Review

Deepshikha Saini*

Synthesis and functionalization of graphene and application in electrochemical biosensing

Abstract: Graphene is a two-dimensional material with amazing characteristics, which grant it the title "wonder material". It has grabbed appreciable attention due to its exceptional electrical, optical, thermal, and mechanical properties. Because of these interesting properties, graphene has found its way into a wide variety of biosensing applications. It has been used as a transducer in electrochemical biosensors, bio-field-effect transistors, impedance biosensors, electrochemiluminescence, and fluorescence biosensors. Functionalization of graphene has further opened up novel fundamental and applied frontiers. The present article reviews recent works dealing with synthesis, functionalization of graphene, and its applications related to biosensors. Various synthesis strategies, mechanism and process parameters, and types of functionalization are discussed in view of biosensor development. Some potential areas for biosensor-related applications of functionalized graphene are highlighted, including catalytic biosensors and bio affinity biosensors. Wherever applicable, the limitations of the present knowledgebase and possible research directions have also been discussed.

Keywords: electrochemical biosensors; functionalization; graphene; synthesis.

1 Introduction

Graphene is composed of single-atom thick sheets of sp^2 bonded carbon atoms that are arranged in a perfect two-dimensional (2D) honeycomb lattice. It is often dubbed

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as "miracle material" for its outstanding characteristics. Graphene is also the fundamental building block of carbon materials such as fullerenes, carbon nanotubes, and graphite [1, 2]. Its unique configuration and structure determine a number of fascinating properties such as a high planar surface area (2630 m^2 g^{-1}) [3], superior mechanical strength with a Young's modulus of 1100 GPa [4], unparalleled thermal conductivity (5000 W m^{-1} K^{-1}) [5], remarkable high carrier mobility (10,000 cm^2 V^{-1} s^{-1}) [6], high opacity (~97.7%), and the ability to quench fluorescence [7]. Graphene is currently, without any doubt, the most intensively studied material for a wide range of applications that include electronics, energy, and sensing outlets [8].

However, tuning physicochemical properties becomes necessary [9] in many graphene applications because of intrinsic graphene properties like zero band gap, high sheet resistance, easy aggregation, and poor solubility, which are the big obstacles to the various applications [10, 11]. Functionalization is one of the efficient ways to tailor the electrical properties of graphene. Furthermore, after modification, the as-synthesized hybrid materials could not only overcome the disadvantages of intrinsic graphene but also be inculcated with new desirable properties. Graphene is thought to become especially widespread in biosensors and diagnostics. The large surface area of graphene can enhance the surface loading of desired biomolecules, and excellent conductivity and small band gap can be beneficial for conducting electrons between biomolecules and the electrode surface. Graphene also has significant potential for enabling the development of electrochemical biosensors based on direct electron transfer between the enzyme and the electrode surface. Moreover, other applications including, but not limited to, synthesizing nanoelectronics [12], high-frequency electronics [13], energy storage and conversion devices [14], field-emission displays [15], and transparent conductors [16] are also proofs of their versatility.

In this comprehensive review, recent research efforts to the synthesis, functionalization of graphene, and its

application in the development of next-generation biosensors are addressed. This covers the latest developments and importantly, offer insights on the underlying detection mechanisms and on the unique advantages of graphene in comparison with other materials. I hope that this article would inspire broader interests across various disciplines and stimulate more exciting developments in this still young, yet very promising, field of research.

2 Synthesis of graphene

As the first step to realize the applications, graphene must be synthesized on a large scale with controlled size and morphology. Controllable and scalable production of graphene is of great importance for its applications in different fields. Graphene has been synthesized in various ways and on different substrates. In the following, we summarize the synthesis methods to obtain high-quality graphene. The methods for graphene preparation can be divided into several groups:

(i)   Mechanical exfoliation of graphene layers from highly oriented pyrolytic graphite [17–19]
(ii) Chemical vapor deposition with hydrocarbon decomposition on a metal [20–24]
(iii) Thermal decomposition of SiC (epitaxial growth) [25–29]
(iv) Electrical arc discharge method [30–33]
(v)   Organic synthesis [34–37]
(vi) Chemical method using dispersion of graphite. [38, 39]

The mainstream methods of graphene synthesis have been summarized in Figure 1.

Table 1 summarizes the relative advantages and disadvantages of the above synthesis methods in terms of the feasibility to scale-up the process for mass production, materials and production costs, and the presence of defects.

2.1 Significance of graphene for electrochemical biosensing

Graphene and sensors are a natural combination, as graphene's large surface-to-volume ratio, unique optical properties, excellent electrical conductivity, high carrier mobility and density, high thermal conductivity, and many other attributes can be greatly beneficial for sensor functions. The superior advantages offered by graphene for electrochemical biosensing are as follows
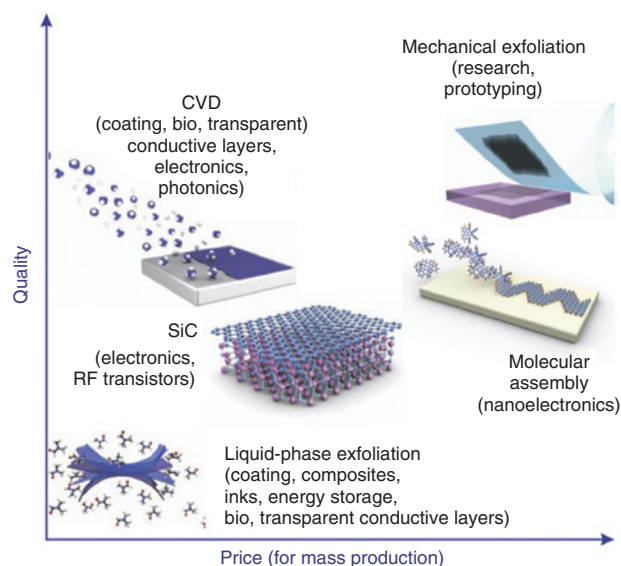
Figure 1: Methods for the mass production of graphene. There are several choices depending on the particular application, each with differences in terms of size, quality (e.g. presence of defects and impurities), and price. Reprinted with permission from Ref. [8] (Copyright 2012 *Nature*).

(i)   Capability of automation and miniaturization
(ii)   High electron transfer rate
(iii) Biocompatibility
(iv)   Easier operation
(v)   Extraordinary carrier mobility and capacitance
(vi)   Excellent transparency
(vii) Exceptionally large surface-to-volume ratio
(viii) Single atom thickness
(ix)   Lower cost
(x)   Robustness and flexibility
(xi)   Excellent conductivity

These properties have also led to significant interest in developing graphene-based electrochemical biosensors [48]. Graphene has been employed as electrode material in various electrochemical biosensors for the detection of a range of analytes like glucose, glutamate, cholesterol, hemoglobin, and more [49]. Interestingly, the large surface area of and excellent conductivity leads to the superior electrochemical biosensing performance of graphene over carbon nanotubes in terms of sensitivity, signal-to-noise ratio, electron transfer kinetics, and stability [50, 51]. Additionally, graphene's properties can be tuned by amendable synthetic conditions, dimensions, number of layers, and doping components [52]. Therefore, graphene-based materials are playing, and will continually play, a significant role in sensing applications.

Table 1: Comparison of different graphene synthesis methods.

Sr. No.	Synthesis methods	Size	Advantages	Disadvantages	Applications	References
1.	Mechanical cleavage	Flakes (5 to 100 μm)	Simplicity, high quality, less defects	Delicate, low yields, uneven films, not scalable	Basic research purpose	[18, 19]
2.	CVD (on Ni, Cu Co)	Thin films ≤75 cm	Scalable, high quality, uniform, high compatibility	High process temperature (>1000°C), high cost, complex transfer process	Touch screens, smart windows, flexible LCDs, OLEDS, and solar cells	[22–24]
3.	Epitaxial growth on SiC	Thin films (>50 μm)	Large-scale production, high quality, no defects	High process temperature (1500°C), high cost, low yields, discontinuous	Graphene electronics	[28, 29]
4.	Chemical reduction of graphite oxide	Nanoflakes/powder (nm to a few μm)	High scalability, high yields, low cost, easily processable	High defect density, low purity	Conductive inks and paints, polymer fillers, super capacitors, sensors	[40–42]
5.	Liquid phase exfoliation	Nanosheets (nm to a few μm)	Simplicity, high scalability, low cost	Moderate quality, impure, low yield	Transparent electrodes, sensors, polymer fillers	[43, 44]
6.	Carbon nanotubes unzipping	Nanoribbons (few microns)	Low-cost, high quality, high yield	Time-consuming, moderate scalability	FET interconnects,NEMs composite s	[45, 46]
7.	Arc discharge of graphite	Nanosheets (100s of nm to >10 μM)	High crystallinity, high purity, low cost, large-scale production	Non-uniform, impure	Novel composite materials	[32, 33]
8.	Carbon dioxide reduction	Few layer graphene	High yield, cost effective	Delicate, time consuming	Nano electronics, sensors, composites	[47]

3 Graphene functionalization

To make full advantage of the superior properties of graphene, it is necessary to functionalize graphene to form multifunctional hybrid materials [39, 53]. Surface functionalization can transform pristine graphene into a chemically sensitive and soluble material, thus, enabling its use in sensing technology [48, 54]. Graphene is more prone to effective, reproducible, and homogeneous functionalization compared to other carbon nanomaterials. Various strategies have been used to functionalize graphene. But mainly two methods are available to functionalize graphene as follows:

(i) The covalent functionalization via the grafting of molecules onto the sp^2 carbon atoms of the π-conjugated skeleton;

(ii) The noncovalent functionalization based on the adsorption of polycyclic aromatic compounds or surfactants via π-stacking and hydrophobic interactions on the carbon framework.

Figure 2 summarizes the most important methods for the functionalization of graphene.

3.1 Covalent functionalization of graphene

Covalent functionalization is the most studied form of graphene functionalization for sensor applications [56]. The structural alteration can take place at the edges and/or on the basal surface. Graphene can be covalently functionalized in two ways:

(i) By reaction with unsaturated π-bonds of graphene [56]

(ii) Heteroatom doping [56]

Covalent functionalization is associated with rehybridization of one or more sp^2 carbon atoms of the carbon network into the sp^3 configuration leading to the introduction of a scattering site in graphene. Further, covalent functionalization can play an important role for controlling the carrier density and band gap engineering [57, 58]. If graphene is covalently bonded to acceptor functional groups, this may give rise to p-type semiconductor properties. Conversely, if graphene is functionalized by donor functional groups, the formation of n-type semiconductor is possible.

Being highly inert and thermally stable, graphene requires high-energy processes to rehybridize its π-conjugated carbon network. The covalent functionalization of graphene can be achieved in four different ways: nucleophilic substitution, electrophilic addition, condensation, and addition [59–69]. This results in the formation of a large density of sp^3 hybridized carbons in the graphene network [70], which disrupts the delocalized π cloud, converting graphene into an insulator. Different kinds of functional groups like amino, hydroxyl, sulfonate, or alkyl groups may be introduced onto graphene through covalent bonding [71, 72]. These groups can further serve as chemical switches to insert functional molecules (proteins, carbohydrates, polymers) on the graphene surface [72].

Advantages of covalent functionalization:

The formation of covalent carbon-carbon bonds involving the basal plane carbon atoms offers key advantages, such as:

1. Greater stability of the hybrid material,
2. Controllability over the degree of functionalization,
3. Reproducibility.

Disadvantages of covalent functionalization:

Although covalent strategies can effectively, stably, and specifically install functionalities, they unavoidably causes

1. A loss of the free, sp^2-associated π electron constituting the π-cloud on graphene.
2. Causing severe decrease in carrier mobility
3. Leading to the introduction of a scattering site in graphene
4. Altering the native electronic structure and physical properties of graphene by converting sp^2 carbons to sp^3 ones.
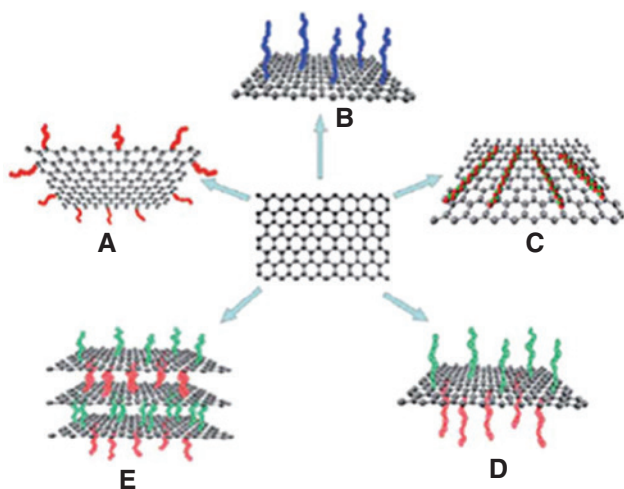
Figure 2: Functionalization possibilities for graphene: (A) edge functionalization, (B) basal-plane functionalization, (C) noncovalent adsorption on the basal plane, (D) asymmetric functionalization, and (E) the self-assembly of graphene. Reprinted with permission from Ref. [55] (Copyright 2014 *J. Mater. Chem. A*).

3.2 Noncovalent functionalization of graphene

Noncovalent functionalization is a simple and economic approach to introduce specific functionalities to the graphene surface. Noncovalent linkages between graphene and other functional groups are mainly achieved through π-π stacking, van der Waals, electrostatic forces, hydrophilic and hydrophobic interactions. Noncovalent functionalization strategies do not affect the transparency or conductivity of the material. This, in turn, introduces scattering sites by creation of high-density electron-hole puddles. Noncovalent strategies also control design-based synthesis of graphene derivatives. For instance, small- molecule aromatics like quinones have been used to functionalize carbonaceous materials and were shown to effectively enhance the performance of energy conversion and storage devices [73].

Various molecules can physically adsorb onto graphene materials without the need of any coupling reagents. Noncovalent functionalization is primarily achieved by polymer wrapping, adsorption of surfactants such as sodium dodecyl sulfate, and hexadecyltrimethylammonium bromide and interaction with metal nanoparticles (e.g. Au, Ag, Pt), porphyrins, or biomolecules such as deoxyribonucleic acid (DNA) and peptides [74–77].

Advantages of noncovalent functionalization:
1. Preserves the intrinsic properties of the graphene
2. Does not destroy the original sp^2-conjugated structure of graphene, which further retains the high electroconductivity
3. Does not create sp^3-hybridized carbons or defects.

Disadvantages of noncovalent functionalization:
1. Physical adsorption is nonspecific
2. There is no control over degree of functionalization
3. It changes the doping density, increases the density of electron-hole puddles, and creates scattering sites.

Different strategies for covalent and noncovalent functionalization of graphene are categorized in Table 2.

4 Current and emerging applications of functionalized graphene in electrochemical biosensors

A biosensor is "a self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological

recognition element which is in direct spatial contact with a transducer," according to the IUPAC definition [109, 110]. Figure 3 schematically depicts the concept of a biosensor. Biosensors can be classified by their biological recognition element or their transducer type.
1. Biological recognition element: Classified into five different major categories. These categories include antibody/antigen, enzymes, nucleic acids/DNA/RNA, cellular structures/cells, and biomimetic. The enzymes, antibodies, and nucleic acids are the main classes of bioreceptors, which are widely used in biosensor applications.
2. Transducers: The transducer plays an important role in the detection process of a biosensor. In case of conducting polymer-based polymer biosensor, the conductive polymer acts as a transducer that converts the biological signal to an electrical signal.

Graphene, a single layer of sp^2-bonded carbon atoms packed into a benzene ring structure, has been demonstrated as an ideal electrochemical platform for the construction of biosensors owing to its large specific surface area, high electric conductivity, good biocompatibility, thermal and chemical stability, and low-cost production [48, 112]. On the other hand, functionalization of graphene covalently and noncovalently can effectively tune its electrical properties and enhance its electrocatalytic performances [113–115]. It is inferred that the electrochemically active sites generated by functional groups triggers the adsorption and activation of analytes, anchoring of functional moieties, and accelerating the charge transfer between electrode and analytes/electrolyte. This, in turn, would be advantages to the enhanced electrochemical biosensing performances [116].

5 Functionalized graphene-based enzyme biosensors

Owing to their unique physical and chemical properties, graphene-based materials have become important candidates for biosensing. The large surface area of graphene can enhance the surface loading of the desired biomolecules and direct electron transfer between enzymes and electrodes. Thus, functionalized graphene could be an excellent electrode material for enzyme biosensors.
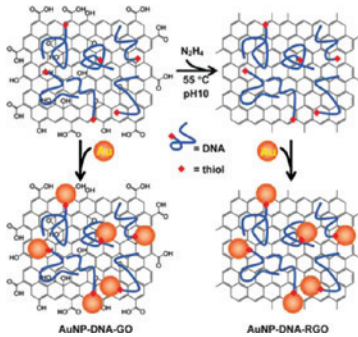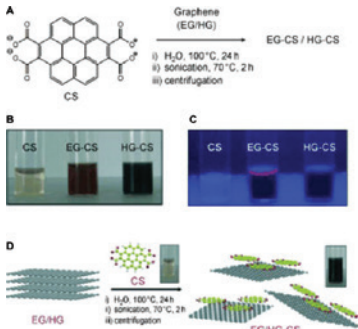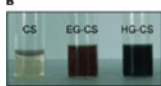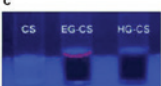
5.1 Glucose biosensor
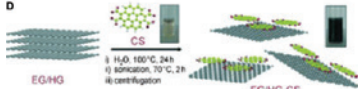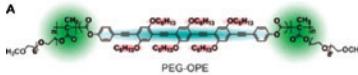
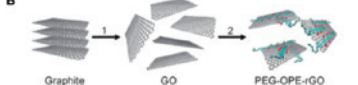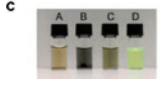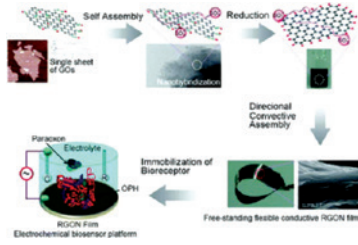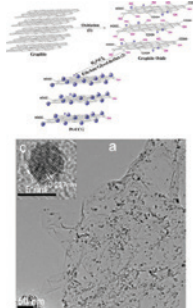The metabolic disorder of diabetes mellitus results in hyperglycemia. It is reflected by blood glucose

Table 2: Different strategies for covalent and noncovalent functionalization of graphene.

Functionalization methods	Procedure of functionalization	Figure/illustration	References
Covalent methods	Addition of free radicals to sp² carbon atoms of graphene		[78–80]
	Addition of dienophiles to carbon-carbon bonds		[66, 81, 82]
	Addition of chromophores		[83–86]
	Covalent linkage to polymers		[87–89]
	Covalent attachments of hydrogen and halogens		[90–93]

Table 2: (continued)

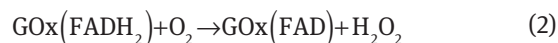| Functionalization methods	Procedure of functionalization	Figure/illustration	References
Noncovalent methods	H-π Interaction		[94–96]
	Π-π Interaction		[97–99]
	Cation-π Interaction		[100–102]
	Anion-π Interaction		[103–105]
	Graphene-ligand noncovalent interaction		[106–108]
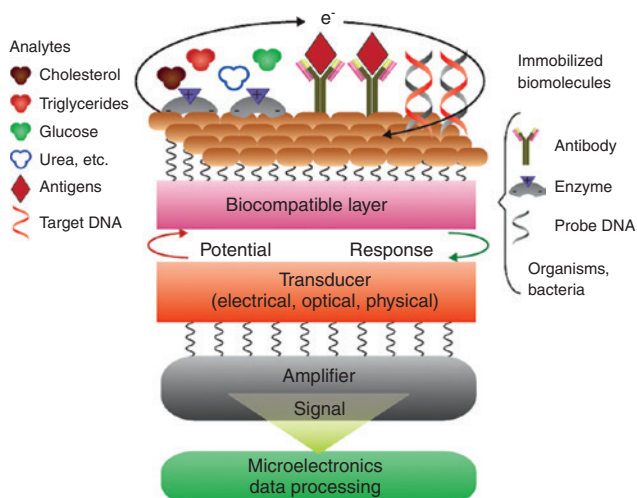
Figure 3: Schematic structure and operating principle of a biosensor. Reprinted with permission from Ref. [111] (Copyright 2011 *Nature*).

concentration higher or lower than the normal range of 80–120 mg dl^{-1}. Persistent hyperglycemia can lead to stroke, coronary heart disease, and circulation disorders [117]. One of the main biomedical applications of graphene in biosensors is to develop glucose concentration-measuring devices for patients suffering from diabetes. Electrochemical detections of glucose have been well demonstrated using glucose oxidase (GOx) as the mediator or recognition element [118, 119]. The redox reaction mechanism is as follows:

In the absence of oxygen:

$$GOx(FAD)+2H^{+}+2e^{-}\leftrightarrow GOx(FADH_2) \qquad (1)$$

In the presence of oxygen:

$$GOx(FADH_2)+O_2 \rightarrow GOx(FAD)+H_2O_2 \qquad (2)$$

$$GOx(FAD)+Glucose \rightarrow GOx(FADH_2)+Gluconolactone \qquad (3)$$
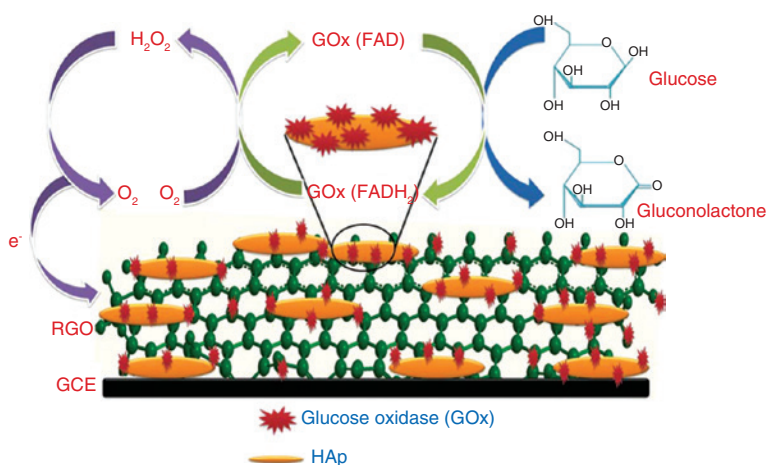
Functionalized graphene-based glucose biosensors can be divided into the following categories:

5.1.1 Enzymatic functionalized graphene-based glucose biosensors

Glucose oxidase (GOx) has been immobilized on graphene sheets for fabricating glucose biosensors via various methodologies [112, 120, 121]. Shan et al. had designed CS-GR/AuNP nanocomposite films for glucose sensing, which exhibited good amperometric responses to glucose with linear ranges of 2–10 mM at -0.2 V and 2–14 mM at 0.5 V [122]. This was attributed to the synergy effect of graphene and the AuNP. G. Bharath et al. [123] have reported the direct electrochemistry of glucose oxidase (GOx) on 1D hydroxyapatite (HAp) on reduced graphene oxide (RGO) nanocomposite-modified glassy carbon electrode (GCE) for glucose sensing (Figure 4). The electrocatalytic and electroanalytical applications of the proposed GOx/HAp/RGO-modified GCE were studied by cyclic voltammetry (CV) and amperometry. The reported biosensor exhibits a superior detection limit (0.03 mM) and higher sensitivity (16.9 mA mM^{-1} cm^{-2}), respectively, with a wide linear range of 0.1–11.5 mM.

Liu et al. [124] fabricated a mono- and multilayered biosensor format with controlled biocatalytic activity. GOx was first modified with pyrene functionalities in order to be self-assembled onto a graphene basal plane via noncovalent interactions. Using an alternate layer-upon-layer of

Figure 4: Schematic illustration for the mechanism of glucose sensing based on an RGO/HAp/GOx nanocomposite. Reprinted with permission from Ref. [123] (Copyright 2015 *J. Mater. Chem. B*).

self-assembled graphene and pyrene-functionalized GOx, mono- and multilayered enzyme electrodes were readily fabricated (Figure 5A). The biocatalytic activity of these enzyme electrodes increased with the number of graphene and GOx layers (Figure 5B). Such multilayered enzyme electrodes with controlled nanostructure exhibited reliable application for the analysis of human serum samples with high sensitivity, good stability, and repeatability.

The disadvantages of enzymatic electrochemical glucose sensors:

The most common and serious problems associated with enzymatic electrochemical glucose sensors are as follows:

(i)   insufficient operational and storage stability
(ii) lower reproducibility
(iii) influence of oxygen limitation.

5.1.2 Non-enzymatic functionalized graphene-based glucose biosensors

Enzymeless sensing of glucose is feasible using metal nanoparticles and nanowires even under neutral pH. In particular, a remarkable detection limit is obtained for glucose (25 nм) using a 3D graphene foam modified with Co_3O_4 nanowires [125]. Another non-enzymatic glucose biosensor based on nickel-polyaniline-functionalized reduced graphene oxide was fabricated by Bing Zhang et al. [126]. They had developed a new class of organic-inorganic hybrid nanostructures, which was applied to efficient non-enzymatic sensing of glucose (Figure 6). This biosensor format included a fast response (~2 s), high sensitivity (6050 µA mм$^{-1}$ cm^{-2}), a linear range from 0.1 µм to 1.0 mм, and a low detection limit (0.08 µм).
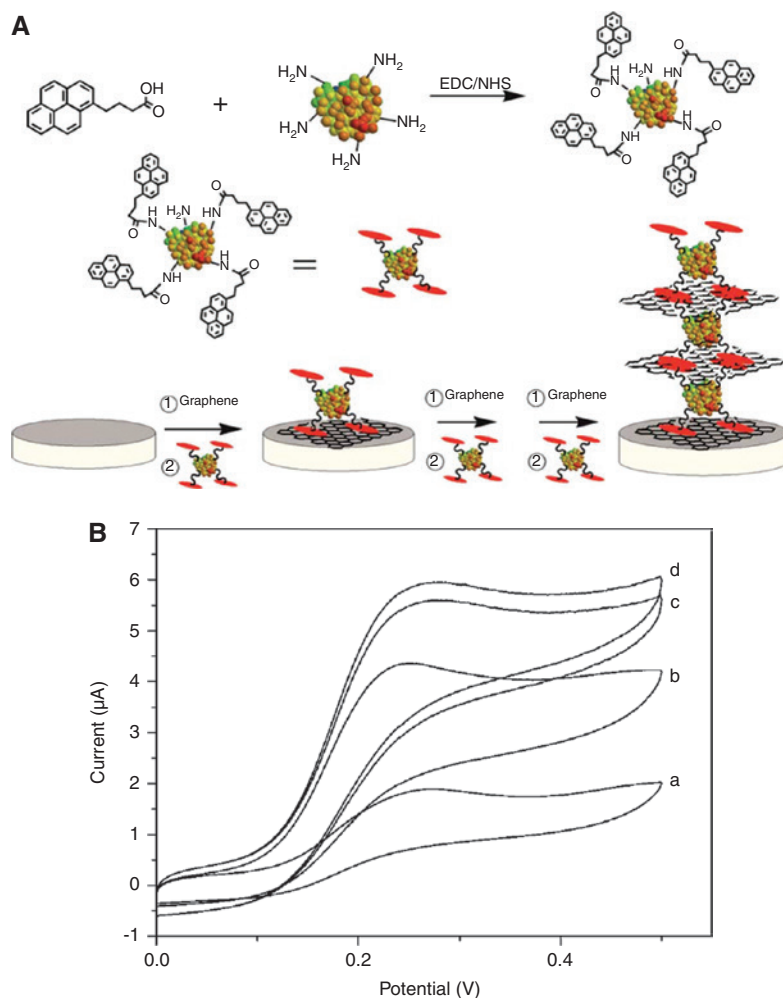
Figure 5: (A) Schematic diagram of the modification of GOD with pyrene and the subsequent fabrication of mono- and multi-layered enzyme electrodes. (B) Cyclic voltammograms of the enzyme electrodes fabricated with mono- and multilayered graphene and pyrene functionalized GOD: (a) one layer, (b) two layers, (c) three layers, and (d) four layers. Data were recorded in 80 mм glucose solution in 0.1 M pH 7.4 phosphate buffer at room temperature and potential scan rate of 10 mV s^{-1}. Reprinted with permission from Ref. [124] (Copyright 2013 *Analyst*).
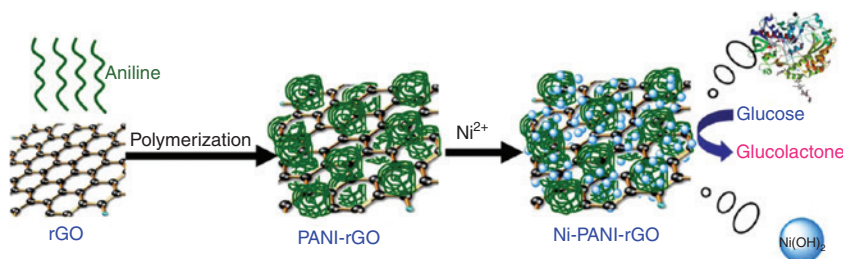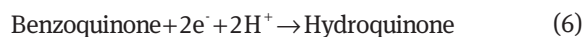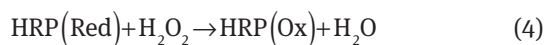
Figure 6: Schematic illustration for Ni-PANI-rGO-based nonenzymatic glucose sensing. Reprinted with permission from Ref. [126] (Copyright 2015 *Microchim. Acta*).

A novel, stable, and sensitive non-enzymatic glucose sensor was developed by electrodepositing metallic Cu nanoparticles on graphene sheets [127]. The Cu-graphene electrode sensor presented a wide linear range up to 4.5 mM glucose with a detection limit of 0.5 µM (signal/noise=3) at a detection potential of 0.5 V in alkaline solution with a very quick (2 s) response. Recently, highly sensitive hybrid structured non-enzymatic glucose sensors were fabricated. Platinum (Pt) nanoparticles were decorated on 3D graphene oxide networks to increase the surface area. Large surface area and high electrocatalytic activity resulted in high glucose sensitivity (137.4 µA mM^{-1} cm^{-2}) and excellent anti-interference characteristics [128].

5.2 Hydrogen peroxide (H$_2$O$_2$) biosensor

H$_2$O$_2$ is also particularly important for the development of biosensing systems. It is a substrate of peroxidases, enzymatic product of oxidases, and an important mediator in clinical as well as pharmaceutical analyses. The detection of H$_2$O$_2$ can be achieved using horseradish peroxidase enzyme (HRP) immobilized onto graphene-based electrodes. The reaction mechanism of the catalytic process can be summarized as follows:

$$HRP(Red) + H_2O_2 \rightarrow HRP(Ox) + H_2O \quad (4)$$

$$HRP(Ox) + Hydroquinone \rightarrow HRP(Red) + Benzoquinone \quad (5)$$

$$Benzoquinone + 2e^- + 2H^+ \rightarrow Hydroquinone \quad (6)$$

Zonghua Wang et al. [129] fabricated platinum nanoparticles/graphene nanohybrids (Pt/G) via *in situ* reduction of PtCl$_6^{2-}$ in the presence of poly(diallyldimethylammonium chloride)-modified graphene (Figure 7). These nanohybrids were used as a matrix for the immobilization of HRP to construct a HRP/Pt/G/glassy carbon electrode (GC)

biosensor for H$_2$O$_2$. The Pt/G hybrid provides an operative environment for maintaining the bioactivity of HRP. It acts as a bridge for the transfer of electrons between the active center of HRP and the electrode surface (Figure 8). Pt/G effectively reinforced the immobilization of HRP and enhanced the utilization of HRP. The linear range for H$_2$O$_2$ was estimated to be from 3.0×10^{-6} mol l^{-1} to 5.2×10^{-3} mol l^{-1} with a detection limit of 5.0×10^{-9} mol l^{-1}.

A sensitive and noble amperometric HRP biosensor is fabricated via the deposition of gold nanoparticles (AuNPs) onto a 3D porous carbonized chicken eggshell membrane (CESM) [130]. Owing to the synergistic effects of the unique porous carbon architecture and well-distributed AuNPs, the enzyme-modified electrode shows an excellent electrochemical redox behavior (Figure 9). Furthermore, the HRP-AuNPs-CESM/GCE electrode, as a biosensor for H$_2$O$_2$ detection, has a good accuracy and high sensitivity with a linear range of 0.01–2.7 mM H$_2$O$_2$ and a detection limit of 3 µM H$_2$O$_2$.

A facile one-pot hydrothermal approach was reported for cerium oxide-reduced graphene oxide CeO$_2$-RGO nanocomposite formation (Figure 10), which is further assembled with HRP for the detection of hydrogen peroxide (H$_2$O$_2$) at trace levels [131]. This biosensor format exhibited a wide linear range of H$_2$O$_2$ from 0.1 to 500 µM with a detection limit of 0.021 µM.

In addition, many novel non-enzyme H$_2$O$_2$ biosensors have been reported [132–134]. For instance, Dye et al. [132] developed a highly sensitive amperometric biosensor based on the hybrid material, which is derived from graphene and Pt nanoparticles for the detection of H$_2$O$_2$ and cholesterol. This sensor shows high sensitivity and fast response toward H$_2$O$_2$ up to 12 mM with a detection limit of 0.5 nM (S/N=3) in the absence of any redox mediator or enzyme. The combination of superconductive graphene sheets and catalytically active Pt nanoparticles accelerated electron transfer for the oxidation of H$_2$O$_2$.
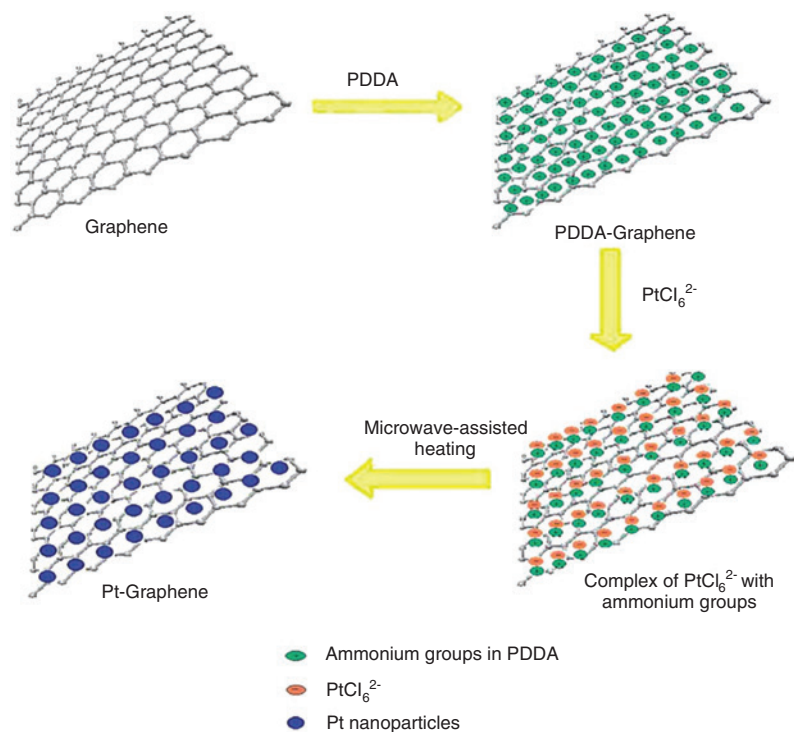
Figure 7: Scheme of the fabrication of Pt/G hybrid. Reprinted with permission from Ref. [129] (Copyright 2013 *Anal. Methods*).
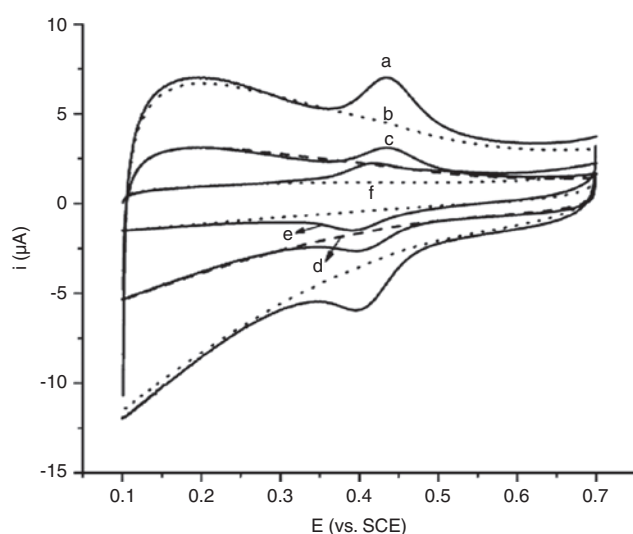
Figure 8: Cyclic voltammograms (CVs) of modified electrodes in 0.1 mol l^{-1} pH ¼ 6.8 PBS, HRP/Pt/G/GC (A), Pt/G/GC (B), HRP/G/GC (C), G/GC (D), HRP/Pt/GC (E), and Pt/GC (F). Reprinted with permission from Ref. [129] (Copyright 2013 *Anal. Methods*).
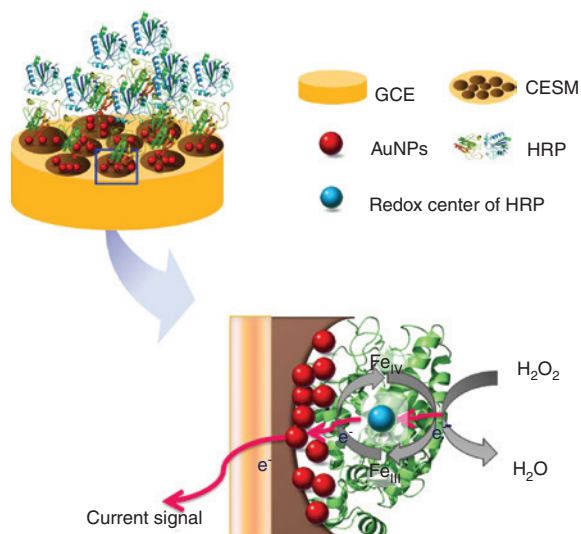
Figure 9: Schematic illustration of the mechanism underlying the detection of H_2O_2 using HRP-AuNPs- CESM-GCE electrochemical biosensor. Reprinted with permission from Ref. [130] (Copyright 2015 *Plos One*).

5.3 Cholesterol biosensor

Cholesterol is an organic molecule. It is an essential structural component of all animal cell membranes [135]. Cholesterol also serves as a precursor for the biosynthesis of steroid hormones, bile acids, and vitamin D. The total blood cholesterol level should be <200 mg/dl in the human body. However, undesired accumulation of cholesterol and its esters causes critical health problems such as hypercholesterolemia, heart diseases, cerebral
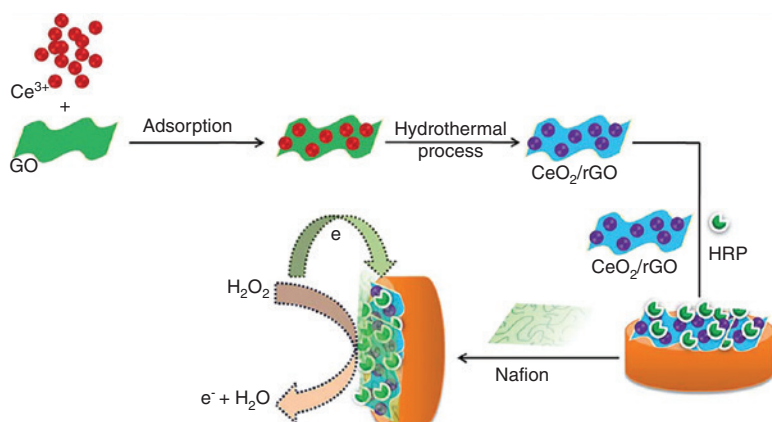
Figure 10: H_2O_2 detection scheme using HRP/CeO$_2$-rGO modified glassy carbon electrode. Reprinted with permission from Ref. [131] (Copyright 2015 *RSC Adv.*).

thrombosis, and atherosclerosis. A smart, quick, accurate determination of cholesterol in blood is an urgent need in clinical diagnosis. The following biochemical reaction occurs as a result of interaction of cholesterol oxidase (ChOx) with cholesterol:

$$\text{Cholesterol} + O_2 \xrightarrow{\text{ChOx}} \text{Cholesterone} + H_2O_2$$
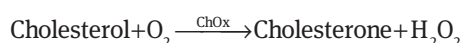
Dey and Raj [136] developed a highly sensitive amperometric cholesterol biosensor based on a hybrid material derived from PtNP and graphene for the detection of H_2O_2 (Figure 11). The cholesterol biosensor was developed by immobilizing cholesterol oxidase and cholesterol esterase on the surface of the GR/PtNP hybrid material. The sensitivity and detection limit of the electrode toward cholesterol ester were 2.07±0.1 μA/μM/cm^2 and 0.2 μM, respectively.

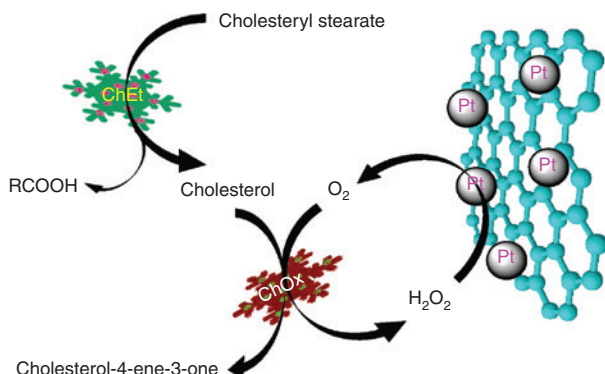Shiju Abraham et al. [137] have reported the development of cost-effective bioelectrodes based on a reduced graphene oxide-functionalized gold nanoparticle hybrid

system (RGO-Fn Au NPs). The electrodes were fabricated by the electrophoretic deposition technique. A synergistically enhanced electrochemical sensing ability of 193.4 μA mm^{-1} cm^{-2} for free cholesterol detection was achieved, which is much higher than that of the traditional RGO system (Figure 12). Moreover, the RGO-Fn Au NP platform promises a wider range of cholesterol detection (0.65–12.93 mM) with lower detection limit of 0.34 mM for free cholesterol.

Deepshikha et al. have fabricated a novel nanobiocomposite bienzymatic amperometric cholesterol biosensor, coupled with cholesterol oxidase (ChOx) and HRP, based on the gold-nanoparticle decorated graphene-nanostructured polyaniline nanocomposite (NSPANI-AuNP-GR) film. The nanocomposite film was electrochemically deposited onto indium-tin-oxide (ITO) electrode from the NSPANI-AuNP-GR nanodispersion [138]. The overall biochemical reaction for ChOx-HRP/NSPANIAuNP- GR is shown in Figure 13.

The gold nanoparticle-decorated graphene-nanostructured polyaniline nanocomposite (NSPANI-AuNP-GR) offers an efficient electron transfer between underlining electrode and enzyme active center. The bienzymatic nanocomposite bioelectrodes ChOx-HRP/NSPANI-AuNPGR/ITO have exhibited wider linearity (35 to 500 mg/dl), higher sensitivity (0.42 μA mm^{-1}), low km value of 0.01 mM, and higher accuracy for testing of blood serum samples than monoenzyme system. The novelty of the electrode lies on reusability, extended shelf life, and accuracy of testing blood serum samples.

5.4 NADH biosensor

β-Nicotinamide adenine dinucleotide (NAD$^+$) and its reduced form (NADH) are a cofactor of many
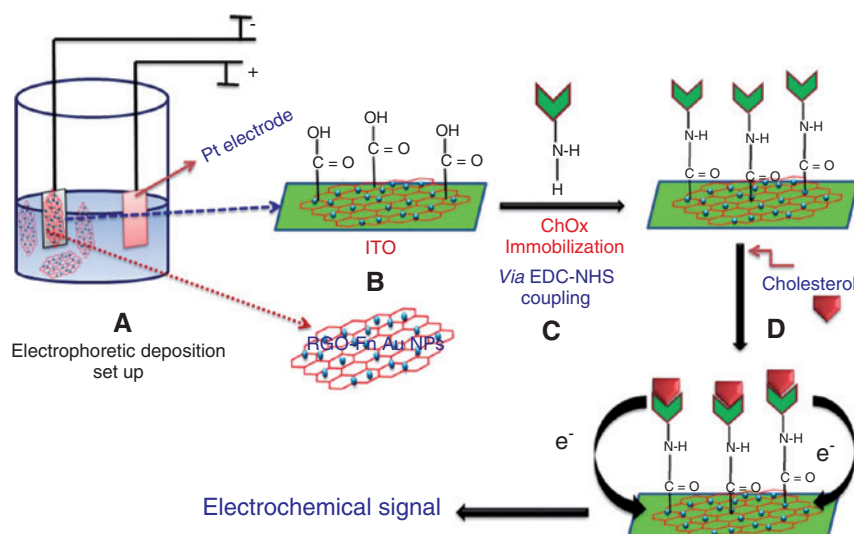

Figure 11: Scheme illustrating the biosensing of cholesterol ester with the GNS-nPt-based biosensor. Reprinted with permission from Ref. [136] (Copyright 2010 *J. Phys. Chem. C*).

Figure 12: Schematic representation of the cholesterol sensing process: (A) EPD set up for the fabrication of RGO and RGO-Fn Au NP thin films; (B) electrophoretically fabricated RGO-Fn Au NP thin film on an ITO substrate; (C) immobilization of ChOx on RGO-Fn Au NPs by EDC-NHS coupling; and (D) the immobilized RGO-Fn Au NP bioelectrode and the electrochemical reaction while adding cholesterol to the electrochemical cell containing the bioelectrode. Reprinted with permission from Ref. [137] (Copyright 2015 *Anal. Methods*).
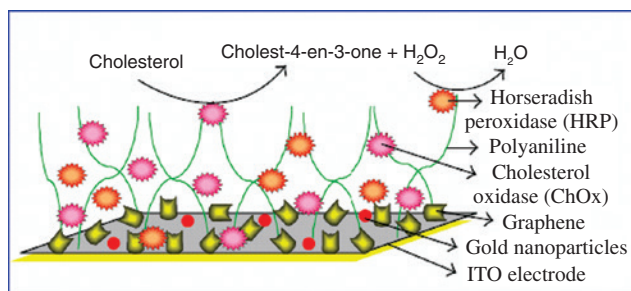
Figure 13: Proposed biochemical reaction on the ChOx-HRP/NSPANI-AuNP-GR. Reprinted from an Open Access Journal Ref. [138], 2012.

dehydrogenases. NAD$^+$/NADH-dependent dehydrogenases have been attempted in the development of biosensors, biofuel cells, and bioelectronic devices. A large overvoltage is necessary for anodic detection of NADH, and electrode fouling is also encountered due to the formation of reaction products. Apparently, graphene shows promise in addressing these two drawbacks. Graphene functionalization with dyes like methylene green (MG) forms a stable complex via noncovalent bonding [139]. The oxidation of NADH on MG-graphene electrode takes place at ~0.14 V, which is much lower than that of pristine graphene [139] and CNTs [140, 141].

Shan et al. [142] showed that graphene electrodes functionalized using ionic liquids could be used for both NADH detection and the biosensing for ethanol. The ionic liquid modified-graphene-based sensor exhibited

acceptable analytical outcomes for the determination of NADH. Moreover, process is convenient, and low-cost preparation is involved. Tang et al. adopted chemically reduced GO (CRGO), which showed a remarkable oxidation shift of about 30 mV compared to graphite and bare GC electrodes [143].

5.5 Dopamine biosensor

Dopamine (DA), an important neurotransmitter, plays an important role in the central nervous, hormonal, renal, and cardiovascular systems. Its deficiency is correlated to Parkinson's disease, which is a degenerative disorder of the central nervous system. The major symptoms of Parkinson's disease result from the death of dopamine-generating cells in midbrain. Therefore, a lot of research works aimed at developing sensitive, rapid, and decentralized sensing devices for its detection. With the excellent electroanalytical properties, graphene-based electrodes have demonstrated excellent performances in detecting DA. Gregory Thien Soon How et al. [144] have reported a reduced graphene oxide/titanium dioxide nanocomposite with highly exposed facets (rGO/TiO$_2$ {001}) for the detection of DA [144]. The modified rGO/TiO$_2$ {001} GCE shows enhanced electrochemical sensing toward DA under the interference effect. This method certainly opens up a new platform of facet manipulation studies for electrochemical applications (Figure 14).
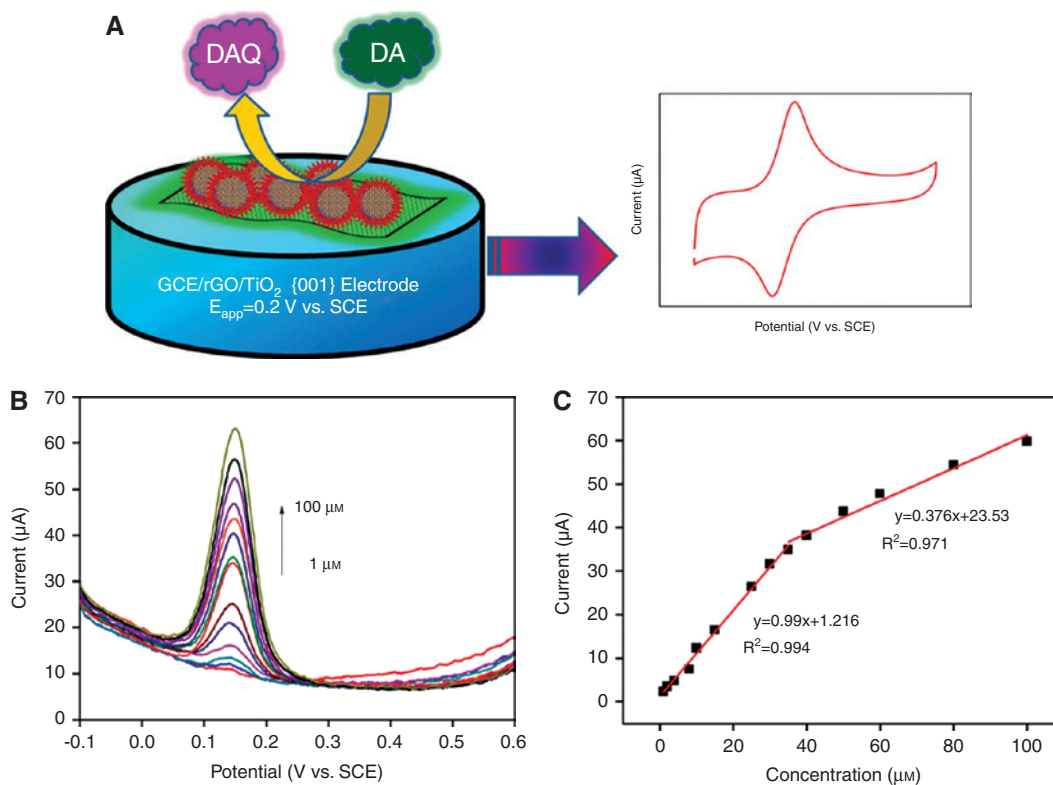
Figure 14: (A) Mechanism for the electrocatalytic oxidation of dopamine at rGO/TiO$_2$ {001} modified GCE. (B) DPV obtained for rGO/TiO$_2$ {001} modified GCEs during the addition of 1, 2, 4, 8, 10, 15, 25, 30, 35, 40, 50, 60, 80, and 100 mM DA into the 0.1 M PBS (pH? 6.5). (C) Calibration plot observed for the oxidation peak current vs. concentration of DA. Reprinted with permission from Ref. [144] (Copyright 2014 *Nature*).
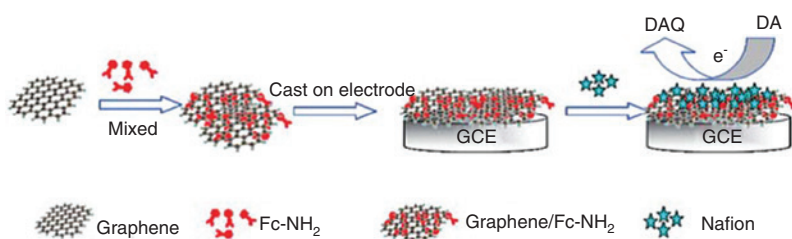
Figure 15: Schematic illustration of the preparation procedures of the Fc-NH$_2$ modified electrode. Reprinted with permission from Ref. [145] (Copyright 2012 *Analyst*).

DA coexists with endogenous electroactive species such as ascorbic acid (AA) and uric acid (UA). They have an overlapping voltammetric response, resulting in poor selectivity and sensitivity of DA. The presence of sp^2 hybridized planes and various edge defects on the graphene surface might attribute to a better sensing performance toward DA [50] and can differentiate DA from AA and UA. Liu et al. [145] have used graphene/ferrocene derivative (graphene/Fc-NH$_2$) nanocomposite to fabricate the Nafion/graphene/Fc-NH$_2$-modified GCE for the detection of DA. The Fc-NH$_2$ embedded on the graphene significantly enhances the electrochemical response toward DA, charge-transport ability, stabilizes the graphene, and

prevent the leakage of ferrocene (Figure 15). In the presence of 1 mM AA and 0.1 mM UA, the electrode showed linear response in the range of 0.1 to 4 μM, and the detection limit was 50 nM (S/N=3).

5.6 DNA biosensors

Electrochemical DNA sensors offer high sensitivity, high selectivity, and low cost for the detection of selected DNA sequences. These also detect mutated genes associated with human disease and provide a simple, accurate, reliable, and inexpensive platform of patient disease diagnosis
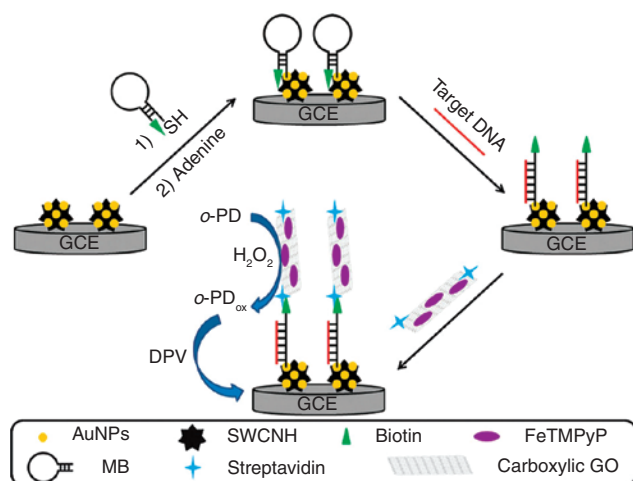
Figure 16: Schematic illustration of graphene-supported ferric porphyrin as a HRP mimicking trace label for electrochemical detection of DNA. Reprinted with permission from Ref. [155] (Copyright 2013 *Chem. Commun.*).

Figure 18: An illustration of the protocol for IgG immunodetection based on the EIS method. (A) A bare DEP electrode modified with CMG material; (B) a CMG material modified DEP electrode after the immobilization of anti-IgG and blocking with BSA; and (C) an anti-IgG and BSA-modified DEP electrode after the incubation with IgG. Reprinted with permission from Ref. [184] (Copyright 2012 *Nanoscale*).

[146, 147]. Graphene, as a perfect 2D material with large surface area, can work as a carrier for DNA molecules loading either by

(i) Direct absorption via π-π interactions [148, 149]
(ii) By other binding approaches via functional groups from modified graphene surface [150, 151].

Furthermore, the excellent conductivity of graphene makes it possible for fast electron transfer between DNA and

Figure 17: A proposed mechanism for an ultrasensitive and highly selective Hg²⁺ sensor based on the modified ERGO. Reprinted with permission from Ref. [160] (Copyright 2013 *Chem. Commun.*).

electrodes. Recently, the electrochemical detection for target DNA molecules has been reported [148–152]. For example, Huang et al. [152] synthesized a tungsten sulfide-graphene (WS$_2$-Gr) nanocomposite for DNA biosensor application. Graphene served as a 2D conductive support for a highly electrolytic accessible surface. However, the electronic conductivity of WS$_2$ is much lower compared to graphene materials, thereby, limiting its application for sensitive detection. On the other hand, DNA detections can also be achieved by non-DNA-modified electrodes [153–155]. For example, Quanbo Wang et al. [155] have designed a novel peroxidase mimic by loading ferric porphyrin and streptavidin onto graphene, which was used to recognize a biotinylated molecular beacon (MB) for specific electrochemical detection of DNA (Figure 16). The biosensor format had exhibited high specificity and excellent anti-interference ability. The biosensing method could discriminate target DNA from single-base or three-base mismatched oligonucleotides.

Table 3: Characteristic features of functionalized graphene-based biosensors.

Sensor type	Sensing material	Detectable product	Linearity range	Detection limit	References
Glucose	N-doped graphene	Glucose	0.1–1.1 mM	0.01 mM	[186]
	RGO/hydroxyapatite	Glucose	0.1–11.5 mM	0.03 mM	[123]
	Graphene/polyaniline/Au	Glucose	4.0–1.12 mM	0.6 mM	[187]
	N-doped graphene/Cu	Glucose	0.004–4.5 mM	1.3 mM	[188]
	Carbon nitride dots-reduced graphene oxide	Glucose	40 μM–20 mM	40 μM	[189]
	Chitosan/AuNP/sulfonates poly(ether-ether-ketone) functionalized ternary graphene	Glucose	0.5–22.2 mM	0.13 mM	[190]
Hydrogen Peroxide	Arginine-functionalized graphene	Hydrogen Peroxide	5 nM–40 μM	1.1 nM	[191]
	CeO$_2$-RGO	Hydrogen Peroxide	0.1–500 μM	0.021 μM	[192]
	PtRu/3D graphene foam	Hydrogen Peroxide	0.005–0.02 mM	0.04 μM	[193]
	AuNP/stacked Graphene nanofibers	Hydrogen Peroxide	0.08–250 μM	0.35 nM	[194]
	MnO$_2$/graphene/CNT	Hydrogen Peroxide	1–1030 μM	0.1 μM	[195]
	PdNP-graphene nanosheets-Nafion	Hydrogen Peroxide	0.1–1000 μM	0.05 μM	[196]
Cholesterol	Pt-Pd-chitosan-graphene	Cholesterol	2.2–5200 μM	0.75 μM	[197]
	Graphene/polyvinylpyrrolidone/polyaniline	Cholesterol	50 μM–10 mM	1 μM	[198]
	PtNP-graphene	Cholesterol	Up to 12 mM	0.5 nM	[136]
	Reduced graphene oxide-functionalized AuNPs	Cholesterol	0.65–12.93 mM	0.34 mM	[199]
NADH	Graphene-TiO$_2$	NADH	1×10^{-8}–2×10^{-3} M	3×10^{-9} M	[200]
	Graphene-Au nanorods	NADH	20–60 μM	6 μM	[201]
	Electroreduced GO-polythionine	NADH	0.01–3.9 mM	0.1 μM	[202]
Dopamine	Trp-functionalized graphene	Dopamine	0.5–110 μM	290 nM	[203]
	N-doped graphene	Dopamine	100–450 μM	0.93 μM	[204]
	Sulfonated-graphene	Dopamine	0.2–20 μM	40 nM	[205]
	N-doped graphene/PEI	Dopamine	1–130 μM	500 nM	[206]
DNA	ssDNA/azophloxine/graphene nanosheets	DNA	1 fM–0.1 pM	0.4 fM	[207]
	ssDNA/AuNP/polyaniline/graphene sheets-chitosan	DNA	10–1000 pM	2.11 pM	[208]
	ssDNA/chitosan–Co$_3$O$_4$ nanorods–graphene	DNA	1.0×10^{-12}–1.0×10^{-6} M	0.43 pM	[209]
	ssDNA-MB/functionalized graphene	DNA	100–350 nM	40 nM	[148]
Heavy Metal Ions	Graphene nanosheets/Au	Pb^{2+}	0.4–20 nM	0.4 nM	[166]
	RGO/Au	Pb^{2+}	50–1000 nM	10 nM	[163]
	Graphene nanosheets/Au	Cu^{2+}	1.5–20 nM	1.5 nM	[166]
	SnO$_2$/RGO	Cu^{2+}	0.2–0.6 μM	0.23 nM	[170]
	SnO$_2$/RGO	Cd^{2+}	NA	0.10 nM	[170]
	Polyvinylpyrrolidone protected graphene/Au	Hg^{2+}	10–60 ppb	6 ppt	[168]
	SnO$_2$/RGO	Hg^{2+}	0.1–1.3 nM	0.28 nM	[170]
	PG modified RGO	Hg^{2+}	0.1 nM–4 nM.	0.1 nM	[160]
Immunosensor	Chemically modified graphene (CMG)	Immunoglobulin G (IgG)	0.3 mg ml^{-1}–7 mg ml^{-1}	–	[184]
	RGO/thionine/gold	Tumor markers	0.01 ng ml^{-1}–300 ng ml^{-1}	–	[185]

5.7 Heavy metal ions biosensors

Heavy metal ions in potable water and food are major health concerns. Their detection is critical to maintaining a safe food supply [156, 157]. Therefore, the highly sensitive, selective, and rapid detection of heavy metal ions has been explored in depth using various analytical methodologies [158–161]. Graphene- based sensors have been employed to detect and monitor the presence of heavy metal ions such as Pb^{2+}, Cd^{2+}, Cu^{2+}, and Hg^{2+} [160, 162–167]. For example, a gold functionalized graphene electrode was produced via noncovalent interaction for the determination of Pb^{2+} and Cu^{2+} [168]. Chunmeng Yu et al. [160] have designed an ultrasensitive and highly selective Hg^{2+} sensor through noncovalent modification of an electrochemically reduced GO (ERGO)-based diode with N-[(1-pyrenyl-sulfonamido)-heptyl]-gluconamide (PG) as the modifier. PG is comprised of a glucose residue and a pyrene residue. The glucose residue can work as multiple-receptor sites for Hg^{2+} in the medium. The large π system in the pyrene can be stably attached on the ERGO surface (Figure 17). The detection limit of this sensor for Hg^{2+} reaches 0.1 nм, which is the best result of electronic sensors and is about 10 times as low as the previously reported results [169]. Alkanethiol-modified graphene field effect transistors exhibited a sensitivity for Hg^{2+} detection at 10 ppm, which provides new opportunities for graphene-based electronics as heavy metal sensors. Sensors based on graphene have also been reported for the detection of Cd^{2+}, Cu^{2+}, and Ag^+ [170–173].

5.8 Immunosensors

Electrochemical immunosensors are miniaturized analytical devices with higher sensitivity and selectivity. Compared with the other methods like enzyme-linked immunosorbent assay (ELISA), immunosensors are attractive because of their high sensitivity, low cost, short analytical time, simple instrumentation, and high specificity of immunological reactions [174]. Immunosensors are capable of detecting nucleic acids [175], viruses [176], antigens [177], and hormone [178] biomarkers. More recently, graphene has been utilized in a number of forms for immunosensor applications [179–183]. Graphene is a material, which has immense potential for the fabrication of immunosensors. A simple electrochemical impedimetric immunosensor for immunoglobulin G (IgG) based on chemically modified graphene (CMG) surfaces was proposed by A.H. Loo et al. [184]. The group had used graphite oxide, graphene oxide, thermally reduced graphene oxide, and electrochemically reduced graphene oxide for fabrication of immunosensor (Figure 18). It was found that thermally reduced graphene oxide has provided the best performance with the linear detection range from 0.3 mg ml^{-1} to 7 mg ml^{-1}.

Simultaneous detection of multiple tumor markers was done by fabricating graphene nanocomposite immunosensor [185]. In this work, reduced graphene oxide/thionine/gold nanoparticles nanocomposites were synthesized and coated on ITO for the formation of an antibody-antigen immunocomplex. Experimental results revealed that the multiplexed immunoassay enabled the simultaneous determination of tumor markers with linear working ranges of 0.01–300 ng ml^{-1}.

Characteristic features of various functionalized graphene-based biosensors are summarized in Table 3.

6 Conclusion

Graphene, as an amazing and wonderful material among the nanocarbon family, has drawn considerable interest in many fields. Its unique structure contributes to its fascinating chemical and physical properties, which lead to a broad range of applications in sensing. In this review, significant advances in functionalized graphene-based biosensors relative to their bulk counterparts have been reported. The unique electronic structure, biocompatibility, and dimensional compatibility with biomolecules indicate that graphene can provide a new generation of smart transduction matrices for the control of a range of biomolecular interactions, from the molecular to the cellular levels. However, graphene, as a newly discovered material, faces several challenges including improving synthesis methods, extensive understanding of graphene surface, and extending the applications in various practical fields.

There are currently two key challenges for the development of large-scale manufacturing of graphene-based products:
1. Production of large quantities and high-quality uniform graphene
2. Tailoring and tuning of graphene properties.

The synthesis and cost of making graphene is relatively much cheaper in comparison to other carbon materials such as CNTs. There have also been significant advances in the preparation of graphene, their functionalization with the desired chemical entities, and the immobilization of biomolecules to the functionalized graphene surfaces for biosensor applications. However, most of the

bioanalytical applications have been demonstrated using multilayered graphene as the preparation of single-layer graphene is highly expensive. Therefore, there is a need for dedicated research efforts to develop cheaper and highly simplified strategies for the development of single-layer graphene.

It is necessary to better understand the chemistry and physics at the graphene surface and also the interactions of chemicals or biomolecules at the interface of graphene when applied in biosensing. Various functionalization chemistries have already been demonstrated to have high biocompatibility without any apparent cytotoxicity. However, further efforts are desired in order to test the cytotoxicity for a relatively longer period of time. An improved understanding of graphene and its interaction with molecules will lead to advances in graphene science and its application in catalysis and sensor development. Improved methods of functionalization are now emerging. An area poised to produce major benefits is the use of functionalized 2D nanostructures to control biomolecular interactions. This suggests that in the near future, the star of the nanocarbon family, "graphene," will find fascinating applications in the biomolecular world.

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