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Graphene and graphene-based nanocomposites: biomedical applications and biosafety

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Graphene is the first carbon-based two dimensional atomic crystal and has gained much attention since its discovery by Geim and co-workers in 2004. Graphene possesses a large number of material parameters such as superior mechanical stiffness, strength and elasticity, very high electrical and thermal conductivity, among many others. It is the strongest and the most stretchable known material, which has the record thermal conductivity and very high intrinsic mobility, as well as being completely impermeable. Numerous favorable properties of graphene make it a potential promising material for applications in biomedicine. A large surface area, chemical purity and the possibility for its easy functionalization allow graphene to provide opportunities for drug delivery. Its unique mechanical properties suggest applications in tissue engineering and regenerative medicine. However, like other nanomaterials, graphene may pose a bio-hazard. In this article, we present a systematic review on the synthesis of graphene, various approaches for the fabrication of nanocomposites of graphene and their applications in biomedicine. A very detailed review is presented on how graphene and its nanocomposites are currently exploited for drug delivery, cancer therapy, gene delivery, biosensing and regenerative medicine. Finally, the safety and toxicity associated with graphene are also discussed.

I. Introduction

Carbon-based materials such as graphite, diamond, fullerenes, nanotubes, nanowires and nanoribbons have been used for various applications in electronics, optics, optoelectronics, biomedical engineering, tissue engineering, medical implants, medical devices and sensors.[1–6] Graphene is an important new

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addition to these carbon family materials with some unique properties. It is the first carbon-based two dimensional atomic crystal and has garnered considerable attention since its discovery by Geim and co-workers in 2004. The structure of graphene represents a one-atom thick planar sheet of sp^2-bonded carbon atoms with a honeycomb crystal lattice arrangement. This sort of arrangement in graphene, with the strong carbon–carbon bonding in the plane, the aromatic structure, the presence of free π electrons and reactive sites for surface reactions, make it a unique material with exceptional mechanical, physicochemical, thermal, electronic, optical and biomedical properties.[7,8] A large number of its material parameters such as mechanical stiffness, strength and elasticity, very high electrical and thermal conductivity, and many others,[9] are supreme. Graphene is the strongest and the most stretchable known material. It has the record thermal conductivity and very high intrinsic mobility, as well as being completely impermeable.

Graphene has a number of properties which make it potentially promising for bioapplications. Its large surface area, chemical purity and the possibility of easy functionalization render it an ideal candidate for drug delivery. Its unique mechanical properties provide opportunities for tissue engineering applications. Also, chemically functionalized graphene might find applications in fast and ultrasensitive measurement devices, capable of detecting a range of biological molecules including glucose, cholesterol, haemoglobin and DNA. Graphene is also lipophilic, which might help in solving another challenge in drug delivery—membrane barrier penetration.

Recently, graphene and its nanocomposites have been widely exploited in biomedicine for drug/gene delivery, cancer therapy, tissue engineering and biosensing. This motivated us to conduct a comprehensive review on this wonder material deployed in biomedicine. In this article we present a systematic review on the synthesis of graphene, various approaches for the fabrication of nanocomposites of graphene and their applications in biomedicine. Finally, the safety and toxicity associated with graphene are also discussed.

Most recent review papers on graphene discuss the synthesis, characterization, and semiconductor applications of graphene materials. Compared with those reviews, this review focuses mainly on the applications of graphene nanomaterials in biomedicine, especially in drug delivery, cancer therapy, gene therapy, biosensing, regenerative medicine, and biosafety. Moreover, especially in the biosensing section, we have included recent applications of graphene in the detection of micro RNAs. In particular, we systematically summarize recent progress in the modification of graphene for these biomedical applications and provide future prospects in the related fields. We expect that our review article will provide a more comprehensive guide for researchers in the field of graphene nanomaterials for biomedical applications.

II. The members of the graphene family

In analogy to carbon nanotubes which vary in wall number (single/multi-walled), surface chemistry and dimensions (length and diameter), graphene nanomaterials vary in layer number, dimension, surface chemistry, quality of sheets and purity. Various members of the graphene family are discussed very briefly in the following section.

II.A. Monolayer graphene (MLG)

As the nomenclature implies, monolayer graphene is of single layer thickness and can be isolated from graphite by repeated mechanical exfoliation of graphite flakes using an adhesive tape,[10–12] or grown on substrates *via* chemical vapor deposition.[13] Pristine graphene of significant lateral dimension is difficult to isolate and to suspend in solvents at high concentrations.

II.B. Few-layer graphene (FLG)

Few-layer graphene consists of about 2–10 flake-like stacks of graphene layers. Originally, it was a byproduct produced during attempts to fabricate monolayer graphene, but later it has become an interesting commercial material. Intercalation of various ions like sulfate, nitrate, *etc.* between the layers of natural graphite, followed by rapid thermal heating, leads to significant internal pressure buildup and hence causes massive expansion of the layered structure of graphite. This thermal exfoliation produces dry powders, which can be dispersed into FLG samples that become reinforcing agents in composite materials. The dry powder product may contain residual intercalants, often sulfur compounds, and offers the possibility of occupational exposure in high-temperature furnace operations not unlike those for carbon nanotubes.[14]

II.C. Ultrathin graphite

Ultrathin graphite is a graphite material with a thickness greater than 3–5 nm but less than 100 nm. In terms of thickness, there is a continuum of material structures from monolayer

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Table 1 Characteristics of graphene synthesized using various methods

Method of synthesis	Characteristics/remarks
Chemical vapor deposition (CVD) method	• Single layer graphene can be obtained on copper catalysts. • Can be scaled up for large area graphene production. • Most promising, inexpensive and feasible method for single layer or multi-layer graphene production. • Graphene produced is of high quality.
Exfoliation and cleavage method	• The simplest and earliest method. • Graphene extracted by micro-exfoliation shows very good electrical and structural quality. • The major shortcoming of this method is its poor scalability and production of uneven graphene films.
Epitaxial growth method	• It produces a multilayered graphene structure. • The number of layers can be controlled by process variables (time and temperature of the heat treatment). • It is difficult to functionalize graphene obtained *via* this route. • Its usage is much less in biomedical application.
Wet-chemistry approach	• It is more versatile than the methods of exfoliation and epitaxial growth. • It is easier to scale up. • It has poor control over the number of layers of graphene produced. • Graphene synthesized *via* this approach may remain partially oxidized, which can potentially change its electronic, optical, and mechanical properties.

graphene to conventional graphite powders, and it is attractive to define ultrathin graphite in this way as lying between FLG and milled graphite powders of larger minimum dimensions, which are not classified as nanomaterials.[14]

II.D. Graphene oxide (GO)

Graphene oxide is a highly oxidized form of graphene, produced *via* harsh oxidation of crystalline graphite followed by sonication or other dispersion methods to produce a monolayer material, typically in aqueous suspension.[15] The structure of GO consists of single-atom-thick carbon sheets with carboxylate groups on the periphery, where they provide a pH dependent negative surface charge and colloidal stability.[15] The basal surfaces contain hydroxyl (–OH) and epoxide (–O–) functional groups, which are uncharged but polar. The basal planes also include unmodified graphenic domains that are hydrophobic and capable of π–π interactions relevant to the adsorption of dye molecules or some drugs.[14] The result is an amphiphilic giant sheet-like molecule that can act like a surfactant and stabilize hydrophobic molecules in solution, or collect at interfaces.

II.E. Reduced graphene oxide (rGO)

Reduced graphene oxide is the product produced upon treating GO under reducing conditions, which include high-temperature thermal treatment and chemical treatments with hydrazine (N_2H_4) or other reducing agents.[14,15] Reducing GO often alters many of its properties such as decreasing its oxygen content, increasing its hydrophobicity, introducing holes or defects in the carbon lattice due to CO/CO_2 liberation, and reducing its surface charge and water dispersibility.[14]

III. Synthesis of graphene

A clear understanding of the different methods for the synthesis of graphene is key to realizing the optimum potentiality of graphene for a large variety of applications, including that in biomedicine. The size and quality of graphene produced depends largely on the approach adopted for its synthesis. In what follows, we discuss various commonly adopted methods for graphene synthesis, their merits and issues (Table 1). The major synthesis routes of graphene are depicted in Fig. 1.

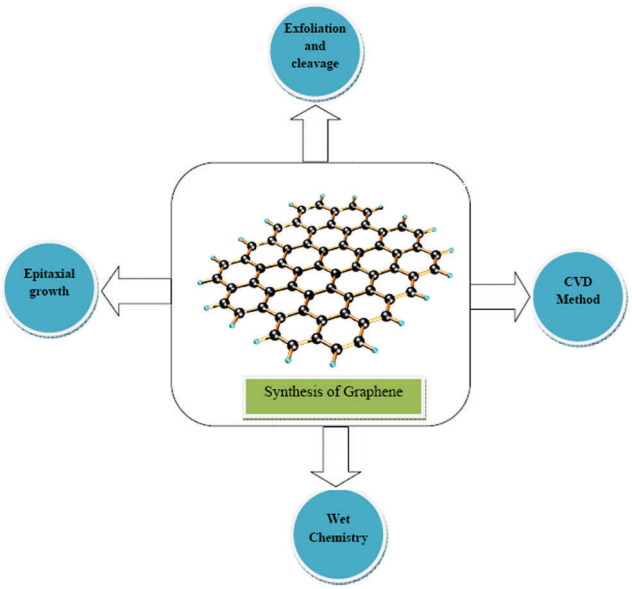

Fig. 1 Schematic representation of the synthesis of graphene.

III.A. Exfoliation and cleavage method

The simplest and earliest method adopted for the synthesis of graphene consists of micromechanical exfoliation of graphite.[10–12] Graphene layers are mechanically peeled off from highly ordered graphite using Scotch tape and then deposited on a substrate (*e.g.* SiO_2). This is a simple and efficient method in which graphene is obtained from highly ordered graphite crystals. Graphene extracted by micro-exfoliation shows very good electrical and structural quality. However, the major shortcoming of this most elementary method is its poor scalability and production of uneven graphene films.

III.B. Epitaxial growth method

It is also possible to synthesize graphene by annealing SiC crystals[13,16] at a very elevated temperature (~2000 K) under

high vacuum. Thermal desorption of Si from the surface layers of SiC crystalline wafer produces a multilayered graphene structure. The number of layers can be controlled by process variables such as time and temperature of the heat treatment. The quality and the number of layers in the samples depend on the face of SiC deployed for their growth.[17] Although the produced structure has a larger area than that yielded by the exfoliation technique, the coverage or area is still below the size required in electronic applications. Moreover, it is difficult to functionalize graphene obtained by this route and hence its usage is much less in biomedical applications.

III.C. Chemical vapor deposition (CVD) method

Graphene acquired using the CVD process has been demonstrated to possess a large area, high quality, controllable number of layers and low defects. The CVD approach has been found to be by far the most effective technique to produce high quality, large scale graphene (Table 2).

The CVD-based graphene synthesis process typically involves a thin layer of a transition metal (usually a few hundred nanometers thick) deposited on a substrate, *e.g.* SiO_2. The substrate is then put into a furnace to be heated up to about 1000 °C in a hydrocarbon gas (*e.g.* methane and hydrogen) environment. The transition metallic layer catalyzes the decomposition of hydrocarbon gas and the dissociated carbon atoms are gradually absorbed into the metal layer or diffuse/remain on the metal surface depending on the metal. Experimentally, many different transition metal catalysts (*e.g.* Ru, Ir, Pd, Ni, Cu) have been used to synthesize graphene.[18,19] The roughness of the metal substrates affects the uniformity of the graphene layers synthesized using CVD. Thinner and more uniform graphene can be synthesized on smoother Ni substrates. Metal-catalyzed graphene synthesis has been very well studied. Yet, the role of H_2 in the growth atmosphere, which is also very crucial for graphene growth, needs to be addressed properly.

By further understanding the growth mechanism and optimization of the growth conditions, it can be foreseen that high quality graphene can be routinely reproduced using this CVD technique. Further improvement of the transfer process is still highly desired to minimize the structural defects and impurities on graphene. On the other hand, direct deposition of graphene onto insulating substrates is still a challenge facing the scientific community. There is definitely an advantage in avoiding the transfer process that can be problematic as mentioned. Besides, the transfer process can be time consuming and is not an environmentally friendly process.

III.D. Wet chemistry approach

A wet-chemistry based approach is also utilized to synthesize graphene *via* the reduction of chemically synthesized graphene oxide. Graphite oxide (GO) is usually synthesized through the oxidation of graphite using oxidants including concentrated sulfuric acid, nitric acid and potassium permanganate. The intercalant is then rapidly evaporated at elevated temperatures, followed by its exposure to ultrasound or ball milling. Exfoliation of graphite oxide readily occurs in aqueous medium due to the hydrophilicity of the former. The subsequent reduction of the exfoliated graphite oxide sheets by hydrazine results in the precipitation of graphene owing to its hydrophobicity.[10] Notably, it is more versatile than the methods of exfoliation and epitaxial growth on SiC and is easier to scale up. However, it has poor control over the number of layers of graphene produced. Graphene synthesized *via* this approach may remain partially oxidized, which can potentially change its electronic, optical, and mechanical properties.

III.E. Other methods

Few additional approaches to synthesize graphene have recently been reported yet definitely need further research to render them commercially viable. An interesting bottom-up approach described surface-assisted coupling of molecular monomer precursors into linear polyphenylenes with subsequent cyclodehydrogenation to create high-quality graphene nanoribbons.[20]

Hackley *et al.*[21] reported a molecular beam epitaxy method to grow chemically pure graphene. However, it is unlikely to be used on a large scale because of its much higher costs compared to CVD methods.

Laser ablation is a potentially interesting growth technique that allows the deposition of graphene nanoplatelets on arbitrary surfaces.[22] This relatively expensive method is in direct competition with the spray-coating of chemically exfoliated graphene, thus it is unlikely to be widely implemented.[23]

IV. Nanocomposites of graphene

The physicochemical properties of polymer matrix nanocomposites depend on the distribution of graphene layers in the polymer matrix as well as interfacial bonding between the graphene layers and polymer matrix. Pristine graphene is not compatible with organic polymers and thus does not form homogeneous composites. In contrast, graphene oxide (GO) sheets are more compatible with organic polymers.[10,24,25] As a result, GO has attracted considerable attention as a nanofiller for polymer nanocomposites. Unlike graphene, graphene oxide is electrically insulating, which makes it unsuitable for the synthesis of conducting nanocomposites.

It should be noted that the physicochemical properties (like polarity, molecular weight, presence of reactive groups, hydrophobicity, *etc.*) of polymers and solvents, apart from graphene, influence the preparation of nanocomposites.[26,27] Various approaches for the synthesis of graphene-based polymer matrix nanocomposites are discussed as follows.

IV.A. Intercalative polymerization

In this method, graphene or modified graphene is first swollen within a liquid monomer. A suitable initiator is added and polymerization is initiated either by heat or radiation.[26] A large number of polymer nanocomposites have been prepared using this method, for example, polystyrene (PS)/graphene,[28,29] polymethylmethacrylate (PMMA)/expanded graphite (EG),[30,31] *etc.*

Table 2 An overview of the drug/gene delivery and biosensing applications of graphene and graphene based nanocomposites

Type of application	Drug/molecules/gene delivered/sensed	Type of graphene	Remarks	Ref.
Drug delivery	SN38	PEG-GO	NGO–PEG–SN38 complex exhibited excellent water solubility while maintaining its high cancer cell killing potency.	43
Drug delivery	DOX and CPT	Folic acid-GO	Exhibited specific targeting and much higher cytotoxicity towards MCF-7 cells.	27
Drug delivery	CPT	PNIPAM-GS	PNIPAM-GS sheets were proven to be practically nontoxic and possess a superior capability of binding CPT.	59
Drug delivery	DOX	PEG-GO	Faster release of DOX was observed.	60
Drug delivery	DOX	Gelatin-GS	The gelatin–GS–DOX complex also exhibited high toxicity towards MCF-7	61
Drug delivery	DOX	Chlorotoxin-GO	Conjugation with chlorotoxin enhanced accumulation of doxorubicin within glioma cells.	64
Drug delivery	Paracetamol and benzocaine	Pyrolytic graphite	The 2D immobilization of pro-drug derivatives *via* a non-destructive physisorption mechanism could prove to be useful for applications such as drug delivery	65
Drug delivery	Ibuprofen	Chitosan-GO	Controlled release of ibuprofen was achieved	66
Cancer therapy/drug delivery	Curcumin	Curcumin-GQD	A synergistic effect of Cur-graphene composites on cancer cell death (HCT 116) both *in vitro* and *in vivo* was observed	76
Cancer therapy/drug delivery	Chlorine6	PEG-GO	The complex showed excellent water solubility and was able to generate cytotoxic singlet oxygen under light excitation for photodynamic therapy.	78
Gene delivery, cancer therapy	Si-RNA, DOX	PEI-GO	Sequential delivery of siRNA and DOX shows a synergistic effect, which leads to significantly improved chemotherapy efficacy.	87
Gene delivery	pGL-3	PEI-GO	Intracellular tracking of Cy3-labelled pGL-3 indicates that PEI-GO could effectively deliver plasmid DNA into cells and be localized in the nucleus.	88
Gene delivery	Plasmid DNA	PEG-BPEI-rGO	The developed photothermally controlled gene carrier has the potential for spatial and temporal site-specific gene delivery.	89
Gene delivery	EGFP gene in HeLa cells	PEI-GO	Low toxicity and high transfection efficiency was observed	90
Drug delivery/gene delivery	Plasmid DNA, CPY	CS-GO	GO-CS nanocarrier is able to load and deliver both anticancer drugs and genes.	91
FET biosensing	DNA hybridizations and negatively charged bacteria	Graphene sheets labeled with gold nanoparticle–antibody conjugate	Field effect transistors based on reduced graphene from graphene oxide or graphene amine have detected DNA hybridizations and negatively charged bacteria	102
FET biosensing	Glucose and glutamate	CVD grown graphene	Glucose or glutamate molecules were detected by the conductance change of the graphene transistor as the molecules are oxidized by the specific redox enzyme.	103
FET biosensing	Streptavidin	Graphene	The peptide-enabled gFET device has great potential to address a variety of bio-sensing problems, such as studying ligand–receptor interactions, or detection of biomarkers in a clinical setting.	105
FET biosensing	DNA	rGO	The developed R-GO FET DNA biosensor showed ultrasensitivity and high specificity, indicating its potential applications in disease diagnostics as a point-of-care tool.	106
FET biosensing	H_2O_2	Graphene-polypyrrole (PPy) nanotube (NT) composite	The FET sensor provided a rapid response in real time and high sensitivity toward H_2O_2 with a limit of detection of 100 pM.	107
Biosensing	H_2O_2	CNTs grown at the graphene surface (CNT/G)	The heme peptide was immobilized on the surface of the CNT/G film for amperometric sensing of H_2O_2	108
Biosensing	Propyl gallate	Composite of graphene and single-walled carbon nanotubes (GR-SWCNTs)	The linear range of the sensor to PG was 8.0×10^{-8}–2.6×10^{-3} mol L^{-1} with a limit of detection of 5.0×10^{-8} mol L^{-1} (S/N = 3).	109
Biosensing	HCV-1b cDNA	Graphene quantum dots (GQDs)	A linear range from 5 fM to 100 pM with a detection limit of 0.45 fM at a signal-to-noise ratio of 3 and showed satisfactory selectivity and good stability	110

IV.B. Solution intercalation

In this approach, the polymer is solubilized in a solvent and graphene or modified graphene layers are allowed to swell.[32] Polymer nanocomposites such as polyethylene-grafted maleic anhydride (PE-g-MA)/graphite,[33] polystyrene (PS)/graphene,[34] polypropylene (PP)/graphene,[35] polyvinylalcohol (PVA)/graphene,[36] *etc.*, have been produced using this method.

IV.C. Melt intercalation

Graphene or modified graphene is mixed with the polymer matrix in the molten state. A thermoplastic polymer is mixed mechanically with graphene or modified graphene at elevated temperatures using conventional methods, such as extrusion and injection molding.[34,35,37] The polymer chains are then intercalated or exfoliated to form nanocomposites. A wide range of polymer nanocomposites, including polypropylene (PP)/expanded graphite (EG),[33] high density polyethylene (HDPE)/EG,[38] polyphenylenesulphide (PPS)/EG,[30,31] polyamide (PA)/EG[39] *etc.* have been prepared using melt intercalation methods.

V. Graphene in biomedicine

Numerous favorable attributes of graphene make it potentially promising for applications in biomedicine. The large surface area, chemical purity and the possibility of easy functionalization of graphene offer opportunities for drug delivery. Its unique mechanical properties suggest the use of graphene in tissue engineering and regenerative medicine.[40] Its ultimate thinness and conductivity impart it as an ideal support for imaging biomolecules in transmission electron microscopy.[41] Also, chemically functionalized graphene may lead to fast and ultrasensitive measurement devices, capable of detecting a range of biological molecules including glucose, cholesterol, haemoglobin and DNA.[42]

Effective drug loading *via* π–π stacking and hydrophobic interaction is facilitated due to the presence of delocalized surface π electrons in graphene. Additionally, the large surface area of graphene enables high-density bio-functionalization *via* both covalent and non-covalent surface modification. Graphene was first deployed in biomedicine in the year 2008.[43,44] Subsequently, various studies on the *in vivo* behavior and bioactivity of graphene have proven it to be a promising material,[45–50] which has the potential to replace the existing materials and devices used as drug delivery vehicles, tissue engineered scaffolds and grafts, biosensors, *etc.* The following sections highlight some of the recent studies using graphene-based materials mainly in the fields of drug delivery, gene therapy, photo therapy, biosensing and tissue engineering.

V.A. Graphene in drug delivery and cancer therapy

There has been a surge of interest in developing graphene for drug loading and delivery as there exists a strong interaction between hydrophobic drugs and aromatic regions of graphene sheets. Since the first report on the use of graphene oxide (GO) as an efficient nanocarrier for drug delivery by Liu *et al.*,[43] much interesting work has been carried out. Graphene oxide (GO) used for drug delivery is usually comprised of 1–3 layers (1–2 nm thick), with sizes ranging from a few nanometers to hundreds of nanometers.[44,51] The unique large and planar sp^2 hybridized carbon domain, high specific surface area (2630 $m^2 g^{-1}$), and rich oxygen-containing groups render graphene with excellent biocompatibility, physiological solubility and stability, capable of loading drugs or genes *via* chemical conjugation or physisorption. Moreover, the reactive COOH and OH groups on the surface of GO facilitate conjugation with various systems, such as polymers[52] and other biomolecules,[52–55] imparting GO with multi-functionalities for diverse biological and medical applications.

Liu *et al.*[43] synthesized PEG-functionalized nanoscale graphene oxide (NGO) sheets loaded with SN 38, a camptothecin (CPT) analogue. This complex (NGO–PEG–SN38) exhibited good water solubility retaining the high potency and efficacy of SN38. The complex also showed high cytotoxicity in HCT-116 cells and was found to be approximately 1000 times more potent than camptothecin. In a separate study, the same group explored the targeted delivery of rituxan (CD20+ antibody) conjugated PEG–NGO.[44] The non-covalent π–π stacking was used to load doxorubicin (DOX) onto a PEG–NGO conjugate. It also revealed that the drug release from the GO surface was pH dependent, suggesting the possibility of pH-controlled drug release. The pH-sensitive drug release behavior from many different GO-based drug delivery systems was also studied later by many research groups.[26,27,56–59]

In an approach for multiple drug therapy in cancer treatment, Zhang *et al.*[26,27] loaded two anticancer agents (DOX and CPT) with folic acid and SO_3H group conjugated GO *via* π–π stacking in a controllable manner. Combined loading of two drugs by GO with a folic acid ligand exhibited specific targeting and a much higher cytotoxicity to MCF-7 cells, human breast cancer cells with folic acid receptors, and more importantly, a remarkably higher cytotoxicity than GO loaded with only a single drug.

An efficient approach to functionalize graphene sheets (GS) with well-defined poly(*N*-isopropyl acrylamide) (PNI PAM) has been reported by Pan and his co-workers.[59] They explored the use of PNIPAM-GS to load the water insoluble anticancer drug, CPT, and studied its release from the PNIPAM–GS–CPT complex in water and PBS at 37 °C. The interaction of PNIPAM with graphene resulted in a hydrophilic to hydrophobic phase transition at 33 °C, which is lower than the low critical solution temperature of a PNIPAM homopolymer (37.8 °C). A superior CPT loading (18.5%) was obtained due to π–π stacking and hydrophobic interaction with graphene sheets and a higher anticancer activity than CPT. The PNIPAM-GS sheets were proven to be practically nontoxic and to possess a superior capability of binding CPT.

However, the methods adopted to functionalize graphene with polymers may affect drug release due to the diffusional barrier properties of polymers. This issue was addressed by Wen *et al.*[60] by fabricating a redox-responsive PEG detachment mechanism in PEGylated nanographene oxide for effective intracellular drug delivery.

Several natural polymers have been conjugated with graphene for drug delivery applications. Natural polymers are biocompatible, biodegradable and have low immunogenicity which can greatly reduce the toxic effects of graphene. Gelatin, as a functionalizing agent, was successfully used by Liu *et al.*[61] to load DOX onto graphene nanosheets. Gelatin–GS showed a higher drug loading capacity due to the large surface area and relatively higher π interactions. The gelatin–GS–DOX complex also exhibited high toxicity towards MCF-7. A stimuli responsive nanocarrier system for the targeted delivery of DOX to the cytosol has also been developed by Kim *et al.*[62]

Recently, an environmentally-friendly approach for the synthesis of soluble graphene using *Bacillus marisflavi* biomass as a reducing and stabilizing agent under mild conditions in aqueous solution has been reported.[63] The authors have reported the cytotoxicity effects of graphene oxide (GO) and bacterially reduced graphene oxide (B-rGO) on the inhibition of cell viability, reactive oxygen species (ROS) generation, and membrane integrity in human breast cancer cells.

Wang *et al.*[64] have developed a glioma-targeted drug delivery system based on graphene oxide. Doxorubicin was loaded onto chlorotoxin-conjugated graphene oxide (CTX-GO/DOX) with a high efficiency (570 mg doxorubicin per gram CTX-GO) *via* non-covalent interactions. Doxorubicin release was pH-dependent and showed sustained-release properties. Anticancer studies revealed that compared with free doxorubicin or graphene oxide loaded with doxorubicin only, CTX-GO/DOX mediated the highest rate of glioma cells death. Furthermore, conjugation with chlorotoxin enhanced the accumulation of doxorubicin within glioma cells. The cellular localization and distribution of DOX, GO/DOX, and CTX-GO/DOX are presented in Fig. 2.

Popoff and Fichou[65] reported that paracetamol and benzocaine molecules with a long aliphatic chain can be mobilized on highly oriented pyrolytic graphite (HOPG). The 2D mobilization of pro-drug derivatives *via* a non-destructive physiosorption mechanism was proven to be useful for drug delivery applications. Rana *et al.*[66] reported the delivery of ibuprofen by using chitosan grafted GO. Furthermore, controlled drug release can be achieved by adjusting the pH values.

Recent studies report the bacterial toxicity of graphene and suggest that it may find future application in antimicrobial product development. Previously, it has been reported that highly purified carbon nanotubes inactivate *E. coli*.[67,68] Akhavan *et al.*[69] investigated the bacterial toxicity of GO and reduced graphene oxide against Gram-negative *E. coli* and Gram-positive *S. aureus* bacteria. Both GO and rGO were effective as antibacterial materials with rGO exhibiting the strongest antibacterial effectiveness. Similar results were obtained by Hu *et al.*[70] when they investigated the antibacterial activities of both GO and rGO towards *E. coli*. Within 2 hours, the *E. coli* cell metabolic activity was reduced to approximately 70% and 13% at concentrations of 20 and 85 mg/ml, respectively. The authors confirmed these results using transmission electron microscopy,[71] which revealed that the bacterial cells lost membrane integrity. These experiments suggest that GO and rGO produce bacterial membrane damage upon contact, although the fundamental toxicity mechanism and its relationship to specific GFM material properties awaits further study.

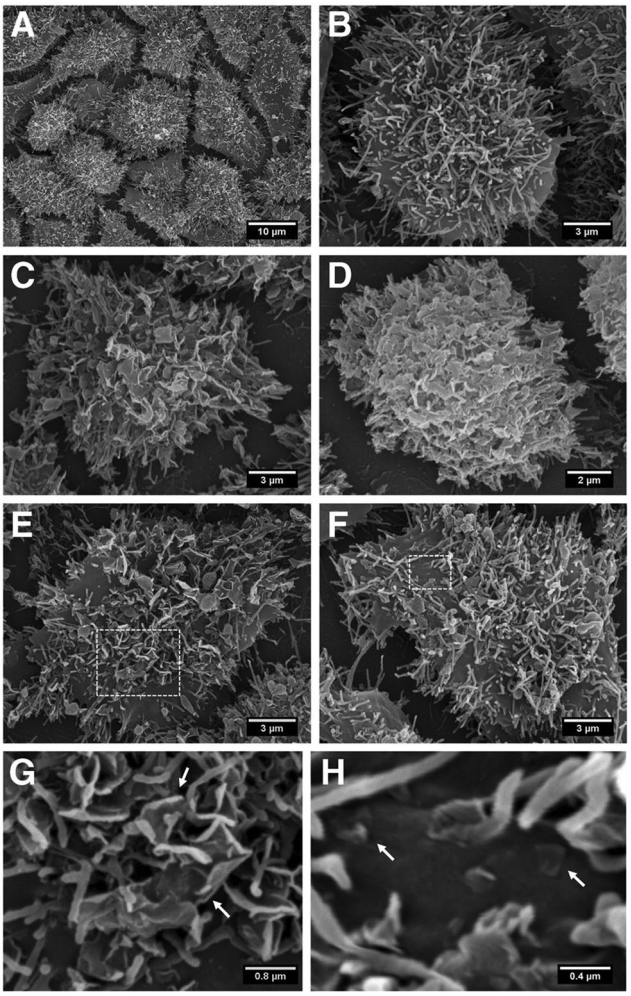

Fig. 2 Cellular localization and distribution of DOX, GO/DOX, and CTX-GO/DOX in C6 cells with the equivalent concentration of DOX (0.5 μg ml^{-1}) for 24 hours of incubation. The nuclei were stained with Hoechst 33258. Scale bar 10 μm. Adapted from ref. 64. Note: The red dots and the arrows are pointing to GO/DOX or CTX-GO/DOX. Abbreviations: DIC, differential interference contrast; DOX, doxorubicin; GO/DOX, graphene oxide loaded noncovalently with doxorubicin; CTX-GO/DOX, chlorotoxin conjugated graphene oxide loaded noncovalently with doxorubicin.

In contrast to these studies, the *Shewanella* family of bacteria are capable of metal reduction and have been shown to reduce GO in suspension cultures with no inhibition of bacterial growth.[72] Microbial reduction of GO provides a unique, nontoxic approach for the synthesis of graphene.

Intercalation of redox active metal ions such as Fe^{2+} between GO sheets may also be exploited for bacterial killing. Natural nanoscale clays containing adsorbed metals have been shown to kill bacteria. This antibacterial activity does not require direct physical contact but depends on aqueous leaching of Fe^{2+}, intracellular transport, and generation of hydroxyl radicals intracellularly resulting in bacterial death.[73] This mechanism could be exploited by designing metal-intercalated GO sheets for

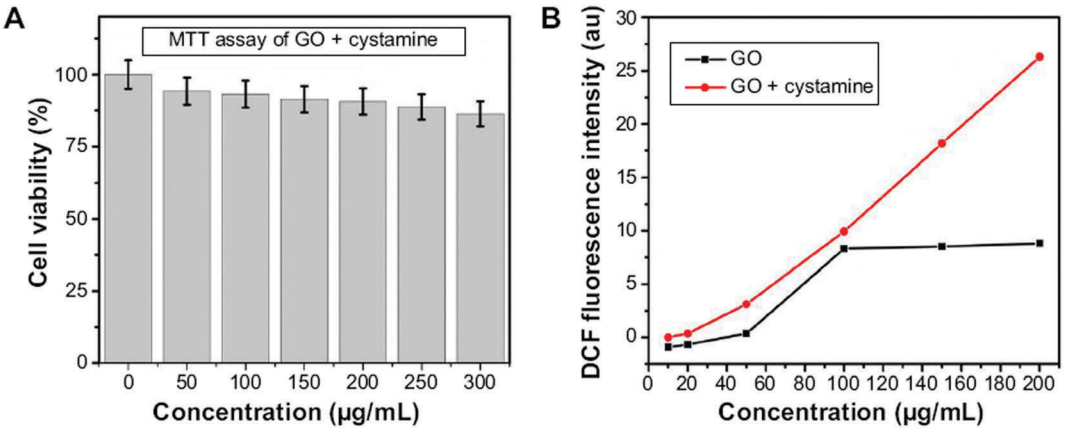

Fig. 3 Cytotoxicity and ROS studies of cystamine conjugated GO. Adapted from ref. 74. Notes: (A) cell viability of cystamine-conjugated graphene oxide (GO). (B) Reactive oxygen species (ROS) studies of GO (black color) and cystamine-conjugated GO (red color). Abbreviations: MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); au, arbitrary unit; DCF, dichlorofluorescein.

external application to treat wounds infected with antibiotic-resistant bacteria.

Cystamine-conjugated GO with low cytotoxicity, but strong reactive oxygen species (ROS) effects and high antibacterial activity was recently reported by a research group from South Korea.[74] Cytotoxicity studies with the squamous cell carcinoma 7 cells (SCC7) indicated that cystamine-conjugated GO caused a dose-dependent decrease in cell viability (Fig. 3A). It was also found that the oxidative stress induced by cystamine-conjugated GO, but not GO, increased at higher concentrations (Fig. 3B). This confirmed that ROS were generated in a concentration-dependent manner when cells were exposed to cystamine-conjugated GO.

Cheng *et al.*[75] recently synthesized a novel pH-sensitive antitumor drug from the chitosan-xanthone-graphene oxide (GCS) nanocomposite. Release of the CS antitumor parts from as-synthesized GCS showed superb pH-dependent properties, because changes in pH resulted in the breakdown of amido bonds between GO and chitosan, leading to the controlled release of CS from the nanocomposite unlike the physical mixture. In addition, GCS exhibited excellent antitumor activities when compared with xanthone and paclitaxel.

Highly efficacious graphene-derivative curcumin composites with antitumor activities were recently reported.[76] Among the graphene derivatives, graphene quantum dots (GQDs) were found to be the best composite, carrying a large amount of curcumin and serving as bioprobes for tumor imaging. The amount of drug loading increased with an increasing number of oxygen-containing functional groups of graphene derivatives. Remarkably, GQDs exhibited an ultrahigh drug-loading capacity of about 40 800 mg g^{-1}. While tumor growth was initially inhibited to some extent in the control group treated with free curcumin, the tumor size did eventually increase (Fig. 4). In striking contrast, the DGO-Cur and GQD-Cur groups exhibited a remarkable inhibition of tumor growth, with GQD-Cur- and DGO-Cur-treated mice surviving more than 14 days with almost no observable increase in tumor size (Fig. 4).

Markovic *et al.*[77] reported graphene-mediated photothermal killing of cancer cells involving oxidative stress and mitochondrial membrane depolarization, thereby resulting in mixed apoptotic and necrotic cell death. Tian and his co-workers[78] used the photosensitizer molecule, chlorine6 (ce6) loaded on PEG-functionalized graphene oxide *via* supra-molecular π–π stacking for its potential application in multifunctional cancer therapy. Shen *et al.*[79–81] used a multifunctional nanocomposite based on graphene oxide (GO-PEG-FA/Gd/DOX) for *in vitro* hepatocarcinoma diagnosis and treatment.

An electrochemically controlled drug release system for the delivery of π-orbital-rich drugs with an amino moiety, like doxorubicin and tetracycline, has recently been reported using a uniform graphene nanodot inlaid porous gold electrode prepared *via* ion beam sputtering deposition and mild corrosion chemistry.[82] The amino groups in the drugs can be easily protonated in acidic medium to become positively-charged, which allowed these drug molecules to be desorbed from the porous electrode surface *via* electrostatic repulsion when a positive potential was applied at the electrode. This study has actually confirmed the promising practical applications of microelectrodes as drug carriers for effective controlled drug delivery *via* embedding in the body.

In order to achieve the efficient, specific and controlled release of doxorubicin, a pH responsive drug carrier has been reported.[83] Graphene oxide (GO) was modified with carboxymethyl chitosan, followed by conjugation of hyaluronic acid and fluorescein isothiocyanate. This conjugate, with high drug loading (95%) and high specificity towards CD44 receptors, was reportedly used as a drug carrier to deliver doxorubicin.

Recently, in one more attempt to deliver doxorubicin, hybrids of chitosan based polyseudorotaxane (as a pH-responsive supramolecular polymer) and mesoporous silica-coated magnetic graphene oxide have been reported.[84] The drug nanocarrier has potential applications in tumor therapy due to good pH-sensitive behavior, improved solubility and high colloidal stability in biological media.

Park *et al.*[85] reported a conjugate of reduced graphene oxide with folic acid (rGO/FA) through a completely noncovalent functionalization method. This conjugate loaded with doxorubicin

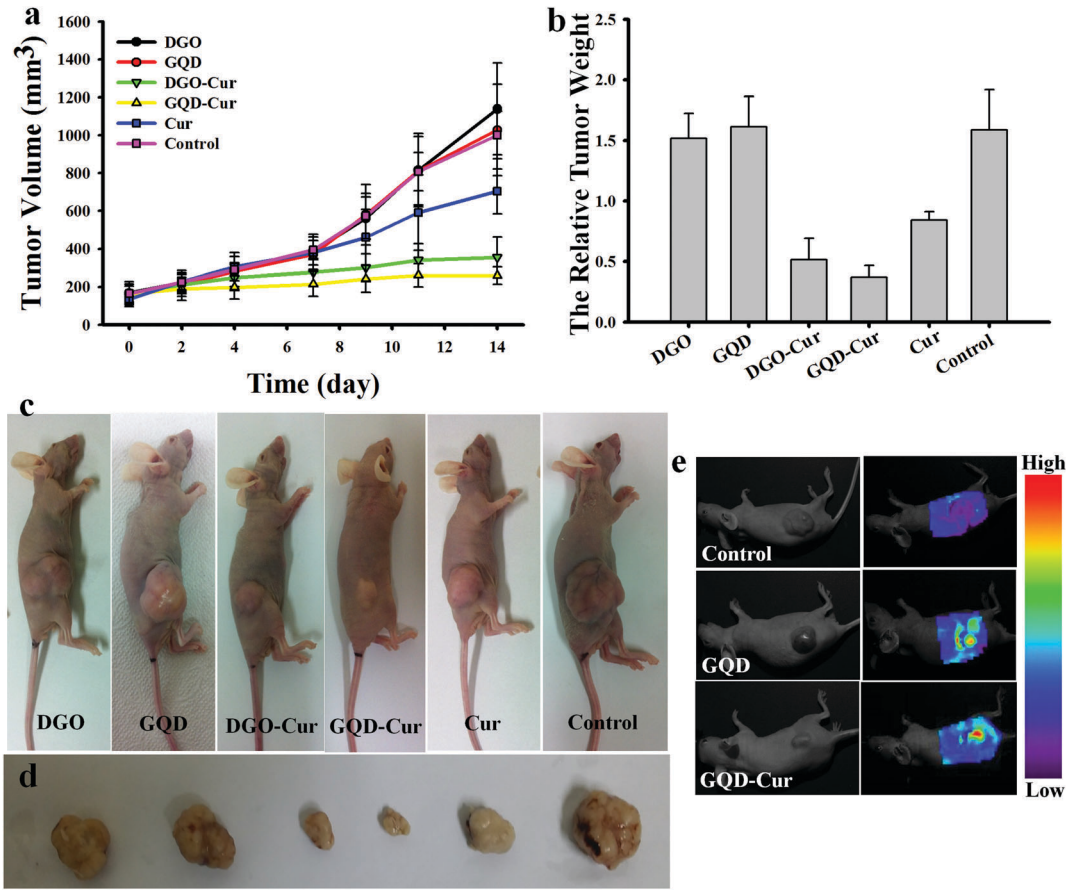

Fig. 4 (a) Relative tumor volumes of mice (n = 5, 6) treated with PBS, DGO, GQD, DGO-Cur, GQD-Cur, and Cur; (b) relative tumor weights of mice (n = 6) treated with PBS, DGO, GQD, DGO-Cur, GQD-Cur, and Cur; (c) photographs of mice treated with PBS, DGO, GQD, DGO-Cur, GQD-Cur, and Cur after 14 days; (d) photographs of tumors after 14 days of treatment with PBS, DGO, GQD, DGO-Cur, GQDCur, and Cur; (e) *in vivo* imaging of tumor-bearing mice after injection of GQDs and GQD-Cur (10 mg kg^{-1}). Abbreviations: PBS, phosphate buffer saline; DGO, double oxidized graphene oxide; GQD, graphene quantum dots; Cur, curcumin. Adapted from ref. 76.

showed specific targeting to MDA-MB 231 cells, excellent drug-release efficiency and cytotoxicity *in vitro*. Considering the simplicity and extendibility of noncovalent functionalization methods, the rGO/FA conjugate can be widely utilized for the design of new graphene-based nanocarriers.

V.B. Graphene in gene therapy

Gene therapy is a relatively new approach to treat various diseases caused by genetic disorders, including cystic fibrosis, Parkinson's disease, and cancer.[86] In 2012, Glybera became the first gene therapy treatment to be approved for clinical use in either Europe or the United States, after its endorsement by the European Commission. Gene therapy involves the use of DNA as a drug to treat disease by delivering therapeutic DNA into the patient's cells. The most common form of gene therapy is to use DNA that encodes a functional, therapeutic gene to replace a mutated gene. Other forms invoke directly correcting a mutation, or using DNA that encodes a therapeutic protein drug (rather than a natural human gene) to provide treatment. In gene therapy, DNA that encodes a therapeutic protein is packaged within a "vector", which is used to get the DNA inside cells within the body. Once inside, the DNA becomes expressed by the cell machinery, resulting in the production of therapeutic protein, which in turn treats the patient's disease. Hence, successful gene therapy essentially requires a gene vector that protects DNA from nuclease degradation and facilitates cellular uptake of DNA with high transfection efficiency.[86]

Graphene functionalized with a cationic polymer such as polyethylenimine (PEI) has been exploited in gene delivery.[87–89] PEI has been extensively investigated as a non-viral gene vector due to its strong electrostatic interactions with negatively charged phosphates of RNA and DNA. It also renders easy chemical modification to achieve increased transfection efficiency, cell selectivity and reduced cytotoxicity. Compared to PEI alone, different molecular weight grades of PEI used to functionalize graphene showed significantly lower cytotoxicity and high transfection efficiency of the PEI–GO complex.[90] A chitosan–GO complex has been exploited for simultaneous drug and gene delivery.[91] It was found that the chitosan–GO complex possesses a superior loading capacity for camptothecin, and in comparison to the pure drug, the complexes showed remarkably high cytotoxicity in HepG2 and HeLa cell lines. Moreover, the complex was found to be suitable to condense plasmid DNA into stable, nanosized complexes, and the resulting

GO–CS/pDNA nanoparticles exhibit reasonable transfection efficiency in HeLa cells at certain nitrogen/phosphate ratios. Amine-terminated PEGylated GO was successfully used to deliver high protein payloads due to non-covalent interactions with the surface of PEG–GO.[79–81] La *et al.*[92] have loaded bone morphogenic protein-2 (BMP-2) onto a Ti substrate coated with alternate layers of positively (GO–NH3+) and negatively (GO–COO–) charged GO nanosheets with a high loading efficiency and preserved bioactivity. Preclinical investigations in mice also showed robust new bone formation with Ti–GO–BMP2 implants compared with Ti or Ti–GO or Ti–BMP2 implants, making the new composite a very effective carrier for therapeutic drug delivery.

However, given the high safety, clinical and regulatory hurdles and long timescales associated with drug development, which are exacerbated when new materials are involved, it is unlikely that products using graphene-based drug delivery technology will be near the market within the next decade.

V.C. Graphene in biosensing

The sensing or detection of bio-molecules is very important for biomedical, environmental, and security purposes, and can be carried out using biosensors. A chemical sensor is a device that quantitatively or at least semiquantitatively converts information about the presence of a chemical species into an analytically useful signal. Usually, sensors consist of two structural components: a receptor and a transducer. A receptor can be any organic or inorganic material with a specific interaction with one analyte or group of analytes. In the case of biosensors, the recognition element is a bio-molecule. The second key element of the sensing platform is the transducer, which converts chemical information into a measurable signal.

Recently, graphene has evolved as a suitable candidate for the sensing of bio-molecules owing to its conductance changing properties as a function of the extent of surface adsorption, large specific area and low Johnson noise.[93–97] The nucleotide bases in single-stranded DNA bind strongly to the graphene surface *via* π–π stacking, which is greatly weakened after DNA hybridization to form double-stranded assisted development of nanoprobes for DNA analysis.[98] Wang *et al.*[97] reported the successful delivery of oligonucleotides (including aptamers) by graphene into living cells for *in situ* probing of bio-molecules. Ultra-high specific surface area and excellent electron mobility renders graphene or graphene-based nano-composites as a promising material to modify electrodes in the electrochemical sensing of various bio-molecules, including glucose, DNA and proteins.[99,100]

The zero-band gap semiconductor property of graphene renders it an ideal candidate for the fabrication of field effect transistor (FET) based biosensors. Recently, Mao *et al.*[101] reported a very sensitive (down to about 2 ng ml^{-1}) and selective FET biosensor using vertically-oriented graphene sheets labeled with gold nanoparticle–antibody conjugates. Field effect transistors based on reduced graphene from graphene oxide or graphene amine have been used to detected DNA hybridizations and negatively charged bacteria.[102] A research group from Singapore[103] demonstrated a CVD grown graphene based FET biosensor for the detection of glucose and glutamate. Glucose or glutamate molecules were detected *via* the conductance change of the graphene transistor as the molecules were oxidized by the specific redox enzyme (glucose oxidase or glutamic dehydrogenase) functionalized onto the graphene film.

Liu *et al.*[104] reported a versatile biosensing platform capable of achieving ultrasensitive detection of both small-molecule and macromolecular targets. The system featured three components: reduced graphene oxide for its ability to adsorb single-stranded DNA molecules nonspecifically, DNA aptamers for their ability to bind reduced graphene oxide but undergo target-induced conformational changes that facilitate their release from the reduced graphene oxide surface, and rolling circle amplification (RCA) for its ability to amplify a primer-template recognition event into repetitive sequence units that can be easily detected. The synergistic release of DNA probes is interpreted to be a contributing factor for high detection sensitivity.

Recently, Khatayevich *et al.*[105] reported a graphene field effect transistor (gFET) biosensor which can detect streptavidin against a background of serum bovine albumin at less than 50 ng ml^{-1}. The reported nano-sensor design allows for the restoration of the graphene surface and the utilization of each sensor in multiple experiments. The peptide-enabled gFET device has great potential to address a variety of bio-sensing problems, such as studying ligand–receptor interactions, or the detection of biomarkers in a clinical setting.

A reduced graphene oxide (rGO)-based field effect transistor (FET) biosensor used for the ultrasensitive label-free detection of DNA *via* peptide nucleic acid (PNA)–DNA hybridization has been reported by Cai *et al.*[106] A detection limit as low as 100 fM was achieved. Interestingly, the fabricated DNA biosensor was found to have a regeneration capability. The developed r-GO FET DNA biosensor showed ultrasensitivity and high specificity, indicating its potential for application in disease diagnostics as a point-of-care tool.

Recent research is now focusing on using carbon nanotube–graphene hybrid materials for biosensing. Many research groups have already reported carbon nanotube–graphene composites for biosensing applications.[107,108] While attempting to develop a highly specific and sensitive FET biosensor for the detection of H_2O_2, Park *et al.*[107] reported a liquid-ion-gated field effect transistor (FET) using a graphene-polypyrrole (PPy) nanotube (NT) composite as the conductive channel. Liquid-ion-gated FETs composed of these graphene nanocomposites exhibited a hole-transport behavior with conductivities higher than those of rGO sheets or PPy NTs. This implies an interaction between the PPy NTs and the rGO layers, which is explained in terms of the PPy NTs forming a bridge between the rGO layers. The FET sensor provided a rapid response in real time and high sensitivity toward H_2O_2 with a limit of detection of 100 pM.

A hybrid film consisting of carbon nanotubes grown at the graphene surface (CNT/G) was used as a conductive nano-scaffold for enzymes.[108] The heme peptide (HP) was immobilized

on the surface of the CNT/G film for amperometric sensing of H_2O_2. Compared with flat graphene electrodes modified with HP, the catalytic current for H_2O_2 reduction at the HP-modified CNT/G electrode increased due to the increase in the surface coverage of HP. In addition, microvoids in the CNT/G film contributed to the diffusion of H_2O_2 to modified HP, resulting in the enhancement of the catalytic cathodic currents.

Xu *et al.*[109] recently reported an imprinted sol–gel electrochemical sensor for the determination of propyl gallate (PG) based on a composite of graphene and single walled carbon nanotubes (GR-SWCNTs). Under the optimized conditions, the linear range of the sensor to PG was 8.0×10^{-8}–2.6×10^{-3} mol L^{-1} with a limit of detection of 5.0×10^{-8} mol L^{-1} (S/N = 3).

A strategy for the highly sensitive electrochemiluminescence (ECL) detection of DNA was recently proposed based on site-specific cleavage of BamHI endonuclease combined with the ECL activity of graphene quantum dots (GQDs) and bidentate chelation of the dithiocarbamate DNA (DTC-DNA) probe assembly.[110] The difference between photoluminescence and ECL spectral peaks suggested that a negligible defect existed on the GQD surface for generation of an ECL signal. Using hepatitis C virus-1b genotype complementary DNA (HCV-1b cDNA) as a model, a novel signal-off ECL DNA biosensor was developed based on the variation of the ECL intensity before and after digestion of the DNA hybrid. This ECL biosensor for HCV-1b cDNA determination exhibited a linear range from 5 fM to 100 pM with a detection limit of 0.45 fM at a signal-to-noise ratio of 3 and showed satisfactory selectivity and good stability, which validated the feasibility of the designed strategy.

Several microRNAs (miRNAs), a class of short non-coding RNA molecules, have already been implicated in common human disorders. It has been shown that the expression levels of some miRNAs are reduced in chronic lymphocytic leukemia, colonic adenocarcinoma, and Burkitt's lymphoma samples providing possible links between miRNAs and cancer.[111] Recently, a stable, sensitive, and specific miRNA detection method on the basis of cooperative amplification combined with graphene oxide (GO) fluorescence switch-based circular exponential amplification and the multimolecule labeling of SYBR Green I (SG) was reported.[112]

The ability to discriminate ssDNA and double-stranded nucleic acid structures, coupled with the extraordinary fluorescence quenching of GO on multiple organic dyes, recently allowed the simultaneous and selective detection of several miRNAs labeled with different dyes in the same solution.[113] In another interesting work, purposefully inserting mismatches at specific positions in DNA (probe) strands showed increased specificity against miR-10b, over miR-10a, which varies by only a single nucleotide.[114] Though the authors have demonstrated the discrimination of miR-10b from miR-10a, the approach can be extended to other short RNA molecules which differ by a single nucleotide. It was also reported that unlocked nucleic acid (UNA) is 50 times more powerful than DNA in discriminating miR-10b from miR-10c.[115]

Peptide nucleic acid (PNA) as a probe for miRNA sensing offers many advantages including high sequence specificity, high loading capacity on the NGO surface compared to DNA and resistance against nuclease-mediated degradation. A Korean research group developed a nanosized graphene oxide (NGO) based miRNA sensor for quantitative monitoring of target miRNA expression levels in living cells.[116] Their strategy was based on the tight binding of NGO with PNA probes, resulting in fluorescence quenching of the dye that is conjugated to the PNA, and subsequent recovery of the fluorescence upon addition of target miRNA.

Circulating oncomiRs like miR-141 and miR-21 are highly stable diagnostic, prognostic, and therapeutic tumor biomarkers, which can reflect the status of the disease and response to cancer therapy. Over-expression of miR-141 is observed in advanced prostate cancer patients; however, miR-21 is significantly elevated in the early stage, but not in the advanced stage of prostate cancer. A recently published work demonstrated the simultaneous detection of exogenous miR-21 and miR-141 from human bodily fluids including blood, urine and saliva using nanographene oxide.[117]

It appears that graphene sensors are superior to CNT-network sensors. This may be attributable to several reasons: (1) the sensitivity of the CNT network is impaired by the presence of metallic tubes; (2) the functionalization of enzymes is more effective and uniform on the flat graphene film than on small nanotubes; and (3) the functionalization steps may alter the tube-to-tube contact in the CNT network or lead to the loss of some nanotubes.

V.D. Graphene in regenerative medicine

Regenerative medicine involves the process of tissue engineering of previously irreparable tissues or organs. This multidisciplinary medical area has gained a lot of momentum due to the recent major progress in cell and organ transplantation. Tissue engineering strategies include three major components: cells, signaling molecules, and natural or artificial scaffolds. Such scaffolds have been developed for use in various tissues such as bone,[118–120] cartilage,[120] muscle,[121] skin[122] and nerve.[123] Scaffolds for use in regenerative medicine provide the base for the repopulation and specialization of stem cells, blood vessels and extracellular matrices.[124] In general, the surface morphology of the scaffold strongly affects the attachment of surrounding cells and tissues after implantation. Nanostructures at the surface of the base material enhance some bioactivities due to quantum size effects and the material's large surface area.[125] Early contact between regenerative cells or tissues and the nanostructures facilitate the tissue-reforming process. Recent studies clearly revealed that graphene-family nanomaterials, such as graphene, GO, or rGO, support the adhesion and proliferation of mammalian cells including human mesenchymal stem cells (hMSCs),[126] human osteoblasts,[126,127] fibroblasts,[128] and adenocarcinoma cells.[129]

A recent investigation suggested the possible application of graphene oxide (GO)-decorated hybrid fiber sheets composed of poly(lactic-*co*-glycolic acid, PLGA) and collagen (Col) (GO-PLGA/Col) prepared *via* dual electrospinning as skin tissue engineering scaffolds.[130]

Baniasadi *et al.*[123] reported the development of conductive porous scaffolds for peripheral nerve regeneration by incorporating conductive polyaniline/graphene (PAG) nanoparticles into a chitosan/gelatin matrix. This work supports the use of a

porous conductive chitosan/gelatin/PAG scaffold with a low amount of PAG (2.5 wt%) as a suitable material having appropriate conductivity, mechanical properties and biocompatibility that may be appropriate for different biomedical applications such as scaffold materials in tissue engineering for neural repair or other biomedical devices that require electroactivity.

Hybrid nanoparticles of graphene sheets decorated with strontium metallic nanoparticles for bone tissue engineering have recently been reported.[131] Strontium-decorated reduced graphene oxide (RGO_Sr) hybrid nanoparticles were synthesized and macroporous tissue scaffolds were prepared by incorporating RGO_Sr particles into poly(ε-caprolactone) (PCL). The PCL/RGO_Sr scaffolds were found to elute strontium ions in aqueous medium. Osteoblast proliferation and differentiation was significantly higher in the PCL scaffolds containing the RGO_Sr particles in contrast to neat PCL and PCL/RGO scaffolds. This study demonstrated that composites prepared using hybrid nanoparticles that elute strontium ions can be used to prepare multifunctional scaffolds with good mechanical and osteoinductive properties.

An artificial matrix (Fn-Tigra), consisting of graphene oxide (GO) and fibronectin (Fn), was recently developed on pure titanium (Ti) substrates *via* an electrodropping technique assisted by a custom-made coaxial needle for possible bone tissue engineering applications.[132] The morphology and topography of the resulting artificial matrix is orderly aligned and composed of porous microcavities. In addition, Fn is homogenously distributed and firmly bound onto GO as determined *via* immunofluorescence and elemental mapping, respectively. Cell proliferation and viability are significantly higher on Fn-Tigra and Tigra than that of cells grown on Ti. Furthermore, enhanced *in vitro* osteogenic differentiation of preosteoblasts cultured on Fn-Tigra over those cultured on bare Ti was observed.

VI. Biosafety of graphene

Theranostic application of any nanomaterial warrants great care to ensure that its toxicities are well characterized. Unfortunately the reported studies related to toxicities with graphene and its composites are significantly less when compared to that for carbon nanotubes.[133] One of the defining characteristics of graphene materials is high surface area, and hence can be expected to be potent sorbents for a variety of small molecule solutes in physiological fluids. Adsorption on carbon surfaces is generally favored for molecules with low solubility, partial hydrophobicity, or positive charge (for the common case of negatively charged graphene materials), and for molecules with conjugated π-bonds that impart planarity and allow π–π interactions with graphenic carbon surfaces. The biological consequences may include micronutrient depletion.[134]

An *in vitro* evaluation of GO on A549 cells suggested no obvious cytotoxicity and revealed the absence of the entry of GO into the A459 cells.[135] However, the authors reported dose-dependent oxidative stress in the cell and the induction of a slight loss of cell viability at high concentrations of GO. In another attempt to evaluate the biocompatibility of graphene on human fibroblast (HDF) cells, Wang *et al.*[136] reported obvious cytotoxicity (at a dose exceeding 50 μg ml^{-1}) such as decreasing cell adhesion, inducing cell apoptosis, entering into lysosomes, mitochondrion, endoplasm, and cell nucleus. Moreover, the authors have also reported that graphene oxide under a low dose (0.1 mg) and middle dose (0.25 mg) did not exhibit obvious toxicity towards mice, but under a high dose (0.4 mg) graphene oxide exhibited chronic toxicity, such as 4/9 mice death and lung granuloma formation, mainly located in the lung, liver, spleen, and kidneys, and almost cannot be cleaned by the kidneys. Liao *et al.*,[137] in an attempt to assess the cytotoxicity of graphene and GO in human erythrocytes and skin fibroblasts, found that at the smallest size, graphene oxide showed the greatest hemolytic activity, whereas aggregated graphene sheets exhibited the lowest hemolytic activity. Coating graphene oxide with chitosan nearly eliminated the hemolytic activity.

Taken together, these results demonstrate that the particle size, particulate state, and oxygen content/surface charge of graphene have a profound impact on biological/toxicological responses to red blood cells. The study also revealed that compact graphene sheets are more damaging to mammalian fibroblasts than the less densely packed graphene oxide. Clearly, the toxicity of graphene

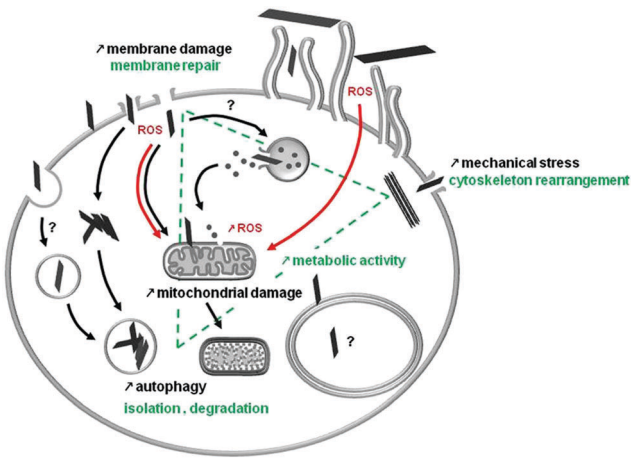

Fig. 5 SEM micrographs of Hep G2 cells after exposure to GO and CXYG for 24 h. Images A and B show SEM micrographs of non-treated cells at 1500× and 5000× magnification. The scale bars in these images correspond to 10 and 3 μm, respectively. Control-cells demonstrate healthy cell morphology with numerous microvilli protruding from the cell surface. Images C and D show SEM micrographs of cells treated with 16 μg ml^{-1} GO and 32 μg ml^{-1} CXYG, respectively. GO and CXYG platelets deposited and formed a layer completely covering the cell surface. The scale bar displayed in C and D corresponds to 3 and 2 μm, respectively. Images E and F show SEM micrographs of cells treated with 8 μg ml^{-1} GO and CXYG, respectively. At this concentration cells were only partly covered with nanomaterials. The scale bars in E and F correspond to 3 μm. The boxed-in areas (white, dotted line) are shown at higher magnification in images G and F, respectively. Image E shows the interaction of micro-sized GO platelets (white arrows) with microvilli. Image F shows the interaction of CXYG nanoplatelets with lateral dimensions between approximately 200 and 400 nm with the plasma membrane (white arrows). The scale bar in G and H is 0.8 and 0.4 μm, respectively. Adapted from ref. 138.

and graphene oxide depends on the exposure environment (*i.e.*, whether aggregation occurs or not) and the mode of interaction with the cells (*i.e.*, suspension *versus* adherent cell types). Recently a research group from Spain[138] confirmed that graphene oxide (GO) and carboxyl graphene (CXYG) nanoplatelets physically interact with Hep G2 cells and cause plasma membrane damage in a dose dependent manner. Exposure to GO and CXYG was furthermore found to induce oxidative stress, alter metabolic activity and cell ultrastructure (Fig. 5). A hypothetical model has also been proposed for possible internalization and cytotoxicity of graphene nanomaterials (Fig. 6). Li *et al.*[139] studied the *in vivo* distribution and pulmonary toxicity of nanoscale graphene oxide (NGO) following intratracheal instillation. Radioisotope tracing and morphological observation demonstrated that intratracheally instilled NGO was mainly retained in the lung (Fig. 7). NGO could result in acute lung injury and chronic pulmonary fibrosis. In addition, the study also revealed that the biodistribution of ^{125}I-NGO varied greatly from that of ^{125}I ions. Therefore, it is possible that nanoparticulates can deliver radioactive isotopes deep into the lung, which may settle and result in mutations and cancers.

Several studies, to assess graphene related toxicities, have reported that the toxicity of graphene-related nanomaterials in

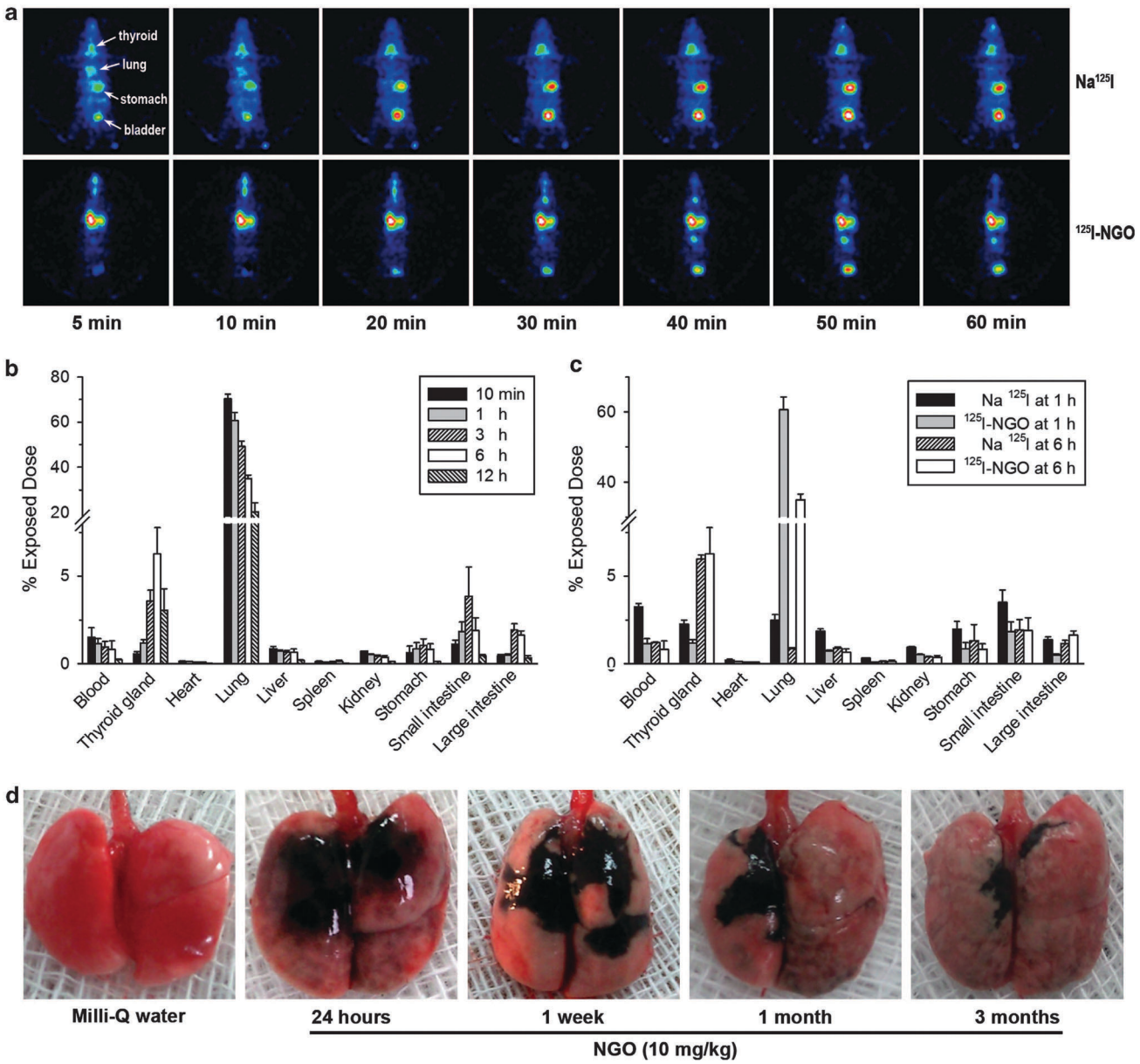

Fig. 6 Hypothetic model of graphene nanomaterial internalization and cytotoxicity. GO and CXYG nanoplatelets, which penetrate through the plasma membrane into the cytosol, are concentrated and encapsulated in intracellular vesicles. Cells respond with the formation of cytokeratin filament bundles to mechanically reinforce the plasma membrane and initiate plasma membrane repair mechanisms. These processes involve an increase in metabolic activity. Exposure to GO and CXYG nanoplatelets results in elevated intracellular ROS levels, perturbation of mitochondrial structure and function, and an augmented number of autophagosomes. Adapted from ref. 138.

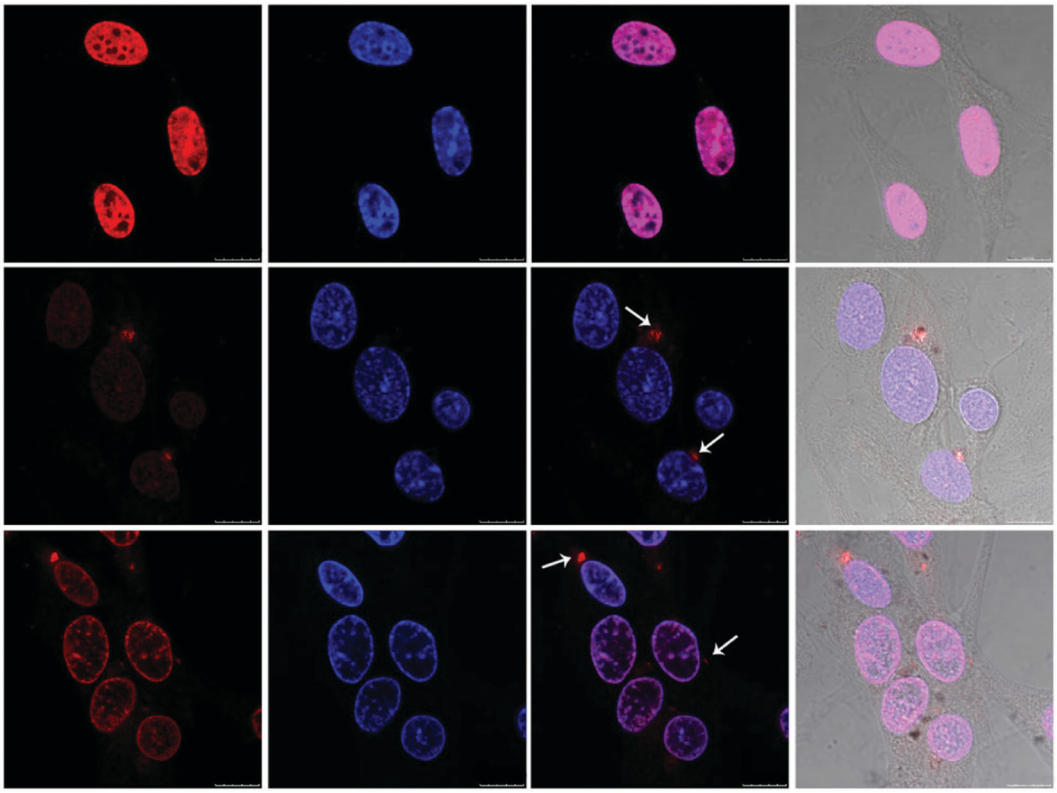

Fig. 7 Biodistribution of NGO after intratracheal instillation. (a) SPECT images of mice at several time points after intratracheal instillation with ^{125}I-NGO or Na^{125}I. (b) Distribution of ^{125}I-NGO in the blood and major organs of mice at five different time points. N = 5 in each group. Values are presented as the mean ± s.e.m. (c) Comparison of Na^{125}I and ^{125}I-NGO distribution in mice at 1 and 6 h after intratracheal instillation. N = 5 in each group. Values are presented as the mean ± SEM. (d) The morphological observation of the lungs from mice instilled with Milli-Q water or 10 mg kg^{-1} NGO. The dorsal view shows the distribution of NGO (black region). Adapted from ref. 139.

biological systems may be influenced by their physiochemical properties, such as surface functional groups and structural defects. GOs prepared using four different oxidative treatments with varied oxygen content/functional groups were investigated by measuring the mitochondrial activity in adherent lung epithelial cells. The results suggest that there is a correlation between the amounts of oxygen content/functional groups of GOs and their toxicological behavior towards the A549 cells.[140]

VII. Conclusions

In the last decade, there has been a very steep increasing trend observed in literature studies regarding graphene nanomaterials in biomedical applications. Graphene family nanomaterials have been exploited, such as carbon nanotubes for small molecule drug delivery, gene delivery, cancer chemotherapy, phototherapy, biosensing and regenerative medicine. There are also reports of graphene nanomaterials exhibiting antimicrobial activity. Functionalization of graphene *via* physical and chemical methods also provides the possibilities and challenges of tailoring the properties of graphene for biomedical applications. This manipulation includes sizes, geometries, band gaps, doping levels, functionalized chemical groups and so on. Despite current and future challenges, graphene research provides huge potential for material and functional applications and it is still progressively active around the world.

Some new two-dimensional graphene derivatives like graphyne, graphdiyne, graphone, and graphane have been proposed recently.[141] Graphyne and graphdiyne are two-dimensional carbon allotropes of graphene with honeycomb structures. Graphone and graphane are hydrogenated derivatives of graphene. Because these materials are close to graphene they deserve further, careful, and thorough studies for their potential in biomedical applications.

Clearly, long-term adverse health impacts must be considered in the design of graphene and its derivatives for drug delivery, tissue engineering, and bio-molecular sensing. Future research is likely to be directed towards exploring more fundamental biological responses to these wonder materials including systematic assessment of their physical and chemical properties related to toxicity. Complete material characterization and mechanistic toxicity studies are essential for the safer design and manufacturing of graphene and its nanocomposites in order to optimize biological applications with minimal risks for environmental health and safety. Due to the high diversity, properties, and advantages of graphene, a multitude of nanocomposite-based applications have been envisioned to be practical. These multifunctional

graphene composites coupled with affordable cost will soon be seen in the global market.

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