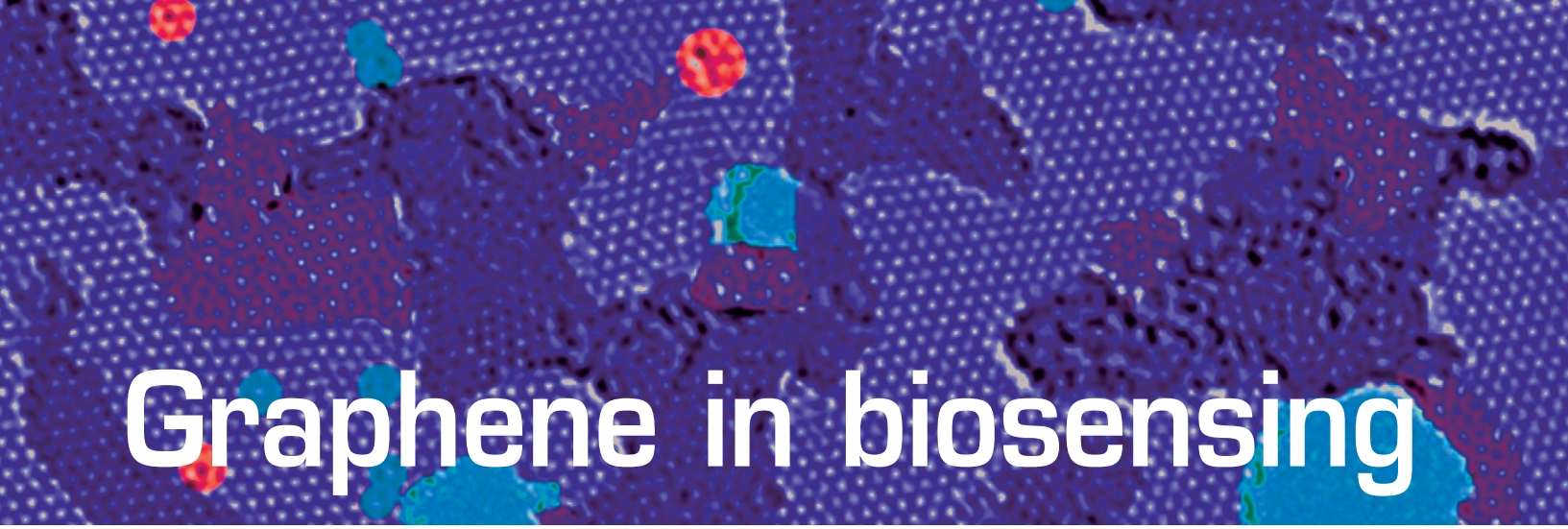

Graphene in biosensing

Biosensing is paramount for improving the quality of human life. Biosensors and biosensing protocols are able to detect a wide range of compounds, sensitively and selectively, with applications in security, health care for point-of-care analyses of diseases, and environmental safety. Here, we describe biosensors and biosensing systems employing graphene. Graphene is a zero-gap semiconductor material, which is electroactive and transparent. Because of its interesting properties, graphene has found its way into a wide variety of biosensing schemes. It has been used as a transducer in bio-field-effect transistors, electrochemical biosensors, impedance biosensors, electrochemiluminescence, and fluorescence biosensors, as well as biomolecular labels. In our review, we describe the application of graphene for enzymatic biosensing, DNA sensing, and immunosensing. We compare different techniques and present our views on the future development of the field.

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Graphene is a one-atom-thick material consisting of sp^2-bonded carbon with a honeycomb structure. It resembles a large polyaromatic molecule of semi-infinite size[1,2]. In the past five years, graphene-based nanomaterials have been the focus of a vast amount of attention. The interesting and exciting properties of single-layer graphene sheets, such as high mechanical strength[3], high elasticity and thermal conductivity, demonstration of the room temperature quantum Hall effect, very high room-temperature electron mobility[2], tunable optical properties[4,5], and a tunable band gap[6] have excited the scientific community especially in the areas of materials, physics, and chemistry. Different, but similarly fascinating properties are exhibited by double-, few-, and multilayer graphene. Because graphene is a conductive yet transparent material, with a low cost and low environmental impact, it is an ideal material for the construction of sensors and biosensor-based devices in various transduction modes, from electrical and electrochemical transduction to optical transduction.

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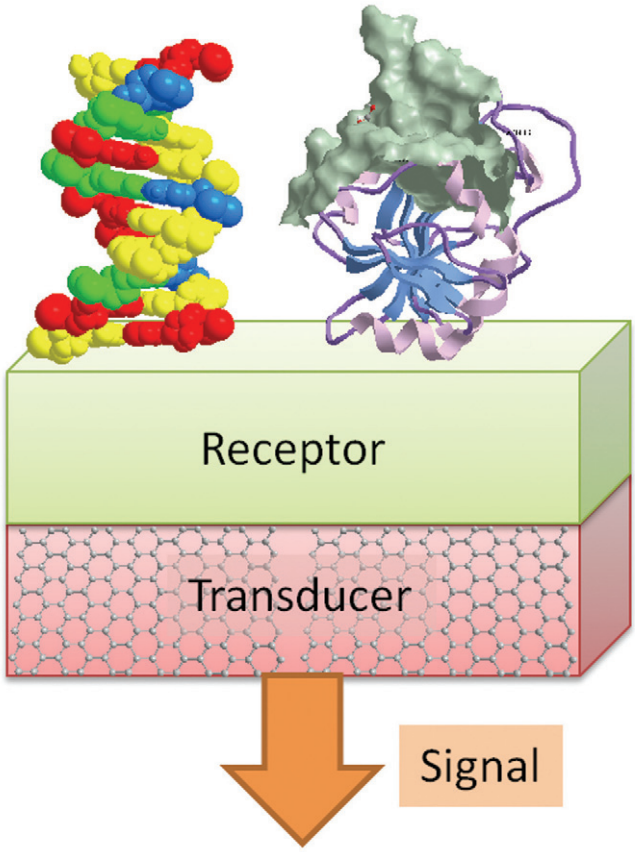

Fig. 1 Scheme of a biosensor. The biosensor consists of a receptor layer, which consists of a biomolecule (e.g., DNA or protein), and a transducer, which is a graphene-based material.

The detection of biologically active molecules is of critical importance from a biomedical, environmental, and security point of view. Such detection can be carried out by biosensor or by bioanalytical protocols. A chemical sensor is a device that quantitatively or semi-quantitatively converts information about the presence of a chemical species to an analytically useful signal[7]. Sensors consist of two elements: a *receptor* and a *transducer* (see Fig. 1). A receptor can be any organic or inorganic material with (preferably) a specific interaction with one analyte or group of analytes. In the case of biosensors, the recognition element is a biomolecule. The second key element of the sensing platform is the transducer, which converts chemical information into a measurable signal. Bioanalytical protocols usually include more than one processing step. In this review, we will describe biosensors and bioanalytical systems that utilize graphene as a key component.

Fundamentals

There are different kinds of graphene-based nanomaterial and their type is closely related to the method of production. Graphene can be produced in many ways; by chemical vapor deposition (CVD) growth, mechanical exfoliation of graphite, or exfoliation of graphite oxide[8].

Neither CVD-produced graphene nor mechanically exfoliated graphene contain large quantities of defects or functionalities. However, bulk quantities of graphene-based nanomaterials are typically prepared by different methods, such as the thermal exfoliation of graphite oxide[9] which leads to a material called thermally reduced graphene (TRGO) or, for example, sono-assisted exfoliation of graphite oxide to graphene oxide (GO)[10]. Graphene oxide can be further reduced chemically or electrochemically. Thermally reduced graphene oxide (TRGO) contains large amounts of defects and significantly differs from pristine graphene, which has a perfect honeycomb lattice structure[11]. The presence of the defects is not disadvantageous. To the contrary, it is well known that heterogeneous electron transfer in the electrochemistry of sp^2 carbons occurs at the edges and defects, and not at the basal plane of graphene sheets[12]. Graphene oxide has a structure that is not fully planar because the sp^2 carbon network is heavily damaged. It contains large amounts of oxygen-containing groups, which can be beneficial to the functionalization through the action of the biomolecules for biorecognition events during biosensing[10]. Graphene oxide can be chemically or electrochemically reduced. Such products have a partly restored sp^2 lattice that also contains some degree of oxygen-bearing groups. The products are typically referred to as chemically reduced graphene oxide (CRGO) or electrochemically reduced graphene oxide (ERGO)[13]. The structures of graphite oxide, graphene oxide, TRGO, and CRGO are shown in Fig 2. Ruoff *et al*. suggested that graphite oxide, graphene oxide, TRGO, ERGO, and CRGO should be termed chemically modified graphenes[8]. Therefore, one could have a large graphene "toolbox" to choose the right type of graphene for the right application and transduction mechanism. When compared to carbon nanotubes (for reviews on carbon nanotube biosensors, see e.g.[14-16]), it is clear that the structural differences, such as tubes vs. sheets, will play a major role in the nanoarchitectonic design of biosensors. From a practical point of view, it is important to note that CNTs are grown from metallic particles which might affect the response of the sensor, while graphene is often synthesized in different ways. In the following text, we will discuss how graphene-based nanomaterials are used in different transduction systems.

Graphene in bio-field-effect transistors

Field-effect transistors (FET) have received a great deal of interest in the area of biosensing as they can provide full electronic detection that is fully integrated into the electronic chips produced by semiconductor companies. Therefore, it is not only academia that is fascinated by these devices, but there is a strong interest (and investment) from industry as well[17]. Field-effect transistor-based biosensors rely on biorecognition events between molecules at the gate of the FET[18,19]. Upon biorecognition between the probe and target biomolecules, the electric charge distribution changes the charge carrier density at the biorecognition layer and thus, the conductivity of the channel between

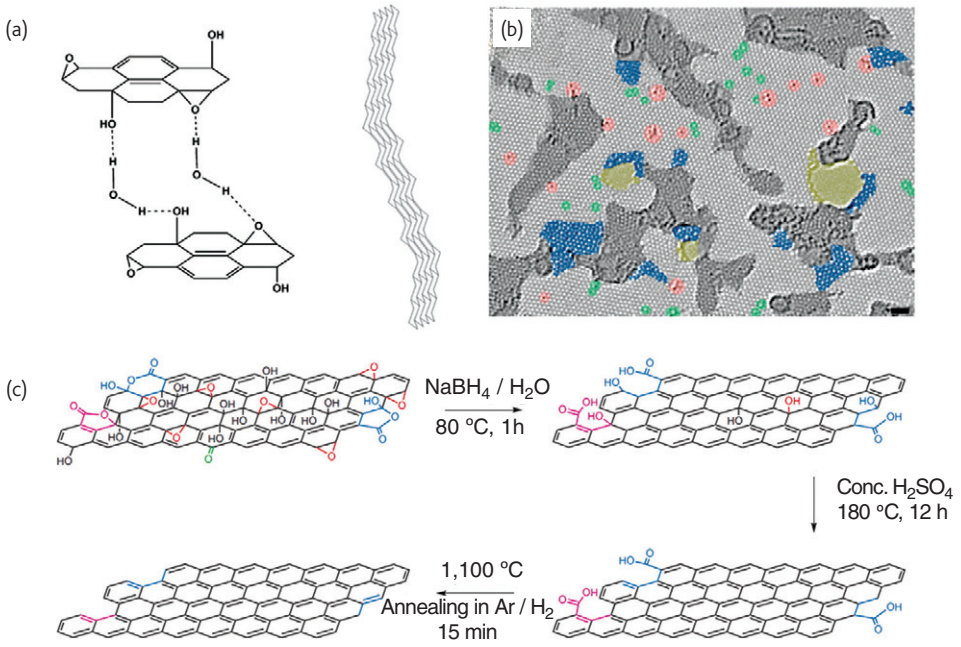

Fig. 2 Structures of different chemically modified graphenes. (a) Chemical and long-range structure of graphite oxide. Reprinted with permission from[9]. © 2006 American Chemical Society. Also reproduced from[10] by permission of The Royal Society of Chemistry. (b) Structure of thermally reduced graphene oxide: atomic resolution, aberration-corrected TEM image of a single-layer reduced-graphene oxide membrane. False color has been added to highlight the different features. The defect-free crystalline graphene area is displayed in light gray. The contaminated regions are shaded in dark gray. The disordered single-layer carbon networks, or extended topological defects, that we identify as remnants of the oxidation-reduction process are blue. Individual adatoms or substitutions are highlighted in red. Isolated topological defects, that is, single bond rotations or dislocation cores, are green. Holes and their edge reconstructions are yellow. Scale bar, 1 nm. Reprinted with permission from[11]. © 2010 American Chemical Society. (c) Procedure for producing chemically reduced graphene oxide, taking into account the five- and six-membered lactol rings (blue), ester of tertiary alcohol (pink), hydroxyl (black), epoxy (red), and ketone (green) functionalities. The relative ratios are likely to be 115 (hydroxyl and epoxy) : 3 (lactol O–C–O) : 63 (graphitic sp^2 carbon) : 10 (lactol/ester/acid carbonyl) : 9 (ketone carbonyl). This model shows only the chemical connectivity and not the steric orientation of these functionalities. Reprinted by permission from Macmillan Publishers Ltd: from[13], © 2009.

the source and drain. Graphene is an ideal material for the construction of FET biosensors because it is a zero-band gap semiconductor, and the band gap can be tuned by surface modification[20]. As mentioned above, FET transistors are ideal for sensing charged molecules. Therefore, graphene-based FETs can be employed for DNA sensing since DNA has a charged phosphate backbone[21]. Chen, Li, and co-workers[22] demonstrated that large-scale chemical vapor deposition (CVD) grown single- and few-layer graphene films are highly sensitive to DNA hybridization (Fig. 3). The shift in gate voltage was found to be sufficiently large for detection even at a concentration of 10 nM of single stranded DNA (ssDNA). The addition of gold nanoparticles to the probe surface led to an extension of the linearity of the response to 500 nM as this increased the amount of probe DNA immobilized on the FET surface. Tamanaha *et al.*[23] used reduced graphene oxide modified with DNA for real-time detection of ssDNA with a detection limit of 10 nM.

It is apparent that the approach of modifying graphene sheets with nanoparticles to increase the number of probe biomolecules at the FET gate is beneficial in terms of the linearity of the response. Such an approach is not only used for sensing DNA but also for immunosensing. Thermally reduced graphene oxide sheets were suspended over the gold electrodes of an FET. TRGO sheets were then modified with gold nanoparticle (Au NP)-antibody (anti-immunoglobulin G) conjugates (see Fig. 4)[24] . This functions as a specific recognition site for the detection of immunoglobulin G (IgG). When a biorecognition event occurs, significant changes occur in the electrical characteristics of the FET which are used as an analytical signal. Yang and Gong used a very simple system that did not employ an FET but only conductivity measurements[25]. Graphene was percolated with anti-prostate-specific antigen and the detection of the prostate cancer marker was followed by measuring changes in the resistance of the graphene film. An aptamer based FET assay was also developed for the detection of IgE[26].

Graphene impedimetric biosensors

Electrochemical impedance (EIS) platforms provide very high sensitivity for biosensing. We were one of the first groups to develop a graphene platform for the detection of DNA hybridization and polymorphism using electrochemical impedance spectroscopy as a detection technique[27]. We compared the performance of three different graphene platforms, and showed how different numbers of graphene sheets can affect detection (Fig. 5). We found that few-layer graphene provided the best sensitivity and we employed this platform for the detection

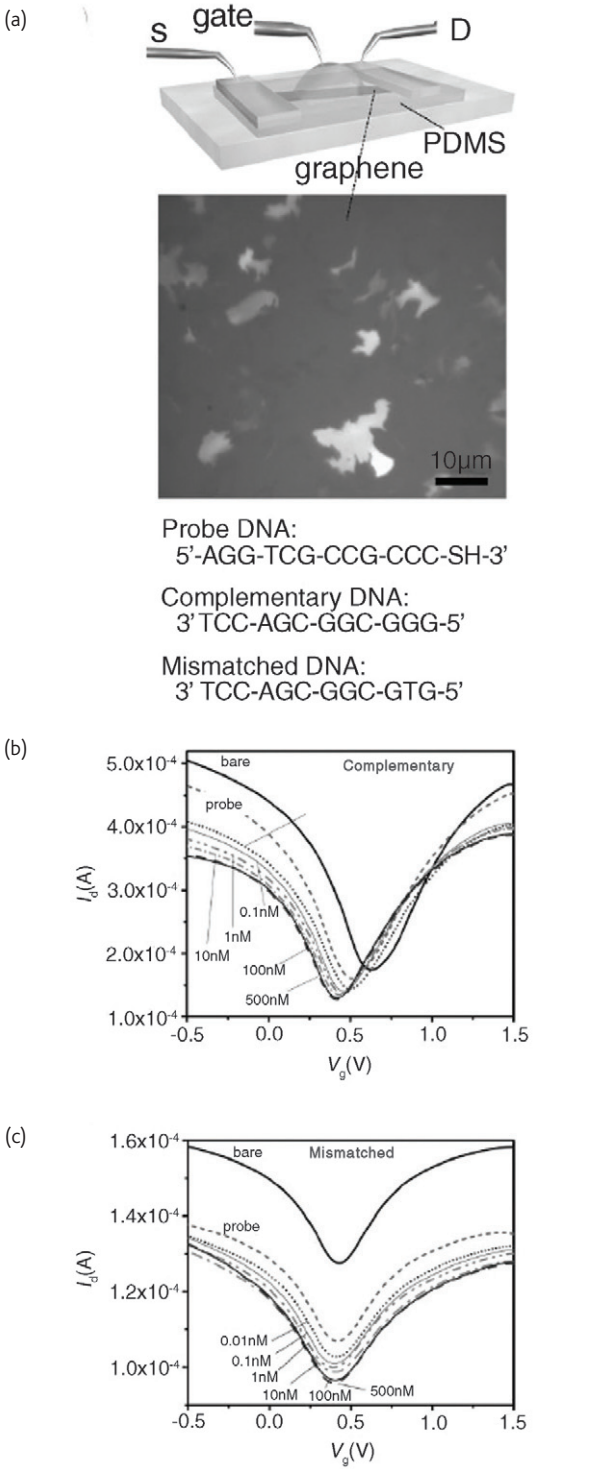

Fig. 3 Bio-field effect transistor for DNA detection. (a) Schematic illustration of the graphene device operated by liquid gating. The middle image is an optical microscopy image of the graphene films. The text below shows the DNA sequences used in the experiments. (b,c) Transfer characteristics for graphene transistors before adding DNA, after immobilization with probe DNA, and after reaction with (b) complementary or (c) one-base mismatched DNA molecules with concentrations ranging from 0.01 to 500 nM. Reprinted from[22]. © Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

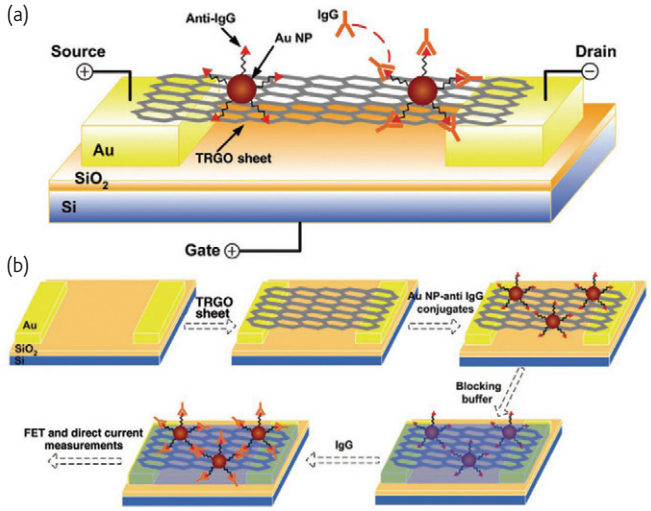

Fig. 4 Bio-field-effect transistor based on graphene decorated with gold nanoparticles for protein detection. (a) Anti-IgG is anchored to the TRGO sheet surface through Au NPs and functions as a specific recognition group for the IgG binding. The electrical detection of protein binding (IgG to anti-IgG) is accomplished by the FET and direct current measurements. (b) Schematic illustration of the TRGO FET biosensor fabrication process. TRGO sheets were first dispersed on the electrodes and then decorated with Au NP-antibody conjugates through non-covalent attachment. Reprinted from[24]. © Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

of a single nucleotide polymorphism. A higher sensitivity was obtained with impedimetric detection compared to that obtained with a similar platform using fluorescence methods. We believe that the graphene-based strategy presented here could be used in the development of an analytical device for point-of-care diagnostic tests and for very sensitive detection of SNPs correlated with various diseases. A number of schemes for the immobilization of single-stranded DNA on graphene surfaces can be used, e.g., a chemical bond between the carboxylic group of graphene sheets and NH_2-modified ssDNA[28]. Upon hybridization with target DNA, the conformation of ssDNA changes from a "lying" structure to a "standing" double helix. This change of DNA conformation and distribution of charges at the surface of the electrode (note that the backbone of DNA is negatively charged due to the presence of phosphate groups) leads to changes in impedance of the electrode surface and to a measurable analytical signal. Hu *et al.*[28] were able to determine low concentrations of the HIV-1 gene.

Graphene in electrochemical biosensors

Electrochemical detection is highly sensitive to electroactive molecules. In addition to sensitivity (which is also a property of electrical detection), it also offers detection selectivity as different molecules can be oxidized/reduced at different potentials. Graphene is an excellent conductor of electrical charge. Heterogeneous electron transfer (the transfer of electrons between graphene and the molecule in the solution necessary for the oxidation/reduction of said molecule) occurs at the edges of the graphene or at defects in the basal plane.

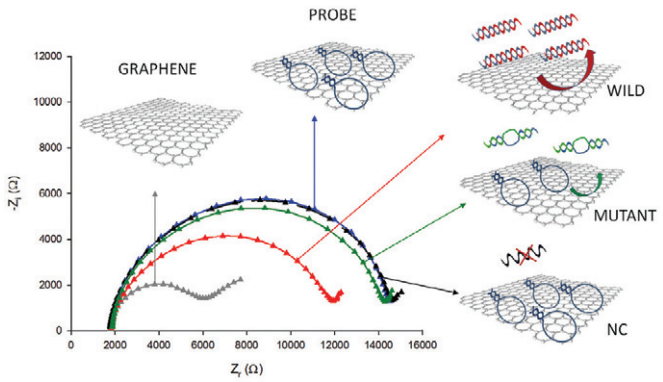

Fig. 5 Impedimetric biosensor based on a graphene platform for Hairpin DNA-based detection. Schematic of the protocol and Nyquist plots, $-Z_i$ vs. Z_r, of the graphene surface (gray), hpDNA (blue), complementary target (red), 1-mismatch target (green), and negative control with a non-complementary sequence (black) (concentration of DNA probes, 1×10^{-5} M; concentration of DNA target, 3×10^{-8} M). All measurements were performed in 0.1 M PBS buffer solution containing 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. Reprinted with permission from[27]. © 2011 American Chemical Society.

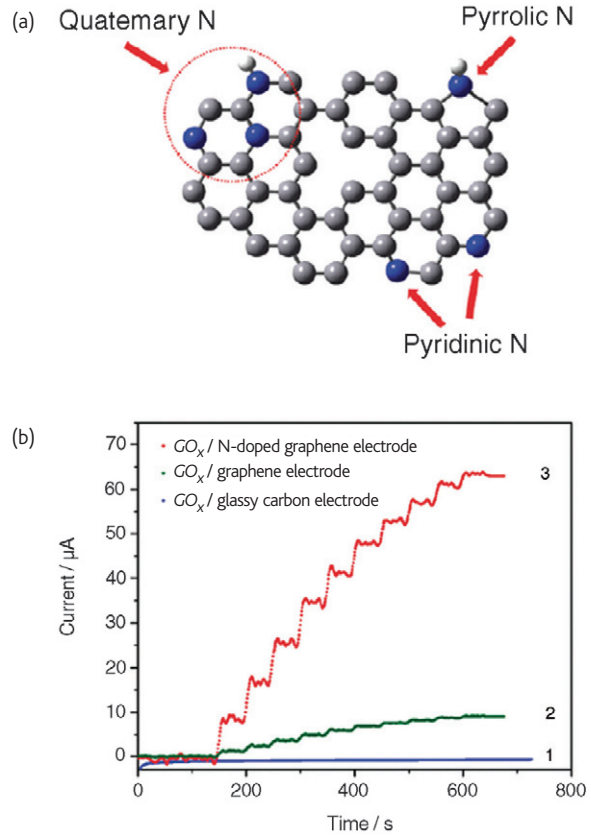

Fig. 6 N-doped graphene for electrochemical biosensing. (a) Schematic representation of N-doped graphene. Gray for the carbon atom, blue for the nitrogen atom, and white for the hydrogen atom. A possible defect structure is shown in the middle of the ball-stick model. (b) Current-time curves for GOx immobilized on (1) a glassy carbon electrode, (2) a graphene electrode, and (3) a N-doped graphene electrode with the successive addition of 0.1 mM glucose. Reprinted with permission from[33]. © 2011 American Chemical Society.

Thus, the high surface area of graphene facilitates large amounts of defects and thus, electroactive sites[29]. Graphene has been employed in many schemes for sensing glucose[30]. This is reflected by the fact that electrochemistry is paramount to sensing glucose for diabetic patients (reflected by the multibillion USD glucosensing market)[31]. The glucose oxidase enzyme is used as a biorecognition element: glucose oxidase oxidizes glucose to gluconic acid and shuffles electrons into the oxygen which is dissolved in the solution, and then reduced to hydrogen peroxide. Hydrogen peroxide is typically detected electrochemically. However, in several examples, direct electron transfer from the enzyme (without the need of O_2 as an electron acceptor) is possible, making this an analytically valuable signal[31]. Ultrathin multilayer graphene platelets (also called graphite nanoplatelets) have been used as a transducing material for the biosensing of glucose[32]. In another example, it was shown that N-doped graphene provides significantly enhanced oxidation currents for the enzymatic detection of glucose, compared to ordinary graphene materials (Fig. 6)[33]. Direct electron transfer from glucose oxidase has been reported by several authors. Glucose oxidase enzyme was immobilized on a Nafion polymer film with graphene nanoplatelets[34]. It was demonstrated that such simple non-covalent bonding enhances the redox current of a ferrocyanide solution and leads to a lowering of the overpotential of hydrogen peroxide[34]. In another example, direct electron transfer was observed in graphene/ionic liquid/glucose oxidase systems[35]. Other schemes for enzymatic detection of glucose have been reported using Au NPs/graphene/chitosan composites[36]. Another biosensor was developed for the detection of pesticides[37] and hydrogen peroxide (using horseradish peroxidase[38] or hemoglobin[39]).

The electrochemical detection of DNA has also attracted a significant amount of attention. It is possible to detect DNA recognition (hybridization) directly using the oxidative signals of DNA bases or by using electroactive labels[40]. Direct detection has an advantage because it is label free, but offers poorer sensitivity than label-based DNA assays. In addition, on traditional carbon materials, such as glassy carbon and graphite, the adenine (A) and guanine (G) bases give analytically useful signals while cytosine (C) and thymine (T) do not provide well-resolved signals. It was shown by Dong *et al.*[41] that chemically reduced graphene oxide provides well-resolved signals of all four A,G,C,T bases (Fig. 7, left panel) with higher sensitivity than with graphite. This is attributed to the high defect density of the CRGO and thus superior electrochemical performance when compared to graphite. This feature was used for the label-free detection of single mutation polymorphism (Fig. 7, right panel). In a similar manner, Loh *et al.*[42] compared the electrochemistry of DNA bases on epitaxially grown graphene (meaning the graphene was grown on a substrate where the basal plane is exposed to the solution; it resembles basal plane pyrolytic graphite with the difference being that the number of graphene layers is smaller). Electrochemical oxidation of pristine epitaxially grown graphene led to the creation of defects on its surface

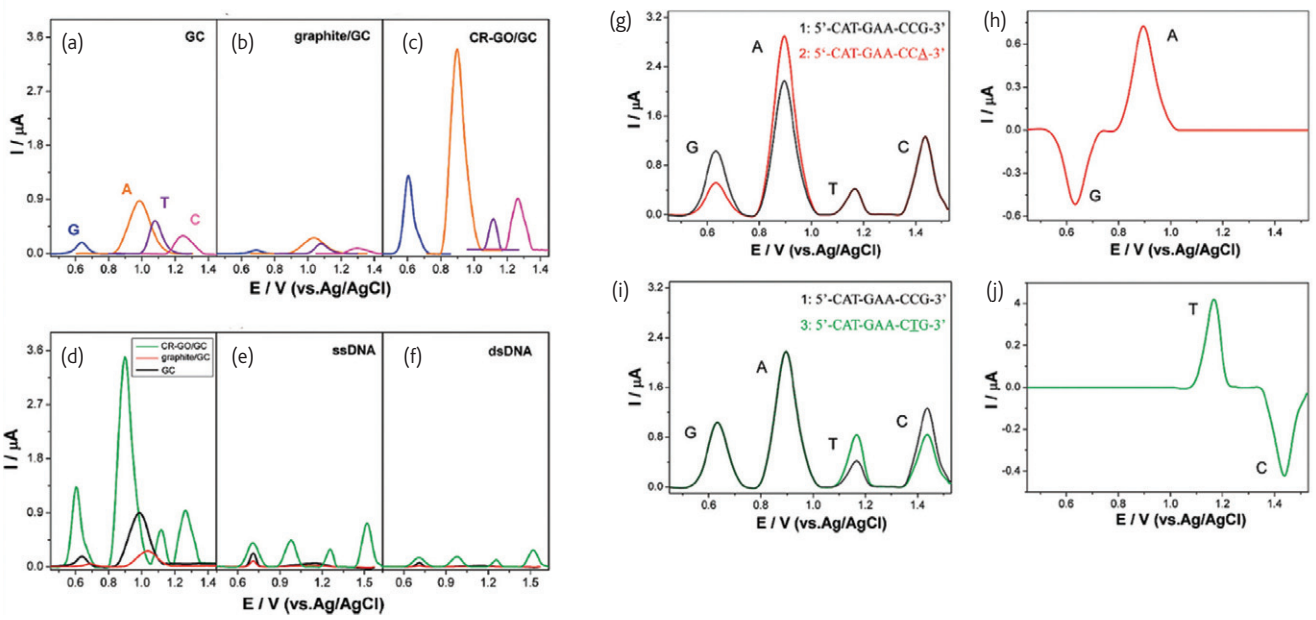

Fig. 7 Electrochemical detection of DNA bases on graphene oxide electrodes. Left panel: (a) differential pulse voltammograms (DPVs) at the glassy carbon (GC) electrode for G (blue), A (orange), T (violet), and C (magenta); (b) DPVs at the graphite/GC electrode for G (blue), A (orange), T (violet), and C (magenta); (c) DPVs at the CR-GO/GC electrode for G (blue), A (orange), T (violet), and C (magenta); (d) DPVs for a mixture of G, A, T, and C at CR-GO/GC (green), graphite/GC (red), and GC electrodes (black); (e) DPVs for ssDNA at CR-GO/GC (green), graphite/GC (red), and GC electrodes (black); (f) DPVs for dsDNA at CR-GO/GC (green), graphite/GC (red), and GC electrodes (black). Concentrations for different species (a-f): G, A, T, C, ssDNA, or dsDNA: 10 μg mL^{-1}. Right panel: Detection of SNPs of oligonucleotides including the sequence from codon 248 of the p53 gene at the CR-GO/GC electrode. (g) DPVs of (1) wild-type oligonucleotide and (2) its single-base mismatch (GfA mutation). (h) Subtraction of the DPVs of (1) and (2). (i) DPVs of (1) wild-type and (3) its single-base mismatch 3 (CfT mutation). (j) Subtraction of the DPVs of (1) and (3). Reprinted with permission from[41]. © 2009 American Chemical Society.

(this has previously been shown with the walls of carbon nanotubes[43]) and leads to a significantly higher response. It was suggested that electrochemically oxidized graphene or graphene with large amounts of defects could be used for highly sensitive electrochemical sensing. This is consistent with a previous publication[41]. Loh *et al.* applied electrochemically oxidized graphene to discriminate between ssDNA and hybridized DNA. We have also shown that large amounts of defects are beneficial for the electrochemical detection of DNA using stacked graphene platelet nanofibers[44]. These nanofibers are the direct opposites of carbon nanotubes because they consist of perpendicularly stacked graphene sheets along the *c*-axis, exhibiting exclusively electrochemically active edges (with the exception of terminal basal planes). Such nanofibers also provide significantly enhanced signals for all four DNA bases when compared to graphite, glassy carbon, or pure carbon nanotubes.

Graphene has also been used for electrochemical immunosensing. In immunosensing, the direct electrochemical detection of antibody-antigen recognition is usually not possible and electrochemically active labels must typically be used. There are two strategies in which graphene can be used. First, graphene can be used as an electrode surface for sensitive detection of a label[45]. This case was employed for the graphene-enhanced detection of α-fetoprotein, which is a cancer biomarker. Graphene sheets were modified with antibodies, then the α-fetoprotein was added and consequently secondary antibodies loaded with microspheres bearing horseradish peroxidase enzyme as a sensitive label (Fig. 8, left panel). The second approach employs graphene as a label-bearing nanocarrier[46]. More specifically, a gold nanoparticle electrode was modified with a probe antibody, to which phosphorylated protein p53 was entrapped. The secondary antibody was conjugated with graphene oxide and horseradish peroxidase to generate large amounts of electroactive molecules and thus a larger signal (Fig. 8, right panel).

Electrochemiluminiscence (ECL) or electrochemically generated luminescence is a type of chemilumininscence where one or more reactants are generated electrochemically. ECL is highly sensitive and is used for the detection of thrombin in the presence of interference on graphene platforms[47].

Fluorescence

Fluorescence is a highly sensitive platform for biomolecular detection. Graphene is applied in various roles as a substrate in fluorescence quenching detection schemes. For example, the quenching principle was used for the aptamer-based detection of thrombin[48]. Aptamer specific to thrombin was labeled with fluorescent dye. Graphene was used as a substrate for the nonspecific adsorption of the fluorescent dye-labeled aptamer for non-covalent assembly. In such a configuration, graphene quenched the fluorescence signal due to a transfer of fluorescence resonance energy from dye to graphene. The addition of thrombin

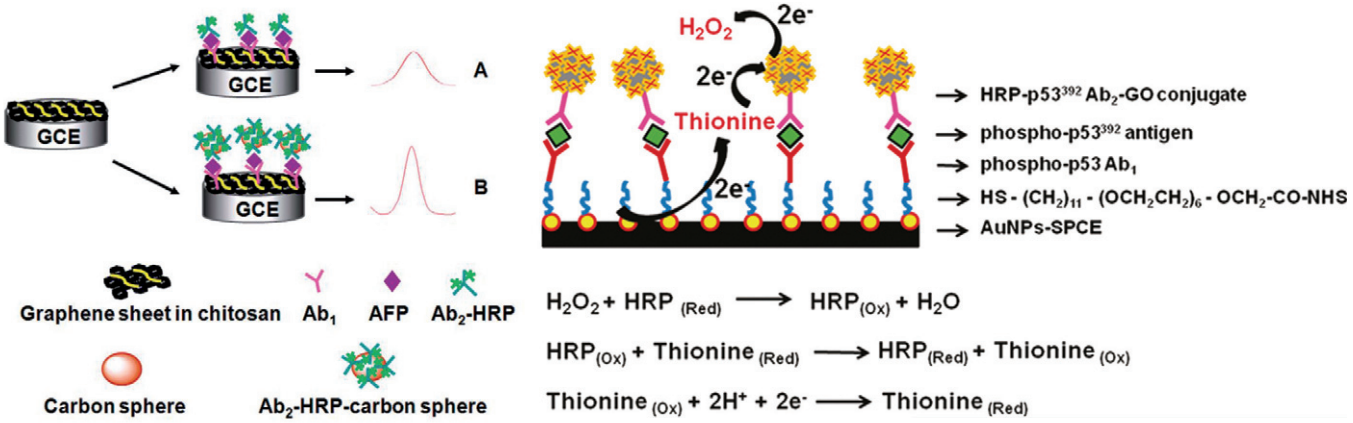

Fig. 8 Schematic illustration of an application of graphene for electrochemical immunosensing. Graphene in the role of sensitive transducer (left panel) and in the role of label carrier (right panel). Reprinted with permission from[45,46] respectively. © 2010 and 2011 American Chemical Society.

results in the formation of quadruplex-thrombin complexes, which have a weak affinity to graphene. The change in conformation leads to a configuration where the dye is no longer in contact with the graphene sheet and thus the fluorescence is no longer quenched (Fig. 9a). This scheme is very sensitive, with detection limits as low as 31 pM. The very high surface area of graphene oxide also enables the application of a similar fluorescence quenching scheme, this time for DNA detection based on the hybridization of the complementary DNA strands, allowing for multiple detection of three different strands[49]. This leads to a multicolor sensor for detecting multiple DNA targets in a single solution (Fig. 9b). Fluorescence detection can be used for virus detection in the format of a graphene microarray[50]. A rotavirus-specific antibody was chemically linked via $-NH_2$ groups to –COOH groups present on graphene oxide. After binding of the rotavirus to a chemically attached

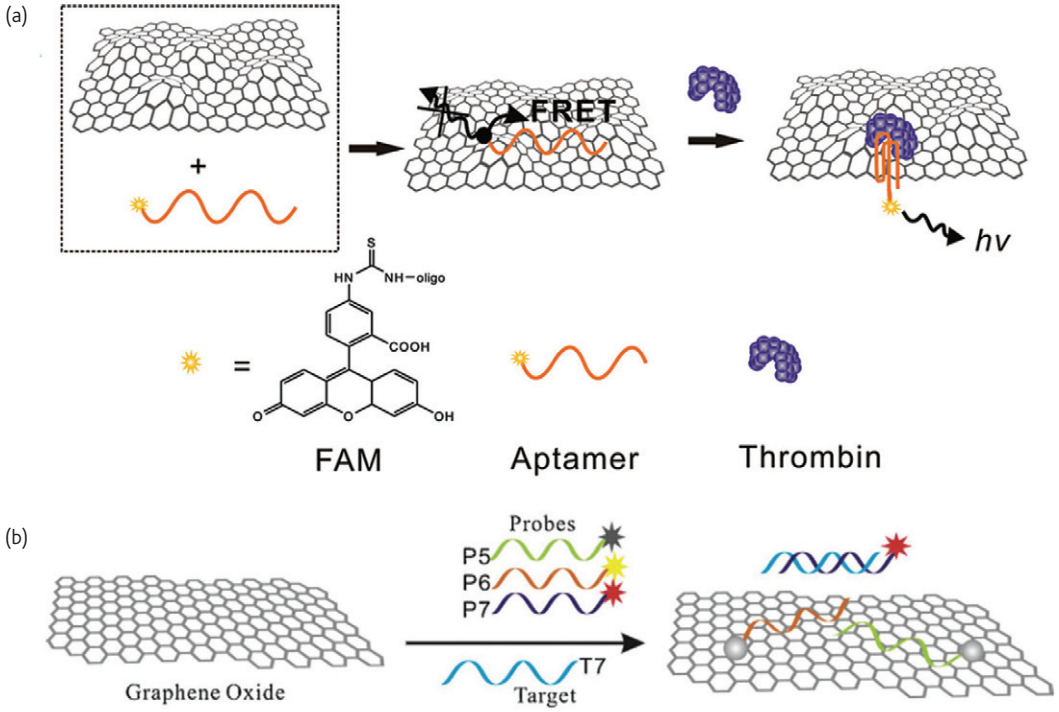

Fig. 9 Fluorescence detection of DNA using a graphene substrate. (a) The fluorescence of dye-labeled aptamer is quenched when aptamer binds to graphene due to fluorescence resonant energy transfer between dyes and graphene. The fluorescence recovers while the thrombin combines with aptamers to form quadruplex-thrombin complexes, which have much less affinity to graphene, causing a fluorescent function-associated molecule to be far away from the graphene surface. Reprinted with permission from[48]. © 2010 American Chemical Society. (b) Scheme for graphene oxide-based multicolor DNA analysis. Fluorescence spectra of mixture probes (P5, P6, P7) in the presence of different targets T5 (blue), T6 (red), and T7 (orange) with excitation wavelengths of 494, 643, and 587 nm, respectively . Reprinted from[49]. © Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

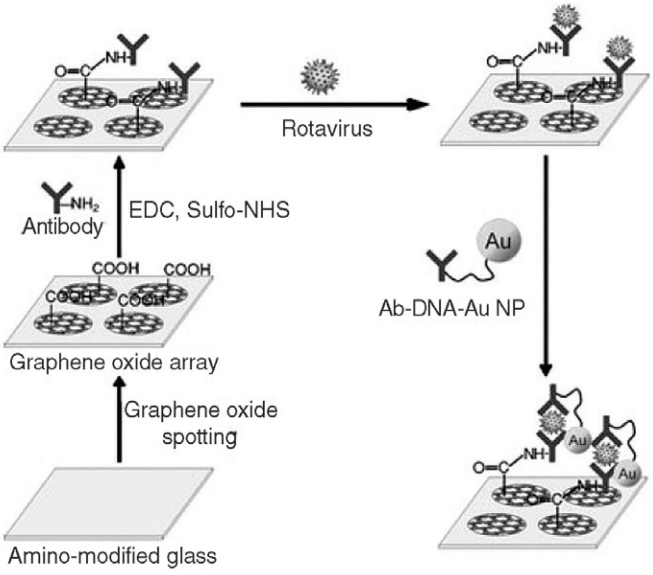

Fig. 10 Fluorescence immunosensing detection of a rotavirus, based on graphene oxide microarrays. Reprinted from[50]. © Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

antibody, a secondary antibody linked to gold nanoparticles was added as a label. The gold nanoparticles act to quench the fluorescence of the graphene oxide (Fig. 10).

Conclusion and outlook

To summarize, we have described how biosensors can benefit from graphene as a transducing material. We have discussed the fundamental differences of the different types of graphene and their influence on applications in biosensing. Because graphene is a zero-gap semiconductor and an electroactive and transparent material, there are many possibilities for its application as a transducer or label in biosensing schemes. We have discussed enzymatic biosensors, genosensors, and immunosensors based on different transduction approaches, such as field-effect transistors, electrochemistry and electrochemiluminiscence, impedance, and fluorescence measurements. Although it would be beneficial to have side-by-side comparison of CNTs and graphene-based biosensors, such reports are very scarce. It is known that graphene nanoribbons, which have the dimensional restriction of a graphene sheet of tens of nanometers, exhibit significantly different electrical properties from large-sheet graphene. This is yet to be integrated in bio-FET design and promises even higher sensitivity and selectivity for FET devices. It is also expected that *zigzag* and *armchair* graphene edges will exhibit different electrochemical properties and, although fabrication of purely *zigzag* or *armchair* edges is difficult, a breakthrough in this area is expected. Because the field is still young, it is expected to branch out into many applications to meet the needs of society in the areas of safety, enhanced health care, and a clean environment. mt

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