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REVIEW

Graphene: a versatile nanoplatform for biomedical applications

Yin Zhang,†[a] Tapas R. Nayak,†[b] Hao Hong[b] and Weibo Cai‡*[abc]

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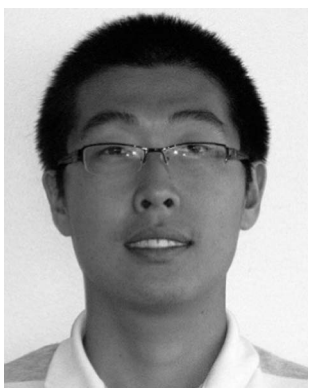
Graphene, with its excellent physical, chemical, and mechanical properties, holds tremendous potential for a wide variety of biomedical applications. As research on graphene-based nanomaterials is still at a nascent stage due to the short time span since its initial report in 2004, a focused review on 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. We then 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.

Introduction

The field of nanotechnology has advanced tremendously during the first decade of the 21st century, with focus 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

[a]*Department of Medical Physics, University of Wisconsin–Madison, WI, USA. E-mail: wcai@uwhealth.org; Fax: +1-608-265-0614; Tel: +1-608-262-1749*
[b]*Department of Radiology, University of Wisconsin–Madison, WI, USA*
[c]*University of Wisconsin, Carbone Cancer Center, Madison, WI, USA*
† These authors contributed equally to this work.
‡ Present address: Departments of Radiology and Medical Physics, School of Medicine and Public Health, University of Wisconsin–Madison, 1111 Highland Ave, Room 7137, Madison, WI 53705-2275, USA.



Yin Zhang

Yin Zhang is a graduate student in the Department of Medical Physics at the University of Wisconsin–Madison. He received a BS degree in Physics from the University of Science and Technology of China (USTC) in 2006 and a MA degree in Physics from the Johns Hopkins University in 2008. He is now pursuing a PhD degree in Medical Physics under the supervision of Prof. Weibo Cai. Design and syntheses of novel multimodal molecular imaging agents for cancer diagnosis and treatment, as well as nanotechnology and its biomedical applications, are his major research interest.

Tapas R. Nayak

Tapas R. Nayak received his MTech degree in biochemical engineering and biotechnology in 2006 from the Indian Institute of Technology, Kharagpur (India) and PhD degree in pharmacy in 2011 from the National University of Singapore. Dr Nayak is currently a Research Associate under the supervision of Prof. Weibo Cai at the University of Wisconsin–Madison. Dr Nayak has authored >10 peer-reviewed articles, >10 conference abstracts, and 2 book chapters. His research at the University of Wisconsin is focused on tissue engineering, stem cell biology, and biomedical applications of various nanomaterials such as graphene and zinc oxide.

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

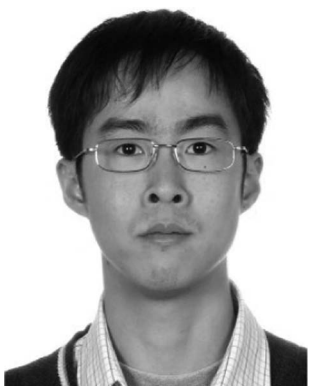
Graphene is an atom thick monolayer of carbon atoms arranged in a two dimensional honeycomb structure, and it is a basic building block for other graphitic materials such as graphite and CNTs.[12–15] Because of their unique and desirable characteristics, graphene, graphene oxide (GO), and reduced graphene 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 are still at a nascent stage and most of the reports have appeared in 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 includes tissue engineering, molecular imaging, and drug/gene delivery. Since graphene-based biosensing has been studied extensively[21–23] and several review articles 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 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. Conversely, MTT colorimetric assays revealed that graphene–chitosan composites were biocompatible to L929 cells that were derived from normal mouse subcutaneous areolar and adipose tissue.[28] Most studies to date have indicated reduced or no 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 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 that plays a critical role in the biocompatibility, since it can help in reducing the strong hydrophobic interaction of graphene or 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.



Hao Hong

Hao Hong received his BS (in 2002) and PhD (in 2008) degrees in Biochemistry and Molecular Biology from Nanjing University, P.R. China. He is currently a Research Associate under the supervision of Prof. Weibo Cai in the Department of Radiology at University of Wisconsin–Madison. Dr Hong has published > 40 peer-reviewed articles and won many awards, including the Susan G. Komen Postdoctoral Fellowship (2009–2011), the Society of Nuclear Medicine Berson–Yalow Award (2012), and multiple Travel Awards to attend international conferences. Dr Hong has authored >30 conference abstracts, 4 book chapters, and co-edited a book.

Weibo Cai

Weibo Cai received his PhD degree in Chemistry from UCSD in 2004 and did post-doctoral research at Stanford University. In 2008, Dr Cai joined the University of Wisconsin–Madison, where his research is focused on molecular imaging and nanotechnology (http://mi.wisc.edu/). Dr Cai has authored >100 peer-reviewed articles, 8 book chapters, >90 conference abstracts, and edited a book. He has won many awards, including the Society of Nuclear Medicine Young Professionals Committee Best Basic Science Award (2007), the European Association of Nuclear Medicine Springer Prize (2011), among many others. Dr Cai is currently the Executive Editor of the American Journal of Nuclear Medicine and Molecular Imaging.

Several *in vivo* studies indicated chronic toxicity associated with GO, which was primarily deposited in the lung after intravenous injections 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 the mice.[35] Similar results were also obtained for PEGylated nanographene sheets, which did not cause appreciable toxicity at a dose of 20 mg kg^{-1} in mice over 3 months, as evidenced by blood biochemistry, haematological analysis, and histological examinations.[37] Aside from graphene and GO, respirable graphene nanoplatelets (which consisted of several layers of graphene sheets) were inflammatory 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] The 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 *in vitro* and *in vivo* toxicity studies mentioned above have paved the way for the future investigation of graphene-based nanomaterials for various applications such as tissue engineering, molecular imaging, drug/gene delivery, and 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 an 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 of cells in this context. In addition, they can serve as structural reinforcement for other scaffold materials that are currently being used for this purpose.[47]

Graphene was shown to be a suitable 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 any notable deleterious effects. In other studies, graphene was found to promote the 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 the 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 the differentiation of these hMSCs into osteocytes in a controlled manner, but also accelerated the differentiation to a rate comparable to that of 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. This contrasted with GO, which did not interfere with the process. These results were 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 interactions with graphene. Together, these findings suggested that graphene exhibits different binding interactions with different growth factors and hence has a different influence on the growth of stem cells and their subsequent differentiation to specific tissues.

This aspect was further confirmed by a recent study on cultured mouse induced pluripotent stem cells (iPSCs) on graphene and GO surfaces, which displayed distinct proliferation and differentiation characteristics.[53] GO enabled better iPSC attachment and proliferation than graphene, owing to the presence of abundant oxygen atoms (*e.g.* OH) on its surface. In addition, GO was found to promote the differentiation of iPSCs towards an endodermal lineage to a higher extent than graphene, although the differentiation towards ectodermal and mesodermal lineages was comparable for both surfaces. This result 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 amount of recent interest in the use of graphene-based nanomaterials for tissue engineering applications has culminated in many exciting and intriguing literature reports 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 consist of *in vitro* studies of specific cells, future *in vivo* investigations will ultimately lead to its use as an implantable tissue engineering material. The continued development of non-invasive imaging techniques will undoubtedly

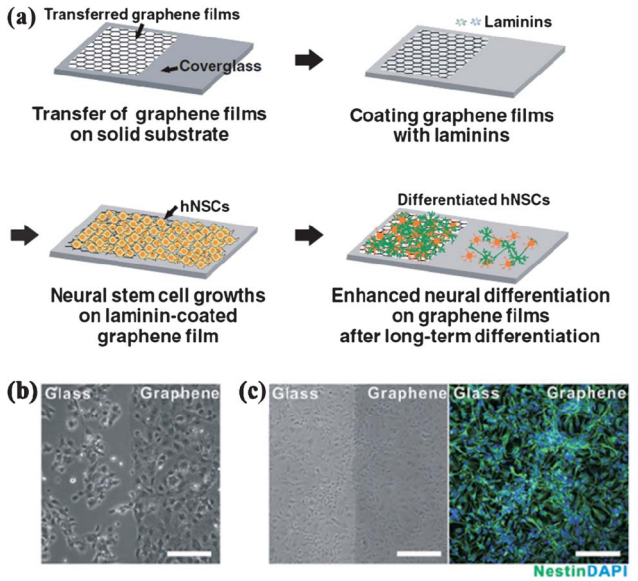


Fig. 1 Tissue engineering with graphene. (a) A schematic diagram depicting growth and differentiation of human neural stem cells (hNSCs) on graphene coated with laminin. (b) A bright-field image of hNSCs at the boundary area between glass (left) and graphene (right) at 10 h after cell seeding. (c) Bright-field (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 with permission.

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 traditional imaging techniques but also introduces molecular imaging agents to measure the expression of indicative markers at different stages of disease.[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 nanometers [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] It exhibited an 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] Another example is trastuzumab-conjugated rGO, which 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 a 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 and 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. In contrast to single-walled carbon nanotubes (SWNTs),[66,67] GO showed much higher accumulation in the kidneys rather than in the RES (*e.g.* liver and spleen) at 24 h post-injection. Successful optical imaging confirmed high tumor uptake of GO after intravenous administration. Photothermal therapy was then carried out with low-power NIR laser irradiation, which efficiently ablated the tumors at a dose of 20 mg kg^{-1} of GO. No short-term side effects were observed in the treated mice, which paved the way for future long-term toxicity and other *in vivo* investigations 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 mice.[37] Radiolabeled GO was found to accumulate mainly in the RES and was 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 that were treated with a 20 mg kg^{-1} dose of GO did not show any noticeable toxic effects in 3 months, suggesting excellent biocompatibility of the PEGylated GO.

Optical imaging is a relatively low-cost method that is primarily suitable 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 concentrations *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 requires tracer concentration many orders of magnitude lower than the pharmacologically active level. The most widely used PET isotopes include

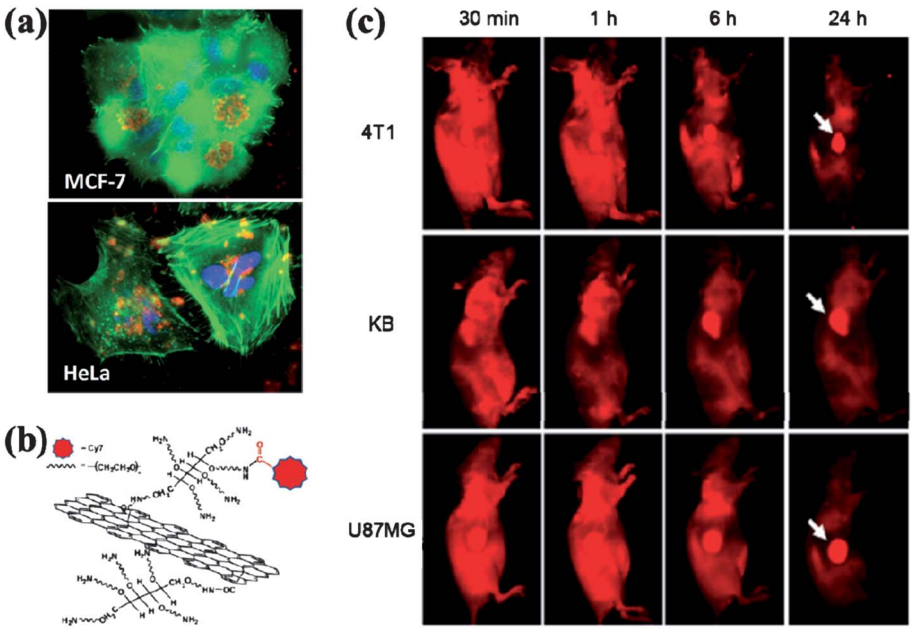


Fig. 2 Optical imaging of graphene-based nanomaterials. (a) Cellular uptake of folic acid-conjugated QD–rGO in human breast cancer MCF-7 and HeLa cells, where QD fluorescence is shown in red-orange. (b) A schematic representation of Cy7-labeled GO through six-arm branched PEG chains. (c) *In vivo* fluorescence imaging of mice bearing different tumors (indicated by arrows) after intravenous injection of Cy7-labeled GO. Adapted from ref. 64 and 65 with permission.

^{11}C ($t_{1/2}$ = 20 min), ^{18}F ($t_{1/2}$ = 110 min), and ^{64}Cu ($t_{1/2}$ = 12.8 h), among others.

To date, most *in vivo* imaging studies that use 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. As a result, 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; Fig. 3). *In vitro* studies demonstrated the 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 the 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 conjugates accumulated quickly in the murine breast cancer 4T1 tumor, with persistent tumor uptake over time. The conjugates were primarily cleared through the hepatobiliary pathway (Fig. 3). Blocking studies with a "cold" dose of unconjugated TRC105 confirmed the CD105 specificity of the TRC105-conjugated GO, which was further validated by biodistribution and histological studies. With the availability of a large number of isotopes that are suitable for PET imaging applications,[86] it is desirable to use an 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 their large surface area and versatile chemistry, graphene-based nanomaterials can be utilized for multimodal imaging, where the same agent can be simultaneously detected by several imaging techniques. Since no single modality is perfect among all of the molecular imaging techniques, the 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 by decomposition of ferrocene and formation of metallic iron nanoparticles on the graphene sheet. The complex was then 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. The multifunctional graphene did not affect the survival rate after microinjection into zebrafish embryos.

In a recent report, a rGO–iron oxide nanoparticle (rGO–IONP) 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 for

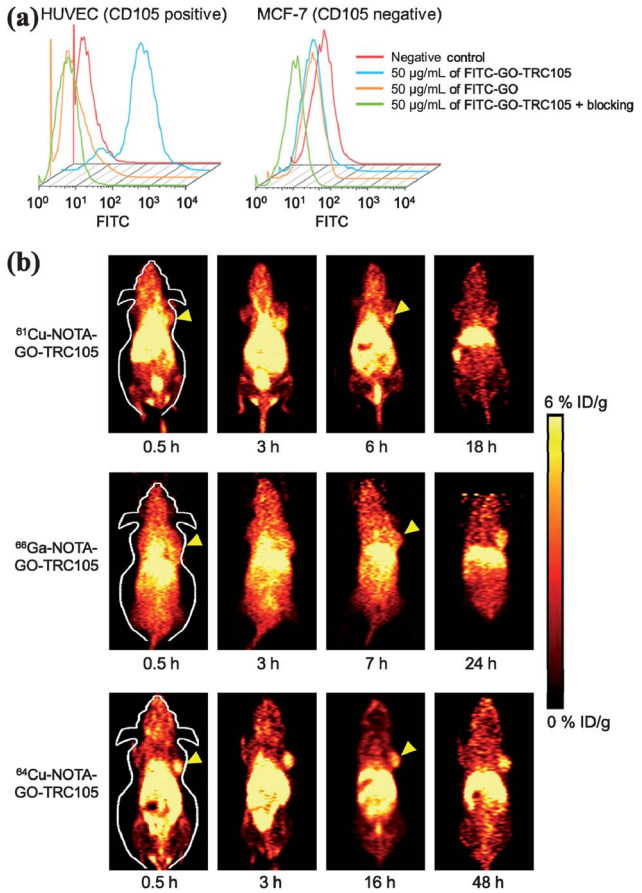


Fig. 3 Positron emission tomography (PET) imaging of radiolabeled GO. (a) Flow cytometry analysis of GO conjugates in CD105 positive human umbilical vein endothelial cells (HUVECs) and CD105 negative MCF-7 cells. (b) Serial PET imaging of 4T1 tumor-bearing mice after intravenous injection of NOTA–GO–TRC105, 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 and ref. 78 with permission.

improved biocompatibility, which then exhibited excellent stability in bovine serum. The pharmacokinetics, biodistribution, and toxicity of the multifunctional conjugate were further studied, as well as the feasibility of its use 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). A biodistribution study revealed a tumor uptake of about 5% of the injected dose per gram of tissue (%ID per g) at 48 h post-injection, which may be further improved in the future by the incorporation of tumor targeting ligands.

Drug delivery with graphene-based nanomaterials

The intrinsic physical and chemical properties, such as ultrahigh surface area and large sp^2 hybridized carbon area, make graphene-based nanomaterials promising carriers for efficient drug and gene delivery. In one of the studies mentioned above, 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 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 using 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, and a 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 better 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. An efficient surface modification method is covalently grafting polymers to graphene or its derivatives, 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. The drug-loaded chitosan-grafted GO was more cytotoxic than CPT alone to human hepatoma HepG2 cells. In another study, PF127, a non-ionic surfactant polyol, was used as a solubilizing agent to coat graphene nanosheets (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. The PF127/GN 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 a nanopenetration mechanism and endocytosis, thereby exhibiting higher cytotoxicity. Further investigation and in-depth comparisons are warranted for better understanding the differences between these carbon-based nanomaterials.

Photodynamic therapy (PDT) is an emerging and promising alternative for non-invasive cancer treatment. Upon uptake of

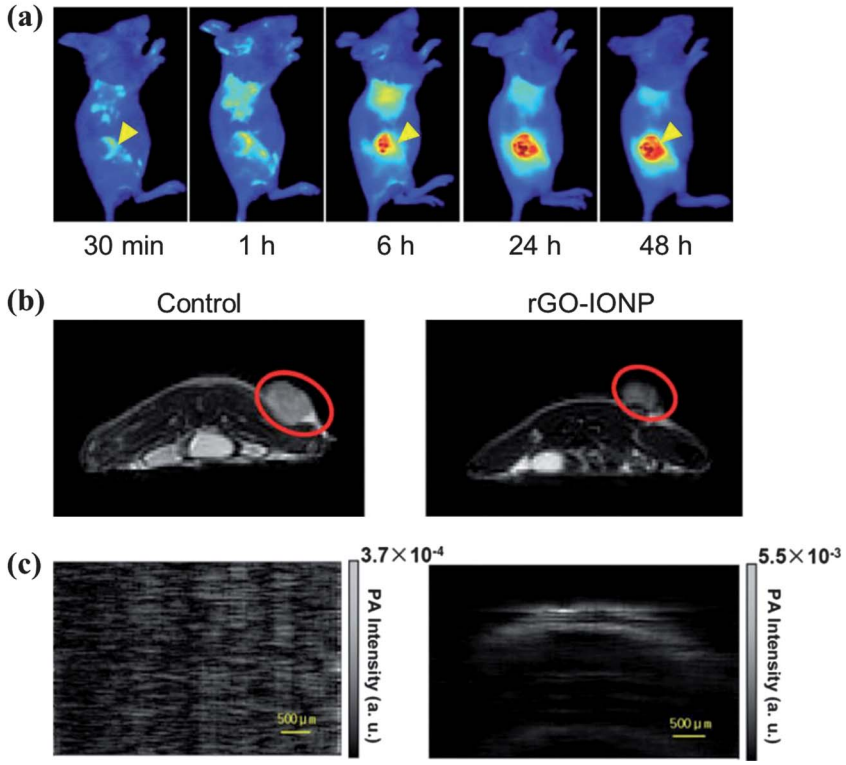


Fig. 4 *In vivo* multimodal imaging of rGO–IONP in 4T1 tumor-bearing mice. (a) Serial fluorescence imaging of Cy5-labeled rGO–IONP after intravenous injection. Yellow arrowheads indicate the tumor. (b) T_2-weighted magnetic resonance imaging of rGO–IONP, where the red circles indicate the tumors. (c)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

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 the 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 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 a combination of chemotherapy and photothermal therapy using DOX-loaded GO (Fig. 5). Furthermore, the side effects of DOX were 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. 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 forms strong electrostatic interactions with the negatively charged phosphate groups of DNA/RNA, and 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 load pDNA for transfection of the enhanced green fluorescent protein (EGFP) gene into 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.

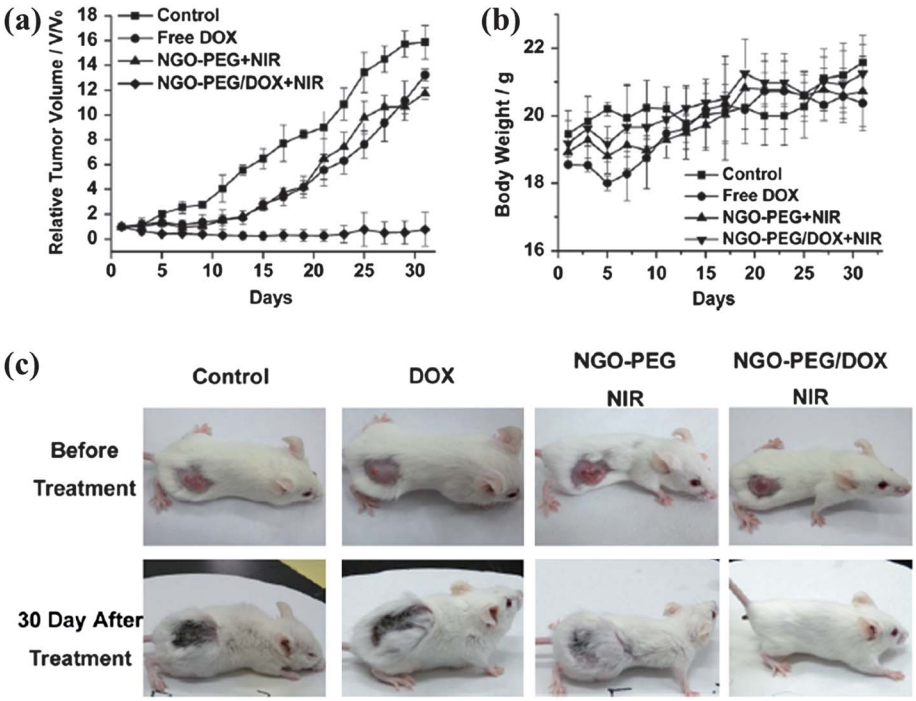


Fig. 5 Synergistic effect of chemo-photothermal therapy with PEGylated graphene oxide. (a) Tumor growth curves of mice in different treatment groups. (b) Mean body weight of mice in different groups after treatment. (c) Representative photos of mice after different treatment. NGO: nano-graphene oxide; NIR: near-infrared photothermal therapy; DOX: doxorubicin. Adapted from ref. 100 with permission.

In another report, it was further demonstrated that with PEI of 25 kDa molecular weight, the transfection efficiency of GO–PEI-25k at the 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 reports have shown that transfection efficiency is reduced by the presence of serum proteins. However, GO–PEI-25k had high gene transfection efficiency even in the presence of 10% fetal bovine serum. In one interesting study, the sequential delivery of Bcl-2-targeted short interfering RNA (siRNA) and anticancer drug DOX using PEI-grafted GO resulted in enhanced anticancer efficacy.[104] One of the reports mentioned above 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 in its infancy. For tissue engineering applications, both graphene and its derivatives have been demonstrated as biocompatible substrates for the 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, indicated the broad potential of these nanomaterials as extracellular scaffolds to guide osteogenesis, neurite outgrowth, and adipogenesis, among others. Graphene and GO can be promising candidates for 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 that combine both therapeutic components and multimodal imaging. The ultimate goal is to develop nanomaterial-based agents that 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 a lower biological barrier for the efficient delivery of nanomaterials than 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 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 may 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 advantageous to attach more than one type of targeting ligand to the surface of graphene or its derivatives. This can enable multi-receptor targeting with a single agent, which may have improved tumor specificity and targeting efficacy.

A few major obstacles for the biomedical application of graphene and its derivatives include the fact that graphene is non-biodegradable and long-term toxicity may be a concern. The *in vivo* behavior of graphene-based nanomaterials with different structure, size, and surface properties still remains unknown. Furthermore, there are many commercial and regulatory challenges to be overcome 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, it should be noted that some highly complex nanoparticles have already reached the clinic for Phase I trials.[107] This indicates 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, multimodal 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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