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Graphene based biosensors

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Reliable data obtained from analysis of DNA, proteins, bacteria and other disease related molecules or organisms in biological samples has become a fundamental and crucial part of human health diagnostics and therapy. The development of non-invasive tests that are rapid, sensitive, specific and simple would allow preventing patient discomfort, delay in diagnosis, and the follow-up of the status of the disease. Bioanalysis is thus a progressive discipline for which the future holds many exciting opportunities. The use of biosensors for the early diagnosis of diseases has become widely accepted as a point-of-care diagnosis with appropriate specificity in short time. To allow a reliable diagnosis of a disease at an early state, highly sensitive biosensors are required as the corresponding biomarkers are generally expressed at very low concentrations. In the past 50 years, various biosensors have been researched and developed encompassing a wide range of applications. This contrasts to the limited number of commercially available biosensors. When it comes to sensing of biomarkers with the required picomolar (pM) sensitivity for real time sensing of biological samples only a handful of sensing systems have been proposed, being often rather complex and costly. Lately, graphene-based materials have been considered as superior over other nanomaterials for the development of sensitive biosensors. The advantages of graphene based sensor interfaces are numerous, including enhanced surface loading of the desired ligand due to the high surface-to-volume ratio, excellent conductivity and small band gap beneficial for sensitive electrical and electrochemical read outs, as well as tuneable optical properties for optical read outs such as fluorescence and plasmonics. In this paper, we review the advances made in the last years on graphene based biosensors in the field of medical diagnosis

Subject area: diagnostics

Keywords: biosensors, graphene; diagnostics;

1. Introduction

Biosensors are analytical devices that convert a biochemical/biological reaction into a measurable physicochemical signal, which is proportional to the analyte concentration. A typical biosensor consists thus of two elements: a surface linked biological component that interacts selectively with the analyte of interest in blood or serum and a transducer for the detection of the analyte binding event on the surface. The major advantage of using biosensors as compared to other conventional biochemical assays such as immunoassays and PCR-based strategies is the fast response time (normally several minutes) along with high

specificity. The first biosensor reported dates back to the work by Leland C. Clark, considered as the father of biosensors. Based on his experience on the oxygen electrode [1], he proposed making electrochemical sensors more intelligent by entrapping enzymes such as glucose oxidase onto the oxygen electrode using dialysis membrane [2]. This glucose analyser became commercially available in 1975 in the form of an amperometric sensor. The idea of fixing antibodies rather than enzymes to the sensor transducer has emerged in the early 80's with the work by Lieberg et al. [3]. This work paved the way for the commercial success of surface plasmon resonance (SPR) biosensors (Biacore Technology, launched in 1990); these devices rely on monitoring the change in the plasmonic signal upon antibody-antigen affinity reactions in real time. Since then various biosensors have been developed encompassing a wide range of applications.

One of the many remaining challenges in biosensor development concerns the efficient and selective capture of the biological recognition event. In order to achieve the often requested picomolar (pM) detection limit for biological analyte, nanomaterials have been intensively investigated as candidates for transducer coatings. The possibility to enhance the quantity of bioreceptors immobilisation with consequently improved signal readout makes this biosensor approach greatly appealing. Among the different nanomaterials considered [4], graphene and its various forms such as graphene oxide (GO), reduced graphene oxide (rGO), graphene nanoribbons (GNR), etc have received worldwide attention for the development of biosensors. Different sensing mechanisms including optical, electrochemical, or electrical can be employed with graphene based biosensors, in the following noted as G-biosensors. In the case of electrochemical (amperometric, voltammetric, impedimetric) G-biosensors and electrical sensing concepts (graphene based field effect transistors (G-FET), the high electron transfer rates, high charge-carrier mobility and low electrical noise levels are of uttermost importance for highly sensitive detection of biomarkers and other biological analytes in serum and blood samples [5, 6]. Furthermore, chemically derived graphene derivatives can exhibit a high density of edge-plane-like defect sites, providing many active sites for electron transfer to chemical and biological species [5]. Also, the high optical transparency of graphene monolayers makes them ideal materials for optical based G-biosensors and highly beneficial to improve the sensing performance of plasmonic sensors [7]. The fluorescence quenching ability of GO resulted in the development of several fluorescence resonance energy transfer (FRET) based G-biosensors [8]. Graphene has also shown to be an emerging material as surface enhanced Raman (SERS) substrate due to its ability to generate strong chemical enhancement [9]. Independent on the detection method adopted, the presence of hydrophobic domains or π -systems on graphene-based transducers renders them excellent supporting layers for biomolecules' immobilization. However, the control of non-specific interactions becomes even more important when compared to other sensing surfaces, especially when analysing biological liquids [10]. Improvement in the fabrication of non-fouling graphene transducers is one of the essential steps in the development of high performance G-biosensors [10].

To obtain a general overview on the results achieved in this field, some of the key works around the development of point-of-care sensing in biological fluids using G-biosensors will

be highlighted here. As the examples of G-biosensors are countless, the focus will be on the direct detection of small molecules such as glucose and dopamine, DNA, and protein biomarkers (e.g. folic acid protein, lysozyme, prostate specific antigen) using immunosensing and pathogen detection. It is hoped that this brief overview of G-biosensors and its interest for the biomedical field will stimulate further research activities together with commercialization of some of proposed concepts to help advancing personalized care.

2. Preparation of graphene based biosensors

A number of different approaches for the synthesis of graphene and its derivatives such as graphene oxide (GO), reduced graphene oxide (rGO), porous reduced graphene oxide (prGO), and graphene nanoribbons (GNRs) are available in the literature (**Figure 1**). Large area single and few layer high-quality graphene nanosheets can be produced by chemical vapour deposition (CVD) methods on nickel or copper and are commercially accessible. Such graphene sheets are nowadays routinely transferred to any transducer interface using mainly PDMS-supported transfer processes [11]. The high quality of CVD graphene and the possibility to obtain mono- and bilayer modified electrical interfaces makes such electrodes advantageous for G-FETs and plasmonic biosensing. The use of chemically derived GO and rGO nanosheets, obtained from graphite precursor through solution-based exfoliation aiming at weakening the van der Waals forces between the graphene layers, is the most commonly used synthetic approach for the construction of G-biosensors. It is a relatively cheap method for obtaining GO/rGO on a large-scale, with the additional benefit of possible modulation of the morphology and porosity of the nanosheets. Furthermore, doping with non-metallic elements such as nitrogen, sulphur, or boron allows to modulate the electronic structure of these materials and leads in general to improved electrical and electro-catalytic properties. Reduction of the GO flake size results in better dispersible structures of 3-20 nm in size consisting of no more than 5 layers; these structures exhibit a high surface area and termed graphene quantum dots (GQDs) [12].

Different techniques such as drop-casting, spin-coating, electrostatic interaction between positively charged interfaces and the negatively charged GO/rGO nanosheets, electrophoretic deposition (EPD) and electrochemical reduction of GO can be employed to coat electrical as well as inert surfaces with the chemically derived graphene materials. The method of choice depends on the thought after application and the employed transducer element. **Table 1** gives a short list of selected biomedical analytes of interest together with the method employed for their detection and the limit of detection which can be reached, most of them being discussed in more details in this review.

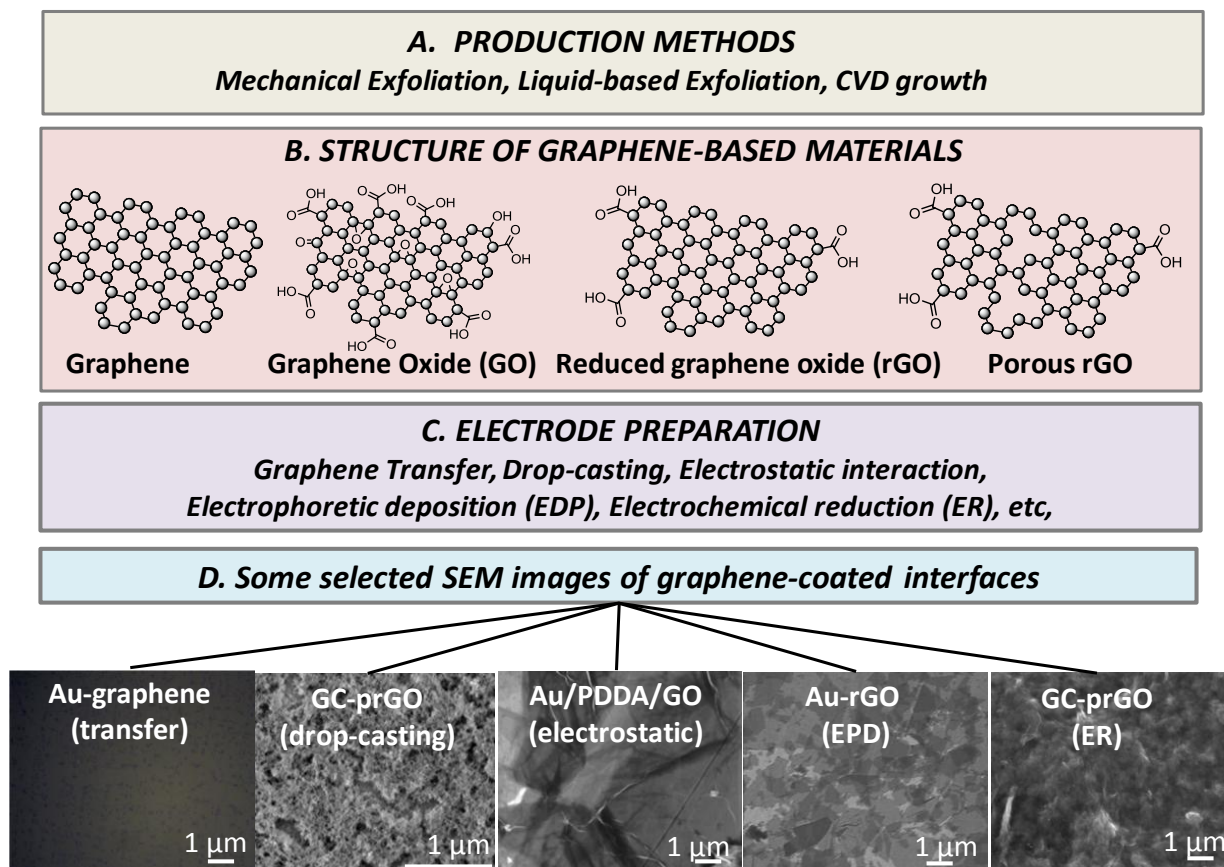


Figure 1: Construction of graphene-based biosensor interfaces: (A) Current preparation methods, (B) Chemical structures of different graphene derivatives widely used for biosensing; (C) Methods for the transfer of graphene-based materials to solid substrates; (B) SEM images of graphene coated interfaces using different deposition methods and different graphene precursors

Table 1: Selected examples of most performing graphene based biosensors for different analytes.

Analyte	Sensor design	detection	LOD	Ref
glucose	3D graphene foam-Co ₃ O ₄ nanowires	EC	20 nM	[13]
glucose	Graphene+GO _x	FET	0.1 mM	[6]
glucose	GQDs-bipyridine boronic acid	fluorescence	1 mM	[14]
dopamine	rGO-polyvinylpyrrolidone	EC	0.2 nM	[15]
DNA	GO and GQD-ssDNA	FRET	75 pM	[16]
DNA	Graphene-Au NPs-ssDNA	SPR	500 aM	[11]
DNA	GO nanowalls	DPV	9.6zM	[17]
DNA	graphene	FET	10 fM	[18]
lysozyme	Au/PDDA-GO- <i>Micrococcus lysodeikticus</i>	SPR	3.4 nM	[19]
Folic acid	Au-rGO	DPV	1 pM	[10]
Folic acid	graphene	SPR	5 fM	[20]
β -amyloid	Magnetic/plasmonic GO	SERS	100 fg mL ⁻¹	[21]
PSA	rGO	FET	1 fM	[22]

<i>E. coli</i>	Graphene-anti- <i>E. coli</i>	FET	10 cfu mL ⁻¹	[23]
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EC: electrochemistry; GO: graphene oxide; GQDs: graphene quantum dots; GO_x: glucose oxidase; Au NPs: gold nanoparticles; DPV: differential pulse voltammetry; PDDA: poly(diallyldimethylammonium); PSA : prostate specific antigen.

3. G-biosensors for glucose and dopamine

One challenging and important molecule to monitor is glucose. Increase in glucose levels is critical for human health as hyperglycemia, defining diabetes, leads to premature death caused by macrovascular and microvascular complications. A close monitoring of the blood glucose concentration can largely help to manage diabetes. Tremendous efforts have been put into the development of efficient and reliable methods for glucose sensing. Graphene based glucose sensors are generally built by immobilizing glucose oxidase (GO_x) onto the graphene surface such as the graphene-FET proposed by Huang at al. (**Figure 2A**) [24]. In this work, GO_x was covalently lined *via* its amine groups to 1-pyrenebutanoic acid succinimidyl ester, where the pyrene end is firmly attached to graphene through π - π stacking interactions. Measuring a change in conductance allowed glucose detection down to 0.1 mM. Although the use of GO_x allows for high selective detection towards glucose, non-enzymatic glucose sensors based on the integration of electro-catalytic sites for glucose, often in the form of nanoparticles, onto graphene have been pursued [13, 25, 26]. While these sensors are less selective to glucose and need to be for the most part operated in an alkaline medium, they display several advantages such as better stability than GO_x-based interfaces and often improved sensitivity with detection limits in the low μ M to nM range. In the case of N-doped porous reduced graphene oxide loaded with Cu NPs (N-prGO-Cu NPs) the improved sensitivity to glucose is attributed to the electro-catalytic behaviour of the CuO/CuOOH couple (**Figure 2B**). In the presence of glucose, the redox peak of Cu(0)/Cu(I) stays unchanged, while the peak of the Cu(I)/Cu(II) transition is decreased reflecting the formation of Cu(I)-glucose complex. The band at +0.4V is strongly increased, in line with the activity of Cu(III) in basic medium (**Figure 2B**) [27].

A different non-enzymatic glucose sensing approach based on the use of graphene quantum dots (GQDs) modified with boronic acid substituted bipyridine ligands, which serve as fluorescence quencher upon electrostatic interaction with GQDs was proposed [14] (**Figure 2C**). When glucose is added to the system, the boronic acid moieties are converted to tetrahedral anion glucoboronate esters, which neutralize the net charge of the bipyridinium, thus greatly diminishing the quenching effect and recovering the fluorescence intensity of the GQDs (**Figure 2C**).

Another small biomolecule of interest is dopamine. It is one of the crucial catecholamine neurotransmitter widely found in serum samples at concentrations between 10 nM and 1 μ M. Abnormal levels can result in a variety of diseases (Huntington's disease, Parkinson's disease, Schizophrenia) with high levels increasing the risk of high blood pressure related diseases. Rapid and accurate detection of dopamine at low cost has thus become of demand in clinical diagnostics. The electrochemical activity of dopamine makes its electrochemical detection rather appealing. However, uric acid (UA), ascorbic acid (AA) and serotine (ST) are coexisting with dopamine in the extracellular fluids of the central nervous system in mammals at even higher concentrations and can be oxidized at a potential close to that of dopamine. The excellent electrochemical properties of graphene for dopamine together with chemical enhancement *via* π - π stacking interactions have enabled the development of dopamine

specific G-sensors even in the presence of high concentrations of AA, UA and ST [28, 29] (**Figure 2D**). Extremely low LOD could be achieved using electrochemically reduced GO in combination with polyvinylpyrrolidone capable of detecting 0.2 nM dopamine in the presence of 1 mM AA [15].

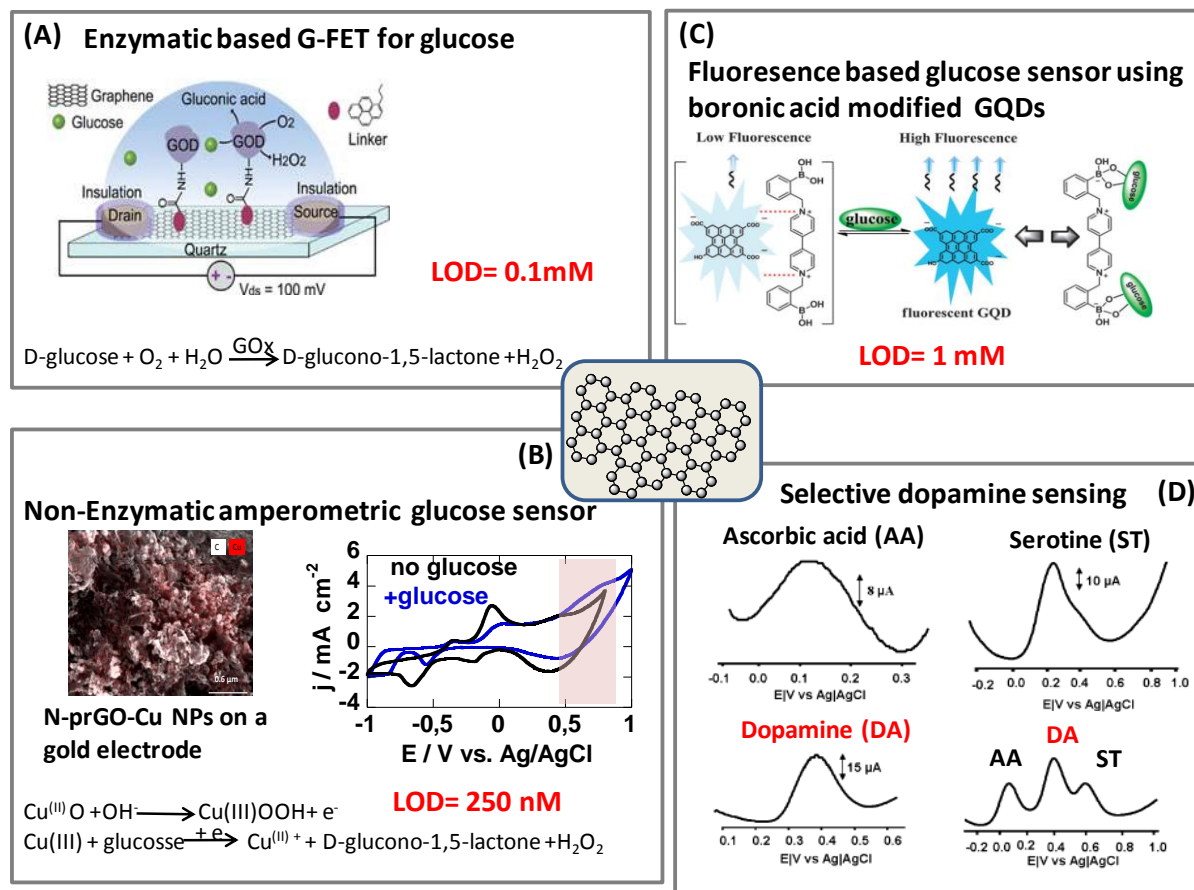


Figure 2: G-based sensors of small molecules such as glucose and dopamine: (A) CVD graphene modified with glucose oxidase (GO_x) using a bifunctional pyrene linker for the construction of a G-FET for glucose (reprint with permission from Ref.[23]); (B) Non-enzymatic glucose sensor operating under basic conditions based on N-doped porous reduced graphene oxide loaded with Cu NPs (N-prGO-Cu NPs) (reprint with permission from Ref. [27]); (C) Graphene quantum dots (GQDs) modified with boronic acid substituted bipyridine ligands for non-enzymatic glucose sensing under physiological conditions (reprint with permission from Ref. [14]); (D) Differential pulse voltammetry of ascorbic acid (1 mM), serotone (1 mM) and dopamine (1 mM) and a mixture of all three (0.1 M) on glassy carbon electrodes modified with hydrazine reduced GO (reprint with permission from Ref.[28]).

4. DNA sensing with G-biosensors

The need for a rapid and sensitive DNA analysis is an important issue in clinical diagnosis. Major studies have focused on the sequence-specific recognition of ssDNA and on the detection of single nucleotide polymorphisms (SNP). SNP is a common form of genomic variation occurring in every 100-300 base pairs and related to many major diseases and disorders, such as Parkinson's disease, Alzheimer's disease, diabetes and various cancers. The development of analytical approaches for selective DNA sensing has consequently been

strongly pursued with the believe that low-cost systems suitable for DNA analysis could revolutionise modern health care. Electrodes modified with graphene oxide nanowalls (GON) with preferred vertical orientation [17] or graphene nanoplatelets [30] are capable to catalytically oxidize the four DNA bases simultaneously, resulting in DNA sensors with a LOD as low as 9.4 zM [17] (**Figure 3A**).

The fluorescence quenching ability of GO was exploited by several research groups for the detection hybridization events. When a dye-labelled ssDNA is immobilized *via* non-covalent binding onto GO, the fluorescence is quenched; this non-covalent interaction is reversible. ssDNA interaction with GO occurs thus *via* π - π stacking, hydrophobic interactions and hydrogen bonding. Even though both, GO and ssDNA, are negatively charged, DNA can be adsorbed on GO in buffers containing a high concentration of salts to screen electrostatic repulsion [31]. In dsDNA the nucleotide bases are hidden in the helical structures, preventing their effective interaction with the GO surface in contrast to ssDNA. In the presence of a complementary DNA (cDNA) target, a duplex is formed, disturbing the GO-ssDNA interaction and resulting in a release of the formed dsDNA upon which fluorescence is restored (**Figure 3B**). This approach has been recently used in combination with ssDNA modified GQDs for fluorescence resonance energy transfer (FRET) based DNA sensing [16] (**Figure 3B**).

This concept was further applied to surface plasmon resonance (SPR) based DNA sensing [11] (**Figure 3C**). Gold nanostars modified with ssDNA were integrated into commercially available graphene coated SPR chips. As dsDNA has a lower affinity to graphene, hybridized targets are released from the graphene surface, resulting in a negative shift of the SPR signal which allows an easy discrimination between mismatched DNA and offers a label-free approach for DNA detection with a LOD of 500 aM over a linear dynamic range up to 10 nM [11].

The feasibility of G-FET based sensors for nucleic-acid based analytes has been shown by Zheng et al. [18]. Peptide nucleic acid (PNA) was non-covalently immobilized to the graphene channel and hybridization with target DNA produced a left-shift in the Dirac point with a LOD of 10 fM [18]. Dontschuk et al has shown in addition the interest of G-FETs for DNA sequencing [32]. They demonstrated experimentally that GFETs are able to measure distinct coverage-dependent conductance signatures upon adsorption of the four different DNA nucleobases, a result that was attributed to the formation of an interface dipole field.

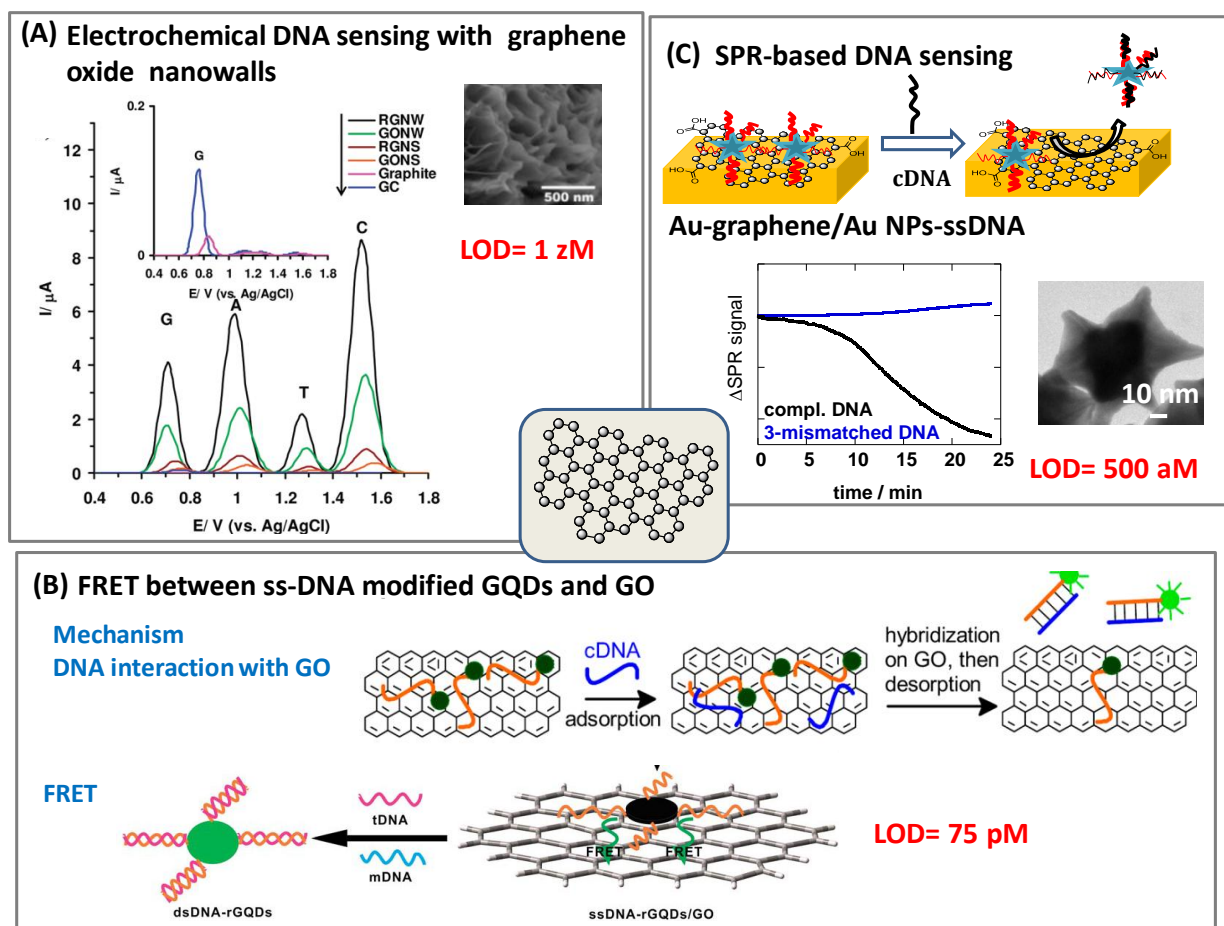


Figure 3: DNA sensing with G-biosensors: (A) Single DNA electrochemical biosensing using graphene nanowalls (GNW): SEM image of GNWs formed by electrophoretic deposition onto graphite rod, differential pulse voltammograms of dsDNA (0.1 μM) in PBS (0.1 M, pH 7) on different interfaces (reprint with permission from Ref.[17]); (B) Mechanism of DNA interaction with GO (reprint with permission from Ref.[31]) and FRET based DNA sensing using graphene quantum dots and graphene oxide (reprint with permission from Ref [16]); (C) Graphene-SPR based DNA sensing: TEM image of a gold nanostructure together with change in SPR signal upon incubation with cDNA and mismatched DNA [11].

5. G-based sensors for protein biomarkers

Protein biomarkers are specific molecules existing in blood or tissues, whose measurement or identification is very critical and efficient in the prediction, diagnosis, and monitoring of cancer and many other diseases. Clinical utility of biomarkers to diagnose disease requires the capability to measure femto- to picoMolar concentrations of these, which is also important to understand cellular processes and to search for new protein biomarkers. The detection limits of the prevailing analytical protocols (e.g. ELISA-enzyme linked immunosorbent assay) for biomarker detection lags far behind the requirements for clinical utility and research. Graphene-based immunoassay platforms, where specific antibodies are immobilized onto graphene to capture selectively the biomarker analyte, have shown on the other hand excellent sensitivity [33] (**Figure 4**). For example, we have recently demonstrated that post-functionalization of rGO modified electrodes by simple immersion into a solution of folic acid allowed for the development of an electrochemical based sensor for folic acid protein with a

LOD of 1 pM [10] (**Figure 4A**) and a plasmonic sensor with 5 fM detection limit [20]. Levels of folic acid protein in serum can increase up to 22 pM in metastatic diseases. Given that human serum is free of folic acid proteins, detection of this protein in serum serves as an early stage cancer diagnostic step.

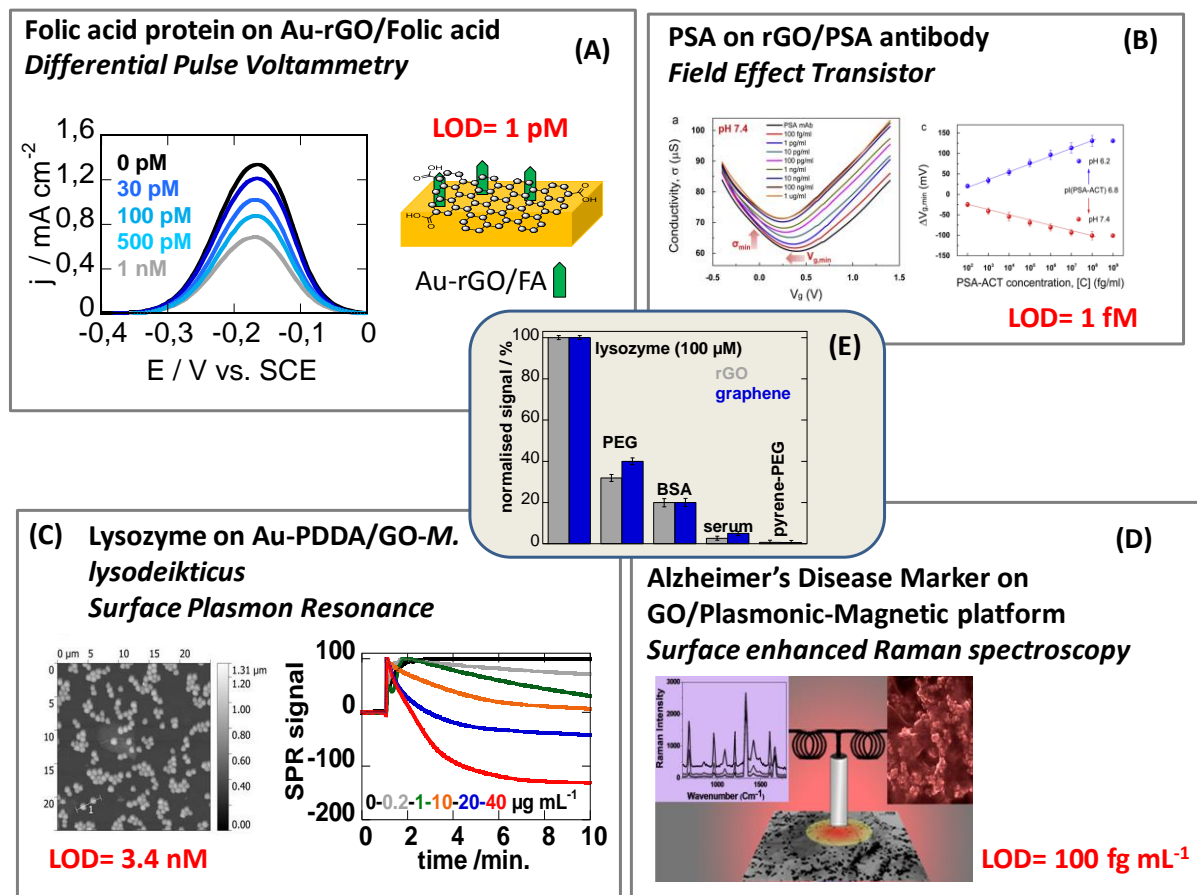


Figure 4: G-biosensors for protein sensing using: (A) Electrochemical sensor for folic acid protein: DPV upon addition of increasing concentrations of folic acid proteins (reprint with permission from Ref. [10]); (B) G-FET for the analysis of prostate biomarkers (reprint with permission from Ref. [22]); (C) Graphene-SPR based sensing for lysozyme: AFM image of Au-(PDDA/GO)₂ interface modified with *Micrococcus lysodeikticus* and change in SPR responses to different concentrations of lysozyme added to fetal bovine serum (reprint with permission from Ref. [19]); (D) Concentration dependent SERS spectra from tau protein conjugated nanoplatform after magnetic separation together with SEM image of core-shell nanoparticle attached hybrid GO and HR-TEM picture (reprint with permission from Ref.[21]).

A rGO based FET modified with prostate specific antigen- α 1-antichymotrypsin (PSA-ACT) was used by Kim et al. to detect fM levels of PSA with a dynamic range over 6-orders of magnitude [22] (**Figure 4B**). Surface plasmon resonance (SPR) based read out was used by Cosnier and co-workers for the detection of cholera toxin on graphene coated gold chips modified with pyrene-nitrilotriacetic acid (NTA) with a detection limit of 5 $\mu\text{g mL}^{-1}$ [34]. We showed recently the suitability of *Micrococcus lysodeikticus* modified GO-coated SPR

interfaces to sense serum lysozyme levels with a limit of detection of 3.4 nM [19] (**Figure 4C**). Lysozyme, an enzyme found in biological fluids is upregulated in leukaemia, renal diseases as well as a number of inflammatory diseases. While lysozyme concentration in serum of healthy people ranges from 27 to 301 nM, patients suffering from Inflammatory Bowel Disease show microMolar levels of lysozyme. Recently, a multifunctional nanoplatform based on magnetic-plasmonic nanoparticles attached to GO allowed for the sensitive detection of Alzheimer disease biomarkers (β -amyloid, tau proteins) down to 100 fg mL⁻¹ [21] (**Figure 2D**). These examples highlight the efficient use of G-biosensor for protein analysis.

One main hurdle of all these sensors when performing tests in human serum samples, often not evoked in the literature, is linked to the high non-specific interaction between the graphene surface and serum proteins. We have compared a number of different strategies to reduce nonspecific binding of clinical serum samples spiked with lysozyme (100 μ M) on rGO [20] (**Figure 4E**). While simple immersion into serum decreased strongly the anti-fouling properties of graphene, rGO modification with pyrene-PEG units have shown to result in the best non-fouling interface [20, 35].

6. Bacteria and Viruses

The specific and sensitive detection of pathogenic microorganisms remains a big scientific challenge and a practical problem of enormous significance. Pathogen diagnosis is currently based on culturing the microorganism on agar plates with the disadvantage of being long (minimum of 24 h) and ignoring viable but non-cultural cells. G-FET biosensors have been successfully applied for the detection of bacteria and their metabolic activities. CVD graphene modified with *anti-E. coli* antibodies allowed *E. coli* concentrations as low as 10 cfu mL⁻¹ to be detected [23]. Graphene oxide in combination with *E. coli* O157:H7 antibody-conjugated quantum dots was used as pathogen-revealing agent by exploiting the universal high efficient long-range quenching properties of GO; a LOD of 3.8×10^3 cfu mL⁻¹ was achieved [36]. Graphene printed onto water-soluble silk and modified with antimicrobial peptides allowed bioselective detection of bacteria at single-cell levels remotely [37].

7. Conclusion and perspectives

We tried to review the most recent advances on graphene based biosensors by selectively highlighting a variety of different examples for the detection of some molecules of biomedical interest. Routinely, such sensors achieve a picomolar detection limit, with some even reaching the low femtomolar concentration range. The possibility that a large range of different detection methods can be employed with graphene based sensors is of high advantage, as depending on the looked after final application, sensor size and read out can be customised at will. There is however still an urgent need for moving beyond research by developing new concepts for achieving even better sensitivity and selectivity, in order to bring some of the current sensors into real biomedical applications. Even though a large amount of sensors reported in the literature exhibit good storage stability and repeatability, important for complex sensors involving nanomaterials and manual step preparations, the performance in

real biological samples is often not reported. As non-specific interactions are of primary concern on graphene based interfaces, this lack of information together with the large-scale reproducibility of the fabrication of graphene biosensor interfaces are probably two of the most crucial limiting factors for current commercialization. When it comes to *in vivo* application of some of the sensing concepts, graphene based biosensors are still in their infancy. Toxicity and biocompatibility issues still need to be addressed carefully to avoid any undesired secondary health effects. Current *in vivo* and *in vitro* assessments of the biostability of the sensors are encouraging and promising for further technological transfer.

Different challenges are still to overcome. The collaboration between material scientists, chemists, physicists as well as engineers and medical personal is of fundamental importance to drive this field further and to propose graphene based biosensors as point-of-care alternatives for patients. The success of any new biosensor material lies in addition on its reproducibility and possible industrial scale production. The emergence of a number of companies providing mono- and bilayered graphene nanosheets on several interfaces, graphene oxide (GO), reduced graphene oxide (rGO) and even modified matrixes has been an additional motivation for using graphene for biosensor applications.

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