Graphene-Based Nanomaterials for Bioimaging

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Abstract

Graphene-based nanomaterials, due to their unique physicochemical properties, versatile surface functionalization, ultra-high surface area, and good biocompatibility, have attracted considerable interest in biomedical applications such as biosensors, drug delivery, bioimaging, theranostics, and so on. In this review, we will summarize the current advances in bioimaging of graphene-based nanomaterials, including graphene, graphene oxide (GO), reduced graphene oxide (rGO), graphene quantum dots (GQDs), and their derivatives. There are two methods to synthesize graphene-based nanomaterials: \textit{in situ} synthesis and binding method. We will highlight the molecular imaging modalities including optical imaging (fluorescence (FL), two-photon FL, and Raman imaging), PET/SPECT (positron emission tomography/single photon emission computed tomography), MRI (magnetic resonance imaging), PAI (photoacoustic imaging), CT (computed tomography), and multimodal imaging. In the end, we will elaborate on the prospects and challenges of their future bioimaging applications.

Graphical abstract

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Keywords
Graphene; graphene oxide; reduced graphene oxide; graphene quantum dots; photoacoustic imaging; PET/SPECT; MRI; bioimaging

1. Introduction

Graphene, an atomically thick sheet of two-dimensional (2D) honeycomb monolayer sp² hybridized carbon atoms, is the basic building block for all other dimensionalities of carbon nanomaterials, such as fullerene, carbon nanotubes, and carbon nanohorns (1–3). Since its isolation in 2004, graphene and its derivatives have gained considerable attention in chemistry, materials, physics and biomedical communities (4). Afterwards, the new members of graphene family, such as graphene oxide (GO) (5, 6), reduced graphene oxide (rGO) (7, 8), graphene quantum dots (GQDs) (9–11), and their derivatives were extensively explored in biosensors, drug delivery, bioimaging, theranostics, and so on (12–16).

Graphene with aromatic structure has many carbon-carbon bonds in the plane, which provide free π electrons and reactive sites for surface reactions (3). GO, graphene’s water-soluble derivative, is a highly oxidized form of chemically modified graphene, which consists of single atom thick layer of graphene sheets with hydroxyl (−OH) and epoxide (−O −) functional groups on the two accessible sides, and carboxylic acid (−COOH) groups at the edges (17, 18). The unmodified areas of graphene in GO containing free π electrons are hydrophobic and capable of drug loading and non-covalent surface modification by π–π stacking and hydrophobic interactions (19, 20). The epoxide, hydroxyl, and carboxylic acid groups of GO are uncharged but polar, allowing weak interactions, hydrogen bonding and other surface reactions (21). By reduction treatment of GO, the oxygen content, surface charge, and hydrophilicity of GO are decreased, and then rGO is produced with restored electrical conductivity and enhanced optical absorbance (8, 22). GQDs are two-dimensional...
graphene fragments sized in 10–60 nm, usually not single-layer but multi-layers (~10 layers of rGO) (23).

The biomedical applications of graphene and its derivatives are extensively investigated for diagnostics, drug delivery, near-infrared (NIR) light induced photothermal therapy and bioimaging (12). Among them, bioimaging plays critical roles in both research and clinical practice, which allows the observation and study of biological processes from the cellular and subcellular levels to small animals (24). By exploiting specific molecular probes or contrast agents, this powerful technique can detect and characterize early stage disease and provide a rapid method to monitor the treatment response (24). Due to their versatile surface functionalization and ultra-high surface area, graphene and its derivatives can be easily functionalized by small molecular dyes, polymers, nanoparticles, drugs or biomolecules to obtain graphene-based nanomaterials for different bioimaging applications (13). Herein, we focus on applying graphene-based nanomaterials for different molecular imaging modalities, including optical imaging (FL, two-photon FL, and Raman imaging), PET/SPECT (positron emission tomography/single photon emission computed tomography), MRI (magnetic resonance imaging), PAI (photoacoustic imaging), CT (computed tomography), and multimodal imaging (Fig. 1). Each imaging modality will be discussed in details in the subsequent sections. In the end, we will briefly discuss the prospects and challenges on their future bioimaging applications.

2. Synthesis of graphene-based nanomaterials

2.1 Synthesis of graphene, GO, reduced GO, and GQDs

Graphene can be synthesized by either bottom-up or top-down strategy (25). The bottom-up strategy mainly involves chemical vapor deposition (CVD), organic synthesis, and solvothermal synthesis (13). The top-down strategy mainly involves mechanical, physical and chemical exfoliation methods (26). GOs are typically produced by the Hummer’s method through the oxidative exfoliation of graphite using KMnO$_4$/H$_2$SO$_4$ (27). Reduced GO can be obtained by treating GO with reducing agents, such as hydrazine, hydrazine hydrate, L-ascorbic acid, and so on (18). GQDs are usually prepared by thermal oxidation of GOs or other carbon precursors (28, 29).

2.2 In situ growth method

GO has been employed as a template to direct the synthesis of inorganic nanomaterials, such as iron oxide (30), gold nanoparticles (31), silver nanoparticles (32), and so on. Some functional groups of GO, such as carboxylic acid and hydroxyl groups, can serve as the binding sites with metal ions, and form nuclei surrounding the interaction sites between metal ions and GO, and followed by the growth of nanomaterials around the nucleus (32). For instance, GO/iron oxide composites can be synthesized by the following methods: Fe$^{3+}$/Fe$^{2+}$ ions in the proper molar ratio (2:1) were mixed with GO, and then were precipitated by alkaline solutions, such as ammonia solution (NH$_3$•H$_2$O), and sodium hydroxide (NaOH) (33, 34). Besides the coprecipitation strategy of Fe$^{3+}$ and Fe$^{2+}$ ions under alkaline condition, hydrothermal reaction was also employed to prepare GO/iron oxide composites by using iron chloride hexahydrate as the iron source (30, 35). The as-prepared
GO/iron oxide composites can be used as MR contrast agents for MRI. GO/gold composites or GO/silver composites can be prepared by incubating metal ions (AuCl₄⁻, Ag⁺, etc.) with GO, and then followed by the reduction of these metal ions (32). With different reducing agents, the size, shape, and morphology of metal nanoparticles can be controlled. The GO/metal composites can be used for X-ray CT imaging. These in situ synthetic routes are simple and effective for cost-effective large-scale production. Additionally, the GO/metal composites prepared by this method is rather stable, due to the strong covalent binding of GO and inorganic nanomaterials.

There are several challenges for the in situ growth method: i) How to obtain GO with uniform size and narrow distribution? Current synthesis methods are usually based on the top down strategy. The bottom up strategy may have potential to make very uniform GO. ii) How to control the functional groups on GO? These groups directly determine the number, density, and location of the nuclei of inorganic nanomaterials on GO, which ultimately influence the quality and properties of graphene-based nanomaterials.

2.3 Binding method

Although the in situ synthetic strategies can directly control the growth of inorganic nanomaterials on the surface of GO/rGO, it often does not provide a good control over the particle size and morphology (36). To precisely control the particle size and morphology, there is an alternative strategy using a two-step binding method: i) prepare the inorganic nanomaterials by their preferred synthetic route. ii) deposit these as-prepared nanomaterials onto GO sheets through physical adsorption, van der Waals forces, electrostatic binding, or charge transfer interactions (37). This strategy doesn’t affect the graphene’s natural structure and appears to be more versatile (36). Meanwhile, this method offers good control over the size, shape, and functionality of nanomaterials. For example, Chen et al. incorporated 11-mercaptoundecanoic acid (MUA) capped-CdSe/ZnS quantum dots (QDs) with amphiphilic poly(L-lysine) modified rGO through the electrostatic interactions (38). Bovine serum albumin (BSA) capped-QDs can also be grafted onto polyethylenimine (PEI) adsorbed rGO (39). In another case, the dodecanethiol-CTAB-capped gold nanoclusters (GNCs) were anchored on the surface of rGO. These as-prepared composites kept their size, morphology, and fluorescent properties of original nanoparticles for FL imaging (40).

The above-mentioned strategy is based on noncovalent binding between GO/rGO with inorganic nanomaterials. Likewise, a number of drugs (doxorubicin (DOX) (41), camptothecin (CPT) (42), etc.), photosensitizers (PSs) (chlorin e6 (Ce6) (17), sinoporphyrin sodium (DVDSM) (43), etc.) can be effectively loaded onto the surface of graphene, rGO or GO sheets via hydrophobic interactions and π–π stacking. Besides the noncovalent binding, the covalent binding is also employed to modify the surface of graphene and its derivatives, which is usually achieved through the reaction with the –COOH and -OH groups (13). For instance, some dyes (Cy7 (44), Cy5 (45), IRDye800 (46), fluorescein (47), etc.), nanoparticles (UCNPs (Tm³⁺/Er³⁺/Yb³⁺)) (48), and biomolecules (folic acid (17), RGD peptide (49), Herceptin (50), transferrin (51), hyaluronic acid (52), β-cyclodextrin (53), TRC105 (54), vascular endothelial growth factor (VEGF) (55), etc.) were covalently linked to GO/rGO for FL imaging and active targeting, respectively. Meanwhile, this approach was
also employed for radionuclide labeling, such as $^{125}$I (44), $^{64}$Cu (54), $^{66}$Ga (56), $^{198,199}$Au (57), $^{111}$In (58), and so on. The radionuclide-labeled GOs can be used for PET/SPECT imaging.

There are also challenges facing the binding method: i) how to obtain GOs with uniform size and narrow distribution? ii) how to control the binding efficiency, density, and location of compounds/nanomaterials on GO/rGO? iii) how to improve the stability of graphene-based nanomaterials, especially for radionuclide labeling in PET/SPECT imaging? iv) how to control the distance between fluorescent components and GO/rGO, especially for fluorescent labeling in FL imaging? It is crucial to avoid FL quenching of fluorophores by GO and keep the original fluorescent property of fluorophores for better performance in FL imaging.

### 2.4 Surface functionalization

Water dispersibility of graphene and reduced GO is rather poor due to the absence of oxygen-containing hydrophilic groups (59). Additionally, due to the charge screening effect, water-soluble GO tend to form aggregates in physiological buffers (13). Moreover, a number of studies have indicated that graphene and GO could cause toxicity in biological systems, strongly depending on their surface chemistry (60). Therefore, the surface functionalization of graphene and its derivatives is a crucial step for further biomedical applications. So far, two main strategies including covalent and non-covalent approaches, using various biocompatible coatings and targeting ligands, have been employed to engineer functionalized graphene-based materials with improved aqueous solubility, biocompatibility, and selectivity (49). For example, a variety of biocompatible and biodegradable macromolecules, such as polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), chitosan, and so on, have been used to modify graphene and its derivatives (26). Furthermore, a variety of biomacromolecules, such as deoxyribonucleic acids (DNAs), enzymes and proteins, were also employed to biofunctionalize graphene and its derivatives (26).

### 3. Graphene-based nanomaterials in bioimaging

#### 3.1 Optical imaging

Optical imaging, a non-invasive technique, uses visible light and the special properties of photons to obtain detailed images of organs and tissues as well as smaller structures including cells and even molecules (24). It has advantages over other imaging modalities including relatively low cost, high sensitivity ($\sim 10^{-9}$–$10^{-12}$ mol/L), nonionizing radiation, real-time imaging; short acquisition time, and multiplexing capability (61). However, this modality suffers from poor tissue penetration (0–2 cm), strong tissue scattering of photons in the visible light region (395–600 nm) (62), and significant background because of tissue autofluorescence and light absorption by proteins (257–280 nm), heme groups (absorbance maximum at 560 nm), and even water (above 900 nm) (63). To address these issues, near-infrared window (NIR, 650–900 nm) (64) and second NIR window (NIR-II, 1000–1700 nm) (65, 66) imaging modalities have been explored with the advantages of reduced autofluorescence, reduced tissue scattering, and greater depth of penetration for \textit{in vivo} imaging.
Graphene-based nanomaterials were actively explored for optical imaging, mainly including FL imaging, two-photon FL imaging (TPFI), Raman imaging, and so on. The dyes, PSs, QDs, GNCs, or UCNPs functionalized GO/rGO have been widely investigated for FL imaging (12). GQDs, due to their intrinsic photoluminescence, can be used as photoluminescence probes for FL imaging. Nitrogen-doped GQDs (N-GQDs) exhibit a strong two-photon absorption cross section as high as 48000 Göppert-Mayer units and are demonstrated as an efficient two-photon fluorescent probe for TPFI (67). More interestingly, graphene and its derivatives also display great potential as efficient quenchers in graphene-based nanosensors for many fluorescent moieties, including small molecule dyes, QDs and conjugated polymers via FL resonance energy transfer or charge transfer (68). Additionally, the intrinsic Raman signals of GOs, G line (the E\textsubscript{2g} mode of sp\textsuperscript{2} carbon atoms) around 1600 cm\textsuperscript{-1} and the D line (the symmetry A\textsubscript{1g} mode) around 1350 cm\textsuperscript{-1}, can be further enhanced by metal nanoparticles (Au or Ag) for surface-enhanced Raman scattering (SERS) application in Raman imaging (32).

3.1.1 Fluorescence imaging—Fluorescence imaging is a non-invasive technique based on photons emitted from fluorescent probes (24, 69, 70). Based on the intrinsic photoluminescence of nano-graphene oxide (nGO), the Dai group for the first time reported B-cell specific antibody Rituxan (anti-CD20) conjugated and polyethylene glycol (PEG) modified GO (nGO-PEG-Rituxan) for Raji B-cells targeted FL imaging using an InGaAs detector under the 658 nm laser excitation (71). However, the quantum yield of nGO-PEG FL is rather low, which limits its further in vivo FL imaging of animals. Therefore, a number of groups utilized organic fluorescent dyes to functionalize GO/rGO for in vitro and in vivo FL imaging. For instance, Liu group developed a NIR dye, Cy7, conjugated nGO-PEG (nGO-PEG-Cy7) for in vivo FL imaging of tumor xenografted mice, showing high tumor accumulation of nGO-PEG-Cy7 based on the enhanced permeability and retention (EPR) effect of cancerous tumors (44). Chen group developed a multifunctional VEGF-loaded IRDye800-conjugated graphene oxide (GO-IRDye800-VEGF) for FL imaging of ischemic muscle tissues in the murine hindlimb ischemia model (46). The fluorescence intensity of ischemic limbs is stronger than that of non-ischemic limbs at all tested time points after intravenous administration, suggesting that GO-IRDye800-VEGF could actively target ischemic muscle, likely owing to the increased permeability of blood vessels in hypoxic tissues. For dye labeling of GO/rGO, the “bridge” linker between fluorescent dyes and GO/rGO, such as PEG, is crucial to prevent FL quenching of fluorophores with graphene sheets (13).

Besides conjugation with organic fluorescent dyes, the direct loading of dyes or PSs on GO/rGO was also investigated for FL imaging. Unfortunately, the FL of Ce6 (17) or 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide-alpha (HPPH or Photochlor®) (72), was drastically quenched by folic acid-conjugated GO and GO-PEG, respectively. Because both Ce6 and HPPH with single porphyrin ring allow them to be in good contact with the plane of GO via strong π–π stacking and hydrophobic interactions, and form tight complexes with GO, leading to effective FL quenching via an energy/charge transfer process. The FL quenching limits these systems for photodiagnostics especially in FL imaging. To solve this problem, a photo-theranostic agent based on sinoporphyrin sodium (DVDMS) loaded
PEGylated GO (GO-PEG-DVDMS) with improved FL property for enhanced optical imaging guided photodynamic therapy (PDT) was developed (Fig. 2A) (43). The FL of loaded DVDMS is drastically enhanced via intramolecular charge transfer. The emission peaks of DVDMS in GO-PEG-DVDMS are shifted to 644 and 670 nm (Fig. 2B). The FL intensities of GO-PEG-DVDMS are about 3–8 times higher than that of DVDMS at different weight ratios of GO-PEG: DVDMS (0.1:1–2:1) (Fig. 2C). As shown in Fig. 2D, fluorescence signal was mostly observed in the tumor area of mice intravenously (i.v.) injected with GO-PEG-DVDMS. As a comparison, free DVDMS group showed high signal throughout the body, especially in the skin. The GO-PEG-DVDMS appears to have potential for real-time FL visualization of in vivo DVDMS delivery and distribution, and imaging-guided PDT.

Due to the potential photobleaching of fluorescent dyes or PSs, inorganic nanomaterials like QDs, GNCs, or UCNPs have been used to integrate with GO/rGO for FL imaging. Chen et al. developed a multifunctional, low-toxicity QD-tagged rGO (QD-rGO) based on electrostatic interactions of positively charged poly(L-lysine)-rGO and negatively charged MUA capped CdSe/ZnS QDs (38). The QD-rGO can serve as an imaging agent in the visible region and as a photothermal agent in the NIR region. This design overcomes the following limitations: the QD toxicity is mitigated by a surfactant coating, and the FL quenching is greatly reduced by introducing MUA and poly(L-lysine), as spacers between QDs and rGO. Wang et al. successfully anchored GNCs on rGO (GNC-rGO) which retained the NIR fluorescence property of GNCs for cellular imaging (40). Zhang et al. developed a multifunctional nanoplatform by covalently grafting core-shell structured UCNPs with nGO via PEG, and then loading phthalocyanine (ZnPc) on the surface of nGO (48). The UCNP-nGO/ZnPc nanoplatform was used for diagnosis as an upconversion luminescence (UCL) imaging probe of cells and whole-body animals with high contrast.

Photoluminescent GQDs, composed of graphene lattices inside the dots, with good photostability, non-toxicity and easy functionalization, have been widely explored for FL imaging through quantum confinement and edge effects (23). The FL mechanism of GQDs may be originated from quantum size effect, electron hole recombination, zigzag sites and defect effect (energy traps). Nahain et al. developed GQD-hyaluronic acid (GQD-HA) with an average size of 20 nm for efficient CD44 targeted delivery to tumor-bearing balb/c female mice, demonstrating bright FL from the tumor tissue (73). The chemotherapy was achieved by releasing doxorubicin under mildly acidic conditions, which was loaded onto the basal plane of GQDs. In 2014, Ge et al. successfully synthesized a few GQDs with a broad absorption spanning the UV region and the entire visible region and strong deep-red emission peak at 680 nm (74). Both in vitro and in vivo studies clearly demonstrated that GQDs can act as PDT agents with a superior singlet oxygen quantum yield (~1.3), phot-and pH-stability, as well as FL contrast agents for in vivo FL imaging. GQDs are good FL contrast candidates for FL imaging. For further bioimaging applications of GQDs, the quantum yield of GQDs still needs to be improved. Meanwhile, further surface modification is also necessary to enhance the optical property of GQDs, and improve the tumor accumulation rate and reduce reticuloendothelial system (RES) retention.
3.1.2 Two-photon fluorescence imaging (TPFI)—Two-photon FL imaging (TPFI) has attracted much attention for its promising applications in both basic research and biomedical diagnostics, owing to the minor autofluorescence background, larger imaging depth (because of low Rayleigh scattering and low tissue absorption of NIR light), reduced photobleaching and phototoxicity than single-photon fluorescence imaging (12). It can provide more detailed analysis of various cellular/subcellular activities in the deep location of biological samples. Compared to one-photon excitation using simple continuous-wave lasers, two-photon nonlinear excitation requires a high reflux of excitation photons, usually by a femtosecond laser. The nonlinear excitation mode generates relatively high level of spatial resolution and decreases photobleaching. Furthermore, the twophoton excitation wavelength is known to be in the range of 700–1350 nm, it’s quite suitable for imaging deep-sited organs and tissues.

Recently, carbon-based nanomaterials, including carbon dots (C-Dots), GO, and GQDs are attracting considerable interest in the field of TPFI. Wang and Gu et al. first reported transferrin functionalized GO-PEG with strong two-photon luminescence (TPL) as a nonbleaching optical probe for three-dimensional TPFI and laser-based cancer microsurgery, using an ultrafast pulsed laser as the excitation source (51). After that, Gong group developed N-GQDs as efficient two-photon fluorescent probes for cellular and deep-tissue imaging (Fig. 3) (67). The N-GQDs with an average size of ~3 nm were prepared by a facile solvothermal method using dimethylformamide as both solvent and nitrogen source. Fig. 3A shows chemical structure of N-GQDs with dimethylamine binding to GO that enables the extraction of smaller $sp^2$ domains from the large GO sheet. As shown in Fig. 3B, upon 800 nm femtosecond pulse laser excitation, the maximum two-photon FL emission wavelength of N-GQD is indistinguishable with that of the one-photon FL spectrum of N-GQD, but the bandwidth is much narrower. The inset of Fig. 3B is the two-photon FL image of the dried N-GQD samples. Fig. 3C displays the setup used for TPFI of N-GQDs in tissue phantom with different thicknesses. As shown in Fig. 3D, even at the depth of 1800 µm, N-GQDs in the tissue phantom can still be identified with appreciable TPL signal, although the FL intensity is dramatically decreased. However, the OPFI (left panel of Fig. 3D) shows the maximum penetration depth of only 400 µm. Obviously, the TPFI using N-GQDs as fluorescent probe is particularly suitable for in vivo investigation of biostructures in the 800–1500 µm region.

3.1.3 Raman imaging—Raman spectroscopy exploits the inelastic scattering of phonons derived from molecular vibrational excitation modes, which is different from the phonon absorption and emission in FL (12). Raman imaging offers a powerful analytical tool that extends the possibilities of vibrational spectroscopy with extremely high signal-to-noise ratio, negligible photo-bleaching, and multiplexing capabilities to solve chemical and biochemical problems in a non-destructive and non-perturbing manner. GOs as Raman tags exhibit intrinsically strong D and G peaks, which can be further enhanced by integrating metal nanoparticles (75–77). By in situ reduction of Ag⁺ on GO, the Ag/GO hybrids displays a singular remarkable surface enhanced Raman scattering effect (32). Folic acid conjugated Ag/GO hybrids were also developed for targeted SERS imaging on folate receptor (FR) positive cancer cells (75). By in situ reduction of AuCl₄⁻ on GO, Au/GO
hybrids incubated Hela 229 cells exhibit much stronger Raman signals and more
distinguishable Raman images than cells incubated with the pristine GO. Ma et al. reported
gold nanoparticles compactly wrapped within nanosized GOs (NGO) as Raman imaging
probe and a drug delivery system (78). The D and G peaks of Au@NGOs were about one
order of magnitude stronger than those of the NGO, revealing sensitive imaging of
internalized Au@NGOs in Hela cells. Liz-Marzán group successfully developed a method
of seed-mediated growth of reduced graphene oxide-gold nanostar (rGO-NS)
nanocomposites and employed them as active SERS materials for anticancer drug
doxorubicin (DOX) loading and release (79). The rGO-NS nanohybrid shows tunable optical
properties by simply changing the condition of growth reaction, improved stability as
compared to bare Au nanostars, and sensitive SERS response toward aromatic organic
molecules. Furthermore, SERS applications of rGO-NS to probe DOX loading and pH-
dependent release are successfully demonstrated.

Besides in situ synthesis of Au/GO hybrids, Zhang group developed Au/GO and Au/rGO
composites by noncovalent attachment of Au nanoparticles pre-modified with 2-
mercaptopyridine to GO and rGO sheets, respectively, via π–π stacking and other molecular
interactions (36, 80). They demonstrated that the Au/GO hybrids are better SERS substrates
than the Au NPs. Afterwards, the same group also loaded 20 nm of Au nanoparticles on the
surface of sub-200 nm of GOs via amide coupling between PEG and DMSA ligands. The
prepared Au/GO hybrids were served as Raman imaging probes for studying cellular uptake
mechanisms in Ca Ski cell line.

Tan group reported the facile synthesis of alkyne-PEG-functionalized AgCu@graphene
(ACG) nanoparticles (NPs) by using chemical vapor deposition (CVD) method to grow a
few layers of graphene on the surface of AgCu NPs (81). The ACG NPs have been utilized
to enhance the unique Raman signals from the graphitic shell, allowing ACG for cell
labeling, Raman imaging, and SERS detection. As shown in Fig. 4A and B, the alkyne
molecule diphenylacetylene, with a high Raman scattering cross section was efficiently
conjugated on the PEG chain. Alkyne-PEG was functionalized on the surface of ACGs
through strong π–π stacking of diphenylacetylene tail and graphene. Compared with the
Raman spectrum of alkyne-PEG, the results clearly demonstrate the successful
functionalization of alkyne-PEG on the surface of ACGs (Fig. 4C). High-resolution Raman
images of MCF-7 cells treated with functionalized ACGs are shown in Fig. 4D−H. All three
modes, i.e., D, G, and alkyne, were utilized for the colocalization of ACGs, demonstrating
good intracellular localization capability. All signals spread throughout the cell cytoplasm
and accurately pinpoint the location of ACGs.

3.2 Radionuclide-based imaging

FL imaging cannot provide quantitative results and sometimes may be interfered by FL
quenching or photo-bleaching of fluorescent dyes, light absorption and scattering of tissues,
annd autofluorescence background. Radiolabeling method would be able to accurately track
the labeled substances in vivo in a quantitative manner with excellent sensitivity (~10−11−
10−12 mol/L) and limitless penetration depth (24). The radionuclide-based imaging mainly
contains PET and SPECT. PET and SPECT images are acquired over a nominally low
background signal and require little signal amplification (63). Graphene-based nanomaterials as promising nanoplatforms are playing an important role in PET/SPECT imaging.

In 2011, Liu group developed a method to label nGO-PEG with $^{125}$I by anchoring iodine atoms on the defects and edges of GO (44). Based on this method, a number of studies have been explored. Cai and co-workers demonstrated in vivo active tumor targeting using $^{64}$Cu labeled nGO-PEG (54). The in vivo PET imaging results evidenced the active tumor targeting by conjugating nGO-PEG with an antibody, TRC105, to target vasculatures in 4T1 murine breast tumors. As shown in Fig. 5A, the GO conjugates was labeled with $^{64}$Cu through 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA). The different time points of postinjection (p.i.) were chosen for serial PET scans. The PET imaging results demonstrated CD105 specific targeting of TRC105 conjugated GO (Fig. 5B). A parallel study used $^{66}$Ga labeled nGO-PEG for tumor-targeted PET imaging (56).

In comparison to PET, SPECT is approximately ten times less sensitive (~$10^{-10}$–$10^{-11}$ mol/L); however, SPECT enables concurrent imaging of multiple radionuclides of different energies (61). Additionally, SPECT, without the need of cyclotron, is more widely available than PET in the clinic, and SPECT radionuclides are simple to prepare and usually have a longer halflife than PET radionuclides. Cornelissen et al explored the use of anti-HER2 antibody (trastuzumab)-conjugated nGO, radiolabeled with $^{111}$In-benzyl-diethyleneetriaminepentaacetic acid (BnDTPA) via π–π stacking, for in vivo targeting and SPECT imaging (58). Radiolabeled nGO-trastuzumab conjugates demonstrated better pharmacokinetics compared to radiolabeled trastuzumab without NGO, with more rapid clearance from the circulation. Fazaeli et al. investigated the $^{198,199}$Au@AF-GO nanostructure for in vivo targeting and SPECT imaging of tumors (57). The $^{198,199}$Au@AF-GO had excellent tumor targeting/ imaging and fast washing out from the body.

### 3.3 Magnetic resonance imaging (MRI)

Compared to optical and radionuclide imaging, MRI, a noninvasive technique without ionizing radiation, has been widely used to image the anatomy as well as function of tissues in a quantitative manner with excellent spatial resolution. However, MRI suffers from its low sensitivity (~$10^{-3}$–$10^{-5}$ mol/L) and long signal acquisition time (24). Because the lattice-spin (longitudinal) relaxation time $T_1$ and spin-spin (transverse) relaxation time $T_2$ of water proton’s magnetic moment are environment-dependent, MRI contrast agents have been actively explored to enhance the relaxivity for improving the $T_1$ or $T_2$ contrast in the observable water pool (83). $T_1$ agents are mainly Gd$^{3+}$-based and provide brighter images, whereas $T_2$ agents produce darker images and are usually iron oxide nanoparticle (IONP).

Ions of paramagnetic metals such as gadolinium (Gd) and manganese (Mn) are generally toxic owing to the non-selective coordination with biomolecules (84). GOs with many oxygenated functional groups and cavities, can be readily coordinated with these ions by chelation or sequestering the ions between graphene layers, which mitigates the toxicity of these ions (12). For example, Gizzatov et al. chelated Gd$^{3+}$ ions with carboxyphenylated graphene nanoribbons (GNRs) for enhanced MRI relaxivity ($r_1 = 70 \pm 6$ mM$^{-1}$ s$^{-1}$ and $r_2 = 108 \pm 9$ mM$^{-1}$ s$^{-1}$), which are 16 and 21 times greater than the current clinically available Gd$^{3+}$-based $T_1$ agents (85). The Gd$^{3+}$-coordinated GNRs exhibit better MRI contrasts in...
both $T_1$-weighted and $T_2$-weighted images. Wei and Ma et al. developed poly(amidoamine) dendrimer-grafted gadolinium-functionalized nanographene oxide (Gd-NGO) nanoparticles as effective carriers to deliver both chemotherapeutic drugs and highly specific gene-targeting agents such as microRNAs (miRNAs) (86). Gd-NGO could be detected by MRI to identify the tumor area and quantify the concentration of therapeutics within the tumor.

Because IONP can be directly grown on graphene or its capped ligands can be linked with graphene, the superparamagnetic GOs/IONP hybrids were widely explored as $T_2$ MR contrast agents (13). For instance, Zhang group developed a GO-based platform for the formation of aggregates of dextran-coated Fe$_3$O$_4$ NPs to induce efficient $T_2$ shortening (87). The dextran-coated Fe$_3$O$_4$ NPs can anchor onto graphene sheet to form clusters or aggregates, affording enhanced MRI contrast compared to the individual Fe$_3$O$_4$ NPs. Liu group prepared rGO/IONP nanocomposite starting from GO and iron chloride hexahydrate via a hydrothermal reaction (Fig. 6A) (35). The rGO/IONP was further functionalized with the amphiphilic C$_{18}$PMH–PEG polymer via hydrophobic interactions for in vivo MRI guided photothermal therapy (PTT) (Fig. 6B). Real-time monitoring of photothermal therapeutic response of tumors by MRI was also demonstrated. Fu et al developed GO/IONP as a theranostic agent to diagnose and treat regional lymph nodes (RLN) metastasis of pancreatic cancer (88). The GO/IONP is able to visualize the regional lymphatic system by dual-modality mapping of MRI, which is helpful for surgeon to make the preoperative plan. The dual-modality mapping is able to intraoperatively distinguish RLN from surrounding tissue, and then the metastatic lymph nodes could be effectively ablated upon laser irradiation.

Besides paramagnetic metal complexes and IONP, fluorinated graphene oxide (FGO) was also explored as non-magnetic-nanoparticle carbon-based MRI contrast agent by creating dipolar C-F bonds as the paramagnetic centers (82). The superconducting quantum interference device (SQUID) characterization of the synthesized FGO showed a linear dependency with the magnetic field, suggesting its paramagnetic behavior (Fig. 6C). As shown in Fig. 6D, FGO showed good $T_2$ contrast.

### 3.4 Photoacoustic imaging (PAI)

The low-energy electromagnetic waves can penetrate deeper than the short wavelengths, radio frequency waves exhibit much lower scattering in the biological samples, which should be suitable for deep tissue/organ imaging. Photoacoustic imaging (PAI) is a hybrid imaging modality based on the PA effect, in which the absorbed short-pulsed electromagnetic energy (nonionizing laser pulses) is converted into heat, resulting in acoustic emission due to a transient thermoelastic expansion (89). In principle, upon pulsed laser irradiation, tissues or contrast agents absorb light and generate a pressure rise by localized thermoelastic expansion, then emit broadband acoustic waves during contraction that can be collected by ultrasound transducers and processed with similar reconstruction algorithms (90). PAI offers optical absorption contrast with the resolution of ultrasound for deep tissue/organ imaging.

In the graphene family nanomaterials (GFNs), rGO plays an important role as PA contrast agents, because rGOs with larger sp$^2$ domains than GOs, can absorb NIR light more efficiently (91). However, the hydrophilicity of GOs is decreased after the reduction, leading
to poor water solubility of rGOs. To address this issue, many methods have been explored. Patel et al. reported direct production of less oxygenated nanosize graphene sheets, which have small lateral size (~10 nm) and can be easily dispersed in water with higher absorption in the NIR region for PA imaging (92). To further enhance the absorption cross-section of GO, indocyanine green (ICG), with strong absorbance in the NIR region, was loaded onto GO by π–π stacking interactions (93). The ICG–GO complex has a high optical absorbance in the NIR region, and promises as an ultrahigh sensitive PA contrast agent.

Besides, GOs can be reduced and stabilized by BSA via one-step reduction method to afford the nanosized, reduced graphene oxide (nano-rGO) with high stability and low cytotoxicity as reported by Cai group (Fig. 7A) (94). The BSA functionalized nano-rGO has small lateral size (~70 nm) and good monodispersity. Fig. 7B shows the photograph of the mouse bearing MCF-7 tumor xenograft. The site and boundaries of tumor was confirmed by ultrasound imaging (Fig. 7C). Before i.v. administration of nano-rGO, the PA image showed observable but weak PA signals in the tumor region (Fig. 7E). At 2 h after administration, the PA signal in the tumor was significantly increased (Fig. 7E). The subtracted image showed prominent PA signal from the tumor area, suggesting the high tumor accumulation of nano-rGO. Region of interest analysis revealed that the tumor PA signal peaked within 0.5 h and then plateaued for at least 4 h (Fig. 7D), suggesting BSA-functionalized nano-rGO as a good PA contrast agent.

3.5 Computed Tomography (CT)

Computed tomography (CT) can provide complementary anatomical information (95). CT measures the absorption of X-rays when they pass through targets. The ability of CT to distinguish tissues is based on the fact that different tissues provide distinct degrees of X-ray attenuation, where the attenuation coefficient depends on the atomic number and electron density of the tissues. Differences in absorption between bone, fat, air, and water produce high contrast images of anatomical structures.

Nanomaterials containing electron dense elements with high atomic number such as iodine, bismuth or gold have been proposed as CT contrast agents (96). CT contrast requires high concentrations of these elements. Zhang et al. used GO@Ag nanocomposite as an X-ray contrast agent for X-ray imaging application (97). Wu and Yang et al. prepared GO/BaGdF$_5$/PEG nanocomposites using a solvothermal method for X-ray CT imaging of the tumor model in vivo, which shows better X-ray attenuation property than Iohexol (98). Liu (99) and Dai (100) groups used gold to endow GO the X-ray attenuation for X-ray and CT imaging, respectively.

3.6 Multimodal Imaging

Recently, the idea of using multiple imaging modalities in conjunction has gained popularity (63). Multimodality imaging refers to integrating the merits of individual imaging modality, and collecting all information from different imaging modalities, which offers higher efficiency and accuracy of diagnosis (101, 102). Meanwhile, combination of multimodal detectability in a single agent would avoid the additional stress on the body’s blood clearance that accompanies the administration of multiple doses of agents (103).
Graphene and its derivatives, due to their large surface area and versatile chemistry, have been utilized as building blocks for the construction of multimodal imaging contrast agents. Liu group developed a probe based on the intrinsic high NIR optical absorbance and strong magnetic property of RGO-IONP-PEG nanocomposite, as well as external labels for triple modal FL, PA, and MR imaging (35). In another case, they reported a dual-nanostructure modified graphene-based nanocomposite by growing a layer of gold on the synthesized GO-IONP, obtaining a GO-IONP-Au nanocomposite, then functionalized with PEG for dual-modal MR and X-ray imaging (99). Recently, Wu and Yang et al. reported a structure with BaGdF$_5$ NPs directly grown on the surface of GO nanosheets in the presence of PEG. The resulting GO-BaGdF$_5$-PEG shows low cytotoxicity, positive MR contrast effect and better X-ray attenuation property than Iohexol, which enables effective dual-modality MR and X-ray CT imaging (98).

Dai group developed poly(lactic acid)(PLA) microcapsules containing GO and AuNPs through a double-microemulsion water-in-oil-in-water (w/o/w) solvent evaporation technique combined with electrostatic layer-by-layer (LbL) technique for photothermal tumor destruction under the guidance of ultrasonic/CT bimodal imaging (100). The PLA microcapsule plays a dual role: it enhances ultrasound imaging and loads GO and Au NPs. Au NPs enhance CT contrast and GO serves as a strong NIR-light absorbing agent, which efficiently converts the absorbed light into heat.

We developed a nanoformula of GO-PEG loaded with photosensitizer HPPH via π–π stacking for PDT of tumors (72). The FL property of HPPH was used for FL imaging. Moreover, we labeled HPPH with a positron emitting radioisotope, copper-64 ($^{64}$Cu, $t_{1/2} = 12.7$ h), for PET imaging. The obtained GO-PEG-HPPH complex allows dual-modality FL and PET imaging. Recently, we used DVDMS-loaded GO-PEG (GO-PEG-DVDMS) for enhanced FL/PA dualmodal imaging and combined PDT and PTT (104). The GO-PEG carrier drastically improves the FL of loaded DVDMS via intramolecular charge transfer. Concurrently, DVDMS significantly enhances the NIR absorbance of GO for improved PAI and PTT. The as-prepared GO-PEGDVDMS is well suited for FL/PA dual-modal imaging and synergistic PDT/PTT.

4. Prospects and challenges

For bioimaging applications, the multiplex roles of graphene and its derivatives have been gradually uncovered: i) as imaging contrast agents due to their intrinsic FL emission, Raman scattering, and NIR absorbance (12); ii) as carriers because they have two accessible sides with large specific surface area (theoretical value of $2.630$ m$^2$ g$^{-1}$) that offer high loading capacity of drugs, dyes, PSs and other inorganic nanomaterials by physical absorption, Van der Waals forces, electrostatic binding, or charge transfer interactions (105–108); iii) as fluorescence quenchers because their $sp^2$ carbon structure is able to quench small molecule dyes, QDs and conjugated polymers via FL resonance energy transfer or charge transfer (109–111); iv) as wrapping materials because their flexible and amphiphilic structures make them suitable for wrapping or encapsulating insoluble nanoparticles, thus improving their water solubility and stability, biocompatibility, and preventing aggregation, degradation or toxicity in biological systems (112–114); v) as building blocks. Their ultra-high surface area
and versatile surface functionalization promise the synthesis of graphene-based composites, opening a new avenue for new materials construction (115–118).

The photoluminescence properties of graphene and its derivatives are different (12). For graphene, GO, and GQDs, their intrinsic photoluminescence is emitted with UV excitation, and tunable emission wavelength is located in the UV-Vis range (12). Their quantum yield (QY) is in the range of 0.02–70.3% (119–121). However, reduced GO shows a strong photoluminescence quenching effect with enhanced absorbance in the NIR region (12), unlike single-walled carbon nanotubes (SWNTs), which has unique photoluminescence in both NIR-I (700–900 nm) and NIR-II (1100-1400 nm) regions (122–124).

The intrinsic Raman characteristic vibrational modes of graphene and its derivatives are located in the range of 1000~3000 cm\(^{-1}\): the D peak, the G peak, and the 2D peak (125, 126). The D peak (the symmetry A\(_{1g}\) mode) at 1350 cm\(^{-1}\) belongs to the breathing mode. The 2D peak (also called as G0 peak) is located at about twice the frequency of the D peak. The G peak (the E\(_{2g}\) mode of sp\(^2\) carbon atoms) at 1600 cm\(^{-1}\) corresponds to the in-plane motion of the carbon atoms. For practical Raman imaging, the G peak is usually selected to determine the amount of graphene and its derivatives (12). Compared with SWNTs, it also has multiple Raman characteristic vibrational modes, including the radial breathing mode (100–300 cm\(^{-1}\)) and the G band (~1580 cm\(^{-1}\)), which correspond to the vibration of carbon atoms in the radial direction and in the tangential direction, respectively (122). However, the resonance Raman scattering of SWNTs is determined by the density of states available for the optical transition (e.g. E\(_{11}\) and E\(_{22}\) transition), which is dependent on its chirality and diameter (127). Unlike SWNTs, the Raman scattering of graphene and its derivatives is not chirality dependent, and the electronic structure of graphene with a small band gap allows a wide range of photons (visible to NIR) to be used for Raman imaging (12).

The photoacoustic property of GOs and GQDs is usually suppressed due to their disconnected small sp\(^2\) domains with oxygenated functional groups (1). The transition energy from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO) is higher than the larger sp\(^2\) domains, resulting in low NIR absorbance. For reduced GO, their small fragments of sp\(^2\) domains are reconnected with increased conductive and NIR absorbance (128). The extinction coefficient of rGO in the NIR region is comparable to that of SWCNTs, which promises efficient photothermal and photoacoustic properties for PTT and PAI.

To further improve the performance of graphene and its derivatives in bioimaging applications, it is essential to precisely control their sizes, components, and surface coatings so that batch to batch variation is minimized. For example, how to obtain GOs with uniform size and narrow distribution? Current synthesis methods are usually based on the top down strategy of stripping graphite. The bottom up strategy may have potential to make very uniform GOs from designed small molecules. Besides, the properties of contrast agents for different modalities are also very important. For example, how to control the distance between fluorescent components and GO/rGO? It is crucial to prevent FL quenching of fluorophores on graphene sheets. Additionally, how to improve the quantum yield of GOs and GQDs for FL imaging? For Raman imaging, how to enhance the Raman signals of GOs
by metal nanoparticles? For PET/SPECT imaging, how to improve the radionuclide labeling efficiency and metabolic stability of the radiolabeled GOs? For MRI, how to improve the relaxivity of graphene-based MR contrast agents? For PAI, how to enhance the optical absorbance of graphene-based PA contrast agents in the NIR region? Overall, there is still a long way before the clinical translation of graphene-based contrast agents.

For biomedical applications of graphene-based nanomaterials, the concern of long-term toxicity is another major concern (59). Systematic assessment of their toxicity, biocompatibility, immunogenicity, pharmacokinetics, and biodistribution from small animal models (e.g. zebra fish, mice, etc.) to large animal models (e.g. pigs, dogs, monkeys, etc.) will be critical (60, 129). Furthermore, more knowledge of their biological behaviors is not only helpful to understand their toxicology, but also useful to improve their bioimaging performances. Optimization of graphene-based nanomaterials aims to improve the tumor accumulation rate with high bioavailability and reduce RES retention by using specific biomarkers (such as peptides, antibodies). Meanwhile, specific surface functionalization of graphene-based nanomaterials is highly desirable to across various biological barriers, such as blood brain barriers, extracellular matrix, mucus, and so on.

5. Conclusions

In this review, we summarized the recent progress of graphene-based nanomaterials as imaging contrast agents for different bioimaging applications. The dyes, PSs, QDs, GNCs, or UCNPs functionalized GO/rGO have been used for FL imaging. GQDs and their derivatives, due to their intrinsic photoluminescence, have also been employed for FL imaging and TPLI. GOs with intrinsically strong Raman signals of D and G peaks have been used for Raman imaging, which can be further enhanced by integrating metal nanoparticles (Au or Ag). Radionuclide-labeled GOs have been explored for PET/SPECT imaging. Ions of paramagnetic metals or IONP functionalized GO/rGO have been developed for MRI. The rGOs or dyes/PSs modified GOs with strong optical absorbance in the NIR region are suitable for PAI. Nanoparticles containing electron dense elements with high atomic number can be combined with GOs for X-ray or CT imaging. By integrating other materials with specific properties onto GOs, multimodal imaging can be achieved in one platform. It is anticipated that graphene-based nanomaterials will play significant roles in bioimaging and understand the fate of the related therapeutics.

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References


Graphene-based nanomaterials, such as graphene, graphene oxide (GO), reduced graphene oxide (Reduced GO), and graphene quantum dots (GQDs) for molecular imaging, including optical imaging (fluorescence (FL), two-photon FL and Raman imaging), PET/SPECT, MRI (magnetic resonance imaging), PAI (photoacoustic imaging), CT (computed tomography), and multimodal imaging.
Fig. 2.
(a) Sinoporphyrin sodium (DVDMS) loaded GO for enhanced FL imaging guided photodynamic therapy. (b) FL spectra of GO-PEG-DVDMS at different weight ratios of GO-PEG: DVDMS. DVDMS at 100 mg/mL, GO-PEG at 10, 50, 100 and 200 mg/mL. (c) FL intensity of DVDMS at 620 nm and different mix ratios by GO-PEG-DVDMS at 640 nm. (d) In vivo FL imaging of mice bearing U87MG tumors intravenously (i.v.) injected with GO-PEG-DVDMS or DVDMS (DVDMS 2 mg/kg) at different time points. Adapted and reproduced with permission from (43), Copyright 2015, Elsevier.
Nitrogen-doped graphene quantum dots (N-GQDs) as efficient two-photon fluorescent probes for cellular and deep-tissue imaging. (A) Schematic illustration of N-GQDs chemical structure. (B) Two-photon-induced FL spectrum of N-GQD solution under 800 nm femtosecond laser excitation. Inset: Two-photon FL image of solid N-GQD (scale bar: 10 µm). (C) Schematic of the setup used for two-photon FL imaging (TPFI) of N-GQDs in tissue phantom with different thickness. (D) Penetration depth of N-GQDs for TPFI (right...
panel) and OPFI (left panel) in tissue phantom (all scale bar: 100 µm). Adapted and reproduced with permission from (67), Copyright 2013, American Chemical Society.
Fig. 4.
(A) Synthesis procedure of alkyne-PEG. (B) Schematic illustration of the alkyne-PEG functionalization of ACGs. (C) Raman spectra of alkyne-PEG with (black) and without (red) ACGs. (D–H) Raman image of MCF-7 cells treated with alkyne-PEG-modified ACGs using 1 s integration/pixel. BF, bright field; scale bar, 10 µm. Adapted and reproduced with permission from (81), Copyright 2014, American Chemical Society.
**Fig. 5.**

*In vivo* PET imaging of radiolabeled nGO–PEG. (A) A scheme of nGO–PEG conjugated with *anti*-CD105 antibody (TRC105) and 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA). The latter was used for $^{64}$Cu labeling. (B) Time-dependent *in vivo* PET imaging of mice bearing 4T1 tumors i.v. injected with $^{64}$Cu–NOTA–nGO–TRC105, $^{64}$Cu–NOTA–nGO, and $^{64}$Cu–NOTA–nGO–TRC105 after a preinjected blocking dose of TRC105. Tumors are indicated by arrowheads. Adapted and reproduced with permission from (54), Copyright 2012, American Chemical Society.
Fig. 6.
(A) A scheme showing preparation and functionalization of PEGylated RGO-IONP nanocomposites (RGO-IONP-PEG). (B) In vivo MRI of 4T1-tumor bearing mice using RGOIONP-PEG as the contrast agent. (C) SQUID magnetization curve of fluorinated graphene oxide (FGO). (D) Spin-spin ($T_2$) relaxation measurements obtained on a 4.7 $T$ MRI for FGO (I: $a = 625$, $c = 500$; II: $a = 313$, $c = 250$; III: $a = 156$, $c = 125 \, \mu g \, mL^{-1}$) and GO (I: $b = 625$, $d = 500$; II: $b = 313$, $d = 250$; III: $b = 156$, $d = 125 \, \mu g \, mL^{-1}$) with the positive control (*) consisting of diluted magnevist (0.5 mg mL$^{-1}$). Adapted and reproduced with permission from (35), Copyright 2012, Wiley-VCH (a and b). Adapted and reproduced with permission from (82), Copyright 2013, Wiley-VCH (c and d).
Fig. 7.
(A) A scheme showing the preparation of nano-rGO from nano-GO using BSA as reductant and stabilizer. (B) Photograph of the mouse bearing MCF-7 tumor xenograft. (C) Ultrasound (gray) image of the tumor region. (D) PA signal in the tumor region as a function of the injection time. (E) Ultrasound and PA dual-modality images of the tumor region using nano-rGO as contrast agent. Adapted and reproduced with permission from [84], Copyright 2013, Elsevier.