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Graphene: a versatile nanoplatform for biomedical applications

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Abstract

Graphene, with its excellent physical, chemical, and mechanical properties, holds tremendous potential for a wide variety of biomedical applications. As research on graphene-based nanomaterials is still at a nascent stage, due to the very short time span since its initial report in 2004, a focused review on this topic is timely and necessary. In this feature review, we first summarize the results from toxicity studies of graphene and its derivatives. Although literature reports have mixed findings, we emphasize that the key question is not how toxic graphene itself is, but how to modify and functionalize it and its derivatives so that they do not exhibit acute/chronic toxicity, can be cleared from the body over time, and thereby can be best used for biomedical applications. Next, we discuss in detail the exploration of graphene-based nanomaterials for tissue engineering, molecular imaging, and drug/gene delivery applications. The future of graphene-based nanomaterials in biomedicine looks brighter than ever, and it is expected that they will find a wide range of biomedical applications with future research effort and interdisciplinary collaboration.

Keywords

Graphene; graphene oxide (GO); reduced graphene oxide (rGO); molecular imaging; tissue engineering; drug/gene delivery; positron emission tomography (PET); nanomedicine

Introduction

The field of nanotechnology has advanced tremendously during the first decade of the 21st century, which focuses on the design, synthesis, characterization, and application of a wide variety of materials with at least one dimension within 100 nm.[1] These nanomaterials possess unique physicochemical properties that can guide the creation of new structures, systems, nanoplatforms, or devices that can have potential applications in a broad range of scientific areas.[2, 3] To date, the most well-studied nanomaterials in biomedicine include quantum dots (QDs),[4, 5] carbon nanotubes (CNTs),[6] nanoshells,[7] paramagnetic nanoparticles,[8] and many others.[9-11] Over the last several years, graphene has emerged as a promising nanoplatform with enormous potential for biomedical applications and translational research, because of its excellent physical, chemical, and mechanical properties. An important milestone in graphene-based research was the 2010 Nobel Prize in

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Physics, which was awarded to two scientists who first described monocrystalline graphitic films in 2004.[12]

As an atomic thick monolayer of carbon atoms arranged in two dimensional honeycomb structure, graphene is a basic building block for other graphitic materials such as graphite and CNTs.[12-15] Because of the unique and desirable characteristics, graphene, graphene oxide (GO), and reduced graphene oxide (rGO) have been extensively studied for a variety of applications such as nanoelectronics, sensors, energy storage, nanocomposites, etc.[13, 16-20] In addition, the improved synthesis and versatile surface modification of graphene has opened up new avenues for research on the nanoscale. Because of the short time span since its initial discovery, biomedical applications of graphene-based nanomaterials is still at a nascent stage and most of the reports appeared over the last several years.

In this feature review, we will summarize the current state-of-the-art for biomedical applications of graphene and its derivatives, which include tissue engineering, molecular imaging, and drug/gene delivery. Since graphene-based biosensing has been studied extensively[21-23] and several review article are already available,[24, 25] we will not include this topic here. Despite the great enthusiasm about biomedical applications of graphene-based nanomaterials, there are some concerns about the potential toxicity and biocompatibility from both the scientific community and the general public, which deserve to be investigated before in vivo studies and potential clinical translation. Therefore, toxicity studies of graphene-based nanomaterials will first be discussed below, followed by the investigation of these intriguing nanoplatforms for tissue engineering, molecular imaging, and drug/gene delivery applications.

Toxicity studies of graphene-based nanomaterials

The toxicological profile of graphene-based nanomaterials is not yet well elucidated, partly because of the surface modifications that are required to render them suitable for biomedical applications. Although graphene derivatives such as GO usually form stable suspensions in water, they generally aggregate in salt or other biological solutions.[17] In addition, it is important to have a proper size control or size separation on various length scales to select uniform batches of graphene sheets, which has been a challenge.

Recently, graphene was reported to elicit concentration dependent cytotoxicity in cell-based studies,[26, 27] by decreasing cell adhesion, inducing cell apoptosis, and entering into various cellular compartments. On the other hand, MTT colorimetric assay revealed that graphene/chitosan composites were biocompatible to L929 cells, derived from normal subcutaneous areolar and adipose tissue of a mouse.[28] Most studies to date have indicated reduced or absence of cytotoxicity for GO in a variety of cells such as L929 cells,[29] HeLa cells,[30] human fibroblasts,[31, 32] A549 human lung cancer cells,[33] and human hepatoma HepG2 cells.[34] These abovementioned studies indicated that several factors/parameters such as concentration, size, shape, type of dispersants, etc. can influence the cytotoxicity of graphene and GO. In particular, surface functionalization is an important factor which plays a critical role in the biocompatibility, since it can help in pacifying the strong hydrophobic interaction of graphene/GO with cells and tissues. Furthermore, it has been shown that functionalization can lead to a reduction of reactive oxygen species, which mediate apoptosis through caspase-3 activation.[27] This phenomenon has been confirmed by improved biocompatibility of functionalized GO (with polyethylene glycol [PEG][29] or dextran[35]) when compared to plain GO.

Several in vivo studies indicated chronic toxicity associated with GO, which was primarily deposited in the lung after intravenous injection and resulted in pulmonary edema and lung

granuloma formation.[32, 36] However, dextran functionalized GO was found to accumulate in the reticuloendothelial system (RES) such as the liver and spleen after intravenous injection, and could be cleared from the mouse body within a week without noticeable toxicity to mice.[35] Similar results were also obtained for PEGylated nanographene sheets, which did not cause appreciable toxicity at a dose of 20 mg/kg in mice over 3 months, as evidenced by blood biochemistry, haematological analysis, and histological examinations.[37] Aside from graphene and GO, respirable graphene nanoplatlets (which are consisted of several layers of graphene sheets) have been found to be inflammogenic in both the lung and the pleural space, which poses risks to the respiratory system after inhalation exposure.[38]

Recently, nanotoxicology has emerged as a new branch of toxicology for studying the undesirable effects of various nanomaterials.[39, 40] Development of graphene-based nanomaterials for biomedical applications must proceed in tandem with the assessment of any toxicological side effects. However, the toxicity of graphene itself may not be highly relevant to the biomedical applications of graphene, for which it will need to be functionalized for any potential use. The literature reports to date clearly indicated that stably functionalized graphene-based nanomaterials are much less toxic than the unfunctionalized counterparts. Quality control of graphene-based nanomaterials and robust chemistry for functionalization are the two most important prerequisites for future biomedical and clinical applications of graphene-based nanomaterials. The key question is not how toxic graphene itself is, but how to modify and functionalize it and its derivatives so that they do not exhibit any toxicity, can be cleared from the body over time, and thereby can be best used for biomedical applications. The abovementioned in vitro and in vivo toxicity studies have paved the way for future investigation of graphene-based nanomaterials for various applications such as tissue engineering, molecular imaging, drug/gene delivery, biosensing, among others.

Tissue engineering with graphene-based nanomaterials

The goal of tissue engineering is to replace diseased or damaged tissue with biologic substitutes that can restore and maintain normal function. Major advances in the areas of cell and organ transplantation,[41] as well as those in materials science and engineering, have aided in the recent development of tissue engineering and regenerative medicine.[42] There has been a continuous on-going search for biocompatible scaffolds with suitable physical, chemical, and mechanical properties for the design of appropriate biomimetic materials.[43] With desirable properties such as high elasticity, flexibility and adaptability to flat or irregular shaped surfaces,[44-46] graphene-based nanomaterials can play key roles in sustained proliferation, proper adhesion, and enhanced differentiation in this context. In addition, they can also serve as structural reinforcement for other scaffold materials that are currently being used for this purpose.[47]

Graphene was shown to be a viable substrate for the growth of mammalian NIH 3T3 fibroblast cells.[48] On a thin film of graphene, the cells were viable and maintained normal adhesion and migration properties. In addition, enhanced cellular functions such as gene transfection and expression were achieved without notable deleterious effect. In other studies, graphene was also found to promote growth, proliferation, and adhesion of mammalian colorectal adenocarcinoma HT-29 cells, human osteoblasts, and mesenchymal stromal cells.[49, 50] The potential of graphene as a biocompatible scaffold was further confirmed by unhindered growth and proliferation of human mesenchymal stem cells (hMSCs) on various graphene-coated substrates (e.g. glass slides).[51] Intriguingly, graphene not only helped in differentiation of these hMSCs into osteocytes in a controlled manner, but also accelerated the rate of differentiation, which was comparable to what could be achieved using specific differentiation factors.

The possible role of graphene acting as a preconcentration platform for osteogenic inducers was reportedly attributed to its non-covalent binding abilities which can guide stem cell differentiation towards osteogenic lineage.[52] However, results from the same study involving adipogenesis showed suppression of differentiation to adipocytes by graphene in contrast to GO, which did not interfere with such process. This was explained by the fact that insulin, a key regulator for fatty acid synthesis, binds electrostatically to GO and maintains its function, but gets denatured due to π-π interaction with graphene. These findings suggested that graphene exhibits different binding interactions with different growth factors and hence different influence on the growth of stem cells and their subsequent differentiation to specific tissues.

This aspect was further confirmed by a recent study of culturing mouse induced pluripotent stem cells (iPSCs) on graphene and GO surfaces, which displayed distinct proliferation and differentiation characteristics.[53] GO, owing to the presence of abundant oxygen atoms (e.g. OH) on its surface, enabled better iPSC attachment and proliferation than graphene. In addition, GO was found to promote differentiation of iPSCs towards endodermal lineage to a higher extent than graphene, although the differentiation towards ectodermal and mesodermal lineages was comparable for both surfaces, which indicated the importance of surface properties of graphene-based substrates in controlling the behavior of iPSCs. The unique surface property of graphene was further established by enhanced differentiation of human neural stem cells (hNSCs) to neurons when compared with glass substrates (Fig. 1).[54] Similarly, in another study involving the growth of mouse hippocampal cells, graphene substrates led to a significant enhancement of neurite sprouting and outgrowth.[55]

The tremendous recent interest in the use of graphene-based nanomaterials for tissue engineering applications has culminated in many exciting and intriguing literature reports over the last few years, clearly indicating that graphene and its related substrates are excellent nanoplatforms for promoting the adhesion, proliferation, and differentiation of various cells such as hMSCs, hNSCs, and iPSCs. Since graphene is a relatively new material, research on its potential applications in tissue engineering and regenerative medicine is still at a nascent stage. Although most of the literature reports are in vitro studies of specific cells, future in vivo investigation will ultimately lead to its utilization as implantable tissue engineering material. The continued evolvement of non-invasive imaging techniques will undoubtedly contribute to significant advances in tissue engineering and regenerative medicine,[56, 57] including those based on graphene and its derivatives.

Molecular imaging with graphene-based nanomaterials

The field of molecular imaging, “the visualization, characterization and measurement of biological processes at the molecular and cellular levels in humans and other living systems”,[58] has expanded tremendously over the last decade. Molecular imaging not only takes advantages of the traditional imaging techniques but also introduces molecular imaging agents to measure the expression of indicative markers at different stages of diseases.[59] Over the last several years, graphene and GO have been explored with many molecular imaging techniques, including magnetic resonance imaging (MRI), optical, photoacoustic, and radionuclide-based (e.g. positron emission tomography [PET]) imaging.

In one early study, nanoscale GO (a few nanometer [nm] in lateral width) was covalently functionalized with PEG star-polymers and the resulting PEGylated GO exhibited photoluminescence in the visible and infrared range.[60] After conjugation to a B-cell specific anti-CD20 antibody, Rituxan, the intrinsic photoluminescence of GO enabled near-infrared (NIR) imaging in live lymphoma cells. In another report, fluorescein was conjugated to GO via a PEG linker for intracellular optical imaging,[61] which exhibited efficient fluorescence signal, pH-tunable fluorescence, as well as good biocompatibility in vitro. Since direct

labeling of GO with fluorophores will lead to efficient quenching of the fluorescence signal by GO, a PEG linker was incorporated in this study to reduce direct interactions between fluorescein and GO.

Similarly, non-targeted rGO was conjugated with QDs via a bridge of bovine serum albumin (BSA) for fluorescence imaging of live HeLa cells,[62] whereas trastuzumab-conjugated rGO was employed for optical imaging in human breast cancer cells that overexpress HER-2 (the antigen of trastuzumab).[63] In a recent report, folic acid (FA) conjugated rGO was tagged with QD through a short spacer (in the nm range) for the imaging of human breast cancer MCF-7 and HeLa cells (Fig. 2a).[64] Furthermore, this nanocomposite could also serve as an optical indicator for the heat dosage of photothermal therapy.

Because of fluorescence quenching by graphene and GO, in vivo optical imaging with fluorescently labeled graphene-based nanomaterials has been challenging. In a pioneering study, Cy7 (a commonly used NIR fluorophore) was conjugated to PEGylated GO for in vivo fluorescence imaging in mouse tumor models (Fig. 2b&c).[65] The PEGylated GO, which has ample amino groups at the termini of six-arm branched PEG chains, exhibited good optical absorption in solution and a blood circulation half-life of 1.5 h in Balb/c mice after Cy7 conjugation. Although it was reported that there were about 14 Cy7 molecules per GO which could lead to self-quenching due to fluorescence resonance energy transfer (FRET), all three tumor models tested in this study exhibited high uptake of fluorescently labeled GO based on the enhanced permeability and retention (EPR) effect alone. Different from single-walled carbon nanotubes (SWNTs),[66, 67] much higher accumulation of GO in the kidneys than in the RES (e.g. liver and spleen) was observed at 24 h post-injection. After successful optical imaging which confirmed high tumor uptake of GO after intravenous administration, photothermal therapy was carried out with low-power NIR laser irradiation which efficiently ablated the tumors at a dose of 20 mg/kg of GO. No short-term side effect was observed in treated mice, which paved the way for future long-term toxicity and other in vivo investigation of GO conjugates.

In a follow-up study, the same PEGylated GO was labeled with ^{125}I ($t_{1/2}$: 60.1 days) to examine its long-term biodistribution and potential toxicity in Blab/c mouse.[37] Radiolabeled GO was found to mainly accumulate in the RES and be cleared from the mouse body by renal and fecal excretion. The blood circulation of ^{125}I-labeled GO exhibited two-phases, with half-lives of 0.39 ± 0.10 and 6.97 ± 0.62 h, respectively. Mice treated with a 20 mg/kg dose of GO did not show noticeable toxic effect in 3 months, suggesting excellent biocompatibility of the PEGylated GO.

Optical imaging is a relatively low-cost method suitable primarily for small animal studies,[68, 69] however the major drawbacks of optical imaging in living subjects are the poor tissue penetration of light and photobleaching of most fluorescent dyes. Initially developed in the mid-1970s,[70] PET has the capability to quantitatively measure radioisotope concentration in vivo with excellent tissue penetration. Currently, PET is widely used in both clinical patient management and clinical/pre-clinical research.[71-76] PET has extremely high sensitivity (down to the picomolar level), thus it only requires tracer concentration many orders of magnitude lower than the pharmacologically active level. The most widely used PET isotopes include ^{11}C ($t_{1/2}$: 20 min), ^{18}F ($t_{1/2}$: 110 min), ^{64}Cu ($t_{1/2}$: 12.8 h), among others.

To date, most in vivo imaging studies using graphene-based nanomaterials were based on the EPR effect alone (i.e. passive targeting). We recently conjugated GO with an antibody and radiolabeled the conjugate for in vivo targeting and PET imaging of tumor vasculature in a mouse model of breast cancer.[77, 78] Mounting literature reports have indicated that one

major hurdle for tumor targeting with nanomaterials is poor extravasation.[79-81] Therefore, we chose to target the tumor vasculature, where extravasation of functionalized GO is not required to achieve tumor contrast/uptake hence the targeting efficiency could be significantly enhanced. One desirable vascular target in cancer is CD105 (i.e. endoglin), which is almost exclusively expressed on proliferating tumor endothelial cells and has been understudied to date.[82] The targeting ligand used in these studies was TRC105, a human/murine chimeric IgG1 monoclonal antibody (mAb) that binds with high affinity to both human and murine CD105.[83-85]

Since NOTA (i.e. 1,4,7-triazacyclononane-1,4,7-triacetic acid) has been extensively studied and can serve as a highly stable chelator for multiple radiometals, the same covalently linked conjugate (i.e. NOTA-GO-TRC105) could be labeled with three different PET isotopes with different half-lives: ^{61}Cu ($t_{1/2}$: 3.4 h), ^{66}Ga ($t_{1/2}$: 9.3 h), and ^{64}Cu ($t_{1/2}$: 12.7 h), respectively (Fig. 3). In vitro studies demonstrated successful covalent conjugation of TRC105 to GO, without compromising the antigen-binding affinity, and excellent stability of the radiolabel in the conjugate in complete mouse serum. All three PET isotopes enabled non-invasive visualization of the GO conjugates in tumor-bearing mice, over a time scale dependent on their decay half-lives. Serial in vivo PET imaging revealed that the GO conjugate accumulated quickly in the murine breast cancer 4T1 tumor, with persistent tumor uptake over time, and was primarily cleared through the hepatobiliary pathway (Fig. 3). Blocking studies with a “cold” dose of unconjugated TRC105 confirmed CD105 specificity of the TRC105-conjugated GO, which was further validated by biodistribution and histology studies. With the availability of a large number of isotopes that are suitable for PET imaging applications,[86] it will be desirable to use the isotope with a decay half-life that matches the circulation half-life of the nanomaterial of interest in future studies of radiolabeled nanomaterials.[5, 87]

Due to the large surface area and versatile chemistry, graphene-based nanomaterials can be utilized for multimodality imaging, where the same agent can be simultaneously detected. Since no single modality is perfect among all molecular imaging techniques, combination of more than one technique can provide synergistic advantages.[88, 89] Recently, multifunctional graphene with interesting fluorescence and magnetic properties was designed and synthesized.[90] GO was reduced by a microwave-assisted process and simultaneously magnetized with decomposition of ferrocene and formation of metallic iron nanoparticles on the graphene sheet. The complex was further covalently conjugated with fluorescein o-methacrylate via a polyacrylic acid linker. It was demonstrated that the multifunctional graphene exhibited excellent biocompatibility in vitro and could be used for optical imaging in zebrafish, which did not affect the survival rate after microinjection into zebrafish embryos.

In a recent report, a rGO-iron oxide nanoparticle (rGO-IONP) based probe was developed for in vivo optical, photoacoustic tomography, and magnetic resonance imaging in the 4T1 tumor model.[91] rGO-IONP was functionalized by PEGylation to obtain improved biocompatibility, which exhibited excellent stability in bovine serum. The pharmacokinetics, biodistribution, and toxicity of the multifunctional conjugate were further studied, as well as the feasibility of using it for photothermal therapy. Accumulation of rGO-IONP in the tumor sites was confirmed by three imaging modalities: optical, photoacoustic, and magnetic resonance imaging (Fig. 4). Biodistribution study revealed tumor uptake of about 5 percentage injected dose per gram of tissue (%ID/g) at 48 h post-injection, which could be further improved by incorporation of tumor targeting ligands in the future.

Drug delivery with graphene-based nanomaterials

The intrinsic physical and chemical properties such as ultrahigh surface area and large sp^2 hybridized carbon area also make graphene-based nanomaterials promising carriers for efficient drug and gene delivery. In one of the abovementioned studies, rituximab-conjugated GO was loaded via π- π stacking with doxorubicin (DOX, a widely used cancer drug) for targeted drug delivery in vitro.[60] Subsequently, it was shown that GO can also be used for loading (via π- π stacking) and delivery of aromatic water-insoluble cancer drugs such as SN38, a camptothecin (CPT) analog.[92] Intriguingly, it was found that the new delivery vehicle exhibited better efficacy than that of irinotecan, a food and drug administration (FDA) approved SN38 prodrug for cancer treatment. These early studies suggested that graphene can be a novel and promising drug delivery platform for cancer therapy with aromatic drugs that have poor solubility in aqueous solutions.

A few years later, combined delivery of more than one anticancer drugs by GO was also reported,[93] which has been challenging for other nanomaterial-based delivery vehicles. Controlled loading of DOX and CPT onto FA-conjugated GO was investigated, where linear correlation was observed between the loading ratio and the concentration of the drugs.[93] In MCF-7 cells, FA-conjugated GO loaded with two drugs showed both target specificity and higher cytotoxicity than that loaded with either drug alone.

Various strategies for chemical modification or covalent functionalization have been investigated for improving the biocompatibility and solubility of graphene. It was found that an efficient method was covalently grafting graphene or its derivatives with polymers, including PEG, poly[2-(dimethylamino)ethyl methacrylate] (PDMAEMA), chitosan, pluronic F127 (PF127), poly(vinyl alcohol) (PVA), polyethylenimine (PEI), poly(N-isopropylacrylamide) (PNIPAM), among others. In one report, chitosan was grafted onto the GO surface through amide linkages.[94] After rendering good aqueous solubility and biocompatibility, chitosan-grafted GO was further loaded with CPT for in vitro drug delivery, which gave higher cytotoxic effect than CPT alone to human hepatoma HepG2 cells. In another study, PF127, a non-ionic surfactant polyol, was utilized as the solubilizing agent to coat graphene nanosheet (GN) via a one-pot process, which included reduction of GO and assembly of PF127.[95] The obtained PF127/GN nanohybrid exhibited high loading efficiency and pH-dependent release of DOX, with higher amount of DOX release from PF127/GN in acidic conditions than in basic and neutral conditions, which showed remarkable cytotoxicity to MCF-7 cells in vitro. Recently, PNIPAM, a thermo-responsive polymer, was also grafted onto GO sheets via click chemistry for loading of CPT and subsequent cancer cell killing in vitro.[96]

In one study, CPT was loaded onto two PVA functionalized carbon nanomaterials, multiwalled carbon nanotubes (MWCNTs) and GO, and the drug delivery efficiency and cytotoxicity of the two composites were compared.[97] Both MWCNT-PVA-CPT and GO-PVA-CPT exhibited higher cytotoxicity in human breast cancer MDA-MB-231 cells than “free” CPT, with the conclusion that MWCNT-PVA-CPT was superior to GO-PVA-CPT. It was suggested that GO-PVA-CPT could only enter the cells through endocytosis, while MWCNT-PVA-CPT could be taken up through both nanopenetration mechanism and endocytosis, thereby exhibiting higher cytotoxicity. Further investigation and in-depth comparisons are warranted to have a better understanding of the differences between these carbon-based nanomaterials.

Photodynamic therapy (PDT) is an emerging and promising alternative for non-invasive treatment of cancer. Upon uptake of photosensitizers (PSs) into cancer cells, irradiation with light of suitable wavelength and dosage can generate reactive oxygen species that can induce

cell death and/or necrosis. Several PSs have been loaded onto GO for PDT, including Chlorin e6 (Ce6) and hypocrellin A (HA). In vitro studies demonstrated that PDT efficacy of HA was better than GO-HA.[98] However, GO-HA exhibited much better stability which is very important for future in vivo applications. In another study, Ce6 was loaded onto FA-conjugated GO, which selectively accumulated in human stomach cancer MGC803 cells and gave good photodynamic efficacy in cell culture, after irradiation with a 632.8 nm He-Ne laser.[99]

Even though these abovementioned studies demonstrated high drug loading/delivery efficiency and promising anticancer effect in vitro, in vivo studies have yet to be carried out. To the best of our knowledge, only one report exists in the literature regarding the use of GO as a drug delivery vehicle in a preclinical mouse model, where the synergistic effect of chemo-photothermal therapy was investigated with GO.[100] DOX-loaded GO, which was capable of combining chemotherapy with external photothermal therapy, significantly improved the therapeutic efficacy. In addition, tumor recurrence was found in mice in the two control groups (DOX or GO photothermal therapy alone), but not in mice treated with combination of chemotherapy and photothermal therapy using DOX-loaded GO (Fig. 5). Furthermore, the side effects of DOX were also significantly reduced when GO was used as the delivery vehicle.

Gene delivery with graphene-based nanomaterials

Gene therapy has attracted much interest over the last several decades for the treatment of a variety of diseases such Parkinson's disease and cancer.[101] A wide variety of nanomaterials have been investigated for gene delivery and gene therapy applications, and one major challenge of gene therapy is the development of a safe gene vector which can protect DNA from degradation and enable cellular uptake of DNA with high efficiency. Graphene has been shown to bind to single-stranded DNA effectively but not double-stranded DNA. Furthermore, graphene can also protect oligonucleotides from enzymatic cleavage. Because of these advantages, graphene has recently been investigated for gene delivery applications, mostly using PEI-functionalized GO for the delivery of plasmid DNA (pDNA).

PEI, with strong electrostatic interactions with the negatively charged phosphate groups of DNA/RNA, is considered to be one of the best cationic polymers for gene delivery. The major obstacle for utilization of PEI in gene delivery is its poor biocompatibility and high cytotoxicity, especially PEI of high molecular weight. Recently, GO was conjugated with PEI of 1.2 kDa or 10 kDa (denoted as PEI-1.2k and PEI-10k, respectively) to bind with pDNA for transfection of the enhanced green fluorescent protein (EGFP) gene in HeLa cells.[102] GO-PEI-10k exhibited significantly lower cytotoxicity than the PEI-10k, but with similar EGFP transfection efficiency. Transfection with GO-PEI-1.2k resulted in much higher EGFP expression than that with PEI-1.2k, which appeared to be ineffective.

In another report, it was further demonstrated that with a PEI of 25 kDa molecular weight, the transfection efficiency of GO-PEI-25k at optimal ratio was comparable to or even higher than that of PEI-25k.[103] Importantly, GO-PEI-25k was also able to deliver pDNA into the cell nucleus, as evidenced by intracellular tracking of the reporter gene. Many literature reports have shown that transfection efficiency can usually be reduced by the presence of serum protein. However, GO-PEI-25k had high gene transfection efficiency even in the presence of 10% fetal bovine serum. In one interesting study, sequential delivery of Bcl-2-targeted short interfering RNA (siRNA) and anticancer drug DOX using PEI-grafted GO was assessed, which resulted in enhanced anticancer efficacy.[104] One of the abovementioned reports also showed that chitosan-functionalized GO could be used to efficiently load and deliver pDNA into HeLa cells.[94]

Conclusions and future perspectives

The exploration of graphene and its derivatives for biomedical applications has witnessed exciting advancement over the last few years, even though this research area is still at its infancy. For tissue engineering applications, both graphene and its derivatives have been demonstrated as biocompatible substrates for promotion of growth and spontaneous differentiation of various stem cells such as hMSCs, hNSCs, and iPSCs. Meanwhile, they have also been found to be amenable to transduction and genetic manipulation. The different surface properties of graphene-based nanomaterials, which have been found to modulate differential behavior of these cells, implicated broad potential of these nanomaterials as extracellular scaffolds to guide osteogenesis, neurite outgrowth, adipogenesis, among others. Graphene and GO can be promising candidates for many applications such as bone tissue repair,[51] cell replacement therapy in acute liver failure/hepatitis,[53] and neural prostheses for restoring the function of impaired neuronal circuits.[54]

For in vivo imaging and therapy applications, the future of nanomedicine lies in multifunctional nanoplatforms which combine both therapeutic components and multimodality imaging. The ultimate goal is to develop nanomaterial-based agents which can allow for efficient, specific in vivo delivery of therapeutic agents (drugs, genes, therapeutic isotopes, etc.) without systemic toxicity, and the dose delivered as well as the therapeutic efficacy can be accurately monitored non-invasively over time. The versatile chemistry of graphene-based nanomaterials in combination with their intrinsic properties can be explored for a wide range of biomedical applications. Furthermore, they can be engineered to bypass many biological barriers to enhance the targeting efficacy. Therefore, graphene-based nanomaterials are promising candidates as multifunctional nanoplatforms for molecular imaging and therapy, where suitably selected components (e.g. drugs, genes, targeting ligands, etc.) are integrated for each individual application. Much research effort will be needed in the near future before this can be a clinical reality.

The most feasible applications of graphene-based nanomedicine will be in cardiovascular diseases, where there is much less biological barrier for the efficient delivery of nanomaterials than to other sites, and in oncology, where the leaky tumor vasculature can allow for better tissue penetration than in normal organs/tissues. It is exciting and encouraging that graphene-based agents have been assessed with a variety of molecular imaging techniques, including optical, MRI, photoacoustic, and radionuclide-based imaging. Tumor targeting efficiency is one of the key challenges facing future biomedical applications of not only graphene-based, but also most other nanomaterials. Passive tumor targeting based on the EPR effect alone is limited by extravasation and may not be ideal. We believe that tumor vasculature targeting is a desirable approach for graphene-based nanomaterials, which does not require extravasation. Currently, only CD105 has been explored as the target for graphene-based nanomaterials. Other vascular markers of tumor angiogenesis can also be targeted in the future, such as integrin $\alpha_v\beta_3$ and vascular endothelial growth factor receptors (VEGFRs).[79, 105, 106] It may also be more advantageous to attach more than one types of targeting ligands to the surface of graphene or its derivatives, to achieve multi-receptor targeting with a single agent, which may have improved tumor specificity and targeting efficacy.

A few major obstacles for biomedical applications of graphene and its derivatives include: graphene is non-biodegradable and the possible long-term toxicity may be a concern; in vivo behavior of graphene-based nanomaterials with different structure, size, and surface properties still remains unknown; among others. In addition, there are many commercial and regulatory challenges to be tackled with the emerging generation of more complex nanomaterials, in part owing to their multicomponent nature. For example, graphene-based

multifunctional nanomaterials will be difficult and expensive to manufacture at large scale with optimal quality, once they reach the stage of clinical investigation. However, some highly complex nanoparticles have reached the clinic for Phase I trials,[107] indicating that complex nanoparticles can be manufactured with current good manufacturing practice (cGMP) compliance and satisfy regulatory requirements, which is very encouraging.

The National Cancer Institute Alliance for Nanotechnology in Cancer (http://nano.cancer.gov), an initiative encompassing the public and private sectors, was formed in 2005 to accelerate clinical translation and application of nanotechnology in personalized cancer medicine. With continued research effort and interdisciplinary collaboration, it is expected that nanotechnology (including those based on graphene) will mature into a clinically useful field in the near future. Big strides have been made over the last several years and many proof-of-principle studies (e.g. in vivo targeting, photothermal therapy, multimodality imaging etc.) have been successfully performed for graphene-based nanomaterials. The future of graphene in biomedical applications looks brighter than ever, yet many hurdles remain to be conquered.

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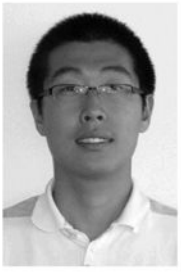
Biographies



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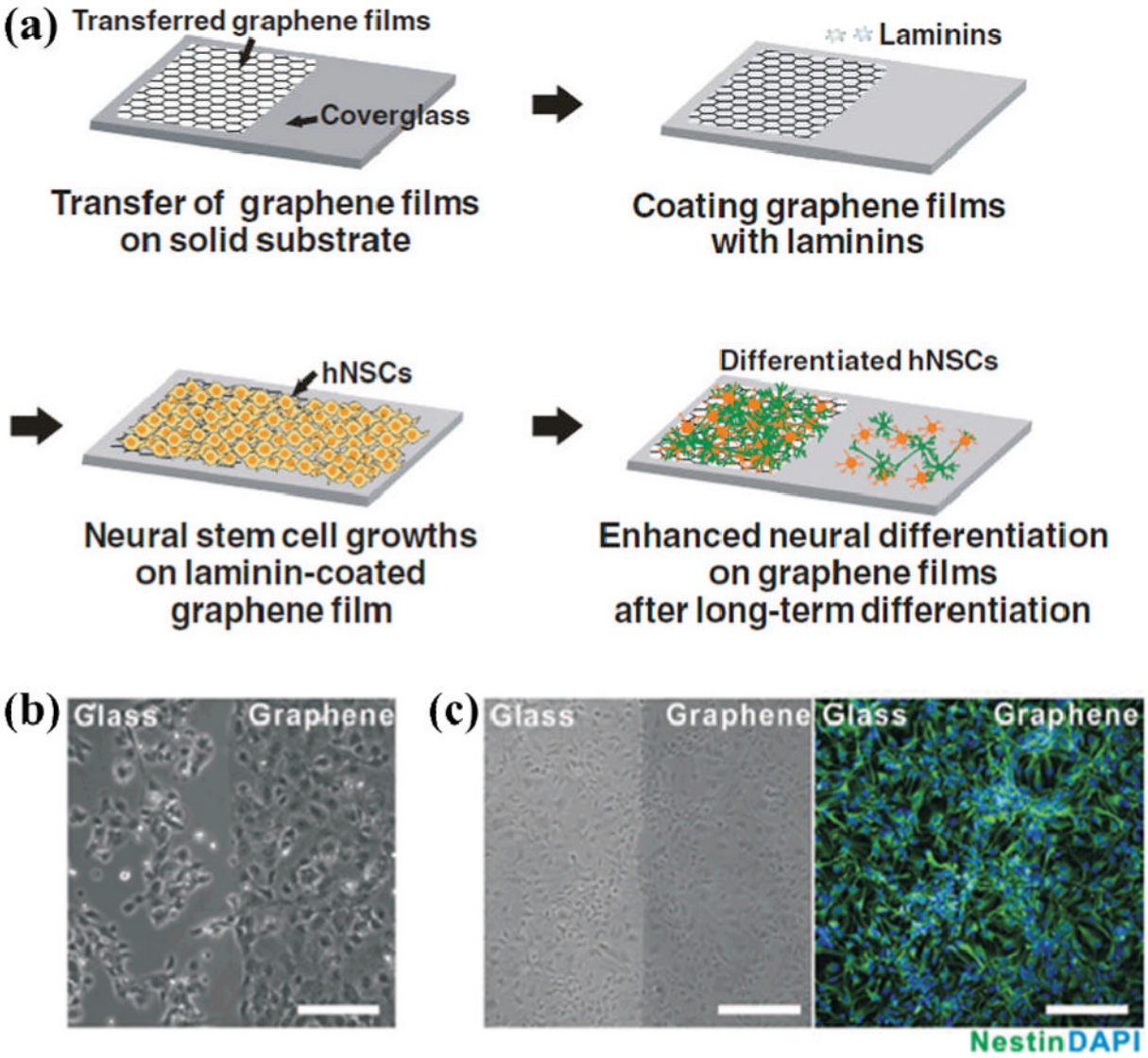

Fig. 1.

Tissue engineering with graphene. (a) A schematic diagram depicting growth and differentiation of human neural stem cells (hNSCs) on graphene coated with laminin. (b) A bright-field image of hNSCs at the boundary area between glass (left) and graphene (right) at 10 h after cell seeding. (c) Bright-field (left) and immunofluorescence (right) images of hNSCs at 5 days after seeding. Green: nestin (a marker for hNSCs); Blue: DAPI (nuclei). All scale bars represent 200 μm. Adapted from ref. [54].

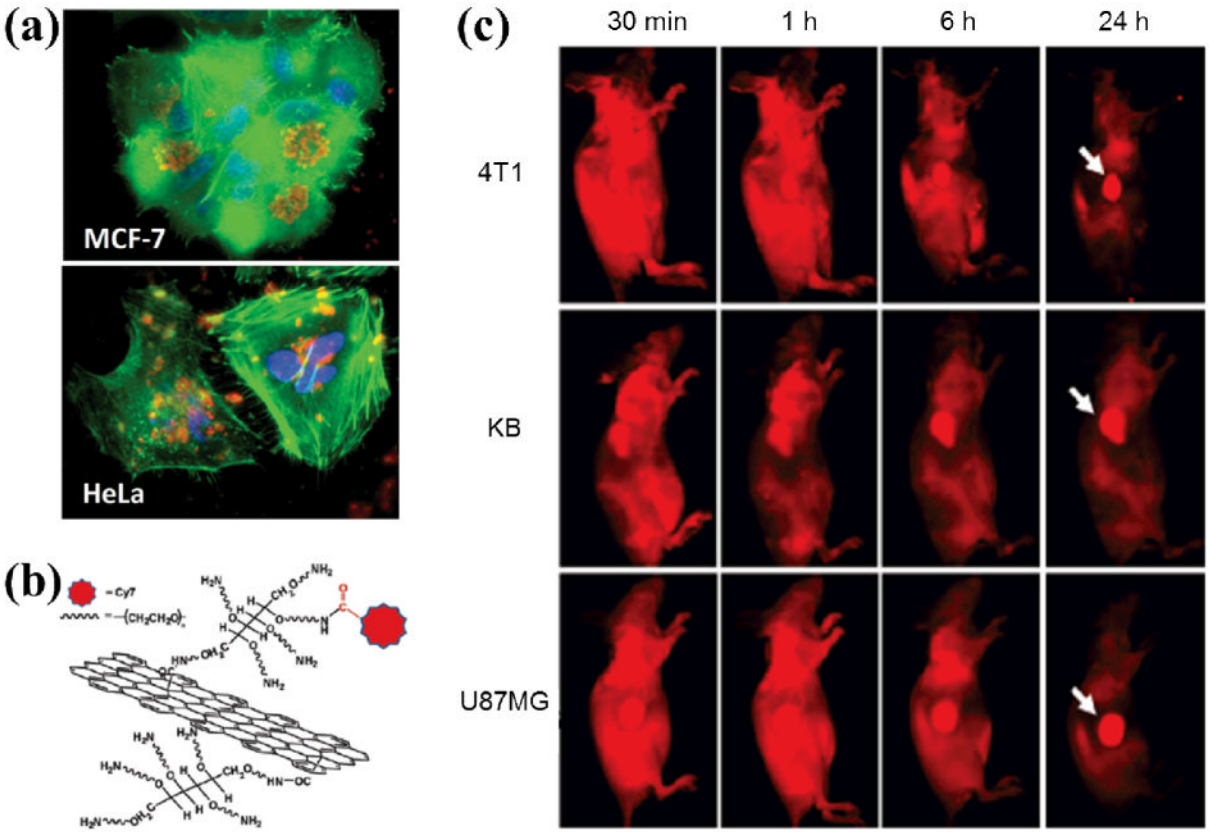

Fig. 2.

Optical imaging of graphene-based nanomaterials. (a) Cellular uptake of folic acid-conjugated QD-rGO in human breast cancer MCF-7 and HeLa cells, where QD fluorescence is shown in red-orange. (b) A schematic representation of Cy7-labeled GO through six-arm branched PEG chains. (c) In vivo fluorescence imaging of mice bearing different tumors (indicated by arrows) after intravenous injection of Cy7-labeled GO. Adapted from ref. [64] and [65].

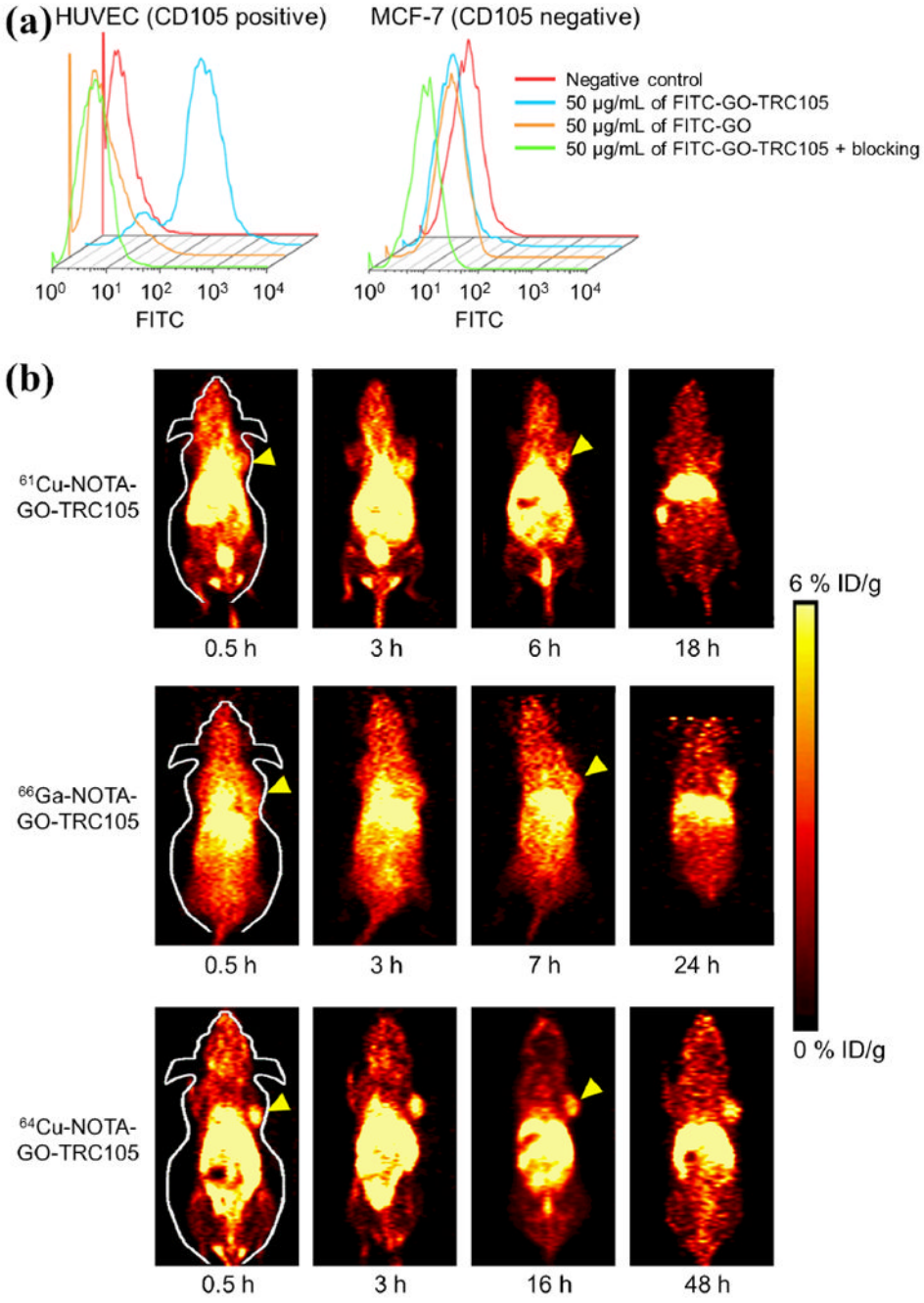

Fig. 3.

Positron emission tomography (PET) imaging of radiolabeled GO. (a) Flow cytometry analysis of GO conjugates in CD105 positive human umbilical vein endothelial cells (HUVECs) and CD105 negative MCF-7 cells. (b) Serial PET imaging of 4T1 tumor-bearing mice after intravenous injection of NOTA-GO-TRC105, labeled with each of the three isotopes: ^{61}Cu, ^{66}Ga, and ^{64}Cu. TRC105 is an antibody that binds to CD105. Arrowheads indicate the tumors. Adapted from ref. [77].

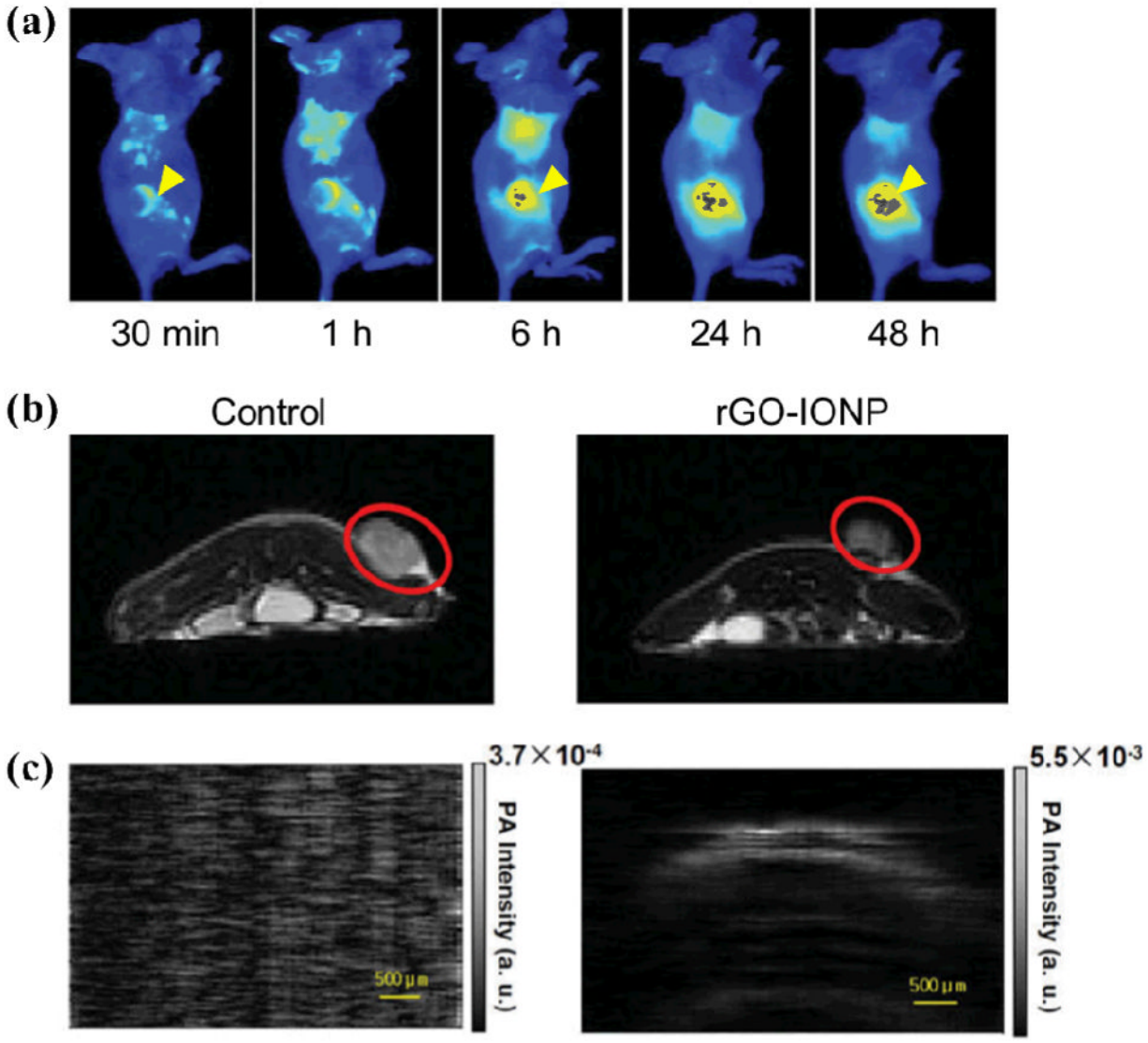

Fig. 4.

In vivo multimodality imaging of rGO-IONP in 4T1 tumor-bearing mice. (a) Serial fluorescence imaging of Cy5-labeled rGO-IONP after intravenous injection. Yellow arrowheads indicated the tumor. (b) T_2-weighted magnetic resonance imaging of rGO-IONP, where the red circles indicated the tumors. (c) Photoacoustic (PA) imaging of rGO-IONP. Adapted from ref. [91].

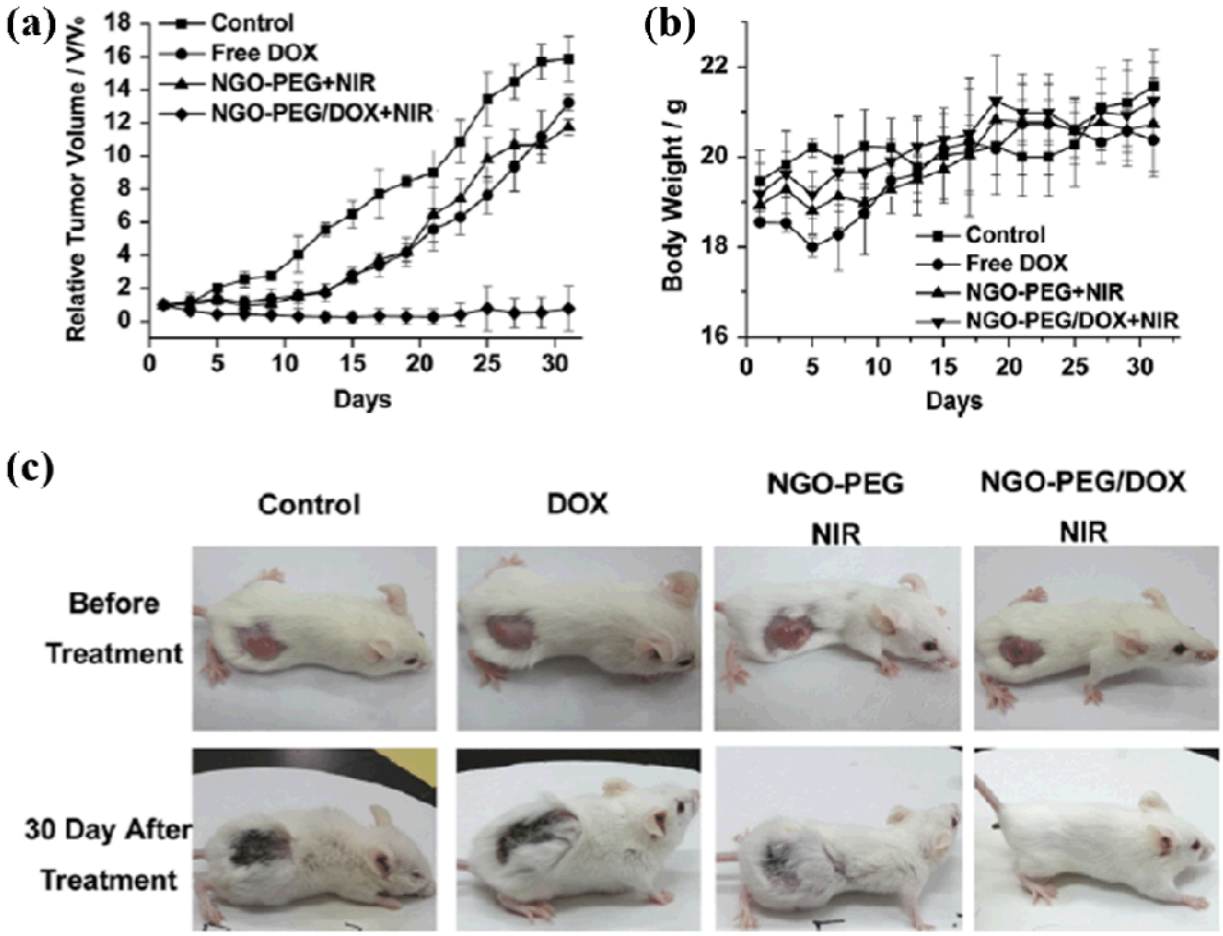

Fig. 5.

Synergistic effect of chemo-photothermal therapy with PEGylated graphene oxide. (a) Tumor growth curves of mice in different treatment groups. (b) Mean body weight of mice in different groups after treatment. (c) Representative photos of mice after different treatment. NGO: nanographene oxide; NIR: near-infrared photothermal therapy; DOX: doxorubicin. Adapted from ref. [100].