) electrochemical anodization treatments [14]. These surface treatments have been extensively used to improve the electrochemical performance of GC [15]. Suggested reasons for activation have been the removal of contaminants from the surface, and the increase in the surface area due to the roughening of the surface or the exposure of fresh carbon edges, microparticles and defects that may be sites for electron transfer. On the other hand, the increase in surface functional groups that may act as electron transfer mediators could play a role. While some of these factors are related to improvements in the electrochemical performance, others are related to both electrochemical and physical features. As an example, the increment in the surface roughness can cause enhancement of the heterogeneous electron transfer rates as the effective area for electron transfer is greater than the geometric area, but can also improve the physisorption of a given molecule. GC is well known for the exhibition of a wide range of functional groups, including carboxylic acids, quinones/hydroquinone, phenols, peroxides, aldehydes, ethers, esters, ketones, and alcohols, which could interact differently with DNA molecules stabilizing the adsorbed molecule, but may also improve the electron transfer, acting as mediators. The activation method most commonly used relies on the electrochemical activation to obtain anodized GC ($GC_{(ox)}$). It was found that the dominant process during electrochemical activation of the GC surface is the formation of a near-transparent homogeneous different phase [15]. The layer was shown to be porous, hydrated and nonconductive, containing a significant amount of microcrystallinity and graphite oxide. Once the film is grown, the surface becomes richer in oxygenated groups that make it more hydrophilic. It is observed that the anodization of the GC induces adsorption: despite the nonconductive nature of graphite oxide, it intercalates aromatic molecules quite well. Only the portion immediately adjacent to the GC substrate seems to be electronically connected to the substrate. The outer

nonelectroactive portion of the layer concentrates the redox species near to the electroactive surface.

PG is made by the pyrolysis of light hydrocarbons onto a hot (800 °C) stage, often followed by heat treatment to higher temperatures. Highly-oriented PG (HOPG) is made from PG by pressure annealing in a hot press at 3000 °C and several kilobars. HOPG has a smooth, shiny basal surface, while PG is mottled and dull [13]. The dominant structural property of PG and HOPG is the long-range order of the graphitic layers (Fig. 1) and the remarkable anisotropy and hydrophobic behavior. HOPG is single-crystal graphite with edge planes and cleavage surfaces (basal plane) that serve as the oriented surface for electrochemical studies. An important advantage of HOPG with respect to other carbonaceous materials is the possibility of performing studies by means of high resolution techniques—even down to the atomic level—by scanning probe microscopy, such as atomic force microscopy (AFM). The rough and complex surface of GC is not suitable for AFM surface characterization. For AFM studies, an atomically flat substrate is required to clearly resolve the molecular adsorbed layer. GC presents a root-mean-square (rms) roughness of 2.10 nm while HOPG surface presents a rms roughness of less than 0.06 nm (both calculated from AFM images in air) [16]. This fact has stimulated the use of HOPG instead of other carbonaceous materials such as GC or CP [17].

Carbon composites result from the combination of carbon with one or more dissimilar materials. Each individual component maintains its original characteristics while giving the composite distinctive chemical, mechanical and physical properties. The capability of integrating various materials is one of their main advantages. Some components incorporated within the composite result in enhanced sensitivity and selectivity. The best composite compounds will give the resulting material improved chemical, physical and mechanical properties. As such, it is possible to choose between different binders and polymeric matrices in order to obtain a better signal-to-noise ratio, a lower nonspecific adsorption, and improved electrochemical properties (electron transfer rate and electrocatalytic behavior).

Powdered carbon is frequently used as the conductive phase in composite electrodes due to its high chemical inertness, wide range of working potentials, low electrical resistance and a crystal structure responsible for low residual currents. A key property of polycrystalline graphite is porosity. Most polycrystalline graphite—such as powdered carbon—is made by heat treatment of high molecular weight petroleum fractions at high temperatures to perform graphitization. The term "graphite" is used to designate materials that have been subjected to high temperatures, and thus have aligned the sp^2 planes parallel to each other.

Regarding their mechanical properties carbon composites can thus be classified as rigid composites [18, 19] or soft composites—the carbon pastes – [20]. The composites are also classified by the arrangement of their

particles, which can be either dispersed or grouped randomly in clearly defined conducting zones within the insulating zones.

The inherent electrical properties of the composite depend on the nature of each of the components, their relative quantities and their distribution. The electrical resistance is determined by the connectivity of the conducting particles inside the nonconducting matrix, and therefore the relative amount of each composite component has to be assessed to achieve optimal composition. Carbon composites show improved electrochemical performances, similar to an array of carbon fibers separated by an insulating matrix and connected in parallel. The signal produced by this macroelectrode formed by a carbon fiber ensemble is the sum of the signals of the individual microelectrodes. Composite electrodes thus showed a higher signal-to-noise (S/N) ratio than the corresponding pure conductors, accompanied by an improved (lower) detection limit.

Rigid composites are obtained by mixing graphite powder with a nonconducting polymeric matrix, obtaining a soft paste that becomes rigid after a curing step [18, 19]. They could be classified according to the nature of the binder or the polymeric matrix, in epoxy composites, methacrylate composites, or silicone composites. Graphite–epoxy composite (GEC) has been extensively used in our laboratories showing to be suitable for electrochemical sensing due to its unique physical, and electrochemical properties.

Soft composites or CPs are the result of mixing an inert conductor (e.g., graphite powder) with an insulating compound (e.g., paraffin oil, silicone, Nujol, mineral oil) [20]. The insulating liquid has a specific viscosity and the paste has a certain consistency. The resulting material is easy to prepare and inexpensive. Compared with other solid materials, CP electrodes have shown some advantages, including wide potential window and low background current. However, these pastes have limited mechanical and physical stabilities, especially in flow systems. Additionally, the pastes are dissolved by some nonpolar solvents.

Fullerenes (C_{60}) (Fig. 3) have a structure similar to that of truncated icosahedron, made out of five- and six-member rings of sp^2 carbons. Higher fullerenes are also made of five- and six-member carbon rings.

In late 1991, the first synthesis and characterization of carbon nanotubes (CNTs) was reported [21]. CNTs are attractive carbonaceous materials with well defined nanoscale geometry. They have a closed topology and tubular structure that are typically several nanometers in diameter and many micrometers in length. CNTs are produced as single-wall Carbon Nanotubes (SWCNTs) and multi-wall carbon nanotubes (MWCNTs). SWCNTs are made out of a single graphite sheet rolled seamlessly with 1–2 nm in tube diameter (Fig. 3). MWCNTs are composed of coaxial tubules, each formed with a rolled graphite sheet, with diameters ranging from 2 to 50 nm. The concentric single-walled cylinders are held together by relatively weak Van der Waals forces with an interlayer spacing of 0.34 nm (Fig. 3). CNTs aggregate

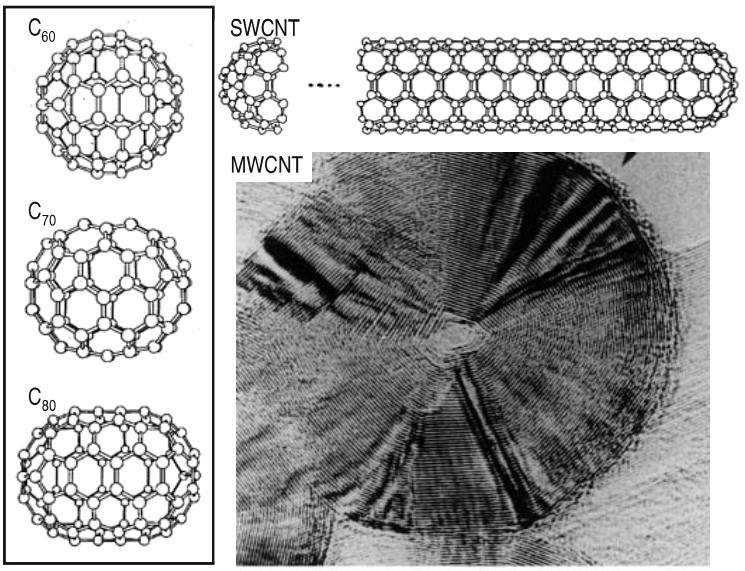

Fig. 3 Structure of fullerenes C_{60}, C_{70}, C_{80} and single-wall carbon nanotube. The figures were taken with permission of Prof. C. Dekker from the image gallery found at http://online.itp.ucsb.edu/online/qhall_c98/dekker/. Transmission electron microscopy image of multi-wall carbon nanotube (*MWCNT*) treated with iodinated and platinate DNA. The figure was taken from [24] with kind permission from Prof. P. Sadler

easily, forming bundles of tens to hundreds of nanotubes in parallel and in contact with each other [22]. CNTs can be grown by the arc discharge method or laser ablation of a graphite rod, as well as by chemical vapor deposition (CVD) [23].

Changes in the winding angle of the hexagonal carbon lattice along the tube (i.e., the chirality) would have a strong effect on the conductive property, resulting in either semiconducting or metallic behavior [23] of CNTs. Mechanically, the CNT is stronger than steel, but lighter. Thermally, it is more conductive than most crystals. Chemically, it is inert everywhere along its length except at the ends or at the site of a bend or kink [24, 25]. It has been shown that while amorphous carbon can be attacked from any direction, CNTs can be oxidized only from the ends. When treated with concentrated oxidizing acid, the ends and surfaces of carbon nanotubes become covered with oxygen-containing groups such as carboxyl groups and ether groups [26]. As graphite is considered to be hydrophobic, CNTs—which correspond to hollow cylinders of rolled-up graphene—and fullerenes are found to have a low solubility in water. The presence of hydrophilic groups (e.g., – OH and – COOH) in the interior of the CNT could play an important role in its properties [26, 27]. Isolated SWCNTs are insoluble in most solvents unless a surfactant is used or chemical modifications to the tubes are carried out.

Such insolubility and the strong Van der Waals attraction between tubes cause them to bundle together as ropes.

Compared with SWCNTs, the much cheaper MWCNTs produced by the CVD method are known to have more defects and can provide more sites for the immobilization of DNA.

CNTs present a larger surface area and outstanding charge-transport characteristics and might therefore greatly promote electron transfer reactions which can dramatically improve electrochemical performance compared to that of other carbonaceous materials [26]. The open end of a MWCNT is expected to show a fast electron transfer rate similar to the graphite edge-plane electrode while the sidewall is inert like the graphite basal-plane (Figs. 1 and 3). Fast electron transfer rate is demonstrated along the tube axis [28]. CNTs are expected to present a wide electrochemical window, flexible surface chemistry, and biocompatibility, similar to other widely used carbon materials [28].

The next section will be focused on the description of the most important features related to DNA adsorption strategies that have found applications in DNA electrochemical analysis.

3
DNA Adsorption Strategies

3.1
Nucleic Acid Structure and Adsorption Properties

Adsorption is an easy way to attach nucleic acids to surfaces, since no reagents or modified DNA are required. Adsorption is a complex interplay between the chemical properties, structure and porosity of the substrate surface with the molecule being adsorbed. Regarding the solid support, the roughness, the size of pores, the uniformity and the permeability, the chemical nature, surface polarity and the presence of chemically reactive functional groups should all be considered. In the case of carbon-based materials, these parameters vary dramatically depending on the nature and the source of carbon: graphite powder composites, graphite leads, PG, GC, CNTs.

The main parameters affecting the adsorption process of a given molecule in solution involve its size, shape, polarity, and chemical structure.

DNA is a structurally polymorphic macromolecule which, depending on nucleotide sequence and environmental conditions, can adopt a variety of conformations. The double helical structure of DNA (dsDNA) consists of two strands, each of them on the outside of the double helix and formed by alternating phosphate and pentose groups in which phosphodiester bridges provide the covalent continuity. The two chains of the double helix are held

together by hydrogen bonds between purine and pyrimidine bases. The sugar–phosphate backbone is responsible for the polyanionic characteristic of DNA. In the double helix structure, the bases exist in a highly hydrophobic environment inside the helix, while the outer, negatively charged backbone allows the dsDNA molecule to interact freely with the hydrophilic environment. The dsDNA is considered a highly hydrophilic molecule. As a negatively charged molecule, it can be easily stabilized on positively charged substrates. While dsDNA only partially shows its hydrophobic domain through its major and minor grooves or through those sites where dsDNA is open and exposing DNA bases, ssDNA has the hydrophobic bases freely available for interactions with hydrophobic surfaces. As such, ssDNA is dual in nature, the highly hydrophilic backbone and the hydrophobic DNA moieties coexisting in the same molecule. These structural and chemical differences between ss and dsDNA are reflected in different adsorption patterns for both molecules. The greater size and the more rigid shape of dsDNA with respect to ssDNA are other parameters affecting the adsorption. Another important compound that should be considered for the adsorption of DNA is its oxidation product 8-oxoguanine that can arise from DNA through the direct attack of reactive oxygen species on chromatin [29]. It is directly associated with promutagenic events and other cellular disorders both in vivo and in vitro. The formation of 8-oxoguanine in the DNA moiety, considered the most commonly measured product of DNA oxidation, causes important mutagenic lesions. In the DNA double helix this adduct pairs more easily with adenine (A) than with cytosine (C). This could lead to the substitution of C in the complementary chain by A, which in turn leads to the substitution of the original guanine (G) by thymine (T) initiating a cellular dysfunction. PNA is an analogue of DNA in which the entire negatively charged sugar–phosphate backbone is replaced with a neutral "peptide-like" backbone consisting of repeated N-(2-aminoethyl)glycine units linked by amide bonds [30]. The four natural nucleobases (i.e., A, C, G, and T) come off the backbone at equal spacing to the DNA bases. Such a structure is not prone to degradation by nucleases or proteases, thus offering high biological stability. The unique chemical properties of the neutral PNA molecule have been extensively studied and compared with the negatively charged DNA counterpart.

Beside the DNA molecule and the carbon substrate, the solvent, normally water, and in particular the ionic strength, pH and the nature of the solutes, play an important role in the adsorption process, mainly in the stabilization of the adsorbed molecule on the substrate.

DNA adsorption properties were first studied using a variety of solid supports for classical analysis methods including Southern and Northern transfers, dot-blotting, colony hybridization and plaque-lifts [31, 32]. Studies of the interactions between nucleic acids and nitrocellulose revealed that molecular weight, finite macromolecular conformation, ionic forces and weaker forces of attraction all play a role. DNA is retained on nitrocellulose only in

buffers of high ionic strength. This may be because increasing salt concentration correlates with decreasing electrostatic repulsion between the phosphate groups of the DNA backbone, yielding more aggregated DNA molecules that are more easily retained on the filter. Nylon membranes are able to bind both native and denatured nucleic acids in buffers of low ionic strength [33]. Positively charged nylon membranes provide an ionic interaction between the negatively charged phosphate groups of the nucleic acid and the positively charged groups of the membrane. Although nylon and nitrocellulose are the most commonly used solid supports in DNA classical analysis, studies of the interaction between DNA and other surfaces such as polystyrenes (microwells, beads), glass, dextran, latex and magnetic beads have also been reported.

Although DNA has been widely attached onto carbonaceous materials, the underlying mechanism of adsorption has not been fully clarified. The next section focuses on the different strategies for the adsorption of nucleic acid (ssDNA, dsDNA, ODN and DNA bases) on carbon-based material.

3.2 DNA Adsorption Methods

The unique practical properties of adsorption have promoted its extensive use in genetic analysis. The disadvantages of adsorption with respect to covalent immobilization are mainly that (1) nucleic acids may be readily desorbed from the substrate, and (2) base moieties may be unavailable for hybridization if they are bonded to the substrate in multiple sites [34]. However, the electrochemical detection strategy based on the intrinsic oxidation of DNA requires the DNA to be adsorbed in close contact with the electrochemical substrate by multi-point attachment. This multi-site attachment of DNA can be thus detrimental for its hybridization but is crucial for the detection based on its oxidation signals.

The common methods for the multi-site adsorption of DNA on carbonaceous-based material can be classified into physical (dry and wet) adsorption and electrostatic adsorption.

Dry adsorption relies on leaving DNA to dry on the carbonaceous surface. Dry adsorption can be assisted by light treatment (except UV, which is able to induce changes in the DNA molecule) or heated until 100 °C. DNA can adopt a variety of conformations depending on the degree of hydration. The most familiar double helix DNA—called "B-DNA"—can turn into the "A-DNA" form if it is strongly dehydrated. A structural alteration occurs due to a greater electrostatic interaction between the phosphate groups, leading to A-DNA. The different structural forms of the double helix promote different dynamic interactions, and the width of the grooves between the strands is important in allowing or preventing access to bases. Both ss- and dsDNA can be adsorbed firmly if it is dried on the carbonaceous surface. When the DNA solution is evaporated to dryness, the bases of DNA which have been dehy-

drated are exposed, and thus the hydrophobic bases are strongly adsorbed flat on the electrode surfaces. Once it is adsorbed, DNA is difficult to re-hydrate. Hence, DNA is not desorbed, no matter how long the adsorbed DNA is soaked in water, characteristic of irreversible adsorption. The "irreversible" behavior of the dry-adsorbed DNA layer has been previously reported [35].

Wet adsorption relies on leaving DNA to interact with the carbonaceous surface through physical forces in the presence of water. During wet adsorption, the stabilization of B-DNA is expected to occur on the carbonaceous surface, by keeping the hydration water of the DNA molecule. As the water is kept on the DNA adsorbed molecule, it can be more easily desorbed from the substrate if soaked in aqueous solutions. The stringency of wet adsorption is related to the use of static or convection conditions. In order to perform DNA adsorption, the convection conditions—which can be achieved by the use of stirring as well as the use of "heated" substrates—prove to be more effective than static conditions. Although a thick or a thin layer of DNA can be attached on the surface during dry adsorption by controlling the concentration of the DNA solution being dried, the wet adsorption normally yields a thin DNA monolayer. During wet adsorption, the substrate is progressively modified with negative charges coming from the DNA being adsorbed, thus repealing the successive DNA molecules that are approaching the substrate. Wet adsorption thus leads to a "self-control" surface coverage and is less stringent than dry adsorption. Depending on the application of the DNA-modified substrate, a thick or thin DNA layer would be necessary. If a stringency control of nonspecific DNA adsorption issues is required, a thick DNA layer is more convenient. However, the yield in hybridization is better on a thin DNA layer.

The electrostatic adsorption can be performed—given the polyanionic nature of DNA molecule—by modifying the ion charge of the carbon substrate by both (1) chemical modification, with polycationic molecules, and (2) by applying a positive potential taking advantages of the conducting properties of the carbon substrate. Both of them are based on the same principle that keeps DNA attached on the widely used, positively modified nylon membranes. The electrostatic adsorption by chemical modification of the substrate is based on the formation of a stable compound between the polycationic molecule that modified the substrate and the polyanionic phosphodiester backbone of DNA, either native or denatured. The potential-driven electrostatic adsorption has been widely used for immobilizing DNA on carbon materials. Taking into account that the DNA bases—A and G—can be oxidized, the applied potentials used to provide the positive charge to the substrate are lower than those producing DNA oxidation. It appears that the adsorption occurs through the negatively charged phosphate backbone, leaving the bases accessible for hybridization reactions in the case of ssDNA and ODNs. There is evidence that the positive potential considerably enhances the robustness and stability of the DNA layers to mechanical stress, through multiple electrostatic interactions between the negatively charged hydrophilic

sugar–phosphate backbone and the positively charged carbon surface. The electrostatic adsorption performed by applying a positive potential is usually driven under stirring conditions in solution (wet adsorption) until full DNA coverage of the substrate is achieved.

The next section will focus on carbonaceous materials that have found applications as transducers for DNA biosensing based on the adsorption of DNA.

4
Adsorption of DNA on Carbon-Based Materials

4.1
Glassy Carbon

Adsorption of dsDNA can be performed on GC surfaces by either dry or electrostatic adsorption procedures yielding a thick or a thin layer of DNA, respectively [36]. The thin layer dsDNA-modified GC electrode was prepared by immersion in a dsDNA solution by applying a potential of + 0.40 V. The resulting DNA layer was non-uniform leaving many bare GC-uncovered regions. The thick layer dsDNA-modified GC electrode was prepared by covering a GC electrode with dsDNA and then transferring it into a solution containing ssDNA for electrochemical conditioning [37]. Briefly, the dsDNA was dry-adsorbed overnight on the GC surface. This led to an almost uniform layer of DNA, 0.1 mm thick when dried, which in aqueous solution swelled to about 1 mm thickness with a gel-like appearance [38]. After drying, the electrode was immersed in ABS and a constant potential of + 1.4 V (vs SCE) was applied for 5 min. It was then transferred to a solution containing ssDNA and differential pulse voltammograms were recorded in the range of 0 to + 1.4 V until stabilization of the peak currents corresponding to A and G electro-oxidation. This procedure produces a thick multilayer of DNA covering the GC surface completely and uniformly with no pinholes or bare GC regions. H-DNA triple helical structure is supposed to occur at the surface of the GC electrode. This thick-layer dsDNA-modified GC electrode allowed the study of DNA interactions and damage by health-hazardous compounds such as metronidazole, mitoxantrone [39], and niclosamide [40] based on their binding properties to nucleic acids. According to the ohmic resistance, there was also evidence that the thick DNA layer on the GC surface is a reasonably good conductor [38]. Additionally, the thick dsDNA layer obtained by dry-adsorption has been demonstrated to be unstable to alkali and to heat, but stable to acid solutions [35, 41]. When the solution containing ds- or ssDNA is evaporated to dryness, dehydrated DNA molecules can be irreversibly adsorbed on the surfaces of GC, which has proved to be very stable for long storage in a dry state [35, 41].

It was also demonstrated that ssDNA is better adsorbed onto the GC electrode than dsDNA. The dsDNA molecule has some difficulty reaching the surface contours of the rough GC electrode surface, while ssDNA can approach closer to the electrode surface because of its greater flexibility.

Although dsDNA can be adsorbed at the GC surface, it is not easily oxidized while ssDNA can be easily adsorbed and oxidized, giving higher oxidation signals, which is attributed to the oxidation of G ($\sim$ 0.8 V) and A ($\sim$ 1.1, vs SCE) respectively [38]. The dsDNA structure had greater difficulty transferring the electrons from the inside of the double-stranded structure to the electrode surface than the flexible ssDNA structure where the bases are in closer proximity to the GC surface.

The electrochemical processes of adsorption and oxidation of ds- and ssDNA on the GC electrode were discussed and studied by in situ FTIR [42]. It was also demonstrated that the well-known oxidation product 8-oxoguanine adsorbs strongly on the GC surface [29]. Adsorbed ssDNA can form a DNA layer which impedes the oxidation product diffusing away, blocking the GC surface [43, 44].

In contrast to the potential dependence observed for the accumulation of ODN at other carbonaceous materials such as CP, both ss- and dsDNA were adsorbed on GC in a broad range of applied potentials (from – 0.60 to + 0.40 V), even when using solutions of different ionic strengths [43, 44]. A slight influence of the GC surface charge was thus observed, indicating that there is a small contribution of the negatively charged phosphate backbone in the adsorption of nucleic acids on the GC surface (especially at high ionic strengths) [44]. Other factors influencing the rate of adsorption of DNA on GC are the size of the ODN, which would produce an easier adsorption of smaller molecules, and the conformation of the nucleic acid in solution prior to the immobilization at the electrode surface [44].

4.1.1 Pretreated Glassy Carbon

The influence of different pretreatment strategies on the adsorption of DNA on the GC surface has been extensively discussed. The sensitivity for ssDNA detection at the GC surface was improved greatly (tenfold) by modifying the electrode surface with an electrochemical oxidation treatment at + 1.75 V (vs SCE) for 300 s in PBS, pH 5.0. The same results were reported when $GC_{(ox)}$ was obtained at (1) 1.60 V (vs SCE) for 15 s in 10% HNO_3 solution with 2.5% $K_2Cr_20_7$, [35] and, (2) 1.20 V (vs Ag/AgCl) in 0.5 M NaOH for 10 min [45].

This improvement was due to an easy adsorption of ss- and dsDNA on the $GC_{(ox)}$ surface [46, 47]. Regarding the nonconductive nature of graphite oxide film formed on the surface during anodization [15], the activation of GC would affect primarily the adsorption process but not the charge transfer of the G and A residues. The ssDNA was preconcentrated on $GC_{(ox)}$ surface

under stirring by means of either its wet-adsorption for 5 min, or its electrostatic adsorption at + 0.3 V (vs SCE) for 90 s. In both cases, the adsorption of DNA on $GC_{(ox)}$ surface are close to the theoretical value of a monolayer. The stirring during the DNA adsorption was critical for enhancing the adsorption while the positive potential was found to accelerate the adsorption process.

Not only was an improvement in the detection for ssDNA at $GC_{(ox)}$ observed, but also for G and A bases [47]. These results suggest that the increased adsorption of DNA on the $GC_{(ox)}$ depends more on the DNA bases than on the phosphate–sugar DNA backbone.

This conclusion is also supported by the fact that, in contrast to ssDNA, the oxidation signal coming from dsDNA is poorly developed at both GC and $GC_{(ox)}$. This is probably attributable to the electroactive A and G residues in dsDNA being inaccessible to the surface, while most bases in denatured DNA can freely interact with the $GC_{(ox)}$ surface. On the other hand, the hydrogen-bonded bases in native DNA are hidden within the double helix, a serious steric barrier to electron transfer between the purine and the $GC_{(ox)}$.

However, when the potential of the pretreatment of the GC exceeded + 1.75 V (vs SCE) or it was driven longer than 300 s in PBS (pH 5.0), the adsorption of ssDNA at the electrode was found to decrease [46], showing that different conditions for obtained $GC_{(ox)}$ were detrimental for the DNA adsorption and oxidation. A similar negative effect was observed when the adsorption of the DNA was performed on polished GC previously exposed to air for a given time [44].

The beneficial effects of the graphite oxide film on the adsorption and oxidation of DNA on $GC_{(ox)}$ seem to be strongly dependent on the thickness of this film, obtained under different conditions (supporting electrolyte, applied voltage, duration of the anodization treatment and pH).

Once the film is grown, the surface becomes richer in oxygenated groups, making it more hydrophilic. It is clear that this increased hydrophilic environment does not favor the adsorption of nucleic acids on GC. The increased adsorption of DNA on the $GC_{(ox)}$ may depend more on the DNA bases than on the phosphate–sugar DNA backbone.

A mixed activation procedure, based on both preanodization and precathodization treatments, respectively, was shown to produce a further 100-fold improvement of the DNA oxidation signal on GC. The electrochemical oxidation was performed at + 1.75 V (vs SCE) for 10 min and cyclic sweep between + 0.3 V and – 1.3 V for 20 cycles in pH 5.0 PBS [48]. The ssDNA was accumulated at the GC surface at an open circuit by wet-adsorption. As previously explained, a dielectric layer is formed on GC during anodic oxidation. Such a graphitic oxide layer possesses insulating properties, is electrochemically inactive and does not contribute to the double-layer capacitance. After the electrode is reduced, the whole layer becomes electrochemically active again, resulting in a significant increase in double-layer capacitance. However, no increase in surface roughness was observed with AFM after being oxidized

and reduced. The oxidation followed by reduction of GC for a very short time produces the C = O functional group on the carbon electrode surface. The adsorptive capacity was thus found to be related to the amount of these surface functional groups and double-layer capacity. The increase in current was not produced by an increased surface area due to porous structure, but by some chemical interaction between the C = O groups and ssDNA. One possible reason for the preferential adsorption of ssDNA on the modified GC could be the positive chemical interaction between the ssDNA and the surface-produced C = O groups. As explained, in ssDNA, all bases can be freely accessible to the electrode surface. Hydrogen bonds can be formed between the more acidic H of nucleic bases in ssDNA and the C = O groups present on the electrode surface [48]. As for dsDNA, the sites that can form hydrogen bonds, have already formed a part of the Watson-Crick hydrogen bonding system, and cannot form hydrogen bonds with the C = O groups on the electrode surface. Therefore, dsDNA cannot accumulate on the modified electrodes as much as ssDNA [48].

4.1.2
Adsorption of DNA Bases on Glassy Carbon

Differential pulse voltammetry and electrochemical impedance have demonstrated that G, A, guanosine, and their oxidation products are electrostatically adsorbed on GC and $GC_{(ox)}$ surfaces [47, 49]. The strength of adsorption of the DNA bases on the GC surface were found to be similar [49]. Strongly adsorbed G dimers were formed on GC between G and the adsorbed G oxidation products, which slowly cover and block the surface. The application of ultrasound led to removal of the adsorbed species. The effect of this was mainly to enhance transport of electroactive species and to clean the electrode in situ, avoiding electrode fouling.

4.1.3
Nature of the Interactions Between Nucleic Acids and Glassy Carbon

To summarize, the adsorption of nucleic acid may involve electrostatic interactions with the negatively charged DNA backbone. However, strong evidence indicates that the adsorption depends mostly on the hydrophobic interactions between the free bases and the surface of GC. The slight influence of the charge of the GC surface during adsorption (especially produced at high ionic strengths) indicates that there is a small contribution of the negatively charged phosphate backbone in the adsorption of nucleic acids on the GC surface. Moreover, the DNA but also DNA bases (without the negatively charged phosphate backbone) are adsorbed on GC in similar conditions. The dsDNA is poorly adsorbed, because its bases are hidden in the interior of the double-helical molecule forming a part of the Watson-Crick hydrogen bond-

ing system. In contrast, ssDNA is highly adsorbed on GC, because its bases are freely accessible for interaction with the surface.

An increased adsorption of ssDNA on GC, (and oxidized/reduced GC) was observed. Taking into account the nature of the film formed on the $GC_{(ox)}$ surface, the higher affinity of ssDNA could be explained by the formation of hydrogen bonds.

4.2
Modified Glassy Carbon

4.2.1
Chemically-Modified Glassy Carbon

An improved adsorption of DNA bases has been observed at a chemically modified electrode based on a Nafion/ruthenium oxide pyrochlore ($Pb_2Ru_{2-x}Pb_xO_{7-y}$ modified GC (CME). Nafion is a polyanionic perfluorosulfonated ionomer with selective permeability due to accumulation of large hydrophobic cations rather than small hydrophilic ones. The Nafion coating was demonstrated to improve the accumulation of DNA bases, while the ruthenium oxide pyrochlore proved to have electrocatalytic effects towards the oxidation of G and A. The inherent catalytic activity of the CME results from the Nafion-bound oxide surface being hydrated. The catalytically active centers are the hydrated surface-bound oxy-metal groups which act as binding centers for substrates [50].

4.2.2
Polymer Surface-Modified Glassy Carbon

GC material was widely modified with conducting (or nonconducting) polymers in order to obtain an improved surface for DNA adsorption and detection. The initial approaches were performed by the physical attachment of nylon or nitrocellulose membranes on GC electrodes [51]. As explained, these membranes were extensively used in classical DNA analysis due to their well-known adsorption properties [33]. Other approaches were performed by the direct adsorption of the polymeric film on the GC surface. Finally, polymeric films were electrochemically grown on the GC substrate. These conducting polymers are particularly promising for the adsorption, but also for inducing electrical signals obtained from DNA interactions.

4.2.2.1
Chitosan-Modified Glassy Carbon

A chitosan oligomer film was used as an active coating for the immobilization of ssDNA at a GC electrode. Chitosan oligomer is a kind of β-1,4-linked

glucosamine oligomer. It is a natural biocompatible, biodegradable and non-toxic cationic polymer that can form a stable complex through its amino groups with the polyanionic phosphodiester backbone of DNA, either native or denatured. Thus, chitosan and its derivatives may represent potentially safe and efficient cationic carriers for gene delivery. Chitosan was dry-adsorbed on the GC surface. The ssDNA was immobilized on the chitosan-modified GC by wet-adsorption [52]. The main advantage of using chitosan as a modifier of GC was that it could form a tight electrostatic complex with DNA which made the immobilization very stable [53, 54].

4.2.2.2
Layer-by-Layer Deposited Film Modified Glassy Carbon

Fabrication of organic thin films based on spontaneous molecular assembly has been considered as one of the powerful approaches to create novel supramolecular systems. In this context, multilayer films were fabricated by layer-by-layer electrostatic deposition techniques based on the electrostatic interaction between dsDNA and the positively charged polymer poly(diallyldimethylammonium chloride) (PDDA) on GC surfaces. A uniform assembly of PDDA/DNA multilayer films was achieved, based on the adsorption of the negatively charged DNA molecules on the positively charged substrate [55].

4.2.2.3
Polypyrrole-Modified Glassy Carbon

Conducting polymers based on polypyrrole (PPy) display many interesting properties such as redox activity, excellent conductivity, and strong adsorptive capabilities towards negatively charged macromolecules such as DNA and ODNs. These interesting adsorptive properties achieved with the positively charged PPy-modified GC have been extensively studied [56]. The PPy film was grown on GC using nitrate [57] or chloride [58] as dopant counter anions. The PPy-coated GC was demonstrated to be sensitive for detecting adsorbed ODN, DNA, and RNA onto the film. Such adsorption behavior was facilitated by electrostatic interactions between the negatively charged nucleic acids and the positive charge density of the PPy backbone. The different response patterns observed in the presence of different dopants hold great promise for the development of multielectrode nucleic acid arrays. The thickness of the PPy film affected the DNA immobilization effectiveness and its own conductivity property. Thicker PPy layers did not improve the hybridization capability or detection sensitivity. It was also possible to dope nucleic acid probes within electropolymerized PPy films. The ODN served as the sole counter anion during the growth of conducting PPy films, and maintained their hybridization activity within the host polymer network [59]. The

anionic ODN was incorporated within the growing film for maintaining its electrical neutrality.

4.2.3
Liposome-Modified Glassy Carbon

Since lipids are known to associate with DNA with high affinity, the adsorption of ssDNA at lipid membranes as a medium for DNA incorporation on a GC surface was extensively studied [60]. Exploiting DNA–lipid interactions, various approaches were designed for the incorporation of ssDNA [61] and dsDNA [62] at a modified bilayer lipid membrane (BLM) GC surface, such as (1) the formation of self-assembled BLMs over ssDNA previously adsorbed on GC, (2) the direct adsorption of ss- and dsDNA [62] into a previously BLM-modified GC and, (3) formation of a BLM with incorporated ssDNA at the GC surface using the monolayer folding technique [61].

The ssDNA was immobilized stronger and faster on the GC surface in the presence of the lipid membrane than on a bare GC surface and using milder conditions [61]. The lipid membrane enhanced the stability of ssDNA towards desorption from the GC surface [61, 62]. Moreover, the adsorption of ssDNA on BLM induced a conductance enhancement due to (1) structural changes (i.e., defect sites) within the membrane and (2) the increase in negative surface charge density of the membrane. The charge of the phosphate groups of ssDNA induced an increase of cation concentration in the electrical double layer [63].

4.3
Pyrolytic Graphite

One of the first attempts to adsorb DNA onto carbonaceous materials was performed on PG [64].

The use of HOPG as a substrate for the adsorption of DNA made a notable contribution to a better understanding of the adsorption process on carbonaceous material due to the use of high resolution image techniques such as AFM.

In preliminary studies, it was found that dsDNA was adsorbed on HOPG more easily by applying a potential of + 0.4 V (vs Ag/AgCl) for 15 min while ssDNA was adsorbed almost equally whether or not this potential was applied. The adsorption of ssDNA was thus only slightly influenced by the potential, suggesting a different adsorption pattern for ssDNA than for dsDNA on HOPG. The dsDNA could be adsorbed on HOPG mainly by phosphate–sugars whereas ssDNA could be attached not only by phosphate–sugars but also by DNA bases. In contrast to other carbonaceous materials such as GC, dsDNA was easily immobilized on HOPG from the solution (by applying a positive potential). Since the HOPG is a smooth single-crystal plane, the less flexible dsDNA molecule would have more contact with the smooth electrode

surface than with a rough surface such as GC [38, 39]. The oxidation products of dsDNA were not easily removed from the HOPG surface, suggesting that these products are strongly adsorbed. In preliminary studies, electrochemical AFM images of dsDNA adsorbed on a HOPG substrate showed that some segments of dsDNA were adsorbed to form a layer on the surface and other parts of the strands form a DNA island above the layer on the surface. The adsorption of dsDNA did not occur with the molecule lying flat against the HOPG surface but rather through some segments [17], perhaps those where dsDNA is open, thereby exposing DNA bases. These preliminary observations have been confirmed using magnetic AC mode AFM [16, 65, 66].

Since the HOPG surface presents hydrophobic characteristics and DNA is a highly charged hydrophilic molecule, the capacity for spontaneous interaction of DNA with the HOPG surface should be reduced. However, both ss- and dsDNA showed a tendency to spontaneously self-assemble from solution onto the HOPG surface and the process was found to be very fast. Magnetic AC mode AFM images in air revealed good coverage of the surface in a film with the aspect of a two-dimensional network, which has been extensively described [16]. For these studies, DNA was first wet-adsorbed in an open circuit on HOPG and then the layer was dried. The immobilization procedure produced A-DNA molecules over the HOPG due to the strong dehydration after adsorption. The continuous dissociation–association of the bases of the dsDNA extremities exposed the hydrophobic core of the DNA helix sporadically. The dsDNA at the surface was thus stabilized through the interaction between the hydrophobic bases and the hydrophobic surface of the HOPG. The interaction of DNA with the hydrophobic HOPG surface induced DNA superposition, overlapping, and intra- and intermolecular interactions. The topography of the ssDNA-modified HOPG suggested that ssDNA interacted and adsorbed more strongly to the HOPG surface than dsDNA. This can be explained because the ssDNA had bases exposed to the solution, which facilitated the interactions with the hydrophobic carbon surface [16].

The application of a positive potential of + 0.300 V (vs Ag wire) to the HOPG surface during adsorption was also studied [16]. The applied potential considerably enhanced the robustness and stability with respect to mechanical stress of the DNA layers through multiple electrostatic interactions between the negatively charged hydrophilic sugar–phosphate backbone and the positively charged carbon surface. The applied potential increased the attractive lateral interaction between adjacent dsDNA helices and caused spontaneous condensation of the dsDNA layer in a complex network on the HOPG surface. The stability of the dsDNA layer was much increased by electrostatic interaction with the positively charged HOPG surface by structural rearrangement of the molecule. During reorientation and equilibration of the DNA on the surface, the helix was destabilized and some phosphate groups detached from the charged electrode, facilitating electrostatic binding on the HOPG surface of the phosphate groups from the same strand and leading

to no formation of helical DNA parts. As a consequence, parts of the phosphate backbone of one strand lay down flat on the surface. The destabilization and local stretching of the DNA duplex may involve a significant loss of base-stacking and hydrogen-bonding. The DNA bases initially protected inside the helix appeared more exposed to the solution and free to undergo intermolecular interactions by hydrogen bonding and base-stacking with bases from other chains that bind nearly on the surface.

As in the case of dsDNA, the application of a potential of + 0.300 V (vs Ag wire), enhanced the strength, robustness, and resistance to mechanical stress of the ssDNA layer. Electrostatic interactions between the negative charges along the dsDNA and ssDNA phosphate backbone and the positively charged HOPG surface were very strong, which increased stability of the molecules on the substrate. Consequently the adsorbed molecules were less compressible by the AFM tip. Many molecules interacted together by hydrogen bonding during equilibration on the substrate, and hydrophobic interactions and van der Waals forces also contributed to adsorption of DNA on the HOPG electrode [16].

The thin layers formed in ABS (pH 5.3) always presented a better coverage of the HOPG surface with DNA molecules than layers formed in pH 7.0 PBS [65]. Comparing the thickness and the electrode coverage of the layers obtained with both ss and ds DNA at different pHs on applying a potential of + 0.300 V it was concluded that the layer obtained at pH 5.3 presented a self-assembled lattice that was more relaxed and extended on the surface. The results that were obtained by AFM corroborate previous observations that the best binding efficiency of dsDNA on hydrophobic surfaces occurs at approximately pH 5.5 [65].

Owing to these characteristics, PG has been extensively used for the adsorption of DNA and its derivatives. DNA was successfully adsorbed on PG by dry-adsorption at 100 °C [67]. The electrodes were stored in TriS buffer at 4 °C without loss of DNA, showing that DNA was firmly adsorbed on PG. It was demonstrated that the adsorbed ODN was also able to be hybridized with its complementary strand, suggesting that although DNA bases are compromised in the adsorption, they are still available for hybridization [67]. A composite film of DNA and the polyanionic perfluorosulfonated ionomer Nafion was cast on PG by the layer-by-layer procedure performed by dry-adsorption [68]. In another approach, the PG surface was electrochemically pretreated at – 1.7 V for 60 s. DNA was then wet-adsorbed at the pretreated electrode surface from solutions containing 0.2 M NaCl, 10 mM Tris– HCl, pH 7.4 , for 1 min followed by rinsing the electrode with distilled water [69, 70].

4.4 Highly Boron-Doped Diamond

The boron-doped diamond (BDD) thin films are particularly attractive for electroanalytical applications due to their unique characteristics, including

chemical inertness, wide potential window, excellent electrical conductivity and extraordinarily low catalytic activity and very low background current within the working potential range [71]. These properties provide superior sensitivity, , reproducibility, and stability of BDD compared to other conventional materials for electroanalysis.

The BDD film was grown on Si(100) substrates [72]. The adsorption and oxidation of ss- and dsDNA has been investigated in ABS (pH 5.0) at a BDD film. Although BDD films are commonly H-terminated, they usually acquire oxygen on the surface during polishing or anodic oxidation processes. The surface termination has been shown to have significant effects on the adsorption and redox processes of ss- and dsDNA. Owing to the difference in the electronegativities of C (2.5), H (2.1) and O (3.4), the surface acquires C – H and C – O dipoles depending on the termination, thus making the surface partially charged [71]. In the case of hydrogen termination, the surface acquires a very small positive polar charge, while the O-terminated surface acquires a relatively high negative polar charge due to the higher dipole moment, causing electrostatic interactions with charged molecules such as DNA. O-terminated diamond was found to repel the DNA molecule, while H-terminated diamond attracted the DNA due to its weak positive charge, enhancing its adsorption on the surface [71]. In contrast, the surface termination did not show much influence on free A and G adsorption. The influence of the negatively charged phosphate-containing sugar backbone in the electrostatic interaction was thus quite obvious. The adsorption of DNA at H-terminated diamond was almost independent of ionic strength, due to the small electrostatic interaction between the H-terminated surface and negative charge of DNA, where the ionic strength did not influence the adsorption much. However, on the O-terminated diamond, a drastic increase in the adsorption would be expected with increased ionic strength, which indicates the masking of surface charge by an increasing number of the positive counter ions in the solution, resulting in a relatively neutral surface. The ssDNA molecule was more firmly adsorbed on the surface of BDD than dsDNA. This difference could be assumed a consequence of the difference in the flexibilities of the DNA. The more rigid dsDNA covered less efficiently the roughness of BDD surface than ssDNA [71].

4.5 Carbon Composites

4.5.1 Soft Carbon Composites. Carbon Pastes

The adsorption of DNA and its derivatives on CP materials has been widely reported. CP for DNA adsorption could be successfully prepared by the

mixing of 70/30 (w/w) graphite powder/mineral oil [73, 74], a composition which yielded the most favorable signal-to-background characteristics.

Of the several ways that nucleic acid could be immobilized on CP surface, electrostatic adsorption proved to be an effective and simple route, and thus was widely used [75]. It was found that the anodic pre-treatment of CP [at + 1.7, 60 s in ABS (pH 5)] greatly enhances the electrostatic adsorption (+ 0.5 V vs Ag/AgCl) of dsDNA, ssDNA, RNA and its derivatives [75]. However, the treated surface did not show electrocatalytic activity. The anodization produced—as in other carbonaceous materials—a substantially larger background current contribution. The electrochemical pre-treatment led to an increase in the density of surface oxygenated groups, a more hydrophilic surface state, and a concomitant removal of organic and pasting-liquid layers from the surface [76]. Such a change in the surface state appeared to facilitate the interfacial adsorption of RNA [73] and DNA [76, 77] on CPs, but not the charge-transfer. Similar behavior was observed at GC, as previously described.

However, after the study of inosine-substituted ODN, there was evidence that the pretreatment improved the electrochemistry of purine bases, with a smaller effect on the interfacial accumulation [78]. The G oxidation signal was strongly affected by the surface pre-treatment of CP. However, the inosine-modified probe response was less affected by this treatment [78], suggesting a lesser effect on adsorption over the electrochemistry of purine base. However, if inosine (a non-purine base) substituted G in the ODN sequence, the stability of the adsorbed probe on CP was similar to that observed with G-containing ODN, i.e., being stable in a stirred PBS for up to 15 min. Such behavior indicated that the inosine substitution has little effect upon the stability of the adsorbed probe [78].

As in other carbonaceous materials, higher electrostatic adsorption efficiencies for short nucleotide sequences (ssDNA) were observed [76]. Shorter ODNs penetrated more readily into the grooves and pores of the rough CP surface. Such behavior increased the accessibility of the base moieties to the graphite particle electron-transfer sites. In contrast, the access of longer oligomers (10 basepairs or more) into the porous surface was restricted. They were unable to follow the contours of the surface and, accordingly, their oxidation currents were smaller [79]. Such interaction with the surface was found to also depend on the flexibility of the DNA molecule, with more rigid molecules following the rough surface less efficiently. Those length-dependent differences on the surface penetration and accessibility of synthetic ODN have shown a profound effect on the adsorption properties. Additionally, it was found that the adsorption and oxidation were influenced not only by the length and rigidity of the ODN, but also by its base content and sequence [79]. However, the less flexible and longer dsDNA molecule was also electrostatically adsorbed at + 0.5 V yielding a high stable layer for the biosensing of pollutants [80].

The coupling of the CP pre-treatment and the electrostatic adsorption results in a stable immobilization layer of ODN. The adsorbed ODN layer on CP remained stable throughout 60 min in stirred solutions of PBS [74]. Moreover, the electrostatic adsorption procedures at + 0.5 V led to a reactive and accessible probe. A comparison study between CP and Hg electrodes showed that no significant hybridization of the DNA adsorbed on Hg was taking place, probably due to a strong interaction of hydrophobic bases with the hydrophobic surface of the mercury electrode. The bases of the probe, interacting strongly with the surface, cannot be accessible to form specific base pairs with the target DNA. Compared with the negatively charged mercury electrode, a different orientation of the adsorbed DNA molecule can be expected at the positively charged CP electrode. The DNA could be attached to the CP surface via the negatively charged hydrophilic sugar–phosphate backbone with bases oriented toward the solution and available for the hybridization with the target DNA. The results of the hybridization experiments matched up to this expectation [81].

This strong adsorption of DNA and its derivatives on carbon materials has made possible the adsorption (and preconcentration) of DNA on CP and its further separation from interferences. It has been shown that low molecular mass substances did not interfere with the analysis of DNA and RNA if the nucleic acid was previously adsorbed at the CP electrode, which was then washed and transferred to a blank electrolyte. This procedure was called adsorptive transfer stripping voltammetry (AdTSV) [75, 82, 83]. Although the electrostatic adsorption results in a strong and irreversible accumulation, the ability to remove DNA layers from CP microelectrodes under potential control was demonstrated. The electrostatic release of surface-confined DNA layer was performed in PBS (pH 7.4) at a potential of – 1.2 V for 2 min. The application of the negative potential at the DNA-modified CP has been shown to electrostatically repel the negatively charged nucleic acid molecules from the CP negatively charged surface [75].

It was also demonstrated that the application of increased temperatures during the electrostatic adsorption results in dramatic enhancement of the oxidation signal, ascribable to an improved electrostatic adsorption at the heated electrode. Forced thermal convection near the electrode surface facilitated the electrostatic adsorption and the use of quiescent solutions [84–86]. The main role of the high temperature in CP was found to be the enhancement of the adsorption efficiency (e.g., through faster localized convection and faster kinetics) but not the preactivation of the surface. The temperature effect strongly depended on the chain length and structure of the nucleic acid molecule [84]. The reason could be a change of structure in the molecule which is suspected to be temperature-dependent. Faster molecular movement and changes in structure could facilitate the adsorption. More electrochemically active sites of the nucleic acid molecules could come into close contact with the electrode surface, thus increasing the signal. In highly complex

molecules such as dsDNA, this effect has proved to be relatively strong compared to that in less complex ones such as tRNA [84].

Another CP pre-treatment that was found to greatly enhance the electrostatic adsorption at + 0.5 V (vs Ag/AgCl) of ODN [87], dsDNA and ssDNA [88, 89] was performed at almost the same conditions (+ 1.7 V, 60 s) but in neutral solutions (PBS pH 7.5). A combination of + 1.5 V, 1 min, PBS pH 7.0 as a pre-treatment step with a further electrostatic adsorption at + 0.3 V was also demonstrated to be successful for immobilizing dsDNA on CP [90].

4.5.1.1 Surface-Modified Carbon Pastes

CP was surface-modified with cetyltrimethyl ammonium bromide (CTAB) by dry adsorption (CTAB/CP) [91]. CTAB could change the surface properties of CP, forming a compact monolayer on the electrode surface with a high density of positive charges. The stabilization of the monolayer was achieved by hydrophobic adsorption of CTAB on the hydrophobic surface of CP. The paraffin oil layer covering the carbon particles had hydrophobic properties similar to those of the CTAB layer. Thus, CTAB could form a stable monolayer on the surface of CP. The CTAB/CP material was applied to the immobilization of dsDNA [91]. The procedure for immobilizing DNA was electrostatic adsorption. With the modification of dsDNA, the CP surface turned from poor to high hydrophilicity and the hydrophilic surface could survive the thorough washing with water, which indicated the tight combination of DNA on the electrode surface [91].

A chitosan-modified CP (ChiCP) material was prepared for the electrostatic adsorption of dsDNA, ssDNA and ODNs [92]. The immobilized ODN could selectively hybridize with the target DNA to form a hybrid on the ChiCP surface.

4.5.1.2 DNA Modifying Carbon Pastes as an Additive

Additives such as polyethylene glycol, cationic antibiotics, polymers, small uncharged molecules, and negatively charged proteins have been used extensively in order to avoid the denaturing of enzymes or to improve the sensitivity and operational stability of biosensors. DNA has been proposed as an additive to improve the response and stability of biosensors based on CP. The biomolecules studied, such as tyrosinase [93], peroxidase [94], cytochrome C [95], have been shown to improve its performance by using adsorbed DNA within CP as an additive.

The presence of DNA in the biosensor improved the durability and greatly increased the sensitivity of the sensor. When the CP-DNA-Tyr was first exposed to the electrolyte, some swelling was observed as a result of hydration

of the DNA. The DNA thus provided a more hydrophilic environment for the enzyme. The direct interaction of DNA with the functional groups of amino acids occurred through hydrogen bonding, with partial displacement of the well-ordered water shell of DNA or with individual water molecules acting as bridges of the hydrogen bonding. Through such interactions, DNA could improve the stabilization of the tertiary structure of the enzyme in comparison with other additives [93]. The DNA molecules have been proved to be an efficient promoter for a direct electron transfer reaction [95], increasing the sensitivity of the biosensors [94]. This behavior was also shown during the evaluation of the Doyle catalyst performance when adsorbed on CP in the presence and the absence of DNA, suggesting a good hydrophilic and conductor character of DNA [96].

4.5.2 Rigid Carbon Composites

Rigid carbon composites have been widely used in our laboratories for the adsorption of ssDNA, dsDNA, ODN and DNA derivatives. In particular, we have used graphite-epoxy composite (GEC) made by mixing the nonconducting epoxy resin (Epo-Tek, Epoxy Technology, Billerica, MA, USA) with graphite powder (particle size below 50 μm). An ideal material for electrochemical genosensing should allow an effective immobilization of the probe on its surface, a robust hybridization of the target with the probe, a negligible nonspecific adsorption of the label and a sensitive detection of the hybridization event. GECs fulfill all these requirements.

Owing to its improved electrochemical characteristics, ss- and dsDNA and ODN have been immobilized by dry adsorption on GEC [97, 98], yielding a thick DNA layer. Beside its improved electrochemical properties, GEC has shown unique and selective adsorption behavior. While DNA is firmly adsorbed under dry conditions, the wet-adsorption of nonspecific DNA, proteins, enzymes or other biomolecules has proved to be negligible under stirring or convection conditions in solution. The DNA-modified GEC surface does not require blocking steps to minimize the nonspecific adsorption on the free sites of the surface. The dual nature of GEC composed of islands of conducting material within the nonconducting and hydrophobic epoxy resin could play an important role in stabilizing the dehydrated A-form of DNA adsorbed on GEC.

Besides thick-layer DNA/GEC surface, a thin-layer DNA/GEC could be achieved by wet-adsorption of ss- and dsDNA and ODN onto a GEC transducer under static conditions [99, 100]. In this case, the hydrated B-DNA form was stabilized over the GEC surface by weaker forces. Unlike the GEC surface modified by the thick DNA layer —produced in dry conditions—the thin-layer DNA/GEC surface required blocking treatment to avoid nonspecific

adsorption. The wet-adsorption procedure produced a less compact DNA layer with wider gaps exposing free GEC surface.

Although DNA can be firmly adsorbed on GEC, it retains its unique hybridization properties, which can be monitored using various strategies [99, 100], suggesting that the DNA bases are not fully committed in the adsorption mechanism.

Moreover, the unique adsorption properties of GEC allowed the very sensitive electrochemical detection of DNA based on its intrinsic oxidation signal that was shown to be strongly dependent of the multi-site attachment of DNA and the proximity of G residues to GEC [100]. The thick layer of DNA adsorbed on GEC was more accessible for hybridization than those in nylon membranes obtained with genosensors based on nylon/GEC with a changeable membrane [99, 101, 102]. Although GEC has a rough surface, it is impermeable, while nylon is more porous and permeable. DNA assays made on an impermeable support are less complex from a theoretical standpoint [7]: the kinetics of the interactions are not complicated by the diffusion of solvent and solutes into and out of pores or by multiple interactions that can occur once the DNA has entered a pore. This explained the lower hybridization time, the low nonspecific adsorption and the low quantity of DNA adsorbed onto GEC compared to nylon membranes.

Compared to GC (a transducer widely used in electrochemical genosensors), the higher sensitivity of GEC can be explained by its higher active surface and rugosity, as is evident from scanning electron microphotographs of both surfaces and also a behavior mimicking that of a random assembly of microelectrodes [97] (Fig. 4). Unlike CP, the rigidity of GEC permits the

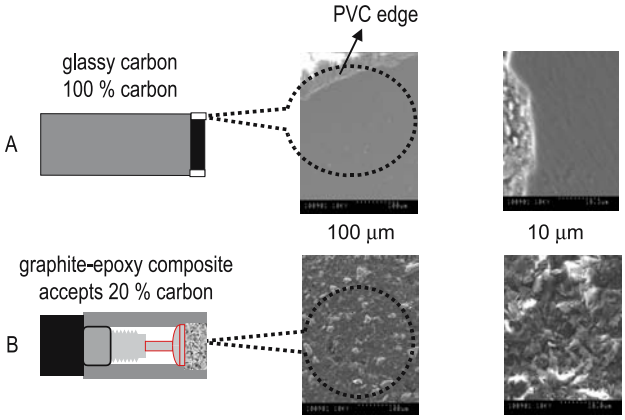

Fig. 4 Scanning electron microphotographs of the surfaces of glassy carbon (GC) (**a**) and graphite epoxy composite (GEC) (**b**). The same acceleration voltage (10 kV) and the same resolution (100 and 10 μm) were used in both cases. Taken from [97]. Reprinted with permission

design of different configurations, and these materials are compatible with non-aqueous solvents.

A new class of sol-gel-based carbon composite material can be formed by homogeneous dispersion of graphite powder in a suitable sol-gel precursor/monomer (mainly based on methanol and methyltrimethoxysilane) [103, 104]. The resulting homogeneous sol-gel CP was allowed to polymerize and left to dry, obtaining the silica sol-gel-derived carbon composite material. Electrodes prepared on the basis of this material have been reported to have various desirable properties for electroanalysis: low background current, good chemical and mechanical stability, easy preparation and wide potential window. CPs proved to have inferior mechanical stability compared with this composite material. At certain and non-stringent conditions, A (but also G) was demonstrated to be electrostatically adsorbed onto this porous material.

4.6 Carbon Inks

DNA has been widely adsorbed on carbon inks. These carbon inks were previously printed onto alumina ceramic plates or other substrates such as polyester for fabricating thick film sensors. Carbon inks are commercially available but normally their composition is not precisely known. As a consequence, it is difficult to predict interactions between DNA and carbon inks, which contain various components such as binders besides graphite. They usually require a curing step at high temperatures. Unlike the CP materials, some carbon inks require a short (10 s) precathodization [at – 2.0 V) prior to the 1 min oxidative activation (1 min at + 1.8 V in ABS (pH 5.0)] [76]. DNA adsorption was performed as in the case of CP by electrostatic adsorption at a potential of + 0.5 V in pH 5.0 ABS (vs Ag/AgCl) [105, 106]. The short anodic pre-treatment was found to enhance the electrostatic adsorption of DNA. The enhanced adsorption was attributed to the increased surface roughness and hydrophilic properties following such treatment [106]. It was also demonstrated that the pre-treated graphite inks at + 1.6 V for 1 min in PBS (pH 7.0) [107] or ABS (pH 5.0) [108, 109] showed lower background currents. The anodization probably removed undesirable compounds from the electrode surface and graphite impurities. Higher oxidation times (up to 60 min) for pre-treatment increased the noise and the background current [107]. The adsorbed nucleic acid layer (ssDNA, dsDNA and ODN) on the carbon inks was demonstrated to be stable for at least 30 min under stirring in ABS (pH 5.0) at + 0.5 V [105].

Besides DNA adsorption driven by a positive potential (electrostatic adsorption) DNA was also wet-adsorbed at an open circuit on a home-made polystyrene-based carbon ink [110]. This ink was prepared by a 2 : 3 mixture of polystyrene and graphite particles in mesitylene, and then printed on a polyester film. DNA was wet-adsorbed over the ink at 37 °C overnight. The nature of the electrode surface (graphite particles embedded in a polystyrene

binder) was found to be a suitable solid phase for the reproducible adsorption of DNA. Moreover, this solid phase led to a negligible nonspecific adsorption of the non complementory ODN probe [110].

DNA modification of a commercial carbon ink without any electrochemical preconditioning by dry-adsorption was also reported. The surface was modified by covering with dsDNA solution and leaving the electrode to dry overnight. A stable, thin (about 100 μm) DNA layer was obtained [111, 112]. The dsDNA was also demonstrated to be stably adsorbed when entrapped in a cellulose-acetate-based film on the surface of a carbon-based ink [113].

4.7
Graphite Pencil Leads

Renewable graphite pencil leads have been demonstrated to be excellent materials for the adsorption of DNA. Various pencil lead materials have been studied [114]. As in the case of GC and CP, the graphite leads were electrochemically oxidized at + 1.4 V for 30 s prior to the DNA electrostatic adsorption, performed at + 0.5 V (vs Ag/AgCl) for 60 s in stirred ABS (pH 5.0) [114, 115]. The adsorption of DNA was shown to be similar to that at a CP electrode. However, substantial differences in adsorption and electrochemical performance were observed at the various types of graphite leads. Such different responses are to be expected considering the different composition and roughness of the various graphite leads containing various insulating polymeric binders and clays besides graphite [116]. However, these non-graphite constituents do not yield a background peak. In the view of the composite nature of the leads, these differences could reflect the differences in the adsorption properties of the nucleic acid, in the kinetics of DNA oxidation processes, or in the effective surface area (roughness) [114].

4.8
Carbon nanotubes

Since it was initially reported [21], several methods have been presented in order to attach DNA onto CNTs, including adsorption. First, transmission electron microscopy showed that the DNA molecules tended to cover the surface of the nanotubes evenly, suggesting a strong interaction with the carbon surface [24].

DNA/SWCNTs interactions were studied by IR and UV spectroscopy and it was found that CNTs could self-organize with DNA molecules during adsorption processes [117, 118]. Moreover, some evidence indicated that SWCNTs influenced the DNA structure more strongly than graphite [119]. The interaction between DNA and CNTs would cause changes in the hydrogen bonds [119] and the partial unwrapping of the dsDNA when dry-adsorbed on CNTs [118]. The SWCNTs could cause A–B transition in some fragments of DNA sugar–

phosphate backbone. This could be in agreement with the model of DNA interaction with SWCNTs based on wrapping the nucleic acid molecule around the CNT [120]. A similar type of DNA behavior occurs in vivo in chromosomes during the process of DNA-assembling by histones.

The regular system of hydrogen bonds in DNA is destroyed in DNA/NaOH solution and the DNA molecule is partly transformed from a double spiral to a chaotic ball [118]. This transformation may promote the interaction of DNA molecules with CNTs. The ssDNA adsorption on CNTs was greater than for dsDNA molecules [117, 118], suggesting that the adsorption of DNA on CNT is presumably via hydrophobic interactions between the nanotubes and the hydrophobic bases on DNA.

4.8.1 Surface-Modified Carbon Nanotubes Approaches

To take advantages of the unique properties of CNTs, a general approach is the immobilization of DNA on CNTs and the further immobilization of the DNA-modified CNTs on an easier-to-handle pure conductor, e.g., GC [26], Pt [121], Au [122]. Another approach consists of the prior modification of the pure conductor (GC) with the CNTs through dry-adsorption and the further DNA or DNA derivatives adsorption on the CNT-modified surface [123–125].

Compared with the bare substrate material (GC, Pt, Au), it was demonstrated generally that the background current of the MWCNTs surface-modified conductor was apparently larger [123, 124]. The surface modification of the conducting material with MWCNTs could thus significantly enhance the effective electrochemical surface area as well as provide a larger surface for DNA immobilization [26]. Unlike the commonly used CP, GC or graphite leads, the CNTs/GC material does not require a surface pre-treatment for enhancing electrochemical signals [123], reflecting a substantial interfacial accumulation onto the CNT-modifier rather than an accelerated electron transfer. Such interfacial adsorption reflects the nature of the MWCNT surface, and its large surface area/volume ratio [123].

As an example, dsDNA was wet-adsorbed on MWCNTs over 24 h. The DNA-modified MWCNT was deposited on Pt and allowed to dry overnight [121]. The thickness of the DNA/MWCNT layer was 160 nm. The modified electrode was stored at 4 °C for 2 months. The good reproducibility and long-term performance can be attributed to the stability of the DNA/MWCNT layer [121]. The DNA molecule was also wet-adsorbed on previously modified SWCNT/GC [124] for 5 min in an open circuit from a PBS (pH 7.2) solution. Under these conditions, the same electrochemical response was obtained for ds- and ssDNA, again suggesting a strong interaction between dsDNA and SWCNTs. As a result of this interaction, the primary redox sites of A and G residues were exposed because of the unwrapping of the DNA double helix and changes in the hydrogen bonds between the bases of the dsDNA

molecule [124]. Moreover, not only dsDNA, but also its oxidation product was strongly adsorbed on the SWCNT–GC surface [124].

Additionaly, the electrostatic assembly of calf thymus DNA on MWCNTs via a cationic polyelectrolyte [poly(diallyldimethylammonium chloride), (PDDA)] was reported [122]. The positively charged PDDA molecule played a key role in the attachment of DNA to MWCNTs, acting as a bridge to connect the negatively charged DNA molecule with MWCNTs, although the direct adsorption of DNA on MWCNTs was observed [122]. By repeating the PDDA/DNA adsorption cycle several times, a PDDA/DNA multi-layer could be formed on MWCNTs.

4.8.2
Bulk-Modified Carbon Nanotubes Approaches

Among the surface-modified CNTs materials, a bulk-modified CNT paste (CNTP) has also been reported [126]. The new composite electrode combined the ability of CNTs to promote adsorption and electron-transfer reactions with the attractive properties of the composite materials. The CNTP was prepared by mixing MWCNTs powder (diameter 20–50 nm, length 1–5 μm) and mineral oil in a 60 : 30 ratio. The oxidation pretreatment [performed in ABS (pH 5.0) for 20 s at 1.30 V, vs Ag/AgCl] proved to be critical in the state of the CNTP surface. Pretreatments improved the adsorption and electrooxidation of both DNA and DNA bases, probably due to the increase in the density of oxygenated groups.

Although the adsorption of DNA at CP was shown to be favored at positive potentials, almost no dependence with the potential was observed at CNTP. No changes in adsorption were observed for dsDNA in the potential range of 0.50 to – 0.20 V, indicating that there is a poor contribution of the negatively charged phosphate backbone in the adsorption of nucleic acids at CNTP, as in the case of GC [44]. Considering the structure of CNTs, it is reasonable to expect that the character of the interaction between the DNA bases and CNTP is mainly hydrophobic [117, 118, 124, 126]. It was also observed that the size of the molecules has a significant effect, producing better adsorption of smaller ODNs on CNTs.

5
Concluding Remarks

A wide range of carbonaceous materials can be modified with a stable DNA adsorbed layer. The multi-site attachment of DNA on carbon surfaces seems to be strongly dependent on hydrophobic interactions between DNA bases and carbon substrates such as GC and $GC_{(ox)}$, HOPG, CNTs and GECs. Al-

though multi-site adsorption was previously claimed to be a disadvantage for hybridization, DNA can be perfectly hybridized with its complementary strand when adsorbed on most of the carbon-based materials. Moreover, adsorbed dsDNA can be easily detected without the need for external markers because multi-site adsorption is known to produce an improved oxidation signal coming from DNA bases moieties.

With regard to its unique properties, a carbon substrate can be considered an excellent alternative material to continuous metal conductors and semiconductors for the construction of DNA sensors and chips.

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References

1. Baltimore D (2001) Nature 409:814
2. Bowtell DDL (1999) Nature Genet [suppl] 21:25
3. Collins FS (1999) Nature Genet [suppl] 21:2
4. Lander ES (1999) Nature Genet [suppl] 21:3
5. Sanders GHW, Manz A (2000) Trends Anal Chem 19:364
6. Pividori MI, Merkoçi A, Alegret S (2000) Biosensor Bioelectron 15:291
7. Southern E, Mir K, Shchepinov M (1999) Nature Genet [suppl] 21:5
8. Paleček E (1958) Naturwiss 45:186
9. Paleček E (1960) Nature 188:656
10. Streat M, Malik DJ, Saha B (2004) Adsorption and ion-exchange properties of engineered activated carbons and carbonaceous materials. In: SenGupta AK, Marcus Y, Marinsky JA (eds) Ion exchange and solvent extraction, chap 1. Dekker, New York
11. Chu X, Kinoshita K (1997) Mater Sci Eng B 49:53
12. Regisser F, Lavoie MA, Champagne GY, Belanger D (1996) J Electroanal Chem 415:47
13. McCreery RL, Cline KK (1996) Carbon electrodes. In: Kissinger PT, Heineman WR (eds) Laboratory techniques in electroanalytical chemistry, chap 10. Dekker, New York
14. Hu IF, Karweik DH, Kuwana T (1985) J Electroanal Chem 188:59
15. Kepley LJ, Bard AJ (1988) Anal Chem 60:1459
16. Oliveira Brett AM, Chiorcea AM (2003) Langmuir 19:3830
17. Wu L, Zhou J, Luo J, Lin Z (2000) Electrochim Acta 45:2923
18. Alegret S (1996) Analyst 121:1751
19. Céspedes F, Martínez-Fàbregas E, Alegret S (1996) Trends Anal Chem 15:296
20. Adams RN (1958) Anal Chem 30:1576
21. Iijima S (1991) Nature 354:56
22. Sherigara BS, Kutner W, D'Souza F (2003) Electroanalysis 15:753
23. Tsui R, Siragusa L, Goronkin H (2000) C R Acad Sci Paris 1:875
24. Guo ZJ, Sadler PJ, Tsang SC (1998) Adv Mater 10:701
25. Xu J (2003) Proc IEEE 91:1819
26. Cai H, Cao XN, Jiang Y, He PG, Fang YZ (2003) Anal Bioanal Chem 375:287

27. Walther JH, Jaffe RL, Kotsalis EM, Werder T, Halicioglu T, Koumoutsakos P (2004) Carbon 42:1185
28. Li J, Ng HT, Cassell A, Fan W, Chen H, Ye Q, Koehne J, Han J, Meyyappan M (2003) Nano Lett 3:597
29. Oliveira Brett AM, Piedade JAP, Serrano SHP (2000) Electroanalysis 12:969
30. Wang J (1998) Biosensor Bioelectron 13:757
31. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York
32. Meinkoth J, Wahl G (1984) Anal Biochem 138:267
33. Reed KC, Mann DA (1985) Nucleic Acids Res 13:7207
34. Rasmussen SR, Larsen MR, Rasmussen SE (1991) Anal Biochem 198:138
35. Pang DW, Zhang M, Wang ZL, Qi YP, Cheng JK, Liu ZY (1996) J Electroanal Chem 403:183
36. Oliveira Brett AM, Vivan M, Fernandes IR, Piedade JAP (2002) Talanta 56:959
37. Oliveira Brett AM, Serrano SHP, Gutz I, La-Scalea MA, Cruz ML (1997) Electroanalysis 9:1132
38. Brett CMA, Oliveira Brett AM, Serrano SHP (1999) Electrochim Acta 44:4233
39. Oliveira Brett AM, Macedo TRA, Raimundo D, Marques MH, Serrano SHP (1998) Biosensor Bioelectron 13:861
40. Abreu FC, Goulart MOF, Oliveira Brett AM (2002) Biosensor Bioelectron 17:913
41. Lu X, Zhu K, Zhang M, Liu H, Kang J (2002) J Biochem Biophys Methods 52:189
42. Wang ZX, Liu DJ, Dong SJ (2001) Bioelectrochemistry 53:175
43. Wang ZX, Liu DJ, Dong SJ (2001) Biophys Chem 89:87
44. Pedano ML, Rivas GA (2003) Biosensor Bioelectron 18:269
45. Tang TC, Huang HJ (1999) Electroanalysis 11:1185
46. Wang HS, Ju HX, Chen HY (2001) Electroanalysis 13:1105
47. Wang HS, Ju HX, Chen HY (2002) Anal Chim Acta 461:243
48. Wang HS, Ju HX, Chen HY (2002) Electroanalysis 14:1615
49. Oliveira Brett AM, da Silva LA, Brett CMA (2002) Langmuir 18:2326
50. Zen JM, Chang MR, Ilangovan G (1999) Analyst 124:679
51. Napier ME, Thorp HH (1999) J Fluorescence 9:181
52. Xu C, Cai H, He P, Fang Y (2001) Analyst 126:62
53. Cai H, Wang Y, He P, Fang Y (2003) Anal Chim Acta 469:165
54. Zhu N, Cai H, He P, Fang Y (2003) Anal Chim Acta 481:181
55. Luo L, Liu J, Wang Z, Yang X, Dong S, Wang E (2001) Biophys Chem 94:11
56. Li Z, Wang H, Dong S, Wang E (1997) Anal Sci 13:305
57. Wang J, Jiang M, Mukherjee B (1999) Anal Chem 71:4095
58. Cai H, Zhu N, Jiang Y, He P, Fang Y (2003) Biosensor Bioelectron 18:1311
59. Wang J, Jiang M, Fortes A, Mukherjee B (1999) Anal Chim Acta 402:7
60. Nikolelis DP, Hianik T, Krull UJ (1999) Electroanalysis 11:7
61. Siontorou CG, Oliveira Brett AM, Nikolelis DP (1996) Talanta 43:1137
62. Tong Y, Han X, Song Y, Jiang J, Wang E (2003) Biophys Chem 105:1
63. Siontorou CG, Nikolelis DP, Tarus B, Dumbrava J, Krull UJ (1998) Electroanalysis 10:691
64. Brabec V, Dryhurst G (1978) J Electroanal Chem 89:161
65. Oliveira Brett AM, Chiorcea AM (2003) Electrochem Comm 5:178
66. Chiorcea AM, Oliveira Brett AM (2002) Bioelectrochemistry 55:63
67. Hashimoto K, Ito K, Ishimori Y (1994) Anal Chim Acta 286:219
68. Rusling JF, Zhou L, Munge B, Yang J, Estavillo C, Schenkman JB (2000) Faraday Discuss 116:77

69. Palecek E, Fojta M, Jelen F (2002) Bioelectrochemistry 56:85
70. Fojta M, Havran L, Kizek R, Billová S (2002) Talanta 56:867
71. Ivandini TA, Sarada BV, Rao TN, Fujishima A (2003) Analyst 128:924
72. Rao TN, Yagi I, Miwa T, Tryk DA, Fujishima A (1999) Anal Chem 71:2506
73. Wang J, Cai X, Wang J, Jonsson C, Palecek E (1995) Anal Chem 67:4065
74. Wang J, Cai X, Rivas G, Shiraishi H (1996) Anal Chim Acta 326:141
75. Wang J, Zhang XJ, Parrado C, Rivas G (1999) Electrochem Comm 1:197
76. Wang J, Cai X, Jonsson C, Balakrishnan M (1996) Electroanalysis 8:20
77. Wang J, Rivas G, Cai X, Dontha N, Shiraishi H, Luo D, Valera FS (1997) Anal Chim Acta 337:41
78. Wang J, Rivas G, Fernández JR, Lopez Paz JL, Jiang M, Waymire R (1998) Anal Chim Acta 375:197
79. Wang J, Kawde AN, Sahlin E, Parrado C, Rivas G (2000) Electroanalysis 12:917
80. Wang J, Chicharro M, Rivas G, Cai X, Dontha N, Farias PAM, Shiraishi H (1996) Anal Chem 68:2251
81. Palecek E, Fojta M, Tomschik M, Wang, J (1998) Biosensor Bioelectron 13:621
82. Cai X, Rivas G, Farias PAM, Shiraishi H, Wang J, Palecek E (1996) Anal Chim Acta 332:49
83. Wang J, Bollo S, Lopez Paz JL, Sahlin E, Mukherjee B (1999) Anal Chem 71:1910
84. Wang J, Gründler P, Flechsing GU, Jasinski M, Rivas G, Sahlin E, Lopez Paz JL (2000) Anal Chem 72:3752
85. Korbut O, Bucková M, Tarapcík P, Labuda J, Gründler P (2001) J Electroanal Chem 506:143
86. Korbut O, Bucková M, Labuda J, Gründler P (2003) Sensors 3:1
87. Erdem A, Kerman K, Meric B, Akarca US, Ozsoz M (1999) Electroanalysis 11:586
88. Erdem A, Meric B, Kerman K, Dalbasti T, Ozsoz M (1999) Electroanalysis 11:1372
89. Meric B, Kerman K, Ozkan D, Kara P, Erensoy S, Akarca US, Mascini M, Ozsoz M (2002) Talanta 56:837
90. Radi A (1999) Anal Chim Acta 386:63
91. Hu C, Hu S (2004) Electrochim Acta 49:405
92. Kara P, Kerman K, Ozkan D, Meric B, Erdem A, Nielsen PE, Ozsoz M (2002) Electroanalysis 14:1685
93. Dantoni P, Serrano SHP, Oliveira Brett AM, Gutz IGR (1998) Anal Chim Acta 366:137
94. Rosatto SS, De Oliveira Neto G, Kubota LT (2001) Electroanalysis 13:445
95. Lee TY, Kim HJ, Moon JO, Shim YB (2004) Electroanalysis 16:821
96. Gil ES, Kubota LT (2000) Bioelectrochemistry 51:145
97. Pividori M I, Merkoçi A, Alegret S (2003) Biosensor Bioelectron 19:473
98. Pividori M I, Merkoçi A, Barbé J, Alegret S (2003) Electroanalysis 15:1815
99. Pividori M I, Alegret S (2003) Anal Lett 36:1669
100. Erdem A, Pividori M I, del Valle M, Alegret S (2004) J Electroanal Chem 567:29
101. Pividori M I, Merkoçi A, Alegret S (2001) Biosensor Bioelectron 16:1133
102. Pividori M I, Merkoçi A, Alegret S (2001) Analyst 126:1551
103. Teh HF, Yang X, Gong H, Tan SN (2004) Electroanalysis 16:769
104. Li J, Chia LS, Goh NK, Tan SN (1999) J Electroanal Chem 460:234
105. Wang J, Cai X, Rivas G, Shiraishi H, Dontha N (1997) Biosensor Bioelectron 12:587
106. Wang J, Rivas G, Cai X (1997) Electroanalysis 9:395
107. Marrazza G, Chianella I, Mascini M (1999) Biosensor Bioelectron 14:43
108. Chiti G, Marrazza G, Mascini M (2001) Anal Chim Acta 427:155
109. Marrazza G, Chianella I, Mascini M (1999) Anal Chim Acta 387:297
110. Azek F, Grossiord C, Joannes M, Limoges B, Brossier P (2000) Anal Biochem 284:107

111. Bucková M, Labuda J, Sandula J, Krizková L, Stepánek I, Duracková Z (2002) Talanta 56:939
112. Labuda J, Bucková M, Heilerová L, Caniová-Ziaková A, Brandsteterová E, Mattusch J, Wennrich R (2002) Sensors 2:1
113. Labuda J, Bucková M, Jantová S, Stepánek I, Surugiu I, Danielsson B, Mascini M (2000) Fresenius J Anal Chem 367:364
114. Wang J, Kawde AN, Sahlin E (2000) Analyst 125:5
115. Wang J, Kawde AN, Erdem A, Salazar M (2001) Analyst 126:2020
116. Wang J, Kawde AN (2001) Anal Chim Acta 431:219
117. Matyshevska OP, Karlash AY, Shtogun YV, Benilov A, Kirgizov Y, Gorchinskyy KO, Buzaneva EV, Prylutskyy YI, Scharff P (2001) Mater Sci Eng C 15:249
118. Buzaneva E, Karlash A, Yakovkin K, Shtogun Y, Putselyk S, Zherebetskiy D, Gorchinskiy A, Popova G, Prilutska S, Matyshevska O, Prilutskyy Y, Lytvyn P, Scharff P, Eklund P (2002) Mater Sci Eng C 19:41
119. Dovbeshko GI, Repnytska OP, Obraztsova ED, Shtogun YV (2003) Chem Phys Lett 372:432
120. O'Connell MJ, Boul P, Ericson LM, Huffman C, Wang Y, Haroz E, Kuper C, Tour J, Ausman KD, Smalley RE (2001) Chem Phys Lett 342:265
121. Wang G, Xu JJ, Chen HY (2002) Electrochem Commun 4:506
122. Guo M, Chen J, Nie L, Yao S (2004) Electrochim Acta 49:2637
123. Wang J, Kawde AN, Musameh M (2003) Analyst 128:912
124. Wang J, Li M, Shi Z, Li N, Gu Z (2004) Electroanalysis 161:140
125. Xu Y, Jiang Y, Cai H, He PG, Fang YZ (2004) Anal Chim Acta 516:19
126. Pedano ML, Rivas GS (2004) Electrochem Commun 6:10