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Graphene-based biosensors: methods, analysis and future perspectives

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Numan Celik ✉, *Wamadeva Balachandran*, *Nadarajah Manivannan*

Centre for Electronic Systems Engineering, Brunel University, Kingston Lane, Uxbridge, UB8 3PH, UK
✉ *E-mail: numan.celik@brunel.ac.uk*

Abstract: Graphene (GN), a single layer two-dimensional structure nanomaterial, exhibits exceptional physical, electrical and chemical properties that lead to many applications from electronics to biomedicine. The unique parameters of GN, notably its considerable electron mobility, thermal conductivity, high surface area and electrical conductivity, are bringing heightened attention into biomedical applications. This study assesses the recent advances in GN-based biosensors and its derivatives in different areas to focus on glucose sensing, DNA sensing, drug and gene delivery, cancer therapy and other related biomedical applications (electrochemical sensors, tissue engineering, haemoglobin and cholesterol sensing), together with a brief discussion on challenges and future perspectives in this rapidly developing field.

1 Introduction

It has been clearly indicated that nanomaterials improve the physiochemical characteristics of bulk materials such as conductivity, strength, reactivity due to the high volume surface ratio and some other physical and electrical properties [1]. Among other nanomaterials, graphene (GN) has received worldwide attention owing to its extraordinary physical, electronic, thermal, optical, chemical and mechanical properties [2–5]. GN is composed of a two-dimensional (2D) single-atom-thick layer of sp^2-bonded carbon atoms arranged in a honeycomb lattice [6]. Geim and Novoselov discovered GN, the thinnest known material with a thickness of 0.35 nm, in 2004 and received the Nobel Prize in Physics for inventing this extraordinary material by a simple mechanical exfoliation [7].

Since then, GN and its related derivatives have attracted the interest of various scientific fields, such as nanoelectronics [8], energy technology (such as supercapacitor, fuel cell, solar panels) [9–11], sensors [12], bioscience/biotechnologies [13–15] due their physiochemical and electric–electronic properties. Particularly, these exceptional values include high surface area (2630 m^2/g) [16], excellent electrical conductivity (1738 S/m) [17], strong mechanical strength (about 1100 GPa) [18], good thermal conductivity (5000 W/m/K) [19], high charge carrier mobility (about 10,000 $cm^2\ V^{-1}\ s^{-1}$) [20], good optical transparency (~97.7%) [21] and ease of biological as well as chemical functionalisation of GN [22]. Due to these excellent physiochemical characteristics, GN-based nanomaterials offer great opportunities for implementing into a wide area of biomedical applications [23]. A summary of the key properties of GN comparing with its related carbon materials has been provided in Table 1 [24].

The review articles [25–30] that have been published during the last three years on GN-based nanomaterials and electrochemical biosensors have highlighted the salient features of GN. Herein, we will give a brief overview of the applications of GN in biomedical fields that is an updated comprehensive work to those described in the reviews mentioned above, rather than attempting to give a systematic and detailed review of this field. In this paper, methods of GN production are briefly explained in Section 2, followed by a basic concept of understanding the principle of biosensing applications including DNA, glucose, haemoglobin (Hb) and cholesterol biosensors in Section 3, and a detailed review on GN for drug and gene delivery and cancer therapy has been presented in Section 4. Section 5 draws an attention regarding the applications of tissue engineering and electrochemical sensors using GN. Furthermore, future trends and possible research directions for applying GN-based electrochemical materials are given at the end of the paper.

2 Overview of GN synthesis

A number of different algorithms to synthesise GN sheets have been demonstrated since first obtained in 2004 [2]. Geim and Novoselov followed the route in which a typical Scotch tape was used to extract thin layers of graphite from highly ordered pyrolitic graphite and then transferred these layers onto a silicon (Si) substrate. This method is called 'mechanical exfoliation', which is one of the procedures to produce GN sheets. Moreover, this technique has produced the best quality GN, so far, in terms of structural integrity and was analysed using underpinning algorithms [20, 31, 32]. However, this method is limited to scientific research, as the size and thickness are not suitable for producing large-scale prototypes and hence applications are limited [33].

Another synthesis of GN is liquid-phase exfoliation that involves solution-based exfoliation of GN oxide (GNO) [34–36]. Specifically, the liquid-phase exfoliation technique is quite promising for large-scale applications such as supercapacitors, composite materials, gas sensors and flexible materials used in biomedical applications [37, 38]. Another production method of GN is to convert SiC to GN via sublimation of Si atoms at very high temperature (generally at ~1300°C) [39, 40]. This method can be referred to as 'epitaxial growth' by the thermal desorption of Si atoms from the SiC surface in different applications [41] and has good potential for large-scale integration of nanoelectronic devices. In recent years, a lot of research has also been done to synthesise GN differently using chemical vapour deposition (CVD) onto metal or Si, Ni and Cu substrates. Among all other strategies to produce GN, CVD on metal substrates has become the most promising approach that has excellent advantages including best quality for large-scale applications and inexpensive production method [42]. The first GN CVD method was applied in 2008 and 2009, using Ni and Cu substrates [43–45], which was followed by many research applications and publications in transition metal substrates [46, 47].

For an overview, the current GN synthesis techniques are listed in Table 2.

Table 1 Comparison of relevant electronic and thermal properties of GN with other related carbon materials (Si, Cu, single wall CNT – SWCN)

	Si	Cu	SWCNT	GN
DC max current density, A/cm^2	–	10^7	$>10^9$	$>10^8$
melting point, K	1687	1357	3800	3800
mobility, cm^2/V s	1400	–	>10,000	>10,000
thermal conductivity ($\times 10^3$ W/m K)	0.15	0.385	1.75–5.8	3–5
mean free path (nm) at room temperature	30	40	$>10^3$	1×10^3

In terms of applications in particular electrochemical biosensors, GN synthesised by solution suspension of GNO followed by chemical reduction, usually referred to as reduced GN oxide (RGO) process, is used most extensively [48]. The GN produced by the RGO process offers smaller sizes, more structural defects, and more functional groups than those produced by other synthesis techniques [49]. Overall, the RGO is a better fit for large-scale applications of small GN sheets, while the CVD technique is more efficient for large-scale applications of high-quality GN. Therefore, the application for which GN is being produced should first be examined before the most appropriate GN synthesis that can be selected (Fig. 1).

3 GN for biosensing

Bioanalysis plays indispensable role in many disease developments and human health diagnostics; thus sensitive and selective detection of proteins, DNA and bacteria are critical to disease diagnosis and therapy. For instance, Alzheimer and various cancers are closely related to DNA damage [51]. Appropriate biosensor is required for early stage diagnosis of the disease as well as disease progression. The GN-based materials have been implemented to construct different types of biosensors based on various sensing mechanisms including optical (fluorescence) and electrochemical sensors [52]. Depending on the specific working principle, GN-based biosensors either use their electrical properties (i.e. high charge-carrier mobility), electrochemical properties (i.e. high electron-transfer rates), or unique structure (i.e. atomic layer thickness and high surface-to-volume ratio) for biomolecule detection [53]. Fig. 2 illustrates different representations of GN-based electrochemical sensors in such biomedical applications.

Table 2 A brief summary of GN production techniques [33]

Synthesis method	Brief description
mechanical exfoliation	• Atomic layer of GN can be seen on ~300 nm SiO_2 substrates • Pristine GN with the highest quality of electrical properties • The size and thickness are uncontrollable, thus limited practical applications
liquid-based exfoliation	• Graphite powders are initially oxidised by chemical modification to be dispersed in solution • Large-scale production for bulk applications, i.e. supercapacitors, composite materials • Serious structural defects
epitaxial growth	• A conversion of SiC substrate to GN via sublimation of silicon atoms on the surface • Done at very high temperature (~1300°C) • Accessibility is limited due to high-end equipment
CVD growth GN	• Most promising, inexpensive and feasible method for single-layer GN synthesis • Using transition metal (Ni, Cu, Si) substrates • Can be scaled up for large area GN production for practical applications

Due to these advantages, GN is selected for synthesis of biological sensors with high sensitivity, selectivity and low detection limit. Using fast electron transportation criteria of GN, the tiny biological information can be converted into an electronic format, thus making the sensors have high sensitivity. Furthermore, high surface-to-volume ratio makes GN easily to conjugate with biomolecules such as enzymes, single-strand DNA (ssDNA), RNA, receptors, aptamers; and detect protein molecules and cancer cells with high efficiency, thus the sensors show the characteristic of low detection limit [55]. In general, biosensors are composed of two parts: a receptor and a transducer. The receptor can be any material that can interact with a target analyte. The biological sensing element connects to a transducer, which does the conversion from biological data to electrical data. The transducer in turn connects to a measuring device translating the electrical signal to a measurable quantity [56]. In GN-based biosensors, GN is used as a transducer element, and changing its electrical characteristics upon interaction with the attached sensing elements.

3.1 GN-based DNA biosensors

In recent years, GN and GNO have emerged as a unique platform for developing DNA-based biosensors, given the DNA adsorption by detection of DNA hybridisation techniques [57, 58] and fluorescence-quenching properties of GNO. Electrochemical GN-based DNA sensors offer high sensitivity, high selectivity, rapid and cost-effective analysis for detecting biomolecules which are significant in clinical diagnosis and treatment.

Major studies on DNA sensing have focused on the sequence-specific recognition and mutation of the ssDNA by various techniques [59, 60]. Double stranded DNA (ds-DNA), which is important for direct visualisation of the genomic information in living cells, and the development of cell-based technology [61]. The electrochemical DNA biosensors can be distinguished into label free (based on intrinsic electrochemical properties of the nucleic acid target) and labelled (where redox active species is used with ds-DNA) ones [62]. Many research groups have shown that GN-based DNA biosensors exhibit high sensitivity and selectivity (the detection limits of 8 nM, 10 fM, 1 pM, respectively) because of the properties of GN [63–65].

Chen *et al.* [66] used GN-based field-effect transistors (FETs) based on large-area monolayer GN synthesised by CVD for label-free electrical detection of DNA hybridisation. The gate materials, buffer concentration and surface condition of GN have been arranged to carry out the detection of DNA sensitivity as low as 10^{-12} mol L^{-1}, which is more sensitive than the existing report based on two-layered GN-based biosensors.

Zhou *et al.* [67] developed a biosensor that chemically reduced GNO (CR-GNO) to provide well-resolved signals of all four bases; A (adenine), G (guanine), C (cytosine) and T (thymine) on the CR-GNO/glassy carbon (GC) electrode are all separated efficiently, showing that CR-GNO/GC can detect four free bases, but neither graphite nor GC can, thus there is a higher electrochemical activity than graphite GC electrodes (GCEs) (graphite/GC) (Fig. 3*a*). These four bases are also separated and detected efficiently in both ssDNA and dsDNA, which are more difficult to oxidise than free bases (Figs. 3*b* and *c*). Moreover, electrochemical detection of single nucleotide polymorphisms (SNPs) was simultaneously realised at physiological pH level (Figs. 3*d* and *e*). This also provides detection of SNP site for short oligomers with a particular sequence at the CR-GNO/GCE without any hybridisation or labelling process, suggesting the potential applications of CR-GNO in the label-free electrochemical detection of DNA hybridisation or DNA damage for further research. The unique electrochemical characteristics of GN, such as single-sheet nature, high conductivity, high surface area; exhibited high electron-transfer rate (low charge-transfer

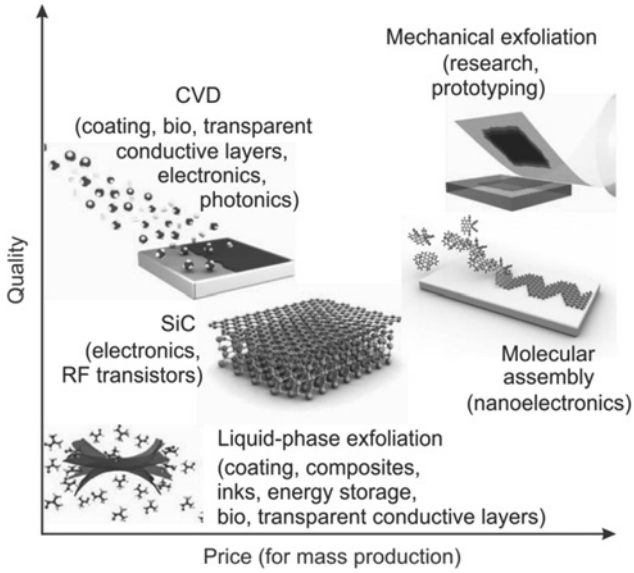

Fig. 1 *Methods for GN synthesis. There are various methods to be chosen depending on the specific application, each one differs from one another with quality and price [50]*

resistance of 160.8 Ω), wide dynamic range and lower oxidation/reduction potentials (0.20/0.10 V), which are much better than graphite/GC and GCEs.

Dan Li *et al.* [68] prepared an electrode material using GNO on which dsDNA was efficiently immobilised, which is used as a sensor for environmental monitoring. The experimental results illustrate the electrochemical activity of DNA on the electrode facilitates the electron transfer between DNA and GNO electrode. When a typical environmental pollutant, hydroquinone, was used in the electrolyte solution, the electrochemical activity of DNA on the GNO electrode was decreased, due to mixing hydroquinone with DNA. Based on this observation, the DNA-immobilised GNO electrode was further developed as the electrochemical biosensor for monitoring hydroquinone.

Tian *et al.* [69] developed a method for sequence-specific DNA detection using functionalised GN (FG) and methylene blue (MB). They found that by adding FG to aqueous analytes when MB was used as an electrochemically active DNA intercalator, DNA could be detected with high sensitivity of 48.15%, which was substantially greater than that (6.02%) obtained without FG. The experiments demonstrate that FG played a critical role in enhancing the sensitivity of DNA detection by mixing MB solution near the electrodes. Their system could also detect single-base-pair mismatches in the sequences of the probe and the target DNA. The fabrication of the system is not only simpler than fabricating GN-electrode, but also involves a probe-immobilisation process.

Bo *et al.* [70] reported fabrication of a DNA biosensor, integrating GN with polyaniline nanowires (PANIw) layer-by-layer with improved sensitivity for DNA detection. The GN/PANIw exhibited efficient differential pulse voltammetry (DPV) current responses for the complementary DNA sequences during analytical performance of the DNA sensor by using the immobilised probe to hybridise with different concentrations of target DNA. The peak currents of the ssDNA/PANIw/GN/GCE are linear with the logarithmic value of the sequence concentration from 2.12×10^{-6} to 2.12×10^{-12} mol L^{-1}. The results of the experiments indicate that the GN and PANIw can provide efficient environment and application for the direct electron transfer at the electrode surface. The ssDNA/PANIw/GN/GCE showed high selectivity and sensitivity towards the complementary DNA sequence.

Zhu *et al.* [71] applied electrochemically oxidised GN on DNA biosensor to discriminate between ssDNA and hybridised DNA based on thionine-GN nanocomposite modified gold electrode. The hybridisation reaction on the modified electrode was monitored by DPV analysis using an indicator. Under optimum conditions, the proposed biosensor showed high sensitivity for detecting complementary oligonucleotide in a wide range ($1.0 \times 10^{-12} - 1.0 \times 10^{-7}$ M) with good linearity ($R^2 = 0.9976$) and low detection limit of 1.26×10^{-13} M ($S/N = 3$).

Yang *et al.* [72] developed an electrochemical biosensor based on RGO and PANI nanocomposite as the sensitive layer of a DNA adsorbent for detecting Hg^{2+} in aqueous solution. Electrochemical impedance spectroscopy results indicated that the electrochemical

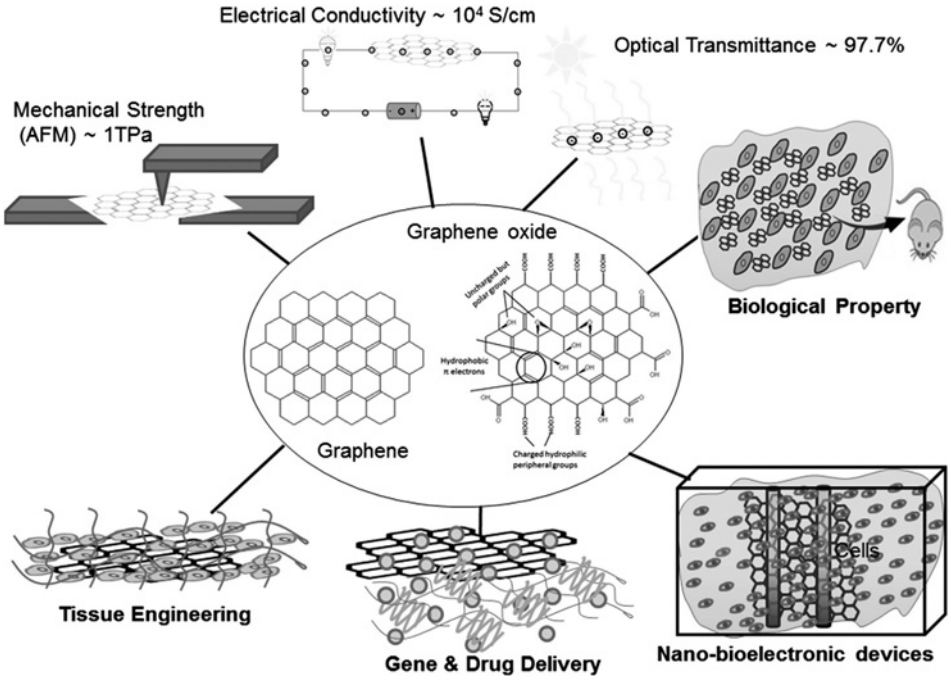

Fig. 2 *Representation of GN-based electrochemical sensors in DNA, protein and cancer cell detection with GN's excellent mechanical, electrical and optical properties [54]*

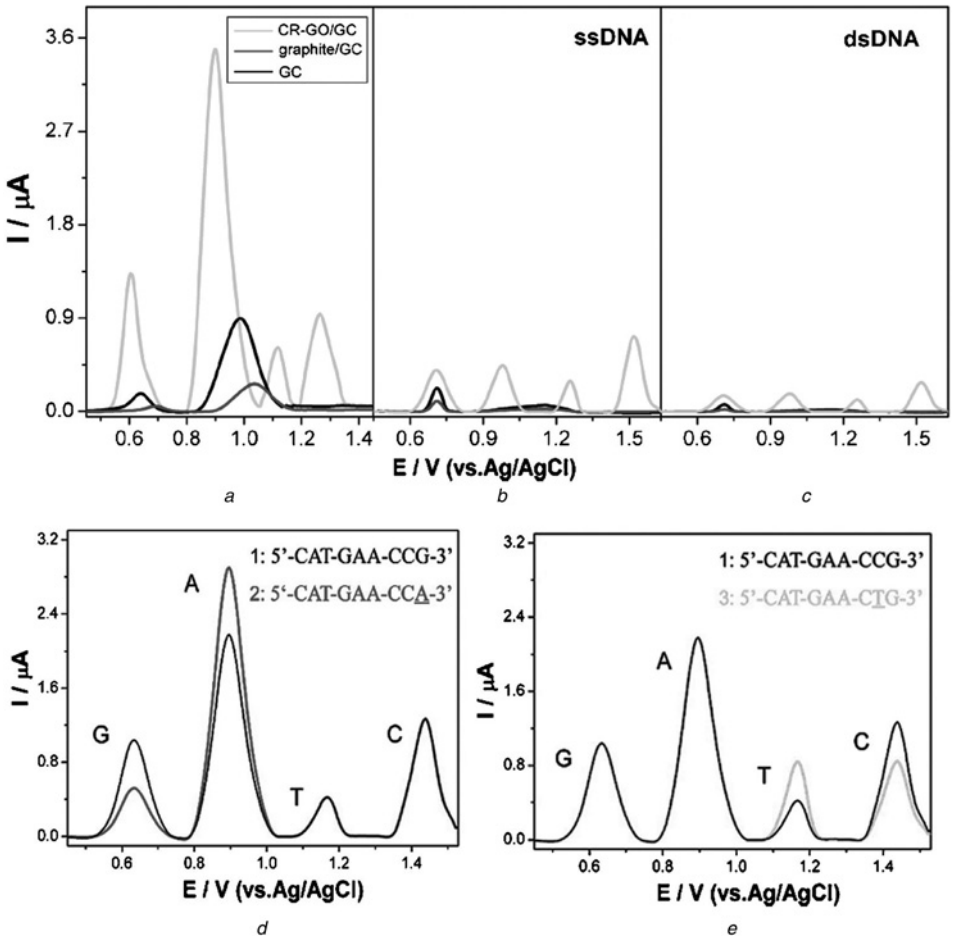

Fig. 3 *DPV curves for*

a Mixture of G, A, T and C
b ssDNA and
c dsDNA at GN/GCE (light grey), graphite/GCE (grey) and GCE (black), respectively; concentration for G, A, T, C, ssDNA or dsDNA: 10 mg/mL. Detection of SNPs of oligonucleotides including the sequence from codon 248 of the p53 gene at the GN/GCE
d DPV curves of wild-type oligonucleotide 1 and its single-base mismatch 2 (G→A mutation)
e DPV curves of wild-type 1 and its single-base mismatch 3 (C→T mutation). Concentration for different oligonucleotides: 1 (1 μM), 2 (1 μM) and 3 (1 μM); electrolyte: 0.1 M pH 7.0 PBS [67])

biosensor showed high sensitivity and selectivity toward Hg^{2+} within a concentration range from 0.1 to 100 nM with low detection limit of 0.035 nM.

Another DNA detection application is managed by Kakatkar *et al.* [73] was based on CVD GN FET biosensors. The presence of DNA and poly-l-lysine are detected by the conductance charge of the GN transistor. A dirac voltage (the voltage at which the GN's resistance peaks) is observed after the GN channel is exposed to a solution containing DNA or poly-l-lysine. This 'dirac voltage' is attributed to the binding/unbinding of charged molecules on the GN surface. It shows that the polarisation of the response changes towards positive direction with poly-l-lysine and negative direction with DNA. This results in detection limits of 8 pM for 48.5 kbp DNA and 11 pM for poly-l-lysine.

Lin *et al.* [74] reported an electrochemical DNA biosensor in which the captured DNA was immobilised on the surface of a GN-modified GCE through π–π stacking (Fig. 4*a*). Gold nanoparticles (AuNPs) modified with single nucleotide probes were then cohybridised on the surface of GCE for the detection of the targeted DNA sequence. Then, the target DNA sequence and oligonucleotide probes-labelled AuNPs were able to hybridise in a sandwich assay format, following the AuNPs-catalysed silver deposition. The deposited silver was further detected by differential pulse voltammetry. Owing to the high DNA loading ability of GN and the distinct signal amplification by AuNPs-catalysed silver staining, the resulting biosensor exhibited a good analytical performance with a wide detection linear range from 200 pM to 500 nM, and a low detection limit of 72 pM. Furthermore, the DNA biosensor could discriminate the target complementary sequence from single-base pair mismatches (Fig. 4*b*).

3.2 GN-based glucose biosensors

Diabetes is one of the most clinically critical diseases in the world and it is important to make a quantitative determination of glucose levels in the blood for the diagnosis of this disorder. This metabolic disorder results in the deficiency of insulin and hyperglycemia and is reflected by blood glucose concentration higher or lower than the normal range of 80–120 mg dL^{-1}. The disease causes many serious conditions including death and disability. Therefore, the diagnosis and treatment of this disease require close monitoring of blood glucose levels. GN supplies highly sensitive and cost-effective material for producing glucose biosensors [75]. Shan *et al.* [76] applied the first GN-based glucose biosensor with GN/polyethylenimine-functionalised ionic liquid nanocomposites modified electrode that indicates wide linear glucose response (2–14 mM, $R = 0.994$), effective reproducibility (normal standard deviation of the current response to 6 mM glucose at 0.5 V was 3.2% for ten measurements), strong stability (response current +4.9% after one week) [76].

Zhou *et al.* [67] produced a glucose biosensor based on CR-GNO. This biosensor applies enhanced amperometric signals for sensing

glucose in the blood: wide linear range (0.01–10 mM), high sensitivity (20.21 μA mM^{-1} cm^{-2}) and low detection limit of 2.00 μM ($S/N = 3$). Linear range in these experiments is wider than the other carbon-based nanomaterials. Furthermore, other differential results (i.e. the detection limit and stability) are also more efficient than any other carbon materials-based electrodes [77, 78]. The response at this CR-GNO and glucose oxidase (GO)-based biosensors indicates that the variation of glucose is very fast (9 ± 1 s to response) and highly stable (91% signal retention for 5 h), that makes this biosensor type suitable for continuously measuring the glucose level in the blood for the treatment of the diabetes.

Kang *et al.* [79] explored the efficiency of chitosan in dispersing GN and constructed glucose biosensors with the desired sensitively. It seemed that chitosan helped to form a well-dispersed GN suspension and immobilised the biomolecules, and the GN-based biosensor showed high sensitivity (37.93 μA mM^{-1} cm^{-2}) and long-term stability for measuring glucose.

GN/metal nanoparticles-based biosensors have also been developed to detect glucose. Qiu *et al.* [80] produced a Pt/PANI/GN-based biosensor for the detection of H_2O_2 (hydrogen peroxide) and glucose. The Pt/PANI/GN-based sensor exhibited a detection limit of 50 nM for H_2O_2. After the immobilisation of GO, the Pt/PANI/GN modified electrode indicated a detection limit of 0.18 μM for glucose.

Viswanathan *et al.* [81] pointed out an approach for fabricating GN-based point-of-care biosensor platform using glucose as an example of target. After immobilising enzymes to an electronically active substrate, enzymatic reactions were transduced by direct electron transport. This functionalised GN-based FET sensor has been attached to a microfluidic chip. The glucose sensor was tested under various flow conditions. The resistance across the GN decreased with increasing glucose concentrations. Three runs with the same device were performed with subsequent experiments showing similar response characteristics, but with reduced signal response corresponding to an increase in resistance at the highest glucose concentration from 2.37 to 3.30 kΩ. Even without using the GN layer chip as a FET, the device showed high sensitivity particularly at low glucose concentrations where the resistance changed by 1 kΩ when the glucose concentration increased from 0.1 to 1.0 mM. The sensor was capable of measuring resistance changes of 180 and 62 Ω for glucose concentrations of 100 mM and 1 M respectively, which are beyond normal variations of around 3–10 mM.

3.3 GN-based Hb biosensor

Hb is the most important component in the blood for transporting O_2 and CO_2 throughout the circulatory system. Change of Hb concentration in the blood can cause various disorders such as anaemia, leukaemia, heart diseases and so on, while its normal level displays the well-functioning of the organism. The normal level of Hb for males is 13.0–18.0 g/dL and for females is 12.0–16.0 g/dL [82] and Hb amount below these levels causes anaemia. It is worth mentioning that about 2 billion people, mainly women and children, worldwide suffer from anaemia. Therefore, the quantitative determination of Hb component in the blood is a clinically significant issue [83].

Xu *et al.* [84] fabricated a chitosan-GN (CS-GN) modified electrode for the electroanalysis of Hb. The cyclic voltammogram (CV) of Hb at the CS-GN/GCE showed well-defined redox peak compared with a CS-GCE. The current response of Hb at the CS-GN/GCE linearly increased from 30 to 150 mV s^{-1}, exhibiting a surface controlled electrochemical process.

Sun *et al.* [85] prepared a new electrochemical biosensor using three-dimensional GN (3D-GN) as the substrate electrode by immobilisation of Hb on the electrode surface with a chitosan film. This electrochemical process exhibited that a pair of well-resolved redox peaks appeared on CV, illustrating the realisation of direct electron transfer of Hb. Based on high conductivity and big surface area of 3D-GN, the electron-transfer coefficient (α) and the apparent heterogeneous electron-transfer rate constant (k_s) were calculated to be 0.426 and 1.864 s^{-1}, respectively. The modified electrode showed efficient electrocatalytic activity to the reduction of trichloroacetic acid (TCA), and also the catalytic reduction peak current had linear response to TCA concentration in the range from 0.4 to 26.0 mM/L with the detection limit of 0.133 mM/L (3σ).

3.4 GN-based cholesterol biosensor

Increasing of cholesterol levels in the arteries can cause serious health problems such as coronary heart diseases, cerebral thrombosis and atherosclerosis [86]. Thereby, the quantitative determination of cholesterol levels in the arteries is clinically important.

Cao *et al.* [87] explored an electrochemical biosensor for detection of cholesterol by using platinum–palladium–CS-GN hybrid (PtPd-CS-GN) nanocomposites functionalised GCE with enhanced sensitivity. The PtPd-CS-GN nanocomposite not only implemented direct electron transfer from the redox enzyme to the electrode surface, but also improved the immobilised amount of cholesterol oxidase (ChOx). Under optimal conditions, the proposed biosensor indicated wide linear values of responses to cholesterol in the concentration ranges of $2.2 \times 10^{-6} - 5.2 \times 10^{-4}$ M/L. The limit of detection is calculated as 0.75 μM/L ($S/N = 3$). The response time <7 s and the Michaelis–Menten constant (K_m^{app}) was found to be

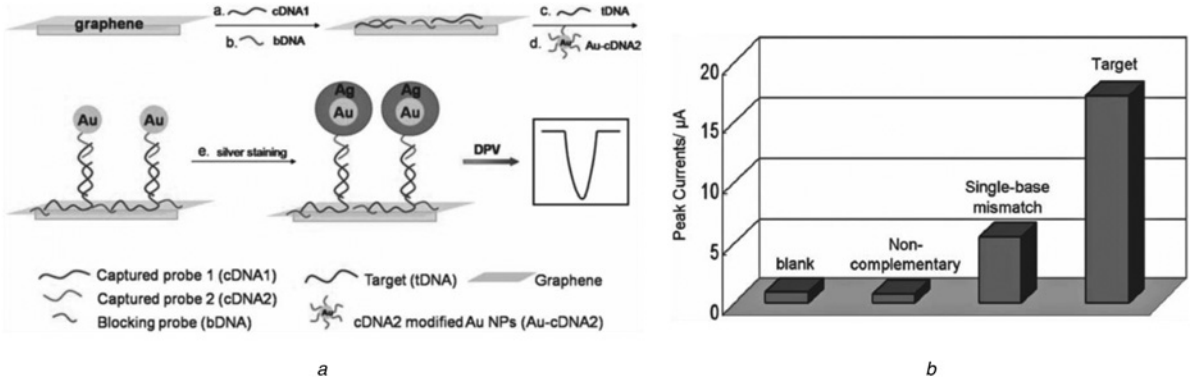

Fig. 4 *Electrochemical DNA sensor*

a Schematic diagram. In a typical experiment, captured probe 1 (cDNA1) was adsorbed on the surface of a GN-modified GCE directly (a), followed by a further adsorption of blocking probe (bDNA) (b). Different target sequences (c) and AuNPs-modified oligonucleotide probes (d) were then cohybridised to the target-active substrates in buffer solution. After silver staining (e) on AuNPs tags as a signal amplification method, a subsequent DPV technique was applied as detection means for deposited silver (silver staining process has been implemented, because oxidation of silver gives a better sensitivity). The magnitude of the anodic peak current, which corresponds to the oxidation of silver particles, reflected the amount of complementary target oligonucleotides bound to the GCE-GR/cDNA1 surface

b DPV responses of the electrochemical DNA sensor in the blank, non-complementary sequence, single-base mismatch sequence, and complementary sequence. Electrolyte: 0.1 M KNO_3. Pulse amplitude: 50 mV. Pulse period: 0.2 s. Silver staining time: 10 min [74]

0.11 mM/L. Moreover, the fabricated biosensor illustrated very efficient values of reproducibility and stability.

Li *et al.* [88] developed a novel cholesterol biosensor by immobilising ChOx on GCE functionalised by CS-GN nanocomposites. The results of transmission electrode microscopy (TEM) and Fourier transform infrared spectroscopy showed that the GNO was successfully prepared and deoxygenised. This cholesterol biosensor was based on direct electrochemistry of ChOx with an apparent rate constant (k_s) of 2.69 s^{-1}. Furthermore, this biosensor indicated that a linear response to cholesterol in the range of 0.005–1.0 mM with a detection limit of 0.715 μM ($S/N = 3$). The apparent Michaeslis-Menten constant (K_m^{app}) was also found to be 17.39 μM, which was much lower than another cholesterol biosensor work (K_m^{app} value of 110 μM) of Cao *et al.* [87]. The small K_m^{app} value indicates that the immobilised enzymes possess high enzymatic activity.

4 GN for drug/gene delivery and cancer therapy

4.1 GN for drug delivery

The development of recent and effective drug delivery systems with the ability to enhance the therapeutic profile and efficacy of therapeutic agents is one of the key issues faced by modern medicine. Delivering medicines and drug to a patient are counted as critical issues in nanomedicine (Fig. 5). The recent discovery and applications of GN have been accompanied by increasing research attention including nanomedicine. GN makes itself an ideal material to drug and gene delivery due to its high surface area (2630 m^2/g), π-conjugated structure of six-atom rings and hybridised sp^2 carbon area, it enables to attach high quantities of drug molecules onto the GN surface. Therefore, it is not surprising that GN has generated remarkable interest in nanomedicine and biomedical applications, where suitably modified-GN can serve as an effective drug delivery platform for anticancer/gene delivery, biosensing, bioimaging, antibacterial applications, cell culture and tissue engineering.

Liu *et al.* [90] first introduced the use of GN as an efficient nanocarrier for delivery of water insoluble anticancer drugs into cells. According to their approach, nanoGN oxide (NGO) was loaded by the anticancer drugs of SN38 (Fig. 6*a*) and doxorubicin (DOX, Fig. 6*b*), onto GN surface via π-stacking. In another work within the same group, in order to target cancer cells for selective cell killing, CD20+ (an activated phosphoprotein, referred as Rituxan, which is over expressed in cancer cells) antibody was further immobilised onto NGO through polyethylene glycol (PEG) molecule [91]. Zhang *et al.* [92] further explored targeted delivery of mixed anticancer drugs, DOX and camptothecin (CPT), onto an NGO surface by π-stacking and hydrophobic interactions (Fig. 6*c*), and then transported into MCF-7 cells and human breast cancer cells. The results indicated that NGO loaded with these two anticancer drugs exhibited excellent higher cytotoxicity than that of NGO loaded with only a single drug.

Fan *et al.* [93] investigated the delivery of anticancer drug 5-fluorouracil (5-FU) into HepG2 cells by developing a GN-carbon-nano-tube-magnetic-nanocomposites (GN-CNT-MNP) (Fe_3O_4). While the high specific surface area of GN allowed for higher drug loading than GN-based drug carriers alone and the iron oxide nanoparticles imparted superparamagentic behaviour to the nanocomposite, the incorporation of carbon-nano-tubes (CNTs) was found to enhance transportation of the GN-CNT-Fe_3O_4 hybrid across the cell membrane. TEM images comparing magnetic CNT nanocomposites (CNT-Fe_3O_4) and magnetic GN nanocomposites (GN-Fe_3O_4) indicated that delivery of the CNT nanocomposites are transported into the cell cytoplasm, but GN nanocomposites remained outside of the cell.

Wojtoniszak *et al.* [94] explored the antitumor activity of the methotrexate-GN-oxide (MTX-GNO) system against MCF-7 cells. Methotrexate (MTX) was covalently grafted onto GNO via amide linkage between the GN carboxyl and the MTX amide groups. The work demonstrated that a significant growth inhibition was found on the MCF-7 cells with the activity depending on the dispersants for stabilising the MTX-GNO.

Yang *et al.* [95] developed a magnetic GN-based nanocomposite to enhance the anticancer effect for a drug delivery system. The experiments illustrated that specific targeting of multifunctional GNO-Fe_3O_4 drug carriers by human breast cancer cells (SK3). After incubating GNO with human breast cancer cells (SK3) at 37°C for 1 h, the cells were observed by fluorescence microscopy. The results showed that much stronger fluorescence can be seen in the SK3 cells after incubation with GNO-Fe_3O_4-FA (folic acid)-FITC (fluorescein isothiocyanate) than with GNO-Fe_3O_4-FITC, which offers specific targeting of multi-functionalised GNO under the leading of FA molecules. The size of multi-functionalised GNO was below 200 nm and the DOX loading capacity was as high as 0.387 mg mg^{-1} in the case of the initial concentration of DOX at 0.238 mg mL^{-1}. The study clearly shows the multi-functional GNO can specifically transport the drugs to SK3 cells and show toxicity to Hela cells after loading.

4.2 GN for gene delivery

Gene delivery generally aims at treating cancer and Parkinson's disease by restoring a mutated gene or replacing a diseased one. Successful gene delivery and therapy require a gene vector that implements for DNA safety from nuclease degradation and facilitates cellular uptake of DNA with efficient transfection [96]. GN-based carriers have become the best candidates for potential vectors of gene delivery due to GN's excellent carbon structure with biomolecules.

Polyethyleneimine (PEI) is a chemically produced cationic polymer generally used as a gene vector, and it has been referenced as the most suitable polymer for the assessment of other fabricated vectors [97]. Chen *et al.* [98] analysed the use of PEI-functionalised GNO for gene delivery using different biomolecular weights of PEI. The study exhibits a pronounced lower cytotoxicity of PEI-GNO complex compared to PEI alone and efficient use of GN as nanogene delivery vector with transfection values. Zhang *et al.* [99] examined the possibility of GNO for delivery of single-stranded ribonucleic acid (ssRNA). The experiments indicated that the RGO was interacted well with ssRNA via π-stacking and delivered into Hela cells. Molecular dynamics simulations illustrated that the interactions of π-stacking between the ssRNA bases and the sp^2 bonds of the GN sheets are the key factors for the packing mechanism.

Zhi *et al.* [100] proposed a functionalised GN by adapting adriamycin and microRNA-21 (miR-21) gene delivery to overcome tumour multidrug resistance (MDR) in vitro. The gene of miR-21 is associated with the advances of MDR in breast cancer, which is a novel target for gene delivery. The miR-21 gene was effectively delivered into MCF-7/ADR cells (an ADR-resistant breast cancer line) by functionalised GN system and successfully silenced the over-expression of the miR-21 gene information. The study showed that the biological efficacy of adriamycin as an improved therapeutic agent.

Combining gene delivery with chemotherapy has been one of the most desired strategies for the early diagnosis of cancer. Whether and how the structure of GN (e.g. size, thickness) would affect the gene delivery efficiency, however, remains a critical point that requires further exploration. Small interfering ribonucleic acid (SiRNA) with therapeutic developments may also be delivered by GN nanocomposites into cancer cells for potential gene therapy [101].

4.3 GN for cancer therapy

For the early diagnosis and cure of cancer, GN plays a very critical role due to its electrochemical and physical properties (i.e. electrical conductivity, high surface area) by distributing GN loaded chemical drugs. Yang *et al.* [102] for the first time investigated the effect of functionalised GN for detecting and treating tumour cells within in-vitro and in-vivo applications. They adapted from photothermal therapy with PEGylated GNO using xenograft tumour in mouse

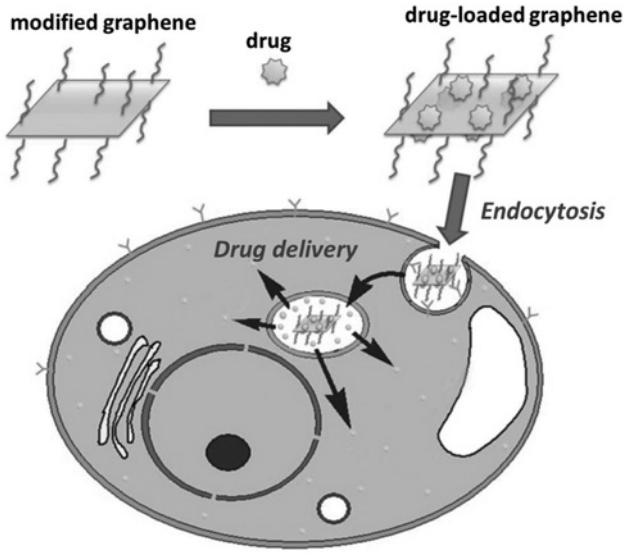

Fig. 5 *Scheme of drug delivery. Functionalised GN loaded with the drug is taken into the cell. The drug then is released into the cytoplasm [89]*

cells. According to this study, a very high tumour uptake of the PEG-GNO is detected using GN's highly effective tumour passive targeting by enhance permeability and retention effect. Furthermore, they observed that tumour destruction was accomplished under the near-infrared (NIR) laser irradiation on the tumour, using efficient absorbance of GNO in the NIR area.

Feng *et al.* [103] reported functionalised GN-based electrochemical aptasensor to find cancer cells by facilitating the high binding characteristics of aptamer AS1411 to nucleolin. The biomedical aptasensor was formed by covalent linking between the functionalised GN and NH_2-modified AS1411. The study indicated that GN-based aptasensor could separate cancer cells from normal ones and detect as low as one thousand cancer cells.

Fiorillo *et al.* [104] explored the therapeutic potential of GNO to target cancer stem cells (CSCs). They illustrated that the GNO can be used to inhibit the proliferative expansion of CSCs, across multiple tumour types. Throughout the study, the tumour-sphere assay was performed to measure the clonal expansion of single CSCs under specific conditions. Specifically, the work exhibited that GNO effectively inhibited tumour-sphere formation in multiple cell lines, across six different cancer types, including breast, ovarian, prostate, lung and pancreatic cancers as well as glioblastoma (brain). Furthermore, they also presented the reduction of the number of CSCs using a panel of specific well-established breast CSC markers (CD44 and CD24), by inducing their differentiation as they begin to express CD24. Importantly, the preliminary results show that GNO treatment does not significantly affect oxidative mitochondrial metabolism, but also suggesting that GNO does not target mitochondria.

An in-depth analysis has been developed by Zhou *et al.* [105] regarding the effect of pristine GN and GNO on inhibiting effectively the migration and invasion of the three cancer cell lines, specifically human breast cancer cells, prostate cancer cells and mouse melanoma cells. The preliminary studies indicated that exposure of cells to GN led to the direct inhibition of the electron transfer chain (ETC), most likely by disrupting electron transfer between iron-sulphur centres, which is due to its stronger ability to accept electrons compared to iron-sulphur clusters through theoretical calculations. The decreased ETC activity caused a reduction in the production of ATP and subsequent impairment of F-actin cytoskeleton assembly, which is crucial for the migration and invasion of metastatic cancer cells. The experiments of the study proved the concept by presenting the evidence that exposure of metastatic cancer cells to subtoxic GN and its derivatives attenuates their migration and invasion effectively (see Fig. 7). GN nanocomposites could enter cells and target lysosomes and mitochondria to fluorescence quenching assay. Then, GN targets to mitochondria might inhibit the activities of mitochondrial ETC complexes by affecting the function of iron-sulphur centres. In further analysis, they provided the direct inhibition of ETC complexes by GN could be ascribed to the larger electron affinity

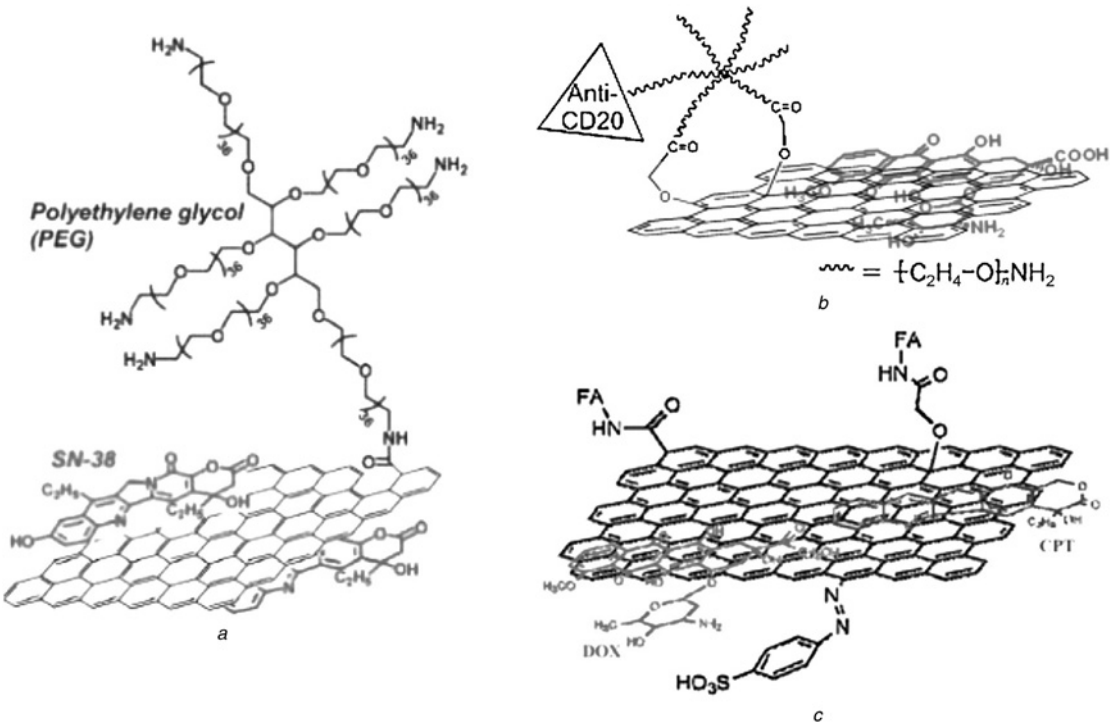

Fig. 6 *Shematic representation of*

a SN38 [90]
b DOX [91] loading onto NGO surface area within PEG Rituxan antibody molecules via π-stacking
c DOX and CPT loading onto NGO surface [25, 92]

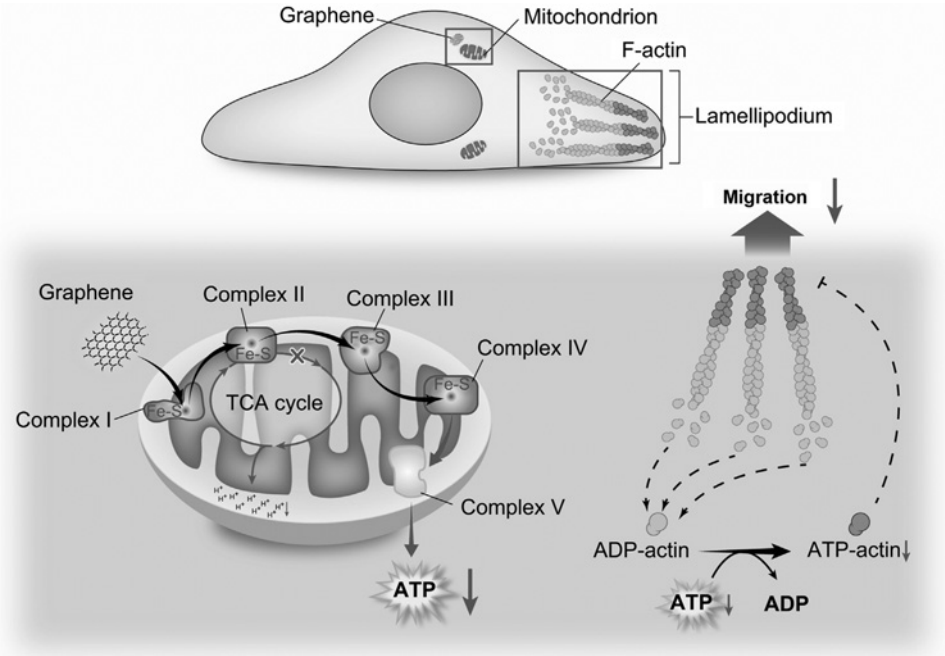

Fig. 7 *Schematic illustration of the influence of GN on the migration and invasion of metastatic breast cancer cells via impairment of mitochondrial energy production. GN directly inhibits the activity of (ETC) complexes by disturbing electron transfer. This leads to decreased mitochondrial membrane potential and reduced ATP synthesis. The migration of cancer cells, an energy consuming process, was thus significantly inhibited due to an insufficient supply of ATP [105]*

of GN compared to those of iron-sulphur clusters. The inhibition of ETC complexes decreased mitochondrial transmembrane potential, and then reduced ATP synthesis. Migration and invasion are the polymerisation of actin filaments during lamellipodia formation, which is an ATP-consuming process. Therefore, reduction in ATP synthesis after GN treatment leads to decreased actin assembly, which in turn attenuates the migration and invasion of cancer cells.

In summary, tumour-initiating cells and CSCs are difficult to eradicate with traditional approaches for detecting and diagnosis of cancer-based cells, such as chemotherapy and radiation. As a result of this, the residual CSCs will be driving the onset of tumour recurrence, and distant metastasis is problematic for treatment of cancer cells. Hence, the use of GN for the treatment of cancer, would be an alternative solution due to having high surface area for loading and delivery of a variety of biomolecules and also being non-toxic nanomaterials that form stable chemical dispersions in a variety of solutions.

5 Other applications

5.1 GN for tissue engineering

Tissue engineering is another biological field that shows promise to propose biomedical substitutes to restore, maintain and improve function, scaffold, cell or tissue of an organ [106]. The scaffold should mimic the properties and structure of the organ it aims to replace and essentially acts as an artificial extracellular matrix to support cell survival and growth. Recently, biosensors have demonstrated high potential for applications in tissue engineering. Tissue engineering is a rapidly growing field in biomedical engineering presenting enormous potential for development of engineered tissue constructs for restoring the lost functions of diseased or damaged tissues and organs [107]. Due to having excellent electrochemical and mechanical characteristics of GN, integration with appropriate biomolecules desired tissue surfaces can be engineered. Thus, GN can be potentially developed as a reinforcement material in hydrogels, biocompatible films and other tissue engineering scaffolds. Lu *et al.* [108] investigated GN-based nanocomposite materials using chitosan-polyvinyl alcohol (CS-PVA) scaffolds containing GN for wound healing. Three different categories (CS-PVA-GN, CS-PVA fibres and control-no scaffold) were studied to see the effect of GN and to realise wound healing potential in mice and rabbit cells. The experimental results indicated that the group of CS-PVA-GN healed completely and at a faster rate than others (the groups without GN) in mice and rabbit. These results were obtained by implementing antibacterial molecules using *E. coli*, *Agrobacterium* and yeast. This study illustrated the growth of prokaryotic cells *E. coli* and *Agrobacterium* was inhibited in the presence of GN with no effect on the growth of eukaryotic yeast cells.

Sayyar *et al.* [109] developed GN-chitosan (GN-CS) through a simple approach using aqueous RGO and lactic acid to create conductive hydrogels that are exhibiting pronounced swelling properties and excellent biocompatibility. The composites could be easily attached into the 3D scaffolds using additive fabrication techniques and fibroblast cells illustrate good adhesion and growth on their surfaces. Preliminary studies demonstrate that the conductivity of the composites increases with increasing addition of conducting chemically converted GN. Addition of just 3 wt% GN improves the conductivity to 1.33×10^{-1} S m^{-1} in composite films using lactic acid. Similar films using acetic acid instead of lactic acid exhibit conductivity less than those made with lactic acid. The reason of having a greater conductivity due to the presence of lactic acid was due to the improved dispersion of GN throughout the polymer matrix, most likely owing to the formation of a greater number of hydrogen bonds among hydroxyl and carboxylic groups of the composite components. Moreover, another interesting point in GN-CS films is that it exhibits high mechanical characteristics. The tensile strength and modulus of the composites in the dry state significantly increase with increasing GN. Addition of only 0.5 wt% GN, the tensile strength is improved by more than 58%, whereas the addition of 3 wt% of GN improved the tensile strength by more than 223% and Young's modulus by more than 135%. These improvements indicate that effective dispersion of GN sheets in the composite matrix and the efficient interaction between GN and the other components of the composite. These GN-CS films are desired to be used as conducting substrates for the growth of electro-responsive cells in tissue engineering.

Fan *et al.* [110] synthesised GN nanosheet (GNS) with hydroxyapatite (HA), which is a major component of natural bone

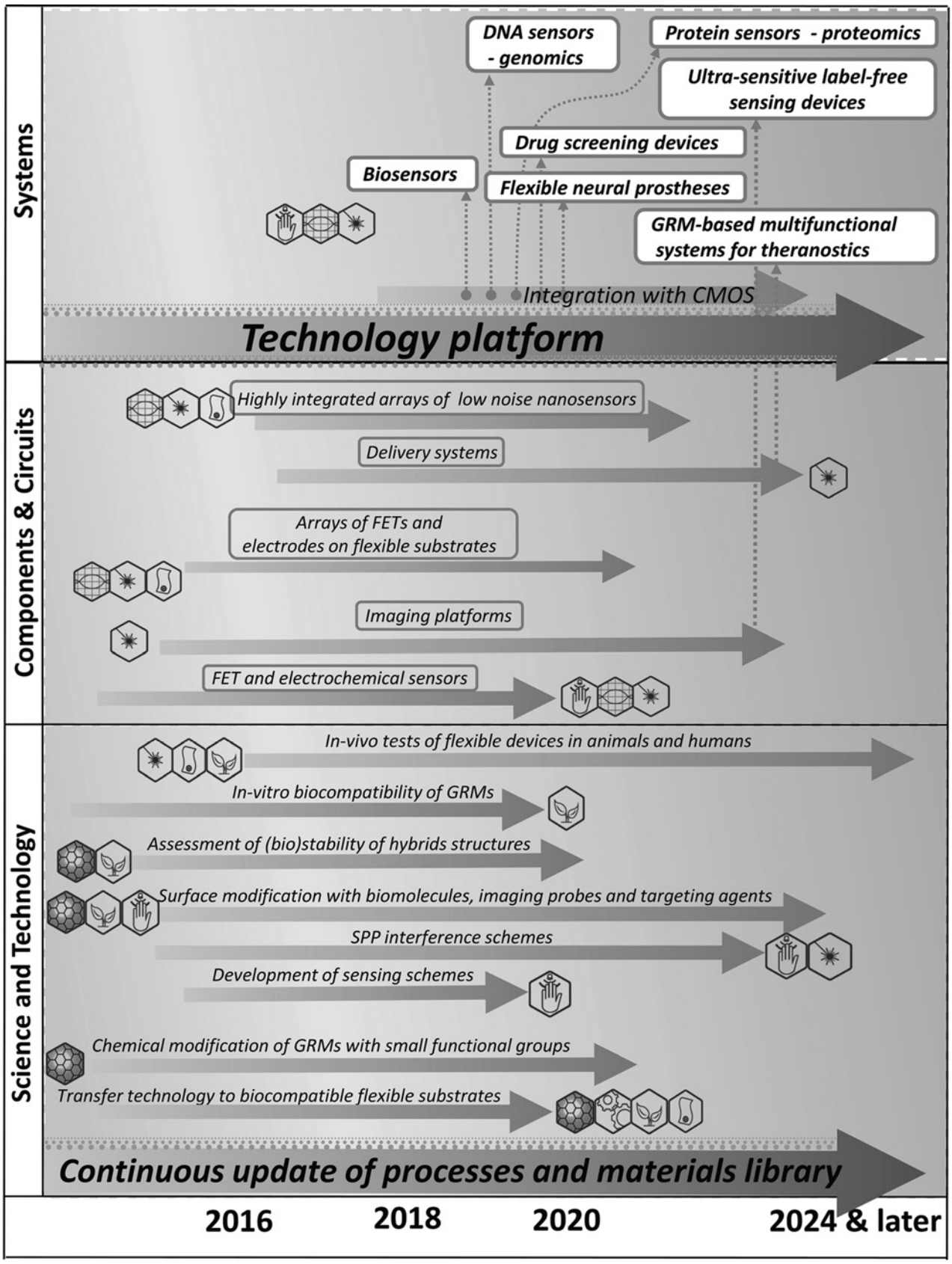

Fig. 8 *Biosensing application timescale [101]*

tissue, to see the effect of GN composites for controlling the morphology of HA and enhancing the strength of HA. Characterisation of GNS/HA composites indicated that it has an average length of 55 nm and a diameter of 13 nm. The synthesised GNS/HA composite containing 40 wt% of HA exhibits higher osseointegration ability with surrounding tissues, better biocompatibility and more superior bone cellular proliferation induction than pristine GNO and HA on their own. The experimental results demonstrated that the potential application of the GNS/HA composites as biomaterials for bone regeneration and

bone replacement as well as for the formation of highly effective tissue engineering applications.

5.2 GN for immunosensors

Immunosensors are analytical devices in which the case of formation of antigen–antibody complexes is detected and converted, by a transducer to an electrical signal, which can be processed and controlled. In immunosensing, the direct electrochemical is not possible and electrochemically active labels must be used [111]. A key reason why immunosensors are popular in clinical studies is due to the characteristic of high selectivity, sensitivity and specificity that an antibody exhibits for its target antigen [112]. For this reason, GN-based bioplatforms have demonstrated a heightened attention to get involved with electrochemical immunosensing due to the unique properties of GN such as high surface volume and structure of carbon atoms. Du *et al.* [113] analysed an electrochemical immunosensor to detect cancer biomarkers α-fetoprotein (AFP). GN sheet was functionalised with carbon nanospheres and labelled with horseradish peroxidase-secondary antibodies (HRP-Ab2) to achieve better sensitivity. The experiments exhibited that an efficient sensitivity was provided for the cancer biomarker detection that is based on a dual signal amplification strategy.

Jia *et al.* [114] developed a label-free electrochemical immunosensor based on GN nanocomposites by performing indium tin oxide (ITO) for simultaneous detection of multiple tumour biomarkers (carcinoembryonic/CEA and α-fetoprotein/AFP). RGO-thionine/Thi-Au nanocomposites were coated on ITO for immobilisation of anti-CEA, while RGO-Prussian blue/PB-AuNPs were proposed to immobilise anti-AFP. The preliminary study indicated that the multiplexed immunoassay enabled the simultaneous determination of CEA and AFP with linear working ranges of 0.01–300 ng mL^{-1}. The limit of detections for CEA is 0.650 pg mL^{-1} and for AFP is 0.885 pg mL^{-1}. In this work, GN played two main roles: first, due to the GN's high surface area, a large number of redox biomolecules (thionine and Prussian blue) and AuNPs were immobilised onto the ITO surface, which is very important for absorbing antigens and signal generation. Second, due to having great electronic characteristics, GN promoted accelerating electron transfer, which helped to achieve the signal amplification.

Recently, Jang *et al.* [115] proposed a novel 3D electrochemical immunosensor capable of high sensitive and label-free determination of prostate specific antigen (PSA), which is important for diagnosing of prostate cancer. This immunosensor was developed by coating a highly conductive GN-based Au nanocomposites within the electrode. The experimental results revealed that this 3D immunosensor operates very well over a broad linear range of 0–10 ng mL^{-1} with a low detection limit of 0.59 ng mL^{-1} using cyclic voltammetry. Moreover, it showed a significantly increased electron transfer and high sensitivity towards PSA due to facilitating GN's electrochemical properties. The GN-based Au nanocomposites were synthesised via aerosol spray pyrolysis. This is a promising technique for clinical diagnostics of prostate cancer biomarkers.

6 Concluding remarks

As a result of the unique structures and superior characteristics of GN and its derivatives, GN-based nanomaterials are amenable to be used in a wide range of applications including biomedical and sensing such as biosensors, drug and gene delivery [50]. Due to high electrical conductivity, high volume surface area, free electron movement on the surface and the availability of fabricating many GN-functionalised nanocomposites, it is favourable for synthesising of high performance electrode materials. These superior properties of GN make it possible to achieve the desired sensitivity, selectivity and reproducibility for several targets in biosensing applications.

This review selectively highlighted a variety of GN-based biosensors for the detection of biological molecules including DNA, protein and biological small molecules, developed in the last five years. Furthermore, we have also reviewed recent advances in drug/gene delivery and cancer therapy due to high specific surface area and π-stacking properties of GN. Several functional groups and free electrons on GN and GNO surfaces offer possibilities for covalent linkages of chemically diverse small molecules and proteins alike [54]. GN-based biomedical applications in tissue engineering and immunosensors were also highlighted. In these electrochemical GN-based biosensors illustrates a promising strategy in functionalised groups within GN synthesis and processing. GN has revealed fascinating performances in direct electrochemistry of enzyme, electrochemical detection of small biomolecules (ssDNA, dsDNA, sRNA), immobilisation of these biomolecules to GN-functionalised surfaces. This has been successfully used to demonstrate the potential of the technology for the diagnostics and treatment of tumour cells and also degenerative nucleic acids. Further work is necessary to extend these ideas to produce biosensors based on GN for early diagnosis and continuous monitoring of disease progression related to various cancers.

Despite these valuable developments, the use of GN-based nanomaterials for biosensors and derivative applications is still in infancy, with many challenges and limitations ongoing. Firstly, the size, electronic band-gap structure, shapes and level of oxidation and thickness properties of GN have pronounced influence on the overall performance of biomolecules immobilisation onto the surface of GN. Fabrication of reliable GN would gain the reproducibility required for accurate biosensors applications within DNA detection and drug/gene delivery. Therefore, alternative methods of synthesising GN sheets should be developed for obtaining high-quality GN-based nanomaterials. The new GN-based biosensors should allow for better control over stem cell differentiation, the distribution and targeting of hybrids in the body, as well as lower limits of achievable sensitivity and selectivity for biosensing. For instance, recently Sarkar *et al.* [116] reported that FET biosensors based on molybdenum disulphide (MoS_2), which provides high sensitivity compared to GN by more than 74-fold in pH sensing applications and also MoS_2 offers easy patternability and inexpensive device fabrication. Secondly, considerable work needs to be assessed about manufacturing portable devices for detection and diagnosis of many diseases with GN-based biosensors. Thirdly, most published papers revealed that only one target could be detected for one DNA-based sensor using GNO in solution environment. If more targets (multiple targets) can be detected by one DNA-based sensor using GNO, then the throughput of detection will be increased. In order to do that, scientists should focus on the surface chemistry during the process of converting GN to GNO, as converting GN to GNO leads to a certain extent disruption of the electron transport properties of GN [55]. Another major issue is to bring limited GN-based biomedical applications such as many applications were restricted to cancer therapeutics and need to be expanded to other therapeutics as well in new studies. Specifically, cardiovascular, brain and neurodegenerative diseases should be analysed using GN-based nanomaterials in new devices due to high electrical conductivity values. A recent development exhibited that GN was able to scavenge amyloid monomers offering protection in neurodegenerative diseases. Furthermore, researchers should also make more effort on how to explore novel biosensor designs with high selectivity in complex conditions. For instance, the most fabricated GN-based electrochemical biosensors were designed at lab-scale and are not suitable for commercial scale production. Therefore, the electrochemical biosensors need be designed and fabricated for commercial scale production with good reproducibility and low cost. In addition to this, future innovative research on GN 2D nanomaterials would couple with other major technological advance, such as lateral-flow, lab-on-chip, and 3D printing techniques for the development of next generation biosensors [117, 118]. Lastly, possible toxicity and biocompatibility issues of GN also need to be addressed to avoid any health risks in the human body. Several functionalised chemical drugs and GN-based nanomaterials have proved that they are biocompatible. However, further studies are encouraged in

order to test the cytotoxicity for a longer period of time [119]. To sum up, Fig. 8 shows the roadmap of GN in biosensing and biomedical applications within a timescale for a long-term period [101].

Overall, the utilisation of GN nanomaterials in life sciences and clinical diagnosis would be extremely promising for improvement of GN-based biosensors, therapy monitoring and its related biomedical applications. There are still challenges to be overcome in this field by effective analysis collaborating from different perspectives and researches including chemistry, physics, biology, medicine and engineering for the treatment and diagnosis of diseases.

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