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Teaser We discuss the bioinspired surface modifications of nanographene oxide for biocompatibility and biostability concerns, and summarize the biomedical applications of graphene oxide derivatives.

Functionalized nanographene oxide in biomedicine applications: bioinspired surface modifications, multidrug shielding, and site-specific trafficking

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Introduction

Graphene is structured by sp^2-hybridized carbon atoms arranged in a honeycomb crystal matrix to form a thick atomic nanosheet [1]. GO is an applicable derivative of graphene materials and has been the focus of research for many years. For example, previous studies investigated the use of graphene and GO in energy storage and electronics [2]. However, their applications in the biomedical field remain limited. Modified GO has been extensively explored in the framework of biomedical applications, such as its application in energy biomaterials, polymer composites, and biosensors [3]. Moreover, novel research into GO in the context of targeted delivery systems has shown that its major physical and biological properties, particularly related to its surface modifications, could have potential for theranostics purposes, including high multi-drug loading and protection, various forms of drug release, and efficient targeting ability [4].

Various strategies have been introduced to improve the ability of GO to act as a nanocarrier for drug delivery. In terms of drug loading, π–π staking is an efficient interaction mechanism for loading various aromatic biomolecules onto the GO surface. Moreover, the presence of oxygen groups on the GO surface results in the formation of hydrogen bonds. These two interactions, π–π staking and hydrogen-bond formation, are the main driving force for GO drug loading. Another issue is related to drug release [5] and centers around how the carrier can discharge its cargo for controlled release in biomedical applications. Stimuli-triggered release is primarily based on the physicochemical differentiation of the microenvironment between tumors and normal tissues, resulting from the acidity, temperature changes, overexpression of specific enzymes, and high redox, reactive oxygen species (ROS) and ATP levels, as well as reactions to external stimuli, such as lasers, ultrasound, and magnets [6]. The integration of GO with biomaterials, such as biodegradable polymers, liposomes, DNA, proteins, and inorganic nanoparticles (NPs), can result in the release of aromatic drugs in a controllable manner.

Even though various current GO-based NPs have demonstrated benefits for anticancer drug delivery, their therapeutic efficacy is restricted mainly by the lack of targeting ability. Moreover, the distribution of chemical drugs in normal tissues by nonspecific delivery can lead to adverse effects [6]. The general strategy to achieve efficient targeting therapy is to modify the vehicles with particular ligands, which bind with high affinity to receptors external or internal to the

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cancer cell surface. Oxygen functional groups on GO serve as active sites and make it possible to conjugate molecules on the GO surface, such as hyaluronic acid (HA), folic acid (FA), lactobionic acid, transferrin (Tf), monoclonal antibodies, peptides, and polysaccharides [7].

Here, we highlight advances in strategies for the surface modification of GO and provide an overview of recent applications of GO-based nanomaterials in biomedicine applications. Although there is much interest in using GO-derived materials in this field, there are still concerns regarding the potential toxicity and biocompatibility of these materials. Therefore, we also critically discuss this concern and propose potential solutions.

Bioinspired surface modification

The structure of GO has been the subject of controversy over the past few years, and even now no clear model exists. Thus, there are four classic structural models of GO proposed, which share the same framework comprising a regular pattern of detached repeat units [8]. The structure of GO significantly influences its physicochemical properties, predominantly because it is planar in shape and contains multiple oxygenated groups, such as epoxy, hydroxyl, and carboxylic groups [9]. This configuration and various functional groups provide GO with ability to conjugate with a range of materials, including drugs, metals, fluorescence probes, and biomolecules.

Noncovalent modifications of nanographene oxide

Two main strategies have been used to modify the GO surface: covalent chemical reactions and noncovalent interactions (Fig. 1) [10]. Noncovalent modifications require moieties that have show hydrophobicity and usually involve Van der Waals forces, π–π interactions [11], hydrogen bonding [12], electrostatic interactions [13], and coordination bonds [14]. In general, noncovalent modifications can be achieved via polymer or biomacromolecule wrapping, and the absorption of various small molecules. The most effective approach to performing this type of functionalization is to use organic molecules and polymers with aromatic groups to form π–π interactions with GO. For example, Liu *et al*. [15] designed a series of pyrene and perylene-diimide-terminated polymers with graphene nanosheets to investigate the π–π stacking interactions between the electron donor and electron acceptor molecules of the two polymers. Both aromatic derivatives of the polymers showed strong interactions with the graphene nanosheets. The resulting hybrids also showed high water dispersion behavior, resulting from the repulsive forces induced between the hybrid nanostructures because of the presence of sulfonic groups on the aromatic molecules. Zhi *et al*. [16] improved the water solubility of GO by removing oxidative fragments resulting from noncovalent electrostatic interactions with L-tryptophan. X-ray diffraction and Raman and Fourier transform infrared spectroscopy (FTIR) were used to show that oxidative debris of reduced GO nanosheets (rGO) were removed following washing with ammonia, improving the water dispersibility by enhancing the π–π interactions between the rGO sheets and L-tryptophan molecules.

Covalent modifications of nanographene oxide

Covalent modifications result from various chemical reactions [17]. The molecules used have amino groups, such as aliphatic and aromatic amines, peptides, and proteins, which attack the epoxy groups and carboxyl groups on GO to form amide bonds. By using a solvothermal process, Wang *et al*. [18] prepared a hydrophilic GO sheet with allyl amines and successfully attached organic molecules to the GO surface to improve its hydrophilic properties. The GO compounds generated were able to dissolve in water and maintained a steady dispersion state. An alternative covalent bonding approach was reported by Zhang *et al*. [19], who exploited the properties of the covalent chemical reaction to modify GO with different amine-terminal silanes via silanization. Such functional GOs are promising materials for further surface modifications.

Generally, functional modification is the main strategy used to improve the performance of GO nanosheets. As mentioned earlier, covalent modifications can be achieved through different types of chemical reaction, including nucleophilic substitution, electrophilic substitution, condensation, and addition reactions. By contrast, noncovalent interactions can be achieved by different techniques, including electrostatic adsorption, surface deposition, aromatic anchoring, and hydrophobic interactions. Noncovalent interactions have little, if any, influence on the structural and electronic properties of GO. In addition, the energy of the covalent bonds is generally higher than that in noncovalent interactions. Importantly, when the dissociation energy decreases, particularly in van der Waals interactions, noncovalent systems become reversible and kinetically adjustable. However, when combined with large surfaces, some noncovalent interactions can compete with covalent bonds in terms of the energy involved. In addition, noncovalent functionalization can be achieved under mild conditions without the need for highly specific laboratory equipment.

Surface modifications for physical stability and biocompatibility

The physical stability and biocompatibility of GO sheets are vital for their applications in biomedicine. However, various studies have revealed chronic toxicity and pathological changes induced by GO *in vivo*. Optimal functionalization renders GO nanosheets applicable for drug shuttling and biomedical applications, and the modified GO structures have fewer adverse effects on metabolism and excretion [20,21]. The main issues associated with GO and its derivatives are solubility and biocompatibility. Even though GO can dissolve in water, it aggregates in the presence of salts, particularly in physiological media, because of charge screening effects [22]. Therefore, to develop a favorable nanocarrier, GO surface modification is required. Various biocompatible polymers have been connected extensively with carbon nanomaterials to enhance their biostability. GO modification using synthetic polymers, such as polyethylene glycol (PEG), poly-L-lysine (PLL), polyvinyl alcohol (PVA), poly ethylene imine (PEI), poly sebacic anhydride (PSA), and poly (*N*-isopropyl acrylamide) (PNIPAM), has been a useful strategy for enhancing GO biostability and biocompatibility [23]. These modified GO nanosheets can be applied in chemotherapy and photothermal therapy as novel carriers of therapeutic agents. At the inchoate stage of GO functionalization, Liu *et al*. [24] introduced PEG to produce a biocompatible PEGylated nanographene carrier. Abhilash *et al*. [25] investigated the effect of PEG modification on the *in vivo* toxicity of GO structures. The injection of few-layer graphene (FLG) and

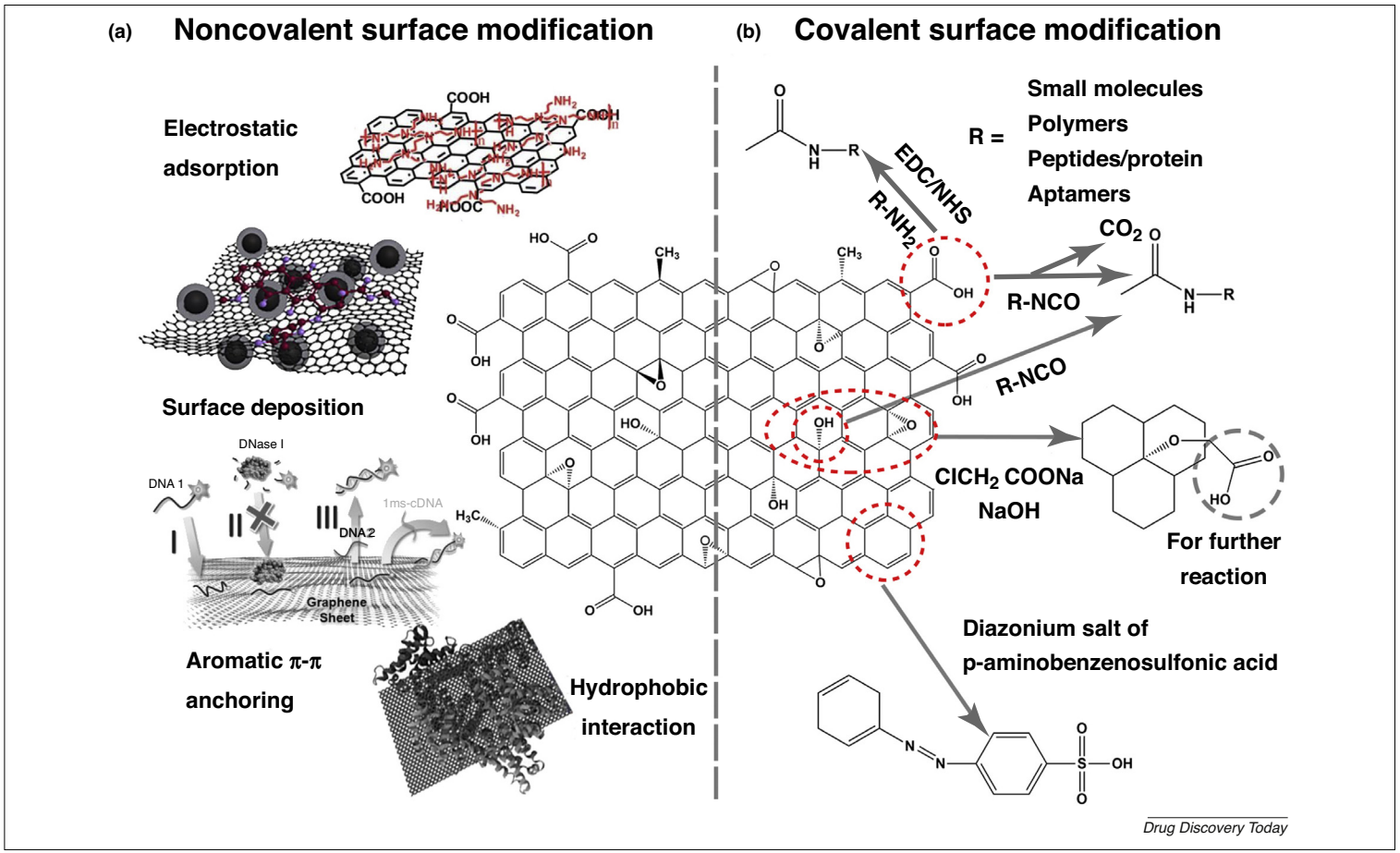


FIGURE 1

Two major strategies for graphene oxide (GO) modification: noncovalent (a) and covalent (b) surface functionalization.

FLG-COOH caused organ damage, such as thicker alveolar walls in lung, loss of the dividing line between red pulp and the marginal zone in spleen, and black spots in the liver; by contrast, FLG-PEG did not cause any injury to tissues, indicating that rational surface modification could significantly improve the biocompatibility of the nanosheets. Pan *et al*. [26] conjugated PNIPAM polymers with GO nanosheets via click-reactions. The complex produced had high drug-loading efficacy with desirable stability and solubility in aqueous solutions. The polymer-modified GO sheets were modified to bear camptothecin and demonstrated no noticeable cytotoxicity at a low camptothecin concentration.

Polysaccharide- and glucose-related polymers, such as chitosan (CS), dextran, and heparin (Hep), are also used to improve the biocompatibility and biostability of GO. Compared with synthetic cationic polymers, CS is a biodegradable, biocompatible, natural polysaccharide with low cytotoxicity and high biocompatibility [27]. Therefore, many studies have demonstrated that biocompatible polysaccharide polymer-functionalized GO is easily cleared from mice without any noticeable toxicity. Zhang *et al*. [28] developed a CS-functionalized nanocomposite to deliver CpG oligodeoxynucleotides for immunotherapy. Positively charged CS and negatively charged GO self-assembled spontaneously into a stable nanostructure via intense electrostatic interactions. Hep is naturally glycosaminoglycan that is expressed on the surface of eukaryotic cells as an anticoagulant. Accordingly, Hep-encapsulated materials were demonstrated to be environment friendly. A novel drug delivery carrier in the form of Hep-grafted GO was reported by Zhang *et al*. [29]. In this study, GO was conjugated with Hep via adipic dihydrazide (ADH) linkers to fabricate a Hep-modified 2D nanostructure for improved stability and blood compatibility.

Biomolecules considered to be efficient surfactant candidates have also been applied to improve the physicochemical properties and biocompatibility of GO nanosheets [30]. As discussed earlier, the GO surface offers opportunities for protein binding via π–π stacking and hydrophobic interactions. However, these interactions can be complicated because of the charge of the surface active groups in proteins, which depend on environmental circumstances, such as pH value and ionic strength [31]. Bovine serum albumin (BSA) is a biocompatible globular protein produced by the liver and can be degraded by cysteine proteases. Therefore, the encapsulation of GO with albumin BSA could offer another option for improving the physicochemical stability of GO; in addition, the enzymatic degradation of the protein could also contribute to efficient cargo release. This approach was recently illustrated by Xing and co-workers [32], who designed GO and albumin/photosensitizer nanohybrids via non-covalent interactions to accelerate the intracellular release of the photosensitizer. The results revealed that the terminal complex was able to protect the photosensitizer from degradation and inactivation before it reached the targeted site.

Noticeably, polymers or biomolecules used for GO modification not only improve the solubility, stability, and biocompatibility of materials, but also act as functional group donors for further drug shuttling. Thus, numerous bioactive components of targeting ligands could be attached to graphene sheets. Moreover, polymer-anchored GO nanosheets could also gain functions including site-specific targeting and multiple drug shielding.

Surface modifications for enhanced drug shielding and flexible release

Even though GO-derived nanomaterials have unique advantages in terms of their drug-loading capacity compared with other NPs, such as iron oxide NPs and gadolinium ferrite NPs, their targeting ability and drug release profile remain to be enhanced. Recently, versatile modifications have been introduced onto the surface of GO NPs to construct advanced drug delivery systems that are responsive to internal microenvironment variations (e.g., in pH, redox, ROS, ATP, and enzyme levels) [33–35]. For example, the low pH of the tumor microenvironment has been commonly utilized as a stimulus to induce drug accumulation and/or to accelerate intracellular drug release with the aid of specific chemical modifications. Based on the physical properties of modified GO, external stimuli, such as heat, light, ultrasound, and magnetic field [36], have also been used to trigger drug release in a controllable manner. The advantage of GO NPs is a pH-responsive drug release that is faster at lower pH, probably because of the improved hydrophilicity of the protonated doxorubicin (DOX) [37]. Given that there is an ATP gradient between the intracellular and extracellular environment, as well as between normal cells and tumor cells, numerus GO NPs have been developed to contain ATP aptamers that can respond to high ATP concentration as a recent approach for controlled anticancer drug release. After GO decoration with ATP-responsive DNA, Ran *et al*. [33] constructed a stimuli-triggered anticancer drug delivery system in which a GO nanoaggregate was cross-linked with two single-stranded (ss)DNAs containing ATP aptamers. This cross-linked structure was able to protect its cargo (DOX) and release it at the targeted site triggered by high ATP concentrations.

The large π–π conjugated structures of various mineral NPs, including Fe_3O_4, Ag, Au, Cd, Pd, TiO_2, Pt, and Cu, have also been used to adapt GO functions. Inorganic NPs have been successfully linked with GO for multimodal therapies, including photodynamic therapy (PDT) and photothermal therapy (PTT) [38,39]. Such graphene/mineral NP complexes have remarkable optical and magnetic properties, combined with capacity for organic drug delivery in response to external stimuli, as well as biosensing functions. Huang *et al*. [40] developed an effective, scalable, and advanced multifunctional targeted delivery structure, in which GO served as the basic structure, with Fe_3O_4 NPs chemically installed onto the GO surface via precipitation. The NP complex obtained demonstrated superparamagnetic behavior and a high payload delivery efficiency.

Site-specific targeting is a crucial issue that needs to be addressed when delivering drug delivery systems. Based on the specific receptors overexpressed on tumor cells, multiple ligands with high binding affinity have been introduced to GO nanostructures, such as FA, transferrin (Tf), and HA. Qin *et al*. [41] conjugated FA with the GO surface to improve its tumor-targeting ability. However, such targeting technology alone cannot guarantee the avoidance of drug leaks while in the circulation. Therefore, multifunctional carriers serving various purposes (e.g., biocompatibility, protective delivery, controlled drug release, and site-specific targeting) are required to overcome existing physiological barriers. Notably, the synergetic combination of targeting delivery and flexible release is essential to avoiding adverse effects. Yin *et al*. [35] successfully applied HA as surface-coating polymer to modify GO via disulfide

bonds and induced drug release in controlled manner via external NIR triggers. HA functioned as targeting ligand to facilitate the accumulation of GO derivatives at tumor sites, as well as to cover the corona to enhance its stability and prevent drug leakage. After arrival at the tumor tissues, the concentrated hyaluronidase degrades the HA corona and the disulfide linker is snipped by glutathione (GHS), which is highly expressed in tumor cells. Moreover, photothermal disruption mediated by NIR also facilitates the escape of the nanocarriers from endosomes and/or lysosomes. Thus, this study successfully combined dual-microenvironment factors (i.e., enzyme and redox effects) and NIR light for efficient targeted drug delivery and release.

Therapeutic platforms for multidrug cellular trafficking

Inspired by developments in the synthesis of graphene, GO and related nanomaterials have been investigated in various fields, including composite materials, electronics, energy technology, and biosensors. However, the bioapplication of GO is a relatively new research area with substantial potential. Since the first influential report on GO as a proficient nanocarrier, various promising strategies have been developed to exploit the GO nanostructure for widespread biomedical applications (Fig. 2), from drug delivery, phototherapy, bioimaging and biosensing to antibacterial approaches [42,43]. Such research has been driven by various properties of the nanosheet structure, including its specific and high surface area (~2630 m^2/g), mechanical strength (~1100 GPa), thermal conductivity (~5000 W/m/K), electronic conductivity (~200 000 cm^2/V/S), intrinsic biocompatibility, and high capability for superficial chemical functionalization.

Chemotherapeutic delivery via functionalized graphene nanostructures

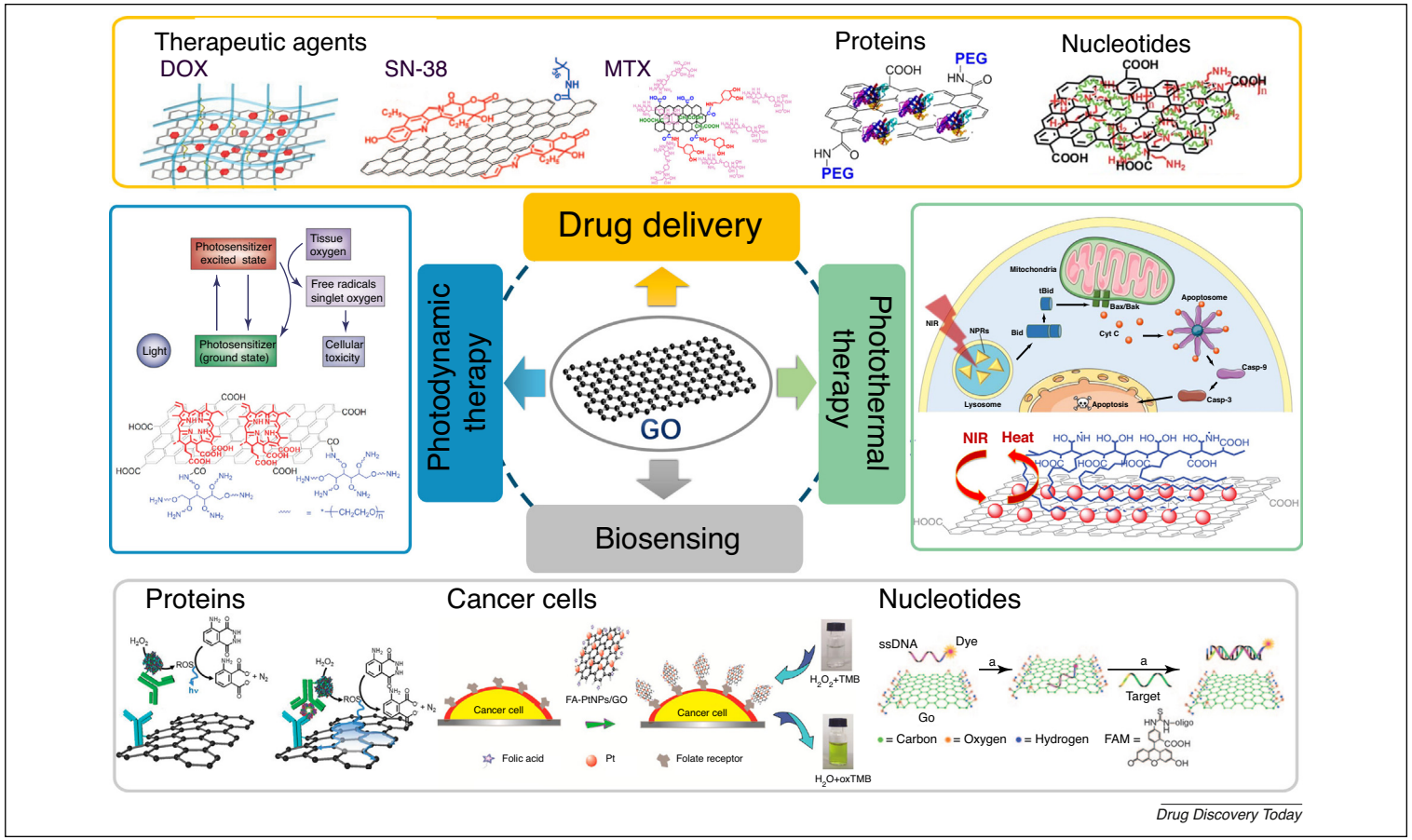
Nanostructures based on graphene have been investigated for various chemotherapeutics, especially small molecules, such as DOX, paclitaxel (PTX), camptothecin (CPT), cisplatin, and methotrexate. Among these active agents, DOX, as an anthracycline antitumor antibiotic, has been the most extensively studied for GO surface loading because of its aromatic structure with high loading efficiency via π–π stacking interactions. DOX-loaded nanosheets have been applied for the treatment of various cancers in humans, including breast, liver, prostatic, and cervical cancer. Transport of DOX by GO nanostructures demonstrated higher cellular uptake efficiency compared with free DOX. As a result, DOX-loaded GO derivatives displayed tumor suppression against DOX-resistant breast cancer, which resulted from pH-sensitive DOX release in endosomes and/or lysosomes because of the protonation of molecules. Apart from inherent pH-responsive drug release, various strategies have been applied to regulate intracellular DOX release profiles, such as redox and ATP-triggered drug liberation. Disulfide linkages have been utilized to construct a sheddable shell for GO nanosheets [35]. When exposed to high concentrations of GSH in cytoplasm, the linkages are snipped by GSH, resulting in accelerated DOX release. Moreover, ATP as the primary energy molecule is another potential trigger for the controlled release of the drug payload. ATP aptamers can be conjugated to fabricate GO-based nanosystems that function as ATP-responsive drug shuttling [33] and molecular channels [44]. Similar to DOX, CPT has a planar pentacyclic ring structure that can also be shielded on the GO surface by π–π stacking interactions. Deb *et al*. [45] synthesized a biocompatible GO-derived vehicle for targeted CPT transport into cancer cells that utilized PEG as a stabilizer and FA moieties as targeting ligands. The GO-PEG-FA-CPT nanocomplexes generated revealed pH-dependent CPT release behavior and greater tumor-suppressive activity by FA-receptor mediated cellular internalization. Given the lack of an aromatic structure, PTX cannot be loaded onto the GO surface. Thus, Angelopoulou *et al*. [46] modified GO with amphipathic PLA-PEG copolymers for stabilization in physical environments. Moreover, the PLA section of the copolymer increased the hydrophobicity of the GO surface, which then provided a reservoir for PTX loading. GO/PLA-PEG composites exhibited a satisfactory drug loading capacity of 9–11%, with highly sustained drug release, which can be regulated by adjusting the proportion of the PLA-PEG copolymer in the composite. For PTX loading on GO derivatives, Xu *et al*. [47] used a covalent conjunction strategy, whereby PTX was connected with biocompatible six-armed PEG, followed by covalent installation onto the GO nanosheets. The generated GO-PEG-PTX nanodrug had a high loading ratio of 11.2 wt %, as well as superior stability under physiological conditions.

The co-delivery of multiple drugs is a synergistic strategy for the reversion of multidrug resistance (MDR). Zhang *et al*. [48] developed a simple preparation of GO as a novel nanovehicle for multidrug loading and tumor-targeting delivery. The nanoscale GO (NGO) was functionalized with sulfonic acid groups, which rendered it stable in physiological solutions, followed by covalent binding of FA molecules to the NGO (FA-NGO) to specifically target human breast cancer MCF-7 cells. Furthermore, DOX and CPT could be loaded onto the functional GO in a controlled manner (Fig. 3a): the drug loading ratio for DOX was higher than that of CPT (Fig. 3b,c), which could be ascribed to the difference in chemical structures. Drug loading of DOX was independent of the presence of CPT (Fig. 3d), and two peaks at 490 and 365 nm in the UV/Vis spectra (Fig. 3e) indicated that DOX and CPT were co-loaded onto FA-NGO. For cell cytotoxicity, FA-NGO-DOX/CPT complexes displayed enhanced anticancer efficacy because of FA-receptor mediated cellular internalization of nanocarriers and the synergistic effects of DOX and CPT (Fig. 3f,g).

Biomolecular delivery via functionalized graphene nanostructures

Graphene and its derivatives share superior properties that render them as effective carriers for not only chemical drugs, but also biomolecules, such as large nucleic acids, antibodies, proteins, and peptides. Even though gene therapy shows potential against genetic diseases [49], it is necessary to design efficient vehicles that protect nucleic acids from nuclease degradation and improve their transfection efficiency [50]. To this end, GO nanosheets with optimal modifications could be used to overcome these issues.

For example, Yang *et al*. [51] investigated the ability of NGO to protect from cellular degradation by using a molecular beacon (MB) as a model oligonucleotide. Compared with free MB, which was fully hydrolyzed in 15 min, MB-adsorbed NGO nanostructure revealed no obvious hydrolysis, demonstrating that DNA can be protected from DNase cleavage by noncovalent adsorption. Feng and coworkers [52] applied branched PEI-modified GO as a gene



Drug Discovery Today

FIGURE 2

Summary of advanced biomedical applications of functionalized graphene oxide (GO), including drug delivery, photodynamic therapy (PDT), photothermal therapy (PTT), and biosensing.

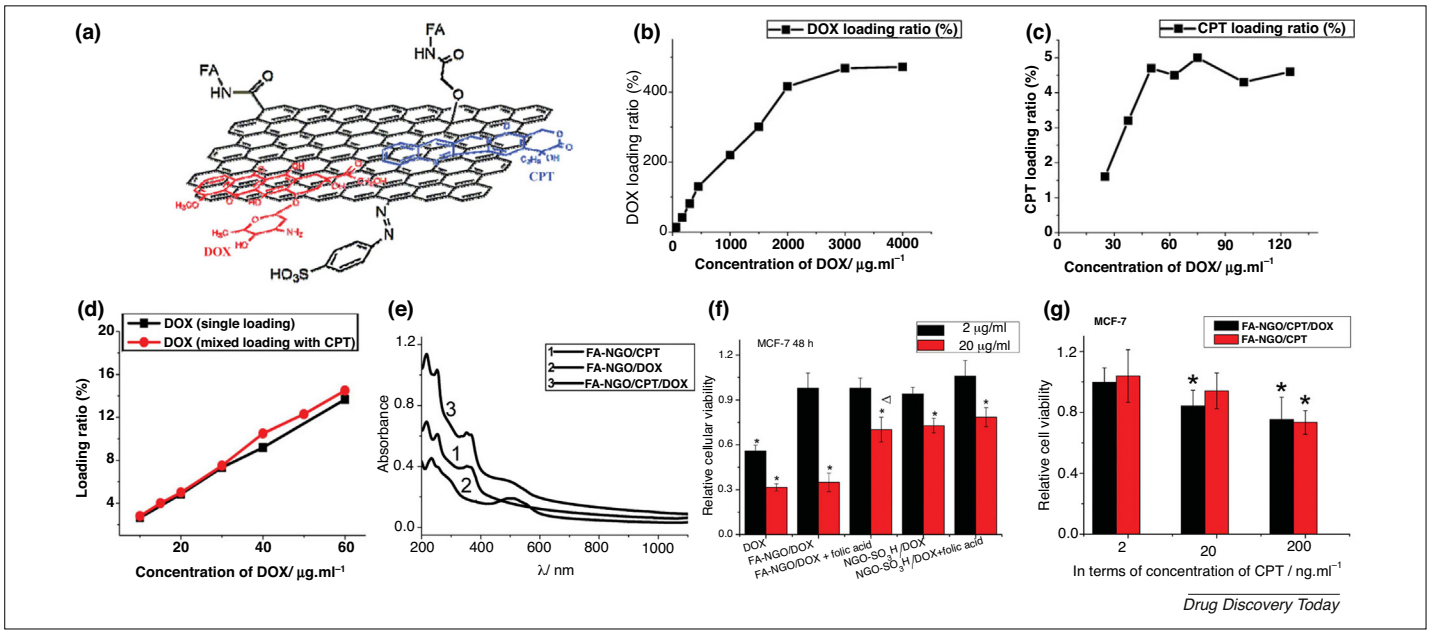


FIGURE 3

Schematic images representing the loading of doxorubicin (DOX) and camptothecin (CPT) onto folic acid (FA) nanosheet graphene oxide (FA-NGO) **(a)** and differences between the loading ratio of DOX **(b)**, CPT **(c)** and a DOX/CPT mixture **(d)** onto FA-GO nanoparticles (NPs). (e) Ultraviolet (UV)/Vis spectra of DOX, CPT, and DOX/CPT loaded onto FA-NGO. The cellular uptake **(f)** and cytotoxicity **(g)** of FA-GO-DOX/CPT composites are also shown.

transfection carrier. PEI-decorated GO materials were confirmed to be both stable and biocompatible. For intracellular transfection, positively charged GO-PEI complexes exhibited high affinity for plasmid DNA via electrostatic interactions and enhanced expression of GFP. By contrast, naked 10-kDa PEI displayed significant toxicity. These results confirmed GO after modification as an innovative gene delivery vector with high transfection efficiency and low cytotoxicity, with wider application for gene-targeted delivery. For enhanced transfection, Feng *et al.* [53] described a light-controllable gene carrier for intracellular trafficking by utilizing the photothermal and gene delivery advantages of dual-functionalized GO. In their study, PEG and PEI were covalently attached to the surface of GO nanosheets via amide bonds (NGO-PEG-PEI). Upon NIR laser irradiation, the resulting nanocarrier demonstrated efficient gene transfection ability as a result of cell membrane permeability caused by mild photothermal effects. Similarly, Yin *et al.* [54] explored a combined photothermal and gene technique directed against pancreatic cancer cells using a PEGylated GO nanocarrier (PEG-GO) for the co-delivery of small interfering (si)RNAs targeting *HDAC1* and the G12C mutant K-Ras gene. As a plasmid DNA delivery platform, Bao *et al.* described GO-CS complexes for the co-delivery of plasmid DNA and CPT. The synthesized GO-CS complexes shared a high drug-loading capacity and the CPT-loaded GO-CS systems exhibited improved cytotoxicity compared with free CPT. Importantly, GO-CS was able to condense plasmid DNA that was protected from enzymatic degradation to enhance DNA transfection efficiency. Moreover, Zhang *et al.* [55] proposed a gene-chemotherapy strategy for overcoming the MDR of breast cancer cells. They constructed a PEI-GO nanocarrier for the delivery of Bcl-2 targeted siRNA and DOX to HeLa cells. The treatment of HeLa cells with PEI-GO/siRNA/DOX complexes significantly decreased Bcl-2 expression levels. Compared with PEI-GO/DOX, the cytotoxicity assay indicated the potency of PEI-GO/siRNA/DOX, decreasing the IC_{50} of DOX from 1.3 μg/ml to 0.52 μg/ml [56]. Recently, a dual-functional GO nanocarrier was developed by the conjugation of an aminated PEG polymer and octa arginine (R8) for the intracellular trafficking of nucleic acids [57]. This dual-functional combined NP demonstrated high pDNA and siRNA condensation ability in the presence of an endosomal buffering environment, and high cellular uptake with superior internalization efficacy in breast cancer cell lines. The few unmodified GO structures reported for nucleic acid delivery resulted because this delivery system required cationic polymers to provide electrostatic interactions between the carriers and cargos.

Here, we provide a comprehensive overview of comparative assays for the cellular uptake and internalization of GO-based gene delivery [28,58,59]. As shown in Fig. 4a, PEG-GO nanocomposites were fabricated with peptide nucleic acid (PNA) to form PEG-GO-PNA complexes, which were then incubated with cancer cells for 3 h. In a fluorescence microscopy assay, the PEG-GO-PNA complexes demonstrated significantly stronger fluorescence signals compared with naked PNA and PNA/GO, indicating acceleration of the cellular uptake of PEG-GO-PNA induced by PEG modification. The nanocomposites of GO fabricated with CS polymers were designed to deliver oligonucleotides into RAW264.7 cells (Fig. 4b). Observed by confocal microscopy, cells incubated with CS-GO-CpG ODNs showed a stronger GFP signal compared with CpG ODNs, indicating the higher cellular localization of CS-GO-CpG ODNs. For cell penetration peptide (PF14-SCO)-functionalized GO nanosheets (Fig. 4c), the resultant complexes achieved high gene transfection rates. Confocal microscopy images demonstrated strong fluorescence signals after treatment, indicating the high cell internalization of GO-SCO-PF14 nanocomposites. In addition, a biodegradable porous silicon NP was encapsulated with GO nanosheets to form a pSi-GO-based gene

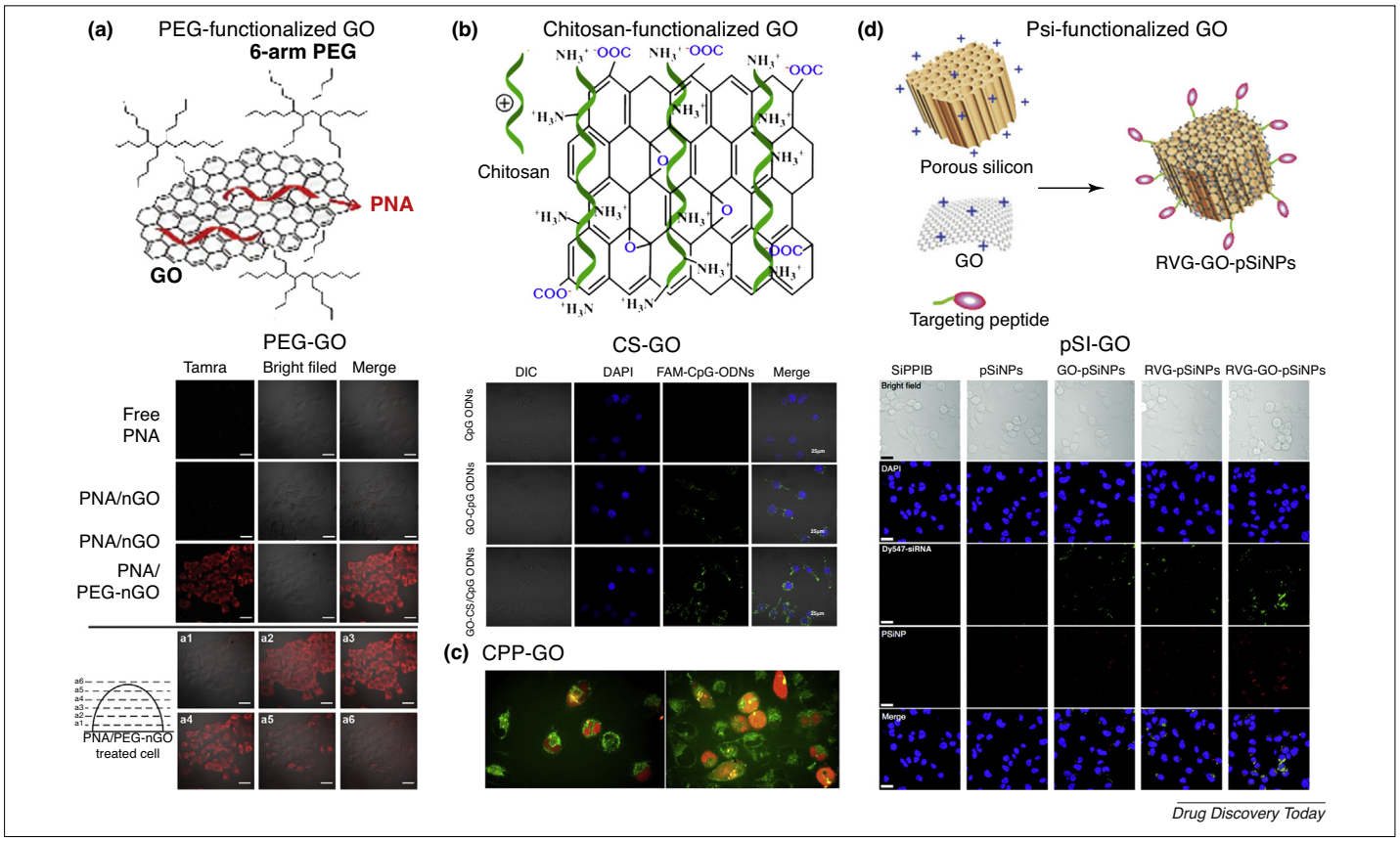


FIGURE 4

Examples of nanostructures and cellular uptake confocal microscopy images of graphene oxide (GO)-based drug delivery systems, including polyethylene glycol (PEG)-GO **(a)**, chitosan (CS)-GO **(b)**, cell penetrating peptide (CPP)-GO **(c)**, and porous silicon (pSi)-GO **(d)**. Abbreviation: NP, nanoparticle.

delivery system for the efficient delivery of genetic molecules into the injured brain (Fig. 4d). Notably, a higher level of specific cellular uptake was detected for pSi-GO/siRNA complexes compared with pSi/siRNA without GO decoration. Based on these results, the versatile functionalization of GO has a significant influence on the cellular trafficking of GO-based gene and immune therapy approaches. Thus, the conjugation of biocompatible materials, including polymers, peptides, and nonmetallic elements, could efficiently decrease electrostatic repulsions, strengthen the gene–cell bounds, and protect the targeted molecules from the endosome via endocytosis.

There have been few studies of the delivery of protein or peptides mediated by GO and its derivatives. However, proteins, as biological molecules, could combine with GO via π–π stacking and hydrophobic interactions because of the abundant π electrons in the GO structure. Shen *et al.* [60] constructed a PEGylated GO (GO-PEG) as a nanovector for the efficient delivery of proteins into cells. The GO-PEG had a high BSA-loading efficiency of 350% (w/w), which was higher than that of other commonly reported carriers, such as liposomes (loading efficiency <90%). Yang *et al.* [61] mixed GO with silk fibroin (SF) via a one-step method for the self-assembly of SF/GO films under mild conditions. They found that SF/GO films had a powerful mechanical capacity and improved the growth, early adhesion, and osteogenic differentiation of mesenchymal stem cells [62]. The co-delivery of therapeutic proteins and/or peptides with chemical drugs has been confirmed as a suitable strategy for cancer treatment. Jiang and colleagues reported the GO nanostructure-mediated co-delivery of protein drugs and anticancer molecules [63]. They conjugated PEG-GO with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), together with DOX co-loading via noncovalent interactions. The GO nanocarriers accumulated at the tumor site preferentially with the specific recognition of TRAIL and the death receptor on the tumor cells, and enhanced permeability and retention effect (EPR) in the tumor sites. The furin on the cell membrane digested the linker between PEG and TRAIL, resulting in the stimuli-responsive extracellular release of protein drugs for enhanced apoptosis induction. The attached DOX combined with the apoptosis-inducing effect of TRAIL indicated optimal synergistic antitumor activity.

Phototherapy and photodynamic applications

Graphene-based materials are considered excellent photothermal agents because of their large specific surface area, abundant functional groups, and, most importantly, unique electronic band structure. Thus, GO materials exhibit plasmonic effects under specific wavelengths, such as NIR, which generate heat through plasmonic photothermal conversion. Yang *et al.* [64] reported GO combined with PTT for the successful treatment of tumors. They indicated that conjugated PEGylated GO complexes showed high accumulation behavior in tumors and obvious photothermal therapeutic efficacy in cancer xenograft mouse models under a

low-power NIR laser. Similarly, Li *et al*. [65] designed rGO NPs coated with transferrin and PEG for enhanced PTT. The functionalized GO nanocomposites produced a microbubbling effect that destroyed cancer cells. *In vivo* results correspondingly indicated the antitumor efficacy of rGO activated by a NIR laser. The PTT enhancement of rGO was mainly the result of the improved π conjugation of electrons in a reduced form compared with its oxidized counterpart [66]. Inspired by GO-based nanomaterials, graphene analogs have been developed, such as transition metal dichalcogenides (TMDs), transition metal oxides (TMOs), MXenes, hexagonal boron nitride, and black phosphorus, which resulted in comparative or improved NIR absorption and light-thermal conversion efficiency.

However, because of the locally heterogeneous distribution of heat induced by PTT, it is impossible to eliminate tumor cells with PTT alone; therefore, the combination of photothermal ablation therapy with other treatments could be vital to enhance the overall therapeutic efficacy. Graphene-based materials can also absorb various chemotherapeutic molecules via their broad surface area to result in specific targeted release behavior to achieve synergistic effects of PTT and chemotherapy. Various anticancer therapeutics have been added onto GO-derived nanocarriers for combined cancer therapy, such as DOX, CPT, PTX, and cisplatin. Zhang *et al*. synthesized PEG-GO nanocomplexes encapsulated with DOX as a targeted anticancer therapy [37]. *In vitro* and *in vivo* investigations revealed that the combination therapy had better tumor-suppressive effects than either PTT or the chemical treatment alone. Similarly, the introduction of active targeting ligands can also enhance PTT efficacy. With the assistance of targeting molecules, such as HA [35] and aptamers [67], enhanced PTT therapy can be achieved with decreased toxicity to healthy tissues following the higher intracellular uptake and site-specific delivery of cargo. The narrow penetration range of NIR (only several millimeters) is a limitation of GO-based PTT; therefore, understanding how to utilize the low power of NIR to achieve efficient photothermal conversion is vital. To address this problem, enhanced photothermal conversion was introduced by anchoring Au NPs onto GO nanosheets [68]. The resulting structure had remarkable light thermal conversion efficiency [69], which was enhanced by adjusting the shape and size of the Au nanostructures. To enhance tissue penetration, magnetic field-induced PTT was combined with chemotherapy to achieve low-power PTT. Lu *et al*. [70] designed a pH-sensitive dual-targeted magnetic nanocarrier for chemophototherapy in cancer treatment. They fabricated a versatile nanohybrid particulate comprising a GO nanosheet and magnetic Fe_3O_4 NPs to enhance the photothermal efficacy and to carry DOX to specific cancer tissues. *In vitro*, the authors demonstrated the targeted killing of CT-26 magnetically, whereas the photothermal effect was clear following irritation of the GO nanohybrid material. The results revealed the significant anticancer effects of magnetic GO NPs, demonstrating a 29-fold increase compared with the combination of chemotherapy with PTT (Fig. 5).

PDT is another useful strategy in clinical cancer therapy, which uses light application as an external trigger for the specific activation of photosensitizers in the targeted tissues. The activated photosensitizer has the capability to generate reactive oxygen species (ROS), such as singlet oxygen, superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide, causing irreversible damage to tumor cells and tissues. To increase the transformation efficiency, PTT and PDT were applied synchronously to increase phototherapy efficacy for tumor ablation. Tian and co-workers [71] efficiently encapsulated Chlorine e6 (Ce6) as a photosensitizer into PEG-functionalized GO nanomaterials via π–π stacking and hydrophobic interactions [71]. The photothermal effects of GO were utilized to increase the accumulation of Ce6 in cancer cells with significant photodynamic effects on malignant cells under a mild NIR laser. To increase the accumulation of Ce6 in specific cancer cells, the application of functionalized GO loaded with photosensitizer agents for targeted PDT [50,72] was recently introduced for cancer treatment. Huang *et al*. designed a novel receptor-mediated specific targeted drug delivery system comprising rGO-PVP-RGD [73]. The anchored RGD peptide on the rGO surface led to improved PDT efficacy compared with Ce6 alone; and PVP decoration not only significantly increased the dispersibility, biostability, and compatibility of the basic sheets, but also provided attachment sites for RGD peptides.

In summary, the structural features and superficial chemical alterations of GO materials offer attractive opportunities for improved phototherapy outcomes with the capability for diverse targeted molecule shielding and shuttling, such as anticancer chemicals, as well as photosensitizers for cancer theranostics. These investigations provide promising strategies for cancer diagnosis and treatment with PTT and PDT.

Biosensing and bioimaging applications via functionalized graphene nanostructures

Biosensors are analytical devices that can detect biomarkers swiftly, selectively, and ultrasensitively, transforming a biological recognition event into a measurable signal (Fig. 6). Graphene and its derivatives, such as GO, rGO, and functional graphene, have been widely exploited as promising biosensors because of their outstanding optical and electrochemical properties. GO-based nanocomposites can be readily engineered for the detection of various targets, including DNA/RNA, enzymes, small molecules, immune components and cells, and can act as efficient fluorescence quenchers or emitters. Examples of significant platforms of GO-based biosensors are summarized in Table 1.

For instance, the initial application of a GO platform for nucleic acid detection was based on complete fluorescence quenching induced by the intense π–π stacking of the GO sheet with dye-labeled ssDNA [74]. The targeted DNA was hybridized with dye-labeled ssDNA and released newly formed double-stranded DNA (dsDNA) from the surface of GO sheets because of poor affinity. The restoration of fluorescence was observed and transformed quantitatively, highlighting this platform as an off-on model to detect specific DNA. This mechanism could also be extended to other uses [75,76]. Shahrokhian *et al*. [77] explored the use of an ultrasensitive label-free electrochemical DNA biosensor-based GO NP to detect a cancer biomarker BRCA1. An electrochemical behavior analysis was conducted to evaluate this modified biosensor electrode, which demonstrated accurate detection of DNA with remarkable sensitivity, selectivity, and reproducibility in blood plasma samples. In addition, there are numerous strategies available to optimize the DNA sequencing by the introduction of GO-based nanodevices in precision medicine [78,79]. For example, Caspase-3 is a member of the cysteine-aspartic acid protease family

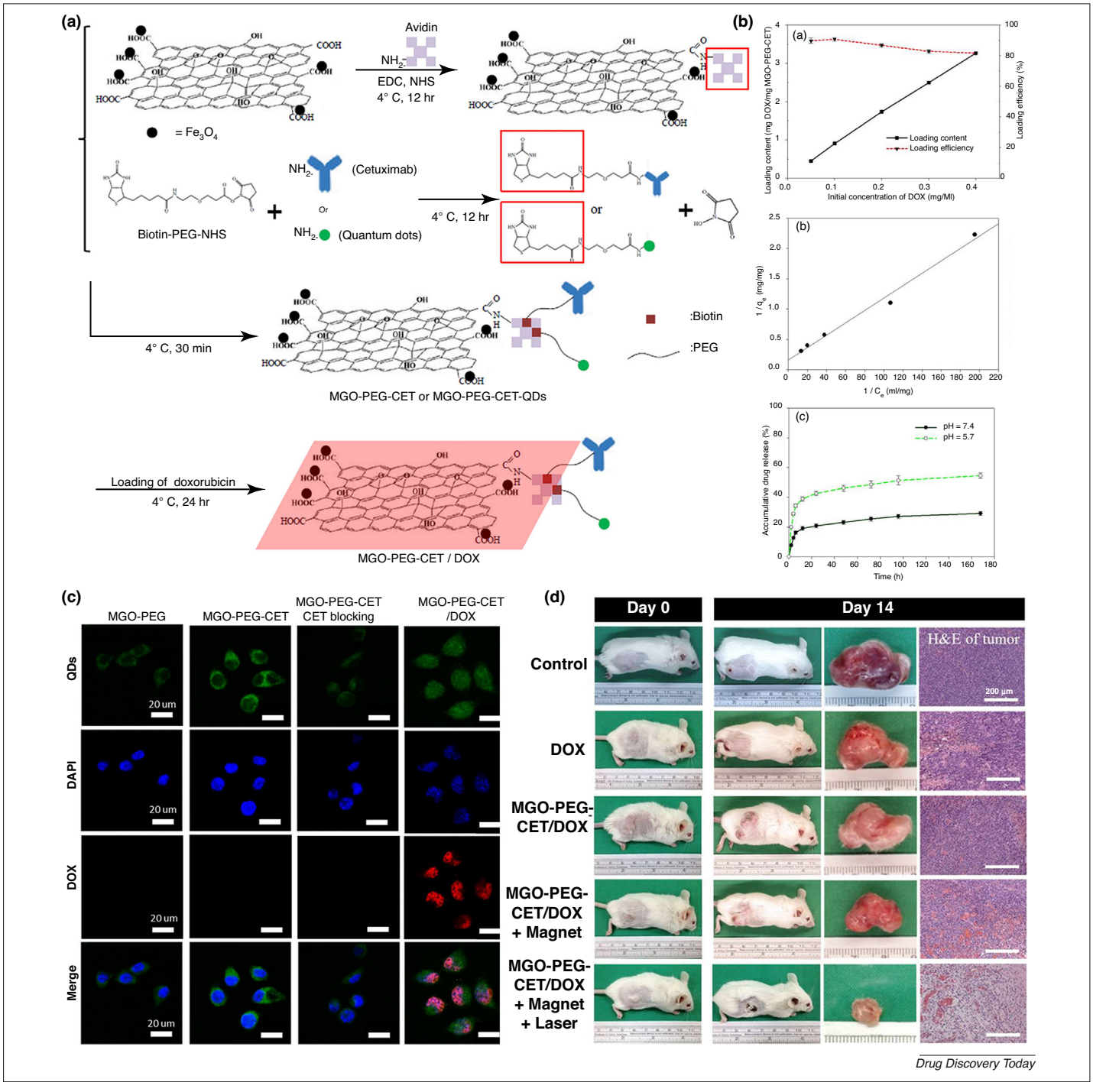


FIGURE 5

Magnetic graphene oxide (MGO) for targeted drug delivery and photothermal therapy. **(a)** Schematic illustration of doxorubicin (DOX)-loaded magnetic graphene oxide (GO) nanostructures of MGO-polyethylene glycol (PEG)-cetuximab (CET)/DOX. **(b)** Drug-loading content and loading efficiency modeled with the Langmuir adsorption isotherm and DOX release profiles. **(c)** Confocal microscopy images of cells treated with various MGO preparations. **(d)** Antitumor efficacy *in vivo* against tumor-bearing BALB/C mice.

[80], the sequential activation of which has an essential role in cell apoptosis. Wang *et al.* [81] constructed a GO nanodevice that could be applied as an intracellular protease sensor to detect the activation of Caspase-3 in cells, with the limit of detection of 7.25 ng/ml. Li *et al.* [82] designed a GO-derived biosensor with a dye-labeled aptamer as a probe for the detection of thrombin based on FRET, which showed excellent specificity with a low detection limit of 31.3 pM.

The development of advanced biosensors to detect pathogens rapidly holds great significance for clinical diagnostics. Jung *et al.* [83] proposed a novel GO immunobiosensor system, in which GO was bound to an amino-modified glass surface via electrostatic interactions. Rotavirus antibodies were arrested on the GO nanosheet surface via amidation reactions to induce specific antigen-antibody interactions between the NP and the rotavirus cells. Then, AuNP-linked antibody complexes (Ab-DNA-AuNP) were

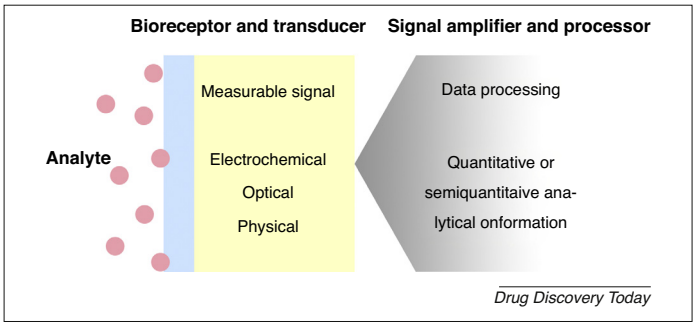


FIGURE 6

Schematic of a typical biosensor system: the analyte interacts with a bioreceptor on a selective biologically affined layer and generates a biological response, which is further transformed to a measurable signal by the transducer. Afterwards, the signal is amplified to provide additional information.

TABLE 1

Applications of GO derivatives in biosensing

Material	Analyte	Biomolecular probe	Fluorescent label	Limit of detection	Refs
GO	ssDNA	ssDNA aptamer	FAM	~2 nM	[74]
		DNA probes	FAM, Cy5, ROX	~100 pM	[75]
rGO	ssDNA	PNA	–	100 fM	[76]
GO	dsDNA	Positively charged dye	PNPB	~1 nM	[88]
	Thrombin	Thrombin aptamer	FAM	31.3 nM	[82]
	Caspase-3	Lysine	FAM	7.25 ng/ml	[81]
Graphene/anti-CEA	CEA	CEA antibody	–	<100 pg/ml	[84]
Ab-DNA-AuNP	Rotavirus	Rotavirus antibody	–	10^5 pfu/ml	[83]
PtNPs/GO	MCF-7	Folic acid	–	–	[89]
AuNPS/GO	MCF-7	MUC1 aptamer	–	0.0375 μg/ml	[90]
GO/Fe_3O_4/Au	ATP	Aptamer	FAM	0.1 nM	[85]
GO nanosheets	ATP (living cells)	Aptamer	FAM	~12 μM	[86]

synthesized to analyze the immune biosensor properties. The results revealed highly selective and sensitive detection between AuNP and the GO sheets. Moreover, Zhou's team proposed another application for immunosensors based on antibody-modified graphene [84]. They constructed a carcinoembryonic antigen (CEA)-decorated graphene field effect transistor (GFET) and showed the specific, ultrasensitive, real-time monitoring of CEA protein (<100 pg/ml). Inspired by the biomolecules discussed earlier, GO nanostructures have also been exploited to detect ATP, H_2O_2, and gases, such as nitrogen dioxide and ammonia. [85–87].

As well as serving as a biosensor, functionalized GO and graphene-based nanocomposites have also been introduced as tools for fluorescence, ultrasound, and MR bioimaging based on their optical and magnetic properties. For example, Dai and coworkers investigated the cellular trafficking of PEG-modified GO nanosheets by exploiting their NIR fluorescence absorbance properties [91]. They prepared PEG-NGO with different particle sizes and properties, which showed outstanding biostability in serum and could be excited to emit fluorescence in the visible to IR spectra. Thus, PEG-NGO could be utilized for living cell NIR imaging. Similarly, another group developed a gelatin-modified GO NP and labeled it with a fluorescence dye for cellular imaging investigations [92]. Moreover, photoacoustic tomography, MR, and Raman scattering-based bioimaging strategies have also been investigated. An example of one such approach is photoacoustic imaging (PAI). Cai *et al.* [93] developed a photoacoustic/ultrasonic dual-mode imaging system, and demonstrated an obvious linear correlation between the photoacoustic imaging signal of the rGO and its concentration. Attempts have also been made to integrate GO with other mineral NPs, such as Ag and Au NPs, for better bioimaging results. Kim *et al.* [94] successfully incorporated the unique properties of GO and AuNPs to construct facile core shell nanohybrid NPs. The nanohybrids obtained were encapsulated with photosensitizers (e.g., zinc phthalocyanine). As a result, the GO-AuNPs shared low toxicity and multifunctional properties for theranostic treatments, such as PTT and PDT in addition to Raman bioimaging. Similarly, by using mineral NP combinations, Qiu *et al.* [95] developed a nanohybrid of Ag-GO NPs via a facile synthesis strategy, which showed potential for use in bioimaging, both *in vitro* and *in vivo*, and for use in cellular biodetection.

Antibiosis applications via functionalized graphene nanostructures

Over the past few years, the extensive use of antibiotics has led to MDR bacteria [96], which require the development of new antibacterial techniques. Graphene nanostructures as novel antibacterial materials exert ideal antibacterial activity resulting from various features, such as their sharp edges, which directly destroy bacterial membranes, the extraction of phospholipid molecules, and the trapping of bacteria followed by photothermal ablation. Moreover, the antibacterial capacity of GO nanosheets is associated with particle size, surface charges, oxygen content, and degree of dispersion. For example, Liu *et al.* [97] evaluated the antimicro-

bial effects of graphene-based nanomaterials on *Escherichia coli*. According to scanning electron microscope (SEM) and dynamic light scattering analyses, GO materials demonstrated high antibacterial efficacy, resulting from their nanoscale size, which enabled them to disrupt the bacterial cell membranes. Likewise, Hu *et al*. [98] confirmed that water-dispersible GO or rGO nanosheets efficiently inhibited the growth of bacteria, with low cytotoxicity. Additionally, graphene-based nanosheets have been complexed with other materials, such as metals and polymers, to overcome the limitations of individual components and to achieve synergistic outcomes. By using AgNP and GO NPs, Lu *et al*. [99] fabricated GO-based nanocomposites with outstanding antibacterial activities towards *E. coli*. Arriagada *et al*. [100] proposed a complex of PLA with either GO or thermally rGO NPs, which were formulated by melt mixing. In addition to their enhanced bioactivity, the complexes, including PLA-GO and PLA-rGO, demonstrated high growth inhibition against both *E. coli* and *Staphylococcus aureus*. Moreover, the antibacterial activity increased when an electrical stimulus was applied to the PLA-rGO complexes.

Concluding remarks

GO-based nanomaterials have been extensively studied as effective nanoplatforms for the diagnosis and treatment of various diseases. Here, we have discussed surface modification strategies for the construction of GO-drug nanosystems with enhanced biostability and biocompatibility. We also highlighted some of the latest developments in the functionalization of graphene materials to regulate their properties in biological systems, resulting in a combinatorial regimen of chemotherapy, gene therapy, imaging-guided therapy, PDT, and PTT. Moreover, we provided a comprehensive overview of recent applications of GO-based nanocarriers in biomedical fields, including drug shuttling, phototherapy, biosensing, bioimaging, and antibiosis.

Despite these significant advances, several issues need to be addressed further. GO nanosheets have excellent drug loading capability compared with other nanocarriers because of their ultrahigh surface area. However, this large surface area could foster interactions between proteins and GO derivatives that could increase their diameter, resulting in their quick elimination from the body. The large-scale synthesis of GO can be achieved at low cost, but the nanoscale sizes of GO derivatives are difficult to control. Particle size is crucial for the physiochemical stability and biological responses of GO derivatives; thus, strategies to control their size require further investigation and standardization. Moreover, thermal effects on the microenvironment surrounding tumors needs further investigation to ensure their therapeutic efficacy and biosafety. Thus, more studies and strategies are required to optimize the physicochemical properties and/or structural modifications of GO nanosheet, to lead to new developments in the field of nanomedicine.

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