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REVIEW

A Comprehensive Review: Development of Biosensors Based on Graphene-Mesoporous Combined Materials

Kamrun Nahar Fatema and Won-Chun Oh✉

Asian Journal of Materials Chemistry

Volume: 4 Year: 2019
Issue: 3-4 Month: July–December
pp: 24–33
DOI: https://doi.org/10.14233/ajmc.2019.AJMC-P76

Received: 24 July 2019
Accepted: 20 September 2019
Published: 26 December 2019

ABSTRACT

Reliable data obtained from analysis of DNA, proteins, bacteria and other disease-related molecules or organisms in biological samples have become a fundamental and crucial part of human health diagnostics and therapy. After a brief summary of the implication of template based ordered mesoporous materials in electrochemical science, the various types of inorganic and organic-inorganic hybrid mesostructured used to date in electroanalysis and the corresponding electrode configurations are described. The development of non-invasive tests that are rapid, sensitive, specific and simple would allow patient discomfort to be prevented, delays in diagnosis to be avoided and the status of a disease to be followed up. The use of biosensors for the early diagnosis of diseases has become widely accepted as a point-of-care diagnosis with appropriate specificity in a short time. To allow a reliable diagnosis of a disease at an early stage, highly sensitive biosensors are required as the corresponding biomarkers are generally expressed at very low concentrations. In past 50 years, various biosensors have been researched and developed encompassing a wide range of applications. This contrasts the limited number of commercially available biosensors. Lately, graphene-based materials have been considered as superior over other nanomaterials for the development of sensitive biosensors. The advantages of graphene-based sensor interfaces are numerous, including enhanced surface loading of desired ligand due to the high surface-to-volume ratio, excellent conductivity and a small band gap that is beneficial for sensitive electrical and electrochemical read-outs, as well as tunable optical properties for optical read-outs such as fluorescence and plasmonics. In this paper, we review the advances made in recent years on graphene-based biosensors in the field of medical diagnosis.

KEYWORDS

Biosensors, Graphene, Mesoporous materials.

Author affiliations:

Department of Advanced Materials Science & Engineering, Hanseo University, Seosan-si, Chungnam 356-706, Republic of Korea

✉To whom correspondence to be addressed:

Fax: +82 41 6883352
Tel: +82 41 6601337
E-mail: wc_oh@hanseo.ac.kr

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INTRODUCTION

Biosensors are analytical devices that convert a biochemical/biological reaction into a measurable physiochemical signal, which is proportional to the analyte concentration. A typical biosensor thus consists of two elements: a surface linked biological component that interacts selectively with the analyte of interest in blood or serum and a transducer for the detection of the analyte binding event on the surface. The major advantage of using biosensors compared with other conventional biochemical assays such as immunoassays and polymerase chain reaction

based strategies is the fast response time (normally several minutes) along with high specificity. The first biosensor reported dates back to the work by Leland C. Clark Jr., who is considered to be the father of biosensors. Based on his experience with the oxygen electrode [1], he proposed making electrochemical sensors more intelligent by entrapping enzymes such as glucose oxidase onto the oxygen electrode using a dialysis membrane [2]. This glucose analyzer became commercially available in 1975 in the form of an amperometric sensor. The idea of immobilizing antibodies rather than enzymes on the sensor transducer emerged in the early 1980s with the work by Lieberg *et al*. [3]. These devices rely on monitoring the change in the plasmonics signal upon antibody-antigen affinity reaction in real time. The possibility of enhancing the amount of baroreceptor immobilization with consequently improved signal read-out makes this biosensor approach greatly appealing. In parallel, recent years have faced the emergence of electrodes modified with ordered porous materials and/or nanostructured porous electrodes, both being prepared by a template route [4-7], following the tremendous efforts made towards the development of synthetic strategies for nanostructured materials with well controlled size, shape, composition and spatial arrangement [8,9]. Using either soft or hard templates, ordered mesoporous non-oxide materials, ordered mesoporous metals, ordered mesoporous carbons and ordered mesoporous polymers. According to IUPAC classification, the mesopores size ranges between 2 and 50 nm, but most of these mesoporous materials exhibit monodisperse pore sizes that can be tuned typically in the 2-10 nm range and even up to 30 nm for large pore mesoporous solids. They offer attractive features likely to be useful to the electrochemical sensors field, such as variable composition, extremely high specific surface areas that are fully accessible owing to the ordered mesostructured, ease of functionalization with various organic compounds (especially silica-based materials), hosting/support properties (for adsorbed species, macromolecules, catalysts, nano-objects or biomolecules), intrinsic electro-catalytic activity, possibility to get materials ranging from isolating (silica and organosilicon) to semi-conducting (metal oxides) to conducting (metal, carbon). Among the different nanomaterials considered [10], graphene and its various forms such as graphene oxide (GO), reduced graphene oxide (rGO), graphene nanoribbons (GNRs) and so on have received worldwide attention for the development of biosensors. Different sensing mechanisms including optical, electrochemical or electrical can be employed with graphene-based biosensors, in the following noted as G-biosensors. In the case of electrochemical (amperometric, voltammetry, impedimetric) G-biosensors and electrical sensing concepts (graphene-based field effect transistors (G-FETs), the high electron transfer rates, high charge-carrier mobility and low electrical noise levels are of utmost importance for highly sensitive detection of biomarkers and other biological analyte in serum and blood samples [11,12]. Furthermore, chemically derived graphene derivatives exhibit a high density of edge-plane-like defect sites, providing many active sites for electron transfer to chemical and biological species [11]. Also, the high optical transparency of graphene monolayers makes them ideal materials for optical-based G-biosensors and highly beneficial to improve the sensing performance of plasmonics sensors [13]. The fluorescence quenching ability of graphene oxide (GO) resulted in the development of several fluorescence resonance energy transfer (FRET)-based G-biosensors [14]. Graphene has also been shown to be an emerging material as a surface- enhanced Raman substrate (SERS) due to its ability to generate strong chemical enhancement [15]. Improvement in the fabrication of non-fouling graphene transducers is one of the essential steps in the development of high-performance G-biosensors [16]. To obtain a general overview of the results achieved in this field, some of the key works around the development of point of care sensing in biological fluids using G-biosensors will be highlighted here.

Preparation of graphene-based biosensors: A number of different approaches for the synthesis of graphene and its derivatives such as GO, rGO, porous-reduced graphene oxide (rGO) and GNRs are available in the literature (Fig. 1). Large area singles and few layer high-quality graphene nanosheets can be produced by chemical vapor deposition (CVD) methods on nickel or copper and a commercially accessible. Such graphene sheets are nowadays routinely transferred to any transducer interface using mainly polydimethylsiloxane supported transfer processes [17]. The high quality of CVD graphene and the possibility of obtaining mono- and bilayer modified electrical interfaces makes such electrodes advantageous for G-FETs and plasmonics biosensing. The use of chemically derived GO and rGO nanosheets, obtained from a graphite precursor through solution-based exfoliation aiming at weakening the van der Waals forces between the graphene layers, is the most commonly used synthetic approach for the construction of G-biosensors. Reduction of GO-flake size results in better dispersible structures of 3-20 nm in size consisting of no more than five layers; these structures exhibit a high surface area and are termed graphene quantum dots (GQDs) [18]. Different techniques such as drop-casting, spin-coating, electrostatic interaction between positively charged interfaces and the negatively charged GO/rGO nanosheets, electrophoretic deposition (EPD) and electrochemical reduction of GO can be employed to coat electrical as well as inert surfaces with the chemically derived graphene materials. The method of choice depends on the use after application and the employed transducer element. Table-1 gives a short list of selected biological analyte of interest together with the method employed for their detection and the LOD which can be reached, most of them being discussed in more detail in this review.

G-biosensors for glucose and dopamine: One challenging and important molecule to monitor is glucose. An increase in glucose levels is critical for human health as hyperglycemia, defining diabetes, leads to premature death caused by microvascular and microvascular complications. Close monitoring of the blood glucose concentration can largely help to manage diabetes. Tremendous efforts have been put into the development of efficient and reliable methods for glucose sensing. Graphene based glucose sensors are generally built by immobilizing glucose oxidase (GOx) onto the graphene surface as graphene-FET [19] (Fig. 1a). In this work, GOx was covalently linked *via* its amine groups to 1-pyrenebutanoic acid succinimidyl ester, where pyrene end is firmly attached to graphene through

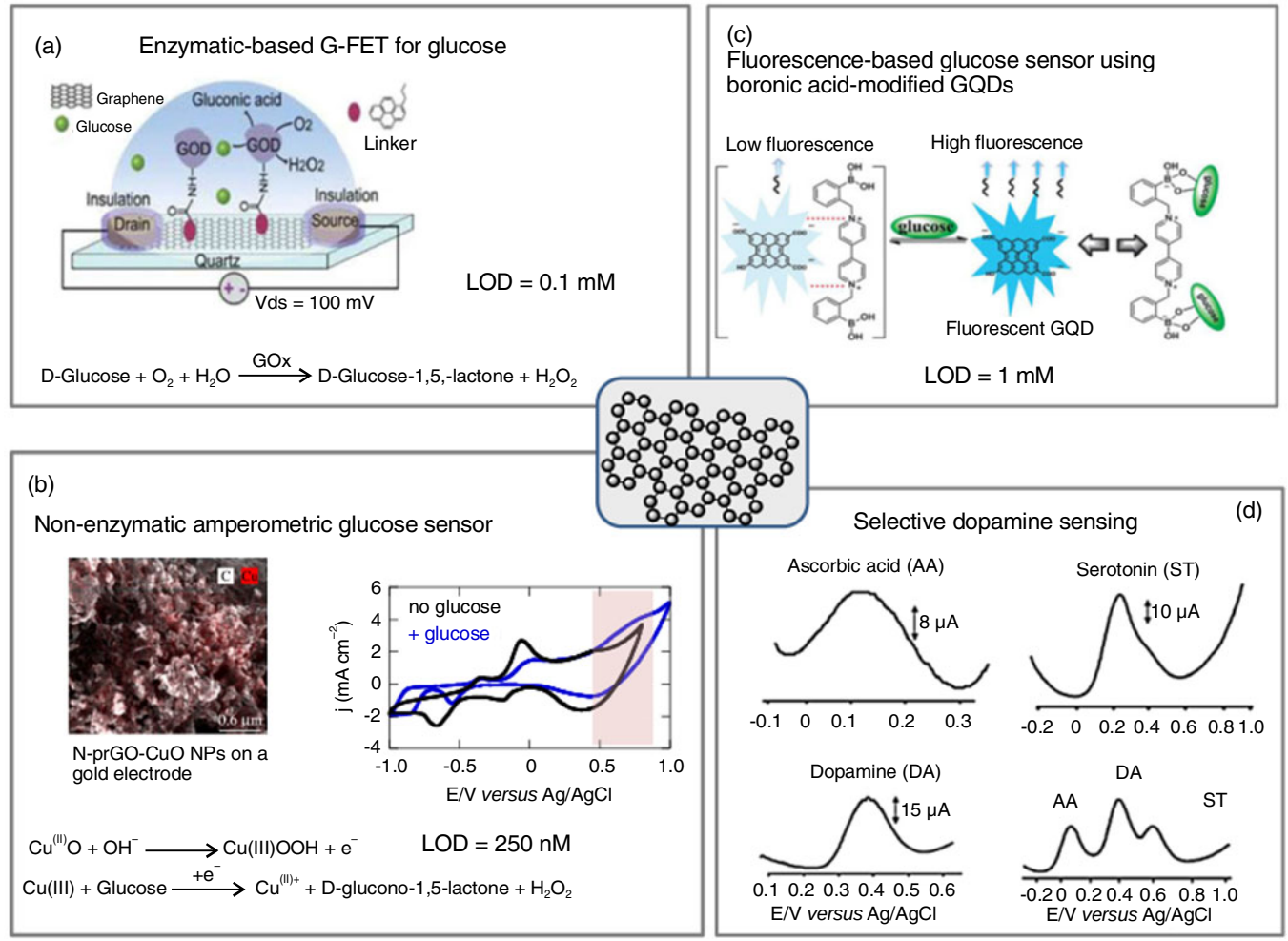

Fig. 1. G-based sensors of small molecules such as glucose and dopamine: (a) CVD graphene modified with glucose oxidase (GO x) using a bi functional pyrene linker for the construction of a G-FET for glucose (reprinted with permission from Huang *et al.* [30]); (b) non-enzymatic glucose sensor operating under basic conditions based on N-doped porous-reduced graphene oxide loaded with CuO NPs (N-rGO-Cu NPs) (c) graphene quantum dots (GQDs) modified with boronic acid-substituted bipyridine ligands for non-enzymatic glucose sensing under physiological conditions (d) differential pulse voltammetry of ascorbic acid

TABLE-1
SELECTED EXAMPLES OF MOST PERFORMING GRAPHENE-BASED BIOSENSORS FOR DIFFERENT ANALYTE. 3D, THREE-DIMENSIONAL; EC, ELECTROCHEMISTRY; FET, FIELD EFFECT TRANSISTOR; FRET, FLUORESCENCE RESONANCE ENERGY TRANSFERS; GO, GRAPHENE OXIDE; rGO, REDUCED GRAPHENE OXIDE; GQDs, GRAPHENE QUANTUM DOTS; GOx, GLUCOSE OXIDASE; AuNPs, GOLD NANOPARTICLES; DPV, DIFFERENTIAL PULSE VOLTAMMETRY; LOD, LIMIT OF DETECTION; PDDA, POLY PSA, PROSTATE-SPECIFIC ANTIGEN; SERS, SURFACE-ENHANCED RAMAN SUBSTRATE; SPR, SURFACE PLASMON RESONANCE; ssDNA, SINGLE-STRANDED DNA (REPRINTED WITH PERMISSION FROM He *et al.* [16])

Analyte	Sensor design	Detection	LOD	Ref.
Glucose	Graphene + GO_x	FET	0.1 mM	[12]
Glucose	3D graphene foam–Co_3O_4 nanowires	EC	20 nM	[20]
Glucose	GQDs-bipyridine boronic acid	Fluorescence	1 mM	[21]
Dopamine	rGO-polyvinyl pyridine	EC	0.2 nM	[22]
DNA	GO and GQD-ssDNA	FRET	75 pM	[23]
DNA	Graphene-Au NPs-ssDNA	SPR	500 aM	[17]
DNA	GO nano walls	DPV	9.6 zM	[24]
DNA	Graphene	FET	10 fM	[25]
Lysozyme	Au/PDDA-GO-*Micrococcus lysodeikticus*	SPR	3.4 nM	[26]
Folic acid	Au-rGO	DPV	1 pM	[16]
Folic acid	Graphene	SPR	5 fM	[27]
β-Amyloid	Magnetic/plasmonics GO	SERS	100 fg mL^{-1}	[28]
PSA	rGO	FET	1 fM	[29]
Escherichia coli	Graphene-anti-*E. coli*	FET	10 du mL^{-1}	[30]

π-π stacking interactions. Measuring a change in conductance allowed glucose detection down to 0.1 mM. Although the use of GOx allows for high selective detection of glucose, non-enzymatic glucose sensors based on the integration of electro-catalytic sites for glucose, often in the form of nanoparticles, onto graphene have been pursued [20,31,32]. In the presence of glucose, the redox peak of Cu(0)/Cu(I) stays unchanged, while the peak of Cu(I)/Cu(II) transition is decreased, reflecting the formation of a Cu(I)-glucose complex. The band at þ0.4 Vis strongly increased, in line with the activity of Cu(III) in basic medium (Fig. 1b) [33,34]. A different non-enzymatic glucose sensing approach based on the use of GQDs modified with boronic acid-substituted bipyridine ligands, which serve as a fluorescence quencher upon electrostatic interaction with GQDs was proposed [21] (Fig. 1c). The driving forces are the strong interaction between the inorganic precursors and the hydrophilic heads of amphiphiles assembled onto the electrode surface under potential control, in order to enable continuous construction of interfacial inorganic-organic assemblies.

DNA sensing with G-biosensors: The need for rapid and sensitive DNA analysis is an important issue in clinical diagnosis. Major studies have focused on the sequence-specific recognition of ssDNA and on the detection of single nucleotide polymorphisms (SNPs). The SNPs are a common form of genomic variation occurring in every 100-300 bp and related to many major diseases and disorders, such as Parkinson's, Alzheimer's diseases, diabetes and various cancers. The development of analytical approaches for selective DNA sensing has consequently been strongly pursued with the belief that low-cost systems suitable for DNA analysis could revolutionize modern health care. The fluorescence quenching ability of GO was exploited by several research groups for the detection of hybridization events. When a dye-labelled ssDNA is immobilized *via* non-covalent binding onto GO, the fluorescence is quenched; this non-covalent interaction is reversible. ssDNA interaction with GO occurs thus *via* π-π stacking, hydrophobic interactions and hydrogen bonding. Even though both GO and ssDNA are negatively charged, DNA can be adsorbed on GO in buffers containing a high concentration of salts to screen electrostatic repulsion [6]. In double-stranded DNA (dsDNA), the nucleotide bases are hidden in the helical structures, preventing their effective interaction with the GO surface in contrast to ssDNA.

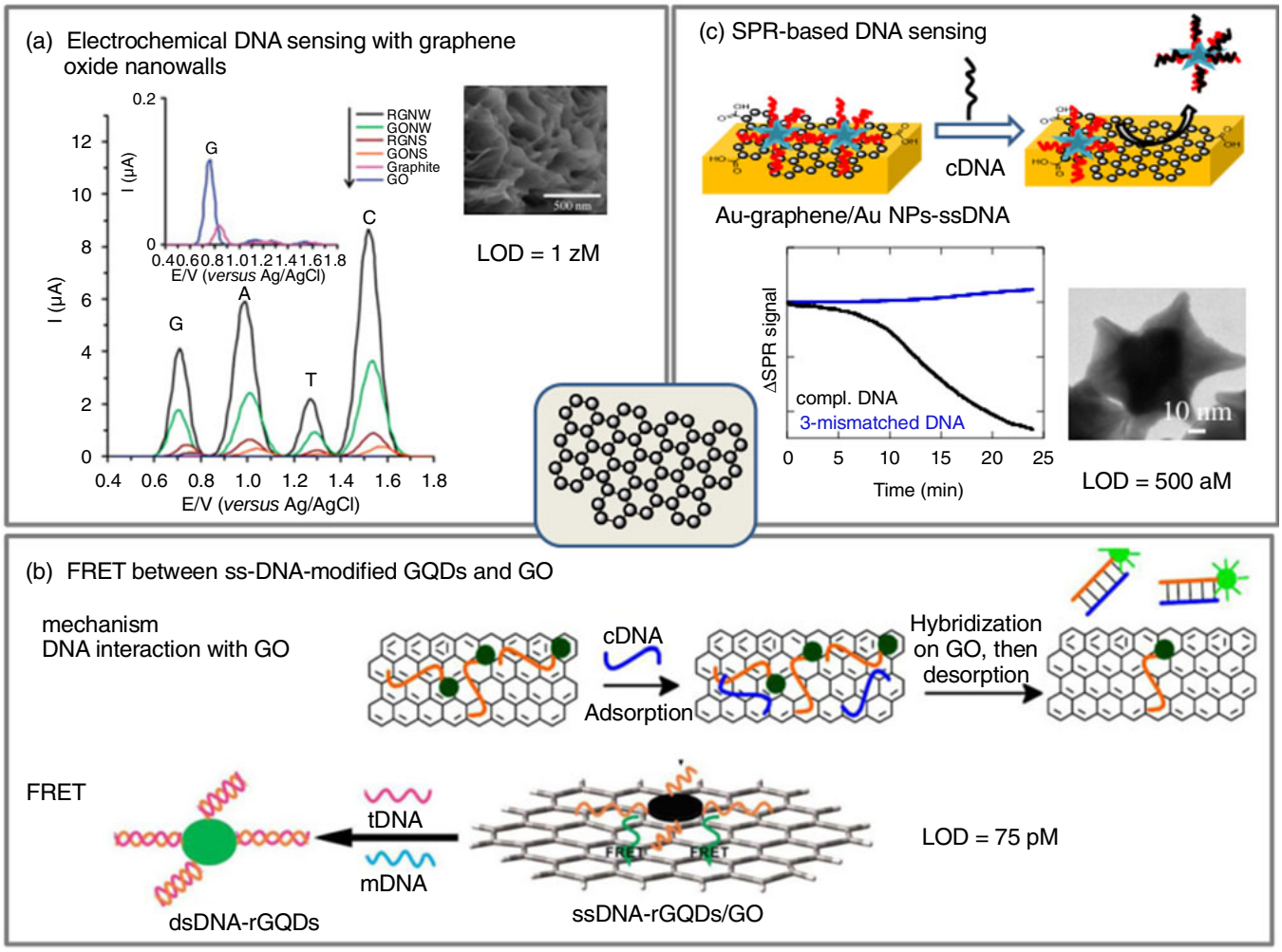

Fig. 2. DNA sensing with G-biosensors: (a) single DNA electrochemical bio sensing using graphene nanowalls (GNWs): SEM image of GNWs formed by electrophoretic deposition onto graphite rod, differential pulse voltammogram of dsDNA (0.1 mM) in phosphate-buffered saline (0.1 M, pH 7) on different interfaces (reprinted with per- mission from Akhavan *et al.* [24]); (b) mechanism of DNA interaction with GO (reprinted with permission from Liu *et al.* [6]) and FRET-based DNA sensing using GQD sand GO (reprinted with permission from Qian *et al.* [23]); (c) graphene–SPR based DNA sensing: transmission electron micrograph (TEM) image of a gold nanostructure together with the change in SPR signal upon incubation with cDNA and mismatched DNA

This approach has been recently used in combination with ssDNA-modified GQDs for FRET-based DNA sensing [23] (Fig. 2b). This concept was further applied to SPR-based DNA sensing [17] (Fig. 2c). Peptide nucleic acid (PNA) was non-covalently immobilized to graphene channel and hybridization with target DNA produced a left-shift in the Dirac point with an LOD of 10 fM [25]. Dontschuk *et al.* [7] have also shown the usefulness of G-FETs for DNA sequencing. Electrodes modified with graphene oxide nanowalls (GONs) with preferred vertical orientation [24] or graphene nanoplatelets [5] are capable of catalytically oxidizing the four DNA bases simultaneously, resulting in DNA sensors with an LOD as low as 9.4 zM [24] (Fig. 3a). In the presence of a complementary DNA (cDNA) target, a duplex is formed, disturbing the GO-ssDNA interaction and resulting in the release of the formed dsDNA, at which point fluorescence is restored (Fig. 3b). They demonstrated experimentally that G-FETs are able to measure distinct coverage dependent conductance signatures upon adsorption of four different DNA nucleobases-a result that was attributed to the formation of an interface dipole field.

Mesoporous materials used in electrochemical sensors: The ordered mesoporous materials are characterized by a regular three-dimensional structure made of mesopores of uniform diameter, thanks to the use of a template in their synthesis. Basically, one can distinguish two cases (Fig. 3a). The first preparation mechanism, which historically originates from the first synthesis of ordered mesoporous silica is the cooperative assembly of ionic or non-ionic surfactant micelles, or other supramolecular amphiphilic compounds (*e.g.*, block copolymers) and inorganic precursors (metal alkoxides, metal ions, carbon source) to form an ordered organic-inorganic composite, the final mesoporous product being obtained after removal of the organic template (Fig. 3b). The second way (*i.e.*, nanocasting Fig. 4) is to use a mesoporous material prepared by the first approach (most often silica) as a hard template that is filled with an appropriate precursor (mostly metal ions or a carbon source such as sucrose), which is then transformed in the interior of such confined space (*e.g.* by reduction of metal ions or carbonization of organic precursor) to produce a mesoporous replica, the final material being obtained by dissolution of hard template. The main characteristics of these materials are a periodic and widely open structure (hexagonal, cubic, lamellar, wormlike, or multimodal), a great porosity (pore volume above 0.7 cm^3 g^{-1} and specific surface area in the 500-1500 m^2 g^{-1} range) with a narrow pore size distribution, typically tunable between 2 and 10 nm (and even up to 30 nm), as well as a good thermal

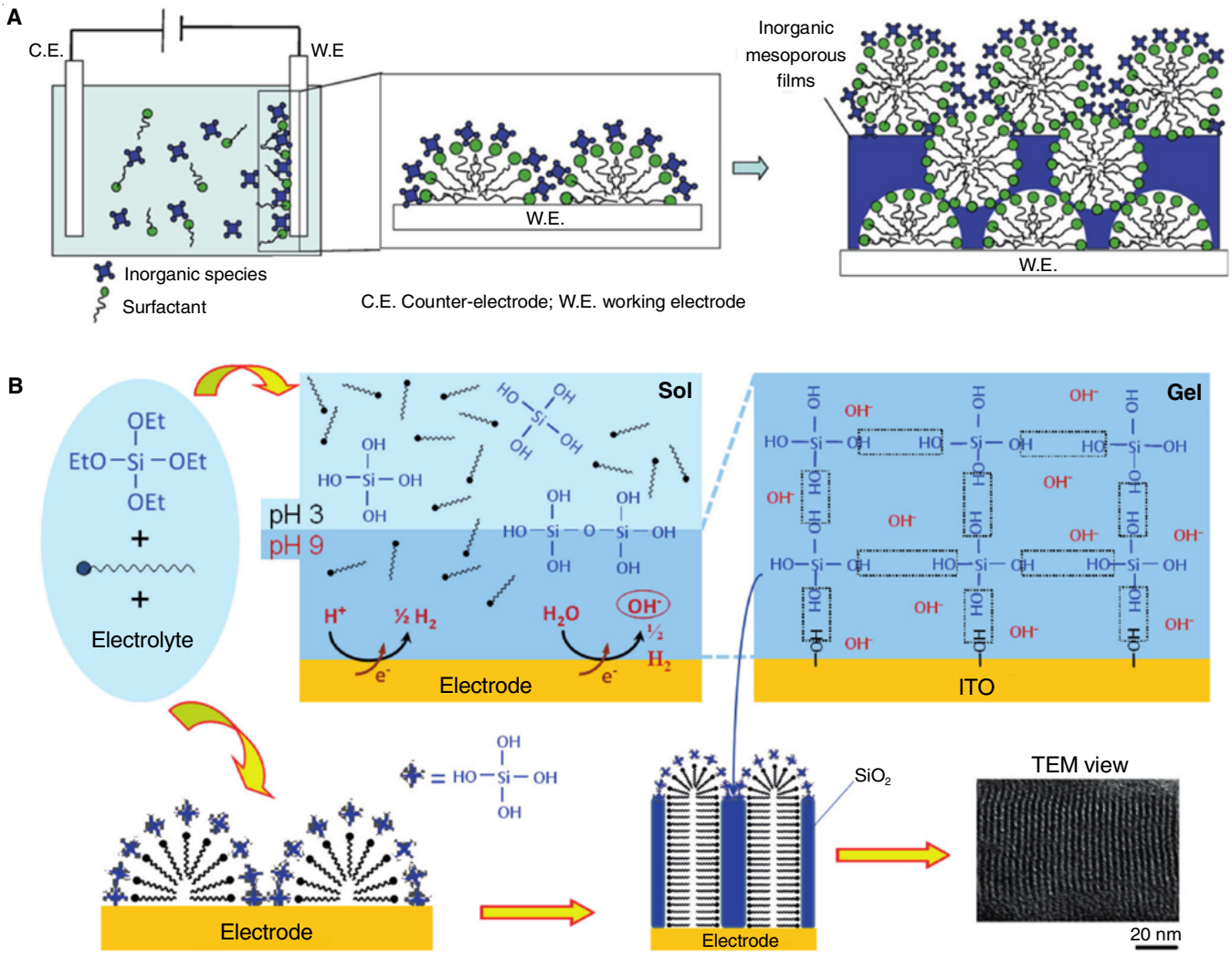

Fig. 3. (A) Schematic representation of an electrochemical interfacial surfactant templating method, (B) Schematic representation of electro-Assisted self-assembly (EASA) method used to generate ordered and vertically-aligned sol-gel-derived mesoporous silica thin films

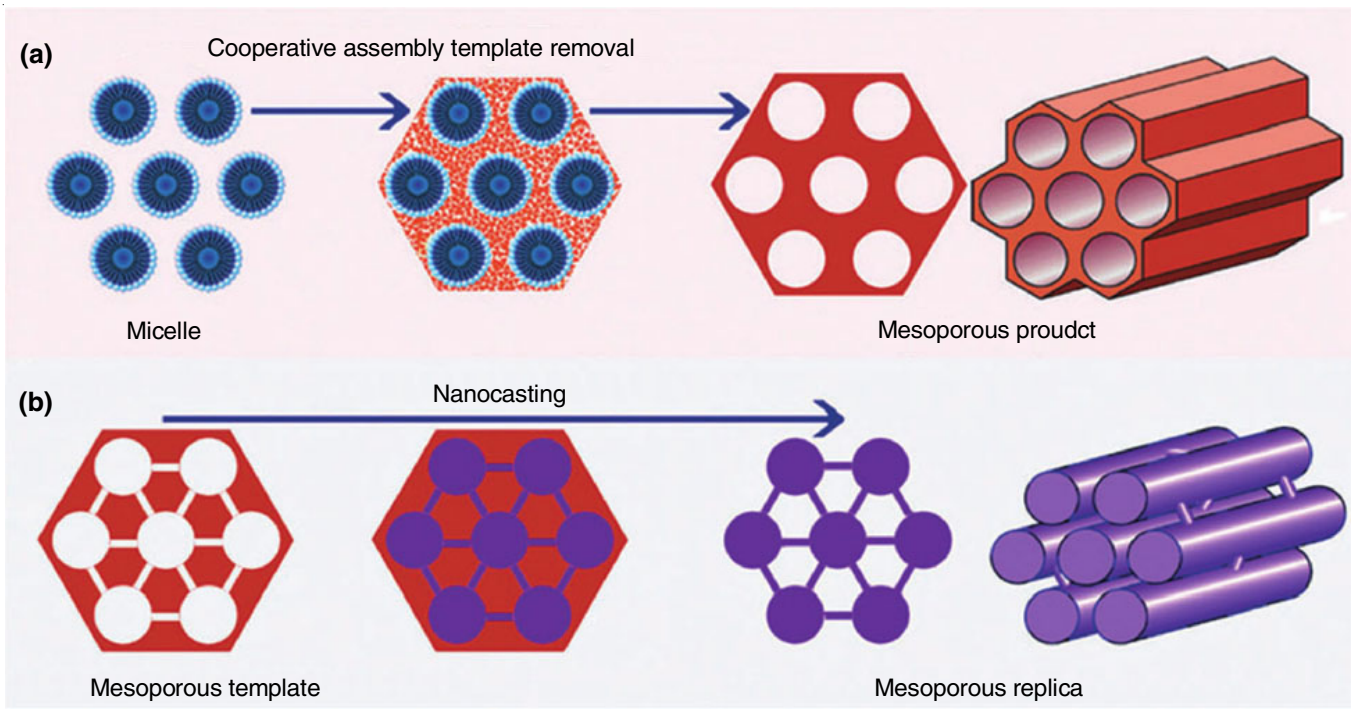

Fig. 4. Scheme of two representative synthesis routes for ordered mesoporous materials: (a) soft-templating method and (b) hard templating (nanocasting)

and mechanical stability. Those materials specifically used in electrochemical sensors are briefly presented below, the reader interested in more detailed information being directed to well-documented reviews, dealing with mesoporous silica-based materials [35-37], metal oxides other than silica, mesoporous non-oxide materials, ordered porous metals ordered mesoporous carbons or mesostructured organic polymers. Among the accessible morphologies, powders and especially thins films [37] have been largely exploited in electrochemistry.

Electrode configurations: A first configuration (Fig. 5a), largely exploited to get electrodes modified with non-conductive mesoporous materials is the dispersion of as-synthesized powders of mesoporous silica or silica-based organic-inorganic hybrids into a conductive composite matrix such as carbon paste (part (a) of Fig. 5b). The method is very simple (just mixing the powdered material, graphite particles and a mineral oil as binder, typically in ratios extending respectively in the range 10-20:60-40:30-40 w:w:w %,) and versatile in the sense it could be applied to any kind of mesoporous material likely to be prepared as powder. In such configuration, the composite electrode surface facing the solution is made of both carbon (where the electron transfer reactions are expected to occur) and the modifier (which is directly contacting the solution and thus likely to interact directly with the target analyte), leading to usually fast responses. The electrode surface can be basically renewed by mechanical polishing (*i.e.* removal of the "dirty" portion of paste and smoothing the new surface on a weighing paper for instance), but this implies that all components are uniformly dispersed in the composite to ensure good reproducibility of the measurements. A second strategy is to deposit the mesoporous particles onto the surface of a solid electrode. This can be performed by simple dropping of a suspension containing the powdered material and let the solvent to evaporate (under quiescent or spinning conditions) to get the particulate film onto the electrode surface (part b of Fig. 5a). Such particulate films can suffer from poor mechanical stability, notably in stirred solutions. Composite deposits have been also reported (mesoporous Fe_3O_4-graphene sheets, mesoporous MnO_2-graphene oxide, mesoporous TiO_2-graphene or OMC-layered double hydroxide. But the most widely applied strategy to coat powdered materials in a durable way onto an electrode surface is to add a polymer in the mesoporous material suspension to be deposited, so that after solvent evaporation the mesoporous particles are embedded within a polymer matrix on the sensing element (part b of Fig. 5c). In all cases, beneficial effects have been reported in terms of enhancing peak currents and/or decreasing over potentials with respect to the bare GCE, but these effects were variable from one analyte to another one (Fig. 6).

G-based sensors for protein biomarkers: Protein biomarkers are specific molecules existing in blood or tissues, whose measurement or identification is very critical and efficient in the prediction, diagnosis and monitoring of cancer and many other diseases. Graphene based immunoassay platforms, where specific antibodies are immobilized onto graphene to capture selectively the biomarker analyte, have shown on the other hand excellent sensitivity [8]. For example, recently the post-functionalization of rGO-modified electrodes is demonstrated by simple immersion into a solution of folic acid allowed for the development of an electrochemical-based sensor for folic acid protein with an LOD of 1 pM [16] and a plasmonics sensor with a 5 fM LOD [27]. Levels of folic acid protein in serum can increase up to 22 pM in metastic diseases. Given that human serum is free of folic acid proteins, detection of this protein in serum serves as an early stage cancer diagnostic step. A rGO-based FET modified with prostate-specific antigen-a 1

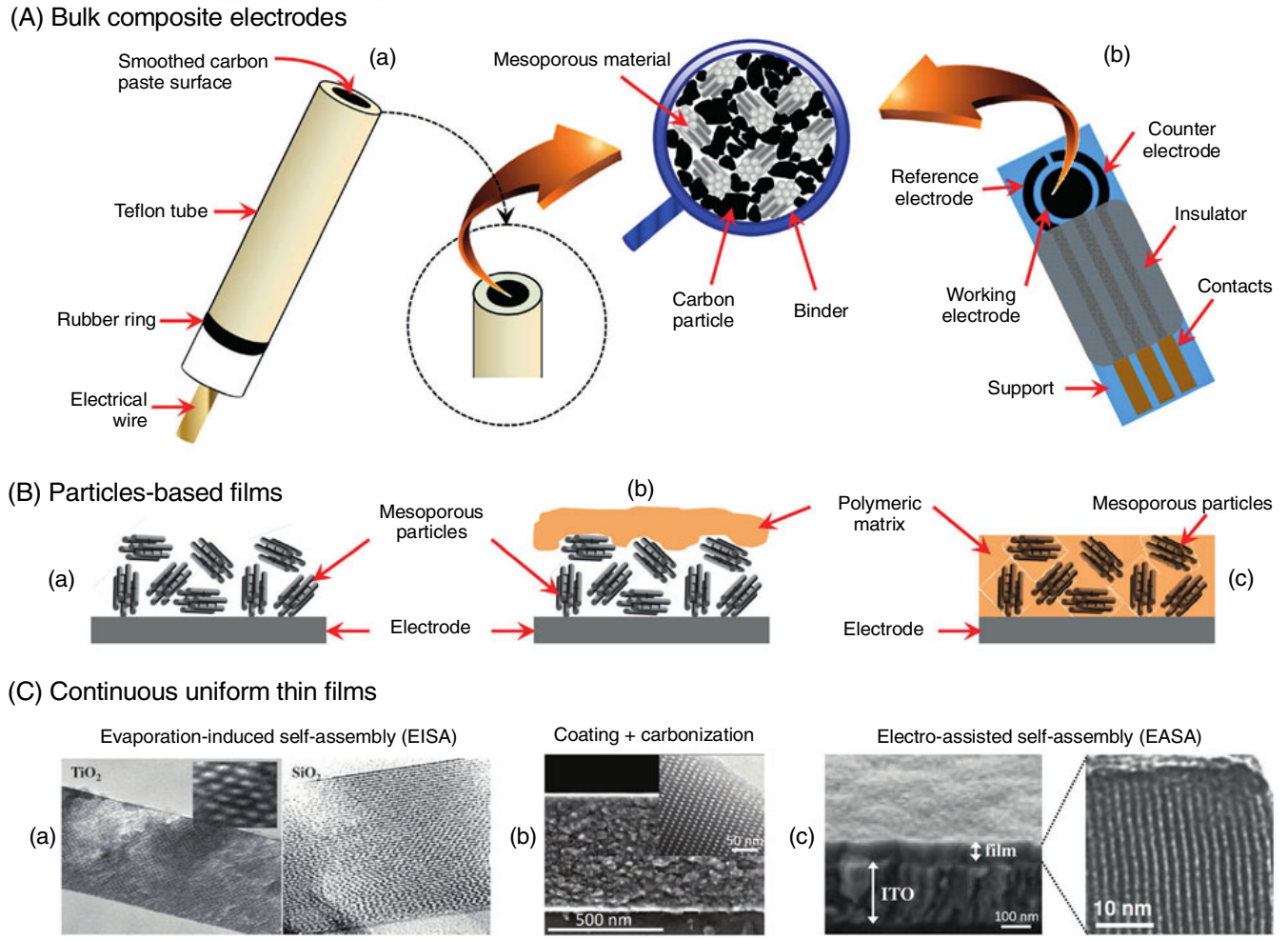

Fig. 5. Illustration of the various configurations of electrodes modified with ordered mesoporous materials. (A) Dispersion of mesoporous powder into a conductive composite matrix: (a) carbon paste electrode and (b) screen-printed carbon electrode. (B) Deposition of mesoporous particles onto solid electrode surfaces: (a) mesoporous particles only; (b) mesoporous particles over coated with a polymer layer; (c) coating made of mesoporous particles dispersed into a polymer matrix. (C) Generation of continuous uniform thin films of mesoporous materials: (a) TEM views of typical mesoporous metal oxide films formed by evaporation-induced selfassembly (b) SEM and TEM (inset) views of a graphitized mesoporous carbon film prepared by coating a carbon precursor and a block copolymer template and subsequent carbonization of the precursor (c) SEM and TEM views of a perpendicularly-oriented mesoporous silica film generated by electrochemically-assisted self-assembly

-anti chymotrypsin (PSA-ACT) was used by Kim *et al*. [29] to detect fM levels of PSA with a dynamic range over six orders of magnitude. An SPR-based read-out was used by Cosnier *et al*. [9] for the detection of cholera toxin on graphene coated gold chips modified with pyrene nitrilotri acetic acid (NTA) with an LOD of 5 pg mL^{-1} [28]. We showed recently the suitability of *Micrococcus lysodeikticus* modified GO-coated SPR interfaces to sense serum lysozyme levels with an LOD of 3.4 nM [26]. Recently, multifunctional nano-platform based on magnetic-plasmonics nanoparticles attached to GO allowed for the sensitive detection of Alzheimer′s disease biomarkers (β-amyloid, tau proteins) down to 100 fg mL^{-1} [28]. These examples highlight the efficient use of G-biosensors for protein analysis. One main hurdle of all these sensors when performing tests in human serum samples, often not evoked in the literature, is linked to the high non-specific interaction between the graphene surface and serum proteins. We have compared a number of different strategies to reduce non-specific binding of clinical serum samples spiked with lysozyme (100 mM) on rGO [27]. While simple immersion into serum decreased strongly the anti-fouling properties of graphene, rGO modification with pyrene polyethylene glycol (PEG) units has been shown to result in the best non-fouling interface [27,35].

Bacteria and viruses: The specific and sensitive detection of pathogenic microorganisms remains a big scientific challenge and a practical problem of enormous significance. Pathogen diagnosis is currently based on culturing the microorganism on agar plates with the disadvantage of being long (minimum of 24 h) and ignoring viable but non culturable cells. CVD graphene modified with anti-*Escherichia coli* antibodies allowed *E. coli* concentrations as low as 10 cfu mL^{-1} to be detected [30]. Graphene oxide in combination with *E. coli* O157:H7 antibody-conjugated quantum dots was used as a pathogen-revealing agent by exploiting the universal highly efficient long-range quenching properties of GO; an LOD of 3.8×10^3 cfu mL^{-1} was achieved [36]. Graphene printed onto water-soluble silk and modified with antimicrobial peptides allowed bio selective detection of bacteria at single-cell levels remotely [37].

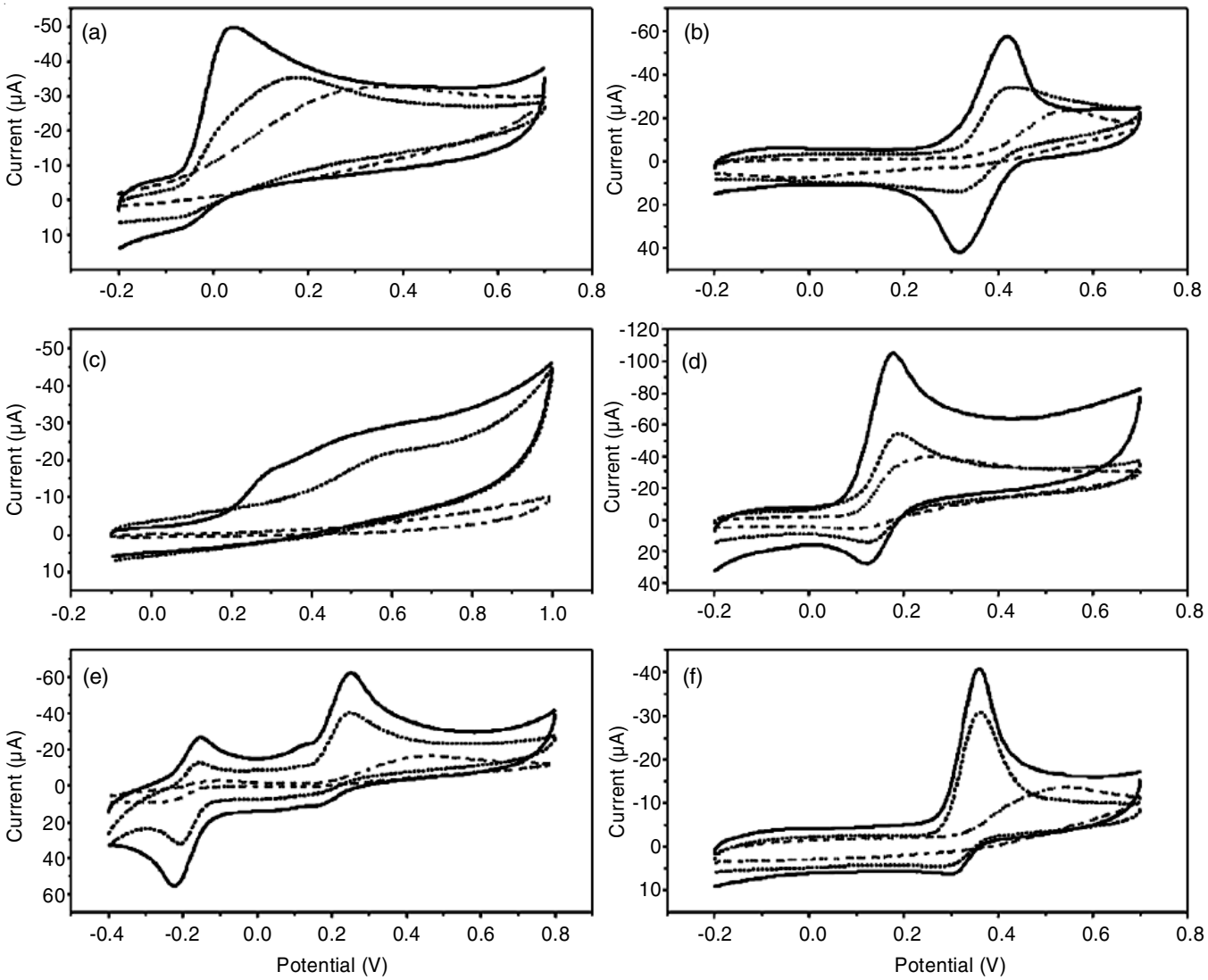

Fig. 6. Cyclic voltammogram of 1 mM ascorbic acid (a), acetaminophen (b), cysteine (c), dopamine (d), epinephrine (e) and uric acid (f) recorded at OMC (solid line), CNT (dashed line) and GP (dotted line) electrodes. Scan rate: 50 mV s^{-1}; supporting electrolyte: 0.05 M phosphate buffer (pH 7.4). OMC, ordered mesoporous carbon; CNT, carbon nanotube; GP, graphite powder

Conclusion

An attempt is made to review the most recent advances in graphene-based biosensors by selectively highlighting a variety of different examples for the detection of some molecules of biomedical interest. The possibility that a large range of different detection methods can be employed with graphene-based sensors is of high advantage, as depending on the looked after final application, sensor size and read-out can be customized at will. There is, however, still an urgent need for moving beyond research by developing new concepts for achieving even better sensitivity and selectivity, in order to bring some of the current sensors into real biomedical applications. Even though a large number of sensors reported in the literature exhibit good storage stability and repeatability which are important for complex sensors involving nanomaterials and manual step preparations, the performance in real biological samples is often not reported. A second one is related to improving the long-term stability of the sensor, which would require stronger and more durable immobilization of the electro catalysts (redox mediators); again a possible direction is the modification of mesoporous carbon materials by electro-grafting of diazonium. Finally, several analytes that have been determined to date using electrodes modified with mesoporous materials are biologically-relevant and/or intermediates of enzymatic reactions, so that these systems are also likely to be of importance for the future development of electrochemical biosensors. Current *in vivo* and *in vitro* assessments of the bio stability of the sensors are encouraging and promising for further technological transfer. Different challenges are still to be overcome. The collaboration between material scientists, chemists, physicists as well as engineers and medical personnel is of fundamental importance to drive this field further and to propose graphene-based biosensors as point-of-care alternatives for patients. The success of any new biosensor material lies in addition in its reproducibility and possible industrial-scale production. The emergence of several companies providing mono- and bilayered graphene nanosheets on several interfaces, GO, rGO and even modified matrices, has been an additional motivation for using graphene for biosensor applications.

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