Carbon nanomaterials for electrochemical and electrochemiluminescent medical sensors

Chapter 6. Carbon nanomaterials for electrochemical and electrochemiluminescent medical sensors

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6.1 Electrochemical properties of carbon nanostructured electrodes

Carbon nanomaterials have attracted a considerable interest as modifiers of electrodes. Some of their unique electrochemical advantages are: the wide potential window, the reduction of overvoltage, the resistance to surface fouling and the increase of both electroactive area and electron transfer rate which produce a considerable enhancement of the sensing response towards the detection of many analytes (Figure 6.1).

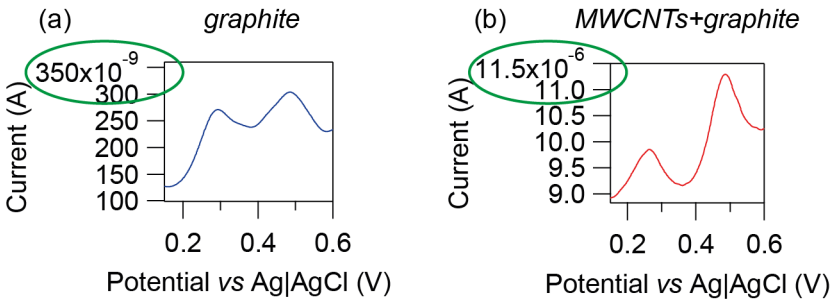

Fig. 6.1 Zoom of oxidation peaks of a metabolite of medical interest, bilirubin (150 μM in 0.01 M phosphate buffer solutions, pH 7.4) at bare (a) and nanostructured (b) electrodes. The green circles underline the increase of orders of magnitude of the peak currents at carbon nanostructured electrodes

Based on the study of Tang *et al.*[1] the electronic structure and the electrochemistry of carbon nanomaterials are beneficial for analytes that react differently at various electrode surfaces. They investigated the electrochemical response of three model redox couples $[Ru(NH_3)_6]^{3+/2+}$, $[Fe(CN)_6]^{3+/2+}$ and $Fe^{3+/2+}$ at graphene-based electrodes. The first analyte is an "outer-sphere" compound, insensitive to

possible defects and impurities of the electrode, $[Fe(CN)_6]^{3+/2+}$ is defect-sensitive but not oxide-sensitive compound and $Fe^{3+/2+}$ is sensitive to both defects and oxygen-containing groups. For all the considered substances, the presence of graphene improves the electron transfer reaction rates of orders of magnitudes with respect to bare electrodes.

It has been well established that the origin of the electron transfer for *highly ordered pyrolytic graphite* (HOPG) comes from the edge plane-like sites and defects. The basal plane of HOPG is electrochemical inert (Figure 6.2 (a)).

Recently, similar considerations have been extended to CNTs and graphene sheets (Figure 6.3 (b) and (c)). Both these materials show an anisotropic electron transfer. Nanotube and graphene peripheral ends exhibit an electrochemical behavior similar to the edge plane-like sites/defects of HOPG. On the other hand, a slow electron transfer characterizes both nanotube sidewalls and graphene sides electrochemically resembling the HOPG basal planes.

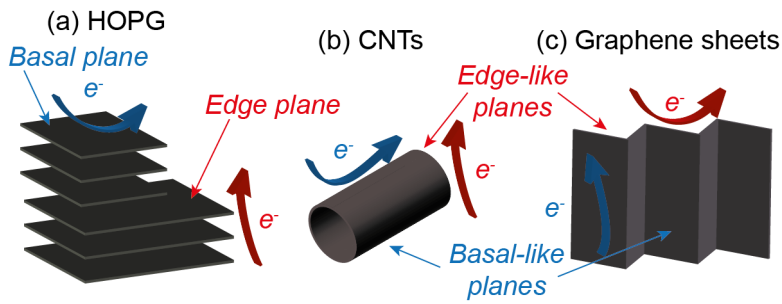

Fig. 6.2 Structure of (a) HOPG , (b) CNTs and (c) graphene sheets. The edge-plane-like sites responsible of a faster electron transfer rate are shown

In separated works, Nugent [2] and Bark [3] investigated the redox reaction of $[Fe(CN)_6]^{3+/2+}$ and found enhanced currents and reduced peak-to-peak separations at MWCNT-based electrodes and at the edge plane of HOPG electrodes. They concluded that the reasons of the electrocatalytic nature of the MWCNTs reside to the open ends that are structurally equal to the edge plane graphite.

This assumption has been validated by comparing the electrochemical properties of $[Fe(CN)_6]^{3+/2+}$ at aligned and randomly dispersed CNTs [4-6]. An ideal reversible behavior was observed at aligned tubes. On the other hand, the peak separation was higher when CNTs were randomly oriented onto the electrode surface and the electrochemical response was significantly reduced. Figure 6.3 shows the calibration curves obtained from the detection of $[Fe(CN)_6]^{3+/2+}$ at vertically aligned and randomly oriented MWCNTs directly grown onto Si substrate [5]. The considerably higher signal of the vertically aligned CNTs was attributed to the higher density of the CNT end-groups with respect to the spread tubes. Moreover, vertically aligned MWCNTs, when used in combination with a particular electrochemical technique explained in the following section, produce a typical response

signal with some features exploited to increase the Signal-to-Noise Ratio (SNR) [7].

Later, Gooding and co-workers [8] provided a strong evidence of the different electrochemical behavior between CNT caps and walls with another study. They cut vertically aligned nanotubes for 2, 4 and 6 hours using an acid solution. The electron transfer rate increased with the cutting time because of the higher amount of edge-like sites produced by a prolonged chemical treatment.

The significant importance of the open tips density in the CNT electrochemistry was also proved by Lawrence and co-workers [9]. CNTs produced by CVD were more electrochemically reactive than ARC-discharge nanotubes because the latter CNTs have a smaller fraction of exposed open tips. Effectively, the electrocatalytic reactivity of ARC-produced CNTs was enhanced by an anodic pretreatment that breaks the CNT caps.

The introduction of defects onto CNTs was proved to increase the electroreactivity of CNTs [5] (Figure 6.3 (b)). Recently, Lim *et al*. [10] have also investigated the effects of the defects introduced onto CVD epitaxial graphene on the electron transfer kinetics. Defects created onto the graphene sides cause superior electrochemical responses.

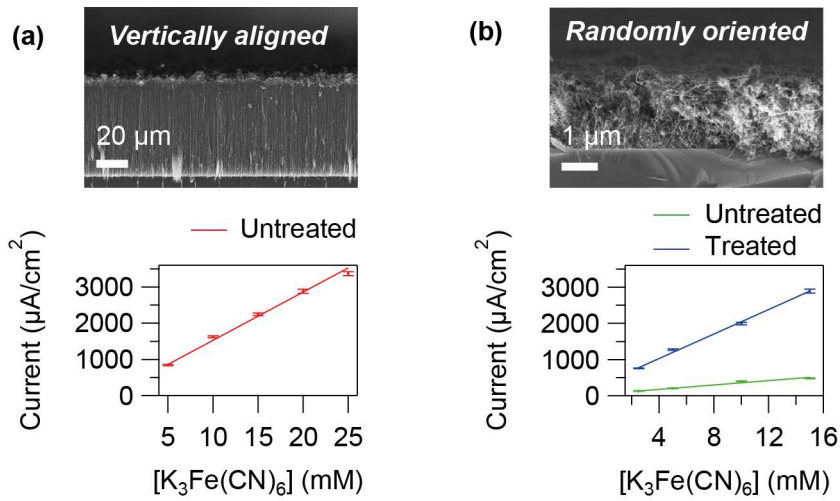

Fig. 6.3 Calibration curves obtained from the detection of $[Fe(CN)_6]^{3+/2+}$ at vertically aligned (a) and randomly oriented (b) MWCNTs. Results before (in green) and after (in blue) a chemical activation are shown for randomly oriented CNTs [5]

The electrochemical role of the oxygen-containing groups at both CNTs and graphene sheets is still under discussion. Certainly, the types of redox systems under study play an important role. For instance, carboxylic groups on CNTs exhibit good electrocatalytic properties for the oxidation of endiols. This is not the case for analytes with hydrophobic sites. Indeed, the oxidation peaks of ascorbic acid,

uric acid and dopamine at electrochemically reduced graphene oxide were better defined than using hydrazine-reduced graphene oxide. The larger amount of functional groups on the hydrazine-reduced graphene determines a nonspecific adsorption/desorption of the molecules [11]. In this context, Guo *et al*. [12] carried out a systematic study concerning the role of defects and oxygen-containing groups localized on tips and on sidewalls of CNTs. They considered the electrochemistry of many redox compounds and obtained completely different trends.

Many others variables can affect the electrochemistry of carbon nanomaterials making a fully understanding of their electrochemical properties even more complicated. For example, it is undisputed the influence of iron impurities on oxidation of hydrazine [13] and on reduction of hydrogen peroxide [14]. Also the ion-doping inside the tubes that follows an acid treatment [8] and the presence of nanographite [15] change the electrochemistry of "pure" carbon nanomaterials.
Few studies compare the electrochemistry of graphene and CNTs. For istance, by testing many electroactive metabolites by voltammetric techniques, Arwarappan [16] found a faster electron transfer rate by using graphene than SWCNTs. Other authors came out with similar results by a comparative study of stacked graphene nanofibers and MWCNTs as electrode materials [17].

6.2 Electrode nanostructuration approaches

The electrochemical response of electrodes based on carbon nanomaterials strongly depends on the type of material incorporated (pristine or treated, oriented or not, single-walled or multi-walled CNTs, multi- or single-layer graphene) and on their incorporation methods. Carbon nanomaterials are commonly integrated onto electrodes by three methods (Figure 6.4):

- the adsorption onto electrodes with or without a polymer (drop casting)
- *via* electrochemical methods (electropolymerization for CNTs, electrodeposition of graphene, anodic oxidation or cathodic reduction of graphite to obtain graphene flakes)
- the directly growth (with or without a transfer) onto the desired electrode surface

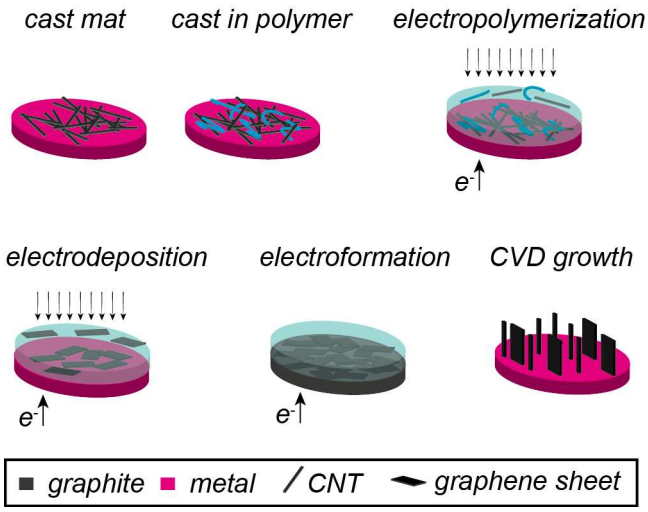

Fig. 6.4 Schematic representation of the principal methods to nanostructure electrochemical electrodes with carbon nanomaterials for biosensing applications.

Casting approaches. One of the major obstacles to the applications of CNTs and graphene for electrochemical biosensors is the difficulty to handle them. Their characteristic insolubility in almost all the solvents reduces the possibility to form stable and homogeneous films. To overcome this problem, carbon nanomaterials are firstly functionalized with hydrophilic groups and then dispersed in a solvent with the help of a long-lasting sonication step. Then, a certain amount of the resulted dispersion is cast onto electrodes and is allowed to dry. To this end, many solvents can be used: ethylene glycol [18], ethanol [19], water [20], N, N-dimethylformamide (DMF) [21]. Additive-assisted dispersions are also employed to further improve the solubility and the stability of the nanostructures. For example, Nafion has been extensively used because of its ion exchange, amphiphile structure and biocompatibility properties [22-26]. The biopolymer Chitosan is also employed because of its excellent film-forming, water permeability, good adhesion and facile surface modification [27-29]. The employment of surfactants [30], ion liquids [31], and other polymers [16, 32] has been widely reported. The predominance of this nanostructuration method is due to the simplicity despite the reproducibly and the film stability is very poor.

Electrochemical methods Considering the high demand of miniaturized medical devices, the confining of carbon nanomaterials onto micro-sized electrodes is of urgent importance. The above-described strategies are not suitable for a selective nanostructuration. Some authors have already reported the use of the casting technique to nanostructure microelectrodes with a microspotter [24]. However, a precise and automatic positioning of the microsyringe is challenging and time consuming. An alternative strategy to prepare nanostructured microelectrodes is the use of electrochemical methods. For CNTs-based biosensors, the electrode modi-

fication can be easily conducted by an electrochemical polymerization. Monomers and CNTs are placed in dispersion. By applying a fixed potential, the hydrogen ions at the electrode interface are reduced to hydrogen and the pH near the electrode surface increases. At higher pH the monomers (usually Chitosan) become insoluble and polymerize at the electrode entrapping CNTs as dopants [33]. Chen's group deposited graphene nanosheets through cyclic voltammetric reduction of a graphene oxide colloidal solution and without the use of any binder [34]. Other electrochemical methods to prepare nanographene flakes involve the application of cathodic or anodic potentials or currents in aqueous or non-aqueous solutions by using a graphite working electrode [35].

Direct growth With casting and electrochemical techniques, carbon nanotubes and graphene result randomly dispersed onto the electrodes often surrounded by binders. An alternative and more challenging approach is to fabricate electrodes with only well-aligned CNTs or nanosheets. An edge-plane-based electrochemical platform offers a high surface area and excellent electrocatalytic properties and could be very promising for enhanced biosensing properties. To this end, various techniques can be used as self-assembly monolayer (SAM) procedures. Unfortunately, nanomaterials are not in direct contact with the underlayer conductive substrates in SAM and the procedure is well-established only for SWCNTs [36]. MWCNTs and nanographene easily aggregate and the selective introduction of thiol groups onto MWCNT tips/graphene edges is very difficult. Consequently, a precise tuning of MWCNT and graphene orientation by SAM is extremely hard [37]. Carbon nanomaterials can be closely coupled with the substrate and precisely aligned by directly growing them *via* CVD processes. The biosensing properties of the as-produced carbon nanomaterials can be tested either directly or after a transfer onto another substrate.

Gao *et al*. [36] prepared aligned MWCNTs on quartz by CVD. Then, a thin gold layer was sputtered onto the tubes followed by a separation of the gold-nanotube electrode from the quartz substrate with an aqueous solution of HF. They employed the constructed electrode for the detection of glucose by an indirect monitoring the H_2O_2 oxidation. In the presence of glucose, H_2O_2 was produced by an oxidase immobilized onto the aligned tubes. Similarly, Gutés [38] fabricated a very sensitive glucose biosensor by using a CVD graphene decorated with Pt nanoparticles. Graphene was transferred from Ni to a GC electrode. Also Wang and co-workers [39] transferred carbon nanosheets grown on Si to a GC electrode for sensing NADH. Unfortunately, the transfer of CVD carbon nanomaterials is affected by a loss of nanostructures that reduces the reproducibility of this modification protocol. Moreover, nanomaterials can easily collapse, losing their original orientation.

Avoiding the transfer of the CVD nanomaterials entails an easier, faster and more reproducible integration method. The biodetection properties of CNTs grown onto insulating materials have been extensively studied [40]. More challenging is the CNT synthesis onto metal electrodes. The growth of CNTs on metals by using a vapor pressure catalyst cannot be applied for selective growth.

Therefore, a pre-deposition of catalyst is the major requirement for a selective CNT growth on metals. The elevated temperatures may activate the diffusion of the catalyst into the substrate inhibiting its activity. To overcome this problem, authors generally utilized thin buffer layers to stop the alloying between the catalyst and the underlayer conductive substrate (Al [41], SiO_2 [42]). Furthermore, for a selective CNT synthesis onto working electrodes, also the problem of the spontaneous CNT growth onto insulating materials should be solved (Figure 6.5).

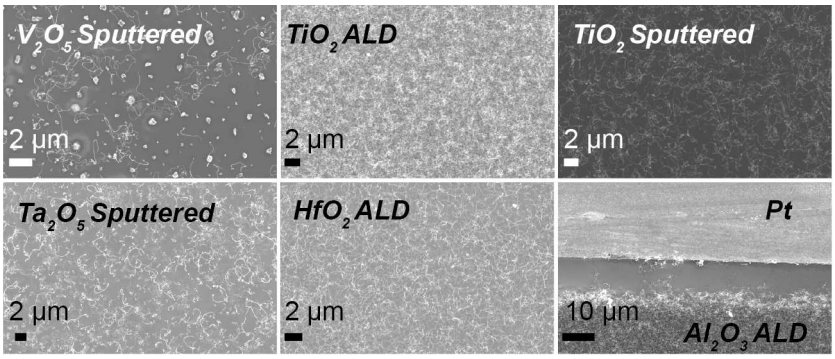

Fig. 6.5 Catalyst-free CVD growth of CNTs onto different dielectric materials.

Up to know, few authors have only reported CNT non-selective growths onto metals without the use of buffer layers for biosensing. Zheng [43] used MWCNTs grown on Ta coated with a thin Co catalyst film to detect electroactive biomolecules. Tominaga *et al*. [44] successfully synthesized CNTs onto Pt plates by CVD using Fe nanoparticles derived from ferritin molecules. They tested CNTs for an enzymatic determination of D-fructose. Lin *et al*. firstly reported a growth of aligned CNTs onto chromium-coated silicon substrate. Then, an oxidase was immobilized onto the broken CNT tips to detect glucose [45].

Metals as Ni and Cu are commonly employed for the CVD synthesis of graphene. Brownson [46] studied the electrochemical properties of commercial graphene grown by CVD on Ni towards the detection of various biological analytes. They proved the principal role of the graphene edges for an efficient electrochemical biosensing by comparing the electrochemistry of CVD graphene-based electrodes with the edge-plane pyrolytic graphite electrodes. Considering this result, we expected that vertically oriented graphene flakes should exhibit the best electrochemical properties. Unfortunately such a kind of orientation can be obtained only by a catalyst-free growth on Si substrate [39, 47] . The electron transfer of vertical carbon nanosheets was extremely fast towards the detection of critical electroactive metabolites [47].

Commonly used nanostructuration techniques namely drop casting and electrochemical methods suffer from disadvantages as low reproducibility and uncontrollability of the nanomaterial orientation. The preparation of a solution is necessary

making the above mentioned approaches time-costly and poorly reproducible. Moreover, these integration methods often employ some binders that inevitably compromise the sensing performance and the stability of the nanostructuration over the time. Moreover, drop casting approaches are hardly-scalable. A very promising method to nanostructure electrodes is the direct growth. It allows us to obtain uniform film as well as a selective nanostructuration of well oriented materials. A CVD synthesis reduces the fabrication steps and benefits from a high reproducibility making it ideal for industrialization and large scale production.

6.3 Direct detection of electroactive metabolites

Many molecules of medical interest are electroactive. For instance, catecholamines, such as dopamine, epinephrine, norepinephrine and serotonin, require an accurate quantitative detection since some mental disorders are due to the change of their concentrations in human fluids. Insulin is another electroactive metabolite of clinical interest because of its central role in diabetes. Cancer drug monitoring has a critical significance in personalized medicine. Also some drugs as etoposside are electrochemically detectable without the use of a mediator bioprobe. A direct electrochemical sensing of electroactive biomolecules is challenging because of three main problems. Firstly, normal concentrations of these compounds are often very low (nanomolar ranges). Consequently, the response at commercially available electrodes is poor or totally absent. Secondly, the overlapping of their oxidation peaks makes difficult the electrochemical discrimination at bare electrodes. Finally, two electroactive biomolecules are normally present in biological fluids, uric acid (UA) and ascorbic acid (AA). All the electroactive metabolites together with AA and UA oxidize at the same potential. Furthermore, the physiological concentrations of AA and UA are in millimolar ranges, orders of magnitudes higher than those related to electroactive biomolecules of clinical interest.

Several techniques have been investigated to selectively detect electroactive metabolites even in the presence of AA and UA. Nafion [23], polypyrrole [32], platinum nanoflowers supported on graphene oxide [48] and stearic acid [49] have been extensively employed as membranes to discriminate dopamine (DA) from AA and UA thanks to their cationic permeability. Unfortunately, these modification protocols are often time-consuming and result in unstable and scarcely reproducible electrode surfaces. It is well known that carbon nanomaterial-modified electrodes resolve the voltammetric peaks of electroactive metabolites (Figure 6.6).

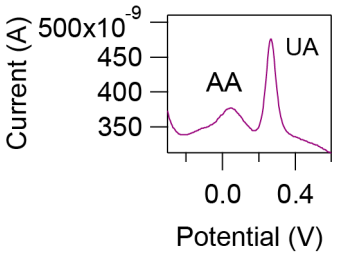

Fig. 6.6 Voltammetric discrimination of AA and UA at carbon nanostructured Pt microelectrode (scan rate: ; PBS 0.01 M pH 7.4)**.**

Higher peak currents shifted towards more negative potentials are observed after the electrode modification. The superior sensing performance of electrodes modified with nanomaterials are due to the defects and edge-plane sites that act as "fast nanoconnectors" of electrons to the underlayer metal substrate. Polymers in conjunction with MWCNTs have been largely employed for the simultaneous detection of DA, UA and AA [50]. The well-shaped peaks are attributed to the electrostatic and hydrophobic interactions metabolites-cationic sites of the polymer and negative charges of the functionalized MWCNTs. The decrease of the overvoltages of certain metabolites, rather than of others, also contributes to the discrimination of various peaks. Also nanographene-modified electrodes are used to separate peaks of a wide range of electroactive analytes [47]. Results are similar to those observed with electrodes-based on CNTs. Preliminary comparative studies demonstrate a higher sensing performance of graphene-based electrodes for a direct biosensing [51]. This result was attributed to the higher conductivity, to the larger active area and to the stronger Π-Π interaction of nanographene with some electroactive molecules.

Recently, another strategy to measure DA level involves its role as catalytic agent. Yuan *et al*. proposed an eletrochemiluminescence sensor based on ECL of peroxydisulfate solution for detecting the dopamine using reduced graphene oxide/multiwall carbon nanotubes/gold nanoparticles (rGO/MWCNTs/AuNPs) hybrid modified glassy carbon electrode (GCE). Under the optimized conditions, the enhanced ECL signal intensity of peroxydisulfate solution was linear with the concentration of DA in the range between 0.20 and 70 μM (R = 0.9902) with a detection limit (S/N = 3) of 0.067 μM. This novel method exhibited sensitive ECL responses to DA even in the presence of a high concentration of UA and AA [52].

6.4 Enzyme-mediated detection

We previously discussed about the determination of molecules amenable to a direct electrochemical detection. Unfortunately, many metabolites are not electroactive at suitable redox voltages. To sense them, specific enzymes are often incorporated into the sensor. For a viable biosensor, the enzyme must be properly attached to the transducer with maintained activity.

Carbon nanostructures represent a good platform for the immobilization of proteins and preserve the protein structure and bioactivity. Moreover, both CNTs and graphene facilitate the electron transfer between the redox center of many enzymes and the electrode. This finding is probably due to the nano-sized structure of CNTs and nanographene or to the defects created after the treatments or to the direct heme core-nanomaterials π- π communications [53].

The protocols for the incorporation of enzymes are similar to those concerning the nanomaterials. Proteins can be immobilized by physical adsorption [18, 20, 21, 29, 30, 54-56], by crosslinking [19], by an embedment in polymeric membranes [57, 58] or by a covalent linking [28, 59, 60] to some carbon functionalities. Figure 6.7 (b) shows the morphology of carbon nanomaterials with incorporated *glucose oxidase* (GODx) by crosslinking with glutaraldehyde. The order of incorporation of nanomaterials and proteins onto the electrode is of crucial importance [61]. For an efficient molecule detection, the enzyme should be immobilized after the nanomaterials since the coimmobilization results in a decreased sensor response [61].

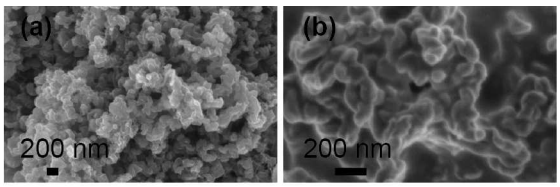

Fig. 6.7 SEM images of carbon nanomaterials without (a) and with (b) GODx immobilized by crosslinking *via* glutaraldehyde.

Enzymatic biosensors are divided into three categories:

- the first generation that consists on the indirect detection of O_2 or H_2O_2, products generated from enzymatic reactions
- the second generation that is based on the use of an electron transfer mediator
- and the third generation that is grounded on the direct electron transfer proteins/electrode

Some examples concerning the three generations of carbon nanostructured biosensors are described below. Glucose has been chosen as analyte of interest because of its clinical importance and because of the availability of many enzymes for glucose sensing.

Wang *et al*. proposed an amperometric microsensor based on CNTs and GODx for the detection of glucose. In the presence of oxygen and glucose, the reaction mechanisms of GODx follow the pathways below ((1) and (2)):

(1) $GODx(FADH_2) + O_2 \rightarrow GODx(FAD) + H_2O_2$

(2) $GODx(FAD) + glucose \rightarrow GODx(FADH_2) + glucolactone$

Then, the product H_2O_2 is detected according to the reaction (3) by applying a suitable potential

(3) $H_2O_2 \rightarrow 2H^+ + O_2 + 2e^-$

The presence of carbon nanostructures decreases the H_2O_2 detection potential [57]. Another way to exploit GODx is described in the work of Jiang *et al*. in which H2O2 produced by GODx-Glucose reaction catalyzes an ECL reaction of luminol labeling carcinoembryonic antigen. The electrode was a dispersion of graphene (GR) and CNTs in chitosan (Ch) decorated with Au and Pt nanoparticles [62]. Alternatively, the consumption of O_2 is monitored as another way to quantify indirectly glucose [54].

Redox mediators are usually coupled with carbon nanostructured electrodes to lower the overpotential of enzymatic reactions and to increase the sensor response. For instance, Qiu proved that electrodes with GODx linked to ferrocene-MWCNTs show better performance than the mediator-less electrodes [27].

It should be noted that biosensors based on *glucose dehydrogenase* (GDH) benefit from measurements independent from oxygen. GDH catalyzes the reaction (4)

(4) glucose + $NAD^+ \rightarrow$ glucolactone + NADH + e^-

and the produced cofactor (NADH) is stoichiometrically related to the glucose in the sample. CNTs and graphene also reduce the overpotential and increase the sensitivity by detecting NADH. GDH-based carbon nanostructured sensors can be used with [55] or without an incorporated mediator [28]. Most works investigate the GDH activity in solutions containing a fixed amount of NADH. A novel and promising approach is to immobilize the cofactor covalently [63] or not [19] together with the enzyme. This strategy would solve the problem of the cofactor adsorption onto most carbon materials during the measurements that is due to the interaction NAD^+ –COO^- groups.

The third generation of biosensors involves the direct electron transfer between electrodes and active redox centers of immobilized enzymes. The advantage of a direct electrodetection is primarily the independence of external redox reactions and the fast time response. In general, the cofactors of enzymes are deeply buried in a glycoprotein shell. Both CNTs and graphene can penetrate the shell acting as nanoconnectors between the enzyme centers and the electrodes. The most common method to immobilize the enzyme for this type of detection is the covalent bonding. Liu [59] and Patolsky [60], in separate works, studied the direct electron transfer of GODx covalently immobilized onto aligned SWCNTs. The rate of electron transfer was significantly higher than using indirect glucose detection. Unlike the result of Liu *et al*. [59], Patolsky [60] found a decrease of the electron transfer rate constant for longer tubes. This result was ascribed to the increase of defects onto the sidewalls that act as barriers to the electron transport. A direct detection was also observed when randomly oriented nanomaterials are used. Nevertheless, the strong hydrophobic interaction GODx/CNT walls or graphene sides may distort the enzyme structure strongly decreasing the rate of electron transfer [20, 21, 29, 30, 47, 64]. In all these works, a pair of reversible and well-shaped redox peaks was observed by cyclic voltammetry between -0.42 and -0.66 V. It

was attributed to the electron transfer process from the active center of GODx to the electrode surface as shown in Figure 6.8.

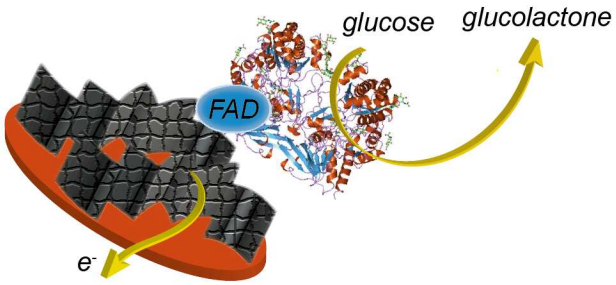

Fig. 6.8 Enzymatic glucose oxidation mechanism for the third generation sensors based on carbon nanostructures

Table 6.1 shows the peak position and the rates of electron transfer of GODx at some CNTs and graphene nanostructured electrodes.

Table 6.1 Electron transfer properties of GODx at CNTs and graphene modified electrodes.

Electrode	Redox Potential (V)	Electron transfer rate (s^{-1})	Analyte	Reference
SAM SWCNTs	-0.422 (*vs* Ag|AgCl)	0.3	NA	[59]
SAM SWCNTs	-0.45 (*vs* SCE)	NA	Glucose	[60]
MWCNTs-surfactant	-0.466 (*vs* SCE)	1.53 ± 0.45	NA	[30]
SWCNTs	-0.441 (*vs* Ag|AgCl)	1.7	Glucose	[20]
CNTs	-0.659 (*vs* Ag|AgCl)	1.61 ± 0.3	NA	[64]
SWCNTs	-0.465 (*vs* SCE)	NA	NA	[21]
Graphene-PFIL	-0.43 (*vs* Ag|AgCl)	NA	NA	[47]
Graphene-Chitosan	-0.477 (*vs* Ag|AgCl)	2.83 ± 0.18	NA	[29]

To the best of our knowledge, there is no systematic study concerning the detection performance of biosensors based on GODx/CNTs and GODx/nanographene. Lu *et al*. [18] nanostructured electrodes with graphite nanoplatelets and Nafion and found a response to glucose up to three times better than that obtained by other research groups employing sensors based on CNTs [22].

Hybrid materials The use of nanoparticles (NPs) is very common to increase the sensor sensitivity. NPs have characteristic catalytic properties towards both oxidation and reduction of the enzymatically generated H_2O_2. NPs can be easily embedded in between carbon nanomaterials. As a result, there are several excellent works regarding the use of carbon nanomaterials decorated with NPs (Au, Pt) to further improve the electrochemical sensing of H_2O_2 [56, 58]. Recently, it has been developed another way to exploit GODx that makes use of NPs and it is described in the work of Jiang *et al*. In this work, H_2O_2 produced by GODx-Glucose reaction catalyzes an ECL reaction of luminol labeling carcinoembryonic antigen (CEA). The electrode is a dispersion of graphene (GR) and CNTs in chitosan (Ch) decorated with Au and Pt nanoparticles. Everything is deposited on the surface of a bare gold electrode. The CEA was determined in the range of 0.1 pg/ml to 40 ng/ml with a limit of detection down to 0.03 pg/ml [65].

6.5 Real sample analysis

CNT- and graphene-based biosensors have been tested for real sample (e.g. blood, urine) analysis. In the majority of these works, the sensor results are validated by a comparison with those obtained with commercial biochemical analyzers. Some examples concerning the determination of UA, AA, DA and glucose (via GODx-mediated detection) are listed in Table 6.2.

Table 6.2 Biosensors based on CNTs and graphene for real sample analysis

Electrode	Analyte	Real sample	Limit of Detection	Reference
MWCNTs-Carbon fiber microelectrode	AA	Rat brain	40 μM	[66]
Poly(3-methylthiophene)-SWCNTs-Nafion-GCE	DA	Serum	5 nM	[25]
MWCNTs-ion liquid paste coated GCE	UA	Urine	5 nM	[31]
GODx-AuPtNPs-Chit-CNTs-GCE	glucose	Blood and Urine	200 nM	[58]
GODx-PtNPs-CNT-graphite	glucose	Blood	NA	[56]
Graphene-Nafion-GODx-GCE	glucose	Serum	NA	[26]

6.6 Electrogenerated luminescence

Electrogenerated chemiluminescence (also called electrochemiluminescence and abbreviated ECL) is the process whereby species generated at electrodes undergo high-energy electron-transfer reactions to form excited states that emit light [67]. Hercules, Bard et al. described the first detailed ECL studies in the mid-1960s [68-70]. After about 50 years study, ECL has now become a very powerful analytical technique and been widely used in the areas of, for example, immunoassay, food and water testing, and biowarfare agent detection. ECL has also been successfully exploited as a detector of flow injection analysis (FIA), high-performance liquid chromatography (HPLC), capillary electrophoresis, and micro total analysis (μTAS) [71]. Luminescence is the generation of light without heat. Light can be emitted *via* a number of luminescent processes [72-74], which include photoluminescence (PL), chemiluminescence (CL), and ECL. ECL is a form of CL, in which both ECL and CL involve the production of light by species that can undergo highly energetic electron-transfer reactions; however, luminescence in CL is initiated by the mixing of necessary reagents and often controlled by the careful manipulation of fluid flow. Luminescence in PL is initiated by a light source excitation (usually a LASER). In contrast, luminescence in ECL is initiated and controlled by changing an electrode potential. As an analytical technique, ECL possesses several advantages over CL and PL. First, in ECL the electrochemical reaction allows the time and position of the light-emitting reaction to be controlled. By controlling the time, light emission can be delayed until events such as immune or enzyme-catalyzed reactions have taken place. Control over position can be used to confine light emission to a region that is precisely located with respect to the detector, improving sensitivity by increasing the ratio of signal to noise. A good example of this is the combination of ECL with magnetic bead technology, which allows bound label to be distinguished from unbound label without a separation step [75, 76]. Control over position could also be used to determine the results of more than one analytical reaction in the same sample by interrogating each electrode in an array, either in sequence or simultaneously using a position sensitive detector [77]. Second, ECL can be more selective than CL, because the generation of excited states in ECL can be selectively controlled by varying the electrode potentials. Third, ECL is usually a nondestructive technique, because, in many cases, ECL emitters can be regenerated after the ECL emission.
Because ECL is a method of producing light at an electrode, in a sense, ECL represents a marriage between electrochemical and spectroscopic methods. ECL has many distinct advantages over other spectroscopy-based detection systems [72, 78]. For example, compared with fluorescence methods, ECL does not involve a light source; hence, the attendant problems of scattered light and luminescent impurities are absent. Moreover, the specificity of the ECL reaction associated with the ECL label and the coreactant species decreases problems with side reactions, such as self-quenching.

6.6.1 Carbon based electrodes for ECL

Obviously, ECL can be used in all of the detection mechanism previously described. Before providing some examples, a unique aspect of ECL when used in combination with carbon-based electrode material.

As reported in the work of Sanginario *et al*. a working electrode made of free standing vertically aligned carbon nanotube (Figure 6.9) has been used for ECL experiment. After a proper preparation, such electrode showed a different behavior if compared with standard gold electrode. In fact, as shown in Figure 6.10, the luminous intensity over hundreds of voltammetric cycles was almost stable and constant [7].

Fig. 6.9 Free standing vertically aligned multi wall carbon nanotube

This fact lets the authors to apply a well-known filtering technique typically applied to periodic signals. By exploiting the averaging algorithm they were able to create a custom algorithm that can increase significantly the Signal-to-Noise Ratio and thus the minimum detectable analyte quantity.

Averaging is one of the most powerful operators for periodic signal processing, of common use in telecommunication. Its use in the present case is possible since light emission occurs always at the same potential value and at a fixed time in the voltammetric periodic cycle. Here, we briefly recall the algorithm mainly to define and contextualize its variables for this case. At time t the quantity x is sampled for the i-th time and its value $x_i(t)$ can be written as:

$$x_i(t) = s(t) + n_i(t)$$

where s(t) denotes the useful signal and n(t) the noise. As the signal s(t) has a period T, if we repeat the measurement after a time j · T (j = integer value) we have:

$$x_j(t + j \cdot T) = s(t) + n_j(t + j \cdot T)$$

By sampling the signal N times at fixed T intervals and averaging we obtain:

that clearly shows how the impact of noise on the measured values is reduced by increasing N. Assuming to have a noise with Gaussian distribution, the associated noise power σ_N^2/N can be calculated from the variance definition. Therefore, the SNR can be written as:

$$SNR = \frac{s(t)}{\sqrt{\frac{\sigma_N^2}{N}}} = \frac{s(t)}{\sigma_N}\sqrt{N}$$

With this approach, we achieve a considerable SNR improvement, as shown in Figure 6.10. In fact, noise, being uncorrelated, is averaged in power domain, while signal is added at each cycle: noise standard deviation is reduced with averaging, while signal confidence increases. We recall that this algorithm can be successfully applied when light emission is periodic and constant over a large number of cycles.

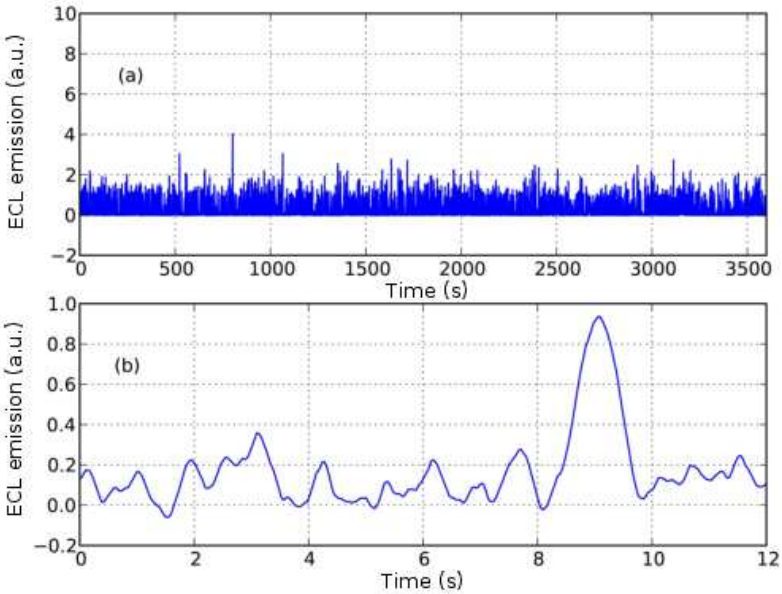

Fig. 6.10 CNT emitted raw signal data (a) and processed signal resulting after gated averaging (b). Scan rate: 500 mV s^{-1}

The increase of SNR is proportional to the square root of the cycle numbers so the more the light emission can remain constant the more the SNR increases. Figure 6.10 shows the emission signal acquired with a concentration of 10^{-7} using the CNT WE, before (Figure 6.10 (a)) and after (Figure 6.10 (b)) that the averaging technique was applied. Figure 6.10 (a) reports the whole experiment of 300 cycles. The useful signal (the light emission) is totally buried in the noise and no reliable value for it can be determined. On the other hand, Figure 6.10 (b) reports the average cycle obtained by applying the time averaging procedure described above. After this processing, the signal neatly emerges from the noise at the proper position).

6.7 Conclusions and future works

We have addressed recent advances in electrochemical biosensor applications of both CNTs and graphene. The key advantages of these nanomaterials are the marked electrocatalytic activity towards small (H_2O_2) as well as big molecules (AA and UA), the lowering of redox potentials, the resistance to surface fouling and the high electroactive area. Their nano-size structure can promote the direct electron transfer of various enzymes as GODx without affecting the macrobiomolecule activity over the time. Modification of electrodes with carbon nanomaterials

mainly focuses on casting methods which results in randomly dispersed nanostructures. Few studies deal with a direct and selective nanostructuration approach namely the CVD growth, which should offer better sensitivity and possibility of mass production.

Future works should aim a better understanding of the electron transfer mechanisms of CNTs and graphene sheets. For electrochemical biosensors, it is our view that CVD carbon nanomaterials directly integrated onto electrodes should perform better than sensors modified by using more conventional methods. Nevertheless, the progress in this area is still limited and there is a lack of fully validated and very robust protocols. Studies demonstrate the efficiency to use carbon nanostructured electrodes for real sample analysis. In this respect, the major difficulty is the easy hydrophobic interactions of several metabolites with carbon materials that leads to an overestimation of the real concentration of metabolites. Moreover, a careful cytotoxicity analysis of these devices needs to be done.

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