Chapter

Fluorescence Imaging Enhanced by Members of the Graphene Family: A Review

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Abstract

Graphene is a two-dimensional allotrope of carbon with a range of highly attractive physicochemical properties suitable for a wide variety of applications. In the context of fluorescence imaging graphene and its derivatives have recently started to gain more attention since they could assist in the enhancement of imaging of cells, tissue, or other biologically relevant samples such as cell organoids for example mitochondria as well as in the imaging of cancer cells, tumors, and various pathogens. This chapter attempts to cover the most relevant, recent advances in this growing research field. Some basic information on the physical and (photo)chemical properties of important members of the graphene family is provided. Additionally, novel approaches involving graphene-based materials (GBMs) in cellular and tissue imaging systems are reviewed. Important examples of contemporary applications of GBMs in cancer detection using fluorescence imaging are also presented. The specific role of graphene (or other GBMs) in each case is explained and analyzed. Finally, future perspectives and novel applications of fluorescent imaging techniques involving GBMs are discussed.

Keywords: graphene, fluorescence, imaging, 2D-materials, microscopy, cellular imaging, cancer detection

1. Introduction

1.1 Graphene-based fluorescence imaging applications

Fluorescence imaging is a non-invasive approach that utilizes fluorescent probes to generate photons and provides more sensitivity, specificity, and less harm than other imaging methods [1]. It can be used to monitor cells, tissues, and living organisms *in situ* and analyze specific biomolecules. This is why fluorescence imaging is considered as one of the most essential tools for biomedical research [2]. The fluorescence imaging probes should not be cytotoxic while they should exhibit adequate resistance to photobleaching caused by the natural features of the biological system. In this regard, graphene-based nanomaterials are presently considered as viable alternatives for fluorescence imaging. However, because graphene is a

zero-bandgap material, fluorescence cannot be detected in pristine graphene [3]. Conveniently, numerous studies have successfully fragmented or functionalized 2D graphene into 0D graphene quantum dots (GQDs) or carbon nanodots (CNDs) [4]. GQDs consist of graphene lattices and are commonly employed in fluorescence imaging due to quantum confinement and edge effects. Not only do they exhibit the distinct physical and chemical characteristics of conventional graphene, but they also display excellent biocompatibility, low cytotoxicity, and easy functionalization opportunities [5]. However, the poor fluorescent quantum yields often encountered along with the non-specificity of GQDs restrict their wide application in fluorescence imaging. Various research attempts have demonstrated that surface modification of GQDs can effectively alter their chemical activity, electrical structure, and quantum yield, consequently improving their photoluminescence capabilities [6, 7]. Since the realization of nitrogen doped GQDs [8], heteroatomic doping of GQDs has proven to be an effective technique for increasing quantum yield and tuning photoluminescence wavelength. For instance, in Wang et al. utilized the solvothermal method to synthesize boron-doped GQDs (B-GQDs) and phosphorus-doped GQDs (P-GQDs) [9]. B-GQDs and P-GQDs have emission wavelengths of 460 and 630 nm, respectively. Due to the matched band structure, rapid energy transfer between the blue-emitting B-GQDs and the orange-emitting P-GQDs can result in efficient fluorescence emission in the P-GQDs when the blue-emitting B-GQDs are excited at the ideal excitation wavelength of 460 nm. Furthermore, with effective energy transfer, the quantum yield of P-GQDs improves to 0.48, which is significantly greater than the quantum yield of pure P-GQDs. Similarly In Gong et al. synthesized nitrogen and bromine co-doped GQDs (NBr-GQDs) that used a facile deflagration approach for the first time. The quantum yield of NBr-GQDs was up to 52%, with low cytotoxicity, great pH resistance, and stable photoluminescence intensity [10]. Theoretical calculations indicate that N and Br co-doping may reduce the band gap between excited singlet states, significantly improving the photoluminescence performance of GQDs. Recently, Li et al. have also synthesized manganese and boron-nitrogen-doped graphene quantum dots (Mn-BN-GQDs) using the hydrothermal synthesis method and employed them in biosensors [11]. The results reveal that Mn-BN-GQDs have excellent fluorescence characteristics and quantum yield, low cytotoxicity, and high biocompatibility, indicating a promising future for the advancement of bioimaging. On the other hand, graphene oxide (GO), one of the most significant graphene derivatives, has a heterogeneous electrical structure that enables it to emit fluorescence in a particular wavelength range. GO exhibits exceptional features including great mechanical strength, strong photostability, simple surface modification, and photoluminescence that is wavelength dependent. The presence of epoxide functional groups on its surface offers an easily chemically modifiable substrate allowing for the conjugation or interaction with a wide range of biomolecules *via* a variety of covalent/non-covalent interactions, electrostatic forces, absorption, and hydrogen bonding [12]. GO mainly consisting of sp2 and sp3 carbons, generate electron and optical band gaps, allowing it to fluoresce over a broad range of wavelengths and serve as a donor for fluorescence resonance transfer. Moreover, GO possesses effective fluorescence quenching characteristics. Most often, GO acts as the energy acceptor, whereas organic dye acts as the donor. Since the donor's emission spectrum overlaps with the acceptor's absorption spectrum, fluorescence resonance transfer can occur between the two, resulting in fluorescence quenching of the fluorescent dye. In particular, the functional group types, localized domains, lateral dimensions, and solvent dopants can significantly impact the electronic energy transition and fluorescence characteristics of GO [13].

Currently, Numerous studies have demonstrated that GO and its derivatives may generate fluorescence signals of various colors. Based on a study by Kalluru, Nano-GO exhibits single-photon excitation wavelength-dependent photoluminescence in the visible and short near-infrared ranges, making it appropriate for multicolor fluorescence imaging *in vivo* [14]. This particular property of GO in a single biological platform makes this nanomaterial a promising candidate for clinical applications in the early diagnosis of a variety of disorders. Wang et al. reported for the first time the metal fluorescence enhancement of GO and discovered that the fluorescence intensity of GO on an Ag substrate was approximately 10 times stronger than that on glass [15]. Furthermore, unlike other fluorescent materials, GO in direct contact with the metal exhibits a high metal fluorescence enhancement without quenching, indicating that GO might be employed as a fluorescent probe for 3D optical imaging and sensing.

The following section provides a comprehensive introduction to the fluorescence imaging mechanism of graphene quantum dots and graphene oxide, as well as some reviews of current achievements in the field of graphene-based fluorescence imaging application. Fundamentally, to increase the performance of graphene and its derivatives in bioimaging applications, it is necessary to properly control their sizes, surface coatings, and components in order to maximize their photoluminescence capabilities. It is anticipated that the unique structure and exceptional capabilities of graphene and its derivatives offer new opportunities for disease detection and clinical treatments, have promising application prospects in fluorescence imaging, and play a crucial role in fostering the growth of the biomedicine industry.

2. GO, GQDs and CNDs in fluorescence imaging: why so important?

2.1 Graphene oxide

Graphene oxide is one of the most commonly used forms of the graphene-based materials (GBMs) family nowadays. This comes as no surprise since it is a very stable multifunctional material with a wide range of superior properties and hence a broad application scope [16]. GO is essentially the oxidized form of graphene which can be produced through a variety of oxidative methods [17–19]. Structurally, GO encompasses a variety of functional groups, the most important being: carboxy, hydroxy, epoxy, and keto- or aldehyde groups. Hydroxy groups can be found both at the edges and the surface of GO, epoxy on the surface areas and the rest mostly at the edges [20]. Their abundance and ratios are sensitive to the preparation method. These functional groups give rise to a quite dipolar character of this GBM and at the same time generate a range of excitation possibilities due to the presence of oxygen as the main heteroatom involving lone pairs of electrons. As a result, transitions such as n-σ^* n-π^* as well as π-π^* are the most important [21]. When it comes to the electronic and emission properties which are of primary interest for applications within fluorescence imaging, GO is known to exhibit a range of very interesting features:

2.1.1 Excitation energy dependent fluorescence

This is a property which is also observed in GQDs. Unlike most of the conventional fluorophores, GO can result in different emission energies (i.e. a variety of colors of emitted light) when photo-excited at different wavelengths (energies) [22]. Specifically, upon increasing excitation wavelength from 325 nm to 650 nm, a red

shift of the GO fluorescent band is observed [23]. This effect in terms of applications in fluorescence imaging is very important since by employing a single material it is possible to obtain a wide range of emitted light colors simply employing different laser energies for excitation.

2.1.2 Fluorescence resonance energy transfer (FRET)

FRET occurs when a donor chromophore, being in its electronically excited state, transfers energy to an acceptor chromophore *via* nonradiative dipole–dipole coupling [24]. The efficiency FRET is proportional to the reciprocal sixth power of the distance between the acceptor and the donor rendering this effect very sensitive to the donor-acceptor distance (see **Figure 1**) [25]. A wide variety of donor and acceptor fluorophore combinations [26] have been reported so far and FRET is considered as an eminent tool for biomolecular research and applications thereof [27, 28]. When it comes to GO, it is well established that GO can function as an efficient energy acceptor (EA) causing the quenching of fluorescence of a variety of energy donor organic fluorophores [29]. The benefits of using GO as an EA are multiple; the variety of functionalization possibilities its structure offers, the large number of binding sites as a result of its size, the high internalization to a wide range of cells, and the very effective energy transfer though a long distance [30] are some of them. In practice, the approach of employing GO, a fluorophore acting as the electron donor, and a functionality, factor, or molecule exhibiting specificity to a target analyte is widely used in many sensor and imaging attempts. Biorelevant analytes and biomarkers such as antibodies, microRNA [31, 32], folate receptor [33] as well as many other examples of analytes [29, 34] have been reported so far.

Additionally, GO can also be employed as an energy donor (ED) in FRET systems involving GO and an acceptor fluorophore. Such systems might involve either organic

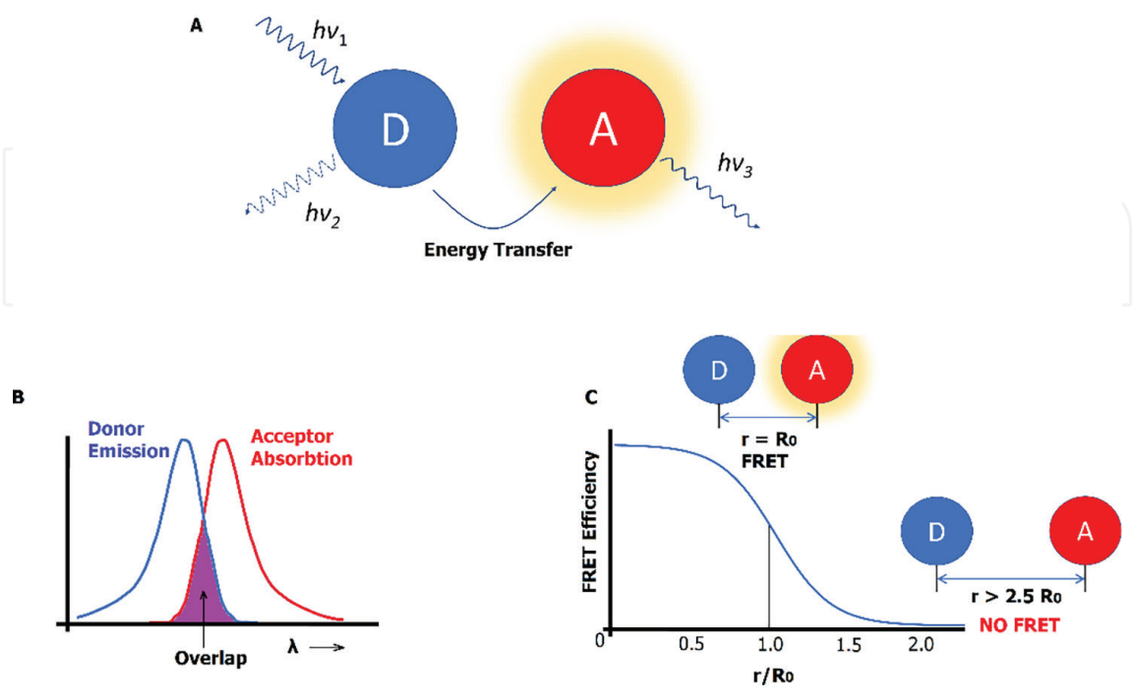

Figure 1.
Fundamental info about FRET. (A) Illustration of the FRET interaction between a donor and an acceptor fluorophore. (B) Example of successfully donor emission/acceptor absorbance overlapping spectra (C) dependence of FRET efficiency on the distance between the donor and the acceptor.

dyes or noble metal nanoparticles acting as EAs corresponding to GO as ED [35, 36]. A variety of bio-relevant sensor applications based on GO/EA FRET systems have been reported so far allowing for the detection of antibodies [35] pharmaceutical screening/imaging, diagnostic tools [36, 37], etc. Some more detailed examples are presented in the next paragraphs.

2.1.3 Fluorescence of GO

The fluorescence band of GO is very broad and thus not suitable for accurate sensor and fluorescence imaging applications. Nonetheless, GO exhibits NIR emission when photoexcited in the NIR region and due to this feature GO is currently finding application in TP microscopy and imaging of biological samples [38, 39]. It is noteworthy that the excitation dependent emission (*vide supra*) allows for use of GO in the two biological wavelength-windows of 1000–1350 nm and 650–950 nm in which biological samples are nearly transparent [40]. This important fact justifies one more important use of GO in biological imaging in addition to FRET applications. Moreover, research attempts to tune the emission colors of GO have been also reported. Mei et al. [41] came out with an oxidative methodology allowing for controlled modifications of GO leading to emissions shifts from brown to cyan with no excitation wavelength alteration. Indeed, by merely varying oxidation reaction times of GO nanosheets was proved to result in controllable and accurate tuning of the emission properties. This approach is thought to introduce new opportunities in cellular imaging as well as in multiplex encoding analysis.

2.2 Graphene quantum dots (GQDs) and carbon nanodots (CNDs)

A very interesting family of materials belonging to the wide carbon family is the Carbon dots family. Two important classes of carbon dots are Graphene quantum dots (GQDs) and carbon nanodots (CNDs). GQDs and CNDs are currently in the forefront of research in the field of bioimaging and specifically within the research and development of novel fluorescence imaging materials and techniques. The ease of production of CNDs and GQDs, their high structural versatility and the wide range of structure-properties modulation opportunities, as well as their biocompatibility and bright fluorescence are the main reasons associated with their high relevance for bioimaging. Up to date a broad range of production methods have been published.

Several methods have been reported for producing GQDs and CNDs, with solvothermal, [42] microwave-assisted, [43] and electrochemical methods [44] being the most commonly used. The choice of the synthetic method used relies on the target batch-size and desired structure and properties of the final materials. It is currently established that for their production inert conditions e.g., heating at temperatures as low as even 120°C are adequate. As a comparison earlier methods employed drastic conditions involving laser ablation [45] or even temperatures higher than 900°C [46]. Nonetheless, it is well agreed by many researchers that milder production conditions can indeed result in very interesting properties since many of the microwave-assisted and hydrothermal methods leave parts of the molecules being subjected to heat or microwaves respectively, unaffected. Hence, the surface of the GQDs and CNDs produced by this "mild processing" can encompass a variety of functional groups [47, 48].

Up to date a wide variety of GQDs and CNDs have been synthesized *via* combining simple compounds such as citric acid, glucose or mono- or di-saccharides and urea, formamide or amino acids [46]. Another trend is to employ natural occurring sources such as fruit extracts, plant specimens etc. which upon treatment (usually microwave or hydrothermal) yield in GQDs and CNDs with a large variety of properties [49, 50]. This research field is highly active which promotes more sustainable and inexpensive production strategies for these vey important class of GBMs.

Why are GQDs and CNDs so important for fluorescence imaging is strongly associated to their outstanding photophysical properties. GQDs and CNDs exhibit photoluminescence (fluorescence emission by many authors) of often high quantum yields in a variety of media. The photoluminescence can be highly dependent on the size and even the shape of the dots [51] but mainly on the surface defects that they exhibit. In fact, without the surface passivation of carbon dots the observed photoluminescence is very limited. Post functionalization of their surface and/or incomplete "termination" reactions/partial carbonization during their production can efficiently enhance their photoluminescent properties [46]. Probably the most remarkable of the properties of GQDs and CNDs is the excitation-dependent photoluminescence [46]. This property (which also GO exhibits, *vide supra*) has a number of important implications on the potential uses of these materials. Specifically, the fact that different emission colors can be obtained by the same material just by choosing a suitable excitation wavelength, is highly appreciated in bioimaging where the use of multiple fluorophores can lead to complications and misinterpretations of the observed phenomena. Indeed, problems such as overlapping emission spectra, photobleaching, and phototoxicity are often encountered [52–54]. These adverse effects can be minimized or even eliminated when using carbon dots. The low toxicity, high water solubility, and very high stability, of carbon dots render this class of GBMs a very attractive option for bioimaging [46]. In the following paragraphs the use of GQDs and CNDs in contemporary fluorescent imaging technologies and applications is discussed.

3. Cellular and tissue imaging systems involving graphene-based materials

Contemporary cell-imaging methods have facilitated the advancement of a wide range of biologically relevant assays aiming at a variety of therapeutic fields and revolutionized the R&D relating to drug design. Cellular imaging encompasses the application of a system or technology required for the visualization of a single cell, cell population, or subcellular structure. Even though a wide range of technologies, methodologies, and molecules enabling cell imaging do exist, there is a constant need for the development of novel systems of higher accuracy, fidelity, specificity, low cost, low cytotoxicity, and high photo- and chemical stability. In terms of fluorescent molecular materials, many of these requirements are fulfilled by members of the wide family of GBMs. In this section, the most recent developments falling in this area of research and technology are reported.

The fluorescence imaging of biomolecules particularly proteins and DNA is an important field of research and technology that can enable the visualization of proteins in cells and tissues with the use of fluorescent probes. Its importance is rationalized as high in terms of the various opportunities for the studies of localization and dynamics of proteins in living cells and tissues that it can offer (e.g. studies of protein–protein interactions, protein folding, and protein degradation) [55–58].

Indeed, graphene and other GBMs have been shown to act as useful platforms for fluorescent sensing of biomolecules including DNA and a variety of proteins and this opens a variety of opportunities in cell and tissue fluorescent imaging [59–62].

Bovine serum albumin (BSA) is a globular protein of animal origin (cow) that is used in a plethora of biochemical applications due to its stability and lack of interference with biological reactions [63]. Kuchlyan et al. performed a thorough study on the interactions of BSA with GO. The group employed a set of spectroscopic methods such as fluorescence correlation spectroscopy (FCS), Fluorescence Lifetime Imaging Microscopy (FILM), and Circular Dichroism (CD). For the study, BSA was labeled with the bright fluorescent Alexa Fluor 488 (AF488). They concluded that GO exhibits a pronouncedly strong interaction with BSA. GO was proved to have a drastic fluorescent quenching effect on AF488-BSA [64]. On the other hand, Yang et al. recently reported on the advancement of a highly sensitive nanosystem based on GO corresponding to microRNA (miRNA) which can be applied in living cells as well as *in vivo* [65]. GO acted as an efficient quenching agent against a molecular beacon labeled with the bright fluorophore cyanine-5 (Cy5). In the presence of the specifically targeted analyte miRNA, fluorescence was recovered allowing detection in cells or tumor tissue samples at very low levels [65].

Very recently Reagen et al. developed a novel class of GQDs exhibiting near-infrared (NIR) fluorescence (emission centered at λ = 860 nm) derived from biomass obtained from organic source and prepared through pyrolysis. The prepared GQDs were tested for cell imaging in two distinct cell lines namely RAW 246.7 (macrophage cells) and MCF-7 breast cancer cell line. The results indicated low cytotoxicity as well as substantial internalization through endocytosis. Moreover, the GQDs exhibited a marked aptitude in detecting Hg^{2+} ions in biological samples enabling NIR fluorescence imaging in cells and toxic heavy metal detection *in vivo* [66]. Such multifunctional GQD-fluorescence imaging systems are currently sought-after. The multifunctional character and multi-use feature of GQDs in cellular imaging has been recently pointed out by a range of research groups [67–69].

Two-photon (TP) microscopy is a fluorescence imaging technique that is particularly well-suited to image-scattering living tissue of up to about 1 millimeter in thickness. It works by shining an intense beam of near-infrared light onto a single point within a sample, inducing simultaneous absorption of two photons at the focal point, where the intensity is the highest [70]. TP microscopy has found nowadays huge applicability in bioscience. Nonetheless, a typical restriction that TP- microscopy/bioimaging techniques exhibit is that they rely on single-color fluorescence changes. Due to the special emission properties of GQDs among a range of other beneficiary properties (*vide supra* paragraph 2 for details) these nanomaterials have become very attractive for fluorescence imaging. Zhao et al. as early as 2016, reported the development of a dual-emission and TP GQD suitable for imaging applications targeting hydrogen peroxide (H_2O_2) as an analyte. The fluorescence response of the TP-GQD towards H_2O_2 was shown to be fast and very specific and renders the mapping of the production of endogenous H_2O_2 in living cells and deep tissues feasible. This probably constitutes the first published example pertaining to a dual-emission, TP-GQD of high specificity and applicability in cell and tissue fluorescence imaging [71].

In Wang et al. reported on the development of some GQDs through a hydrothermal method utilizing 1,3,6-trinitropyrene and $(NH_4)_2SO_3$. The resulting GQDs encompassing amino as well as sulfate groups were evaluated in terms of their TP fluorescence efficiency in the context of cellular imaging. A very high TP absorption cross-section was observed and evaluated as significantly higher as compared to

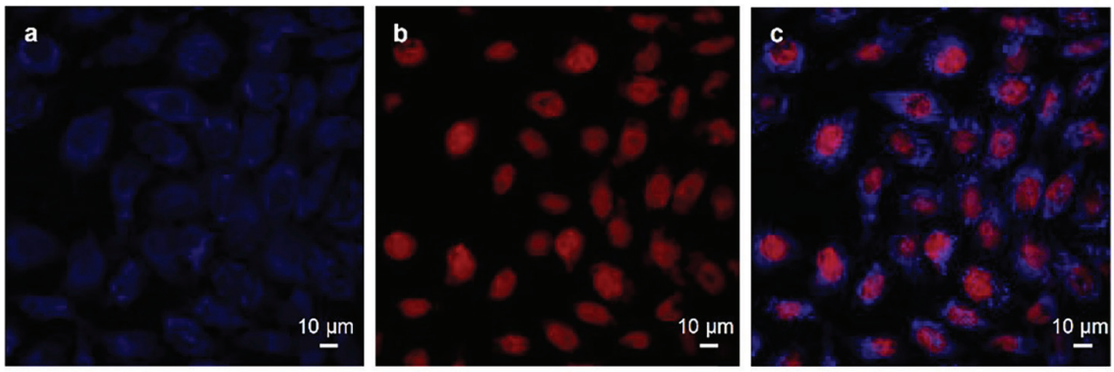

Figure 2.
(a) Image of GQDs at 405 nm excitation. (b) Image of nuclear view red dye at 633 nm excitation. (c) Merged image (obtained with permission from supplementary information of Ref. [72]).

traditional/conventional fluorophores. The research group further performed tests in a cell line (HeLa cells) and found out that the GQDs were internalized in the cytoplasm providing very bright and clear cell images (see **Figure 2**) [72].

Earlier Sapkota et al. reported the synthesis of GQDs of tunable size and explored their capacity in fluorescence imaging. It was found that GQDs with a size between 15 and 35 nm exhibit vivid fluorescence (quantum yields of 0.64 by average) as well as high TP absorption (TPA) cross sections, which renders these GQDs excellent candidates for fluorescence imaging. Indeed, their use in cellular imaging was evaluated on living epithelial cells and even though internalization was observed, entering the nucleus was not possible [73].

Chen et al. as early as in 2015 advanced an aptameric sensor with nano dimensions based on graphene which is capable of inducing/enhancing the fluorescence activation imaging of cytochrome c (Cyt c; a major mediator in cell apoptosis released from mitochondria) [74]. In order to achieve this, Chen et al. connected a fluorophore-tagged DNA aptamer on graphene nanosheets modified with PEG polymer chains. The fluorescence of the fluorophore is inhibited due to the presence of graphene. Yet, dissociation of the a fluorophore-tagged DNA aptamer from graphene occurring immediately after cytosolic release of Cyt C, triggers the fluorescence and empowers real-time visualization of the Cyt c release kinetics. This nanosensing technology is envisioned to exhibit potential applications in visualization of key molecular factors in apoptotic signaling which are critical for cell biology and clinical theranostics.

Wang et al. on the other hand developed an innovative nanosensor employing graphene quantum dots (GQDs) which were conjugated to gold nanoparticles (AuNPs). The nanosensor was found to efficiently serve as a sensor of endogenous biological cyanide ions. This graphene-based nanomaterial further exhibits efficient TP excitation and exploits the drastic quenching efficiency of AuNPs and thus it can accomplish detection cyanide limits as low as 0.52 µM. This, combined with the potential of deep penetration depth of approx. 400 µm render this nanomaterial a perfect candidate for tissue imaging of cyanides [75].

Similarly, Hong et al. also employed a combination of AuNPs and a GBM (GO) for the development of fluorescent imaging/sensing system with specific applicability in monitoring intracellular telomerase activity. Their fluorescence imaging is applicable to a variety of living cells and it was tested toward the aptitude to distinguish normal-form cancer cells [76].

One of the pioneering works reporting GQDs-based nanosystems utilized in TP-induced fluorescence imaging was that by Liu et al. published as early as 2013. The research group produced some N-doped GQDs employing a solvothermal method in which dimethylformamide (DMF) was used both as solvent as well as N-source. The reported GQDs exhibit a marked TP absorption cross-section of nearly 48 kGM (GM: Göppert Mayer units) and were proved to function well even at imaging depth in tissue samples as large as 1800 μm. The development of these GQDs has been an early example of the immense potentials of GQDs in fluorescence bioimaging with applications in the broad biological and biomedical research fields [77].

4. GBMs in cancer detection through fluorescence imaging

Cancer currently constitutes one of the major mortality causes for humans. Ensuring enhanced cancer therapies requires improvement of cancer diagnostic techniques. In recent years, GBMs have found applicability in cancer detection. The wide range of suitable properties and attributes of this broad range of materials are considered for efficient cancer imaging.

Campbell et al. recently reported on the use of GQDs involving amino, hydroxy as well as carboxy functional groups and doped with nitrogen, nitrogen and boron or sulfur, in spectrally distinguishing among healthy and various types of cancer cells. In addition to this, the authors evaluated the pH-responsiveness of the investigated GQDs exploiting their wide range of emitted light wavelengths spanning from the visible to near-infraredred (NIR) part of the electromagnetic spectrum (**Figure 3**) [78].

Wu et al. on the other hand developed a new type of graphene-based nanomaterial functionalized with Anti-EpCAM antibodies and galactose-rhodamine-polyacrylamide

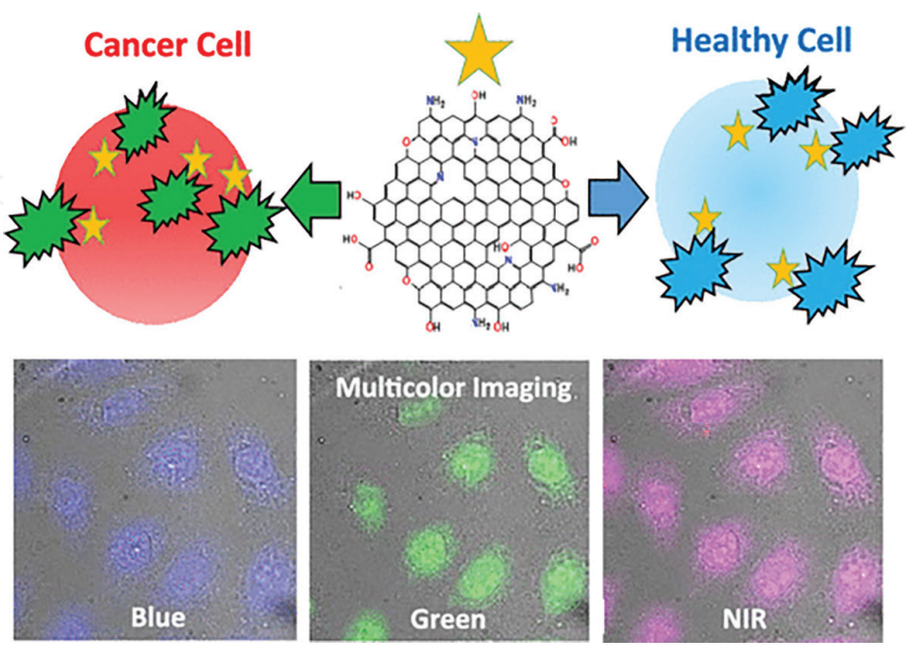

Figure 3.
Illustration depicting the structure of GQDs developed by Campbell et al. and the possibility of distinguishing cancer from healthy cells. Lower images depict the variety of colors emitted by the GQDs as a result of their excitation at different wavelengths (excitation-dependent emission). Obtained with permission from supplementary information of Ref. [78].

nanoparticles with a high aptitude to recognition of hepatocellular carcinoma cells (HCC-CTCs). The presence of graphene is dual here, acting both as a nanoparticle carrier platform as well as a strong quencher of rhodamine's fluorescence. Upon capturing and endocytosis of the aforementioned nanoparticles, fluorescence is recovered and the fluorescence imaging of HCC-CTCs can thus be efficiently achieved (**Figure 4**) [79].

It is important to mention that the aforementioned strategy involving graphene as a quencher of fluorophores connected to agents that can efficiently/specifically bind cancer cells or other bio-targets, has been repeatedly proposed within the context of fluorescence bio-imaging. A variety of recently published attempts towards bioimaging targets of biologically relevant analytes such as DNA [80], ions, [81] antibiotics [82], etc. have recently been reviewed [83].

Nurannabi et al. as early as 2014, developed GQDs bearing -OH and -COOH functionalities with an average size of 5 nm exhibiting significant red photoluminescence when excited at 655 nm (**Figure 5**). Their photoluminescence behavior was shown to enhance visualization of deep tumor tissues in experimental animals. Moreover, the described GQDs were tested *in vivo* as photodynamic therapeutic agents against MDA-MB231 cancer cells and it was proved that they exhibit a marked phototherapeutic activity [84].

In Pramanik et al. reported on the use of aptamer-conjugated GO in TP imaging (TPI) of breast cancer cells (specifically of SK-BR-3 cells). The developed GBM displays a drastic 2-photon absorption and marked photostability after even long irradiation sessions [38].

In Narasimhan et al. reported on the use of GQDs produced through laser ablation for use in both *in vitro* and *in vivo* fluorescence imaging of MCF-7 breast cancer cells. The reported GQDs were bearing hydroxyl and carboxylic acid groups at the edges as well as on their surface and it was observed that uptake of the GQDs by the

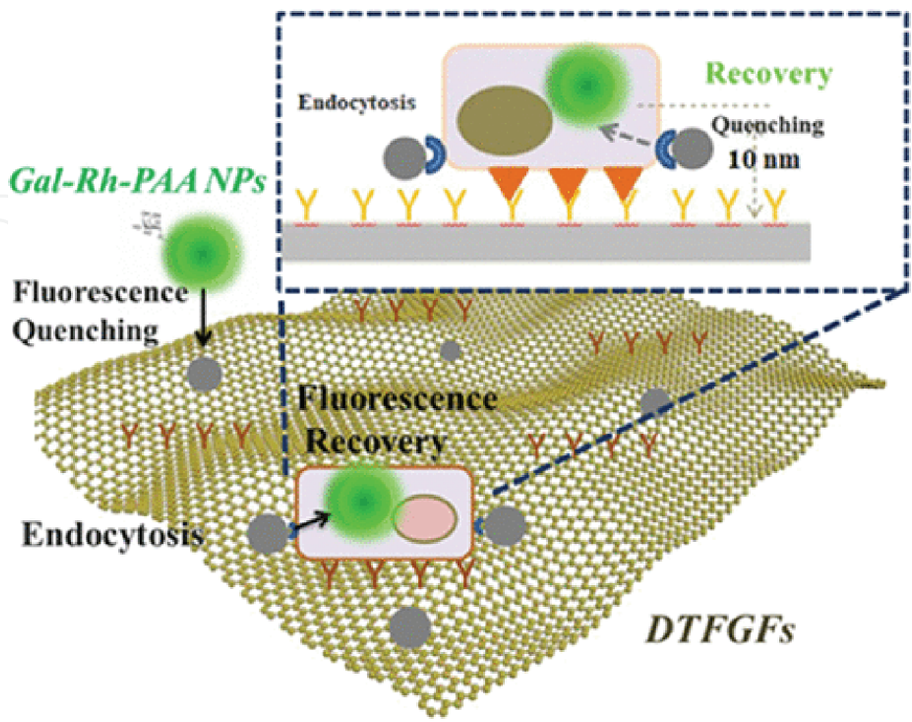

Figure 4.
Illustration of graphene-based nanomaterial developed for recognition of hepatocellular carcinoma cells (HCC-CTCs). Obtained with permission from supplementary information of Ref. [79].

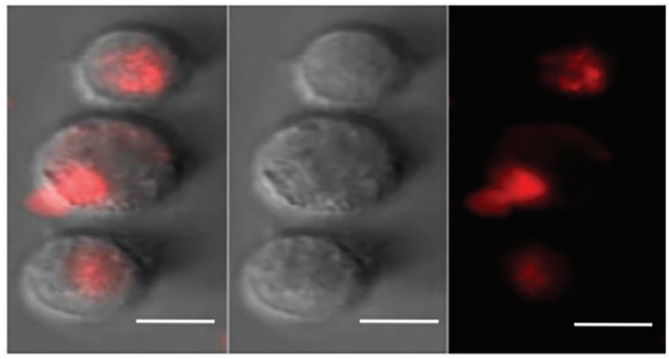

Figure 5.
Confocal laser scanning images of MDA-MB231 cells after treatment of cGdots (100 μg/mL). Scale bar: 20 μm (obtained with permission from supplementary information of Ref. [84]).

MCF-7 cells was feasible through endocytosis rendering imaging of implanted cells in mice possible through an intense red fluorescence observed upon excitation at 610 nm [85].

In Liu et al. proposed the use of graphitic carbon nitride nanosheets as a scaffold allowing for the detection of hyaluronase (HAase). Similarly to the already described strategies for cancer cell/tissue imaging, a cancer marker is targeted as an analyte (in this particular case HAase). Imaging of HAase in cancer tissues can be achieved through the activable two-photon fluorescence of the developed graphitic carbon nitride nanosheets in presence of HAase [86].

Some years earlier Park and coworkers utilized hyaluronic acid (HA) instead of HA-ase to accomplish efficient target-specific delivery of GQDs. In their strategy, the researchers tethered HA to GQDs and thus synthesized brightly fluorescent nanoparticles with an approximate size of 20 nm [87]. These interesting HA-GQD conjugates were tested and it was found that they exhibit strong fluorescence in CD44 overexpressing A549 cells as well as in *in vivo* experiments involving CD44 receptor overexpressing tumor-bearing balb/c female mice. CD44 antigen is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration. It is the receptor for hyaluronic acid and can also interact with other ligands e.g. matrix metalloproteinases (MMPs) etc. [87]. Its specificity to HA explains the very good emission response of the HA-GQDs by Park and coworkers. As a step further the efficiency of HA-GQDs to specifically curry chemotherapeutic drugs such as doxorubicin to cancer cells was evaluated as high rendering this smart nanomaterial both a good nanosensor and a drug nanocarrier.

In a similar fashion, Goreham et al. investigated the role of GO modification with folic acid in fluorescence lifetime imaging of HaCaT cells. Since folate receptor is an important recognized biomarker [88] currently considered for new diagnostic tools for cancer, modification of graphene oxide with folic acid was considered in this study. The water solubility green fluorescence (upon photoexcitation at 305 nm) and the evaluated low toxicity of GO indicated the high potentials of these graphene family members in cancer fluorescence imaging and corresponding diagnostics [89].

Liu et al. also utilized GO as a perfect platform on which a molecular beacon (a type of oligonucleotide hybridization probe which is capable of specifically detecting the presence of nucleic acids) having a couple of Cyanine-5 (Cy-5) fluorophore units at its both ends. The fluorescence of Cy-5 was quenched both due to self-quenching and fluorescence resonance energy transfer (FRET) caused by GO. With this

nanosystem, the researchers achieved detection of microRNAs even at concentrations a low as 30 pM. This nanoimaging technique was found to be applicable to a variety of cancer tissues and cells [65].

Additionally, Kumawat et al. reported the use of GQDs obtained *via* a green chemistry approach in fluorescence nuclear imaging in a variety of cell lines. It was observed that the reported GQDs even deprived of further support of external targeting agents (such as folic acid, hyaluronic acid etc.) exhibited a drastic propensity to self-localize into cell nuclei [90].

Moreover, Fan et al. investigated the use of some pH-responsive GQDs (pRF-GQDs) which were synthesized *via* an electrochemical method. The pRF-GQDs were found to undergo a fluorescence color transition between green and blue by varying the pH and developed a method which enable distinguishing between healthy and cancer tissues [91]. These are two important recent examples of the immense potentials of GQDs in bioimaging of cancer and cancer diagnosis advancements.

While fluorescence can be readily used in the detection and imaging of a variety of tumor cells, Magnetic resonance imaging (MRI) techniques enhanced through the use of GQDs appear to be very attractive due to the ease of production, size, easy structure modulation as well as multi-purpose character of GQDs [92]. Zhang et al. nearly a decade ago reported the use of GO-gadolinium (GO-Gd) complexes for the enhancement of and quality improvement of the MR-imaging of cancer. They showed that the developed GO-based material not only can operate as an enhancing agent for MRI but furthermore serves as a material for fluorescent imaging with anticancer-drug delivery aptitude (**Figure 6**). This early finding clearly showcases the immense possibilities and versatility of GBMs in bioimaging [93].

More recently and in a similar fashion, Yang et al. reported on the use of surface-modified GQDs by polyethylene glycol (PEG) which were functionalized with the Gd-DOTA complex (where DOTA stands for tetraazacyclododecanetetraacetic acid) in cancer imaging through MRI and fluorescence imaging. The research group managed to significantly increase the relaxivity by regulating the length of the PEG linkers and hence advanced a novel MR contrast agent with immense potentials within cancer-imaging [94].

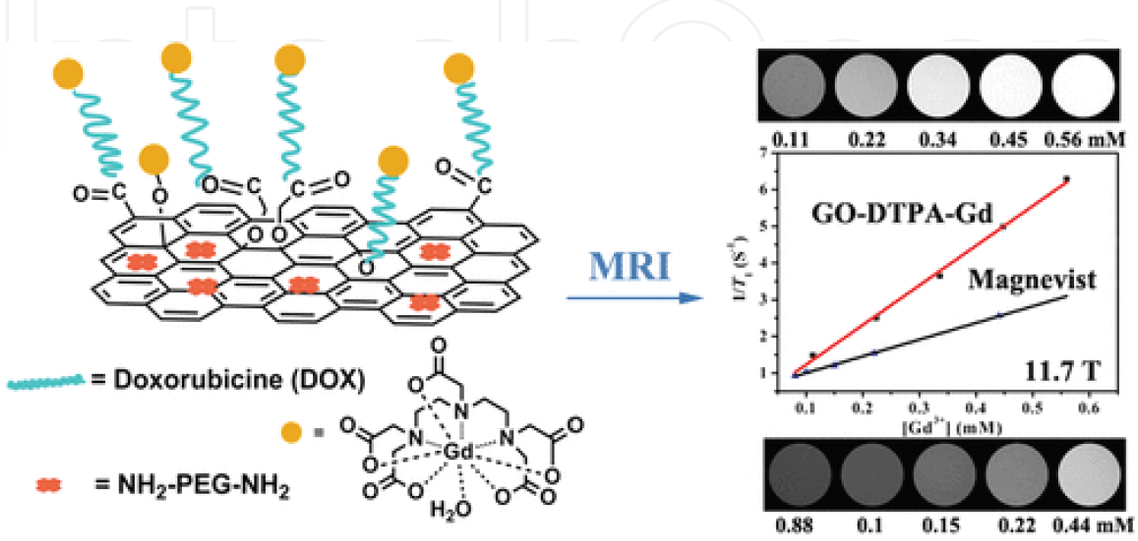

Figure 6.
Illustration of the Gd-bearing GO-based platform developed by Zhang et al. allowing for enhanced MR-cancer imaging as well as for targeted drug delivery applications. Obtained with permission from supplementary information of Ref. [93]).

5. Conclusions and future perspectives and uses of fluorescent imaging involving GBMs

There is a wide range of current and potential future applications of fluorescence imaging, two of the most promising being fluorescence-guided surgery (FGS) [95] and robotic-assisted fluorescence surgery (RAFS) [96]. The applications are numerous while probably the most relevant ones lie in the field of surgical oncology [95, 97]. The idea of using tumor-targeted imaging agents in the context of this developing research and technology area is considered as a promising strategy for intraoperative cancer detection. In this regard, the broad family of GBMs and their attributes can demonstrate an important role as potent fluorescent/emissive materials of low toxicity, biocompatibility and tunable optical properties [46]. A variety of GQDs have been reported so far labelling tumor cells and tissue thus potentially facilitating tumor surgery [91, 98, 99]. Particular interest has been placed on the use on pH-responsive fluorescent GQDs (pRF-GQDs) as transition in the emitted light color depending on the pH can help distinguish healthy tissue from tumors (extracellular microenvironment of tumors exhibits lower pH) [33, 91]. Based on the so-far published research works, it becomes apparent that the multifunctional character of GBMs could facilitate tumor FGS in the future and in general bioimaging and the detection of disease [100, 101].

Acknowledgements

The authors are grateful to Dr. K. Tzavlaki for fruitful discussions on bioimaging.

Conflict of interest

The authors declare no conflict of interest.

Acronyms and abbreviations

AuNPs	gold nanoparticles
BSA	bovine serum albumin
CNDs	carbon nanodots
CD	circular dichroism
CD44	antigen is a cell-surface glycoprotein
Cyt c	cytochrome c 1
DMF	dimethylformamide
EA	energy acceptor
ED	energy donor
FGS	fluorescence guided surgery
FRET	fluorescence resonance energy transfer
FCS	fluorescence correlation spectroscopy
FILM	fluorescence lifetime imaging microscopy
GBMs	graphene based materials
GM	Göppert Mayer units
GO	graphene oxide

GQDs	graphene quantum dots
HA	hyaluronic acid
HAase	hyaluronase
HaCaT	a spontaneously transformed aneuploid immortal keratinocyte cell line
HCC-CTCs	hepatocellular carcinoma cells
MRI	magnetic resonance imaging
NIR	near infrared
RAFS	robotic-assisted fluorescence surgery
pRF-GQDs	pH-responsive fluorescent graphene quantum dots
PEG	polyethylene glycol
SK-BR-3	a breast cancer cell line
TP	two photon
TPA	two photon absorption

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