Environmental Applications of Carbon-Based Nanomaterials. Acetylcholinesterase Biosensors for Organophosphate Pesticide Analysis

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Introduction

Natural resources of the world beginning to get exhausted, agriculture has to accomplish an important role in a sustainable world. Taking into account the limited availability of water and land resources, achievements in agriculture can be obtained using new technologies. Nanotechnologies can offer the ways to make crucial changes in the agricultural sector in environmental engineering and in water resources.

Improving air, water and soil quality represent an important challenge of the 21st century. Identifying and treating the environmental contaminants and preventing pollution are obligatory steps in environmental protection, each step involving important contributions of materials science. The progress in materials science increased exponentially in the last decade, arriving in our days at a large variety of nanomaterials, which can deliver new environment technologies, due to their immensely powerful capacity.

Organophosphate pesticides (OPP) include parathion, malathion, methyl parathion, chlorpyrifos, diazinon, dichlorvos, phosmet, tetrachlorvinphos, triazophos, oxydemeton and azinphos methyl. OPP obtain their toxicity from the ability to inhibit cholinesterase, causing neurotoxicity, but apart from the toxic effects long term effects include their ability to disrupt the endocrine systems in organisms [1]. It must be noted that many pesticides are transformed in the environment through physical, chemical and biological processes, being sometimes transformed in much more toxic products [2]. At this moment, there are alternative methods of detection investigated, using enzymes for detection purposes seeming a very promising route. These enzymes are incorporated into biosensors, these miniaturized devices being very promising for monitoring pesticides in agriculture. Nanotechnology offers new solutions in pollution sensing and prevention by using adequate nanomaterials with unique properties. A multitude of applications of nanomaterials in environmental pollutions and pollution sensing in agriculture are already published, but nanotechnology has to move in a more practical regime, making its presence really felt in improved agricultural production and in environmental protection.

Types of Nanomaterials used in the Biosensor's Construction. In recent years, nanomaterials and their functional derivatives began to have environmental applications in treatment and remediation, pollution sensing, detection and pollution prevention, the most important ones being in sorption of environmental contaminants and in environmental sensing.

Environmental applications benefit from the unique properties of nanomaterials and in particular, their size, shape and surface area; molecular interactions and sorption properties; electronic, optical and thermal properties. Molecular manipulation implies control over the structure and conformation of a material. For carbon nanomaterials this includes size, length, chirality and number of layers.

Elucidating the molecular interactions, sorption and partitioning properties governing nanotubes, graphene and graphitic nanoplatelets is a joint effort between theorists and experimentalists [3].

There are a lot of criteria of classification of nanomaterials, function of their composition, crystalinity, dimensions, shapes and forms [4]. One of the mostly used classifications of the nanomaterials is based on their composition in Fig. 1.

Function of the number of characteristic dimensions, nanomaterials can be classified in 0-D (nanomaterials with spherical shape) [5], 1-D (nanowires, nanorods, nanotubes) [6], 2-D (nanocoatings and nanofils) [7] and 3-D (nanocrystalline and nanocomposite materials with no bulk dimensions) (Fig. 2).

Among carbon-based nanomaterials, depending on the hybridization states we can include: nanodiamonds, fullerenes, carbon onions, single and multi-walled carbon nanotubes and grapheme [8], with different physical, chemical and electronic properties.

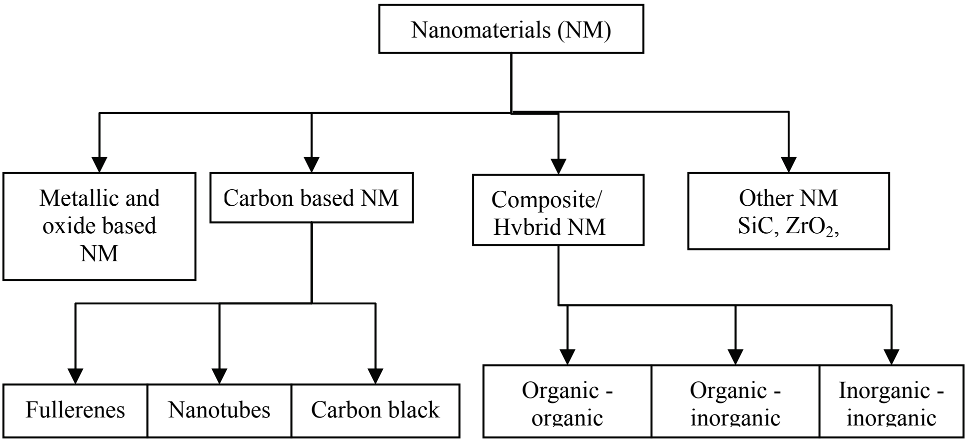


Fig. 1. Nanomaterials classification function of the nature of the components.

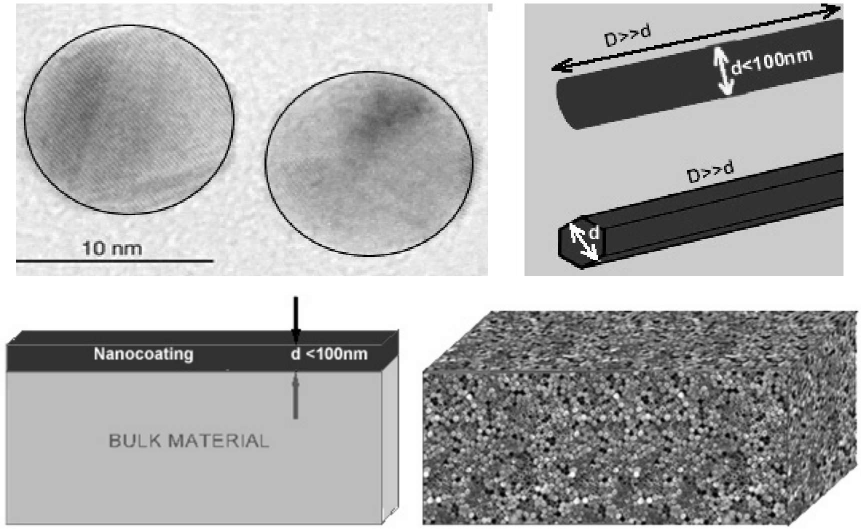


Fig. 2. Nanomaterials classification function of the number of characteristics dimensions (reproduced with permission).

Recently, graphene have attracted attention, due to its novel mechanical, thermal and electronic properties [9], material processing being till the last year the rate-limiting step in graphene applications. The scotch tape method [10] or the micro-mechanical cleavage discovery encouraged the research in this field. Other methods include various chemical combinations and reductions, important amounts of graphene for commercial use becoming possible. Graphite nanoplatelets represent also a solution for inexpensive filler substitutes for carbon nanotubes with interesting properties [11]. It is essential to mention that the sorption of organic molecules is improved on a basal plane of graphite where the molecules adsorb laying flat in comparison with the sorption on the curved tube wall, the effect of the carbon nanotubes nanoscale curvature on the surface adsorption being largely explained in [12].

As far as costs, graphene-based materials are cheaper than the carbon nanotube counterpart, displaying similar properties. Furthermore, toxicity issues are reduced because graphene based materials are one nanometer thick, being less likely to cause cancer [13]. Taking into account these advantages, graphene entrapped in biosensors construction prove to be efficient solutions for improving the characteristics of the biosensors such as: the linear range, the detection limit and the correlation coefficient values in comparison with other electroanalytical techniques.

Engineered Carbon-Based Nanomaterials (ECNM) unite the distinctive properties of sp^2 hybridized carbon bonds with the unusual characteristics of physics and chemistry at the nanoscale. From the electrical conductivity of a single nanotube to the adsorptive capacity of bulk nanomaterials, both single molecule and bulk properties offer potential advances in environmental systems. Carbon nanomaterials are generally consistent with traditional physical-chemical models and theories including electrostatics [14], adsorption, hydrophobicity [15]. Molecular modeling has provided an interpretation of physical-chemical processes occurring at the nanoscale that are otherwise inaccessible through experimental techniques, though computational demands limit the range of length-scale, chirality and layers feasible in the molecular modeling of heterogeneous nanotube and graphene samples [16].

The potential energies of interaction between carbon nanomaterials are described by the classic Lennard-Jones continuum model. The Lennard-Jones model accounts for both van der Waals attractive forces (Kessom, Debye and London forces) and Pauli repulsion originating from overlapping electron orbitals at very short separation distances [17]. Geometry-specific empirical constants provide strong correlation to the theory of a universal graphitic potential when geometries are considered.

Functionalization via covalent or supramolecular techniques reduces aggregation through steric hindrance and the introduction of polar functional groups that confer hydrophilicity to the otherwise hydrophobic nanofillers [18]. Hydrophobicity relates the strength of water-water interactions to water-particle and particle-particle interactions. A hydrophobic molecule will interact less favorably with water than two solute molecules interact with each other, causing the liquid to withdraw from the surface and form a vapor layer. Molecular simulations of CNTs in water suggest that the primary barrier to dissolution is the energy required to disrupt water-water bonds when forming a cavity for the CNT [19].

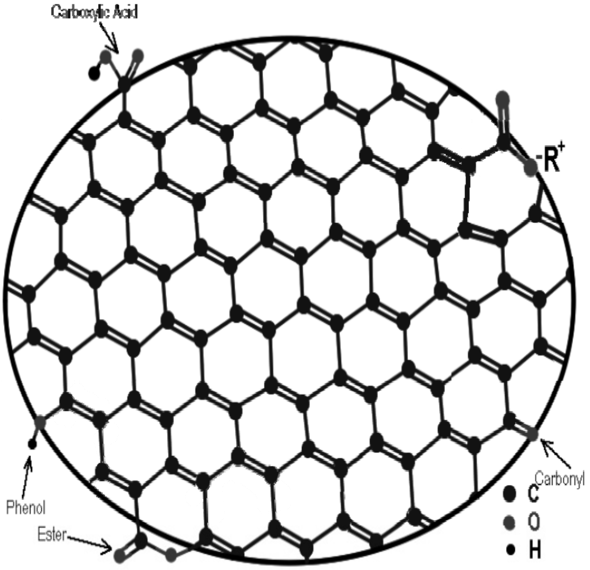


Fig. 3. Functionalization through oxidation of the graphene edges.

Modification of the surface morphology is important in enhancing the capability of graphene, the functional groups being created through chemical functionalization. Usually, the surface characteristics are altered due to the introduction of new functional groups such as: COOH, HO-, lactones, C = O through oxidation, which later on can be further functionalized [20].

In the literature of the last three years, there were published several sensors and biosensors based on graphene. A sensing platform for ultrasensitive determination of cadmium was presented based on the Nafion-graphene nanocomposite film modified electrode [21]. The graphene is dispersed in the Nafion solution, the interfusion of graphene into the Nafion film exhibiting excellent stripping performance for trace analysis of heavy metals, based on the advantages of the graphene nanosheets. The sensor responds on a concentration range from 0.2 to 15 μg L^{-1}, with a detection limit of 0.005 μg L^{-1}, using a 500 s preconcentration.

Types of Sensors Based on Carbon Nanomaterials. Removal of contaminants from the environment and from agricultural areas should firstly use methods to determine the presence of these chemicals.

Rapid and robust sensors used in the detection of pollutants at the molecular level can enhance the environmental protection. The process control of the industrial production, the ecosystem monitoring and environmental decision will be improved if more sensitive and cheaper techniques for the detection of contaminants would be easily available Very important will become in these conditions the continuous monitoring devices that can detect pollutants at trace level.

Different sensors are used operating on different principles. Among them it can be mentioned: solid state electrochemical sensors as chemical gas sensors very good in what concerns sensitivity and reproducibility, but with poor selectivity [22].

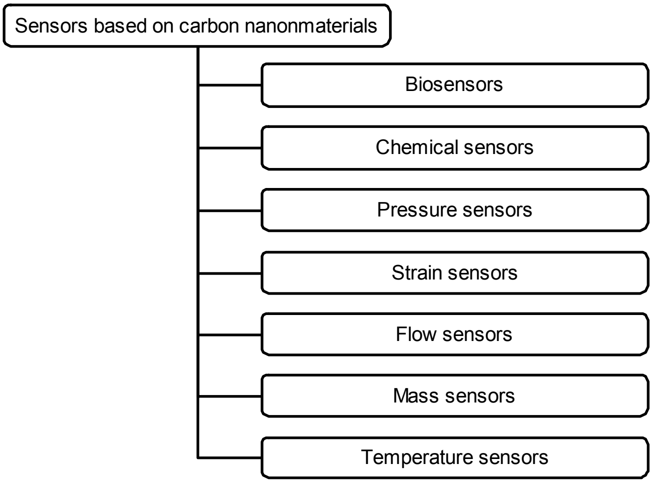


Fig. 4. Types of sensors based on carbon nanomaterials.

Experimental investigations suggest that mechanical deformation can change the electrical conductance of metallic and semiconducting CNTs. [23, 24]. This provides the foundation for the application of CNTs as high-sensitivity **electromechanical sensors**. Reports by Srivastava *et al.* [25] found that the chemical reactivity of SWCNTs can be significantly increased by local strain on SWCNTs. Several researchers have proposed the use of CNTs for measuring **strain** and **pressure** at nanoscale [26, 27].

Flow sensors are devices used for measuring the flow rate or quantity of a moving liquid or gas. A CNT flow sensor is based on the generation of a current/voltage in a bundle of SWCNTs when the bundle is kept in contact with flowing liquid.

Micromechanical resonators such as microcantilevers are based on the detection principle that the resonant frequency of the cantilever depends on the inverse square root of the cantilever mass. Therefore, a change in the mass of the resonator is detected as a shift in resonant frequency. The small size and extraordinary mechanical properties of CNTs make these nanostructures promising candidates for replacing cantilever structures in a **mass sensor**. The principle of mass sensing is based on the resonant frequency shift of a CNT resonator when it is subjected to changes in attached mass or external loading. The resonant frequency is sensitive to the resonator mass, which includes the self-mass of the resonator and the mass attached on the resonator. The key issue of mass detection is in measuring the change in the resonant frequency due to the added mass.

Biosensors contain biological materials such as proteins (*e.g.*, cell receptors, enzymes, antibodies), oligo- or polynucleotide, microorganisms, or even whole biological tissues [28], and is used to monitor biological processes or for the recognition of biomolecules.

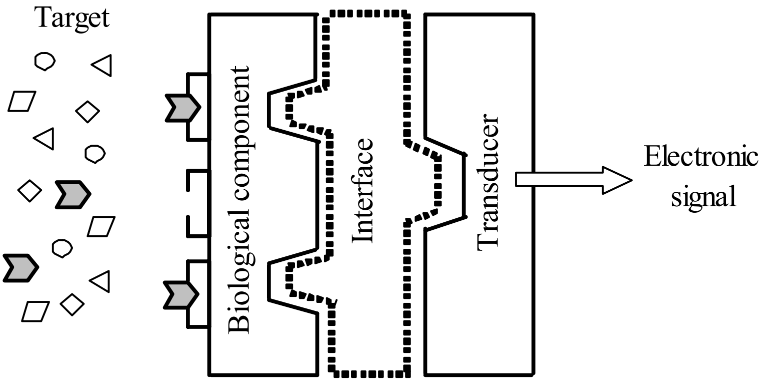


Fig. 5. General schematic representation of biosensors.

The integration of biomolecules with CNTs has resulted in hybrid systems, in which CNTs are used as nanoscale electrode elements (*e.g.*: enzyme electrodes), as electronic elements (*e.g.*: CNT-Field effect transistors) and as platforms upon which biomolecules can be attached.

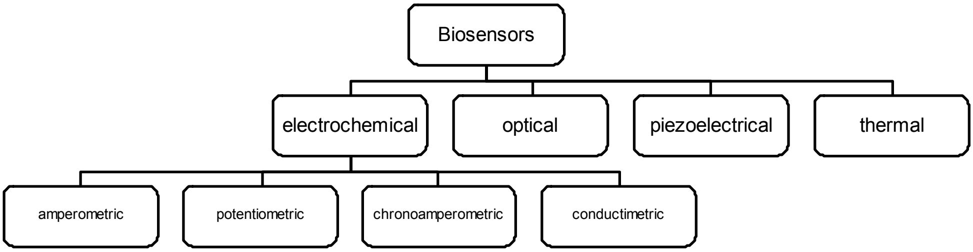


Fig. 6. Types of biosensors using carbon based nanomaterials.

Amperometric Biosensors are based on the measurement of a steady state current produced when a constant potential is applied. The current recorded is related to the oxidation or reduction of an electrochemical species in function of the rate at which it is consumed or produced by a biological element immobilized at the electrode surface.

Potentiometric transducers are based on measurement of a change in potential, the magnitude of which is dependent on the concentration of the analyte.

In **chronoamperometric biosensors**, the enzyme reaction is allowed to proceed for a short period before the potential step is applied.

Conductimetric Biosensors involve a biocomponent immobilized between two closely spaced electrodes and are based on the overall change in conductivity in a solution induced by the consumption or production of ionic species in a reaction.

The basis of **optical biosensors** is the change in optical phenomena such as absorption, fluorescence, luminescence, refractive index or scattering that occurs when light is reflected at a sensing surface.

A **piezoelectric** transducer is a device which transforms one type of energy to another by taking advantage of the piezoelectric properties of certain crystals or other materials (*e.g.*: *Quartz Crystal Microbalance*, QCM).

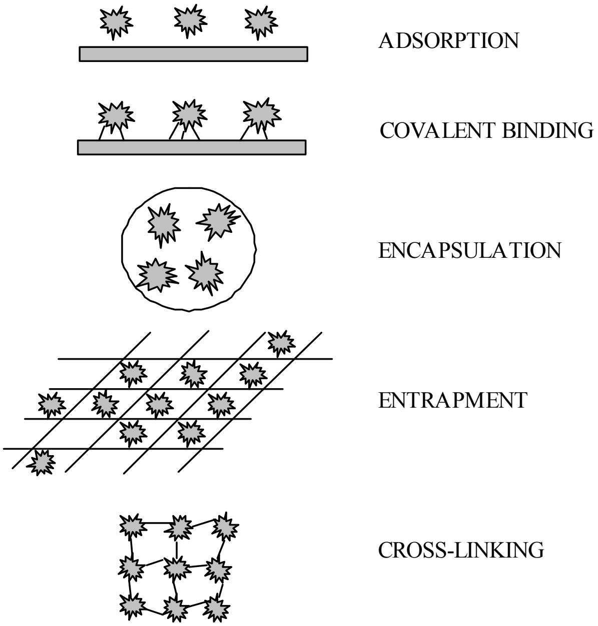


Fig. 7. Principal methods of immobilization.

It is important to be noticed that if the electroanalytical values would be compared with the values obtained using chromatographic techniques and values obtained using organic phase enzyme electrodes based biosensors without nanomaterials, carbon-based nanomaterials improved characteristics of the methods and simple real time analysis.

Immobilization by **adsorption** involves reversible surface interactions between enzyme and support material. The forces involved are mostly electrostatic, as Van der Waals forces, ionic and hydrogen bonds.

Covalent Binding involves the formation of covalent bonds. Functional chemical groups belonging to amino acid residues on the surface of the enzyme may be attached covalently at the chemically activated supports (glasses, cellulose, synthetic polymers).

Encapsulation of receptors can be achieved by enveloping the biological components within various forms of semipermeable membranes. The enzymes are free in solution, but restricted in space.

Immobilization by **entrapment** involves that enzymes are free in solution, but restricted by lattice structure of the entrapment system. There are three general methods: entrapment behind a membrane, entrapment of biological receptors within *Self Assembled Monolayers* (SAMs) or *Bi-Layer Lipid Membranes* (BLMs), entrapment of biomolecules within polymeric matrix membranes.

Cross-linking procedure is support-free and involves joining the receptor to each other to form a large, three-dimensional complex structure this being achieved by chemical or physical methods.

Applications of biosensors based on carbon nanomaterials in pesticides analysis in agriculture. Pesticides are widely used in agricultural practices as insecticides, fungicides, rodenticides, etc… but the toxicological problems connected to their persistent residues were noticed after years of use [29]. There are already mentioned health risks due to their accumulation and the increased risk of cancer. Nanotechnologies can offer improved detection using sensor based on nanomaterials as well as complete degradation of many of them.

Carbon nanotubes based enzyme biosensors. Conventional electrochemical biosensors use glassy carbon electrodes (GCE) or metal electrodes (Au, Pt, Cu) for voltammetric or amperometric analyte detection. Carbon based nanomaterials [30, 31] were introduced in the construction of these electrodes for testing their new achieved sensing properties [32]. CNTs can improve some of these electrodes characteristics [33] such as poor sensitivity and high overpotential for electron transfer reactions [34] taking into account their property to undergo fast electron transfer and the resistance of CNTs to surface fouling. The selectivity and sensitivity can be improved using immobilized enzymes, CNTs facilitating the connection between the enzyme and the substrate.

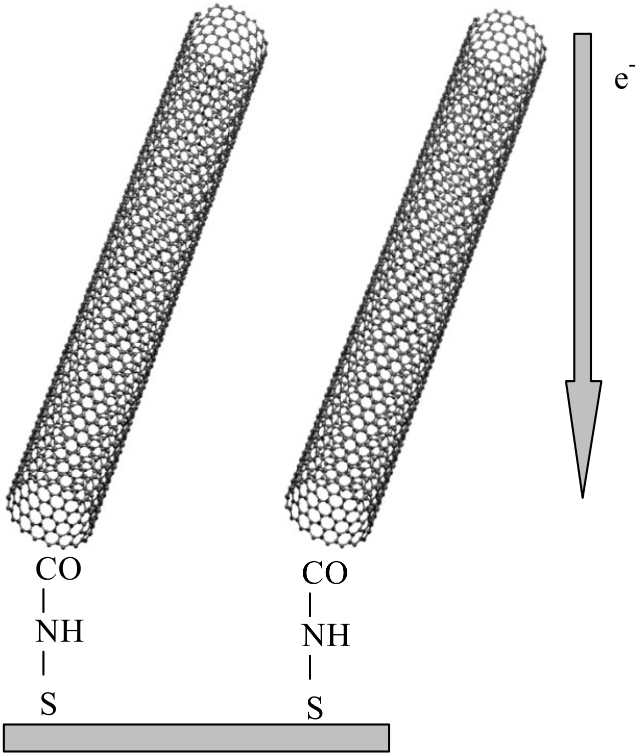


Fig. 8. Scheme of the connection between the enzyme and the substrate on the surface of the CNTs.

Among the examples of **CNT biosensors used for the detection of organophosphate compounds** there can be mentioned: disposable biosensor for organophosphosphate nerve agents based on carbon nanotubes modified thick film strip electrode was proposed by Joshi *et al.* [35]. Biosensors based on self-assembling acetylcholinesterase on carbon nanotubes for flow injection/amperometric detection of organophosphate pesticides and nerve agents were designed by Liu *et al.* [36]. andimalla *et al.*, proposed another technique of enzyme immobilization through Binding of acethylcholinesterase to multiwall carbon nanotube-cross-linked chitosan composite for flow-injection amperometric detection of organophosphate insecticide [37] **Amperometric biosensors for organophosphate compounds by absorbing OPH onto a SWCNT- or MWCNT modified GCE** were realized by Deo *et al.* [38].

Table 1. Characteristics of carbon-based nanomaterials acetylcholinesterase biosensors for pesticides analysis

Immobilization method	**Electrode type**	**Technique**	**Pesticide**	**Detection limit, M**	**Ref.**
Physical adsorption	MWCNT/SPE	Amperometric	Paraoxon	0.5×10^{-9}	[39]
Covalent immobilization using glutaraldehyde	AChE/MWCNT-Chi/GCE	CV	Triazophos	0.01×10^{-6}	[40]
Physical entrapment	MWCNT/GCE	CV	Triazophos	5.0×10^{-9}	[41]
LBL self assembling technique	PDDA/AChE/MWCNT/GCE	FIA	Paraoxon	0.4×10^{-12}	[36]

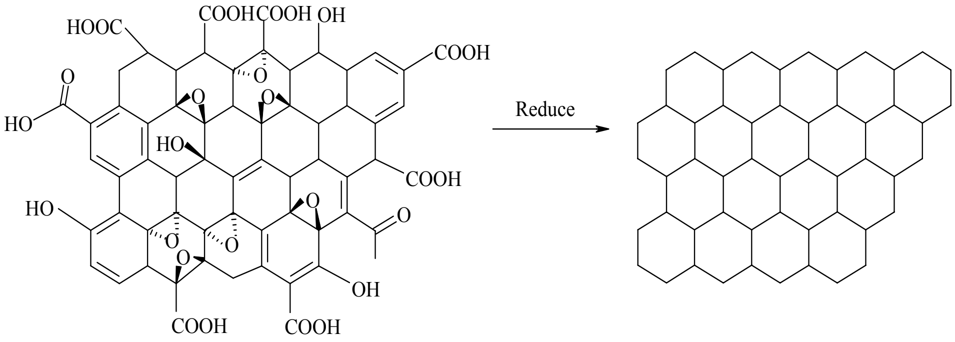
CV–cyclic voltammetry; FIA–flow injection analysis; GCE–glassy carbon electrode; AchE–acetylcholinesterase; Chi–chitosan; MWCNT–multiwalled carbon nanotubes

Table 2. AChE inhibition-based biosensors with CNTs for chlorpyriphos (CPF) detection

Inhibition	**Enzyme**	**Technique**	**Sample**	**LOD**	**Ref.**
CPF	AChE	Voltammetric	Wine	< 300 ng mL^{-1}	[42]
CPF	Butyrylcholinesterase (BChE)	Voltammetric	Grape juice	2×10^{-8} M	[43]
CPF	AChE	Voltammetric	Aqueous sample	3×10^{-8} M	[44]
CPF	AChE	Voltammetric	Aqueous samples	1.58×10^{-10} M	[35]

Graphene based biosensors for environmental sensing. New class of sensors were developed after the discovery of carbon based nanomaterials, beginning with 2007, after Geim *et al.* [45] Some of the key factors influencing graphene electrical and optical properties need to be further studied in order to establish if these really improve the characteristics of graphene based biosensors in comparison with CNTs based ones [46, 47]. This rather new nanomaterial will probably give raise in the future to lots of differently fabricated biosensors and platforms [48] of graphene-based biosensors, but at this moment there are relatively few reports in this area.

An important observation is that graphene needs to be functionalized [49, 50, 51] to modify its electrical properties [52], stronger adsorption onto graphene involving the role of impurities or vacancies [53]. It is also important how the electrode is constructed and how the signal is built on [54, 55]. When focusing on the construction of graphene-based biosensors, the number of graphene layers plays an important role, opening up the possibility to hone the graphene's electronic properties [56, 57].



graphene oxide

reduced graphene oxide

Fig. 9. Scheme of GO reduction process to RGO.

Based on the electrocatalytic activity of graphene and the performance for direct electrochemistry of glucose oxidase, graphene proved to be till this moment a good electrode material for oxidase biosensors [58]. There were reported several glucose biosensors in the last two years. Lu *et al.* [59], reported the first example of glucose biosensor based on graphitic nanoplatelets (xGnP) with good properties and these properties were lately improved by introducing metal nanoparticles on the graphitic nanoplatelets and keeping in this way the nanoparticles extremely small and well distributed [60, 61]. Chitosan was used by Kang *et al.* [62] for a better graphene dispersion and a better immobilization of the of the enzyme molecules. Shan *et al.* [63], a composite film on gold electrode with enhanced performances due to the large surface area and good electrical conductivity of graphene.

Table 3. Graphene based biosensors for glucose sensing

Electrode	Method	Sensitivity	Detection limit (μg L-1)	Ref.
Exfoliated graphite nanoplatelets/glucose oxidase/Nafion	Voltammetry	14.17 μA(mM^{-1}.cm^{-2})	10 μM (S/N=3)	[64]
Glucose oxidase/graphene/chitosan	Direct electrochemistry	37.93 μA(mM^{-1}.cm^{-2})	0.02 mM	[63]
Pt-Au,graphene/glucose oxidase/Nafion	Voltammetry		1 μM	[65]
Pt/glucose oxidase/graphene/chitosan	Amperometric sensor		0.6 μM	[62]

Based on their distinctive properties, graphene offer potential applications in environmental systems. Size, shape, surface area, sorption and electronic properties play an important role in the sorption of environmental contaminants and in environmental sensing.

A new acetylcholinesterase (AChE) biosensor based on the immobilization of exfoliated graphitic nanoplatelets (xGnPs) in chitosan and glutaraldehyde for organophosphate pesticides was proposed by Ion *et al.* [66], Glutaraldehyde is used as cross-linker to bonded AChE to a composite of cross-linked chitosan and xGnPs leading to a new acetylthiocholine iodide (ATCI) sensor. The presence of xGnPs on the electrode surface leads to enhanced electron transfer rate with reduced surface fouling [67]. xGnPs are highly conductive nanomaterials with interesting possible future application in biochemical sensing. The proposed sensor combines for the first time the highly conductive and electroanalytic behavior of xGnPs with the biocompatibility of chitosan, leading to good stability and increased sensitivity for detection of ATCI. It will be further applied to analysis of organophosphate pesticides for environmental monitoring. The detection limit of this sensor was 1.58×10^{-10} M, with a simple fabrication, a fast response and an acceptable stability.

Networked sensing systems can monitor environmental parameters and providing data maintaining water and soil quality. For example, CNTs based sensors present advantages in sensor platforms in simultaneous determinations of several kinds of on-field contaminants [68, 69, 70]. The improved characteristics of these sensors lie in covalent and supramolecular functionalization with enzymes, metals and chemical groups. The environmental applications of CNTs based biosensors were presented in several reviews [47, 71, 72]. Based on the models offered by CNTs (considered as enrolled graphene), graphene opens the way of ultra-sensitive and ultra-fast electronic sensors due to their low electrical noise materials. Even if CNTs have almost ideal properties for electronic applications, they have one dimensional structure which is not suitable in electronic devices, but this problem was solved after the discovery of grapheme [73] that is 2D structure of one atomic thick carbon. Together with the interesting properties of CNTs, graphene can be considered as very challenging materials for environmental sensors.

Detection of mixtures of pesticides in real samples. The major research into the detection of pesticides took place using mostly chromatographic methods of analysis, where each pesticide in the mixture can be determined. In real environmental samples there are more than one pesticide in one sample, the effect of the sample matrix having an important influence. Where a mixture of pesticide is present, the inhibition of AChE will represent the total anticholinesterase effect, so only using chromatographic methods each pesticide from the mixture will be detected.

Detection of the presence of pesticides in real samples was carried out by several research groups, in the following table a comparison between results obtained using AChE- CNM based biosensors and chromatographic techniques being presented.

Table 4. Characteristics of nanomaterial based AChE sensors with chromatographic techniques

Immobilization method	**Electrode type**	**Techniques [Incubation time]**	**Organophosphorus pesticide (linear conc. in M)**	**Detection limit in M [correlation coefficient]**	**Ref**
Physical adsorption	MWCNTs/SPE	Amperometry [30 min]	Paraoxon (1.0×10^{-9} to 6.9×10^{-9})	0.5×10^{-9} [0.9859]	[74]
LBL self assembling technique	PDDA/AChE/PDDA/MWCNT/GCE	FIA [6 min]	Paraoxon (1×10^{-12} to 0.1×10^{-9})	0.4×10^{-12}	[42]
Covalent immobilization using glutaraldehyde as cross linking agent	AChE/MWCNTs-Chi/GCE	CV [10 min]	Triazophos (0.03×10^{-6} to 7.8×10^{-6} and 7.8×10^{-6} to $32\times\times10^{-6}$)	0.01×10^{-6} [0.9966, 0.9960]	[75]
Physical entrapment	MWCNTs/SiSG/GCE	CV [12 min]	Triazophos (0.02×10^{-6} to 1×10^{-6} and 5×10^{-6} to 30×10^{-6})	5.0×10^{-9} [0.9957 and 0.9986]	[76]
		Gas chromatography (GC)	Methyl parathion Malathion Triazophos	0.04×10^{-6} 0.03×10^{-6} 0.08×10^{-6}	[77]
		Liquid chromatography – atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS)	Malathion Paraoxon Triazophos	0.02×10^{-6} 0.08×10^{-6} $0.\ 02\times10^{-6}$	[78]
		Matrix Solid-Phase Dispersion (MSPD) and GC	Parathion methyl Malathion	4.0×10^{-9} 9.0×10^{-9}	[79]

In the area of biosensors, stabilized enzymes provide the tools for the development of complex analytical instruments. The most important purpose of the stabilization techniques is to decrease the tendency of enzymes to unfold, by increasing its rigidity. Recent studies using carbon nanoporous materials have shown that it is a stabilization effect when enzymes are introduced into nanosized cages [80, 81]. The improved performances of CNTs based biosensors are attributed to this effect, but their higher price discourages the application at a large scale. The development of inexpensive nano sized graphene sheets may develop affordable biosensors with high sensitivity and fast response.

Legislation onto pesticides. National governments establish regulatory limits to minimize the contamination with pesticide residues of the environment and of the agricultural areas. Hamilton *et al.* [82] give a comprehensive overview of regulatory limits for pesticides in water issued by WHO, Australia, USA, New Zeeland, Japan, Canada, the European Union and Taiwan. Only the European

Union (EU) has a different approach to regulatory limits a maximum limit of 0.1 μg L^{-1} being set for individual pesticides and a combined maximum limit of 0.5 μg L^{-1} for total pesticides. These limits can be a problem in the application of enzymatic detection methods, because methods there are not able to detect pesticides at the legal limits will have very limited applications.

Conclusions

The real time analysis in field conditions is much needed for robust performance of carbon-nanomaterials–based electrochemical sensors, but only few sensors based on these materials have reported real sample analysis. Among these few examples where multiwalled carbon nanotubes were used, some of the obtained performances of the sensors are in good agreement and even superior in comparison with chromatographic methods. Taking into account the rapid analysis procedure, these nanomaterials prove to be very promising for real sample analysis and comparing the much lower prices of graphene and their improved characteristics with carbon nanotubes, graphene and graphite nanoplatelets will be probably the materials of the future in sensors for environmental and agriculture real field applications, becoming very clear that nanotechnology can offer fundamentally new technologies in environmental detection, sensing and remediation and in agriculture technology.

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