Graphene quantum dots (GQDs) have attracted tremendous attention in recent years. In this review, the recent developments are summarized in the synthesis, edge functionalization, and cytotoxicity of GQDs, followed by presenting a focused overview on their current applications for in vitro fluorescence imaging in cells and tissues as well as in vivo macroscopic and microscopic imaging in animals. Challenges and opportunities in the development of GQDs for bioimaging are also discussed.

1. Introduction

Fluorescence refers to the property of certain materials to emit light at a particular wavelength upon absorption of light with a shorter wavelength for a brief interval known as the fluorescence lifetime. The phenomenon of fluorescence is now indispensable for developing imaging technologies for both biological research and biomedical diagnosis. Comparing with other imaging techniques, such as X-ray computed tomography (CT), magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), positron emission tomography (PET), fluorescence imaging offers a unique approach that can be used to visualize morphological details for bio-species, ranging from living cells to animals, without any harmful radioactivity.

In fluorescence bioimaging, fluorescent probes are used to play an important role in labeling the molecules of interest and amplifying the fluorescence signal. The quality of fluorescence imaging depends strongly on the nature of fluorescent probes. Therefore, the development of efficient, photostable, and biocompatible fluorescent probes has long been a hot research topic. Over decades, small organic molecules, rare-earth doped up-conversion nanoparticles, and carbon nanomaterials for effective bioimaging. Among them, semiconductor QDs were considered as ideal fluorescent probes due to their superior photostability, broad excitation wavelength, narrow range of emission, and ease with which they can be functionalized with surface reagents, including biomolecules. However, most of semiconductor QDs (e.g., CdHgTe, CdTe@CdSe, CdTeSe@CdZnS, and PbS) contain heavy metals, leading to undesired biological and environmental hazards that limit their biological applications.

As a recent addition to the carbon family, graphene quantum dots (GQDs), along with nanodiamonds (NDs) and carbon dots (CDs), have emerged as a new class of carbon-based fluorescent nanomaterials. NDs typically possess a sp$^3$-hybridized diamond-like core with a small amount of graphitic carbon on the surface while GQDs mainly possess graphene lattices inside the dots smaller than 100 nm in size and less than 10 layers in thickness. Generally speaking, CDs are usually quasi-spherical carbon nanoparticles with a size less than 10 nm. In comparison with common semiconductor QDs, GQDs are made up of carbon, which is the most abundant element in biological systems and are usually biocompatible.

The unique structure and property characteristics of GQDs make them attractive for bioimaging. Although tremendous progress has been achieved and several review articles focusing on the synthetic strategies, physical properties, and energy-related applications of GQDs along with some mini reviews on the general applications of GQDs in biosensing and cell imaging, have recently appeared, no systematic review on GQDs for bioimaging has been reported as far as we are aware. In this article, we will present a focused review on the design, synthesis, and applications of GQDs for in vitro fluorescence imaging in cells and tissues as well as in vivo macroscopic and microscopic imaging in animals. We will also discuss some challenges and opportunities for the use of GQDs in biomedical diagnosis and therapy.
2. Synthesis and Edge Functionalization of GQDs for Toxicology Studies

2.1. Synthesis

Commercial applications of GQDs will not be realized if there is no facile and large-scale fabrication capability for GQDs with a well-defined size and shape. To date, various synthetic approaches have been developed for the synthesis of GQDs, which can be classified into two strategies. One is the top-down approach involving the cleavage of carbonaceous materials via physical, chemical, or electrochemical processes. For instance, various carbon materials, including graphite, graphene, graphene oxides, carbon nanotubes, carbon fibers, carbon black, and even coal have been used as starting materials for the synthesis of GQDs via acidic oxidation, hydrothermal treatment, electrochemical exfoliation, to name a few.[27] Alternatively, GQDs can be synthesized via a bottom-up approach, involving solution chemistry, cyclodehydrogenation of polyphenylene precursors, or carbonizing certain organic precursors.[28]

The optical properties of GQDs are the basis of application in in vitro and in vivo imaging. Different synthetic often lead to GQDs with different sizes, functional groups, and edge defects, which play important roles in regulating optical properties. GQDs usually show typical absorption bands in the UV region, with an absorption peak around 230 nm due to the $\pi-\pi^*$ transition of aromatic sp² domains and a long tail extending into the visible range.[29] However, another broad band over 270–390 nm associated with the n–$\pi^*$ transition of C=O could also appear.[29] More importantly, most of the GQDs reported in the literature exhibit photoluminescence emissions, which make them useful for fluorescent bioimaging. Till now, GQDs with a wide range of PL emission colors from ultraviolet to near-infrared have been synthesized via various approaches (Figure 1). Some of the GQDs are reported to even possess upconversion photoluminescence properties[27,28,31] attractive for multi-photon-induced bioimaging.[32] For detailed discussions on the various synthetic approaches to GQDs GQDs and their optical properties, the interested readers are referred to specialized review articles.[27,28] Here, we give only a brief summary in Table 1[33–46] while the photoluminescence of GQDs for different bioimaging applications will be described in the subsequent sections.

![Different emission spectra of graphene quantum dots. Reproduced with permission.](image)

**Figure 1.** Different emission spectra of graphene quantum dots. Reproduced with permission.[37b] Copyright 2013, Royal Society of Chemistry.

2.2. Edge Functionalization for Toxicology Studies

GQDs synthesized by the above-mentioned approaches often contain various hydrophilic groups (e.g., carboxylic, hydroxy,
and amino), and thus could be dispersed well in water. These edge functional groups could not only improve the biocompatibility of GQDs, but also allow GQDs for covalent bonding with biomolecules for bio-recognition in bio-systems. In this context, Zheng et al.,[38] have synthesized insulin-conjugated GQDs for specific labeling and dynamic tracking of insulin receptors in 3T3-L1 adipocytes. On the other hand, Zhu et al.,[47] prepared various edge functionalized GQDs by connecting GQDs with alkylamines (m-GQDs) or reducing GQDs with NaBH₄ (r-GQDs) (Figure 2a), and found a very low toxicity to MC3T3 cells for both GQDs and r-GQDs with a distinct dose-dependent cell viability for m-GQDs (Figure 2b). These results also support the notion that the edge functionalization plays a critical role in the cytotoxicity of GQDs. In addition, Nurunnabi et al.,[26b] performed both in vitro and in vivo toxicity evaluations on carboxylated GQDs prepared from carbon fibers. In the in vitro toxicity evaluations, the GQDs (up to 500 µg mL⁻¹) were introduced to KB, MDA-MB231, A549, and MDCK cells, and were found to not impose considerable toxicity to the selected cell lines. In the in vivo toxicity evaluations, no obvious organ damage or lesions were observed for the mice after 21 d of administration of GQDs at 5 mg kg⁻¹ or 10 mg kg⁻¹ dosages (Figure 3). By preparing nitrogen-doped GQDs (N-GQDs) from GO, Liu et al.,[48] performed co-incubation of HeLa cells with N-GQD for 12 and 24 h, respectively, and found that different doses of N-GQDs (5–400 µg mL⁻¹) did not weaken the cell activity compared with the control group.

Apart from cytotoxicity studies of cells and animals, the genotoxicity of GQDs is also very important to evaluate the potential hazards of GQDs to biological systems because there is a close correlation of DNA damage with mutation and cancer. It has been demonstrated that carbon nanotubes,[49] nanodimonds,[50] and graphene oxides[51] could cause DNA damage even when they showed a low toxicity to cells. However, no study on the genotoxicity of GQDs has been reported so far. Therefore, more comprehensive and thorough cytotoxicity and genotoxicity studies are needed to exploit the full potentials of GQDs for biomedical applications, though GQDs are more biocompatible than other fluorescence probes, such as semiconductor QDs.

### 3. GQDs for In Vitro Cell Imaging

The photoluminescence is one of the fascinating features of GQDs that makes them attractive for bioimaging. Consequently, considerable research efforts have been dedicated to exploit GQDs as fluorescent probes for in vitro and in vivo imaging. For instance, Zhu et al.,[52] have prepared GQDs with strongly green fluorescence emission from graphene oxides via one-step solvothermal method (Figure 4a). The resultant GQDs were demonstrated to exhibit a good biocompatibility, which could be used as excellent biolabeling agents for imaging cells (Figure 4b).

Using polycyclic aromatic hydrocarbon as the precursor, Zhou et al.[53] synthesized GQDs via a bottom-up approach. The resultant GQDs were then efficiently internalized by MCF-7...
cells and accumulated in the cytoplasm (Figure 5a). Compared with Alexa fluo 488 and fluorescein that are commonly used fluorescence probes for cellular imaging, these GQDs exhibited a much higher photostability (Figure 5b).

By conjugating insulin with GQDs, Zheng and co-workers revealed insulin receptor dynamics in live cells using a total internal reflection fluorescence microscopy (TIRFM).[38] These authors observed small discrete clusters of GQDs under TIRFM after pre-incubating adipocytes with insulin-GQDs (Figure 6a) and tracked the constant lateral movement of GQD-enlightened clusters to the cell membrane and vertical movement between the inner cytosol and the plasmalemmal region by following the GQDs fluorescence. Based on the real-time tracking of individual clusters during the imaging period, four subpopulations were identified (Figure 6b–e), demonstrating great potential of the edge functionalized GQDs in cell imaging, particularly for investigating dynamic cellular processes.

The basis for the use of GQDs as fluorescent probes for in vitro imaging in above-mentioned and other studies[54] is the one-photon-excited photoluminescence emission. In the one-photon-excited fluorescence, the fluorescence emission is usually induced by UV or blue excitation, which are harmful to living cells or bio-systems. Therefore, the multi-photon induced fluorescence of GQDs has been studied for cellular imaging. In particular, Zhu et al.[47] performed two-photon fluorescence imaging of MC3T3-E1 cells, using GQDs as fluorescent probes and near-IR excitation (Figure 7). Using nitrogen-doped GQDs with a two-photon absorption cross-section as high as 48 000 GM as two-photon probes, Liu et al.[48] have obtained a large imaging depth of 1800 µm in tissue phantom (Figure 8).

4. GQDs for In Vivo Imaging of Animals

In most cases of fluorescence imaging for animals, significant background signal associated with autofluorescence of tissues of the animals is observed.[55] The short-wavelength light (either the excitation light or the emission light) could also be easily absorbed by water in the biological tissues and scattered due to the Rayleigh scattering effect, which is not desirable for animal imaging. Thus, the quality of fluorescence imaging for small animals depends strongly on the emission wavelength of fluorescent probes. Following an intravenous injection of carboxylated GQDs with green fluorescence, Nurunnabi et al.[26b] performed in vivo fluorescence imaging of mice, in which the fluorescence signal of GQDs was observed at the tumor site at 12 h post-injection, indicating that GQDs could be used for superficial tissue imaging to detect, for example, skin cancer (Figure 9). However, the fluorescence signal of GQDs in the deep tissue or organs, such as heart, liver, or spleen, could not be detected in vivo due to the short-wavelength excitation and emission of the GQDs used in this particular case. When GQDs with near infra-red photoluminescence were used for in vivo imaging, however, the near infra-red fluorescence signal

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Figure 3. Histology study to measure toxicity. Histological evaluation of the major organs of the mice at 22 d after intravenous injection of the GQDs. No symptoms of inflammation and/or lesion were observed in the images. Tissues of 5 and 10 mg kg⁻¹ GQDs treated mice are similar with that of tissues of saline-treated mice. Images were taken at 160× magnification with hematoxylin and eosi n staining. Reproduced with permission.[26b] Copyright 2013, American Chemical Society.

Figure 4. a) PL (at 375 nm excitation) spectra of the GQDs aqueous solution (inset: photograph taken under UV light). b) Fluorescence imaging of MG-63 cells treated with GQDs (at 405 nm excitation). Reproduced with permission.[52] Copyright 2011, Royal Society of Chemistry.

Figure 5. a) Merged image of fluorescence imaging and bright-field imaging of MCF-7 cells after incubation with GQDs for 2 h. b) Time-dependent fluorescence intensity ratio (/I₀) of P-GQDs, Alexa fluo 488, and fluorescein. I₀ and I are the emission intensities of GQDs, Alexa fluo 488, and fluorescein without and with laser illumination for different time, respectively. Reproduced with permission.[53]
of GQDs could be detected around the heart, liver, spleen, and kidneys at 8 h post-injection (Figure 10). Ge and co-workers synthesized GQDs using a hydrothermal method with polythiophene derivatives (PT2) as the carbon source. The GQDs thus produced exhibited a broad absorption in the UV–vis region, along with a strong emission peak at 680 nm useful for in vivo bioimaging. In addition to bioimaging, these authors found that these GQDs could produce \( ^{1}O_2 \) via a multistate sensitization process (Figure 11a) with a quantum yield of about 1.3, which is the highest yield reported for photodynamic therapy (PDT) agents. Consequently, they performed in vivo fluorescence imaging and PDT in mice models (Figure 11b).

Along with the in vivo fluorescence imaging for animals in macroscopic scale, GQDs also show promise for in vivo imaging of microcosmic structures in animals. By using two-photon scanning microscope and GQDs as fluorescent probes, Qian et al. monitored the flow distribution and clearance of GQDs in ear blood vessels of mice (Figure 12). It was found that GQDs with a large two-photon absorption cross-section and long-wavelength emission hold great potentials for in vivo imaging of microcosmic structures, such as brain blood vessels.

5. Conclusions and Perspectives

GQDs have attracted tremendous attention in many fields, including chemistry, physics, and biomedicine. It was noted that in many recent publications various carbon-based nanostructures were named as “GQDs,” however, a closer look at the data often reveals that those particles were actually CDs. In this article, we focus on the applications of real GQDs, which possess graphene lattices inside the dots smaller than 100 nm in size and less than 10 layers in thickness. We have presented a focused review on the design, synthesis, and applications of GQDs for in vitro fluorescence imaging in cells and tissues and in vivo macroscopic and microscopic imaging in animals. Even this short review article has revealed the versatility of GQDs for bioimaging, biomedical diagnosis and therapy. With so many synthetic approaches already reported, and more to be developed, there will be many opportunities for developing various new GQDs with tailor-made structures and well-defined fluorescence properties desirable for bioimaging. However, the applications of GQDs for bioimaging are still at its early stage with many important issues remained to be addressed:

1. Although GQDs with emission wavelengths ranging from UV to near infra-red have been synthesized via various bottom-up and top-down approaches, the quantum yields of GQDs reported so far are much lower than conventional semiconductor QDs. There is an urgent need to improve the quantum yield of GQDs. GQDs with bright red/near infra-red emissions are considered as ideal nanoprobes for bioimaging.

2. Targeted imaging of tumors based on GQDs has rarely been demonstrated in vivo. The next generation of cancer diagnostic agents for small animals requires improvement...
in their accumulation efficiency in tumor tissues. Edge functionalization of GQDs with antibodies or peptides for targeted imaging of specific cancers needs to be further explored.

3. Development of multifunctional nanoprobes to cover a broad spectrum of imaging modalities is always an interesting topic. It is still a major challenge to design GQDs-based nanoprobes for simultaneous optical imaging, MRI, and CT monitoring. In these cases, the optical and radioactive properties of GQDs will be promising for simultaneous imaging and therapy.

4. By optimizing the multi-photon excitation and emission properties of GQDs, their novel applications in biomedical areas, such as neurobehavioral analysis, gene therapy in the brain, and barrier penetration through brain blood capillaries, could be exploited.

5. Like many other new biomedical technologies, there are concerns about the possible side effects derived from the use of GQDs. Cytotoxicity and genotoxicity studies of GQDs with different components, sizes, shapes, and surface coatings need to be investigated. Bio-distribution studies of GQDs in various animal models are also needed.

Continued research and development efforts in this emerging field is of great value. This will revolutionize the way in which future biomedical tests and clinical diagnoses to be performed that could affect many aspects of our lives.

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Figure 11. a) Schematic illustration of the $^1\text{O}_2$ generation mechanisms by conventional PDT agents (left) and GQDs (right). b) Photographs of mice after various treatments on the 1st, 9th, 17th, and 25th d. (PDT: GQDs + light irradiation; C1: GQDs only; C2: light irradiation only.) Reproduced with permission.\textsuperscript{[41]} Copyright 2014, Macmillan Publishers Ltd.

Figure 12. In vivo two-photon scanning and one-photon confocal luminescence imaging of intravenously injected GQDs in a blood vessel of a mouse ear at various time points after sample treatment. Scale bar: 50 μm. Reproduced with permission.\textsuperscript{[32]}


