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Graphene as a signal amplifier for preparation of ultrasensitive electrochemical biosensors

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Abstract

Early diagnostics of diseases performed with minimal money and time consumption has become achievable due to recent advances in development of biosensors. These devices use biorecognition elements for selective interaction with an analyte and signal readout is obtained via different types of transducers. Operational characteristics of biosensors have been reported to improve substantially, when a diverse range of nanomaterials was employed. This review presents construction of electrochemical biosensors based on graphene, atomically thin 2D carbon crystals, which is currently intensively studied nanomaterial. The most attractive directions of graphene applications in biosensor preparation are discussed here including novel detection and amplification schemes exploiting graphene's unique electrochemical, physical and chemical properties. The future of graphene-based biosensors is most likely bright, but there is still a lot of work to do to fulfill high expectations.

Keywords

graphene; biosensors; nanomaterials; electrochemistry; affinity; immunoassays

Introduction

Since the first study describing the glucose oxidase biosensor more than 50 years ago (Clark & Lyons, 1962), the original idea of exploiting biorecognition elements integrated within an electrochemical transducer has greatly evolved. The growing interest in electrochemical biosensors was driven by their perspective applications in medicine, biotechnology and environmental sciences, with a need to analyze quite complex samples with high accuracy. Typical biorecognition elements, i.e. antigens/antibodies, enzymes, lectins/glycans, DNA or aptamers are highly specific what allows high selectivity of assays (Bertok *et al.*, 2013; Bučko *et al.*, 2012; Hushegyi & Tkac, 2014; Klukova *et al.*, 2014; Luo & Davis, 2013; Paleček & Bartošík, 2012; Šefčovičová & Tkac, 2014). Thus, a complicated sample pretreatment is not necessary and the whole analysis can be quick and cost-effective. In general, biosensors can rely on electrocatalytic activity of enzymes or on a specific affinity

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of nucleic acid, aptamers, lectins and antigens/antibodies towards relevant analyte. Since affinity-based biorecognition molecules generally do not contain directly detectable redox centers, the readout signal is usually obtained using an additional electrochemical probes/labels introduced into an assay protocol.

In 2004, the first paper describing graphene was published by Geim´s lab (Novoselov *et al.*, 2004). Authors observed that one atom thick, planar carbon crystals prepared by a physical exfoliation of graphite are very stable under normal conditions. Besides, they observed an extremely fast in-plane electron transfer through graphene´s highly ordered system of conjugated π-π bonds. Later on, it was proved that unusual electronic, optical and chemical properties of these two dimensional nanocrystals can be easily tuned by adjustment of parameters of a graphene fabrication method (Ambrosi *et al.*, 2014; Chen *et al.*, 2010). High purity and extremely conductive graphene sheets are typically prepared by a physical exfoliation from graphite or by chemical deposition techniques. Nevertheless, it is much cheaper to prepare “graphite oxide” by oxidation of graphite, which can be also obtained in inexpensive and sustainable ways (Akhavan *et al.*, 2014). Direct exfoliation of graphite oxide results in isolated “graphene oxide” (GO) flakes (Fig. 1) in which the conductive system of conjugated π-π bonds is disrupted by the presence of surface oxygen groups. To restore conductivity, GO sheets must be reduced, either chemically, thermally, or electrochemically (Fig. 1). The obtained nanomaterial is usually labeled “reduced graphene oxide” (rGO) since it exhibits rather different properties compared to pure graphene (Pumera, 2013; Wang *et al.*, 2011b). In fact, the substantial difference between graphene, GO and rGO is in the amount of oxygen-containing functional groups within this nanomaterial. While graphene sheets, by definition (Fitzer *et al.*, 1995), should not contain any oxygen, its total amount can reach up to 30% in GO. By reduction, oxygen amount is decreased approximately to 5 – 10% in rGO. Presence of oxygen-rich moieties does not only have impact on graphene´s conductivity, but it is also responsible for substantial differences in hydrophobicity and in interfacial charge of different graphene-based materials (Ambrosi *et al.*, 2014).

Such features of graphene attracted scientific attention for development of various bio/electrochemical devices, including biosensors, and this effort has been summarized in several excellent reviews (Filip & Tkac, 2014; Kochmann *et al.*, 2012; Liu *et al.*, 2012; Pumera, 2011; Wang *et al.*, 2013d). Furthermore, different aspects of employment of these nanomaterials in biosensor field were reviewed with a focus on comparison with carbon nanotubes (Yang *et al.*, 2010), on introduction of various detection methods (Bonanni *et al.*, 2012c) or to list potential analytes (Hernandez & Ozalp, 2012; Zhu *et al.*, 2012). All authors agreed that an inexpensive and easy-to-prepare rGO possesses high conductivity, favorable interfacial electrocatalytic properties and high active surface area, similarly to carbon nanotubes. This set of features allowed development of electrode interfaces capable of hosting high amount of biorecognition units what enhances sensitivity of the biosensor devices. Lower conductivity of GO compared to graphene can be applied in devices based on impedimetric or field-effect sensing transducing schemes. Carboxyl and other oxygen-contaning moieties of GO or rGO can be also used for covalent attachment of biorecognition molecules, when biosensor surface is covered by GO or rGO nanoparticles. This feature

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makes GO nanoparticles almost ideal for fabrication of various electrochemical labels (sandwich assays).

What is crucial for fabrication of biosensors is a fact that either oxygen-containing moieties or the edge sites within GO or rGO flakes can increase heterogeneous electron transfer rate during redox transformation of a broad range of biologically relevant molecules (Pumera *et al.*, 2010; Wu *et al.*, 2014a) and enzymatic cofactors. This has been employed for development of robust amperometric sensors with high sensitivity of detection and for development of enzymatic biosensors with enhanced performance of analysis, as well.

The aim of this review is to introduce and summarize detection and amplification principles of amperometric biosensors employing a diverse range of graphene-based nanomaterials. Since there is a vast amount of papers describing applications of graphene in amperometric biosensors, here we would like to provide only a brief introduction to graphene´s potential to amplify electrochemical response rather than to provide a detailed list of all devices fabricated.

Biosensors based on interfacial graphene electrochemistry

Enzymatic biosensors

Excellent features of graphene-based electrode interfaces were most often tested by immobilization of glucose oxidase (GOx), a “model” oxidoreductase containing flavin adenine dinucleotide (FAD) cofactor. Under normal conditions, FAD is reduced by glucose and reoxidized by oxygen which is, in turn, reduced to hydrogen peroxide (Zhu *et al.*, 2012). Concentration of both O_2 and H_2O_2 in a thin layer on the electrode surface is thus related to GOx activity and an amperometric detection of either of these two compounds provides information about glucose concentration (Fig. 2). Due to the enhancement of electron transfer rate, both analytes are detected with higher sensitivity when graphene-based nanoparticles are present on the biosensor interface compared to an unmodified one. The same is valid also for electrode-assisted regeneration of electron mediators, electrochemically active compounds that “shuttle” electrons between an electrode and enzymes instead of oxygen.

It was revealed that GOx physisorbed on rGO with low level of oxygen groups exhibits direct electron transfer (DET) between the active site and the electrode (Zhang *et al.*, 2014) what allowed direct amperometric detection of glucose in mediatorless arrangement. Contrary, when GOx was immobilized on the surface with high amount of oxygen functionalities (i.e. GO), the amperometric response of the bioelectrode in presence of glucose was generated by reduction of H_2O_2 (Zhang *et al.*, 2014). An efficient DET between GOx and chemically rGO loaded with gold nanoparticles (AuNPs) and polyaniline (PANI) nanocomposite allowed analysis of glucose in 15 μl of blood covering clinically relevant concentration (Kong *et al.*, 2014). Besides GOx, other oxidases were used in construction of biosensors with integrated graphene-based nanoparticles including cholesterol oxidase (Parlak *et al.*, 2013), oxalate oxidase (Devi *et al.*, 2013) and xanthine oxidase (Zhang *et al.*, 2010).

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It is very difficult to establish DET between GOx and an electrode, but there are other enzymes with a structure favoring this mode of operation (Filip & Tkac, 2014; Shleev *et al.*, 2005; Tkac *et al.*, 2009), which were also integrated with graphene-based electrodes. For example “multicopper oxidases” were easily conjugated with electrochemically reduced GO (ErGO) to fundamentally study their properties (Filip *et al.*, 2013; Świetlikowska *et al.*, 2013) and also for biosensing purposes (Wu *et al.*, 2010). Other enzymes i.e. alcohol dehydrogenase (Guo *et al.*, 2011) can exhibit DET mode of operation when immobilized on the surface patterned by a chemically reduced GO (CrGO), as well.

“Signal-on” affinity biosensing

In affinity amperometric biosensors, an amperometric response is generated by a redox transformation of either an analyte or an electrochemical probe. “Signal-on” assay describes an arrangement when obtained signal increases with increased analyte concentration (Fig. 3).

Direct electrochemical DNA detection—In 2005, Wu et al. reported an amperometric detection of DNA strands on a graphite oxide/PANI (PANI=polyaniline) nanocomposite electrode (Wu *et al.*, 2005) and later Zhou et al. revealed individual voltammetric peaks assigned to a direct oxidation of each of four bases in DNA on CrGO-modified electrode (Zhou *et al.*, 2009). These four separate peaks were not observed when graphite or unmodified glassy carbon electrodes were used under the same experimental conditions. According to the authors, it was presence of numerous edges and edge-like defects together with high intrinsic conductivity of CrGO that allowed detection of all four DNA bases (Zhou *et al.*, 2009). The importance of rGO edges in detecting DNA bases was further studied and confirmed by Loh´s group (Lim *et al.*, 2010), but an ultimate exploitation of this kind of assay was reported by Akhavan (Akhavan *et al.*, 2012). Authors succeeded in increasing the edge-sites density on an electrode surface using deposition of “rGO nanowalls”. Such vertical arrangement of CrGO sheets instead of conventional horizontal configuration of sheets allowed detection of DNA down to unprecedented zM level (Akhavan *et al.*, 2012). Interestingly, authors compared these results also with the ones obtained using non-reduced “GO nanowalls” and even though all four DNA bases were detected, the sensitivity of detection was lower. This comparison supports importance of edge-like sites for oxidation of DNA bases while the effect of oxygen moieties on DNA bases oxidation was not that important. Other studies reported lower overpotential for guanine oxidation on CrGO-modified electrode compared to non-modified glassy carbon electrode (Wang *et al.*, 2011a).

DNA detection via electrochemical probes—An alternative to direct electrochemical detection of DNA is an employment of electrochemical probes. Such probes are typically intercalated into double stranded DNA (dsDNA) chains that are formed on the electrode surface upon hybridization of target single-stranded DNA (ssDNA) with an immobilized capture probe (Fig. 3). Choice of a proper intercalator is a key element to avoid possible interferences while achieving high sensitivity and thus a low limit of detection (LOD). Exceptionally low LOD of 0.4 fM was obtained when a captured ssDNA was covalently attached to a pyrenebutyric acid-modified CrGO and methylene blue was used as the intercalator (Zhang *et al.*, 2013). Authors ascribe excellent LOD to high conductivity of

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CrGO and high density of capture probe attached to the surface. The later feature was achieved by modification of rGO with pyrenebutyric acid which allowed covalent immobilization of ssDNA without affecting conductivity of rGO. This way of stable, but non-covalent attachment of capture probe can be seen as another way how to improve performance of graphene-based biosensors.

Another “signal on” detection of DNA is possible by application of a soluble electrochemical probe. Redox transformation of such electrochemical probe on an electrode surface can be effectively blocked by a capture ssDNA chains adsorbed on the surface resulting in a small electrochemical signal observed. Upon hybridization of a capture probe with target DNA, the formed dsDNA is partially desorbed from the surface leaving it more accessible for the electrochemical probe. Thus an increase in electrochemical signal was observed (Yang *et al.*, 2012) and rGO deposited on the biosensor surface was in this case used to enhance redox signal of the electrochemical probe.

Typically, “signal-on” genosensors could detect DNA down to sub-pM level (Tab. 1) and are able to distinguish between fully complementary ssDNA chains and single-mismatched oligonucleotides (Sun *et al.*, 2011b; Zhang & Jiang, 2012). Such performance makes rGO very promising nanomaterial for further development of amperometric devices with possible future applications in routine DNA analyses.

Protein detection via DNA aptamers—“Signal-on” detection has been applied also for detection of proteins like thrombin and lysozyme using DNA aptamers, i.e. protein-binding DNA oligonucleotides (Guo *et al.*, 2013).The detection is based on electrode interface modified with CrGO sheets conjugated with the redox probe via hydrophobic interactions, since this modification allowed retention of good conductivity of nanomaterial modified biosensor and favorable electrochemistry of a redox probe. Such interface, further modified by selective aptamer, responded very sensitively to the captured analyte protein; the whole DNA-protein complex was desorbed from the electrode surface after biorecognition resulting in increased current generated by the adsorbed electrochemical probe (Guo *et al.*, 2013).

An interesting “signal-on” aptasensor was reported, based on low conductivity of GO sheets (Fig. 4) (Yuan *et al.*, 2012). These nanoparticles anchored to the electrode surface via ssDNA conjugated with DNA aptamer, were “shielding” electrochemistry of GOx-hemin-mediator redox system present on the electrode interface. Upon incubation of such interface with analyte (thrombin), the ssDNA-GO nanoconjugate was displaced from the surface. As a result, an increase of the amperometric signal with increased analyte concentration was observed with LOD of the aptasensor down to sub-pM level (Yuan *et al.*, 2012). There are only few examples of “signal-on” label-free immunosensors (see Tab. 1), since this detection system is more convenient for DNA biosensors.

“Signal-off” affinity biosensing

Graphene-based biosensors were also constructed in a “signal-off” arrangement where a decrease of an amperometric signal with increased concentration of an analyte was observed. Contrary to “signal-on” devices, this technique is more convenient for detection of

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proteins, because of their insulating properties. Examples of typical “signal-off” label-free affinity biosensors using all three kinds of biorecognition molecules are given in Tab. 2.

DNA detection—Pumera and coworkers constructed DNA biosensor with GO employed as an redox probe i.e. reduction of oxygen functional groups of GO was applied for electrochemical signal generation (Bonanni *et al.*, 2012b). In their device, biosensing was based on different affinity of GO to fully hybridized dsDNA as compared to partially hybridized dsDNA (i.e. hybridization of single nucleotide mismatched ssDNA with capture probe DNA) and ssDNA (non-hybridized capture DNA probe). Thus, the highest redox signal was observed after incubation of GO with the biosensor exposed to non-complementary ssDNA followed by incubation of GO with the biosensor exposed to single nucleotide mismatched ssDNA and the lowest signal was observed after incubation of GO with fully complementary ssDNA forming hybridized dsDNA, which could be detected down to pM level (Bonanni *et al.*, 2012b). Nevertheless, this interesting idea suffers from the fact that reduction of GO´s oxygen groups usually occur at unfavorably negative potential.

Protein detection via DNA aptamers—An interesting aptasensor was constructed based on aptamer immobilized on CrGO/AuNPs-modified surface (Deng *et al.*, 2013). On such interface, GOx was deposited functioning as a redox probe and a blocking agent decreasing nonspecific interactions at the same time. DET between the GOx and the modified electrode was applied for signal generation and current obtained decreased with increased concentration of protein platelet-derived growth factor which could be detected with LOD of 1.7 pM (Deng *et al.*, 2013).

GO/methylene blue nanoparticle-based labels were also employed in aptameric “signal off” biosensor. Chen et al. conjugated these labels with capture probes and the labels were decoupled from capture probes once the analyte (thrombin or ATP) was incubated with the biosensor (Fig. 5) (Chen *et al.*, 2013a). In such arrangement, LODs of 110 and 15 pg ml^{-1} were achieved for thrombin and ATP, respectively.

An approach based on one electrochemical probe and an aptamer with two binding sites was introduced recently (Du *et al.*, 2012). Aptamer-modified surface was firstly incubated with a redox probe by electrostatic interactions and when an analyte was bound to the aptamer, redox probe was released from the surface, resulting in a signal decrease (Fig. 6). When both analytes were bound to aptamer, a whole complex together with aptamer was released from the surface with lower signal obtained compared to situation, when only one analyte was bound (Du *et al.*, 2012). The whole detection system was immobilized on CrGO-modified electrode which improved dramatically amperometric response of the redox probe.

Hérnandez and coworkers used electromotive force, a kind of a potentiometric assay, for non-labeled determination of *Staphylococcus aureus* cells on a surface modified by GO or rGO (Hernández *et al.*, 2014) with immobilized aptamers responsible for selective capture of the cells. Analysis was more reliable on rGO with LOD down to a single CFU ml^{-1} (CFU-colony forming unit) with response obtained within 1 min compared to GO-based biosensor. Such a simple device with an outstanding performance is an excellent example how flexible and feasible can be graphene applied for biosensing purposes (Hernández *et al.*, 2014).

Immunosensors—Typical "signal-off" protein detection employs antibodies immobilized on an electrode surface modified with graphene-based nanoparticles. After incubation of the biosensor with analyte, the current response generated by an electrochemical probe is decreased due to "shielding" of the surface towards redox process by presence of additional layer of proteins (analyte) on the electrode surface (Wei *et al.*, 2010). The same detection principle was successfully applied also in aptasensors (Sun *et al.*, 2011a; Yuan *et al.*, 2011).

Electrochemical probes immobilized on the electrode surface (Wei *et al.*, 2010), supplied into an electrolyte (Huang *et al.*, 2011) or generated by a co-immobilized enzyme (Chen *et al.*, 2011a) in "signal-off" immunoassay platforms of detection were successfully used for determination of proteins (Eissa *et al.*, 2012; Huang *et al.*, 2011; Wei *et al.*, 2010), cancer biomarkers (Kong *et al.*, 2011; Li *et al.*, 2013; Mao *et al.*, 2012), toxins (Srivastava *et al.*, 2013), hormones (Li *et al.*, 2011) or viral surface antigens (Huang *et al.*, 2012b) with LOD being between 3 and 170 pg ml^{-1}. All reported biosensor devices have in common their composition; typically the antibodies are deposited on an electrode modified by rGO sheets which secure much more effective redox transformation of the applied redox probe compared to unmodified substrate electrode. Concentration-related decrease of the redox probe-generated current response is observed after the analyte molecules were recognized with surface-attached antibodies. Additional modification of rGO sheets by AuNPs (Huang *et al.*, 2012a; Huang *et al.*, 2011) or an employment of N-doped rGO (Li *et al.*, 2013) was also reported in such immunosensors.

Amazing LOD of 50 ag ml^{-1} for carcinoembryonic antigen (a cancer biomarker) was achieved, when GO, thionine and AuNPs were employed for modification of electrode surface instead of above discussed rGOs (Han *et al.*, 2013). It seems that GO is a suitable electrode modifier for enhanced loading of positively charged redox labels – thionine molecules, most likely via **π- π** stacking and electrostatic interactions and that initial low conductivity of GO was not a crucial parameter. Thus, enhanced loading of redox labels present on the electrode surface is behind high sensitivity of protein detection by this immunosensor.

Affinity biosensors using electrochemical probes in combination with rGO or GO-based electrodes can be arranged in a way to simultaneously detect more than one analyte. Typically, two different antibodies, each one conjugated with distinct redox probe, were immobilized on the electrode interface (Jia *et al.*, 2014; Kong *et al.*, 2013). Each antibody and redox probe could detect its analyte and since redox probes have different redox potentials,both analytes could be detected simultaneously in one measurement at different potentials (Jia *et al.*, 2014; Kong *et al.*, 2013).

Sandwich-based biosensors

Very powerful tool to improve operational characteristics of biosensors is an employment of electrochemical labels in a sandwich arrangement (Pei *et al.*, 2013). As is illustrated in Fig. 7, the sandwich arrangement relies on a selective attachment of tracer probes on the electrode surface. This binding is selective and is performed via affinity of a secondary biorecognition molecule conjugated with the tracer probe to already bound analyte to the

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biosensor surface. Thus each single captured molecule of the analyte cause attachment of one tracer probe particle, which contains copious number of molecules of either an electrochemical probe or a catalyst, most often enzyme, generating electrochemical signal.

There are two ways how to employ graphene-based nanomaterials in sandwich affinity biosensors: 1) (r)GO is deposited on a surface of the electrode (Fig. 7A) to improve electrochemistry of the tracer probe involved in the detection system and 2) graphene-based nanoparticles are integrated with redox probes/catalysts and are employed as the tracer probes (Fig. 7B). In the second approach conductivity of tracer probe is not required and GO can be very effectively used for this purpose since it contains functional groups for efficient immobilization of secondary biorecognition elements and redox probes. Both ways of applications of graphene-based nanomaterials in sandwich biosensors are discussed in the following sections.

Sandwich-based biosensors based on interfacial graphene electrochemistry

Sandwich-based biosensing can be performed in two distinct ways using either redox probes (quantum dots, ferrocene derivatives etc.) or using enzymes, which upon enzymatic action produce redox active probes. Examples of geno-, apta- and immunosensors employing this amplification technique are given in Tab. 3.

Biosensors based on redox probes—All sandwich-type biosensors can be constructed with tracer probes bearing only a limited amount of redox probes such as quantum dots (Wu *et al.*, 2013a), ferrocene (Wang *et al.*, 2013a) or methylene blue (Wang *et al.*, 2014a). The performance of these devices relied on rGO-modified electrode surfaces where the amperometric signal was amplified due to favorable interfacial redox properties of rGO. Sensitive sandwich analysis of DNA was performed using capture ssDNA probe immobilized on rGO and AuNPs modified electrode (Wang *et al.*, 2014a). After analyte DNA was bound to the biosensor, a tracer probe containing methylene blue and signal ssDNA hybridizing to analyte DNA was injected to complete a sandwich configuration. This biosensing approach allowed detection of target DNA with LOD of 0.35 fM (Wang *et al.*, 2014a).

Intact cells can be very effectively integrated into a sandwich assay protocol since after cell binding to the immobilized primary biorecognition element numerous ligands are present on the surface of the cell, which can be detected by a secondary biorecognition element (Bertók *et al.*, 2013; Klukova *et al.*, 2014; Paleček *et al.*, 2014). An interesting approach how to sensitively detect SKOV-3 human ovarian cancer cells by employment of DNA molecules was introduced recently (Xia *et al.*, 2012). Cells were bound to a primary antibody immobilized on GO-modified electrode. In the next step tumor marker HER2 on the cell surface was recognized by a secondary antibody conjugated to ssDNA. After formation of a duplex by introduction of complementary ssDNA, redox probe daunomycin was introduced into the system to intercalate into dsDNA. The immunosensor detected cancer cells with LOD as low as 5 cells ml^{-1} (Xia *et al.*, 2012). It is interesting to note that GO was not reduced prior further modification by a biorecognition element.

Biosensors based on enzyme labels—On the other side, very efficient systems based on enzymatic turnover reactions with horseradish peroxidase (HRP)-catalyzed transformation of H_2O_2 were developed. These HRP-based tracer probes were employed in genosensors (Liu *et al.*, 2013), aptasensors (Peng *et al.*, 2012) and immunosensors (Cai *et al.*, 2011), providing LOD of 3.4 fM, 650 aM and 4.9 pg ml^{-1}, respectively. Besides HRP, alkaline phosphatase catalyzing ascorbate generation from ascorbic acid-phosphate was applied to amplify electrochemical signal with LOD of 2.7 fM for thrombin (Wang *et al.*, 2012a). Very low LOD of 60 fM for microRNA was achieved using a "biobarcode" strategy relying on a tracer probe based on AuNPs containing two types of DNA molecules, one type for binding to the analyte and the second for signal amplification via HRP (Fig. 8) (Zhou *et al.*, 2012).

Sandwich-based biosensors with tracer probes containing GO or rGO

In Tab. 4 there are listed operational features and composition of typical sandwich-based biosensors in which nanoparticles of graphenic materials were used for fabrication of tracer probes. In further sections application of GO *vs.* rGO is discussed in more details providing basic insight into amplification mechanisms developed with these nanomaterials.

GO can be applied for preparation of tracer probes, when for this application a coupling capability of nanoparticles is appreciated more than conductivity. GO was employed for effective anchoring of redox probes (Jiang *et al.*, 2013; Shiddiky *et al.*, 2012) or biocatalysts (Liu *et al.*, 2011; Wang *et al.*, 2012a) together with secondary biorecognition elements. To achieve this, a simple coupling via activated carboxyl moieties of GO and amine groups of the anchored molecules was employed (Qu *et al.*, 2011; Shiddiky *et al.*, 2012). Alternatively, electrostatic interactions could be used for preparation of tracer probes by coating of GO sheets with ionic polymer (Jiang *et al.*, 2013; Liu *et al.*, 2011).

Biosensors based on small catalytic molecules immobilized on GO—GO was also conjugated with hemin (molecule with a peroxidase-like activity) without any GO treatment (Zhou *et al.*, 2014). The microRNA biosensor employed hairpin DNA molecular beacons as capture probes and DNA "biobarcodes" for signal amplification provided LOD of 0.17 pM for its analyte (Fig. 9) (Zhou *et al.*, 2014). Ferric porphyrine, another molecular complex with peroxidase-like activity, was also reported to conjugate with GO sheets by simple adsorption (Wang *et al.*, 2013c). Such conjugated nanoparticles were used as tracer probes for analysis of DNA down to aM concentration range (Wang *et al.*, 2013c). Combination of GO and small catalytic molecules is efficient because 1) catalytic units after deposition on GO surface are active and strongly attached and 2) because these molecules are smaller than HRP and thus higher amount of them can be loaded on a particular GO sheet. This fact is illustrated by 3.5-fold larger amperometric response obtained with tracer probes bearing porphyrine compared to the same device employing HRP (Wang *et al.*, 2013c).

Biosensor based on intrinsic catalytic properties of GO—An interesting "signal-on" sandwich-based immunosensor was fabricated with GO-based tracer probe (Qu *et al.*, 2011). A primary antibody was immobilized on a gold electrode coated with an insulating

SAM layer. After the analyte, a platelet-derived growth factor BB, was bound to a primary antibody, it was coupled with a tracer probe containing a secondary antibody-GO conjugate. GO was applied for in-situ formation of Ag nanoparticles (AgNPs) and such structure restored conductivity of the surface and thus a soluble redox probe could be detected (Qu *et al.*, 2011). The biosensor offered LOD of 5 pg mL^{-1} for its analyte. It is important to note that the electroactive AgNPs were not generated without GO.

Biosensors based on metal nanoparticles deposited on rGO—Quite favorable is modification of rGO with various nanoparticles that allow strong and facile physisorption of secondary recognition molecules (antibodies, DNA aptamers) and signal amplification probes such as Ag nanowires with HRP (Tang *et al.*, 2011), AuNP with thionine and DNA "biobarcodes" (Bai *et al.*, 2014), catalytically active hollow Pt-Co nanoparticles synthesized on rGO sheets (Wang *et al.*, 2011c), CdS quantum dots on rGO sheets (Yang *et al.*, 2011) or AuNPs with immobilized enzyme and two enzyme-like catalysts (Yi *et al.*, 2014). Application of nanoparticles in combination with rGO as a tracer probe allowed detecting cancer biomarkers by the immunosensor with LOD of 5 pg mL^{-1} for a carcinoembryonic antigen (Tang *et al.*, 2011) or with LOD of 3 pg mL^{-1} for a prostate specific antigen (Yang *et al.*, 2011). Moreover, thrombin by the aptasensors could be detected with LOD of 0.34 pM (Wang *et al.*, 2011c) or 0.3 pM (Yi *et al.*, 2014).

A quite complex signal amplification strategy is behind ultrasensitive detection of lipopolysaccharide endotoxin based on aptamer biorecognition with LOD of 8.7 fg ml^{-1} (Fig.10) (Bai *et al.*, 2014). Sheets of rGO coated with thionine, AuNPs and DNA aptamer form a tracer probe and low LOD of the biosensing device is a result of a recycling amplification process (Bai *et al.*, 2014). Moreover, rGO/thionine complex might be behind low LOD for endotoxin as suggested from a previous study (Wei *et al.*, 2010).

Sandwich-based biosensors with rGO-containing tracer probes—In a recent study it was concluded that employment of rGO sheets provided higher current response of the immunosensor compared to control device based on a bare unmodified electrode (Yang *et al.*, 2011). Moreover, the study also showed that the biosensor device based on rGO sheets applied for electrode modification and for construction of a tracer probe to complete a sandwich configuration was 50-fold more sensitive compared to the biosensor constructed from GO. Control experiments with 1) the biosensor constructed with rGO as an electrode modifier with GO applied for preparation of a tracer probe or with 2) the biosensor constructed with GO as an electrode modifier with rGO applied for preparation of a tracer probe have not been performed (Yang *et al.*, 2011). Thus, it is sure if rGO improved performance of the biosensor when applied as 1) an electrode modifier; 2) a part of a tracer probe or 3) an electrode modifier and as a part of a tracer probe. Moreover, sheets of rGO were also employed for fabrication of tracer probes applied in immunosensors (Tang *et al.*, 2011; Yang *et al.*, 2011) and aptasensors (Bai *et al.*, 2012; Wang *et al.*, 2011c).

It seems that the size and/or hydrophobicity/hydrophilicity of graphene-based nanomaterial for preparation of tracer probes really matters. When for example small graphene flakes with the size of few tens of nanometers were conjugated with nanoparticles the final tracer probe consisted of aggregated nanocomposite (Fig. 11) (Zhong *et al.*, 2010). When such tracer

probe was applied for biosensing, a cancer biomarker could be detected with LOD of 0.01 ng/mL (Zhong *et al.*, 2010). Larger GO flakes with the size exceeding few micrometers were applied for preparation of a tracer probe, utilized in analysis of another cancer biomarker (Shiddiky *et al.*, 2012). Large GO flakes conjugated with nanoparticles formed non-aggregated flakes (Fig. 12). When the tracer probe based on large GO sheets was applied in biosensing, the analyte could be detected down to concentration of 0.1 pg/mL (Shiddiky *et al.*, 2012), what is 2 orders of magnitude lower LOD compared to the device constructed with tracer probe based on small graphene flakes (Zhong *et al.*, 2010). It is important to prove if hydrophilicity/hydrophobicity or the size of graphene-based materials is crucial to achieve high sensitivity of biosensing.

Simultaneous analysis of several analytes by sandwich-based biosensors

Sandwich-type biosensor configuration allowed simultaneous detection of several analytes. An electrode surface could be labeled with two (Chen *et al.*, 2013b; Wang *et al.*, 2014a; Wang *et al.*, 2014b) or even three (Zhu *et al.*, 2013) types of biorecognition molecules and tracer probes containing redox labels, which can be detected at different potentials. Alternatively, two analytes could be detected with the same redox label in case the electrode surface is divided into more parts with each one being modified by different primary antibody (Wang *et al.*, 2013b). Simultaneous detection of two different analytes/antigens directly on the surface of low-abundance tumor cells is possible (Wu *et al.*, 2013b). In this case, cells were attached to antibody immobilized on rGO-modified electrode surface and after the target cells were attached, two different tracer probes (differing in secondary antibody and a redox probe) were introduced into the system. Since two different redox probes could be determined at different potential, both antigens on the cell surface could be detected simultaneously (Wu *et al.*, 2013b).

Intriguing immunosensor for a simultaneous detection of four cancer markers was developed, as well (Wu *et al.*, 2014b). An array of screen printed rGO-coated electrodes was developed, each one decorated with a relevant capture antibody. After the analyte was bound, secondary antibody bearing a reactive group formed a sandwich configuration. In the next step, these reactive moieties were applied for growth of a 3D nanostructure containing epoxy groups for effective immobilization of HRP. The enzyme generated electrochemical signal and all four cancer biomarkers could be detected down to sub-pM level (Wu *et al.*, 2014b).

It should be noted that multiple-assay systems described in the previous paragraphs do not illustrate fundamentally new principles of signal amplification performed by graphene-based materials and this is why further details of their construction are no discussed. Such devices have potential to be applied for point-of-care routine analysis of disease biomarkers or clinically relevant analytes with some degree of multiplexing.

Impedimetric biosensors

Another label-free detection method relaying on an electrochemical transformation of probes on an electrode surface is electrochemical impedance spectroscopy (EIS). In EIS measurements, electrodes are biased with a potential oscillating around the redox potential

of the applied probe (most often a mixture of $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$) and measured current response is used for calculation of an impedance of the system. The whole scale of frequencies is applied and a correlation between the frequency and the calculated impedance is used for determination of a charge transfer resistance (R_{CT}) value. This value is related to a rate of heterogeneous surface electron exchange what makes it strongly dependent on surface properties. Thus a detection of changes in R_{CT} is possible once the analyte is conjugated with the capture probe on the surface what is schematically illustrated in Fig. 13.

There are two main issues to be considered when constructing graphene-based impedimetric biosensors. The first is a composition of graphenic material affecting its electrochemical properties. It was revealed that density of oxygen-containing functional groups strongly affects impedimetric assays, since typical redox probe is negatively charged and higher negative charge of the graphenic surface means higher initial R_{CT} (Ambrosi *et al.*, 2011). Pumera´s group investigated the effect of number of rGO (oxidation level not specified) layers on a DNA biosensor performance. They found that interface with 3-4 rGO layers provided more robust platform for physisorption of hairpin DNA capture probe and the impedimetric determination of complementary ssDNA compared to monolayer and multiple rGO layers (Bonanni & Pumera, 2011). In another study sensitivity of DNA biosensor prepared from anodized epitaxial growth graphene was compared to the device constructed from highly oriented pyrolytic graphite (Dubuisson *et al.*, 2011). The former device completely outperformed graphite-based biosensor by exhibiting 3 orders of magnitude lower LOD due to lower capacitance noise. Furthermore, it was found that R_{CT} dramatically dropped after electrochemical introduction of numerous edge-like defects into epitaxially grown graphene electrode (Dubuisson *et al.*, 2011). Such studies indicate that various properties of graphenic nanoparticles can dramatically influence performance of impedimetric biosensors and that the effect of composition of graphenic nanoparticles has to be optimized in order to achieve robust biosensing.

The second important aspect to be optimized is the effect of composition of graphene-related nanomaterials on immobilization of biorecognition elements. Stable hydrophobic-based interaction between six-member carbon rings of graphene and ssDNA backbone has been reported (Chen *et al.*, 2011b; Wang *et al.*, 2011d). This interaction is disrupted upon hybridization with target DNA resulting in easier access of the redox probe to the surface with measurable drop of R_{CT} (Yang *et al.*, 2013b). Contrary, Wang et al. reported that dsDNA remained on ErGO-modified electrode after hybridization as can be judged from increased R_{CT}, suggesting that composition of graphene-based nanoparticles play some role in affinity towards dsDNA (Wang *et al.*, 2011d). To improve performance of such kind of impedimetric genosensors, Chen et al. employed enzyme-induced “digestion” of target DNA i.e. once the target molecule hybridized with a capture probe (hairpin DNA), it was released by a specific endonuclease allowing it to hybridize with another hairpin DNA molecule (see Fig. 14). Thus one analyte molecule could “open” and “digest” significantly more than just one hairpin DNA which led to a substantial drop of R_{CT}. LOD of such amplified biodetection system is 20–500-fold lower compared to other EIS-based detection strategies” (Chen *et al.*, 2011b).

Another way is to attach the probes covalently o the interface, preferably using carboxyl groups of graphenic surface. Interestingly, in Pumera´s study (Bonanni *et al.*, 2012a) it was revealed that ErGO sheets provided the highest sensitivity and reproducibility of simple impedimetric DNA detection as compared to devices employing GO and CrGO. It was suggested that the electrochemical reduction did not remove surface carboxyl groups, only the net electrochemical properties are improved as compared to other GO derivatives. Chemical modification of rGO (Hu *et al.*, 2012) or GO sheets can be alternatively employed in order to introduce moieties for chemical coupling of DNA probes. It is also important to note that unlike in the case of physically attached capture probes, covalently bound strains remain on surface even after the hybridization with target and thus an increase of R_{CT} is typically detected with increased concentration of the analyte (Hu *et al.*, 2012; Yang *et al.*, 2013c). These two immobilization methods i.e. covalent grafting vs. physisorption were compared by Dubuisson et al. with results suggesting that the first method allows more molecules to be immobilized which led to the biosensor with better performances compared to the biosensor employing physisorbed capture probes (Dubuisson *et al.*, 2011).

To provide basic information about efficiency of impedimetric DNA sensing, there are listed selected impedimetric genosensors in Tab. 5. Besides these, also aptasensors and immunosensors are introduced in Tab. 5.

Protein detection via DNA aptamers—The methods of conjugation of capture probes with graphene derivatives and other features of these materials were applied also in development of aptasensors (Feng *et al.*, 2011) and immunosensors. In this field, interesting approach was employed by Erdem et al. who used GO/chitosan composite for modification of pencil graphite electrode. Thus numerous functionalities for covalent immobilization of capture probe (aptamer) were introduced and at the same time improved electrode redox properties were achieved. This allowed fabrication of very cheap and effective single-use impedimetric bioelectrode for detection of lysozyme with satisfying LOD of 380 ng ml^{-1}.

In another studiy, *Salmonella* species could be detected with LOD of 3 CFU ml^{-1} using simple GO-modified electrode decorated with AuNPs serving for chemical attachment of thiol-terminated aptamer strand (Ma *et al.*, 2014). Even though authors did not investigate the role of GO, it can be assumed that in this case it helped to increase the electrode surface area. Furthermore, GO has be previously proved to be appropriate platform for in-situ generation of metallic nanoparticles (Qu *et al.*, 2011). Worthy to mention is also a study of Loo et al. (Loo *et al.*, 2012b) who tested several kinds of graphenic materials i.e. graphite oxide, GO, TrGO (thermally reduced GO) and ErGO for fabrication of impedimetric aptasensor with GO turning out to be the most promising candidate for immobilization of thrombin-specific aptamer (Loo *et al.*, 2012b).

Finally, Feng et al., 2011, have described rGO-based impedimetric aptasensor for detection of cancer cells (Feng *et al.*, 2011). These cells could be easily released from the surface by hybridization of DNA complementary to the aptamer to complete regeneration of the biosensor surface (Feng *et al.*, 2011). Since reusability is a desirable feature for every biosensor, this study provides a simple way for biosensor regeneration applicable for other detections systems.

Immunosensors—Interestingly, when antigens were immobilized on different graphene derivatives in order to fabricate impedimetric IgG biosensor, TrGO-modified electrode outperformed graphite oxide-, GO- and ErGO-modified electrodes in terms of response sensitivity achieved (Loo *et al.*, 2012a). This is most probably the result of different binding between protein antigen and IgG compared to binding of protein to DNA aptamer. Also a spatial "bulkiness" of the as-formed antigen/antibody conjugate may require lower density of surface capture probes compared to the biosensor with immobilized aptamers for protein detection.

Impedimetric biosensors could also take an advantage of sandwich-based amplification. Hou et al. (Hou *et al.*, 2013) reported an CEA sandwich-based immunosensor with a tracer probe consisting of GO sheets covalently grafted with secondary antibody and HRP. The enzyme was responsible for precipitation of 4-chloro-1-naphthol which deposited on the electrode surface and consequently was detected as the increase of R_{CT}. This arrangement took advantage of the already discussed high biocompatibility of GO sheets allowing usage of high amount of HRP molecules. While in quite many studies graphene-modified electrodes were used, Wang et al., 2013 prepared an impedimetric immunosensors using a free-standing CrGO paper electrode modified with in-situ synthesized AuNPs and physically adsorbed streptavidin/biotinylated antigen conjugate (Wang *et al.*, 2013e). The device offered LOD of 1,500 CFU ml^{-1} for *E. coli* O157:H7 and these results are quite promising in the terms of a reproducible fabrication of cheap bioanalytical devices.

Conclusions

Going through recent literature, one should be really impressed with enormous interest in application of graphene-based materials in electrochemical biosensors. Such interest is fully justified when considering advantageous properties of graphene and its derivatives for construction of highly sensitive biosensor devices. The most obvious advantage is very low price of starting material - graphene oxide (GO), which can be at least one order of magnitude lower compared to the price of graphene´s "rival" carbon nanotubes (Xie *et al.*, 2012). Additional advantage of GO compared to carbon nanotubes is high solubility of GO, what simplifies its processing/handling, resulting in high reproducibility of surface patterning. Moreover, GO does not contain typical impurities of carbon nanotubes such various metal-based catalysts significantly affecting redox behavior of carbon nanotubes (CNTs). Graphene is more compatible with microfabrication techniques than CNTs, a feature essential for construction of various devices including biosensors (Yang *et al.*, 2010). Moreover, graphene-based biosensors exhibit lower noise compared to CNTs-based ones, what should result in sensitive assays by graphene-based biosensors (Yang *et al.*, 2010).

An obvious disadvantage of GO such as low conductivity can be solved by diverse range of reduction protocols available. Thus, final properties of rGO could be effectively tuned by the reduction process i.e. overall hydrophobicity/hydrophilicity, nature of oxygen groups remaining, the size of graphene sheets and density of edge-like defects. While on one side this flexibility is really welcome for construction of biosensors on the other size this flexibility in graphene preparation might be a nightmare to understand what a particular feature of the rGO is behind high performance of a biosensing device prepared or to

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construct the same biosensor device in other laboratories. Furthermore, it is important to keep in mind that even though GO is a starting material for modification of an electrode surface, short exposure to electric field at a particular working potential can significantly change density of oxygen functional groups present in GO producing partly rGO with different degree of C/O ratio.

Despite all these facts, we can make some conclusions about application of various graphene-based nanomaterials for construction of amperometric biosensors. Primarily, edge plane defects in graphene sheets are catalytically active providing high heterogeneous electron transfer rate for various electrochemical probes, redox enzymes and DNA. Thus, highly conductive graphene (rGO) nanomaterial with edge defects should be preferentially applied for modification of electrode surfaces for fabrication of affinity biosensors such as aptasensors, immunosensors and genosensors. Secondarily, large surface area of individual GO sheets with high density of oxygen-containing reactive functional group is an ideal support for hosting high density of redox probes and for covalent immobilization of large amount of secondary biorecognition molecules to prepare tracer probes for highly robust biosensing in a sandwich configuration.

Even though really impressive numbers of studies describing construction of graphene-based biosensors have been published within a decade since graphene discovery, it is anticipated that only an ongoing fundamental material research will lead to more efficient exploitation of graphenes´ properties in these devices. Moreover, in future it is of high importance to provide additional data with graphene characteristics along protocols describing biosensor construction to design devices with really robust performance.

To conclude, graphene-based materials were successfully used for fabrication of nanostructured interfaces as key elements of highly sensitive catalytic and affinity–based electrochemical biosensors for analysis of a wide range of analytes. Moreover, in some papers multiplexed format of analysis was addressed, but still substantial effort has to be put to challenge graphene-based biosensors with real samples of environmental, forensic and biomedical origin, what is a prerequisite for commercialization of these kinds of biosensors. Even though graphene-based biosensors have been applied in the fields of genomics and proteomics, utilization of graphene-based biosensors in glycomics is still awaiting.

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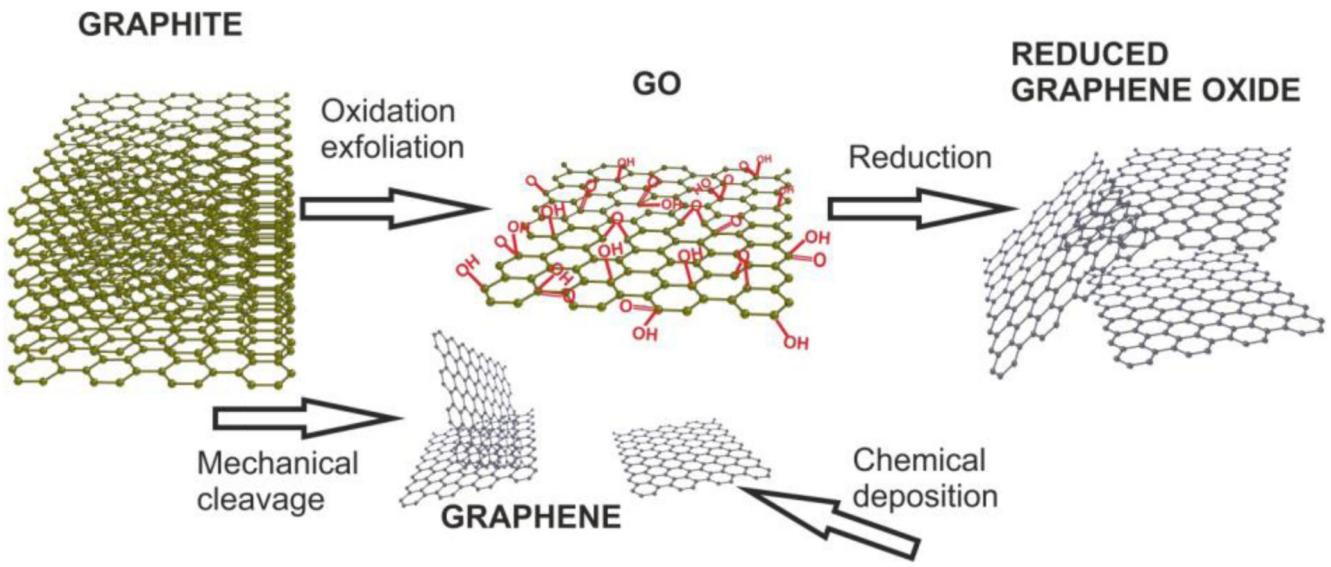

Fig. 1.
Scheme of the preparation of different graphenic nanomaterials.

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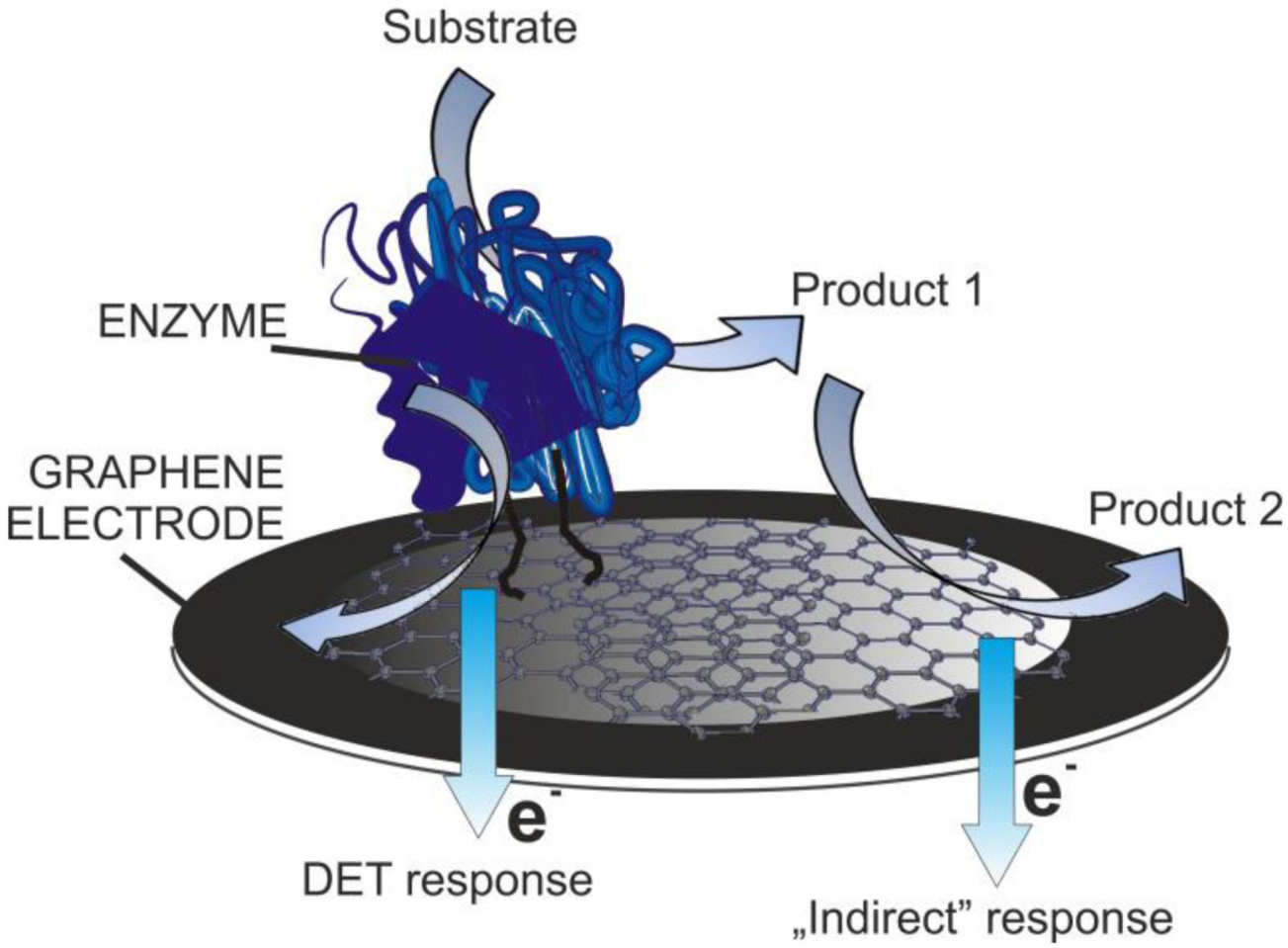

Fig. 2.

Schematic illustration of graphene-based enzyme biosensors operating either in a direct electron transfer (DET) mode or in "indirect" mode, when product of the enzyme i.e. H_2O_2 is detected.

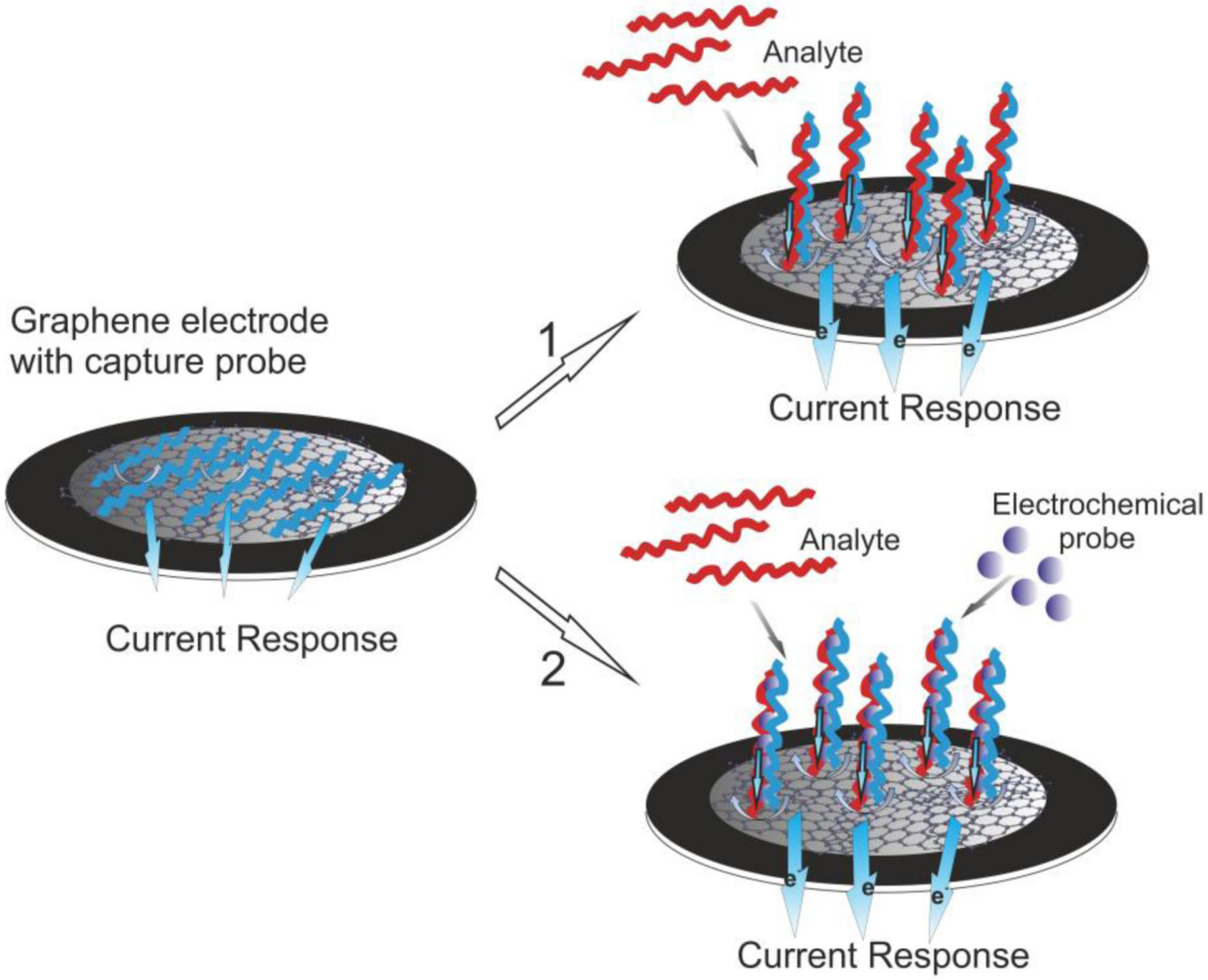

Fig. 3.

Schematic illustration of DNA “signal on” detection with 1) direct electrochemical DNA detection via oxidation of DNA bases or 2) using electrochemical probe intercalated into double-stranded DNA (dsDNA).

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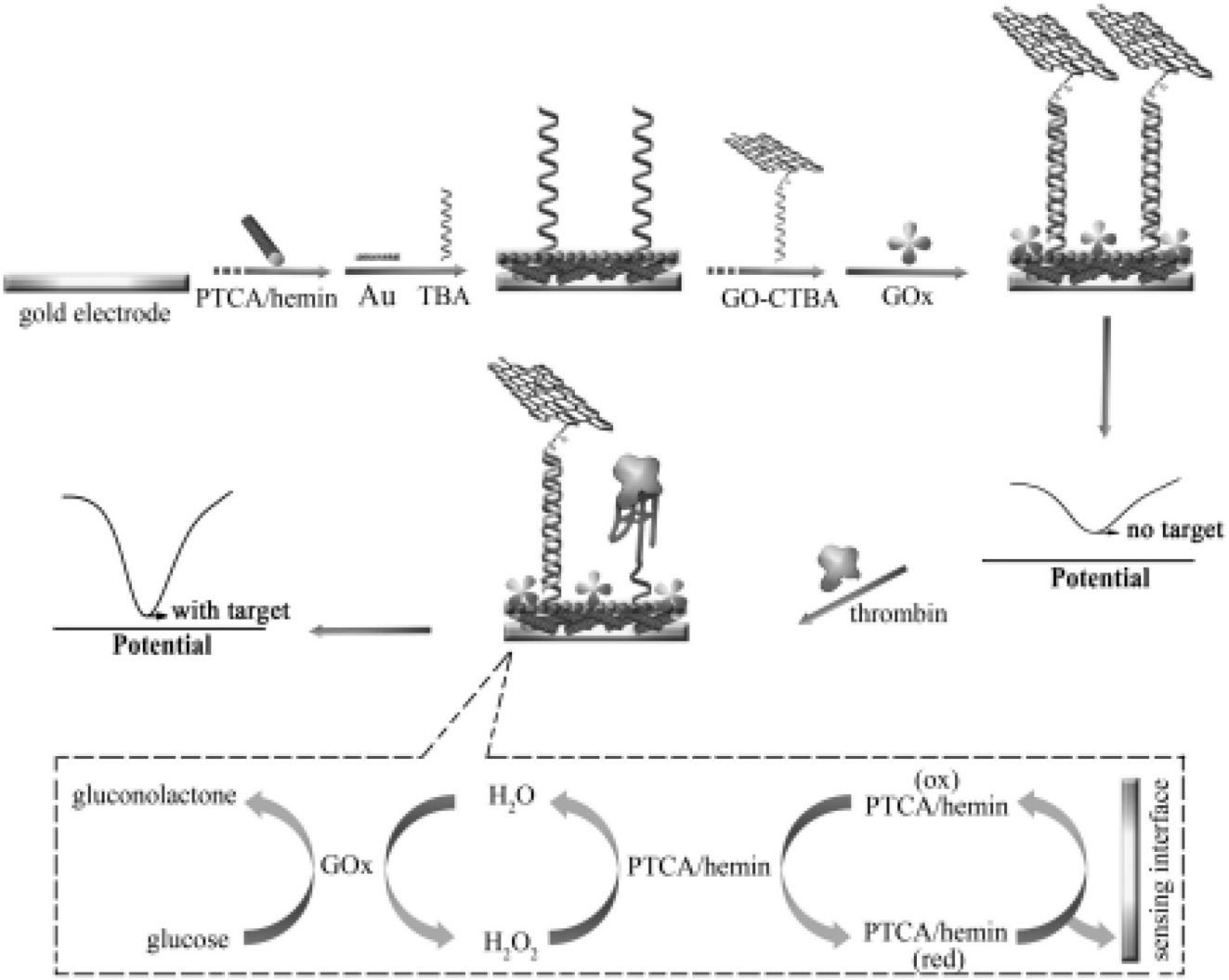

Fig. 4.

Scheme of “signal-on” detection by aptasensor, when GO sheets are released from the electrode upon affinity interaction between aptamer and thrombin, increasing glucose diffusion to immobilized GOx biocatalysts, generating redox species. Adapted from (Yuan *et al.*, 2012).

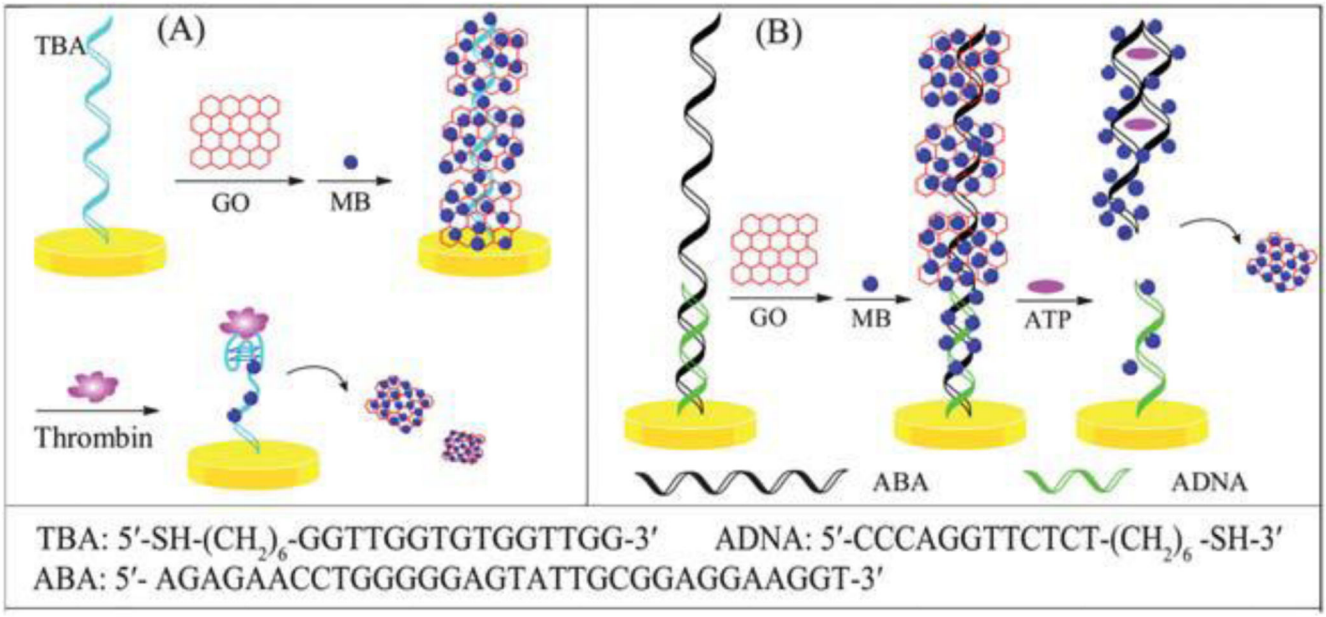

Fig. 5.

"Signal-off " detection of thrombin (A) and ATP (B) by aptasensors using GO-methylene blue (MB) conjugates for a current response generation. Adapted from (Chen *et al.*, 2013a).

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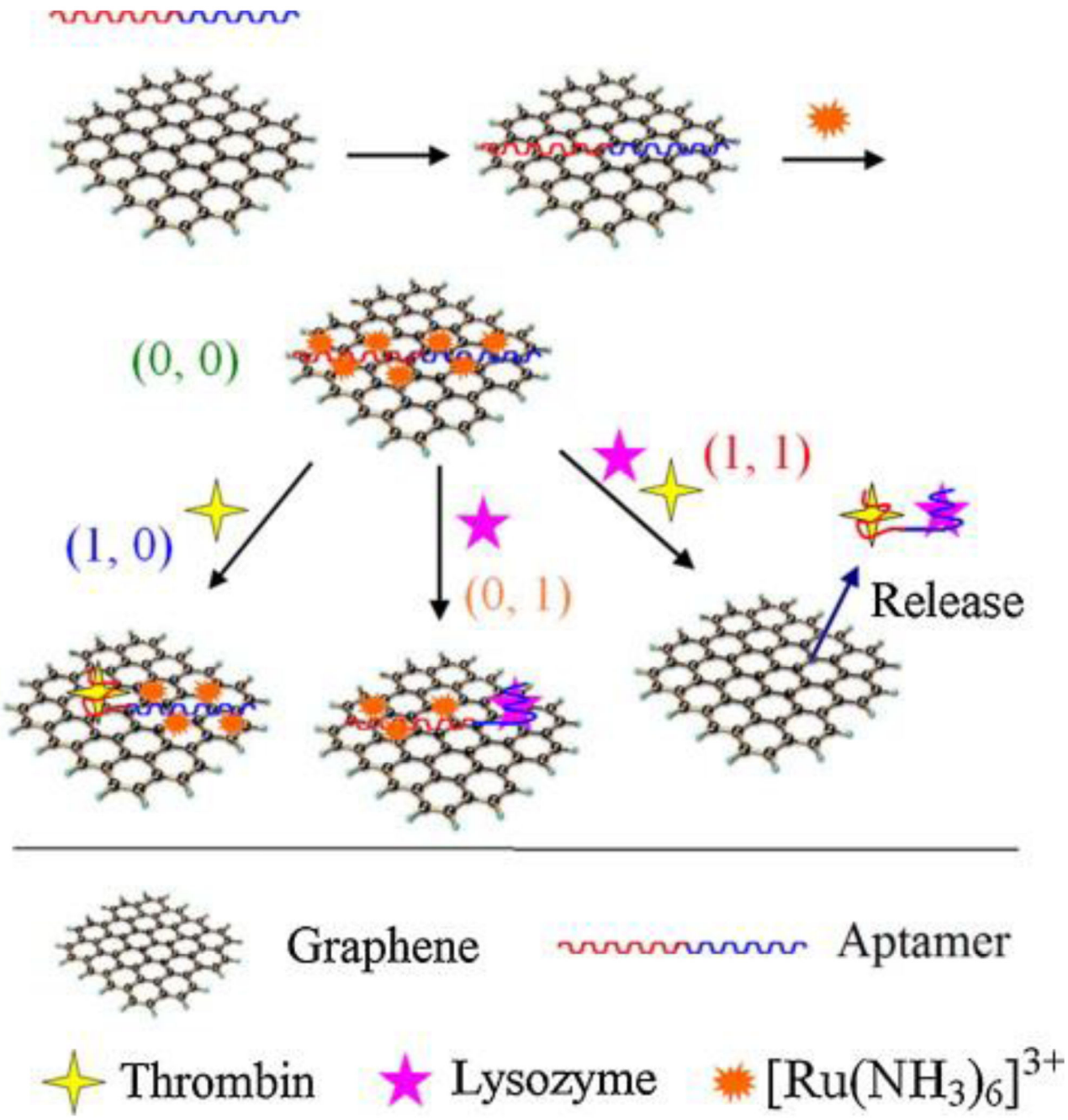

Fig. 6.

Scheme of a “signal-off” logic aptasensor for detection of thrombin and lysozyme based on an aptamer with two binding sites. Adapted from (Du *et al.*, 2012).

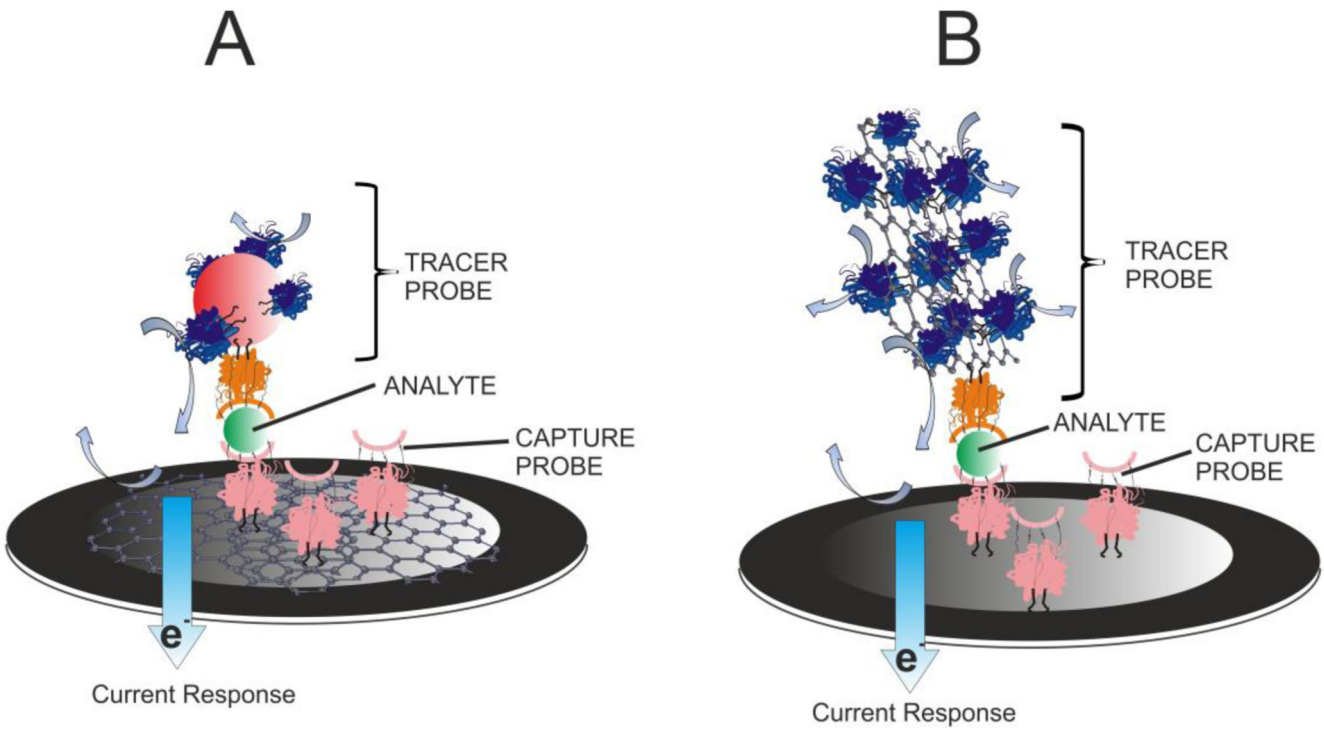

Fig. 7.

Schematic illustration of sandwich-type affinity biosensors with graphene-based electrode (A) and graphene-based tracer probe (B) conjugated with enzymes. Alternatively tracer probe can be loaded with redox probes, not only with enzymes.

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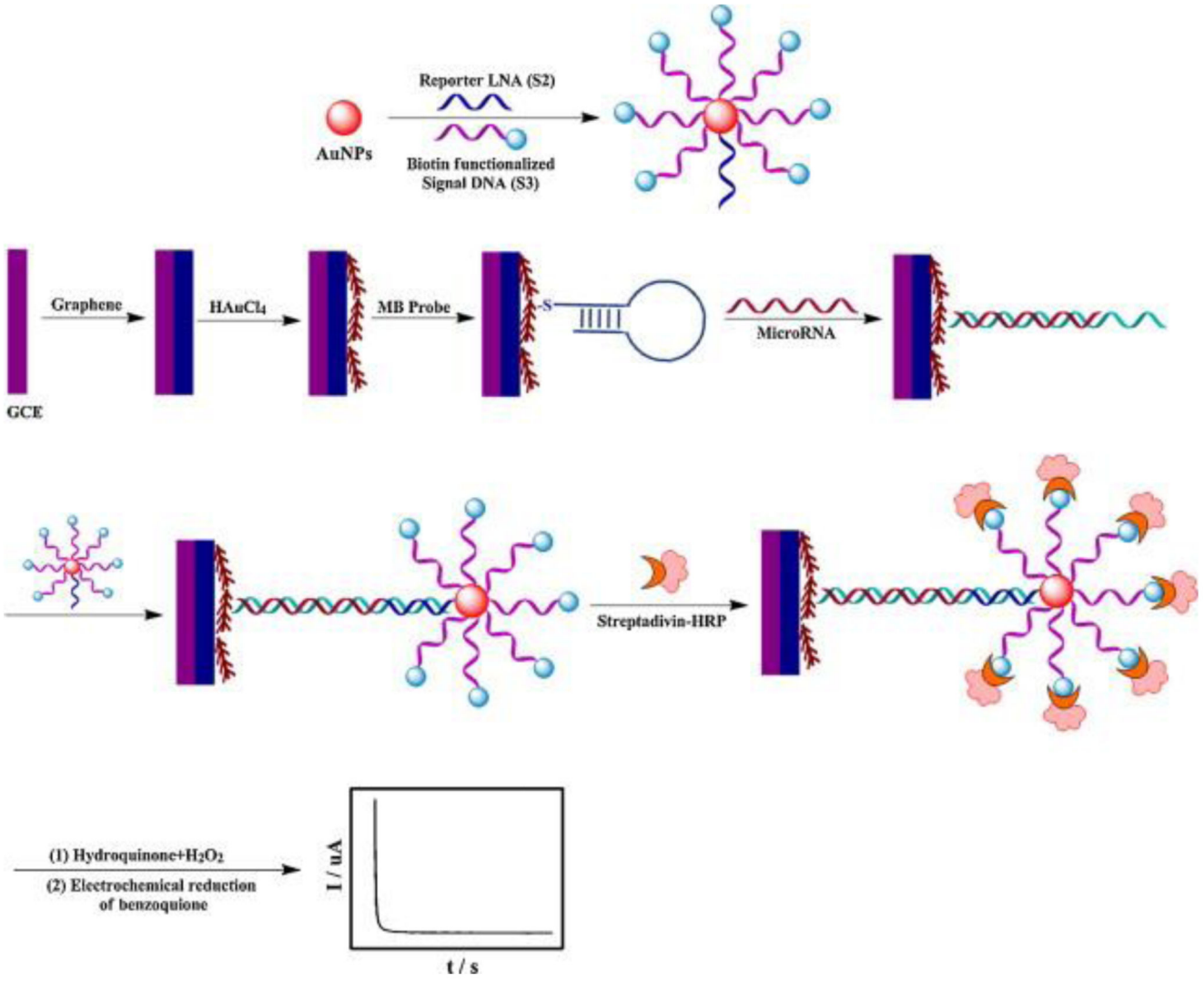

Fig. 8.

Schematic illustration of a biobarcode detection of microRNA. Capture probe is a “hairpin DNA” probe whose conformation is “opened” upon incubation with microRNA. Finally biobarcode nanoparticles containing probe DNA loaded with HRP enzymes form a sandwich configuration and an electrochemical signal is generated in presence of hydroquinone and H_2O_2. Adapted with permission from (Zhou *et al.*, 2012). Copyright 2012 Elsevier.

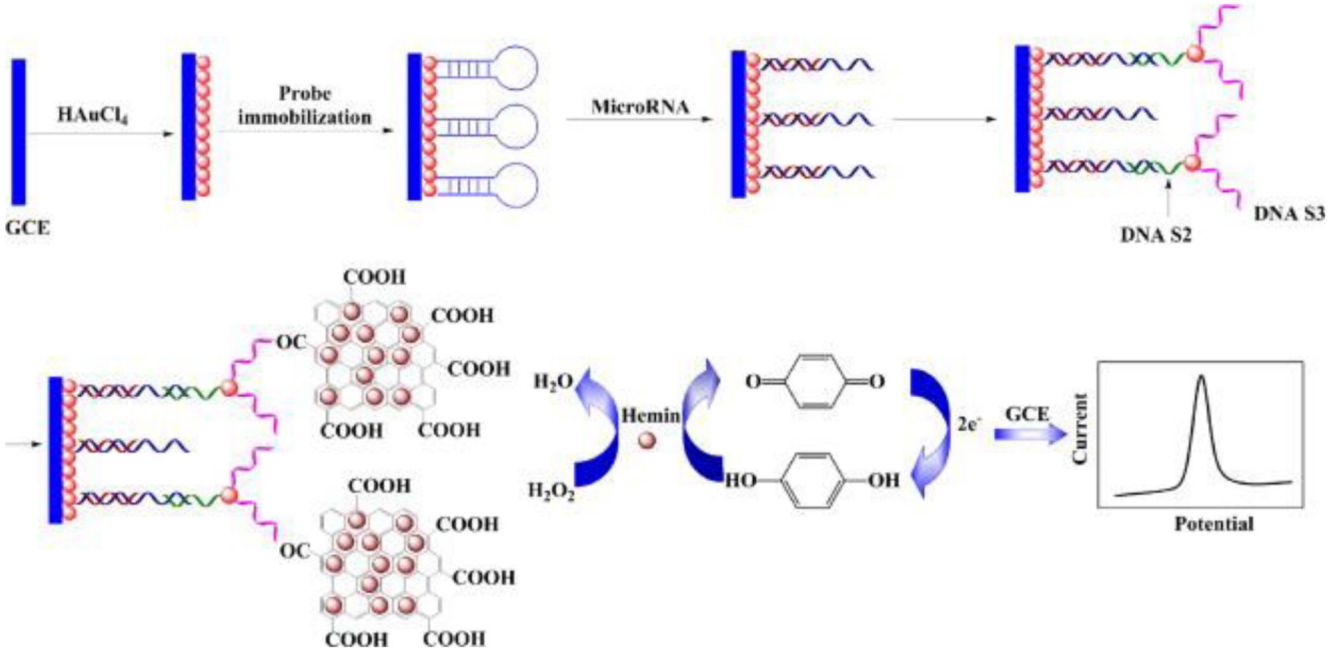

Fig. 9.

Schematic illustration of dual amplification-based microRNA biosensor. The probe immobilized on AuNPs-modified GCE forms a stem-loop structure, which unfolds upon hybridization with target microRNA. The residual single stranded fragment of probe was further hybridized with DNA S2 on biobarcode functionalized AuNPs, leading into introduction of amino functionalized DNA S3. After activation of amine of DNA S3, carboxylic graphene–hemin complex was covalently linked to the surface. Carboxylic graphene–hemin complex effectively catalyze the hydroquinone oxidation in the presence of H_2O_2 to form benzoquinone, which generate an electrochemical signal. Adapted with permission from (Zhou *et al.*, 2014). Copyright 2014 Elsevier.

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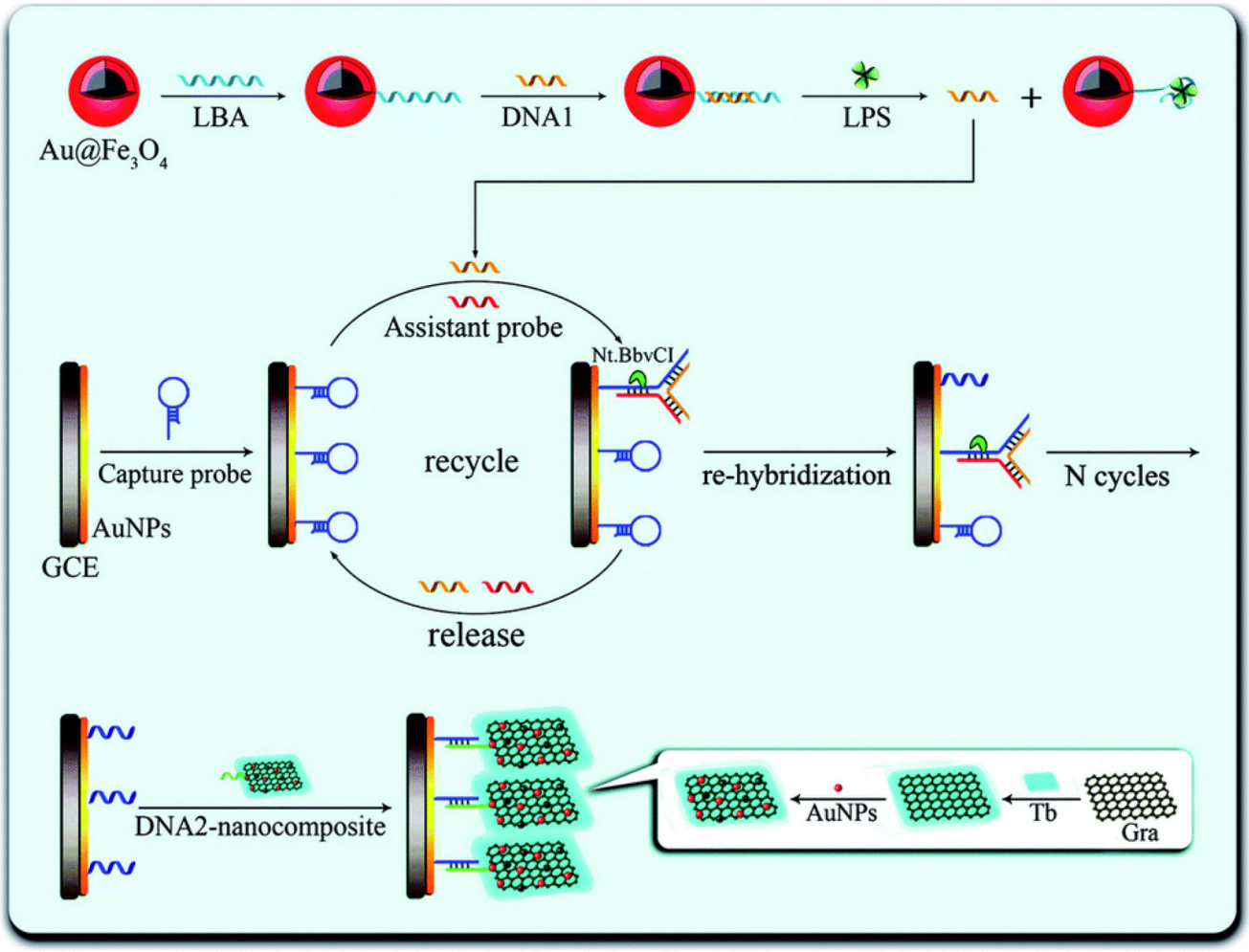

Fig. 10.

Schematic illustration of an amplification method for sensitive analysis of LPS (lipopolysaccharide) using LBA (LPS binding aptamer). By the help of DNA1, which is associated with the concentration of analyte (LPS), the capture probe hybridizes with DNA1 and the assistant probe to form a ternary “Y” junction structure. The DNA1 can be released from the structure in the presence of nicking endonuclease (Nt.BbvCI) to initiate the next hybridization process. Then an increasing amount of cleaved capture probe produced in the cyclic process can bind with DNA2–nanocomposite providing an electrochemical signal. Reprinted from (Bai *et al.*, 2014). Copyright 2014 Royal Society of Chemistry.

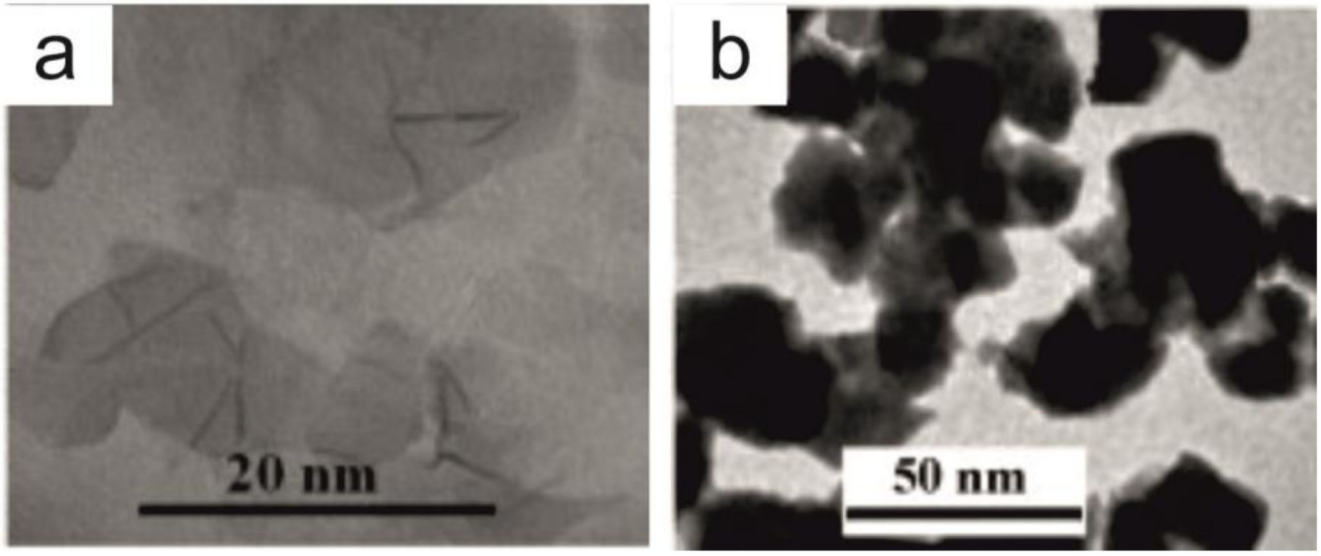

Fig. 11.
TEM images of chitosan-protected graphene (a) and of nanogold-enwrapped graphene (b). Adapted with permission from (Zhong *et al.*, 2010). Copyright 2011 Elsevier.

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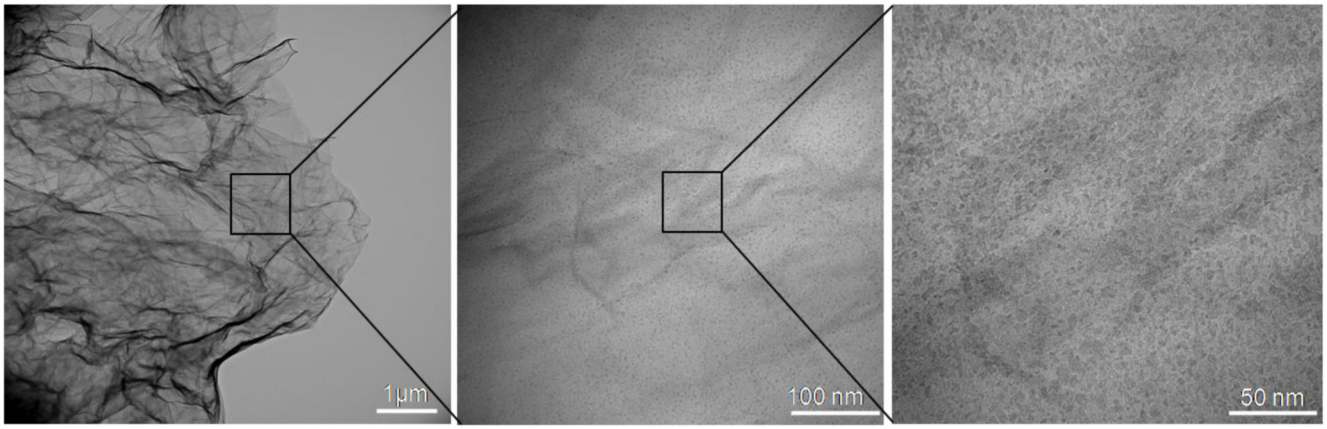

Fig. 12.

TEM images of quantum dots modified rGO-based tracer probe. Adapted with permission from (Shiddiky *et al.*, 2012). Copyright 2012 Elsevier.

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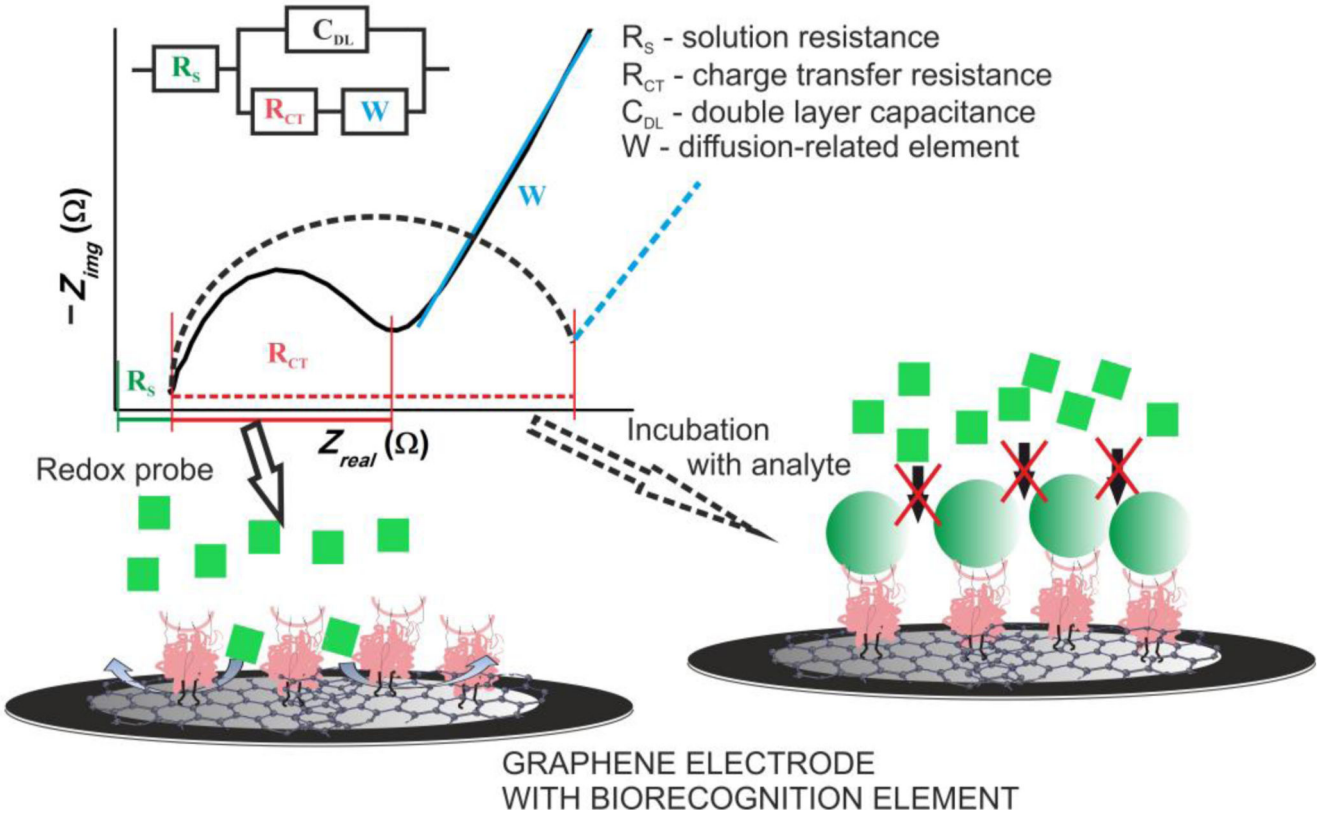

Fig. 13.

Scheme of an impedance spectroscopy assay and Nyquist plot as a typical outcome of the method. Inset of the graph is Randles circuit usually used for fitting of the obtained data.

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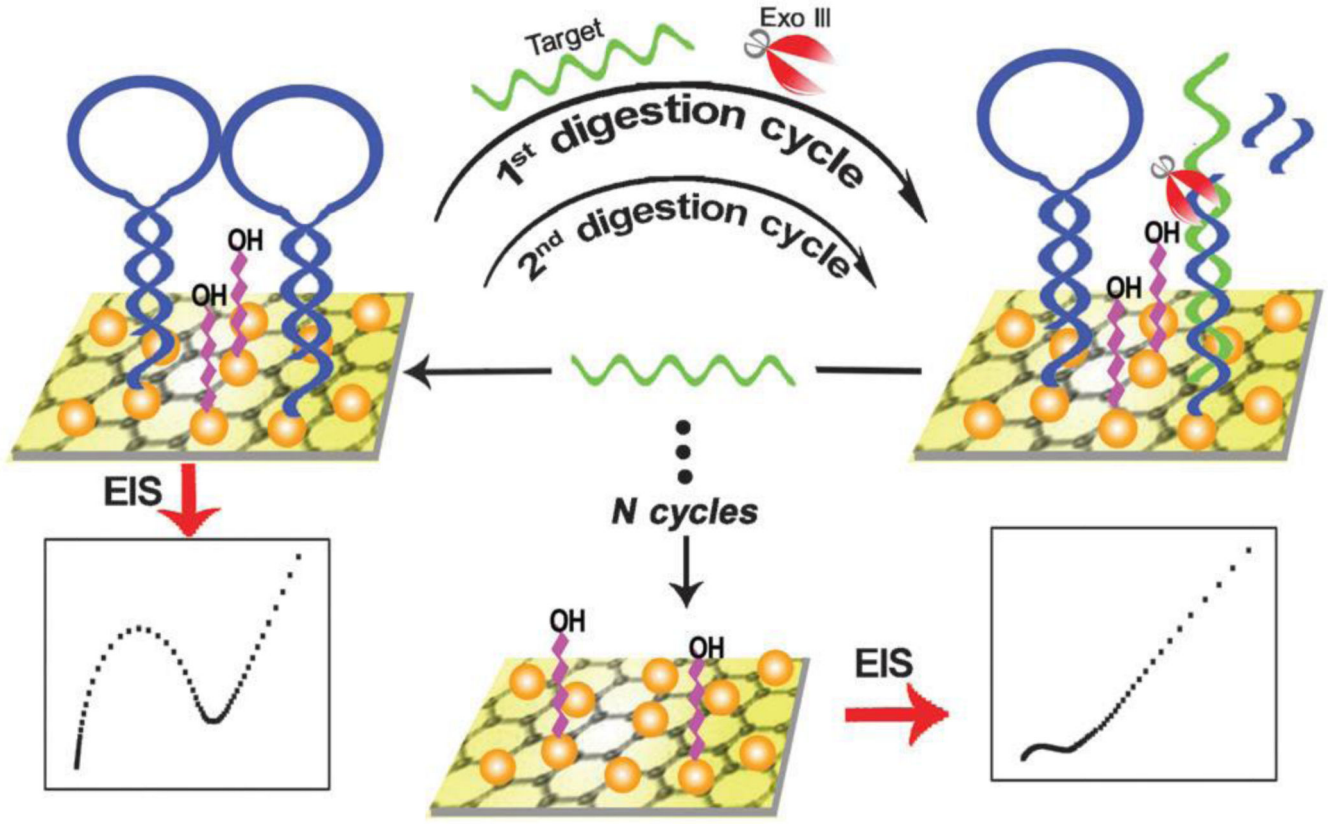

Fig. 14.

Scheme of amplification method based on an enzyme-induced digestion of target DNA. The target DNA after hybridization with the hairpin probe forms dsDNA. At the same time, Exo III specifically cleaves the open, hybridized hairpin DNA and releases the target DNA. Upon enzymatic cleavage of the probe, the released target DNA again hybridizes with the remaining hairpin DNA probe in the subsequent recycling step. Therefore, a small amount of target DNA efficiently removes a large number of hairpin DNA probes from the electrode surface, leading to a substantial decrease in R_{CT} monitored by EIS, proportional to the concentration of the target DNA in the testing samples. Reprinted form (Chen *et al.*, 2011b). Copyright 2011 Royal Society of Chemistry.

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Tab. 1

Operational characteristics of “signal-on” label-free affinity biosensors

Interface	Detection/probe	LOD	Dynamic range	Ref.
DNA biosensors without redox probes				
Anodized epitaxial graphene	DPV/dsDNA[a]	1 mg ml^{-1}	NA	(Lim *et al.*, 2010)
Reduced graphene nanowalls	DPV/dsDNA[a]	9.4 zM	0.1 fM-10 mM	(Akhavan *et al.*, 2012)
DNA biosensors with redox probes				
GO-PANI nanowires	DPV/daunomycin	0.32 pM	2.12 pM-2.12 μM	(Bo *et al.*, 2011)
rGO-AuNPs-ssDNA	DPV/adriamycin	0.04 pM	0.1 pM-10 nM	(Zhang & Jiang, 2012)
rGO-pyrenebutyric acid-ssDNA	DPV/MB	0.4 fM	1 fM-5 pM	(Zhang *et al.*, 2013)
Aptasensors				
rGO-Orange II-TBA	DPV/Orange II	350 fg ml^{-1}	1-400 pg ml^{-1}	(Guo *et al.*, 2013)
rGO-Orange II-LBA	DPV/Orange II	1 pg ml^{-1}	5-700 pg ml^{-1}	(Guo *et al.*, 2013)
PTCA/hemin-AuNP-TBA-GOx + CTBA-GO	CV/GOx-hemin[b]	1.0 pM	5 pM-20 nM	(Yuan *et al.*, 2012)
Immunosensors				
CrGO-anti Aflatoxin B1 Ab	CV/$[Fe(CN)_6]^{3-/4-}$	120 pg ml^{-1}	12.5-100 ng dl^{-1}	(Srivastava *et al.*, 2013)
GPE-Nafion-Cys-AuNPs-anti HBs Ab	DPV/$[Fe(CN)_6]^{3-/4-}$	0.1 ng ml^{-1}	0.5-800 ng ml^{-1}	(Huang *et al.*, 2012a)

DPV – differential pulse voltammetry; CV – cyclic voltammetry; PANI – polyaniline; AuNPs - gold nanoparticles; MB – methylene blue; PTCA – perylene tetracarboxylic acid; TBA – thrombin-binding aptamer; LBA – lysozyme-binding aptamer; HBs – Hepatitis B surface antigen; Cys – L-cysteine; Ab – antibody

[a] – direct detection of DNA

[b] - GOx in the presence of glucose produces hydrogen peroxide which is oxidized by "pseudoenzyme" hemin.

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Tab. 2

Operational characteristics of typical “signal-off” label free affinity biosensors

Interface	Detection/probe	LOD	Linear range	Ref.
	DNA biosensors			
ErGO-PANI nanofibres	DPV/$[Ru(NH_3)_6]^{2+/3+}$	30 fM	0.1 pM-0.1μM	(Du *et al.*, 2012)
ZrO_2–ErGO	DPV/MB	12 fM	0.1 pM-0.1μM	(Yang *et al.*, 2013a)
	Aptasensors			
rGO/PEI-PTCA-AuNP-TBA	CV/PTCA	200 fM	1 pM-40 nM	(Yuan *et al.*, 2011)
CrGO-chitosan-TBA	DPV/$[Fe(CN)_6]^{3-/4-}$	450 aM/2.11fM [c]	up to 100 fM	(Wang *et al.*, 2012b)
	Immunosensors			
GO/thionine-AuNP-anti CEA Ab	SWV/thionine	50 ag ml^{-1}	100 ag ml^{-1}-1 μg ml^{-1}	(*Han et al.*, 2013)
Aminated rGO-anti ovalbumin Ab	DPV/$[Fe(CN)_6]^{3-/4-}$	830 fg ml^{-1}	1 pg ml^{-1}-500 ng ml^{-1}	(Eissa *et al.*, 2013)

DPV – differential pulse voltammetry; CV – cyclic voltammetry; PANI – polyaniline; SWV – square wave voltammetry; MB - methylene blue; PTCA – perylene tetracarboxylic acid; TBA – thrombin-binding aptamer; CEA – carcinoembryonic antigen.

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Tab. 3

Operational characteristics of typical sandwich-assays in affinity biosensors employing graphene-based electrode interface

Interface	Tracer probe	LOD	Linear range	Ref.
	DNA sensors			
rGO-AuNP-capture probe ssDNA	signal DNA-MB	0.35 fM	0.1 μM-1.0 fM	(Wang *et al.*, 2014a)
rGO-dendritic Au - hairpin DNA probe	AuNP-secondary DNA probe signal DNA/HRP conjugate	0.06 pM	0.1-70 pM	(Yin *et al.*, 2012)
ErGO-AuNP-capture probe ssDNA	signal DNA-HRP	3.4 fM	50-5000 fM	(Liu *et al.*, 2013)
	Aptasensors			
rGO-PTCA-AuNP-TBA	HoPtCoNP-Thi-PtNP-TBA2-HRP	0.65 fM	1 fM-1 nM	(Peng *et al.*, 2012)
GO-TBA	GO-ALP/AuNP conjugate-TBA2	2.7 fM	8 fM-15 nM	(Wang *et al.*, 2012a)
	Immunosensors			
GO-anti HER2 Ab	Ab2-dsDNA/daunorubicin conjugate	5 cells ml^{-1}	6-65000 cells ml^{-1}	(Xia *et al.*, 2012)
rGO-AuNP-anti IgG Ab	Ab2/ferrocene conjugate	0.4 ng ml^{-1}	1-300 ng ml^{-1}	(Wang *et al.*, 2013a)
rGO-anti ractopamine Ab (RAC)	AgPdNP-RAC Ab2	1.25 pg ml^{-1}	0.01-100 ng ml^{-1}	(Wang *et al.*, 2013b)
rGO-anti salbutamol Ab (SAL)	AgPdNP-SAL Ab2	1.44 pg ml^{-1}	0.01-100 ng ml^{-1}	(Wang *et al.*, 2013b)
rGO-anti clenbuterol Ab (CLE)	AgPdNP-CLE Ab2	1.38 pg ml^{-1}	0.01-100 ng ml^{-1}	(Wang *et al.*, 2013b)
rGO/PVP-thionine-anti BRCA1 Ab	SBA 15-BRCA1 Ab2-HRP	4.9 pg ml^{-1}	0.01-15 ng ml^{-1}	(Cai *et al.*, 2011)

MB – methylene blue; PTCA - perylene tetracarboxylic acid; TBA – thrombin binding aptamer; TBA2 – secondary thrombin binding aptamer; Ab, Ab2 – antibody, resp. secondary antibody; HoPtCoNP – hollow Pt-Co nanoparticles; Thi – thionine; ALP – alkaline phosphatase; PVP – polyvinylpyrrolidone; BRCA1 – breast cancer 1 protein; SBA 15 - amine-modified silica nanoparticles dispersed with help of ionic liquid.

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Tab. 4

Operational characteristics of typical sandwich-assays in affinity biosensors employing graphene-based tracer probes

Interface	Tracer probe	LOD	Linear range	Ref.
	DNA biosensors			
SWCNH-AuNP-hairpin DNA capture probe	carboxylic GO-FeTMPyP	22 aM	100 aM-10 pM	(Wang *et al.*, 2013c)
AuNP-primary capture DNA probe	AuNP-"biobarcode"/GO-hemin-sec. capture probe[a] GO-hemin	0.17 pM	0.5 pM-1 nM	(Zhou *et al.*, 2014)
	Aptasensors			
HoPtCoNP-chitosan-TBA	PEI-reduced GO-HoPtCoNP-Thi-HRP-TBA2	0.34 pM	1 pM-50 nM	(Wang *et al.*, 2011c)
GO-TBA	GO-ALP/AuNP conjugate-TBA2	2.7 fM	8 fM-15 nM	(Wang *et al.*, 2012a)
	Immunosensors			
Au-anti PDGF BB Ab	GO-PDGF BB Ab2-AgNP deposition[b]	5.0 pg ml^{-1}	0.01-100 ng ml^{-1}	(Qu *et al.*, 2011)
AuNP-anti *E. coli* Ab	GO/PDDA-AgNP-Ab2	10 CFU ml^{-1}	20-1.0x10^{8} CFU ml^{-1}	(Jiang *et al.*, 2013)
ITO-anti EpCAM Ab	carboxylated GO-CdSe QDs-Ab2	0.1/1[c] pg ml^{-1}	NA	(Shiddiky *et al.*, 2012)

SWCNH – single walled carbon nanohorns; FeTMPyP – iron(III)meso-tetrakis(N-methylpyridinum-4- yl)porphyrin; HoPtCoNP – hollow Pt-Co nanoparticles; PEI – polyethyleneimine; ALP – alkaline phosphatase; PDGF BB – platelet-derived growth factor BB; ITO – indium-tin oxide; EpCAM - epithelial cell adhesion molecule; CdSe QDs – CdSe quantum dots;

[a] – an arrangement of two tracer probes, when AuNP-biobarcode is conjugated with the captured target probe and on the other side it conjugates with GO/hemin-based tracer probe;

[b] – in situ and GO-catalyzed synthesis of AgNPs with their consequent voltammetric detection;

[c] –value in buffer/spiked serum.

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Tab. 5

Composition and operational characteristics of typical impedimetric affinity biosensors employing graphene-based electrodes

Interface	LOD	Linear range	Ref.
	DNA sensors		
ErGO/poly(xanthurenic acid)-capture ssDNA	4.2 fM	10 fM-10 nM	(Yang *et al.*, 2013c)
PANI-ErGO-capture ssDNA	0.25 fM	1 fM-10 nM	(Yang *et al.*, 2013b)
anodized EG-ssDNA (covalently bound)	20 fM	50 fM-1 μM	(Dubuisson *et al.*, 2011)
ErGO-AuNP-hairpin ssDNA capture probe [a]	10 fM	50 fM-5 nM	(Chen *et al.*, 2011b)
	Aptasensors		
CrGO-PTCA/Nafion-NSA	794 cells ml^{-1}	$10x^3$-$10x^6$ cell ml^{-1}	(Feng *et al.*, 2011)
GO-TBA (physisorbed)	NA	10-50 nM	(Loo *et al.*, 2012b)
GO-AuNP-SSA	3 CFU ml^{-1}	2.4-2.4x10^3 CFU ml^{-1}	(Ma *et al.*, 2014)
	Immunosensors		
ErGO/PBA-AuNP-anti aflatoxin B1 Ab	1 fg ml^{-1}	3.2-320 fg ml^{-1}	(Linting *et al.*, 2012)
ErGO paper-AuNP-anti *E. coli* Ab	150 CFU ml^{-1}	150-15x10^6 CFU ml^{-1}	(Wang *et al.*, 2013e)
TrGO-anti IgG Ab	NA	0.3 μg ml^{-1}-7 μg ml^{-1}	(Loo *et al.*, 2012a)

EG – epitaxially grown graphene; PTCA - perylene tetracarboxylic acid; NSA - nucleoline-specific aptamer (nucleoline – a surface marker of cancer cells); SSA - salmonella specific aptamer;

[a] – endonuclease-induced “digestion” of analyte ssDNA applied to gain the biosensor performance.