



Functionalized fluorescent carbon nanostructures for targeted imaging of cancer cells: a review

Meghdad Pirsaeheb¹ · Somayeh Mohammadi¹ · Abdollah Salimi^{2,3} · Mehrdad Payandeh⁴

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Abstract

This short review (with 72 refs.) summarizes the state of the art in fluorometric methods for targeted imaging of cancer cells and tumor tissues in order to differentiate between normal cells and cancer cells. Following an introduction into the field and after presenting an overview on the most commonly used carbon dots and graphene quantum dots, we describe methods based on peptide based targeting, aptamer based targeting, antibody based targeting, and ligand-based targeting. A concluding section summarizes the current state and challenges, and discusses future perspectives.

Keywords Carbon based materials · Covalent and non-covalent modification · Heteroatom doping · Polymer passivation · Folate receptors · Cell imaging · Cancer cells

Introduction

Cancer treatment is still extremely difficult, so design of powerful and effective biodevices for early diagnosis of cancer are critical. The main challenge in cancer diagnosis and treatment is fast and effective differentiation between normal and cancer cells. To differentiate diseased and healthy cells, unique molecular signatures on the cells are needed. In most cancers, the differentiation of normal cells from tumor and benign (noncancerous) from malignant (cancerous) is subtle. The early detection of cancer cells by identifying of cellular signatures is a major hurdle for effective cancer therapy; the earlier identification of these signatures will allow more effectively they can be treated. The differences between cancerous cells

and non-cancerous ones are based on their intracellular or extracellular biomarkers. In order to design detection methods for the specific recognition of intracellular biomarkers (e.g. DNA/RNA/Proteins) and extracellular (cell surface) biomarkers, it is necessary to have previous knowledge about these biomarkers [1].

Developing efficient method for targeting cancer cells and tumors and delivering therapeutics to specific locations in the body are a primary objective in basic biological research and cancer medicine. Fluorescence imaging of cancer cells and tumors can enable a possibility of monitoring of the cancer treatment and diagnosis. Fluorescence bioimaging is great important for visualizing the expression and activity of specific molecules as well as cells and biological processes that influence tumor behavior and/or response to therapeutic drugs. For the bio-imaging study of cancer on both cellular and animal levels, robust fluorescence probes are required. A wide range of fluorescent probes including quantum dots [2], upconversion nanoparticles [3], silica nanoparticles [4], gold nanoparticles [5], and fluorescent metal nanoclusters [6, 7] have been applied for cell imaging systems. These fluorescent nanomaterials dominate some drawbacks of organic fluorophores such as the poor photostability for their practical applications in long-term bioimaging. Size-dependent variation of emission color, high brightness and long-term photostability of semiconductor quantum dots (semi-QDs) make them as the promising alternative to organic fluorophores. But the low solubility of semi-QDs in water and their toxicity

✉ Somayeh Mohammadi
smhmohammadi4@gmail.com

¹ Research Center for Environmental Determinants of Health (RCEDH), Kermanshah University of Medical Sciences, Kermanshah 67198-51351, Iran
² Department of Chemistry, University of Kurdistan, Sanandaj 66177-15175, Iran
³ Research Center for Nanotechnology, University of Kurdistan, Sanandaj 66177-15175, Iran
⁴ Regenerative Medicine Research Center, Kermanshah University of Medical Sciences, Kermanshah 67148-69914, Iran

limits their application in bioimaging. Gold nanoparticles and upconversion nanoparticles (UCNPs) have good photostability and biocompatibility, but exhibit low fluorescence quantum yield [8]. On the other hand, the major disadvantages of silica nanoparticles are oxidative biodegradation in biological systems. Silver and gold NCs have been indicated that be a safe choice for the bioimaging application, but they are costly and lack good photo stability [9].

Carbonaceous fluorescent nanomaterials have found particular interest owing to their superiority in terms of their low cytotoxicity, favorable biocompatibility, good chemical stability and ease of functionalization [10]. To date, numerous types of carbon-based nanomaterials including carbon nanotubes (CNTs), graphene and its derivatives, fullerene, nanodiamond, carbon dots (CDs) and graphene quantum dots (GQDs) have been investigated for potential applications in the field of biology [11]. Nanodiamond shows exceptional biocompatibility as well as special optical and chemical properties. There are some challenges including specific targeting of structures in biological samples, colloidal stability, and brightness against auto-fluorescence. These challenges can limit nanodiamonds interaction with living organism [8]. Also, nanodiamond needs costly preparation and laborious separation procedures. Among them, CNTs, graphene and its derivatives are weakly luminescent and poorly dispersible in water. Recently, CDs and GQDs as well as their nanocomposites, as new emerging carbonaceous nanomaterials, are subjects of essential research in bioimaging process [11].

Overview on carbon dots (CDs) for use on fluorescence imaging of cancer cells

For classification of the wide group of carbon-based fluorescent nanoparticles in subgroups, aspects such as crystalline structure, the arrangement of carbon atoms, starting materials for synthesis and the dimensionality should be considered [12]. Carbon dots (so-called carbon quantum dots (CQDs), C-dots or CDs) recently emerged as a new type of zero dimensional fluorescent carbon nanostructure. CDs were discovered by Xu et al. during purification of single-walled carbon nanotubes (SWCNTs) using gel electrophoresis in 2004 [13]. CDs are small and quasi-spherical carbon nanoparticles with diameters smaller than 10 nm [14]. Nuclear magnetic resonance data indicate that the inner part of CDs is mainly consisted of sp^2 hybridized carbon atoms, while outer part composed of sp^3 hybridized carbon atoms [11, 14]. CDs gradually became exciting photoluminescent carbonaceous nanomaterials owing to their several favorable characteristics including low cost for synthesis/fabrication, high photostability, tunable emission, good intracellular solubility, chemical inertness and nontoxicity which resulted in their numerous possible applications, especially in bioimaging fields.

The absence of heavy metals and toxic anions in CDs make them highly promising, eco-friendly much safer for biological applications than inorganic semiconductor quantum dots, which often contain toxic heavy metals [15–17]. The concept of CDs has been broadened to carbon nanodots (CNDs), polymer dots (PDs) and graphene quantum dots (GQDs) etc. [18]. CNDs are defined comprehensively as spherical carbon nanomaterials which do not have a crystal lattice [19]. PDs represent the CDs those are composed of linear polymers or monomers during the cross-linking and dehydration [18, 19]. PDs dimensions can be manipulated from a few nanometers to tens of nanometers with different component ratios [20]. GQDs is defined as the products obtained from graphene monolayer cutting into small pieces (disks) with dimensions of a few nanometers (2–20 nm) that have quantum confinement effect and edge effects. GQDs are crystalline which contain mostly of sp^2 -hybridized carbon [21, 22].

The surface structures of CDs depends on precursors used for synthesis and the preparation procedures [23]. The precursors used for synthesis of CDs are carbon nanomaterials with crystalline structure (such as carbon nanotubes) or various organic molecules (such as melanin [24], phenol [25], glycerol [26], citric acid [27–34], EDTA [35], glycine [36], ATP [37, 38], hyaluronic acid [36, 39], glucose [40–43], carbon fiber [44], dandelion leaf [45], aconitic acid [46], active dry yeast (ADY) [47], L-cysteine [29, 31], α -cyclodextrin [48], folic acid [49–52], urea [50], starch and L-tryptophan [53], thiourea [32], L-aspartic acid [42, 43] whereas GQDs can be synthesized from graphene-based materials [54–56], graphite rod [57], carbon black [58] and carbon fiber [59].

The luminescence properties of CDs are mainly depending on the synthetic procedure and the kind of surface passivation. In the past few years, considerable researches have been directed to use the luminescence properties of CDs which is urged by the demands in the advanced field of optical imaging [60]. CDs can emit photoluminescence colors from blue to red as can be seen in Fig. 1. However, most of them emit in blue light region rather than other longer wavelength lights (i.e., green- to red-light) (as shown in Tables 1, 2, 3, 4 and 5) [61]. Some latest reported CDs have displayed fluorescent emission in the near-infrared (NIR) region recognized as ‘biological window’. In the near-infrared (NIR) region, the autofluorescence from organisms is minimum, thus signal-to-noise ratio can be enhanced [60]. Therefore, the design of multicolor and long wavelength emission CDs with high aqueous solubility, photo-stability and high interference resistance is strongly desirable for further application in bioimaging [61]. Pan et al. reported the successful synthesis of full color CDs using citric acid formamide solution as source. The full color CDs shows unusually comparable emission intensities as changing the excitation wavelengths from 330 to 600 nm, and the corresponding emissions nearly cover the entire visible spectrum (Fig. 1) [62]. Liu et al. prepared three kinds CDs by

microwave heating of phenylenediamine isomers (including o-phenylenediamine (oPD), m-phenylenediamine (mPD) and p-phenylenediamine (pPD)). The m-CDs, o-CDs and p-CDs exhibit blue, yellow and orange fluorescence under 365 nm UV irradiation in ethanol solutions, respectively [63]. Therefore, it has been identified that CDs features require be continuously improving and tuning by the selection of precursors during synthesis, specific targeting moieties and surface passivation and modification.

Targeting moieties used for specific imaging of cancer cells

There are many different types of targeting moieties including peptides (such as transferrin), aptamers, antibodies, as well as small molecules (such as folic acid (FA)) which have been used to incorporate on the surface of nanoparticles. Such

targeting moieties can provide the internalization of nanoparticles into cancer cells and tissues through a ligand-receptor interaction. High specific cancer cell targeting and efficient distribution avoid the side effects coming from the nonspecific bonding. Functionalization of nanoparticles surface by targeting ligands can simplify active targeting of NPs to receptors which are on surface of cells, result in enhancing cellular internalization and/or specific uptake through receptor-mediated endocytosis. Identifying new biomarkers and their appropriate ligands for use in targeted drug delivery is major interest. Binding of NPs to analytes, pathogens, and biomarkers can lead to amplify their signal for detection and molecular imaging [64].

The folate receptor (FR) is a membrane-bound protein with molecular weights ranging from 38 to 40 kDa. Due to over-expression of FR on a large number of cancer cells (nearly 40% of cancer cells), including epithelial, ovarian, lung, breast, kidney, colorectal, brain, endometrial and renal cancer,

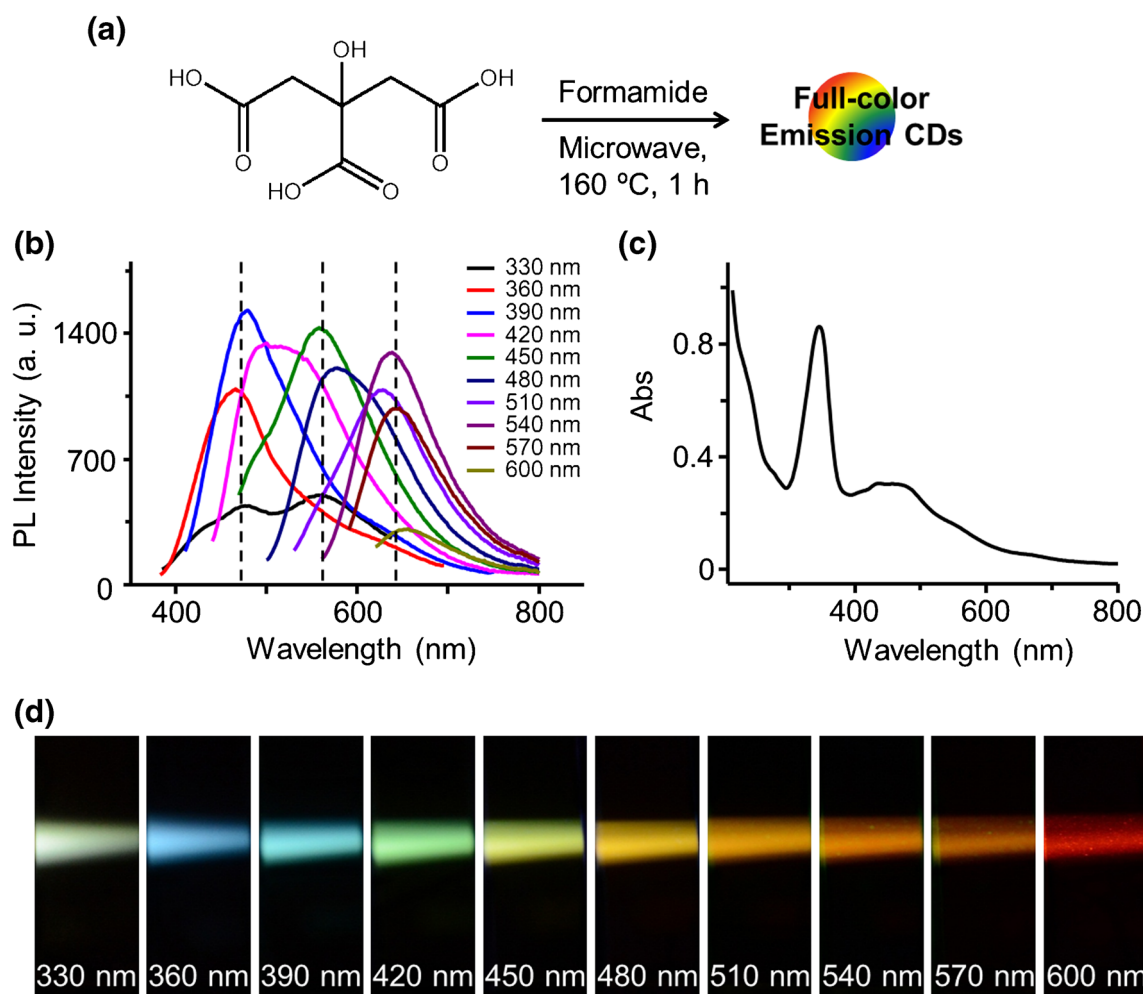


Fig. 1 a A schematic illustration of the preparation of the full-color emission CDs. b FL spectra of the F-CDs under different excitation wavelengths. c UV-vis absorption spectra of the F-CDs. d FL emission

photographs of the F-CDs recorded from 330 to 600 nm in 30 nm increments. All spectra and photographs were obtained in deionized H₂O. Reproduced from [62] with permission Wiley

Table 1 Peptides based target-specific bioimaging applications

Precursors	Type of modification	Targeting ligands	Receptor	Functionalization ligand to CDs	Excitation/Emission Wavelength (nm)	Emission color	Size (nm)	QY	Ref
Melanin	–	Peptide iRGD	$\alpha_v\beta_3$ and NRP-1	Non covalent	440 to 615/570 and 645	Green, Yellow, Red	3.3 ± 0.57	–	24
Phenol/ formaldehyde resins	Oligomeric polymer chains	Transferrin (Tf)	Transferrin	Covalent	360/450 420/500	Blue, Blue	1.5–3	–	25
Graphite Rods	–	Peptide nanofibers (PNFs)	–	Non covalent	320/550 315/438	Green Blue	0.5–2.5	–	57

Table 2 Aptamers based target-specific bioimaging applications

Precursors	Type of modification	Targeting ligands	Receptor	Functionalization ligand to CDs	Excitation/Emission Wavelength (nm)	Emission color	Size (nm)	QY	Ref
glycerol and 2-mercaptoethanol	Thiol terminated	maleimide-terminated TTA1 (mal-Tnc) aptamer		Thiol-maleimide reaction	360/460	Blue	4.5	–	26
Citric acid & ethylene diamine	–	AS1411 aptamers	Nucleolin	Electrostatic interaction	400/470	Blue	~2.5–10	0.54	27
Carbon black	–	AS1411 aptamers		Covalent	480/530	Green	–	–	58

Table 3 Antibodies based target-specific bioimaging applications

Precursors	Type of modification	Targeting ligands	Receptor	Functionalization ligand to CDs	Excitation / Emission Wavelength (nm)	Emission color	Size (nm)	QY	Ref
Citric acid and iohexol	Iodine doped/ Polyethyleneglycole(PEG)	C225 (also known as cetuximab)	EGF	EDC/NHS Covalent	380/450 nm	Blue	~3.7	18%	28
Citric acid and ethylenediamine	-	CA125 Ab	-	Covalent	350/453	Blue	3.2	-	33
Resorcinol	-	CA125 Ab	-	Covalent	230/326	Blue	2.5	29%	34
Citric acid	-	CA153 Ab	-	-	230/446	Green	3.2	52%	-
EDTA, triethylenetetramine and MnCl ₂	Mn	anti-HE4 mAb	-	EDC/NHS Covalent	360/450	Blue	4.6 ± 0.9	90.8%	35
Glucose and glycol	PEI	CEA8 Ab	-	EDC/NHS Covalent	360/475	Pale blue	~7.5	-	69

Table 4 Hyaluronic acid based target-specific bioimaging applications

Precursors	Type of modification	Targeting ligands	Receptor	Functionalization ligand to CDs	Excitation/ Emission Wavelength (nm)	Emission color	Size (nm)	QY	Ref
Hyaluronic acid and glycine	Nitrogen doped	Hyaluronic acid	CD44	Autoclave	360/470	Blue	~2.3	-	36
ATP	Polyethylenimine (PEI)	Hyaluronic acid	CD44	Electrostatic interaction	310/400	Blue	2.2	-	37
polyethylenimine (PEI) and hyaluronate (HA)	Nitrogen doped PEI	Hyaluronic acid	CD44	Covalent	350/470	Blue	2.25	-	39
Starch and L-tryptophan	N doped, MIP shell	glucuronic acid (GlcA)	hyaluronan (HA)	Non covalent	371/452	Blue	~3-10	(25.1 ± 2) %	53
Graphene oxide	-	Hyaluronic acid	CD44	EDC/NHS Covalent	360-480/540	Green	~5	~14%	55
Carbon fiber	-	Hyaluronic acid	CD44	Non covalent	318/450	Blue	20	27%	59
HA and PEI	PEI	Hyaluronic acid	CD44	-	345/470	Blue	32.5 ± 0.5	-	71

Table 5 Folic acid based target-specific bioimaging applications

Precursors	Type of modification	Targeting ligands	Receptor	Functionalization ligand to CDs	Excitation/Emission Wavelength (nm)	Emission color	Size (nm)	QY	Ref
graphite nanoparticles	–	FA	Folate	Covalent EDC/NHS	475/540	Green	–	0.54%	17
Citric acid monohydrate and L-cysteine	PEI-folic acid	FA	Folate	Electrostatic interaction	–	–	8	–	29
Citric acid (CA) and SiO ₂ spheres	(3-aminopropyl) trimethoxysilane (APS)	FA	Folate	EDC/NHS Covalent	360/435	Blue	2.8±0.3	7.1%	30
sodium citrate, N-acetyl-L-cysteine, Na ₂ S, and S, N, and Gd tri-element	doped	FA	Folate	EDC/Sulfo-NHS	360/450	Blue	4.3±0.9	12.8%	31
GdCl ₃	B, N, S doped	–	–	–	360/450	Blue	3–4.5	28%	32
ATP	PEI	FA	Folate	Electrostatic interaction	300/385	Blue	1.5–3	–	38
Glucose (Glu) and Poly(acrylate sodium) (PAAS)	Poly(acrylate sodium) (PAAS)	FA	Folate	Hydrogen-bond interaction- Non covalent	410/520	Green	3.5	~5.4%	40
D-(+)-Glucose and 4,7,10-Trioxa-1,13-tridecanediamine (TTDDA)	TTDDA	FA	Folate	N,N'-dicyclohexylcarbodiimide and NHS Covalent	360/460	Blue	2–7	–	41
D-Glucose and L-aspartic acid (Asp)	–	–	–	–	440/524	Green	2.49±0.42	–	42
D-glucose and L-aspartic acid	–	–	–	–	405 488 555	Blue Green Red	2.28±0.42	7.5%	43
Carbon fiber powder	PEI	FA	Folate	EDC/NHS Covalent	430/520	Green	~3	–	44
Dandelion leaf and EDA	NH ₂ terminated	FA	Folate	EDC/NHS Covalent	396/512	Blue	3.5	13.9%	45
Aconitic acid and EDA	N doped	FA	Folate	Non covalent	360/450	Blue	1.6	56.5%	46
Active dry yeast (ADY) and phosphoric acid	P doped	FA	Folate	EDC/NHS Covalent	360/516	Blue	3.4	11.6%	47
α-cyclodextrin	Poly(ethylene glycol) diamine	FA	Folate	EDC Covalent	360/450	Blue	4.5±0.2	2.1%	48
Folic acid	N doped	–	Folate	–	360/~450	Blue	3.5±0.6	9%	49
Folic acid, Urea	N doped	–	Folate	–	380/460	Blue	4	25%	50
Folic acid	N doped	–	Folate	–	320/400	Blue	5.4±2.2	94.5%	51
Folic acid (FA) and polyethyleneimine (PEI)	N doped	–	Folate	–	370/452	Blue	5.8	42%	52
Graphene oxide	DTPA	FA	Folate	Covalent EDC/NHS	350/520	Green	–	–	56

it is recognized as biomarker for differencing of tumor cells. Therefore, a number of studies has been used the FR as biomarker for many diagnostic tools to allow imaging of cancer cells. Vitamin FA is one of the useful ligands that are exploited for targeting tumor cells. The essential role of FA has been proven in human growth and development, and at the cellular level in cell division and DNA synthesis, for the metabolism of specific biochemical reactions. Due to high affinity ($K_d \sim 10^{-10}$ M) of FA for the FR, FA has become a popular tumor-specific ligand for the cellular and/or intracellular delivery of various covalently attached therapeutic agents. So, the non-specific detection can be decreased using the receptor based methods [41]. In this review, efficient method based on targeting cancer cells and tumors highlights differentiation between normal and cancer cells as the key principles of cancer diagnosis. We have provided an overview on the applications of CDs and GQDs in target specific bioimaging and distinguish cancerous cells from normal cells. Several classes of ligands (including aptamers, peptides, antibodies), especially small molecules (such as FA)) have been reported for functionalizing of CDs and GQDs and their interactions with receptors on cancer cells both in vitro and in vivo.

Surface passivation and functionalization operations

Carbon dots (CDs) can be modified either in the core structure by partial substitution of carbon with other elements (i.e., doping by nitrogen, sulfur, or boron) or by surface functionalization.

The heteroatoms doping

Most CDs and GQDs have relatively low fluorescence efficiency compared to the conventional semiconductor quantum dots. Various surface passivation methods are needed to improve the electronic and optical properties of CDs. Doping of heteroatoms, including nitrogen, sulfur, boron, phosphorous and silicon, is considered as a most promising passivation methods [18]. The effect of single- and co-doping of different elements on the photoluminescence (PL) properties of the resulting CDs is studied. The most widely used element for doping is nitrogen owing to the close similarity between nitrogen and carbon [65]. Recently, it has demonstrated that nitrogen-doped CDs (NCDs) showed heterogeneous multi-layered structures, which they can correspond the characteristic planes between graphite and graphite oxide [36]. NCDs synthesized using FA as a single precursor and without any passivation agents. FA-derived CDs exhibited strong PL with quantum yield (QY) up to 94.5%. FA has rich nitrogen and functional groups of $-\text{NH}_2$, $-\text{OH}$, and $-\text{COOH}$ which allows polymerization and carbonization to prepare high PL NCDs [51]. Xiao et al. prepared N-CDs, possessing some advantages including high QY (> 80%), long fluorescence

lifetimes of 15.0 ns, and also excellent photostability in wide pH range solutions (4–11), high ion strength (2 M KCl), longtime UV light irradiation (4 h continuously), and favorable biocompatibility and low cytotoxicity to human nasopharyngeal carcinoma cells CNE-1 and human embryonic kidney cells HEK-293 T, which are of crucial importance for bioimaging applications [66]. Citric acid and iohexol used for synthesis of iodine doped carbon quantum dots (I-CQDs) which show excitation-dependent PL behavior with the fluorescence QY of 18% [28]. S, N, and Gd tri-element doped magnetofluorescent carbon quantum dots (GdNS@CQDs) exhibited excellent fluorescent, magnetic properties, high stability at ionic strength and physiological conditions [31]. Boron, nitrogen, sulfur doped in carbon quantum dot (BNSCQD) with maximum QY (28%) was synthesized with a precursor ratio, citric acid: thiourea: *m*-aminophenyl boronic acid = 0.5: 0.5:0.3. The synergistic presence of sulfur and nitrogen along with boronic acid provides molecular recognition sites for specific uptake in liver cancer cell (HepG2) [32]. It is found that doping heteroatoms into CDs matrix (X-CDs) can modulate the band structure of CDs. Heteroatom doping not only improve photoluminescence properties of CDs but also impart further functionality to the doped CDs. Thus these features can expand their applications in fluorescent bioimaging [18, 65]. Like CDs, GQDs also can be modified via many methods. Modification of the GQDs can not only enhance their luminescence properties but also allow them to be used more widely in biological applications [54–59].

Passivation of CDs surface with various polymer chains

The CDs possess various functional groups (such as hydroxyl, carboxyl, amine and so on), which provide the possibility to be passivated by inorganic, polymeric and organic materials. Passivation or functionalization of CDs surface is essential to enhance the fluorescence QY. During the passivation process, some reactive sites (for example, hydroxyl, amine and carboxyl groups) were introduced on the CDs surface for functional modification purpose [67, 68]. Through these reactive groups, different specific inorganic, organic, polymeric, or biological materials can be link to the CDs surfaces via covalent bonds, electrostatic interactions and hydrogen bonds, serving as platforms for specific sensing, drug delivery and other specific tasks [68].

In general, the surface modification of CDs with various polymeric can afford high surface density of reactive sites for facilitating of attachment of functional moieties on CDs and increasing solubility in water [38]. The photoluminescence of CDs is improved upon surface passivation with oligomeric polymer chain. Depending on the photoluminescence properties of CDs, various polymer chains can be used for surface passivation. Li et al. investigated the effect of conjugating polyethylene glycol (PEG) chains, polyethylenimide-*co*-

polyethylene glycol-*co*-polyethylenimine copolymer, and 4-armed PEG molecules. The cellular internalization and cytotoxicity of the resulting CDs were evaluated which they exhibited no apparent cytotoxicity [25]. Su et al. reported surface passivated iodine doped CQDs (I-CDs) with diamine-terminated oligomeric poly-(ethylene glycol) [28]. Wang et al. reported the application of (3-aminopropyl) trimethoxysilane (APS) as surface passivation agents to generate high PL QY and also as bridge to enable the further conjugation of ligands onto CDs surface for imaging purpose [30]. polyethylenimine (PEI) is a cationic polymer with high density of amino groups which facilitates attachment of functional moieties on CDs and also provides adsorption possibility of anionic material (such as FA) via non-covalent adsorption or wrapping [38]. The passivation agents containing nitrogen (such as NH_2 terminated PEI) acted as auxochromes that can dramatically improve the PL of CDs [29, 37–39, 44, 69]. CDs are modified with PEI through electrostatic interactions between the positively charged amino groups of PEI and the carboxyl ($-\text{COOH}$) groups of CDs surface [29, 37, 44]. Poly (acrylate sodium) (PAAS) was used as a new passivating agent for synthesis of green fluorescence CDs. Moreover, CDs functionalized with FA via kind of non-covalent bond – hydrogen-bond [40]. The passivation agents, 4,7,10-Trioxa-1,13-tridecanediamine (TTDDA), possesses active amino group. The comparison of QY of CDs before and after passivation demonstrated that the passivation increased the luminescence efficiency of CDs [41]. Choi et al. exhibited that fluorescence efficiency and biocompatibility of CDs enhanced when poly(ethylene glycol) diamine (PEG) utilized for passivating CDs surface [48]. So, passivation of CDs can provide an effective strategy for further modification of the CDs to different ligands and biomolecules in biological applications, including *in vivo* and *in vitro* bioimaging.

Main methods for functionalization of the surface of carbon dots

In order to broaden CDs applications, further chemical modifications are required. Many applications of carbon nanomaterials depend on effective strategies to conjugate specific materials onto the surface of carbon nanomaterials which are mainly categorized into two classes including covalent and non-covalent methods (electrostatic interactions, hydrogen bonds, π - π interaction and van der Waals force).

Non-covalent methods

The development of novel CDs with both capacity of active tumor targeting and red shift fluorescence is very important. For improvement of uptake and tumor targeting of nanomaterials, different ligands have been used for

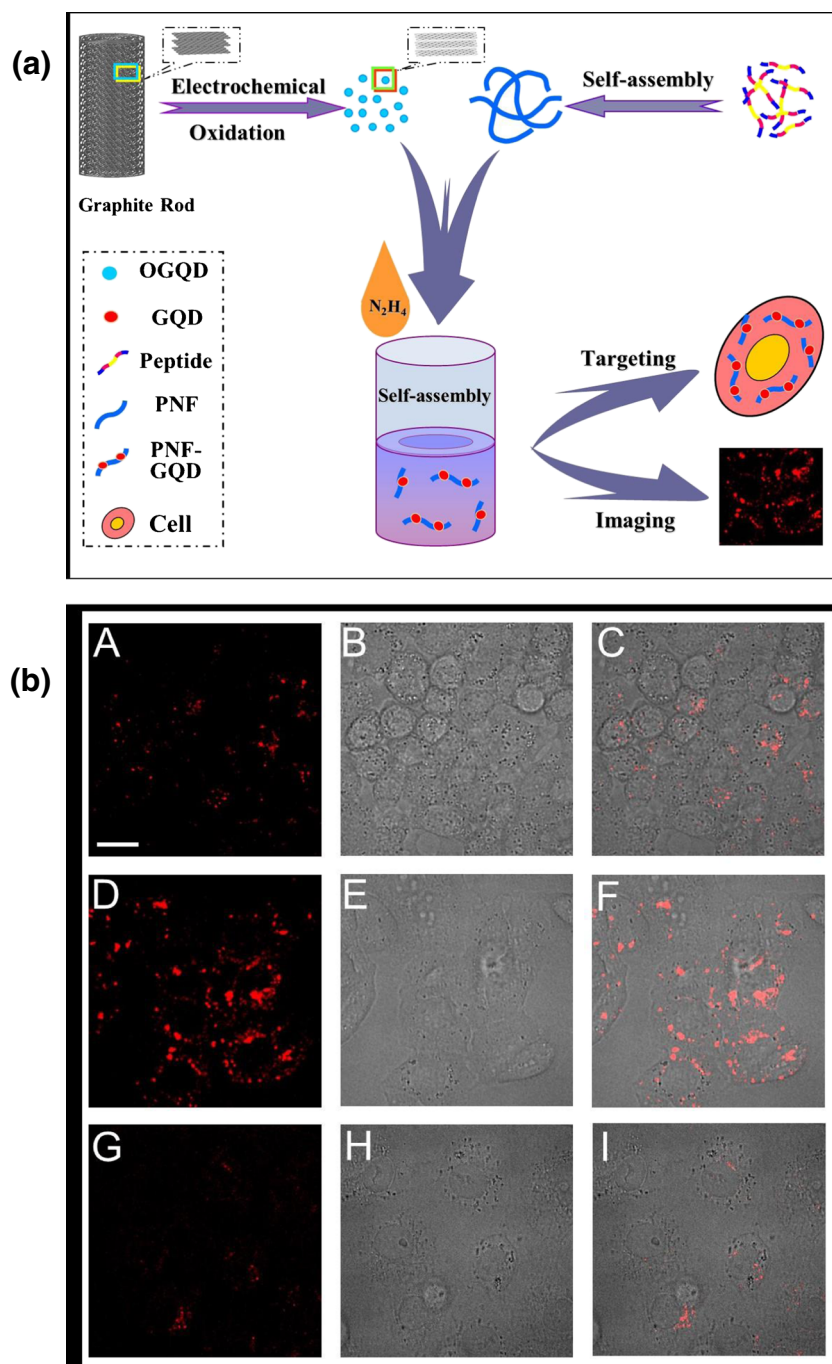
functionalization of nanomaterials. Yang et al. demonstrated that physically (non-covalent) decoration with iRGD peptide can significantly increase penetration of CDs in tumor tissue [24]. Charged CDs can be conjugated by various ligands with opposite charges through electrostatic interactions. Motaghi et al. reported a signal-on spectrofluorometric for cancer cell detection using CD-aptamer nanoconjugate. The electrostatic interaction between positively charged CDs and negatively charged DNA aptamers resulted in the coating of aptamers around CDs [27]. Wang et al. indicated that PEI conjugated FA (PEI-FA) can coat onto CDs surface via electrostatic interaction under neutral condition [29]. Gao et al. reported multi-functional CDs-based composite as a turn-on theranostic fluorescent nanoprobe. Multi-functional CDs-based composite were prepared in several steps: First, CDs are capped with positively charged PEI (CDs-PEI) via electrostatic interaction, and then hyaluronic acid (HA) covalently conjugated with doxorubicin (Dox) (HA-Dox) by EDC/NHS. And finally, electrostatic self-assembly method was used for functionalization of CDs-PEI with HA-Dox [37]. Lei et al. reported a turn on fluorescent probe for targeting and imaging FR positive cancer cells via non-covalent conjugation of FA on PEI-CDs. Conjugation of FA onto PEI-CDs was confirmed by comparing zeta potential amounts (-10.2 mV for CDs, $+8.39$ mV for PEI-CDs, and $+3.16$ mV for FA-PEI-CDs). In PBS solution (pH 7.4), the $-\text{NH}_2$ groups of PEI ($\text{pK}_b = 3.0\text{--}5.0$) are protonated and the $-\text{COOH}$ groups in FA ($\text{pK}_a = 3.55$) are dissociated. Therefore, there is electrostatic interaction between the $-\text{NH}_3^+$ group of PEI and the $-\text{COO}^-$ group of FA [38].

The targeting ligands with specific function can also be conjugated on CDs surface via a kind of non-covalent bond – hydrogen-bond. For example, Liu et al. reported a novel turn-on fluorescence imaging probe for recognizing of special cancer cells through hydrogen-bond interactions between carboxyl coated CDs and FA. For preparation of fluorescent probe, FA was added to CDs solution and dialyzed against pH 7.4 phosphate buffer for 2 h [40]. Su et al. developed a novel nanohybride structure which based on designing a novel peptide (RGDAEAKAEAKYWYAFAEAKAEAKRGD) with three functional motifs. Domain 1 (RGD) have a high binding affinity toward integrin-rich tumor cells; domain 2 (AEAKAEAK) enables the facility of nanofibers forming; and domain 3 (YWYAF) possesses the tendency to bind to carbon-rich surfaces, i.e., GQDs. PNF-GQD nanohybrids were constructed by attachment of GQDs onto the surface of PNFs via noncovalent forces such as electrostatic or π - π stacking interactions (Fig. 2a) [57].

Covalent methods

For CDs and GQDs, the covalent modifications are mostly used through chemical reactions like amidization and silylation reactions. This is discussed in the following sections.

Fig. 2 **a** Schematic depiction of the fabrication of PNF–GQD nanohybrids and the subsequent cellular imaging. **b** Confocal microscopy images of cultured cells. HeLa cells were incubated with A–C) GQDs or D–F) PNF–GQDs in DMEM for 2 h; COS-7 cells were incubated with G–I) PNF–GQDs in DMEM for 2 h. Shown are A,D,G) confocal fluorescence, B,E,H) bright field, and overlay C,F,I) images. Scale bar: 20 μm . Reproduced from [57] with permission from Wiley



Amidization method for modifications CDs and GQDs surface

Amidization method using ethyl(dimethylaminopropyl) carbodiimide(EDC)/N-hydroxysuccinimide (NHS) was widely used for covalent conjugation of various ligands onto nanomaterials surface which based on reaction between carboxyl or amino onto nanomaterials surface with amino or carboxyl terminated ligands.

Modifications amino-terminated CDs and GQDs via amidization method

Amino groups on CDs surface can be engineered for the further conjugation with activated carboxyl groups of transferrin [25], cetuximab [28], FA [44, 45, 47] through covalent bond–amid bond formation. For example, Li and coworkers covalently conjugated transferrin onto the NH_2 terminated CDs surface using EDC chemistry for in Vitro targeted imaging

studies [25]. Su et al. modified PEG-coated nanodots (I-CQDs-NH₂) with cetuximab using a modification of the standard EDC/NHS reaction [28]. Due to remaining of free amine groups on SiO₂ surface, the SiO₂@CDs can be further conjugated with FA using EDC/NHS cross-linking reaction. SiO₂@CDs-FA utilized as fluorescent probes for biological imaging in vitro [30].

Zhang and co-workers synthesized HA and PEI functionalized CDs (HA/PEI-CDs) using a bottom-up procedure for tumor targeting and gene delivery. Dehydration and polymerization between amino groups of PEI and carboxyl groups of HA may form special construction structure similar to amphiphilic polymer structure [39].

Song et al. reported direct conjugation of FA to the TTDDA passivated CDs surface was unsuccessful even in basic media, maybe owing to their low reactivity in the present system. To overcome this problem, carboxyl groups of FA activated by NHS, and then FA-NHS can be covalently bound to amino groups of the TTDDA-CDs [41].

Huang et al. made a multifunctional nanocarrier for simultaneous receptor-specific targeting, dual-modality bioimaging, and cancer chemotherapy. This multifunctional nanocarrier consist of paramagnetic GQDs, folate, and doxorubicin (Dox), utilized as delivery vehicles, a targeting ligand, and a chemotherapeutic drug, respectively. GQDs can covalently bond with different molecules due to the presence of chemically reactive groups at the surface and edges of GQDs. Amino-terminated GQDs was firstly conjugated with folate after preliminary activation of the carboxyl groups of folic acid by cross-linker reagent (EDC or NHS). Subsequently, for chelation of the paramagnetic compound, DTPA reacted with folate-GQDs by using EDC as the activating agent. The folate-GQD/DTPA was then chelated with paramagnetic Gd³⁺ [56].

Modifications carboxyl-terminated CDs and GQDs via amidization method

The -COOH groups on the surface of CDs and GQDs were further cross linked by EDC/NHS to conjugate to various biomolecules containing -NH₂ groups. Chiu et al. reported the surface functionalization of the -COOH groups of GdNS@CQD with -NH₂ groups of FA using EDC/Sulfo-NHS chemical reaction [31].

Manganese(II)-carbon dots (Mn-CDs) hybrid was synthesized using EDTA, triethylenetetramine and MnCl₂ via stable coordination between Mn²⁺ ions and CDs. The Mn-CDs hybrid showed high fluorescence QY of 90.76%. Anti-HE4 mAb as the targeting agent was covalently immobilized onto the surface of Mn-CDs through coupling the amino groups of the mAb with the carboxyl groups of Mn-CDs in the presence EDC/NHS [35].

HA and GQDs modified human serum albumin (HSA) nanoparticles were synthesized for bioimaging and targeted delivery of gemcitabine to pancreatic cancer. Conjugation of GQDs with HSA was the result of the appearance of the characteristic peaks, a peak at 1670 cm⁻¹ which corresponds to amide bond (CO-NH) formation between the carboxyl groups of GQDs and amino groups of HSA-NPs [55].

Wang et al. have reported a novel active targeting theranostic agent, made up of just two components of GQDs and aptamer AS1411. Carboxyl groups on the edge of the GQD converted to amine-reactive NHS esters by EDC/NHS coupling agents. Then, the NHS ester modified GQDs covalently functionalized with the amine group of AS1411. Upon irradiation with two various lasers, GQDs-AS1411 demonstrates high ability of specific tumor cell imaging and synergistic cytotoxicity towards cancer cells [58].

Modifications via silylation reaction

Silylation modification is a reaction between silane and active hydrogen on the surface of CDs [23]. Mesoporous silica nanoparticles (MSNPs) have the unique features, such as nontoxicity, large surface area, and excellent biocompatibility as well as good chemical stability [70]. Silica nanoparticles can act as a silicon shell to modify fluorescent components together to construct multicolored and nontoxicity fluorescence probes. It also can enhance the specific surface area to make CDs have good dispersion [23].

Wang et al. synthesized SiO₂@CDs composites from citric acid and SiO₂ spheres using a one-pot hydrothermal route in the presence of APS. APS not only acted as surface passivation agents to produce high photoluminescence CDs, but also provided the further covalent conjugation of the SiO₂@CDs composites with FA for targeted imaging of cancer cells [30].

Gui et al. synthesized N doped GQDs by carbonization of citric acid with ammonia, and then N-GQDs encapsulated with silica nanoparticles using tetraethyl orthosilicate (TEOS) and 3-aminopropyl triethoxysilane (APTES) through silylation reaction to obtain N-GQDs@SiO₂ [70].

Target-specific bioimaging

Peptide based targeting

Yang et al. reports an active tumor targeting imaging system using peptide iRGD decorated onto red shift emissive CDs. Cellular uptake and cell toxicity of iRGD-CDs were investigated by 4 T1 cells. The results indicated that decoration of iRGD peptide increases the permeability of CDs in tumor vessels and tumor tissue. Consequently, they improve the targeting and imaging efficiency of CDs [24]. Li et al. demonstrates that CDs can be utilized as wavelength-tunable

optical nanoprobe and their photoluminescence properties can be tuned by surface conjugation with oligomeric polymer chains. The authors investigated zeta potential of CDs to verify whether they can be bonded to the membrane surface via electrostatic interactions. Moreover, CDs were coupled with human transferrin (Tf) to evaluate their ability as biocompatible nanoprobe for addressing disease cells such as cancer cells [25]. Su et al. synthesized oxidized graphene quantum dots (OGQDs) by electrolysis of graphite rods in alkaline media. Then, OGQDs was reduced to GQDs using hydrazine (Fig. 2a). They introduced a novel nanohybride structure constructed by conjugation of protein nanofiber (PNF) with GQDs and used the nanohybride for labeling of HeLa cells. The demonstration was such that the GQDs showed similar cellular internalization as PNF-GQDs; PNF-GQDs can be preferentially penetrated in cancer cells avoiding side effects on normal cells as suggested by results from confocal microscopy (Fig. 2b) [57]. Some peptide based target-based bioimaging applications are listed in Table 1.

Aptamer based targeting

Small molecules like aptamers are attractive candidates for targeting tenascin C (Tnc), nucleolin, and Muc1 proteins. Various nanoparticles functionalized with aptamers have been successfully applied for specific targeted delivery in diagnosis and therapy of different diseases including cancers and immunological disorders [27].

Lee and coworkers presented microwave irradiation technique for synthesis of thiol-terminated C-Dots from glycerol (SH-g C-Dots) that applied for conjugation with maleimide-attached targeting molecules. Significant physical properties of g C-Dots including particle size, fluorescence properties, pH sensitivity and structural properties were not affected by thiol passivation. Since the SH-g C-Dots did not show significant toxicity in cancerous and normal cells; they can be used in *in vitro* and *in vivo* studies. Furthermore, a less toxic fluorescence probe, SH-g C-Dots labeled mal-Tnc aptamer, which can specially bind to glioma and cervical cancer cells for targeting cancers. SH-g C-Dots labeled aptamer will provide a useful targeting imaging probe for various cancers and diseases [26].

AS1411 is a 26-base guanine-rich oligodeoxynucleotide aptamer that targets and binds to nucleolin. Nucleolin is a nucleolar phosphoprotein, located normally in the nucleolus and also found in the cytoplasm and on the membrane of cells, which is overexpressed on the surface of certain cancer cells. Motaghi et al. introduced CDs-AS1411 aptamer as a probe for targeted detection of several types of cancer cells. In this study, CDs-aptamer suspensions were incubated with target cancer cells including mouse breast 4 T1, human breast MCF7, and human cervical HeLa cancer cells in the presence of control human foreskin fibroblast cells (HFFF-PI6) and the fluorescence intensity signals were measured [27].

GQDs-AS1411 was synthesized by Wang et al. exhibits capability of specific tumor cell labelling and photothermal therapy. During conjugation GQDs with AS1411, their optical absorption in visible and NIR range increased. Bare GQDs and AS1411-GQD solution emit green fluorescence upon excitation at 480 nm. In order to verify the cancer targeting specificity of AS1411-GQD, confocal microscopy was conducted using various cell lines. 4 different tumour cell lines (A549, HeLa, MCF-7, and HepG-2) and two normal cell lines (COS-7 and HEK293) were incubated with 5 μ M of AS1411-GQDs for 5 h. all of the 4 different tumor cell lines showed bright fluorescence signals with different values whereas no obvious fluorescence signal was observed in either of the two normal cell lines [58] (Fig. 3).

Aptamers based target-specific bioimaging applications are listed in Table 2.

Antibody-based targeting

Owing to the excellent fluorescent features, the CDs can be applied to target specific cells by cell-specific antibody or other reagent. C225 (also known as cetuximab) can specially bind to EGF receptor, which is overexpressed among many cancers, such as lung cancer and head and neck cancers and inhibit the growth of the cells. Targeting properties of Cetuximab-conjugated iodine doped CDs was investigated *in vitro* using HCC827 cells (over-expression of EGFR), and H23 and HLF cells (low expression of EGFR) using laser scanning confocal microscopy. Cetuximab-conjugated iodine doped CDs was exhibited high sensitivity and excellent spatial resolution for targeted imaging of lung cancer cells that it can be used to further improve this system for *in vivo* analysis. Success in the use of folic acid functionalized CDs for targeting cancer cells has also been demonstrated in several reports [28].

Hamd-Ghadareh et al. explored efficiency of CDs in imaging by evaluating of cell cytotoxicity of CA125 Ab conjugated CDs in human ovarian cancer cell line OVCAR-3 using MTT assay. A strong yellow, blue and green colored photoluminescence on the cell membrane and cytoplasm of OVCAR-3 cells exhibited that the CDs penetrated into the cells and maintained their fluorescent characteristics in the cellular environment, while no photoluminescence is observed at nucleus. These results proved that the rod-shaped OVCAR-3 cells can be successfully labeled by CDs [33].

Hamd-Ghadareh et al. reported that CDs had higher uptake in cancerous cells compared to the non-cancerous cells. Short incubation time (2–3 h) is required for the cellular uptake of CDs. The cellular cytotoxicity assessment of CDs using MTT assay of CD-1 toward OVCAR-3 cells and CD-2 toward MCF7 showed no side effects or cytotoxicity in living cells [34].

Anti-HE4 monoclonal antibodies (anti-HE4 mAb) coated onto Mn-CDs hybrid nanoprobe was introduced by Han et al. for optical and MR imaging and targeting of ovarian cancer

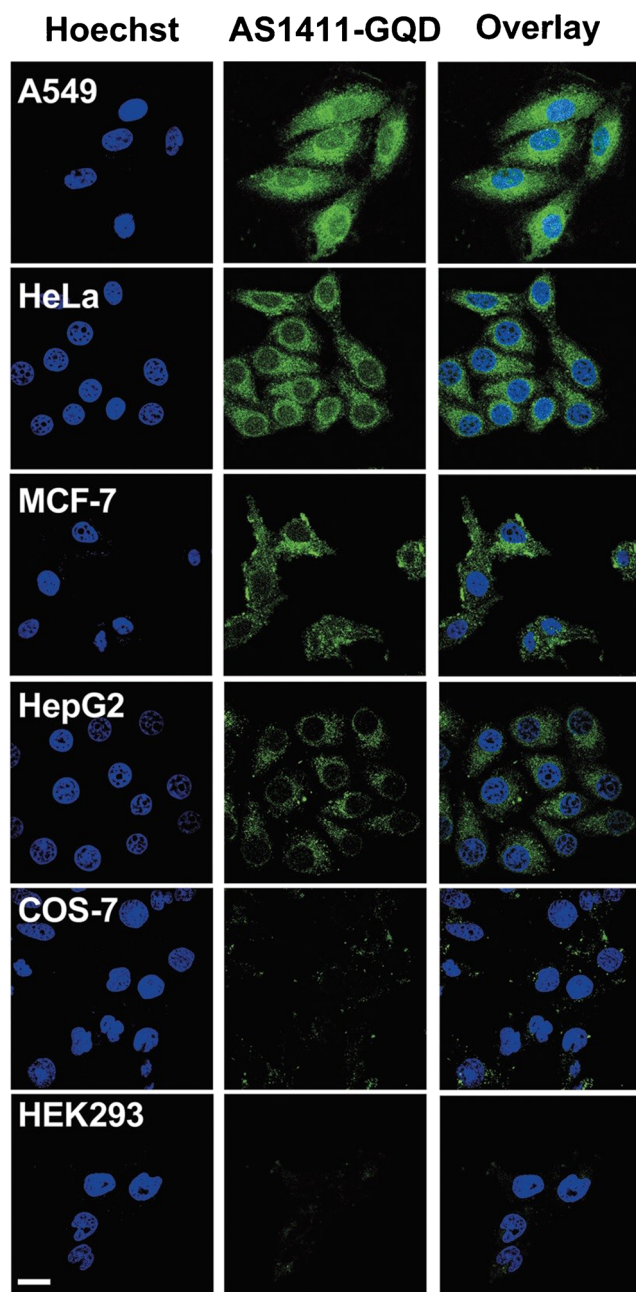


Fig. 3 Specific labelling of cancer cells with AS1411-GQD conjugates. Confocal laser scanning microscopy of four different cancer cell lines and two normal cell lines incubated with AS1411-GQDs (5 μ M) at 37 °C for 5 h. Scale bar is 20 nm. Left column: fluorescence image with Hoechst 33342 nucleus staining. $\lambda_{\text{ex}} = 405$ nm. Middle column: GQD fluorescence image. $\lambda_{\text{ex}} = 488$ nm. Right column: overlay of the corresponding images. Reproduced from [58] with permission from Royal society of chemistry

cell. In vitro and in vivo examinations indicated that the nanoprobe had high bio-compatibility, excellent biodistribution and tumor-targeting ability, superior MR and fluorescence imaging effects [35].

Han et al. synthesized PEI functionalized fluorescent CDs using glucose as carbon source in water-glycol medium.

Then, PEI-CDs can conjugate with CEA8 antibodies and use as a probe to label and image HeLa cells. Images of HeLa cells incubated alone (a), co-incubated with CDs (b) and co-incubated with CDs-CEA8 (c) are shown in Fig. 4. The image of control HeLa cell did not exhibit any autofluorescence with UV excitation (Fig. 4a). HeLa cells incubated with CDs without CEA8 antibody also did not exhibit any fluorescence (Fig. 4b). The images of HeLa cells labeled with antibody conjugated CDs (0.75 mg/mL, incubated for 1.5 h) was apparent in Fig. 4c [69].

Bioimaging applications of antibodies conjugated CDs are listed in Table 3.

Ligand-based targeting

A number of small molecules (such as hyaluronic acid, folic acid) have been used to functionalize the surface of nanoparticles. Such targeting moieties are attractive tools for targeting cancer cells due to their high selectivity to target receptors. In this section hyaluronic acid and folic acid-based targeting will be discussed in more details.

Hyaluronic acid (HA) based targeting

So far, three types of cell receptors have been determined for hyaluronic acid (HA) including CD44, receptor for HA-mediated motility (RHAMM) and intercellular adhesion molecule-1 (ICAM-1) [53].

Hyaluronic acid was used for functionalization of nitrogen-doped carbon quantum dots (HA-CQDs) that provide robust fluorescent dots for use in confocal microscopy and flow cytometry. The results proved that HA-CQDs can be used as a new biocompatible cell-specific targeting probe for imaging of CD44 receptor-over expressed in cancer cells [36].

Evaluation of Hyaluronic acid (HA) can be significant for constructing of fluorescent nanoprobe in vivo drug delivery and cancer diagnosis and therapy. Polyethylenimine (PEI)-modified CDs (PEI-CDs) and Hyaluronic acid (HA)-conjugated doxorubicin (Dox) was applied for targeting and penetrating in cancer cells due to high affinity HA to CD44 receptors overexpressed on many cancer cells. In vitro and in vivo experiments exhibited that cancer diagnosis and therapeutic efficiency of PEI-CDs and HA-Dox synergistically improved compared with free DOX anticancer drug [37].

Zhang et al. synthesized CDs using PEI as transfection motif and hyaluronate (HA) as targeting ligand. HA-PEI-CDs have been successfully applied for gene delivery, tumor targeting, and intracellular imaging. It was demonstrated that the labelled cells were brightly illuminated owing to strong fluorescence emitting from HA-PEI-CDs which distributed in cytosol [39].

Core-shell CD-MIPs templated with glucuronic acid (CD-MIPGlcA) were utilized for biotargeting and bioimaging

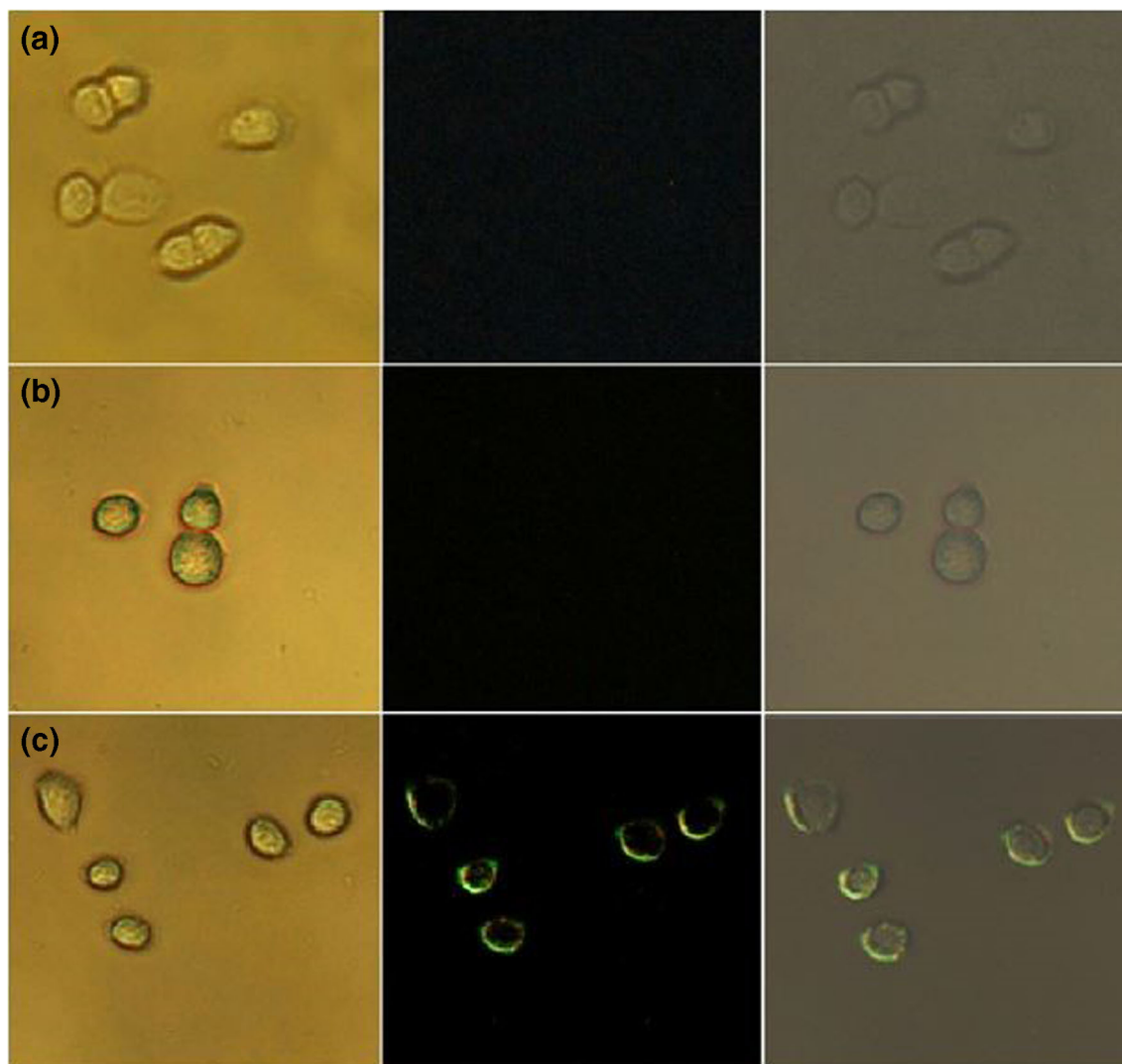


Fig. 4 Image of HeLa cells incubated alone (a), co-incubated with CDs (b) and co-incubated with CDs-CEA8 (c). In the three panels, the left row is imaged in bright field; the central row represents the fluorescence

images excited by UV; the right is the overlay of the image in the left and central row. Reproduced from [69] with permission from Elsevier

hyaluronan in human cervix adenocarcinoma cells (HeLa) and human keratinocytes (HaCaT). CD-MIPGlcA was capable of distinguishing between cells with various hyaluronan amounts, such as healthy and tumor cells [53].

The *in vivo* targeting ability of GQD and the efficiency of employing of HA as a target molecule to deliver GQD was also studied. MDCK as CD44 negative and A549 as CD44 overexpressed cell lines were selected for investigation of *in vitro* imaging and cellular uptake of GQDs-HA. The fluorescence behavior of GQD and GQD-HA in MDCK and A549 cells after incubation for 4 h is shown in Fig. 5. Insufficient fluorescence intensity has been observed from MDCK cells incubated with GQD and GQD-HA (Fig. 5a). The bright fluorescence signals were detected from A549 cells for GQD-HA compared to GQD (Fig. 5b). The loading and release kinetics of the hydrophobic drug doxorubicin from HA-GQD under mildly acidic conditions indicated that HA-

GQDs can be regarded as a novel drug carrier, while the MTT assay supports the nontoxic behavior of HA-GQDs that can be considered as a biocompatible material [59].

In order to investigate the bioimaging ability of the HA-CDs, two selected materials (HA-CP5 and HA-CP10) were first incubated with HeLa and HepG2 cells and then seed by confocal laser scanning microscopy (CLSM) analysis. As shown in Fig. 6 A, bright fluorescence signal observed in both cell lines due to the strong fluorescence emitted from HA-CDs. Under different excitation wavelengths, HA-CDs can stain cells with different colors (blue and green under λ_{ex} of 405 and 488 nm, respectively). It can be concluded that after microwave irradiation, some HA residues remained on the surface of the HA-CDs. Therefore, HA-CDs have targeting imaging capability towards cells overexpressing CD44 (such as HeLa and HepG2 cells). The targeting imaging ability of HA-CDs in HeLa and HepG2 cells was further evaluated using an HA competition assay. The

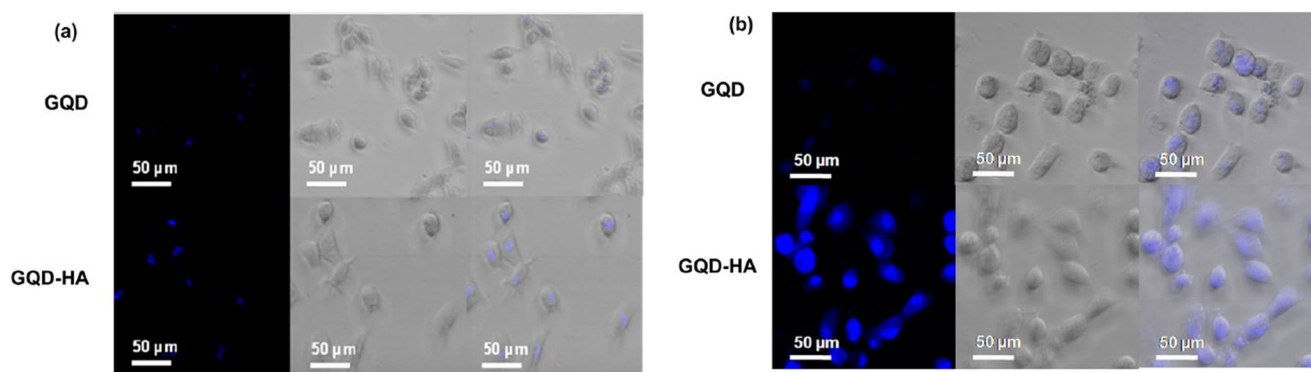


Fig. 5 **a** and **b** CLSM images of GQD and GQD-HA in (a) MDCK and (b) A549 cells after incubation for 4 h. The left column shows the bright field images, the middle column the gray images, and the right column the

merged images. The scale bar is 50 μm . Reproduced from [59] with permission from American Chemical Society

results shown in Fig. 6b revealed that the fluorescence intensity of the HA-CDs decreased in both preincubated cells with free HA. This reduction in fluorescence intensity may be due to the competitive binding of the free HA to the receptors on the cell membrane [71].

Bioimaging applications of hyaluronic acid conjugated CDs are listed in Table 4.

Folate (folic acid) based targeting

Developing a method that can distinguish between cancer cells and healthy cells is very important. Folate receptor (FR) can be used as a target for monitoring the overexpressed molecules in cancer cells [41]. Among targeting ligands, folic acid (FA), possesses high affinity (K_d : 0.1–1 nmol L^{-1}) to the folate receptor (FR) [45]. The targeting of cancer cells through detecting FR has attracted great attention.

Some researchers have described the synthesis of CDs using FA as carbon source for targeting and detecting cancer cells [49–51]. Bhunia and coworkers exhibited that competition experiments and biophysical measurements both confirmed the specific targeting and enhanced uptake of CDs by the folate receptor-expressing cells. The results validated that such cells were easily recognizable in fluorescence microscopy imaging [49].

Yang et al. reported CDs without further modification for the imaging of cells. Confocal images demonstrated that discrimination of FR-positive cancerous cells from normal cells can be due to specific interaction between FA of CDs and folate receptors in the HeLa cancerous cell membrane [50].

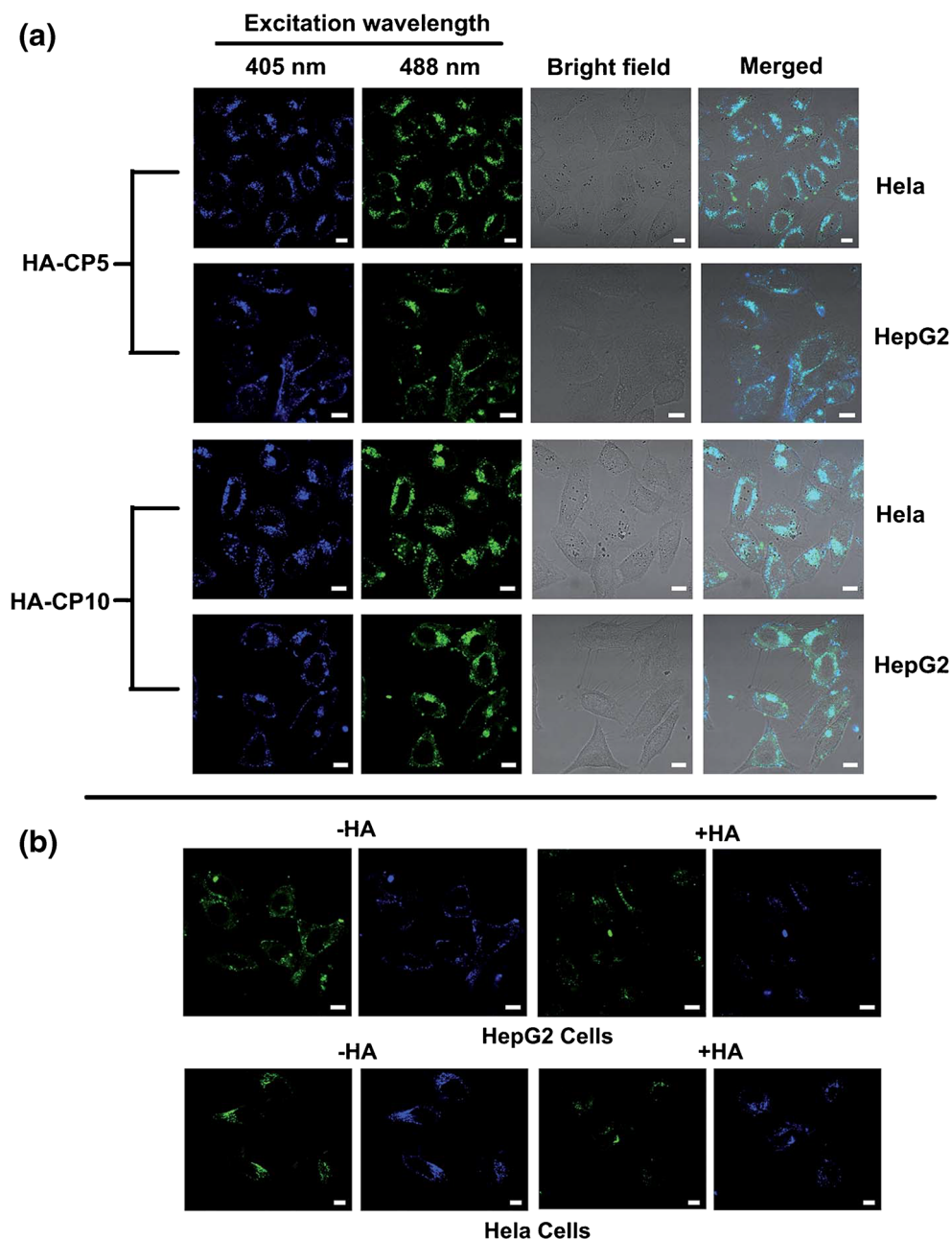
Ultrahigh fluorescent FA-derived CDs synthesized by importing FA as single precursor. The residuals of FA on CDs can specially target FR, and cancerous cells with FR expressing can be easily recognized (Fig. 7) [51].

Fluorescent CDs functionalized with folic acid has been widely applied for molecular and cellular imaging both

in vitro and in vivo, and such strategies have also been used as an important route to targeting cancer cells.

Liu and coworkers evaluated the feasibility of turn-on fluorescence imaging probe for recognition cancer cells with overexpression of folate receptor (FR). FA-CDs nanocomposite was synthesized as a turn-on fluorescence probe for targeting imaging of HeLa cells (human cancer cells with overexpression of FR), HepG2 cells (human cancer cells with a high-expression of FR), and HEK-293 cells (human normal cells with negative FR). The ability of FA-CDs nanocomposite for long-term fluorescence imaging was investigated. CDs exhibits excellent photostability after 120 min continuous irradiation with laser-scanning confocal microscopy. So, FA-CDs probe would have great potential significance for fluorescence assisted surgical resection and obtaining real-time information about tumor cells [40]. The study by Song et al. indicated that fluorescent carbon nanodots conjugated with FA is a good candidate for distinguishing folate-receptor-positive cancer cells from normal cells. Analyzing of model cell mixture of NIH-3 T3 and HeLa cells demonstrated that the method has great potential for cancer diagnosis studies [41]. Also, Aiyer and coworkers reported that FA functionalized green fluorescent CQDs (GCQDs) have high potential applications in cancer cell imaging. They evaluated FA as a targeting ligand in vitro cell targeting and bio-imaging performance of FA-GCQDs on MCF-7 breast cancer cells [44]. Zhao et al. synthesized green fluorescence CDs by the natural carbon source dandelion and the nitrogen source EDA. In this study, FA-CDs is able to make different FR-positive (FR+) HepG-2 cells from normal cells and clearly demonstrate the expression level of FR on the cancer cytomembrane [45]. Recent papers indicated that FA-CDs possess both bright green fluorescence and targeting capability and can distinguish FR-positive cells from the mixed cells. FA-CDs, as a nanoprobe, were introduced by Zhang and co-workers for recognizing of folate receptor-positive cancer cells. Experimental outcomes demonstrated that the resultant FA-CDs noninvasively entered into cancer

Fig. 6 **a** Representative fluorescence microscopic images of HeLa and HepG2 cells incubated with $10 \mu\text{g mL}^{-1}$ of HA-CDs. **b** HA competition assay of HA-CP10 ($10 \mu\text{g mL}^{-1}$) in HeLa cells and HepG2 cells (-HA: without HA. +HA: addition of 100-fold excess of HA to the cells for 2 h before the treatment with HA-CDs). Scale bar = $10 \mu\text{m}$. Reproduced from [71] with permission from Royal society of chemistry



cells via receptor-mediated endocytosis and can differentiate FR-positive HepG2 cells from a cell mixture by fluorescence imaging [47].

The functionalization of polymeric materials on fluorescent nanomaterials is a key strategy for facilitating adsorption or wrapping ligands on the surface fluorescent nanomaterials.

In order to investigate the transfer of CDs-PEI-FA to targeted cancer cells, Wang et al. selected KB and A-549 cell lines as the positive and negative control models, respectively. The fluorescence images of CD-PEI-FA incubated folate receptor- positive KB cells were bright, while the CDs incubated A-549 cells showed an inadequate

signal. The results exhibited that CD-PEI-FA has greater recognition and binding ability towards KB cells. Also, the microscopy images showed that the internalization of CD-PEI-FA into FR-positive cells was higher than FR-negative cells. The fluorescence signal in FR-positive cells was produced via receptor mediated endocytosis of CD-PEI-FA [29]. Lei et al. demonstrated that PEI-assisted strategy is a useful approach for non-covalent modification of FA on PEI-CDs. FA-PEI-CDs indicated turn-on fluorescence signal toward folate receptor (FR)-positive cancer cells. FA-PEI-CDs capability explored for recognizing FR-positive cancer cells and distinguishing FR-positive

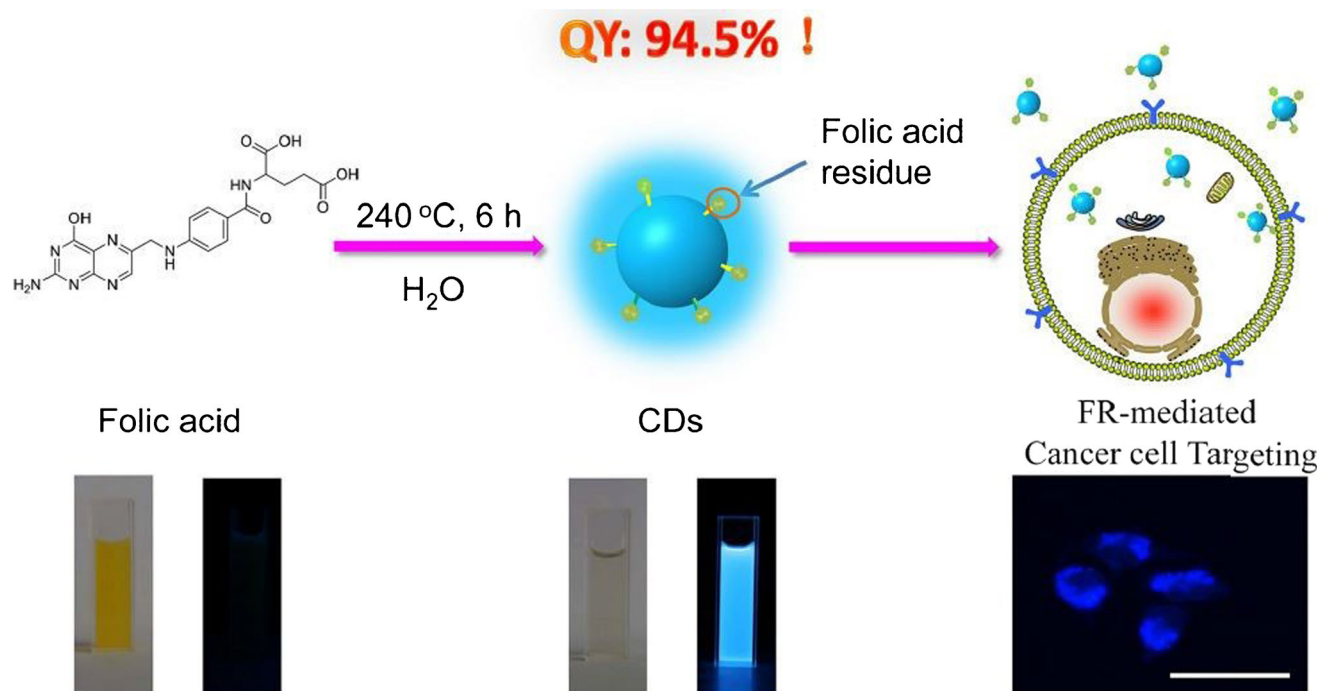


Fig. 7 Schematic illustration of the synthesis of folic acid (FA)-derived CDs and their application for folate receptor (FR)-mediated cancer cell targeting. Reproduced from [51] with permission from Nature

and FR-negative cancer cells that incubated 2 types of FR-positive cancer cells and 1 type FR-negative cancer cell with FA-PEI-CDs for 10 min at 37 °C [38]. Choi et al. represented a procedure for functionalization of PEG-passivated CD (CD-PEG) with FA to make available CD-PEG-FA for the targeting of FA-positive cancer cells. FA is a perfect ligand for folate receptors that are overexpressed in different human cancer cells due to its stability and high affinity to cancerous cells [48]. Also, photoluminescent CDs using FA and PE utilized for selective imaging of folate receptor (FR)-positive cancerous cells from normal cells. The cytotoxicity of PEI can be reduced by conjugation PEI on CDs [52].

Wang et al. used silica spheres as carriers for synthesizing CDs and then SiO₂@CDs composites modified with FA for targeted bioimaging of cancer cells. The cytotoxicity of SiO₂@CDs and SiO₂@CDs-FA composites was examined by MTT assay with 293 T cells which the results confirmed that these composites had high biocompatibility and had no adverse effect on 293 T cells [30].

S, N, and Gd tri-element doped magnetofluorescent carbon quantum dots (GdNS@CQDs) was successfully synthesized by Chiu et al. for simultaneous *in vitro* cell imaging, targeting, pH-sensitive controlled drug release in HeLa cells and also *in vivo* fluorescence imaging, by zebrafish as an animal model. Magnetofluorescent GdNS@CQDs indicated excellent fluorescent properties, and high stability at physiological conditions and ionic strength, and low toxicity [31].

Qian and coworkers synthesized AA-CDs using aconitic acid (AA) as a precursor. The conjugated interaction of FA and AA-CDs was utilized for a weak fluorescent nanoprobe (FA-AA-CDs) fabrication. The capability and potential FA-AA-CDs were tested for targeting imaging of HeLa, SMMC-7721, and A549 cells [46].

Huang et al. developed a multifunctional nanocarrier composed of three constituents: paramagnetic GQDs, FA and diethylene triamine pentaacetic acid (DTPA) utilized as fluorescent probes for intracellular imaging and core material for coating different functionalities, targeting ligand and chelating agent for binding polyvalent metal ions, respectively. The paramagnetic GQDs, FA-GdGQDs, were successfully synthesized by covalently conjugating diethylenetriaminepentaacetic acid gadolinium and FA onto the GQDs surface. The capability of the FA-GdGQDs with strong luminescence emissions was investigated for targeted imaging of HeLa cells. *In vitro* and *in vivo* evaluation of FA-GdGQDs toxicity showed negligible cytotoxicity and high biocompatibility [56].

Water-soluble and low cytotoxic graphene oxide quantum dots (GoQDs; oxidizedGQDs) were synthesized by Goreham et al. using acid exfoliation of graphite nanoparticles. Subsequently GoQDs modified with folic acid using carbodiimide crosslinking chemistry (covalent interaction). Fluorescence lifetime imaging (FLIM) in combination with multiphoton microscopy (MPM) was used (in combination) to image HeCaT cells exposed to GoQDs, resulting in a superior method for bioimaging [17].

Bioimaging applications of folic acid conjugated CDs are listed in Table 5.

The reported studies demonstrated that CDs have potential applications in optical imaging. The most CDs emitted ultraviolet or short-wavelength visible lights as exhibited in Tables 1, 2, 3, 4 and 5. UV lights have weak penetration ability in tissue. On the other hand, almost all tissues will generate autofluorescence under ultraviolet or visible light excitation. The existing CDs are commonly more fluorescent in short wavelength regions (blue-green), which are less favorable for their applications as bioimaging agents. Therefore, development of high luminescent CDs in the deep red or near-infrared region will be necessary to accelerate the application of CDs in clinical imaging [26, 72].

Since synthesis of multifunctional CDs including, improving quantum yield, targeting, and diagnostic agents is a burdensome process. Thus, preparations of highly bright CDs that combined with targeting function in single step process or had intrinsic targeting capability toward cancer cells are highly desirable.

Conclusion and future perspectives

The remarkable properties of CDs and GQDs, such as unique photoluminescence, photostability, excellent biocompatibility, water dispersibility, good intracellular solubility, and high cell permeability have opened new horizon for their advanced biomedical applications. In this review, we focused on many reports of different modification methods aimed at manipulating the structural characterizations and improving optical properties of CDs. Modification methods tailored new functional groups or target molecules on the surface of CDs and improved the optical efficiency of CDs or provided various options to fulfill the requirements of specific bioimaging or biomedical applications. Various ligands (peptides, antibodies, aptamers or small molecules) and targeting strategies have been used to improve the tumor targeting, penetration of nanomaterials, and direct them to tumors. The effective targeting of cancer cells depends on the number of cell surface receptors and their availability of targeting molecules that specifically bind to cancerous cells. The choice of the ligand type is usually based on numerous considerations including availability and ease of production, variety, affinity, applied conjugation approaches, immunogenicity and cost. Each ligand has its own advantages and disadvantages even though a number of them have found their way for clinical application. As compared to antibody, peptides sometimes exhibit lower binding affinity to receptors that is its drawback in the clinical application. The concern about immunogenicity is one of the

disadvantages of antibody as targeting agent. Several potential advantages of aptamers over antibodies include low cost synthesis methodology, more stable to biological degradation and physical stresses such as heat, pH and organic solvents, lack of immunogenicity and smaller size that provide better penetration in solid tumors. The number of studies that used the folic acid ligand for conjugation CDs is more than others, which can be attributed to FA characteristics including small molecular size, good stability, non-immunogenic, cost effective, high availability and high affinity for FR on tumor cell with rapid internalization into tumor cells and minimizing non-specific targeting and toxicity make it ideal candidate for cancer targeted imaging.

However, there still exists a major challenging subject for the future scope of research in further development and applications of CDs as a bioimaging agent. PL colors of most of the reported CDs to date are blue to green. For the further development of CDs, the study in expanding the spectral coverage of CDs to all visible wavelengths and especially red/near- IR spectral regions will play an important role in the future investigations. Near IR emission photons are more effective in tissue penetration and decreasing the interference from background fluorescence. The major challenge for most of bioimaging fields is to enhance the quantum yields of CDs in the deep-red or near infrared region, where new synthetic procedures are still under extensive development. Also, CDs synthesized using variety methods exhibit large size distribution, excitation-dependent photoluminescence (PL) behavior, and the complex and time-consuming separation and purification severely limit their further applications. Therefore, the large scale synthesis of CDs with controlling particle size and fast purification remains a challenge.

The fascinating features and perspectives of CDs will continue to attract significant research attentions from various fields. Challenges along with CDs require further research to improve their biomedical applications as long as we would like to make full use of them. The developments of robust large scale synthesis of red fluorescence CDs with tuning of wavelength emission, active tumor targeting capacity, uniform sizes and high quantum yield for better potential applications are the future scopes of research.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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