EXPERT OPINION

The application of graphene oxide in drug delivery

Yongzheng Pan, Nanda Gopal Sahoo & Lin Li†
†*Nanyang Technological University, School of Mechanical and Aerospace Engineering, Singapore*

Introduction: As a shining star in material science, graphene oxide (GO) and its derivatives possess potential applications in a variety of areas. Among them, the application of GO to drug delivery has attracted ever-increasing interest in the past few years.

Areas covered: In this article, the authors summarize the latest progress of utilizing GO in the field of drug delivery. In particular, the functionalization of GO, cytotoxicity of GO and its derivatives, *in vitro* and *in vivo* drug delivery and the comparison with carbon nanotube-based delivery systems are discussed. Future perspectives and possible challenges in this emerging field are briefly described.

Expert opinion: GO and its derivatives are highly attractive for the application to drug delivery due to their exceptional physiochemical properties and unique planar structure in spite of some existing challenges, such as the reproducibly smart functionalization of GO and the investigation of its long-term toxicology.

Keywords: carbon material, drug delivery, functionalization, graphene oxide

Expert Opin. Drug Deliv. (2012) 9(11):1365-1376

1. Introduction

Graphene, a single layer of sp^2-hybrirdized carbon atoms packed in a honeycomb crystal lattice, not only has attracted a great deal of research interest and generated great impact on scientific community, but also has revealed a cornucopia of potential applications in various fields since its discovery [1-3]. Due to its unique two-dimensional (2D) structure and geometry, monolayer graphene possesses remarkable physicochemical properties including high Young's modulus (~ 1100 GPa), high fracture strength (~ 125 GPa), excellent electrical (~ 100 S/cm) and thermal conductivity (~ 5000 W/mK), fast mobility of charge carriers (~ 200,000 cm^2/Vs) and large specific surface area (theoretically calculated value, 2630 m^2/g) [3,4]. These remarkable properties of graphene provide essentially infinite possibilities for various applications in many areas such as electronics, energy storage and conversion, biotechnology and nanocomposite materials. Perfect graphene does not exist naturally, but bulk and solution-processable functionalized graphene materials including graphene oxide (GO) can now be easily prepared [5]. Oxygen functional groups (e.g., hydroxyl, epoxide and carbonyl groups) attached on the basal planes and edges of GO sheets significantly alter the van der Waals interactions between the layers of graphene and impart the desired solubility in water and some organic solvents. Additionally, these groups enable GO to be functionalized through covalent and non-covalent approaches, thus making it a building block for the fabrication of versatile functional graphene-based materials [6,7].

Recently, it is found that the planar structure and π-conjugated structure of GO endowed it with excellent ability to immobilize a large number of substance, including metals, drugs, biomolecules and fluorescent molecules [8]. More recently, a number of research groups have explored GO's application as a molecular carrier for *in vitro* and *in vivo* drug delivery, as well as the successive cancer therapy. Dai

10.1517/17425247.2012.729575 © 2012 Informa UK, Ltd. ISSN 1742-5247

Article highlights.

- The latest progress in the field of GO's application to drug delivery.
- Various functionalizations of GO for its application to drug delivery.
- Opinions on future development of GO-based drug delivery system.

This box summarizes key points contained in the article.

and collaborators group did the pioneering work on the use of polyethylene glycol (PEG) functionalized GO (PEG-GO) as a nanocarrier to bind water-insoluble anticancer drugs and evaluated its cytotoxicity to human colon cancer cell [9]. Subsequently, GO and GO derivatives have been intensively used in drug delivery [10]. **Figure 1** shows the continuously increasing research efforts for applying GO in the field of drug delivery in the past 5 years. The critical need for building versatile drug carrier systems lies on the high-capacity loading, efficient delivering and specific targeting [11-14]. GO-based nanomaterials could be one of the most favorable nanocarriers for the efficient drug loading and delivery due to their large specific surface with unique optical and electrochemical properties.

In addition to the application to drug delivery, GO and its derivatives have a wide range of potential applications in the biomedical field, ranging from fluorescence resonance energy transfer (FRET) biosensor, living cell detecting and imaging, antibacterial materials, to biocompatible scaffolds for cell culture. Shen *et al.* [15], Wang *et al.* [16], and Feng and Liu [17] have reviewed the various routes for functionalization of GO and the impressive achievements of the applications obtained in the biomedical area, which provide a comprehensive profile and potential challenges in this newly emerging research field. The present review summarizes and discusses in detail the recent development of GO's application specifically in the field of drug delivery during the past few years. Functionalization of GO through both covalent and non-covalent approaches, and the toxicity of GO and its derivatives are discussed first. After that, drug delivery efficiency of GO-based systems for different cancer drugs in terms of *in vitro* cell uptake and *in vivo* cancer therapy are discussed. Finally, the authors will compare the GO-based drug delivery systems with the systems based on carbon nanotubes (CNTs) before drawing conclusions and offering their opinion.

2. Functionalization of GO

GO, without any surface functionlization, usually has sheet dimensions of hundreds of nm and tends to aggregate in physiological solutions with salts and proteins due to screening of electrostatic charges and non-specific binding of proteins. As a result, size control and individual separation are necessary for GO to suitably interact with a biological system *in vitro* and *in vivo*. As well, rational functionalization is essentially needed to offer GO with better compatibility and stability in the physiological environment [18]. To achieve these purposes, two strategies, covalent conjugation and non-covalent physisorption, have been reported.

2.1 Covalent functionalization

GO sheets have a lot of chemically reactive oxygen-containing groups on their surfaces. Therefore, a wide range of reactions can be triggered from either the carboxylic acid groups at their edges or the epoxy and hydroxyl groups on their basal planes. In the past few years, different waster-soluble and biocompatible polymers or molecules [19], including PEG [9,20-23], poly(vinyl alcohol) (PVA) [24], polyethylenimine (PEI) [25,26], poly(*N*-isopropylacrylamide) (PNIPAM) [27], polysebacic anhydride (PSA) [28], chitosan (CS) [29-31], amphiphilic copolymers [32,33], sulfonic acid groups [34] and amino groups [35], have been grafted on the surfaces of the GO sheets through covalent methods to increase their biocompatibility. With increasing solubility and stability in physiological solutions, the polymer-grafted GO nanocarriers could indirectly enhance the dissolution of the water-insoluble anticancer drugs.

The covalent functionalization of GO for its application in drug delivery was initiatively carried out by Dai and collaborators in 2008 [9], which was motivated by their success of CNT-based drug delivery systems. They synthesized PEGylated nanoscale graphene oxide (PEG-GO) and used it as a nanocarrier to load variant anticancer drugs via non-covalent physisorption, and evaluated its photoluminescent property and *in vitro* cellular uptake capacity. PEG is widely used in various pharmaceutical preparations due to its water solubility and low toxicity. As-prepared GO is soluble in water but tended to aggregate severely in physiological solutions that were rich in salts or proteins, such as cell medium and serum, whereas the PEG-GO exhibited an excellent stability in these solutions (**Figure 2A and B**). Due to the sonication steps during synthesis, the lateral size of PEG-GO was reduced to 5 – 50 nm (**Figure 2C**), which was much smaller than that of as-prepared GO sheets (50 – 500 nm) (**Figure 2D**), thus making it easier for PEG-GO to cross cellular barriers. In the light of these finding, the authors synthesized a series of polymer-grafted GO nanocarriers for water-insoluble anticancer drug delivery [24,27,31,33]. The incorporation of polymers onto GO sheets endued the resulting nanohybrids with a high solubility and stability in physiological solutions. Taking advantages of the recent progress on the preparation of well-defined copolymers, the authors made an efficient approach to functionalize GO sheets with a well-defined PNIPAM via click chemistry [27]. The PNIPAM-grafted GO sheets (PNIPAM-GS) consisted of about 50% polymer, which endowed the sheets with a good solubility and stability in physiological solutions, as well as a practical non-toxicity.

However, the polymer coatings on the surface of GO sheets may result in a diffusion barrier effect during the intracellular drug release procedure and adversely affect the successive

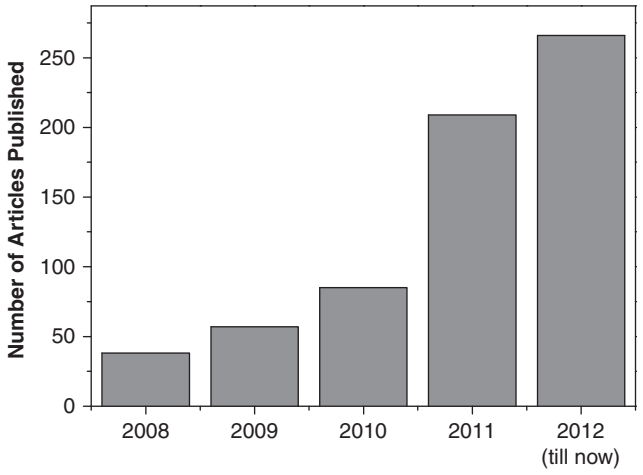

Figure 1. Number of articles published in the last few years pertaining to GO-based drug delivery applications.
Based on data taken from www.sciencedirect.com on 18 July 2012 using 'graphene' and 'drug delivery'.

therapy [36,37]. If the polymer coatings can be cast off on a specific stimulus, such as a reduction environment on the tumor site, the loaded drug will be released rapidly when the delivery systems arrive in target cells, thus leading to a more effective therapy. Shi and collaborators developed a unique GO-based nanocarrier for drug delivery, and the underlying mechanism of GO design is shown in **Figure 3**. This system consisted of GO sheets with a sheddable PEG shell attached via a disulfide linkage (GO-SS-mPEG, where mPEG is methoxy PEG) that can respond to glutathione (GSH) changes for intracellular drug delivery [21]. The cytosolic GSH concentration in some tumor cells was found to be at least four times higher than that in normal cells, so it could be used as an ideal stimulus for the PEG detachment. The synthesis route of GO-SS-mPEG is shown in **Figure 3**. Amino-terminated PEG bearing a disulfide bond (mPEG-SS-NH_2) was synthesized by coupling the succinate-activated mPEG-COOH with cystamine, and then conjugated with GO sheets to obtain the GO-SS-mPEG, which displayed a high solubility and stability in both phosphate buffered saline (PBS) and cell medium. The disulfide bond (-S-S-) between grafted PEG and GO sheets were stable in plasma but could be cleaved quickly by reducing agents like GSH on the tumor site, hence making GO-SS-mPEG a smart drug delivery system with high efficiency.

In addition to the synthetic polymers, a great deal of efforts has been made to use natural biopolymers as reagents for the functionalization of GO sheets because of their better biocompatibility, biodegradability and low immunogenicity. Gelatin is a linear polypeptide that is composed of different amounts of 18 amino acids, which is the thermally and hydrolytically denatured product of collagen [38]. Recently, Liu *et al.* successfully utilized gelatin as both the reducing and functionalizing agents to synthesize gelatin-grafted GO nanosheets (gelatin-GNS) [39]. The GO with a concentration of 0.2 mg/ml substantially aggregated in PBS and fetal bovine serum (FBS), whereas the gelatin-GNS with the same concentration exhibited an excellent stability and no aggregation was observed in 24 h, indicating the good biocompatibility and stability of gelatin-GNS. Moreover, gelatin had a mild reductive effect to GO because of its abundant amine pendant groups. Hence, gelatin-GNS had a relatively larger π-conjugated size, which endowed it with a higher drug-loading capacity. Another natural biopolymer used for functionalization of the GO sheets is CS, a naturally occurring linear cationic polysaccharide, which has found widespread applications in drug and gene delivery, tissue engineering and as a pharmaceutical ingredient. The authors reported the functionalization of GO by a low molecular weight CS via a facile amidation process [31]. The solubility of CS-grafted GO sheets (CS-GO) in PBS and cell medium was substantially enhanced due to the benign solubility of low molecular weight CS. As a nanocarrier, CS-GO was proved to sequentially deliver camptothecin (CPT) drug and a report DNA (pRL-CMV) into human cancer cell lines, leading to the significantly enhanced anticancer efficacy. It is interesting to note that a similar conception was reported by Ha and collaborators at the same time [30]. They demonstrated the successful loading and controlled release of an anti-inflammatory drug, ibuprofen (IBU) and an anticancer drug, 5-fluorouracil (5-FU) by using CS-GO sheets as vehicles.

The covalent functionalization for GO sheets can also be realized by introducing small molecules onto the GO sheets. In a recent study done by Zhang *et al.*, GO sheets were functionalized with sulfonic acid groups (SO_3H), followed by a covalent grafting of folic acid (FA) molecules to the GO sheets [34]. The FA-conjugated GO (FA-GO) could be well dispersed and maintained stable for several months in D-Hanks buffer, a physiological solution. Yang *et al.* prepared FA-GO-Fe_3O_4 nanohybrid by conjugating GO-Fe_3O_4 with FA with the aid of (3-aminopropyl)triethoxysilane (APS) [35]. The combination of GO and FA provides a novel molecular-recognition strategy to specifically carry anticancer drugs into folate-receptor-positive malignant cells, which paved the way for the development of smart drug delivery systems.

2.2 Non-covalent functionalization

GO sheets also exhibit non-covalent binding with some molecules via hydrophobic interaction, π-π interaction or van der Waals interaction on the sp^2 networks that are not oxidized. These molecules include amphliphilic polymers, aromatic compounds and conjugated polymers. Amphiphilic polymers are widely used to increase the solubility, drug-loading capability and antibiofouling ability of GO. Recently, Hu *et al.* prepared a graphene derivative by non-covalently functionalizing GO sheets with pluronic F127, an amphiphilic triblock copolymer with excellent biocompatibility [32]. In this study, GO is reduced to GNS in order to increase

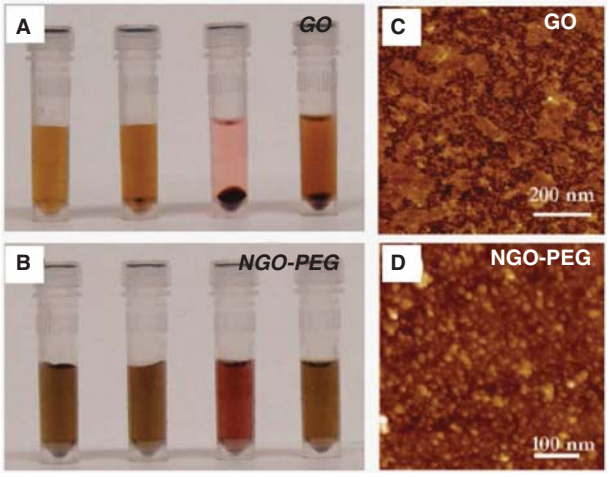

Figure 2. PEGylation of graphene oxide: photos of GO (**A**) and PEG-GO (**B**) in different solutions recorded after centrifugation at 10,000 *g* for 5 min. GO crashed out slightly in phosphate buffered saline (PBS) and completely in cell medium and serum (top panel). PEG-GO was stable in all solutions; AFM images of GO (**C**) and PEG-GO (**D**) [9].
Reproduced with permission from [9]. Copyright (2008) American Chemical Society.

π-conjugated size before coated by F127. The poly(propylene oxide) (PPO) segments in F127 bound to the surface of GNS through hydrophobic interactions and the poly(ethylene oxide) (PEO) segments imparted F127/GNS nanohybird the excellent solubility and stability in both of aqueous solutions and physiological environment. The precipitation test showed that the suspension of F127/GNS in a cell medium could keep steady for more than 1 week. The combination of GO and FA, which provides a molecular-recognition strategy as is mentioned above, can also be fulfilled by non-covalent functionalizations. Liu *et al.* constructed a nanosupremolecular assembly of non-covalently linked FA-modified β-cyclodextrin (FA-CD) and GO using an adamantine-grafted porphyrin [40]. The resulting quarternary supramolecular assembly showed the benign solubility in physiological conditions and can be employed as a targeted drug delivery system.

However, the adsorption of polymers onto GO surface via a non-covalent way is not as strong as the covalent linkage and vulnerable to the variation of external environment, which makes the drug delivery systems not so stable when interacting with biological systems *in vitro* or *in vivo*. On the other hand, non-covalently functionalized GO may load less quantity of aromatic drugs because conjugated areas of the GO sheets are partially occupied by coated polymers. Zhang *et al.* prepared PEI-GO by a non-covalent adsorption method via the electrostatic and hydrogen-bonding interactions between PEI and GO, and compared its drug-loading efficacy with PEI-GO via covalent way [25]. They found that the non-covalently prepared PEI-GO was not as stable in saline as the PEI-GO made by a covalent method. In 10% NaCl solutions, it completely precipitated in 24 h, probably due to the gradual desorption of the attached PEI, thus limiting its further application to drug delivery.

3. Cytotoxicity of GO and functionalized GO

The cytotoxicity of GO and its derivatives must be extensively investigated *in vitro* and *in vivo* if they are employed as drug nanocarriers. Several research groups have deliberatively explored this critical issue.

Most of the *in vitro* experimental results to date have suggested that as-prepared GO was a safe material at low concentrations in a variety of cells such as human fibroblasts [41,42], HeLa cells [43], L929 cells [44], human hepatoma HepG2 cells [45] and A549 human lung cancer cells [46]. Some research groups even demonstrated the applications of GO papers being the substrate for the cell growth [47,48]. Wang and collaborators carried out a comprehensive investigation on the *in vitro* toxicity of GO by examining the influences of GO on the morphology, viability, mortality and membrane integrity of A549 cells [46]. The results suggested that GO did not enter the cells and had no obvious cytotoxicity. However, it was uncovered that GO aroused oxidative stress and induced the slight decrease of the cell viability at a high GO concentration (200 mg/l). The concentration-dependent toxicity of GO was also found by Wang *et al.* GO with a concentration less than 20 mg/l did not exhibit toxicity to human fibroblast cells, whereas the concentration of more than 50 mg/l exhibits obvious cytotoxicity such as decreasing cell adhesion, inducing cell apoptosis, entering into lysosomes, mitochondrion, endoplasm and cell nucleus [42].

On the other hand, *in vivo* studies indicated severe chronic toxicity associated with GO, especially at a high dose. Zhang *et al.* systematically studied the distribution and biocompatibility of GO in mice by using radiotracer technique and a series of biological assays [49]. When mice were exposed

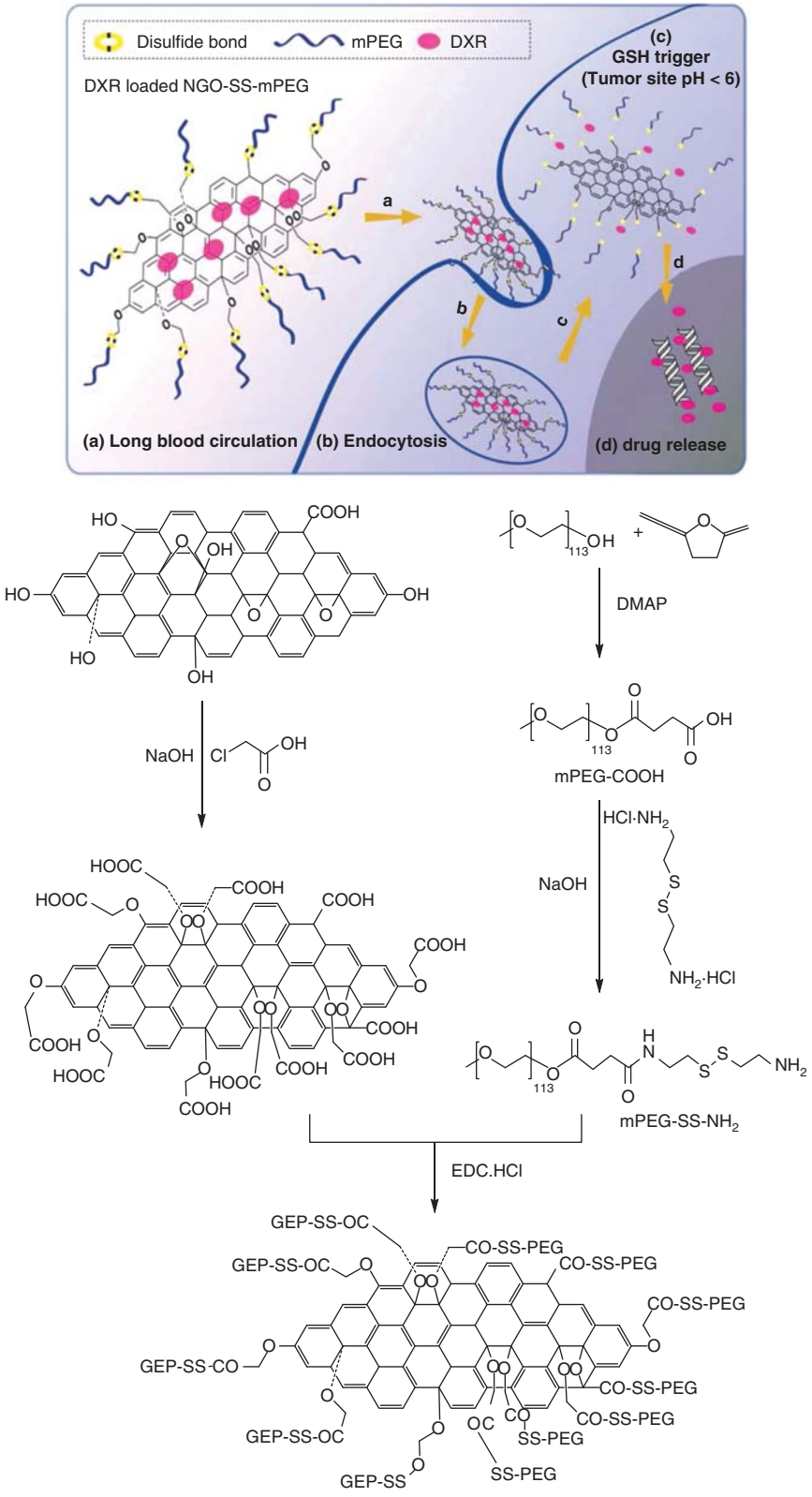

Figure 3. Upper: Schematic diagram showing antitumor activity of redox-sensitive DOX-loaded GO-SS-mPEG. Lower: Synthesis route of disulfide linked GO-SS-mPEG [21].

Reproduced from [21] with permission of John Wiley and Sons.

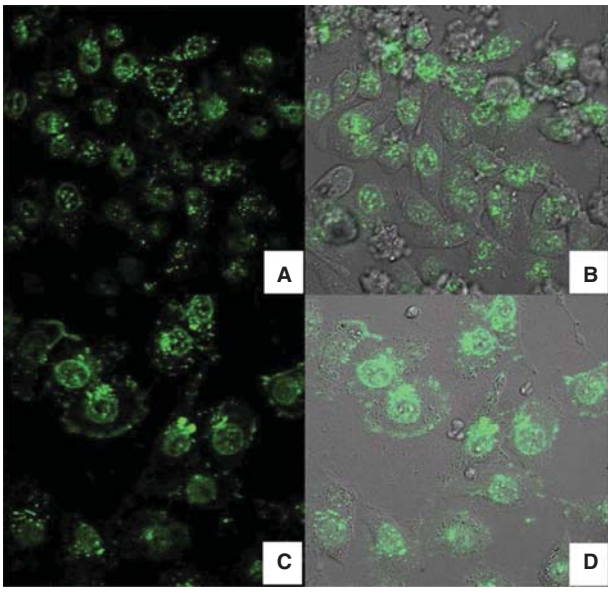

Figure 4. Laser confocal fluorescence micrographs of HepG2 cells treated with 0.25 mg/ml DOX (**A, B**) and DOX-loaded GNC-rGO (**C, D**). (**A, C**) Fluorescence micrographs. (**B, D**) Overlay of the morphological and fluorescence images after incubation for 14 h. Images were acquired at 400-fold magnification [8].
Reproduced from [8] with permission of John Wiley and Sons.

to 1 mg/kg body weight of GO for 14 days, no pathological changes were observed in examined organs, indicating that GO might be non-toxic and suitable for biomedical applications at a low injection dose. However, it is revealed that GO at a relatively higher injection dose (10 mg/kg) showed dominant accumulations in the lungs after being intravenously injected into rats or mice and were hardly excreted for long periods of time, leading to serious toxic effects and significant pathological changes, such as inflammation cell infiltration, pulmonary edema and granuloma formation. The *in vivo* study examined by Wang *et al.* also uncovered that the chronic toxicity of as-prepared GO was closely related to the injected dose [42]. Injection dose of GO at 0.1 and 0.25 mg per mouse did not cause mortality of exposed mice, and showed no obvious clinical toxic signs, whereas four of nine mice treated with 0.4 mg per mouse died and serious lung granuloma formation was found from histopathology of lung tissue. GO was widely found in lung, kidney and liver in the form of severe agglomeration, and could hardly be cleaned, which were mainly due to the screening of electrostatic charges and non-specific binding to protein in bloodstreams, as well as the large lateral sizes.

Functionlization, either covalent or non-covalent, can effectively improve the biocompatibility of GO and almost eliminate its toxicity *in vitro* and *in vivo*. In particular, functionalization is capable of reducing the strong hydrophobic interaction between GO and cells. It has been proved that functionalization can lead to a reduction of reactive oxygen species, which mediates cell apoptosis through caspase-3 activation [50]. For example, Liu *et al.* reported that PEG-GO showed negligible *in vitro* toxicity to various cell lines even at high concentrations up to 100 mg/l [9]. Singh *et al.* also reported that the positively charged GO-NH_2 is more biocompatible than GO, since they found that GO-NH_2 neither demonstrated stimulatory action toward platelets nor induced pulmonary thromoembolism in mice [51]. More recently, Liu and collaborators found that 125I-labeled PEG-GO exhibited negligible lung accumulation after intravenous injection, and could be gradually excreted from mice, although the exact clearance mechanisms required further investigation [20]. Blood biochemistry, hematology and histology examinations revealed no noticeable toxicity of PEG-GO to the treated animals at the dose of 20 mg/kg over 3 months. Similar results were also obtained for dextran functionalized GO (DEX-GO), which did not exhibit appreciable toxicity at a dose of 20 mg/kg in mice for 7 days [52]. No apparent abnormality was found in various organs including the lung of the DEX-GO injected mice, in remarkable contrast to the obvious pulmonary toxicity caused by the as-prepared GO [49]. So far, the literature reports have indicated that the toxicity of GO is closely associated with its biocompatible functionalization and stably functionalized GO derivatives are much less toxic than the unfunctionalized counterparts. However, most currently reported animal experiments are conducted on rodent models, which are different from primates and humans. As well, the long-term safety of the GO-based nanomaterials needs further observation over a long period. Hence, many more pre-clinical toxicity studies are quite necessary before GO-based cancer therapy can be translated into the clinic.

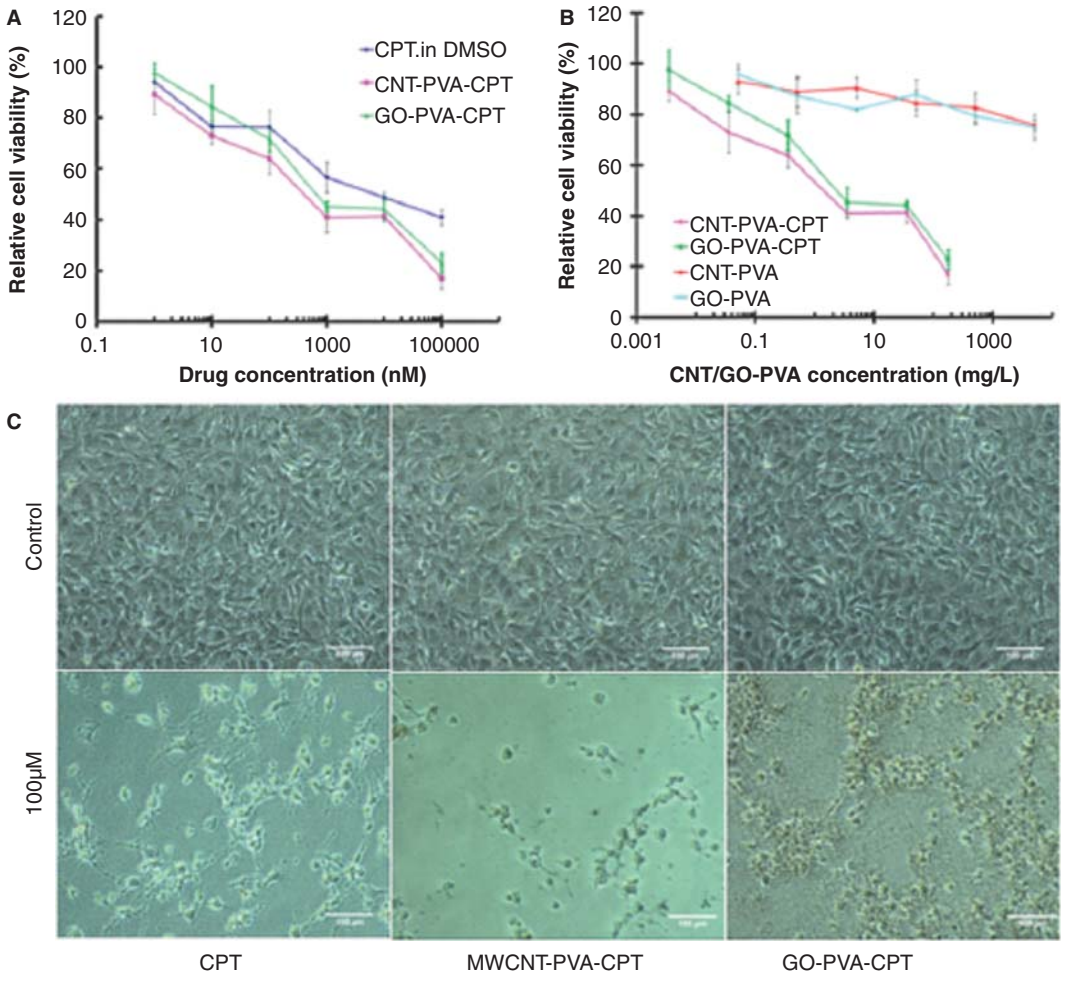

Figure 5. (**A**) Relative cell viability of MDA-MB-231 cells cultured with free CPT, PVA-MWCNT-CPT and PVA-GO-CPT at different concentrations of CPT, respectively; (**B**) relative cell viability of MDAMB-231 cells cultured with PVA-MWCNT and PVA-GO in the presence of and the absence of CPT, respectively; (**C**) optical images of MDA-MB-231 cells after culturing with CPT, PVA-MWCNT-CPT and PVA-GO-CPT, respectively [24].

Reproduced from [24] with permission of The Royal Society of Chemistry.

4. Delivery of drugs

The graphene surface with delocalized π electrons can be utilized for effective loading of aromatic anticancer drugs such as doxorubicin (DOX) and CPT via π–π stacking and hydrophobic interactions. The extremely large surface area of graphene, with every atom exposed on its two sides, is allowed for ultra-high drug-loading efficiency. In this section, the authors summarize and compare the recent research and development of GO-based cancer drugs delivery.

4.1 Doxorubicin

DOX has been widely used in treating different kinds of cancers as an anthracycline antibiotic because of its DNA intercalating effect. However, its traditionally intravenous administration may induce its uneven distribution and possible failure in crossing cellular barriers. To overcome this drawback, a drug carrier, which can immobilize drug molecules and transport them through cellular barriers, is urgently demanded. GO has not only hydrophilic groups on the edges but also a largely hydrophobic basal plane. The large π conjugated structure of GO can form π–π stacking interaction and hydrophobic effect with the quinine portion of DOX. Additionally, the hydroxyl and carboxyl groups on the GO sheets can form a strong hydrogen bonding with hydroxyl and amino groups in DOX. The hydrogen bonding interaction and π–π stacking interaction can be formed between them when they are mixed in aqueous solutions, thus rendering GO an ideal carrier for loading and delivering DOX.

Chen and collaborators investigated the *in vitro* loading and release behaviors of DOX on GO sheets [53]. A simple mixing in an aqueous solution with the aid of sonication is able to facilitate loading of DOX on GO sheets. Of interest is the pH-dependent loading and releasing behavior of the

DOX–GO system, which were due to the different degree of hydrogen bonding between GO and DOX. The high loading capacity is observed under the neutral condition, rather than acidic or basic conditions. The maximum loading of DOX on GO could reach 2.35 mg/mg at the initial DXR concentration of 0.47 mg/ml at pH 6. On the contrary, the release of DOX is more efficient under basic and acidic conditions than under neutral condition; 25 and 71% of the total bound DOX could be released after 30 h at pH 10 and 2, respectively. Depan *et al.* also showed that the loading and release of DOX were strongly depended on pH and hydrogen-bonding interaction between GO and DOX [29]. In this research, DOX was first loaded to GO surface via non-covalent π–π stacking interaction, followed by encapsulation of GO with folic acid conjugated chitosan (CHI-FA). They found that the nanohybrid system exhibited the higher drug release (35%) at pH 5.3 as compared with that (11%) at pH 7.4, which was attributed to the reduced interaction between DOX and the drug carrier. The hydrogen-bonding interaction between DOX and GO was more prominent under the neutral conditions, resulting in a controlled release. When the amine (-NH_2) groups of DOX became protonated, which resulted in the partial dissociation of hydrogen-bonding interaction under acidic conditions, the amount of released DOX from GO was much higher. Hu *et al.* carried out the loading of DOX on pluronic F127 and GNS (F127/GNS) at different initial DOX concentrations with the same concentration of F127/GNS. It was found that the loading efficiency could reach as high as 289% (w/w) at the DOX concentration of 0.9 mg/ml [32]. Then, the *in vitro* cytotoxicity of the non-covalent DOX/F127/GNS nanohybrid in MCF-7 human breast cancer cells was studied. The drug release amount under acid condition was higher than that under neutral and basic conditions. The cytotoxicity of F127/GNS-loaded DOX against MCF-7 was higher than that without DOX compared with free F127/GNS.

To enhance the anticancer effect, Wang *et al.* modified reduced GO (rGO) by gold nanoparticle clusters (GNC) onto their planes through non-covalent physisorption [8]. It was found that DOX-loaded GNC-rGO inhibited HepG2 cell growth more strongly than DOX and GNC-rGO alone, which suggested that DOX was more effectively transported into the cell cytoplasm by the GNC-rGO than free DOX (**Figure 4**). To enhance the effect of targeted drug delivery, Yang *et al.* prepared a superparamagnetic GO-Fe_3O_4 nanohybrid via a chemical precipitation method, and then FA was conjugated onto Fe_3O_4 nanoparticles via imide linkage with amino groups of APS-modified GO-Fe_3O_4 nanohybrid [35]. DOX was loaded onto the surface of this multi-functionalized GO via π–π stacking. The *in vitro* experiments indicated that DOX-loaded GO-Fe_3O_4 nanohybrid exhibited much higher cytotoxicity to SK3 human breast cancer cells than that without loading of DOX, but lower than that of GO-Fe_3O_4-FA-DOX at the same drug concentration. These results suggest that it is possible to use GO as an ideal multi-functionalized drug carrier for tumor combination therapy. Moreover, GO sheets have also demonstrated a great potential both in photothermal therapy and drug delivery. Zhang *et al.* developed a PEG-GO-DOX for the antitumor effect *in vitro* and *in vivo* by combination of photothermal- and chemotherapies in one system [54]. The results revealed that the combined chemophotothermal therapy exhibited a synergistic effect that led to better cancer cell killing effect than chemotherapy or photothermal therapy alone.

4.2 Delivery of other cancer drugs

CPT, a cytotoxic quinoline alkaloid, has been proved to be effective against a broad spectrum of tumors by inhibiting the DNA enzyme topoisomerase I (Topo1) [55,56]. But CPT has a low bioavailability due to its poor water solubility which limits its clinical applications. The functionalized GO as a nanocarrier can help improving not only its solubility but also its cellular uptake by enhancing its ability to go across the target cell membrane. Dai and collaborators explored for the first time functionalized nanoscale GO (NGO) as a novel and efficient nanocarrier for delivery of SN38, a CPT analog into cells [9]. In their approach, NGO was first functionalized with biocompatible PEG to increase aqueous solubility and stability in physiological solutions including serum. Then SN38 loaded onto NGO surface by simple non-covalent adsorption via π–π stacking. The PEG-functionalized NGO loaded with SN38 exhibited highly potent cancer cell killing *in vitro* with a human colon cancer cell line HCT-116 cells. The cytotoxicity was 1000-fold more potent than CPT-11 and similar to that of free SN38 dissolved in dimethyl sulfoxide (DMSO). Later, the authors investigated the cellular uptake and cytotoxicity of CPT, when loaded onto both CNTs and GO as drug carriers [24]. In this research work, the authors have functionalized multi-walled carbon nanotubes (MWCNTs) and GO with highly hydrophilic and biocompatible PVA in order to increase their aqueous solubility. The CPT was loaded onto PVA-MWCNT and PVA-GO through π–π interactions and investigated its capability to kill human breast (MDA-MB-231) and skin cancer (A-5RT3) cells. The cytotoxic activities of the PVA-MWCNT-CPT and PVA-GO-CPT complexes were significantly higher than that of the CPT alone (**Figure 5**). This may be due to the fact that both MWCNT and GO have more available surface area for π–π interactions with the aromatic ring of CPT, which led to the enhanced cell killing efficiency. Recently, the authors first functionalized graphene sheets (GS) by grafting a thermo-responsive PNIPAM via click chemistry, followed by loading of CPT onto PNIPAM-GS through π–π stacking and hydrophobic interaction between PNIPAM-GS and CPT [27]. The PNIPAM-GS–CPT complex showed a high potency of killing A-5RT3 cancer cells compared with free CPT *in vitro*. The authors also covalently functionalized GO sheets with hydrophilic and biocompatible pluronic F38 (F38), Tween 80 (T80) and maltodextrin (MD) for loading and delivery

of a poorly water-soluble antioxidant and anticancer drug, ellagic acid (EA) [33]. The EA release rate from the functionalized GO was found to be pH dependent in an increasing order: water (neutral pH) < pH 4 < pH 10. It was also observed that that EA loaded onto the functionalized GO exhibited a better cytotoxic activity than free EA dissolved in DMSO.

Apart from cancer drugs, presently GO-based drug delivery is elaborated to other drugs for non-cancer diseases treatment. Rana *et al.* synthesized chitosan-functionalized graphene oxides (FGOCs) and studied the delivery of an anti-inflammatory drug, IBU [30]. The IBU drug was released up to ~ 19% in PBS from FGOCs. They showed that there was no serious toxicity of the CEM cell line for various concentrations of FGOCs GS without drug loading, which suggested that FGOCs sheets had a very good biocompatibility. But FGOCs/IBU samples showed higher cell killing effect compared with FGOCs.

Although significant progress has been made in understanding how GO cross the cell membrane, the proposed mechanisms are still being debated. To date, two major intracellular uptake mechanisms have been proposed: i) endocytosis/phagocytosis and ii) nanopenetration. Liu *et al.* [9] and Zhang *et al.* [34] suggested that the functionalized GO enters cells through the endocytosis mechanism. The main advantage of utilizing functionalized GO as a drug nanocarrier compared with free drug is the ability to target delivery for selective destruction of certain types of cells without increasing the toxicity to non-targeted cells.

4.3 Co-delivery of multi-drug

With specific design of molecular structure, a GO-based delivery system can also realize co-delivery of multiple drugs or genes such as DNA and RNA, which will possess an enhanced chemotherapeutic efficacy. Zhang *et al.* implemented the simultaneous loading of two anticancer drugs, DOX and CPT, onto the FA-GO via π–π stacking and hydrophobic interactions [34]. The co-delivery of two drugs by FA-GO exhibited the better targeting efficacy and higher cytotoxicity than GO loaded with either DOX or CPT only.

Cationic polymers such as PEI are widely used for gene delivery due to their strong electrostatic interactions with the negatively phosphate groups of DNA and RNA. But the poor biocompatibility and high cytotoxicity of PEI are the main obstacles for its further biomedical application [57]. Using covalent coupling and non-covalent methods respectively, both Zhang *et al.* and Liu *et al.* fabricated PEI functionalized GO (PEI-GO) for plasmid DNA (pDNA) transfection. PEI-GO showed lower cytotoxicity and higher transfection efficiency at the optimal mass ratio as compared with the pure PEI. Inspired by these findings, it is anticipated that the integration of GO and PEI can simultaneously load aromatic drugs on GO sheets via π–π stacking interactions and bind DNA or RNA around PEI chains via electrostatic interactions. Recently, Zhang *et al.* developed a PEI-GO nanocarrier for co-delivery of small interfering RNA (siRNA) and DOX, and applied it to treat HeLa cells *in vitro* [25]. This co-delivery led to a synergistic effect on cancer cell killing and a significantly increased therapeutic effectiveness. The authors also developed CS-GO as a nanocarrier to separately deliver CPT and pRL-CMV into human cancer cell lines [31]. CS-GO apparently exhibited many positive characteristics required for *in vitro* gene delivery, such as the ability to condense pDNA to form small complexes, and low cytotoxicity. Meanwhile, the incorporation of GO bestows the nanocarrier and excellent drug loading and release capability. Although the research on utilization of cationic polymers with GO sheets for the drug–gene co-delivery is still in the initial stage, it has demonstrated an advantageous chemotherapeutic efficacy and paved a new way for future studies focusing on the simultaneous delivery of anticancer drugs and therapy genes to boost the therapeutic efficacy.

5. Comparison of GO with CNT-based delivery system

Application of CNT to the field of drug delivery has attracted increasing research interest in the last decade [11]. A variety of strategies have been developed to bind different drugs on CNTs via either covalent linkage or non-covalent adsorption. Pristine CNTs are hydrophobic and cannot be dispersed homogeneously in most solvents and physiological solutions. Therefore, functionalization of CNT is required for a good solubility and a benign biocompatibility. Bianco *et al.* reviewed the achievement of CNT in drug delivery, with a specific emphasis on the different approaches to the biofunctionalization of CNT with bioactive peptides, proteins and nucleic acids [58]. In comparison with CNT, GO exhibits some merits like low cost, two external surfaces, facile fabrication and modification and absence of toxic metal particles [59]. GO with all sp^2 carbon atoms exposed on its surface has an ultrahigh surface area for efficient drug binding [60]. Moreover, the planar GO sheets can be easily complexed with functional nanoparticles for potential multi-modal imaging and therapeutic applications. The authors carried out a comparative study on the drug-loading capacity and the cytotoxic activity of CPT-loaded CNT- and GO-based nanocarriers [24]. It was found that 1 g of PVA-CNT was able to load about 0.1 g of CPT, whereas 1 g of PVA-GO loaded 0.12 g of CPT, verifying the higher loading capacity of GO for CPT. It is revealed that the PVA-CNT-CPT had a higher cytotoxic efficiency than the PVA-GO-CPT under the same conditions from the cancer cells studies *in vitro* [24]. The authors estimated that PVA-GO entered the cells through the endocytosis mechanism only, whereas PVA-CNT entered the cells likely through both nanopenetration and endocytosis mechanisms, which resulted in the relatively higher cytotoxic effect of PVA-CNT-CPT. However, the dominated intracellular uptake mechanisms are still debatable and await further study. Liu *et al.* compared behaviors of the PEG-CNT and

PEG-GO in mice by *in vivo* fluorescence imaging for the first time [60,61]. In contrast to PEG-CNT, PEG-GO showed distinctive *in vivo* behaviors, such as reduced reticuloendothelial accumulation, notably improved tumor passive targeting effect and much higher tumor uptake. They attributed the enhanced permeability and retention (EPR) effect for tumor passive uptake to the unique 2D structure and small lateral size of GO sheets, as well as the biocompatible PEG coating.

6. Conclusions

The application of GO and its derivatives to the biomedical field has attracted enormous attention in the past several years. This article reviews the recent achievements in the application of GO to drug delivery and anticipates the promising future of the area. It covers the aspects of interest such as the functionalization of GO, cytotoxicity of GO and its derivatives, *in vitro* and *in vivo* drug delivery and the comparison with CNT-based delivery systems. Along with the great achievements, there also exist a lot of challenges for the GO-based delivery system and further investigation is needed. Based on the literature review, the authors provide some opinions on the perspectives for future research in this field.

7. Expert opinion

Accounting for the fascinating natures of GO, including its unique 2D structure, remarkable physiochemical properties and ease of funcionalization, it is believed that GO can provide a great deal of important advantages for biomedical applications. GO and its derivatives show high load capacity for various small molecule drugs, which can be physically adsorbed or chemically conjugated. The toxicity of GO-based nanomaterials is always the concern in the first priority, whereas there are still conflicting and unclear results obtained by various researchers. A consensus has been gradually reached that functionalization of pristine GO sheets must be conducted to attain high biocompatibility and low toxicity. So far, a number of studies have reported that well-functionalized GO sheets are stable and safe for *in vitro* drug delivery, and they can be readily excreted through the renal route and show to be non-toxic *in vivo* to mice.

Despite the encouraging pre-clinical results shown by some research groups, there are many challenges in front that must be addressed before GO can be practically integrated into biomedical devices and technology. These challenges and advances required in the future, in the authors' opinions, include the following:

i) Facile methods to produce GO-based nanomaterials with accurate control over dimension and quality. For example, it is important to have proper size control or size separation on various length scales to select uniform batches of GO sheets.

ii) Efficient functionalization of GO for benign biocompatibility and low toxicity. As compared with the noncovalent functionalization, covalent methods are proved to be more favorable and applicable with regard to the stability and robustness of drug carrier systems. On the other hand, biological functionalization of GO can realize the integration of GO with biomolecules, such as nucleic acids, peptides and proteins, which points out a new way to fabricate GO derivatives with improved biocompatibility, solubility, stability and tumor-targeting efficiency.

iii) Smart functionalization of GO to increase targeting efficiency of GO-based drug carrier systems. For example, it is of fundamental advantage to develop delivery systems with a molecular-recognition strategy, in which one or more therapeutic agents are carried with recognition capacity and optical, fluorescent or electric signals for imaging or specific targeting.

iv) Efforts to further evaluate the long-term toxicology of the GO-based nanomaterials in various animal models and establishment of international guidelines for determining the toxicity of GO-based nanomaterials, which should strictly comply with in all circumstances.

v) Further investigation and thorough understanding of mechanisms of interactions of GO–drug complexes with physiological systems such as the *in vivo* behaviors of GO-based nanomaterials, intracellular uptake mechanisms and intracellular metabolic pathway.

vi) For the further applications to cancer therapies, GO-based delivery system may be developed along with other therapies like photothermal therapy and gene therapy in order to achieve improved efficacy.

vii) The non-biodegradable nature of GO and its derivatives is still the biggest obstacle for their possible clinic use in the future. At present, there are few publications in this regard.

The application of GO-based nanomaterials for delivery of drugs is a significant development in the field of biomedicine and nanotechnology. A number of studies have presented the unique advantages, such as versatility for functionalizations, high loading efficiency and low toxicity, and the promising future for GO-based drug delivery systems. However, further research is needed in this area, and it is believed that opportunities and obstacles co-exist with regard to the real clinic use of GO-based nanomaterials.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Stankovich S, Dikin DA, Dommett GHB, et al. Graphene-based composite materials. Nature 2006;442:282-6
2. Geim AK. Graphene: status and prospects. Science 2009;324:1530-4
3. Service RF. Carbon Sheets an Atom Thick Give Rise to Graphene Dreams. Science 2009;324:875-7
4. Geim AK, Novoselov KS. The rise of graphene. Nat Mater 2007;6:183-91
5. Stankovich S, Dikin DA, Piner RD, et al. Synthesis of graphene-based nanosheets via chemical reduction of exfoliated graphite oxide. Carbon 2007;45:1558-65
6. Dreyer DR, Park S, Bielawski CW, et al. The chemistry of graphene oxide. Chem. Soc. Rev 2010;39:228-40
7. Cai W, Piner RD, Stadermann FJ, et al. Synthesis and solid-state NMR. structural characterization of 13C-labeled graphite oxide. Science 2008;321:1815-17
8. Wang CS, Li JY, Amatore C, et al. Gold nanoclusters and graphene nanocomposites for drug delivery and imaging of cancer cells. Angew Chem Int Ed 2011;50:11644-8
9. Liu Z, Robinson JT, Sun XM, et al. PEGylated nanographene oxide for delivery of water-insoluble cancer drugs. J Am Chem Soc 2008;130:10876-7

•• **This is the first study that explores the application of graphene to drug delivery and biomedical field.**

10. Liu Z, Robinson JT, Tabakman SM, et al. Carbon materials for drug delivery & cancer therapy. Mater Today 2011;14:316-23

• **This paper summarizes the current efforts toward the development of carbon materials for drug delivery and cancer therapy.**

11. Vashist SK, Zheng D, Pastorin G, et al. Delivery of drugs and biomolecules using carbon nanotubes. Carbon 2011;49:4077-97
12. Liu ZH, Wang YT, Zhang N. Micelle-like nanoassemblies based on polymer-drug conjugates as an emerging platform for drug delivery. Expert Opin Drug Del 2012;9:805-22
13. Pahuja P, Arora S, Pawar P. Ocular drug delivery system: a reference to natural polymers. Expert Opin Drug Del 2012;9:837-61
14. Szuts A, Szabo-Revesz P. Sucrose esters as natural surfactants in drug delivery systems-A mini-review. Int J Pharm 2012;433:1-9
15. Shen H, Zhang LM, Liu M, et al. Biomedical applications of graphene. Theranostics 2012;2:283-94
16. Wang Y, Li Z, Wang J, et al. Graphene and graphene oxide: biofunctionalization and applications in biotechnology. Trends Biotechnol 2011;29:205-12
17. Feng L, Liu Z. Graphene in biomedicine: opportunities and challenges. Nanomedicine 2011;6:317-24
18. Liu Y, Yu D, Zeng C, et al. Biocompatible graphene oxide-based glucose biosensors. Langmuir 2010;26:6158-60
19. Kuila T, Bose S, Mishra AK, et al. Chemical functionalization of graphene and its applications. Prog Mater Sci 2012;57:1061-105

• **This article offers a comprehensive overview on the chemical functionalizations of GO for different applications.**

20. Yang K, Wan J, Zhang S, et al. In vivo pharmacokinetics, long-term biodistribution, and toxicology of PEGylated graphene in mice. ACS Nano 2010;5:516-22
21. Wen HY, Dong CY, Dong HQ, et al. Engineered redox-responsive PEG detachment mechanism in PEGylated nano-graphene oxide for intracellular drug delivery. Small 2012;8:760-9
22. Ma XX, Tao HQ, Yang K, et al. A functionalized graphene oxide-iron oxide nanocomposite for magnetically targeted drug delivery, photothermal therapy, and magnetic resonance imaging. Nano Res 2012;5:199-212
23. Shen AJ, Li DL, Cai XJ, et al. Multifunctional nanocomposite based on graphene oxide for in vitro hepatocarcinoma diagnosis and treatment. J Biomed Mater Res A 2012;100A:2499-506
24. Sahoo NG, Bao H, Pan Y, et al. Functionalized carbon nanomaterials as nanocarriers for loading and delivery of a poorly water-soluble anticancer drug: a comparative study. Chem Commun 2011;47:5235-7

• **A comparison of delivery efficiency and cytotoxic activity between GO- and CNT-based drug delivery carriers is elaborated in this article.**

25. Zhang LM, Lu ZX, Zhao QH, et al. Enhanced chemotherapy efficacy by sequential delivery of siRNA and anticancer drugs using PEI-Grafted graphene oxide. Small 2011;7:460-4
26. Chen BA, Liu M, Zhang LM, et al. Polyethylenimine-functionalized graphene oxide as an efficient gene delivery vector. J. Mater. Chem 2011;21:7736-41
27. Pan YZ, Bao HQ, Sahoo NG, et al. Water-Soluble Poly(N-isopropylacrylamide)-graphene sheets synthesized via click chemistry for drug delivery. Adv Funct Mater 2011;21:2754-63
28. Gao J, Bao F, Feng LL, et al. Functionalized graphene oxide modified polysebacic anhydride as drug carrier for levofloxacin controlled release. RSC Adv 2011;1:1737-44
29. Depan D, Shah J, Misra RDK. Controlled release of drug from folate-decorated and graphene mediated drug delivery system: synthesis, loading efficiency, and drug release response. Mater Sci Eng C 2011;31:1305-12
30. Rana VK, Choi MC, Kong JY, et al. Synthesis and drug-delivery behavior of chitosan-functionalized graphene oxide hybrid nanosheets. Macromol Mater Eng 2011;296:131-40
31. Bao H, Pan Y, Ping Y, et al. Chitosan-functionalized graphene oxide as a nanocarrier for drug and gene delivery. Small 2011;7:1569-78

• **This article demonstrates a simultaneous drug delivery and gene transfection using a GO-based delivery system.**

32. Hu HQ, Yu JH, Li YY, et al. Engineering of a novel pluronic F127/graphene nanohybrid for pH responsive drug delivery. J Biomed Mater Res A 2012;100A:141-8
33. Kakran M, Sahoo NG, Bao H, et al. Functionalized graphene oxide as nanocarrier for loading and delivery of ellagic acid. Curr Med Chem 2011;18:4503-12

34. Zhang LM, Xia JG, Zhao QH, et al. Functional graphene oxide as a nanocarrier for controlled loading and targeted delivery of mixed anticancer drugs. Small 2010;6:537-44
• **The authors firstly demonstrate the co-delivery of multi-drug and present a novel functionalized GO with tumor-targeting delivery capability.**
35. Yang XY, Wang YS, Huang X, et al. Multi-functionalized graphene oxide based anticancer drug-carrier with dual-targeting function and pH-sensitivity. J Mater Chem 2011;21:3448-54
36. Li SD, Huang L. Stealth nanoparticles: high density but sheddable PEG is a key for tumor targeting. J Control Release 2010;145:178-81
37. Hatakeyama H, Akita H, Harashima H. A multifunctional envelope type nano device (MEND) for gene delivery to tumours based on the EPR effect: a strategy for overcoming the PEG dilemma. Adv Drug Deliv Rev 2011;63:152-60
38. Li N, Xu JZ, Yao H, et al. The direct electron transfer of myoglobin based on the electron tunneling in proteins. J Phys Chem B 2006;110:11561-5
39. Liu KP, Zhang JJ, Cheng FF, et al. Green and facile synthesis of highly biocompatible graphene nanosheets and its application for cellular imaging and drug delivery. J Mater Chem 2011;21:12034-40
40. Yang Y, Zhang YM, Chen Y, et al. Construction of a graphene oxide based noncovalent multiple nanosupramolecular assembly as a scaffold for drug delivery. Chem Eur J 2012;18:4208-15
41. Liao KH, Lin YS, Macosko CW, et al. Cytotoxicity of graphene oxide and graphene in human erythrocytes and skin fibroblasts. ACS Appl Mater Interfaces 2011;3:2607-15
42. Wang K, Ruan J, Song H, et al. Biocompatibility of graphene oxide. Nanoscale Res Lett 2011;6:8
43. Lu CH, Zhu CL, Li J, et al. Using graphene to protect DNA from cleavage during cellular delivery. Chem Commun 2010;46:3116-18
44. Wojtoniszak M, Chen XC, Kalenczuk RJ, et al. Synthesis, dispersion, and cytocompatibility of graphene oxide and reduced graphene oxide. Colloids Surf B 2012;89:79-85
45. Yuan JF, Gao HC, Sui JJ, et al. Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and graphene oxide on human hepatoma HepG2 cells: an iTRAQ-Coupled 2D LC-MS/MS proteome analysis. Toxicol Sci 2012;126:149-61
46. Chang Y, Yang S-T, Liu J-H, et al. In vitro toxicity evaluation of graphene oxide on A549 cells. Toxicol Lett 2011;200:201-10
47. Li N, Zhang XM, Song Q, et al. The promotion of neurite sprouting and outgrowth of mouse hippocampal cells in culture by graphene substrates. Biomaterials 2011;32:9374-82
48. Lim HN, Huang NM, Lim SS, et al. Fabrication and characterization of graphene hydrogel via hydrothermal approach as a scaffold for preliminary study of cell growth. Int J Nanomed 2011;6:1817-23
49. Zhang X, Yin J, Peng C, et al. Distribution and biocompatibility studies of graphene oxide in mice after intravenous administration. Carbon 2011;49:986-95
50. Sasidharan A, Panchakarla LS, Chandran P, et al. Differential nano-bio interactions and toxicity effects of pristine versus functionalized graphene. Nanoscale 2011;3:2461-4
51. Singh SK, Singh MK, Kulkarni PP, et al. Amine-modified graphene. Thrombo-protective safer alternative to graphene oxide for biomedical applications. ACS Nano 2012;6:2731-40
52. Zhang S, Yang K, Feng L, et al. In vitro and in vivo behaviors of dextran functionalized graphene. Carbon 2011;49:4040-9
53. Yang X, Zhang X, Liu Z, et al. High-efficiency loading and controlled release of doxorubicin hydrochloride on graphene oxide. J Phys Chem C 2008;112:17554-8
54. Zhang W, Guo ZY, Huang DQ, et al. Synergistic effect of chemo-photothermal therapy using PEGylated graphene oxide. Biomaterials 2011;32:8555-61
55. Slichenmyer WJ, Rowinsky EK, Donehower RC, et al. The current status of camptothecin analogs as antitumor agents. J Natl Cancer Inst 1993;85:271-91
56. Li QY, Zu YG, Shi RZ, et al. Review camptothecin: current perspectives. Curr Med Chem 2006;13:2021-39
57. Jager M, Schubert S, Ochrimenko S, et al. Branched and linear poly(ethylene imine)-based conjugates: synthetic modification, characterization, and application. Chem Soc Rev 2012;41:4755-67
58. Bianco A, Kostarelos K, Prato M. Applications of carbon nanotubes in drug delivery. Curr Opin Chem Biol 2005;9:674-9
59. Banks CE, Crossley A, Salter C, et al. Carbon nanotubes contain metal impurities which are responsible for the "Electrocatalysis" seen at some nanotube-modified electrodes. Angew Chem Int Ed 2006;45:2533-7
60. Yang K, Zhang S, Zhang G, et al. Graphene in mice: ultrahigh in vivo tumor uptake and efficient photothermal therapy. Nano Lett 2010;10:3318-23
• **This study successfully uses GO derivatives for efficient in vivo photothermal therapy by intravenous administration and tests the in vivo toxicology of GO derivatives for the first time.**
61. Liu Z, Cai WB, He LN, et al. In vivo biodistribution and highly efficient tumour targeting of carbon nanotubes in mice. Nat. Nanotechnol 2007;2:47-52

Affiliation

Yongzheng Pan[1], Nanda Gopal Sahoo[1] & Lin Li[†2]
[†]Author for correspondence
[1]Nanyang Technological University,
School of Mechanical and Aerospace Engineering,
50 Nanyang Avenue,
639798, Singapore
[2]Professor,
Nanyang Technological University,
School of Mechanical and
Aerospace Engineering,
50 Nanyang Avenue, 639798,
Singapore
Tel: +65 6790 6285;
Fax: +65 6791 1859;
E-mail: mlli@ntu.edu.sg