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A Review of Promising Selected Agents Combined with Carbon Dots for Biomedical Applications

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Abstract

Carbon dots (CDs) are a new type of QD that has attracted a great deal of attention in recent years because of their multiple fascinating characteristics and advantages resulting from physical and optical properties, fluorescence, water solubility, low toxicity, facile synthesis, favorable quantum yield, long thermal storage resistance and photostability. CDs offer promising applications in therapeutics, diagnostics, optoelectronic devices etc. In this review conjugation and composite formation at nanoscale level between CDs and other materials for altering their properties for the desired therapeutic uses are discussed. CD in order to be suitable for its application as a therapeutic agent it needs desired particle size, size distribution – as they affect the in-vivo distribution, stability, drug loading and drug release ability, uptake by cells, biological fate, toxicity, and targeting ability to a range of cellular and intracellular targets, easy mobility and capacity to cross the blood-brain barrier etc. Conjugating agents of biogenic origin, organic material, synthetic drugs and antibiotics as well as inorganic heteroatoms are discussed.

Keywords: *Conjugated CDs, Drug, Antibiotics, Nanomedicine, Biogenic conjugate.*

1. INRODUCTION

Many allotropes of carbon having graphitic structure (honey-comb like arrangement of carbon atoms) at nanoscale have been fabricated, investigated for example zero-D carbon dots, fullerene and diamond nanocrystals; one-D CNT; two-D graphene, graphene oxide and graphene QD (GQDs). All of them have shown their potential for various applications in theranostic, electronic devices to biosensors and bioimaging agents. This review deals with zero-dimension CDs only. Although CDs and GQDs exhibit similar quantum-confined fluorescent carbon materials; are mainly composed of sp^2 carbon, oxygen and nitrogen elements and other doped heteroatoms [1, 2]; the different spatial arrangements of carbon atoms exhibit distinctive physical and chemical properties [3]. Moreover, GQD has perfect crystal structure, which CDs do not [4]; CDs exhibit luminance at size <10 nm, whereas GQDs of size up to 100 nm [5, 6] are luminescent. Generally, CDs display strong optical absorption in the UV region, with a tail extending out into the visible range i.e., 280–360 nm [7]. Other properties that have made CDs more applicable for biomedical applications are that the absorption band could be regulated via surface passivation or modification. CDs show excitation-dependent emission

properties in wavelength and intensity, their low cost, high quantum yield, abundant source of precursor, low cytotoxicity, superior chemical and photo stability has offered extensive applicability [8]. Considering all these, we are concentrating this review on CDs only.

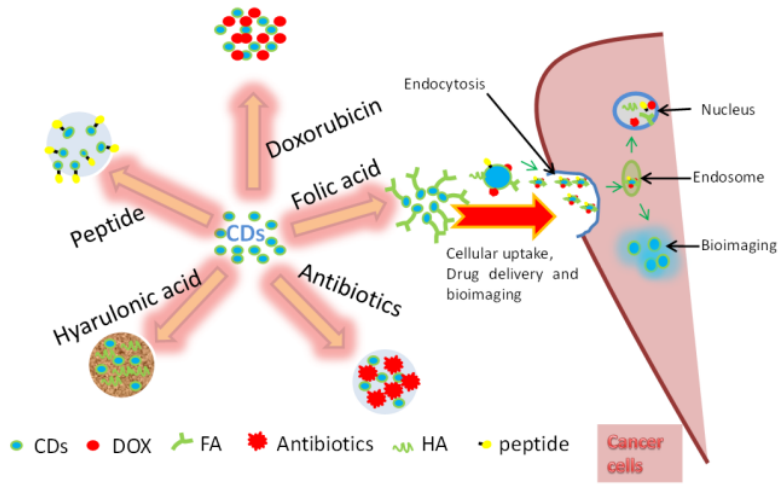
The dimensions of fluorescent nano-sized CD particles range from 10 to 20 nm [9, 10]. CDs are currently substituted for traditional heavy metals-based quantum dots [11]. In addition, they have recently attracted broad research interest due to their desirable optical properties with favorable quantum yield (QY), easy and facile synthesis methods from renewable raw materials, high thermal and photon resistance, tuneable nature of their excitation and emission, effortless surface functionalization, and nontoxic behaviour with excellent biocompatibility [12, 13].

The role of conjugation substrate with CDs is crucial to enhance the biological performance which those modified CDs possess. The conjugated or functionalized CDs have multiple uses, including self-congregation [14], biosensing [15], bioimaging [16], and therapeutic efficacy [17, 18] The first prepared CDs with fair photoluminescence were accidently obtained from the purification of single-walled carbon nanotubes (SWNTs) by Xu *et al*. (2004) [19]. After that, Sun et al. in 2006 standardized the robust photoluminescence of these CDs via laser ablation from an assembly of combination of graphite powder and cement [20]. Since then, the CDs have been appealing functional materials. The preparation of CDs has been progressive for almost 20 years. Nowadays, CDs are mostly prepared from natural resources. Depending on used precursors, synthetic methods of CDs are generally categorized into top-down and bottom-up approaches. Details are further explained in preparation and properties section [21]. Thus, a lot of researches have focused on their synthesis process and specific precursors. The fluorescence of CDs generally originates from complex hybridized carbon atoms of sp^3 and sp^2 (π - π^* transition of C=C), varied particle sizes, surface imperfections, and different functional groups on their edges. The formation of fluorescence mechanism of CDs is formed when they excited at higher wavelengths, related to the existence of a variety of heteroatoms in physicochemical structure of CDs [22]. Photoluminescent CDs have attracted attention due to various scientifically useful applications, for example, fluorescence bioimaging [23], nanocarrier agents [24] [21]for drug delivery system via conjugation and controlling release purposes, clinical theranostics [25], ion detection [26], biosensors [27], optical/electrochemical sensors [28], light-emitting diodes [29], energy conversion and storage [30], electro and photocatalysis [31-34].

Therefore, in this article, we systematically summarized the potential of particular combining agents-assisted CDs, their optimal performance of CDs alone, and conjugated or incorporated CDs efficacy for biomedical applications. Thus, it is hoped that this review article opens a new route to explore effective multiple agents-coupled CDs.

2. PREPARATION OF CARBON DOTS

Since CDs have been discovered, numerous synthetic methods for preparation of CDs to enhance the quality have emerged. Two common synthetic approaches have been tried to synthesize CDs i.e., top-down and bottom-up approaches (**Figure 1**). Top-down method is used to prepare CDs from bulk carbon materials like carbon nanotubes, carbon soots, graphite, and nanodiamonds. Top-down approaches are based on arc-discharge, laser ablation of graphite, Pulsed Laser Irradiation of carbon source and electrochemical exfoliation [35, 36]. These methods are tedious and complex to obtain desired amount of CDs due to tough processing time and synthetic conditions, and rare carbon sources.



Scheme 1. *The diagram of selected agents coupled with CDs for enhanced cellular uptake and fast drug delivery efficacy for cancer cells diagnosis.*

Thus, the top-down method has received relatively less attention for the preparation of CDs. On the other hand, bottom-up approaches, such as hydrothermal treatment, thermal decomposition (Combustion and Thermal Oxidation Method), and microwave irradiation are easy to use for facile and eco-friendly preparation following one-pot procedure and readily available precursors [36].

In top-down process the macromolecule is cracked down to become nano-sized CDs, using physical or chemical transforming methods; whereas the bottom-up approach follows polymerization and carbonization of a chain of tiny molecules to produce CDs through chemical reaction [37, 38]. CDs are successfully prepared using bottom-up approaches from low-cost materials to high-valued functional products. Many precursors come from organic biogenic compounds rich in hydrocarbons [39], carbohydrates [40], polysaccharides [41], glucose [42], chitosan [43], cellulose [44], lignin [45], citric acids [46], and other carbon sources of organic product, including mango peel [47], orange juice [48], fruit extract [49], organic waste [50], banana peels [51], pigments from peels of *Trapa bispinosa* [52], Camphor [53], Neem-gum [54]and so on. Moreover, solvothermal method, plasma treatment, opening of fullerene cage, ultrasonication, chemical and supported synthetic procedure are also being used to produce CDs [55].

3. PROPERTIES OF CARBON DOTS

The major unique properties of CDs are fluorescence, size-dependent photoluminescence, narrow tuneable emission, upconversion, large absorption coefficient and size-dependent absorption, broad excitation spectra, multiphoton excitation, good conductivity, ease of functionalization, good biocompatibility, good solubility in aqueous and polar solvents, chemical inertness, photo-chemical stability against photobleaching and photo-blinking, biocompatibility and low toxicity; has garnered tremendous attention. These properties are well established and published in hundreds of articles. In the following paragraphs we touch upon the optical properties that are useful in application of CDs for biolabeling, bioimaging, as vehicle for drug, antibiotic as well as navigational molecules delivery.

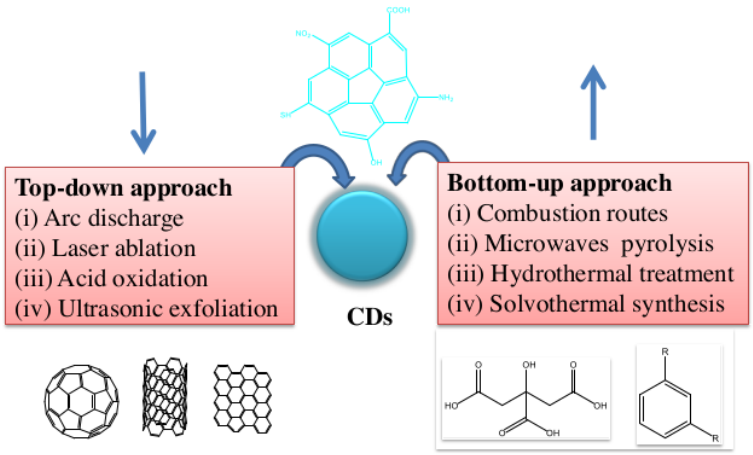


Figure 1. *The schematic diagram of two mainly synthetic approaches.*

3.1. Absorbance

Optical properties are the interaction of electromagnetic radiation (i.e., γ-rays, X-rays, UV rays, visible light, infrared rays and radio waves) with matter, which may result in reflection, refraction (transmitted) or absorption. Usually CDs synthesized via top-down method show size-dependent absorption properties [56, 57]. Generally, CDs shows peaks in the UV-visible region, as estimated, π-π* up-conversion of sp^2 coupled carbon and n-π* shifting, which is hybridized with heteroatoms such as N, S, P.[58, 59] The absorbance nature could be engineered by passivating and modifying the carbon dots sites with some molecules or organic acids [60]. Jiang et al. explored multi-color emissions which released red, green, and blue luminescent CDs that were synthesized from three isomers of phenylenediamines through hydrothermal treatment. The synthesized CDs demonstrated the same UV-visible absorption spectra pattern. In particular, those three CDs even showed red-shift emission with tuneable excitation, suggesting that the smaller electronic bandgaps from three CDs were due to the smaller than their analogous precursors [61]. CDs after functionalization with amino acids, showed a red shift in absorption spectra [52]. CDs prepared using the same precursor (Glucose) but different methodologies (microwave-assisted, ultrasonic and hydrothermal or thermal procedures) show peaks around 250–300 nm in absorption spectra [62-64].

3.2. Photoluminescence (PL)

Fluorescence and PL are luminescence where the energy is supplied by electromagnetic radiation. Term PL is used to mean "luminescence from any electromagnetic radiation," while fluorescence is often used only for luminescence caused by ultraviolet light, although it may also be used for other photoluminescence. PL property of CDs has resulted in its acceptance in many biomedical applications. Moreover, PL property of CDs along with their flexibility in modification, high solubility in water, nontoxicity, good photo stability, and excellent biocompatibility are also applied in bio sensing as biosensor carriers for their flexibility in modification, high solubility in water, low or nontoxicity, good photo stability, and excellent biocompatibility. The biosensors based on CDs could be used for visual monitoring of cellular copper, glucose, pH, and nucleic acid. A general example is about nucleic acid lateral flow assays. The discriminating tags on the amplicons are recognized by

their respective antibodies and fluorescence signals provided by the attached CDs [65].

CDs show size-dependent PL and upconversion luminescence properties due to the multiphoton processes that lead to anti-Stokes type emission. However, the optical behaviour of CDs may not only be due to particles of different sizes, but also distribution of different emissive sites on each CDs. Mechanistically, PL is attributed to the presence of surface energy traps, which become emissive upon surface passivation [66].

There must be a quantum confinement effect of emissive energy traps on the surface in order for CDs to exhibit strong PL upon surface passivation. The surface states of CDs which are formed from the interactive hybridization of carbon networks, and functional groups have preliminarily provided promising fluorescence [67]. The surface state of CD describes the existence of multiple electronic states on the site of CDs' surface; it is due to various moieties on that surface. Since functionalized CDs generate multiple surface states (electronic effects), the excitation primarily moves to the energy absorption band of a specific surface state after entering a series of non-irradiative energy emission processes such as vibrational relaxation. After that, the excited electrons go towards reinstating the former state to relink with the holes while releasing the absorption energy light [68].

Thus, the surface of CDs consisting of surface functional groups, defects, heteroatom doping and particle size, can influence the photoluminescence of CDs [69]. On the other hand, adding some molecules to CDs can reduce photoluminescence. One of the extensively studied anti-cancer drug molecules, which Doxorubicin (DOX) has been studied by many researchers and yielded some interesting and thought-provoking results related to the PL behaviour of CDs.

Yang and co-workers prepared and studied the PL spectra of DOX-CDs conjugates and as it can be seen in **Figure 2a**, they observed a new fluorescence shoulder peak located at 605 nm for DOX-CDs conjugates, while no peak was seed in the spectrum of bare CDs [70].

In a similar study using CD and DOX conjugate Kong *et al*. in 2018 compared the photoluminescence properties of CDs and CDs-DOX. The CDs displayed a strong absorption at 360 nm, while a sharp decreased peak of the CDs-DOX complexes was seen, suggesting that the DOX molecules certainly quenched the CDs fluorescence to a certain extent [71]. Such different observations do arise the question that what were the other parameters that varies giving different results. Such studies need thorough considerations of all the parameters involved in synthesis, conjugation, as well as specific properties of the product. Keeping that in mind Yu *et al*. (2020) prepared DOX-loaded CD along with polyethyleneamine (PEI) as passivating agents, so as to enhance the photoluminescence of DOX loaded CDs.

The PL spectra of CD before and after binding to PEI were analyzed, and change in PL intensity was observed. They suggested that it is likely due to a low absorption of CD-PEI, which displayed strong PL intensity at 490 nm emission, (**Figure 2b**). Whereas, the CD-PEI-DOX complex exhibited two emission peaks with a blue shift, indicating successful loading of DOX on CD-PEI [72].

A possibility of impact of synthesis method has also been envisaged therefore, considering that Zhang and co-workers prepared hyaluronic acid-functionalized CD (or as they mentioned CQD) CQDs (HA-CQDs) through hydrothermal carbonization for tumor-targeted bioimaging. The highest fluorescence exhibited emission peaks at 470 nm when excited from 360 nm, which are likely due to their distinctive surface defect states of functional HA ligands as shown in **Figure 2c**. Thus, the prepared nanomaterials enhance fluorescence intensity for the

labelled cells which were detected by confocal microscope and flow cytometry [73].

Expecting that navigating molecule like folic acid (FA) for cancer cell; Zhang et al. 2018 synthesized folic acid-conjugated green luminescent carbon dots, as a nanoprobe to point out folate receptor-positive cancer cells, and the fluorescent intensity peaks of carbon dots are shown in **Figure 2d**. Here, folic acid did not adversely affect the PL of FA conjugated CDs. After FA-modification, only a little change occurred between fluorescence spectra of the FA-CDs and the CDs. The FA-CDs exhibited nearly unchanged fluorescence intensity in the range of pH 3 to 11. Also treating with NaCl salt concentration was not impact significantly on varied treated CDs solutions. Good photostability was also appeared up to 6 hr under UV lamp and still increased up to 24 hr at ambient temperature under daylight, confirming excellent photostability [74].

From some published articles on loading of DOX on CDs e.g., HA-CDs and other agents-loaded carbon dots for biomedical applications, it can be clearly seen that DOX or HA or other agents can slightly quench the PL of CDs, however; not completely quenched while exposing to the labelled cells by confocal analysis. Contrarily, FA exhibited no significant impact on PL intensity of CDs to a greater degree when FA and CDs are covalently conjugated.

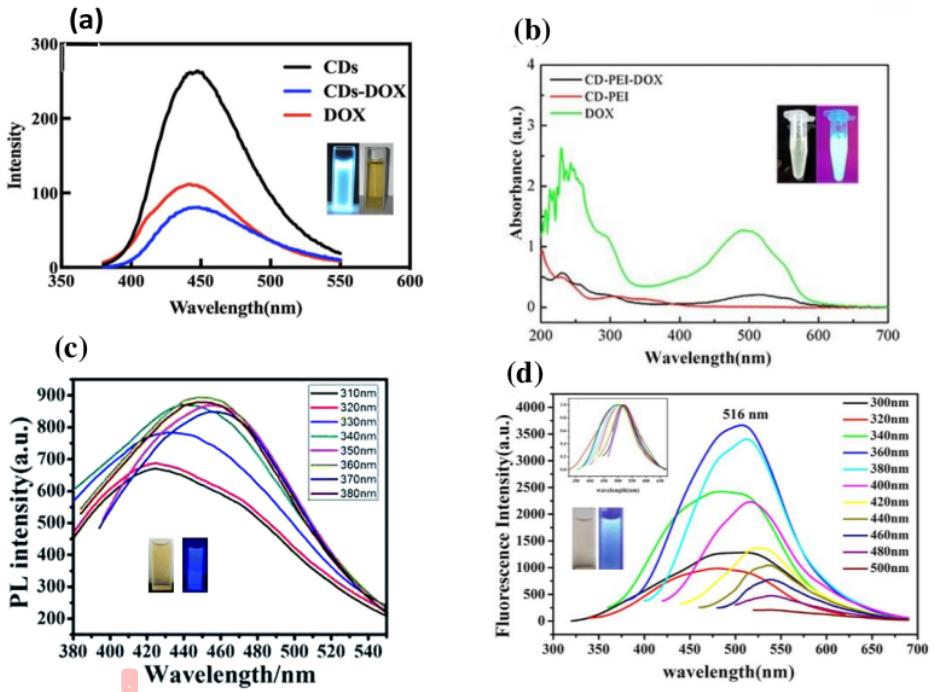


Figure 2. *(a) Photoluminescence spectra of CDs, loading of doxorubicin (DOX) and CDs-DOX (Insets: images of CDs solution in UV light and daylight conditions, respectively). (b) Fluorescence spectra of CD-PEI (Inset; photographs of the CD-PEI solution in nature light and UV light from 365-nm excitation). (c) PL spectra of HA-CQDs at different excitation states in the range of 310 - 390 nm. (Insets: Images of HA-CQDs solution at nature light and UV light). (d) PL peak intensities of CDs when excited from 300 to 500 nm. (Inset: images of CDs solution under daylight, 365 nm UV light, and the normalized PL spectra of that CDs. Reprinted with the permission of respective references [70-74].*

Nevertheless, if versatile conjugating agents and CDs would be combined, it will provide its dual use i.e. bioimaging and therapeutic efficacy against various cancerous cells.

4. CONJUGATION AND INTERACTION BETWEEN CARBON DOTS AND CONJUGATED AGENTS

First let's look at why conjugation of CD is needed? A QD or nanoparticle on its own is rarely used. To be suitable as a theranostic agent it is conjugated with different moieties in such a way that it retains its desired size and size distribution and properties in such a way that they affect the *in-vivo* distribution, remain stable, drug loading and drug release ability, uptake by cells, biological fate, toxicity, and targeting ability of these delivery systems, availability to a range of cellular and intracellular targets, easy mobility and capacity to cross the blood-brain barrier following the opening of endothelium tight junctions by hyper-osmotic mannitol, to treat neurogenerative diseases and brain tumors [75]. Smaller particles have larger surface area-to-volume ratio, hence can attach more drugs and, being near to the particle surface, release drug faster [76]. Thus, tuning of CD particle size provides a means of controlling drug release. Moreover, the surface charge property of CD (which is characterized by the zeta potential) is another important criterion that indicates positive charge on the surface of CDs.

Since CDs have above ±30 mV zeta potential they are stable in suspension, because the surface charge prevents its aggregation. Hence Chowdhary *et al*. (2012) [77], with the help of zeta potential values could determine whether CD is encapsulated within the center of the conjugate or conjugate is attached to the surface of CDs. CDs are usually synthesized to provide the fluorescence imaging which is required for disease detection and treatment in clinical applications. Interestingly, CDs have the promise for tunable full-color emission due to the benefits of their versatile starting materials, chemical conjugation, surface modification and heteroatomic doping via respective synthetic methods [78].

4.1. Types of Carbon Dots Conjugates and Their Applications

The common conjugates that are attached to CDs includes Biogenic compounds (such as proteins/peptides, DNA, RNase, lipids, Fe-amino-clay, dihydroxylipoic acid, folic acid protoporphyrin); Iinorganic nanomaterials (gold, silica, ZnO, Mn^{2+}, ZnS, Fe_2O_3 and Europium); Organic materials (covalently conjugated dyes and polymers) anticancer drugs; anti-neurodegenerative drugs; antibiotics and MWCNT for the purpose of delivering drugs to the desired site and/or tracking them. Moreover, for all these attachments, linkers or drug carrier linkages may be covalent or noncovalent and conjugates based on thiols, hydrophobic interactions, electrostatic interactions, and avidin-biotin complexes have been investigated. Conjugating molecules like folic acid, doxorubicin, antibiotics, hyaluronic acid, peptides, and some molecules are used as versatile loaded agents on CDs in accordance with the principle of covalent bonding [79], electrostatic interaction [80], hydrogen bonds or van der Waals forces [81, 82].

4.1.1. Carbon Dots Conjugated with Biogenic Materials

Biogenic compounds are products of metabolic activity of living organisms that may be organic with at least one C-H bond, (carbohydrates, lipids, peptides/proteins) or inorganic with no C-H bond (skeletons, shells, silica compounds, etc.). Peptides and proteins are attached to CDs by (i) using EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, condensation to react carboxyl groups on the CD surface to amines;(ii) Directly binding protein or peptide to the CDs surface using thiolated peptides or poly-

histidine residues; or (iii) By adsorption or noncovalent self-assembly of engineered proteins.

4.1.1.1. Peptide

Peptide is used for attaching intracellular antibiotics Streptavidin and delivering it to mammalian cells. A successful attachment of Streptavidin was achieved by linking the C-terminus of the peptide (biotin-C6-(L-Arg)9) peptides to CDs and conjugates by Lagerholm [83] and delivering Streptavidin in many mammalian cells such as embryonic mouse fibroblasts (Swiss 3T3), human endothelial cells (HeLa), and human osteoblast-like cells (MG63) where The CDs accumulated in intracellular vesicles (characteristic of endosomes and lysosomes). For use of CD as imaging agent there is a need to enter cell and the nucleus. Accumulation of CDs in the cell as well as the nucleus was demonstrated by [84, 85] by conjugating CDs with membrane translocation peptides TAT (a human immunodeficiency virus-derived protein). TAT not only facilitated the translocation of the tissue by overcoming the cellular membrane barrier but also enhanced the intracellular labelling efficiency.

It has been established that carbon-based dots provide the compatible efficacy both *in vivo* and *in vitro* systems [86]. However, there are still some limitations in their investigation, including short excitation/emission that was not enough to penetrate into deep tissue cells, low photoluminescence, sometimes no signal for antibacterial assessment, and low-irradiated multiple colors. As a consequence, their surface site's modification could be attempted by conjugating with peptides to strive for the antimicrobial efficiency [87]. Mazumdar *et al*. in 2020 explored the potential HSER–CQDs from the preparation of the (*Homo sapiens retinoic acid receptor*) peptide of retinoic acid receptor responder protein 2 of *Homo sapiens*, and subsequently coupled with prepared single CDs to exert the antibacterial response. Noticeably, the peptide and CDs do not exhibit highly effective power to inhibit bacterial cells. The antibacterial activity of HSER-CDs was highly found to disrupt cell membrane via genomic DNA (gDNA). According to cytotoxicity investigation against normal human epithelial cells, the HSER-CDs conjugate showed cell viability over 80%, approving no toxicity at 62.5 μg mL^{-1} of HSER-CDs.

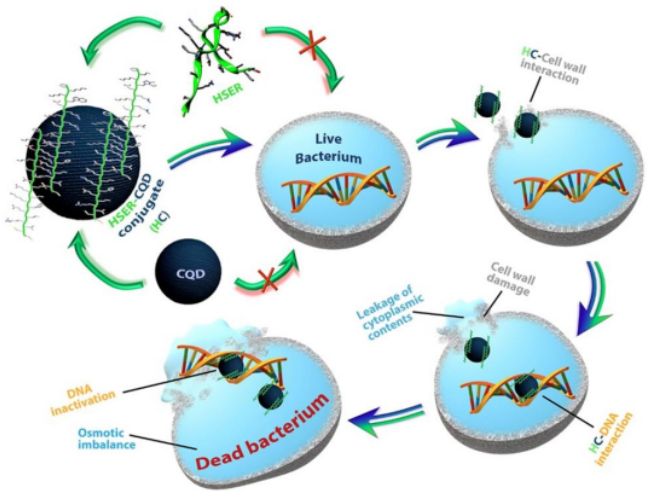


Figure 3. *The schematic diagram of peptide-conjugated Homo sapiens retinoic acid receptor (HSER) for promising antibacterial agent [88]. Reprinted with the permission of cited reference [88].*

They concluded the combined nano-materials might be utilized as a promising antibacterial candidate because of good stability, safety and potent antibacterial response [88]. The schematic diagram is shown in **Figure 3**. Moreover, another work reported by Xia *et al*. was the synthesis of carbon dots from sucrose, ranging 6 nm in narrow size distribution through hydrothermal pyrolysis. Then, the synthesized CDs in the presence of several surface sites were conjugated with amyloid beta 42 peptides. The combined CDs with Aβ42 peptides inhibited the self-fibrillation of that peptides, confirming the homogeneity of CDs and Aβ42 peptides. Thereafter, the Aβ42 peptides were further covalently coupled with the CDs surface via EDC/NHS coupling system so that the attachment of CDs and amyloid fibrils could be improved. They found that the modified CDs are promising fluorescent agents for amyloid imaging [89].

Gao *et al*. 2020 developed potential antimicrobial peptide-conjugated biochar nanocomposites to fight infectious diseases of broad-spectrum superbugs. They designed biochar combined with α-defensin human neutrophil peptide-1 (HNP-1), human β-defensin-1 (hBD-1), and human cathelicidin LL-37 antimicrobial peptide. This work highlighted that the effective human host defense antimicrobials could be designed from a naturally abundant resource of biochar with some modifications. The result showed that the as-prepared antimicrobial agents can be applied for the removal of various model bacterial cells toward Gram-negative and Gram-positive bacteria [90].

Moreover, the sweet lemon peel-derived CDs are preliminarily conjugated with polyamidoamine (PAMAM) dendrimers to become CDs-PAMAM. Interestingly, CDs-PAMAM further conjugated it with RGDS peptide to find the site of integrin, overexpressed in triple negative breast cancer (TNBC) without low toxicity. CDs-PAMAM was conferred that a potential gene carrier tool for TNBC gene therapy according to their bioassay results [91].

The peptide-conjugated CDs provide increasing target specificity, reducing drug side effects and improving the drug bio-availability and the resistance. According to some of those collected published research mentioned above, the peptide-loaded CDs could potentially be done by adding some therapeutic or anticancer agents together to destroy DNA and deactivate nucleus. Thus, the anticancer activity for therapeutics will be improved on the experimented cells.

4.1.1.2. Protein

Zheng *et al*. [92] has done real-time molecular tracking in live cells by fluo-rescent tags that are bright, photostable, biocompatible, and of molecular size so that it can minimize physical hindrance using oxygenated graphene QD (GQD) that is very similar to CDs; conjugated with Protein (insulin) without impairing the functionalities of the protein or largely altering the size, weight, and charge state. Many other proteins i.e. neuropeptide Y, bovine serum albumin, immunoglobulin G, concanavalin A and nerve growth factors have also revealed the real-time dynamics, i.e., distribution, internalization, and recycling.

Duan *et al*. in 2022 [93] proposed bovine serum (BSA) derived CDs combined with DOX to point out the inhibition of cancer growth and distribution. One of the most popular proteins, BSA is used as a drug delivery system for providing cancer therapy. BSA@CDs-DOX showed prolonged high fluorescence according to confocal microscopic analysis. Concerning the toxicity of BSA@CDs-DOX against cancer cells, there was over 80% of cell viability conferring as a good fluorescent drug-loading system for chemotherapy of tumors [93]. Moreover, Sahua and Khan 2020 prepared BSA capped boron-doped CDs to detect lead divalent ion. Lead divalent ion was significantly quenched under the range of detection limit of 1 ppb

to 10 ppb. This research was supportive for the generation tool for bionsensors produced from CDs [94].

4.1.1.3. DNA

DNA is another biogenic moiety used for conjugation with CD, because there are many applications for DNA-conjugated CD such as for drug delivery, for detection of antigen, DNA and micro-RNA, for sensing and bioimaging. The advantage of using CDs-DNA conjugate is that it functions as a fluorescent vehicle to deliver drugs.

Milosavljevic *et al*. [95] checked the interaction between CDs when conjugated with single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) and their impact on the fluorescent behaviors of CDs. They used CDs synthesized from the citric acid coated with PEG. No influence of the interaction of ssDNA with CDs was found on the fluorescence intensity of CDs; whereas with dsDNA, significantly increased the fluorescence intensity of CDs. This result will be beneficial for utilized in biosensing. Hence, Xu's group (2012) [96] designed an assembly of CDs and aptamer-based fluorescent detection of thrombin. *Aptamers* are single-stranded DNA or RNA (ssDNA or ssRNA) molecules and are extremely versatile and bind targets with high selectivity and specificity. They attached two thrombin aptamers on the surfaces of CDs and silica nanoparticles (CDs got attached around the surface of silica nanoparticles due to the specific aptamer–thrombin interaction) to produce two separate assembling units, which detect presence of thrombin.

CDs conjugated with DNA have been used for creating a hybrid hydrogel for sustained release of drugs by Singh *et al*. (2017) [97]. Another application of DNA-conjugated CD is perceived for gene therapy. Wang *et al*. [98] postulated that positive charges are the most suitable surface charges for nanoparticles to be used in gene delivery. So, they used Alkyl-PEI2k CDs for surface passivation and gene delivery, because after passivation the zeta potential of CD (-29 mV) became 17.33 ± 1.97 mV. Moreover, they found that the nano-vector possesses good stability, Monodispersity with narrow size distribution, high biocompatibility, low cytotoxicity, high gene delivery efficiency with the necessary feature of visualizing the delivery and photoluminescence. The gene binding was attributed to the electrostatic interactions between the amine groups of the polymer and phosphate groups of genes, which eventually help form polyelectrolyte complexes. Since then many uses of CD and DNA conjugates have been researched [98].

4.1.1.4. RNase and SiRNA

To combat the challenges of using CD for molecular imaging and *in-vivo* molecular tracking; it is required to enhance the fluorescence intensity of CD. A new way of synthesizing CD with high quantum yields (24.20%) was demonstrated by Liu *et al*. [99] by using multifunctional ribonuclease A (RNase A) as a biomolecular templating agent (under microwave irradiation) by adjusting microwave reaction time and power, the fluorescent color of the RNase A@CD can be adjusted. CD conjugated with folate and passivated withreducible polyethylenimine passivated CD (fc-rPEI CD) has been shown to be a theranostic agent for gene delivery in lung cancer therapy [99]. Utility of siRNA conjugated CD has been shown to be an efficient siRNA nanocarrier for gene therapy of gastric cancer cells [100].

4.1.1.5. Lipid

Rather than getting conjugated with CDs; lipids are used for encapsulation. However, covalent attachment of CDs to phospholipids has also been explored [55]. CDs-modified phospholipids had an advantage that they get readily incorporated within biomimetic membranes (including solid-supported bilayers),

vesicles, and into actual cellular membranes. Hence, CD-phospholipid probe has been used for the effects of polymyxin-B (a cytolytic peptide), valproic acid (a lipophilic drug), and amyloid-beta (a peptide associated with Alzheimer's disease) upon bilayer fluidity and lipid dynamics have been studied [55].

4.1.1.6. Folic Acid

FA provides CDs for both fluorescence and cell uptake via folate receptor-mediated drug targeting when CDs treated with the cancer cells [101]. Moreover, CDs and agents of substrates or molecules are mixed together in order to apply for biomarker because they intrinsically showed dual uses of fluorescence and remedy for cancer cells via endocytosis. Thus, the carbon dots conjugated with corresponding substrates by means of covalently linkage on the CDs surface site, aiming to deeply affect the potential on cancer cells. However, the quantum yield of the conjugated CDs are generally less than that of CDs free, that means CDs alone is higher fluorescence than carbon dots- folate conjugates [68].

Generally, the folate receptor is considered as a desirable biomarker for tumor cells [102]. It provides drug uptake and smooth drug delivery in the given cells. As depicted in **Figure 4**, CDs conjugated with folate acid to show high affinity binding sites on cancer cells. It has a great interest in distinguishing human tumor cells by means of overexpressing the folate receptor-α (FR-α) on their membrane; subsequent FR-α was vividly appeared in the site of the tumour cells via cytoplasm [103]. Folate receptor-α is tie up to the cell surface through a glycosyl-phosphatidylinositol molecule, and provides the important cellular growth and propagation from the living cells mediated by folic acid or B9 vitamin. In tumor cells, the activation of folic acid stimulates the action of the transducer and activator of transcription 3 (STAT3) pathways via FR-α. Due to the significant role of folic acid receptors in carcinogenesis, FR-α was immediately characterized as a desirable therapeutic target [104]. Knowing that the folate receptor abundantly occurs in tumor tissues, its capability for the assembly of folate acid derivatives has been recognized as a potential drug delivery target to enhance folic acid-conjugated drugs [105-107].

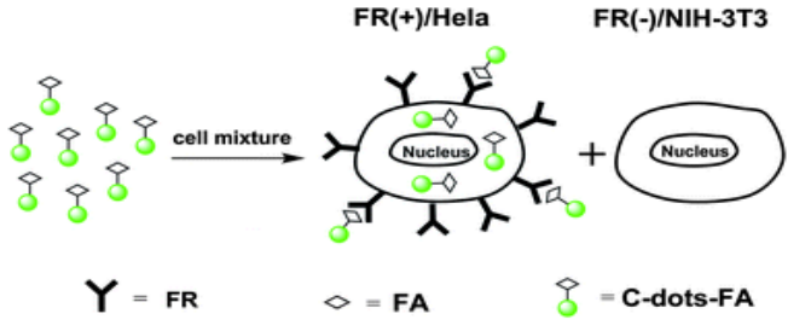


Figure 4. *The proposed FA conjugated carbon dots for cancer diagnosis. Reprinted with the permission from reference [108].*

To visualize those biomarkers on the cells, most common used targeting ligands, such as transferrin, folic acid (FA), and peptides are intentionally conjugated with fluorescent carbon dots which interact with the presented biomarker that is overexpressed on cancer cells [109]. Folic acid, a low-molecular weight ligand, is

essential for cell uptake and high affinity to folate receptor on cancer cells via receptor-mediated endocytosis.

Theoretically, the $-NH_2$ functional groups on the CDs' surface were covalently conjugated with the -COOH groups of FA via a classical cross-linking reaction [110]. Hence, cost-effective, readily available, and non-toxic ligands of FA for visual detection in the prognosis of cancer are satisfactorily demanded. However, as far as CDs coupled with folate receptor-targeted fluorescent CDs has been known, their quantum yield holds less than 10%. Thus, it would be challenging to develop targeted fluorescence of FA-CDs with facile preparation and good luminescence.

Song *et al*. in 2012 [108] proposed fluorescent carbon dots assembled with folic acid (CDs-FA) to distinguish the cell survival and cell uptake on cancer cells from normal cells using the facile microwave pyrolysis and all results are as shown in **Figure 5**. Doing early passivation of CDs with 4,7,10-trioxa-1,13-tridecanediamine (TTDDA) has promoted active site of amino groups for easy conjugation with FA. That surface passivation or defects with smaller size distribution might support a high luminescence, which can be used for bioimaging. Interestingly, to promote the reactivity between FA and amino groups on passivated CDs, a more active amino group on N-hydroxysuccinimide (NHS) was first assembled with FA, and the resulted FA–NHS could further be covalently and easily bound to the passivated CDs surface. Thus, FA was successfully conjugated on the passivated CDs. With regard to toxicity of passivated CDs against Hela cancer cells, cell viability is over 85% up to 200 μg/mL, suggesting good biocompatibility and very low toxicity as shown in Figure 5 (d) and (e). Importantly, the loading efficacy of CDs-FA into Hela cells showed a fairly weaker fluorescence than that of free FA due to receptor-mediated survival cells of CDs–FA [108].

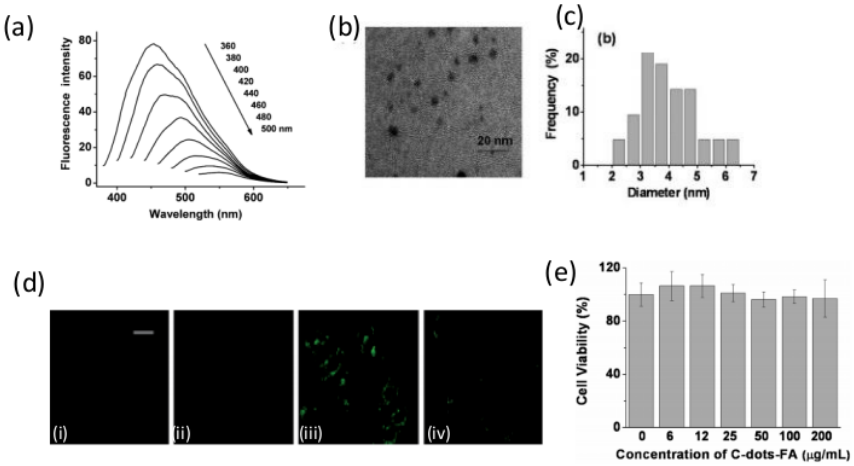


Figure 5. *(a) PL emission spectra (movable excitation from 360 nm to 500 nm) of the CDs functionalized with TTDDA. (b) TEM image. (c) Histogram of the TTDDA passivated CDs. (d) Fluorescence photographs of HeLa cells at various situations - (i) HeLa cells (as a control), (ii) HeLa cells treated with FA alone at 37 °C for 6 h (next control), (iii) HeLa cells tested with CDs–FA (50 mg mL^{-1}) at 37 °C for 6 h, and (iv) HeLa cells pre-treated at 37°C for 1.5 h with increased FA for FR saturation, thereafter; incubated with CDs– FA (50 mg mL^{-1}) at 37 °C for 6 h. (e) The cell viability study of CDs–FA with different concentrations.[108] Reproduced with the permission of reference [108].*

Nasrin et al. in 2020 [78] reported multi-emission carbon dots (CDs) from the derivative of vitamin B1 like thiamine pyrophosphate or ThPP though the hydrothermal treatment as shown in **Figure 6a**. The prepared multi-photon emissive CDs derived from the rich-heteroatom precursors. To develop the imaging efficacy and photosensitization on the human melanoma cancer cells, subsequent FA conjugation with CDs was conducted. Intriguingly, those CDs-FA probes provide better spectral image and targeting in a deep tissue cell. They suggested that the CDs-FA showed no toxicity, exceptional biocompatibility, photostability and enhanced survival cells by folate receptor positive (FR+) cancer cells [78]. There would be deduced from phosphorus doping carbon dots could conjugate with FA to provide fluorescence and therapeutic efficacy.

In 2020, Sara Lee and Kangwon Lee recently prepared pH-sensitive folic acid conjugated Alginate nanoparticle (AF-NPs) to inducing the cancer-specific fluorescence image as shown in **Figure 6b**. The research has attracted much attention on the preparation of cost-effective water-in-oil emulsion system, specific delivery efficacy of prepared NPs to the target site and long stability of the formulated alginate-conjugated folic acid nanoparticles (AF-NPs) in aqueous suspension regardless of aggregation and no toxicity. Their research outcomes demonstrated that the synthesized AF-NPs did not show degradation with the effect of enzymes or other external substances until they were delivered into cancer cells, and thus the conjugated fluorescence NPs can penetrate cancer cells smoothly without any disturbances to produce photoluminescence [111].

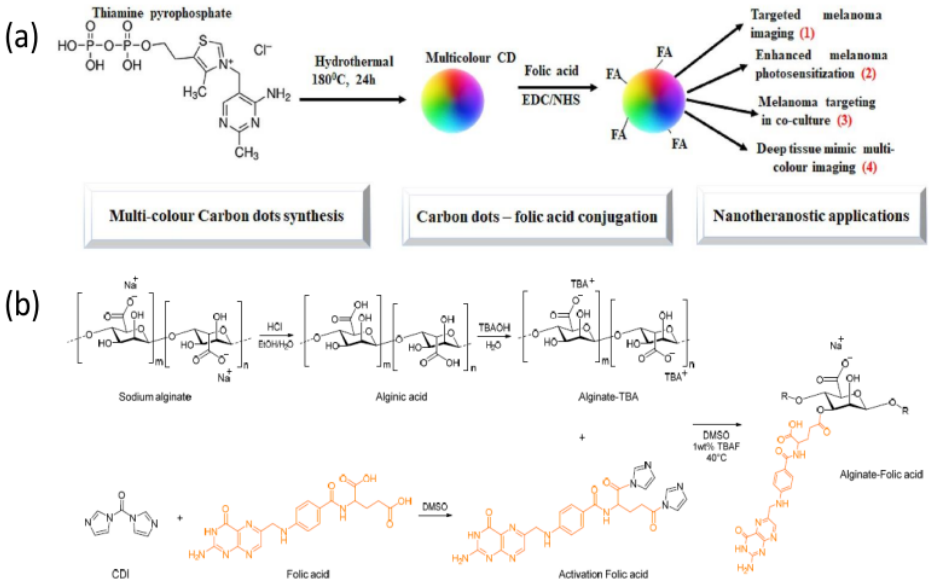


Figure 6. *(a) The proposed thiamine pyrophosphate-based carbon dots conjugated with folic acid.[78] (b) The presented schematic diagram of folic acid conjugated alginate [111]. Reprinted with the permission of respective references [78, 111].*

Table 1: *The proposed FA conjugated carbon dots for their respective uses.*

Starting materials	Conjugated agents	Size	Method	Cell lines	Applications
Active dry yeast	Folic acid	2–6 nm	Facile and fast microwave method	HepG2 cells	To trace cancer cells [74]
Polyethylenimine	Folic acid	9 nm	Microwave pyrolysis	lung cancer cells (H460)	As drug delivery agents in lung cancer therapy [17]
Cationic Ag_2S near-infrared quantum dots (NIRQDs)	Folic acid, Polyethyleimine and doxoroubicin	27.0 ± 2.9 nm	Using a PEG bridge and loaded with DOX.	A549 cells	As optical imaging and selective doxorubicin delivery agent to HeLa cells[112]
Citric acid & diethylamine (N-GQDs)	Folic acid	5 nm	Hydrothermal method	MCF-7 cells	As fluorescent probes to detect cancer cells [113]
Poly(amidoamine) (PAMAM-NH_2) dendrimer	Folic acid	30 nm	Hydrothermal approach	-	For Pt (IV) detection [114]
Dandeliand ethanediamine (EDA)	Folic acid	3.5 nm	Hydrothermal treatment	HepG-2, and MCF-7 cells	As fluorescent probe for targeting cellular cancer cells [115]
Water-soluble CdTe quantum dots	Folic acid	-	By means of an inert gas such as nitrogen or argon	A549 cells	For cancer cells targeting [116]

In the ***Table 1***, potential results of carbon dots conjugated with folic acid or other conjugated agents are presented.

The published papers of particularly selected agents-loaded carbon dots are promising for their respective applications as mentioned above. Specifically, there is a deduction that folic acid-loaded carbon dots are beneficial for applicability in vitro for cancer cells with favorable cell viability because FA intrinsically exhibited folate receptor which showed affinity to the CD4 receptor and other coreceptors or targeting cellular site-mediated respective receptors on cancer cells. As a consequence, the conjugated CDs can easily enter cellular sites via endocytosis. However, folic acid should be preferable in combination with molecules or organic acid with multiple functional groups since strong photoluminescence was provided by those organic acids or molecules with different moieties. Thus, the efficacy of entering the cellular site and exhibiting strong photoluminescence on targeting sites of cancer cells would contribute to handling cancer diagnosis.

4.1.1.7. Chitosan

Chitosan is a natural polymer, used for oral as well as subcutaneous delivery of drugs for treating cancer and diabetes. It is also used as precursor for synthesis of CDs [117]. CDs

CDs prepared from chitosan shows different properties based on the pH at which they are prepared and the passivating agents used [55]. CDs conjugated with Chitosan is not only used for the adsorption and controlled release of drugs systems but also has been tried as scaffold for cell and tissue growth. Pawar et al. 2019 synthesized chitosan-CDs for a fluorescent probe. Chitosan-CDs showed weak fluorescence in organic solvent due to photoinduced electron transfer between negatively charged pyrrole nitrogen in chitosan and positively charged in electron, whereas in continuous water addition it exhibited high fluorescence intensity with water detection limit 0.0023% (v/v) in DMSO [118].

4.1.1.8. Digitonin

Digitonin (DG)-conjugated CDs is used for detection of atherosclerosis. This probe

can selectively bind to a cholesterol-rich area in a solution that can be imaged by fluorescent microscopy and IVIS (*In Vivo* Imaging System Spectrum) images [119]. Krishma *et al*. in 2016 prepared CDs modified with digitonin (DG) to become CDs-DG. CDs-DG was further conjugated with methotrexate (MX) to attain CDs-MX. According to cellular uptake studies, the blue fluorescence of CDs-DG and CDs-MX enter the cell via endocytosis showing blue fluorescence. Regarding its toxicity, all prepared samples exhibited cell viability over 70% against cancer cells. The multi-combined drugs of this study could be helpful for theranostic probes application [120].

4.1.1.9. Hyaluronic Acid

Hyaluronic acid (HA) is a kind of natural mucopolysaccharide which has drawn several benefits, such as eco-friendliness, non-immunogenicity, easy availability, biocompatibility, chemical versatility, non-toxicity, biodegradability, and strong hydrophilicity [119]. Hyaluronic acid-conjugated carbon dots or other nanoparticles (HA-NPs) have been one of the popular projects of scientific researchers, especially in the field of biomedical society because of targeted drugs carrying capability and potential imaging probes. Currently, hyaluronic acid can be not only used as a carbon source for self-targeting when synthesizing CDs, but it is also applicable for conjugated or loaded agents on prepared nanoparticles to mediate a novel drug carrier into tumor cell-specific targeting. Moreover, HA can be used as a guaranteed ligand to bind CD44- and CD168- i.e. HA overexpressed binding receptor in tumor cells, including Hela cells, HT29 cells and SCC7 cells [121]. There are some superiorities of HA-based drug delivery carriers. First, HA is considerable to enhance the stability of the used therapeutic agents in physiological circumstances [122]. Secondly, HA can overcome the current issue of low specificity of anticancer drugs when overexpressed receptors in which HA can be bound easily [123]. Finally, HA can readily receive the chemical modification by other molecules because of multi-functional groups on its surface edge. Specifically, the glucuronic acid with the carboxyl groups and the N-acetyl-D-glucosamine with the hydroxyl groups are commonly chosen to chemically modify to produce HA-based nanocarrier [124, 125].

Wang *et al*. in 2017 prepared the HA-CDs from the combination of hyaluronic acid (HA) and polyethylenimine (PEI) as a passivating agent using microwave irradiation to use for drug delivery agent and cell imaging as shown in **Figure 7**.

The HA-CDs exhibited tuneable mult-color emissions depending on varying excitation movements. According to the MMT assay study, the result showed that the loaded HA on CDs enhanced targeted cell imaging in CD44 overexpressed cells without significant toxicity even at a high weight ratio of 14 in contrast with PEI alone which was used a control [126]. PEI plays a role in smooth targeting of drug delivery to cells, but PEI passivated HA-CDs become more toxic. Thus, adding PEI into HA-CDs has disadvantage and advantage for biomedical application.

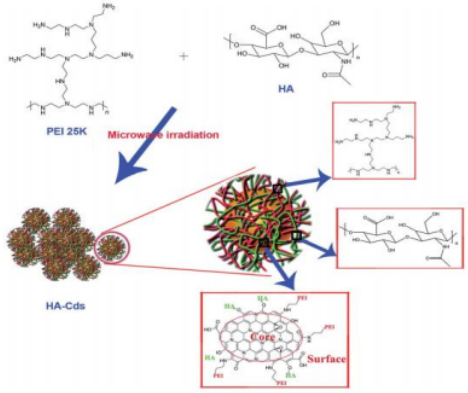


Figure 7. *The schematic diagram of HA-loaded CDs for cell imaging and gene delivery [126]. Reproduced with the permission from reference [126].*

Moreover, nanoparticles synthesized from semiconductors-based precursors also showed good fluorescence, however it brings some side effects. So some modifications of those nanoparticles are required to relieve those inconvenient effects. Researchers are thus approaching conjugation, modification, and function-alization to search for toxicity levels

Gold nanoparticles (Au-NPs) have their outstanding ability to anchor selected functional groups, such as amine and thiol groups, and their optical emissions can be tuned due to their size and configuration and quenching system. On the other hand, Au-NPs exhibited their drawbacks, indicating poor stability, low reactivity and efficacy to coat with hydrophilic drugs. Thus, functionalizing the surface site of Au-NPs with is in high demand to overcome that issue [127]. The functionalization of Au-NPs by hydro-phillic drugs has become advantageous since they increase excellent physicochemical and biological properties. Moreover, CDs were synthesized taking 4-nitrophenol (4-NP) as a catalyst to enhance the adsorption of 4-NP on CDs via π-π stacking interactions for the reduction of 4-NP from its environment pollution issue. It showed that the best advantage of catalytic activity of 4-NP coated CDs for decreasing 4-NP pollution issue. The distinctive disadvantage of selected agents on Au NPs is sometimes leading to aggregation, becoming not well-dispersed solution. On the other hand, researchers sometimes choose both CDs and Au NPs to synthesize for biorelated application using a catalyst of 4-NP which must break up with Au NPs and will pass back into the solution, thus no reaction would happen. This is a crucial aspect on the presence of CDs for Au NPs [121]. HA-conjugated Pheophorbide-A(PheoA) and Au NPs were potentially prepared to provide mutli-useable theranostic vehicles. At first, they made conjugation of Thiolated HA (MW 7 kDa) with PheoA, afterwards, encapsulating Au NPs into post-prepared PheoA-HA. The result of synthesized nanotheranostic agents was found to be lower toxicity than Au NPs only good colloidal stability and long resistant photoactivity in intracellular parts. Moreover, tumor targeting drug delivery and therapeutic capacity were specific in tumor-bearing mice [129].

Another reported by Hayward et al. 2016 was hyaluronic acid-conjugated liposome nanoparticles (HA-LNPs) for carrying target point to CD44 overexpressing glioblastoma (GBM) cells as shown in **Figure 8a**. HA free LNPs ranged 95.0 ± 0.7 nm in hydrodynamic diameter, whereas the HA-encapsulated LNPs became large in size to 126.6 ± 5.62 nm because excessive functional groups of HA exist on the surface of the liposomes as depicted in **Figure 8b**. The fluorescence nature appeared to be analyzed by high magnification confocal microscopy according the fluorescent signal in the lipid bilayer as shown in **Figure 1c**. They found that the HA-LNPs nanomaterial could go well into GBM cells provided by an active ligand on the surface site of HA with high cell viability. They explored the final result that the novel CD44 targeted lipid-based nanoparticles could serve as good nanocarriers, assisting site-specific carrying drugs through CD44 receptors in GBM cells [130].

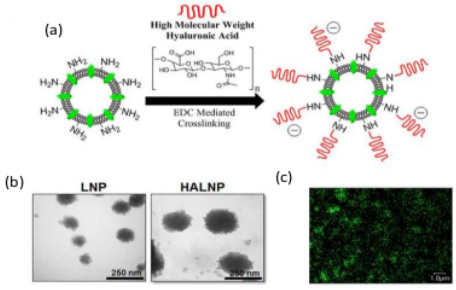


Figure 8. *(a) Lipid nanoparticles (LNPs) were surface functionalized with HA (HA-LNPs) via EDC facilitated amide bond formations. (b) TEM pre and post HA surface crosslinking to ensure the surface decoration of LNPs or HA-LNPs. (c) High magnification confocal microscopic image of the HALNPs [130]. Reprinted from the permission of reference [130].*

Moreover, HA-coated rGO nanosheets were potentially produced by means of coating cholesteryl-2-aminoethylcarbamate (CAEC) with the carboxyl group on HA [124]. The prepared nanomaterials were used as nanoprobes which demonstrated enhanced stability and good biocompatibility due to loading from the hydrophilic HA. Thus, those HA-loaded nanoprobes could be used to target cancer photothermal therapy (PTT) [125]. Moreover, the HA-loaded carbon-based nanosheets, including HA-single-walled carbon nanotubes (HA-SWNT), HA-graphene oxide (HA-GO), and HA-fullerene (HA-C60) were considerable for anticancer effect which exhibited improved water-soluble, non-toxic, biocompatible and tumor-targeted efficacies [119].

Duan *et al*. in 2020 updated the report on the important contribution of hyaluronic acid-loaded CDs to produce CDs-HA-Hep/DOx complex and the results are shown in **Figure 9**. The carbonyl-amino groups on the prepared CDs played a key role to stabilize the hyaluronic acid (HA) and heparin (Hep) condensation reaction to become CDs-HA-Hep complex nanomaterials. Then, DOX was added to these complex nanomaterials to promote the therapeutic capability. CDs-HA-Hep/DOX nanomaterials range relatively increased in size; it is likely due to being encapsulated. More interestingly, drug carrying efficacy of CDs-HA-Hep/DOX was distinctively excellent in the presence of HAase. Thus, drug release replied on HA and pH value as dual-responsive agents. Besides, the hemolysis observation showed that the CDs-HA-Hep/DOX provided considerable performance.

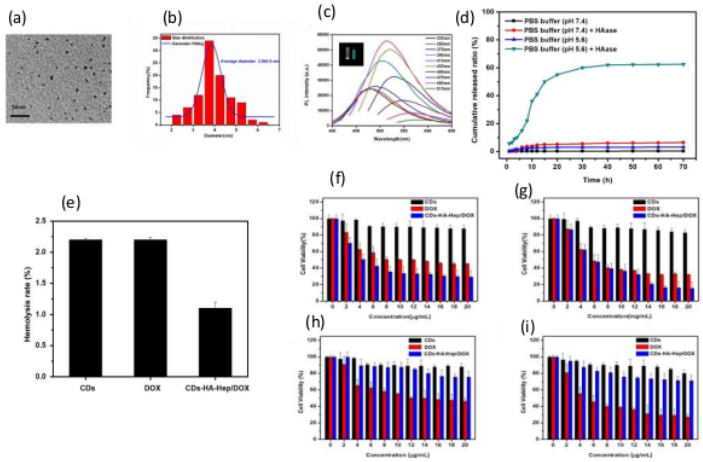


Figure 9. *(a) TEM photographs of CDs (scale bar: 50 nm). (b) Histogram of CDs. (c) Fluorescence emission spectra of CDs at varied excitation wavelengths (Inset: images of CDs aqueous solution under daylight (left) and 365 nm UV light (right)). (d) Drug release curves of CDs-HA-Hep/DOX at varied situations (conducted by controlling the absorbance of DOX at 480 nm). (e) Hemolysis rates of CDs, DOX and CDs-HA-Hep/DOX, respectively. (f) Cell viability studies of CDs, DOX and CDs-HA-Hep/DOX at varied concentrations against MGC-803 cells for 24 h. (g) The cell viability result of CDs, DOX and CDs-HA-Hep/DOX at varied concentration levels against MGC-803 cells for 48 h. (h) The cytotoxicity results of CDs, DOX and CDs-HA-Hep/DOX at varied concentration conditions against GES cells for 24 h. (i) The cytotoxicity results of CDs, DOX and CDs-HA-Hep/DOX at varied concentration conditions against GES cells for 48 h.[132] Reproduced with the permission from references [132].*

Moreover, CDs-HA-Hep-DOX could distinctively inhibit cancer cells on MGC-803 cells, but as in normal GES cells, the cell viability of CDs-HA-He/DOX is medium high (over 70%) at 20 μg mL^{-1}. Therefore, it could be concluded that the prepared CDs-HA-He/DOX can intensively hinder the cancer cell propagation and no toxic effect on normal cells [132].

The HA-loaded CDs might result in the formulation of encapsulating nanoparticles, and thus the size dimensions of HA-loaded CDs are possibly larger than the CDs alone due to being encapsulated by HA molecule or other combined agents. Moreover, concerning the insight of the internalization of the loaded nanoparticles was in accordance with the interaction of HA with CD44 receptor on surface of tumor cells since changed efficacy level of HA-CDs has a very strong affinity for the CD44 receptor overexpressed on cancer cells. In this way, the existence of HA on the nanohybrid surface facilitated considerable cellular uptake by active targeting of ligand-receptors.

4.1.2. Carbon Dots Conjugated with Inorganic Heteroatoms

Recently, conjugation or doping of CDs with several inorganic materials that have organic photovoltaic properties for photocatalytic activities for CO_2 conversion or the degradation of organic dyes [133, 134] has been reported; such as metal (Cu, Zn, Gd, Au, Ag), non-metal (Si, B, P,N, S) and heteroatoms (atoms apart from carbon or hydrogen are also called heteroatoms); because they enhance the properties of CDs (such as increased quantum yield in far red and near IR, decreased photodamage) and have found application in biosensing, bioimaging, and theranostics. They have been extensively reviewed by Xu *et al*. [135].

Conjugation of CDs with inorganic nanosystem has found applications in two-photon imaging and biosensing of pH variations and drug delivery. Sharon's group has shown shape dependent suitability of gold nanorods [136], gold nanotriangles [137] and gold nanospheres [138] for drug delivery. They have also shown that the conjugate of microparticles of mesoporous silica oxide and CDs (mesoSiO2/CDs) have profound application in bioimaging [139] Attachment of ZnO to CDs could improve the charge separation and the reduction of charge recombination; thus, have shown to enhance *photocatalytic* efficiency [140] CD-doped CdS (CDs/CdS) microspheres have also been shown to enhance the *photocatalytic* activity [141].

Many algae metabolize the production of high-energy molecules such as fatty acids and triglycerides. A conjugate of nanoparticles of SrO with CDs is used as *catalyst* for fatty acid methyl esters production using *Chlorella vulgaris* as feedstock [142]. Another metal that is being researched for fabricating dual fluorescence MRI probes for biomedical applications in 2-3 nm size Gadolinium (III)-doped CDs [143]. This conjugate is monodispersed, it forms stable dispersions in water and exhibits bright fluorescence, strong T1-weighted MRI contrast and low cytotoxicity. CDs conjugated with Europium have been used [144] for the detection of tetracycline. Tetracycline is an antibiotic used to treat a number of infections. However, it has damaging effects like renal toxicity and haemolytic anaemia.

Doping of CDs with non-metal heteroatoms like nitrogen and sulphur that are nontoxic and biocompatible and are being researched for traceable drug delivery system [145].

4.1.3. Carbon Dots Conjugated with Organic Material

Intrinsically CDs are not cytotoxic, but often the use of passivating agents increases their toxicity [146]. Therefore, there are attempts to conjugate CDs with polymers that have found application in medicinal science, some of which are (i)

PEG (polyethylene glycol) which is used as a base in laxatives and an excipient in many pharmaceutical products; are conjugated with CDs for gene therapy, helps in nuclear targeting and cancer therapy [147]. (ii) PEI (polyethylenimine) has limited medicinal uses because it is toxic at higher concentrations, though it is used in cell culture as an attachment promoter of weakly anchored cells and as a transfection agent [148].

As it can selectively recognize Cu^{2+} ion and give a very sensitive signal response [149] it has found applications in chemical sensing of metal ions. However, multi-functionalized CDs derived from glucose and PEI have shown both antibacterial (to both Gram-negative and Gram-positive bacteria) and gene delivery properties. For gene delivery [150], (iii) α-Cyclodextrin in conjugation with CDs has been used in molecular recognition and optical sensing with the help of electron transfer mechanism [151]. (iv) Cysteamine is an aminothiol containing an amine, which is used to treat cystinosis. Our group has prepared CD from phenylalanine and conjugated with cysteamine-HCl was used as a linker to attach and controlled release kinetics haloperidol (an antipsychotic drug) [152].

Moreover, CDs can get selective access to cells due to their large interactions with biological membranes [153], avoiding unwanted internalization. (vii) CDs have also been conjugated with polyamidoamine *(PAMAM)* dendrimers for drug delivery and molecular imaging [154, 155].

4.1.4. Carbon Dots Conjugated with Antibiotics

There have been numerous protocols to effectively conjugate antibiotics with NPs for the therapeutic purposes. Specifically, silver [156], or gold nanoparticles (GNPs) [157] or carbon-based nanoparticles [158], coupled with antibiotics would be the potential antibacterial or antimicrobial agents because they are intrinsically antimicrobial or antibacterial [159].

For example, ampicillin loaded NPs were prepared for administration of salmonella infection in mouse, decreased the amount of drug by 40 folds in contrast with free ampicillin. Sometimes, the dose for killing the pathogens is much lower than the prepared antibiotics amounts, and thus affected serious side effects. Balancing dose between bacteria and antibiotics is, therefore, required. When antibiotics conjugate with NPs, some passivation or functionalization agents such as amino acids, glutathione and polyethylene glycol were used as a mediator to those antibiotics conjugated NPs. However, this assisted agents often used to interfere with drug stability and reduce the antibiotics capacity. Interestingly, without any addition of those assisted agents, only antibiotics could directly conjugate NPs, avoiding the disturbance of functionalizing agents on biological system [160].

Thomas *et al*. in 2020 prepared silver nanoparticles conjugated with antibiotics to enhance antimicrobial effect as shown in **Figure 10** [161]. Consequently, there are the following works conducted by some researchers concerning the conjugation of CDs or other nanoparticles with versatile antibiotic agents for the antibacterial and antimicrobial killing.

Moreover, Thakur et al. in 2014 prepared antibiotic conjugated fluorescent carbon dots using gum arabic (GA) and ciprofloxacin for triple uses of drug release, bioimaging, and striven antimicrobial agents.

The broad-spectrum antibiotic, ciprofloxacin was conjugated with CDs (Cipro-CDs conjugate). The Cipro-CDs conjugate demonstrated high antimicrobial response against both models of microorganisms. Moreover, the Cipro-CDs conjugate showed satisfied bioimaging feature and good nanocarrier agents with enhanced antimicrobial effect, indicating that that Cipro-CDs probe is potential for theranostics [158].

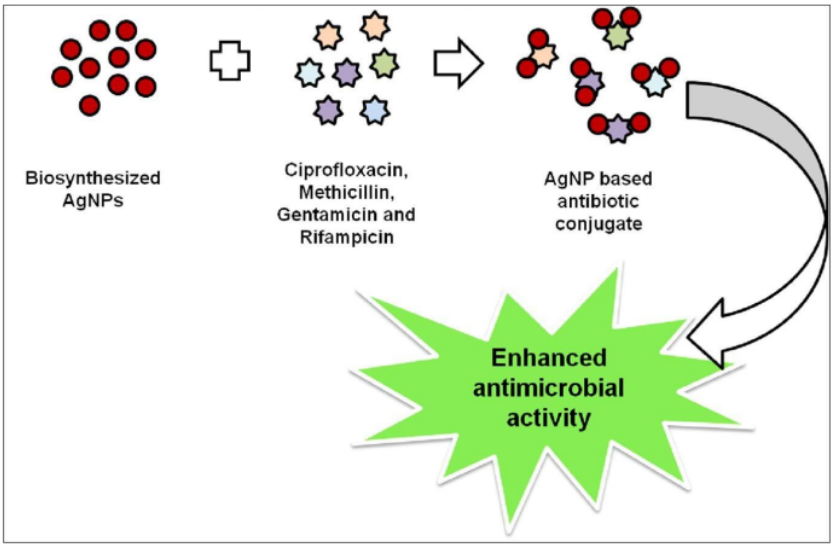


Figure 10. *The proposed antibiotics-conjugated silver nanoparticles for enhanced antimicrobial activity.[161] Reprinted from the permission of reference [161].*

Payne *et al*. in 2016 introduced the effective gold nanoparticles-based antibacterial vehicles through a one-step synthesis of antibiotic (kanamycin)-loaded on the surface of gold nanoparticles (Kan-AuNPs) using the combination of reduced and capped systems of kanamycin. The prepared Kan-AuNPs supported a high response to both Gram-positive and Gram-negative bacteria. Moreover, the minimum inhibitory concentration (MIC) of Kan-AuNPs more significantly exhibited a reduction than Kanamycin alone against the treated bacterial strains. It was conferred that the conducted Kan-AuNPs would contribute as the robust antibacterial candidate to fight bacteria [162].

Masri *et al*. in 2018 proposed that silver-based nanoparticles conjugation with clinically approved drugs, such as Cephradine and Vildagliptin improve the antibacterial capacity. To assess the antibacterial activity when treated with free drugs and drugs-conjugated with silver nanoparticles against a large number of Gram-negative and Gram-positive bacteria for example, neuropathogenic *Escherichia coli* K1, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus cereus* and *Streptococcus pyogenes*. They found that the silver-based nanoparticles conjugation with both Cephradine and Vildagliptin exhibited higher antibacterial potential than the drugs alone. They concluded those clinically approved drugs-conjugated silver nanoparticles showed considerable antibacterial effects [156].

Dong *et al*.[163] 2017 designed the carbon dots in combination with other antimicrobial reagents of H_2O_2, Na_2CO_3, and AcOH (acetic acid). Before the combination, the carbon dots were first passivated with 2, 2'-(ethylenedioxy) bis(ethylamine) (EDA). They explored that the carbon dots combined with H_2O_2 rather exhibited the significant synergistic effects in contrast with other combined agents toward both Gram negative bacteria *E.coli* cells and Gram positive bacteria *Bacillus subtilis* cells. They showed that antimicrobial agents could tune the level of inhibitory concentration index. Thus, carbon dots combined with H_2O_2 could inhibit bacterial growth even in lower concentration, reducing

environmental development of microbial resistances [163].

Perveen *et al*. 2018 introduced silver nanoparticles produced from a natural lychee fruit peel waste by means of green synthesis. Then, the silver nanoparticles were conjugated with selected antibiotics (Cefixim, Streptomycin, and Amoxicillin) to evaluate the synergistic effects of versatile antibiotics with green nanoparticles against bacteria. The results showed that the highest antibacterial activity was found in the conjugates of amoxicillin and cefixim against Gram-negative bacteria i.e *Alcaligenes faecalis*, whereas the amoxicillin conjugates exhibited the highest reduction in minimum inhibitory concentration against Gram-positive strains i.e. *Enterococcus faecium*. They concluded that the green silver-based nanoparticles conjugated with selected antibiotics demonstrated the promising antibacterial vehicles [164].

Moreover, Mohsen *et al*. 2020 recently reported the silver nanoparticles which were loaded on the antibiotic ciprofloxacin using two step ways to improve the antibacterial effect against both Gram-negative (Escherichia coli) bacteria and Gram-positive (Staphylococcus aureus) bacteria. Early silver nanoparticles preparation was the combination of sodium borohydride, lactose and sodium citrate using one-year aging so that the nanoparticles shape could be uniform. Then, the ready-made silver nanoparticles were formulated by ciprofloxacin to form homogeneous silver nanoparticles ciprofloxacin (AgNPs-CIP). Their results approve that AgNPs-CIP composites revealed higher antibacterial activity against Gram-positive than Gram-negative bacteria [165].

As a deduction from the above-presented literatures, the role of selected antibiotics plays an important efficacy of nanoparticles against numerous bacteria. Specifically, functionalized nanoparticles with corresponding antibiotics depicted multiple functional groups on the surface site, enhancing the efficacy of antibacterial activity. Silver or gold nanoparticles intrinsically exhibited antibacterial properties; capping antibiotics to those nanoparticles will allow bacteria inhibit and kill by means of their cell membrane disruption.

4.1.5. Carbon Dots Conjugated with Anti-Cancer Drugs

Anti-cancer drugs that have arrested the attention of scientists for its conjugation with CD are Doxorubicin (DOX) and Cisplatin. Need of conjugation of anti-cancer drug with CD demands multiple functionalization of its surface in order to attach a functional group so that a particular anti-cancer drug can be attached that can be stored and delivered for treatment and diagnosis, keeping the fluorescence property of CDs undisturbed. Doxorubicin is a nucleus targeting anti-cancer drug used for chemotherapy by injecting through the vein.

It is an anthracycline drug extensively used as multiple cancer treatments, including gastric, thyroid, lung, breast, sarcoma, multiple myeloma, and pediatric cancers [166]. Cancer is one of the leading diseases, which yearly threatens many lives all over the world [167]. The traditional therapy for cancer disease is chemotherapy, which negatively affects very low specificity and causes other significant side effects [168-170].

However, to control drug distribution at unwanted sites and to decrease significant harm to healthy organs, potential carriers of theragnostic agents were developed, which are the combination of targeting, imaging, and therapeutic functions into a single carrier. Targeting functions are responsible for directing the smart carriers to select and accumulate them at tumor sites. Imaging directs to track tumor drug localization, drug concentration, and drug metabolism. Aforementioned multi-combined nanocarriers provide doctors to treating disease and realizing drug release rate [171]. So drug nanocarrier

formulations have recently drawn specific effectiveness to improving cancer cells survival and decreasing side effects.

Thus, CDs with good biocompatibility, active surface site, and optimal cellular uptake are currently considerable for popular nanocarriers for reliable delivering drugs. Specifically, multi-functional groups (−NH2, −OH or –COOH) present at the edge of CDs are potential to be able to carry different therapeutic agents through electrostatic interaction or covalent bonding [172]. In addition to use folic acid conjugation with CDs to provide the optimal uptake cell, doxorubicin (DOX), the so-called anti-tumor drug, also exhibits favorable antitumor effect, penetrating the nucleus to protect the nucleic acid synthesis and thus destroying DNA. In addition, delivery efficacy of DOX formed intrinsic fluorescence, which can provide a dual-emission delivery setup with CDs.

Duan et al. designed fluorescent carbon dots as carriers integrated with DOX to track drug delivery capability toward cancer cells [168]. Concerning its toxicity, the CDs-DOX showed barely more toxic behaviour than CDs alone. It is likely due to the DOX effect on CDs. The positively charged CDs reacted with negatively charged DOX through electrostatic interaction as shown in **Figure 11**.

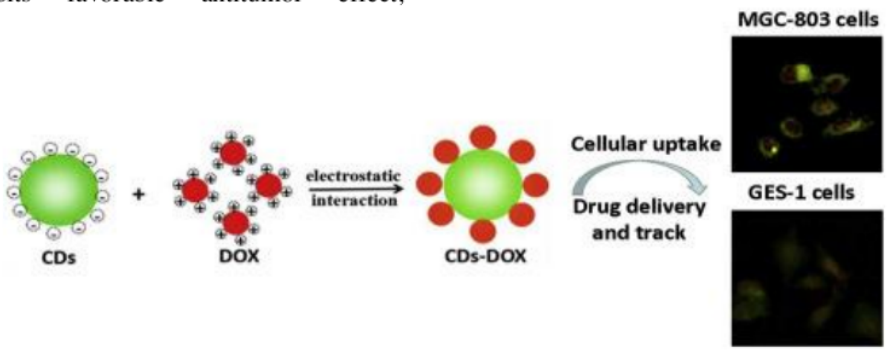


Figure 11. *The schematic diagram of DOX-incorporated CDs via electrostatic interaction for drug delivery agent and tracing cancer cells.[168] Reproduced with the permission of reference [168].*

Yang *et al*. 2016 prepared doxorubicin conjugated CDs for the drug/gene delivery target and the improvement of cancer therapeutic efficacy. They did not conduct the direct conjugation of CDs with DOX. The prepared CDs were firstly functionalized with a nuclear localization signal peptide (NLS-CDs) to carry DOX into cancer cells for increased antitumor activity. To reach their goal, DOX was further conjugated with NLS-CDs through an acid-labile hydrazone bond. Their result demonstrated that the DOX-CDs efficacy in an A549 xenograft nude mice model as *in vivo* therapeutic system was capable of inhibiting tumor growth in contrast with free DOX. They concluded that the DOX conjugated with CDs would contribute to potential drug delivery agents in cancer therapy [70].

Mewada *et al*. in 2014 prepared the fluorescent sorbitol-based CDs for cell imaging. Preliminary modification of CDs with Bovin serum albumin (BSA) was crucial for high affinity of drugs and biocompatibility to labelled cells, subsequent DOX was added to positively change the CDs surface site. Consequently, folic acid was used to navigate the CDs-DOX due to the presence of folate receptors in cancer cells. Their result showed that the internalization of the CDs–FA–DOX goes into a tumor cell at a 7.2 pH specific release condition. Meanwhile,

the drug delivery efficacy would be successfully mediated by DOX.

Sun *et al*. in 2020 reported the efficient CDs from the carbon source of citrate and urea, subsequently conjugated with DOX for bioimaging and promising intercellular drug delivery through a facile hydrothermal treatment and results are as shown in Figure 7. Exceptionally, there is a high quantum yield of 93% of DOX-CDs with low toxicity and good photostability. FTIR analysis showed multi-functional groups (-OH, -COOH, -NH_2) which are promising for conjugating with positively charged DOX. Moreover, the DOX-CDs displayed long conjugation wavelength due to DOX coupling. The resulted excitation-independent emission PL was likely due to the surface states of CDs and heteroatom doping. The cell viability was significantly low against labeled cells, suggesting that the DOX-CDs are excellent anticancer vehicles. As depicted as **Figure 12 (i)**, as in the merged form, the green fluorescence CDs influenced the cytoplasm, while the red fluorescence DOX just appeared in the cell nuclei, suggesting that the DOX did not entrap the CDs and directly connected to the cell nuclei due to high affinity with the DNA [166].

It can be inferred from the above-mentioned insight that DOX was covalently conjugated on the multifunctional site groups of prepared CDs for favorable drug delivery; ligands on DOX were coupled with amino groups on the carbon dots. Moreover, literatures show that DOX generally behaves as positively charged, while carbon dots exhibited negatively charged particles,thus they are conjugated via electrostatic interaction.

Other comparatively less studied for conjugation with CDs is an anti-cancer drug Cisplatin [Pt $(NH_3)_2Cl_2$], which is intravenously administered. It interferes with DNA replication and kills the fast-growing cancer cells. Cisplatin was conjugated with CDs along with folic acid for photo-controlled targeted delivery.

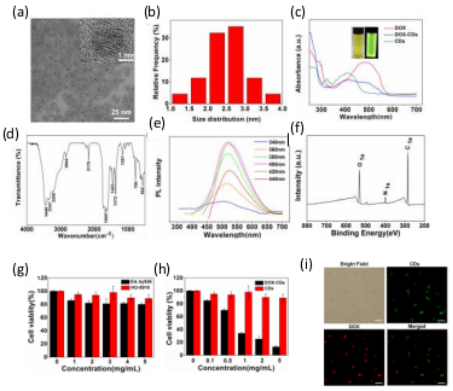


Figure 12. *(a) TEM photographs of CDs (inset: high-resolution TEM photograph). (b) Histogram of CDs. (c) UV-vis spectra of DOX, CDs, and DOX-CDs (insets: the CDs under daylight and UV light). (d) FTIR stretching bands of CDs. (e) The PL emission peaks of CDs under excitation wavelengths from 340 nm to 440 nm. (f) XPS analysis of CDs. (g) The cell viability of CDs against HO-8910 and EA.hy926 cells. (h) The cell cytotoxicity graph of DOX-CDs and CDs against HO-8910 tumor cells (Values are presented as mean ± SD, (n = 3)) (i) The confocal microscopic image of DOX-CDs against HO-8910.[166] Reproduced with the permission from reference [166].*

The Cis-platin-FA@CDs conjugates did not show cytotoxicity even in the presence of reducing glutathione, but when it was photoactivated by either UV or visible light, it produced a similar level of cytotoxicity [173] another platinum-containing anti-cancer drug that is used for colorectal cancer therapy is Oxaliplatin. It has been integrated with CDs to be used as a theranostic agent. CDs-Oxaliplatin is synthesized by condensation reaction between the amino groups on the surface of CDs and the carboxyl group of the oxaliplatin derivative Oxa (IV)-COOH. This conjugate integrates the optical properties of CDs and the anticancer function of oxaliplatin, thus can be used for simultaneous drug delivery and fluores-cent tracking [174].

4.1.6. Carbon Dots Conjugated with Anti-Neurodegenerative Drugs for Central Nervous System (CNS)

For delivery of drugs to the CNS or brain, it is necessary to pass the blood-brain barrier (BBB) that is created with the capillary endothelial cells. The obstruction due to BBB has resulted in the use of increased concentration of drugs and overdosing to achieve the desired treatment <20 nm size have higher permeability coefficient; also, amino-Q-dots were found to be more actively transported through the *in-vitro* BBB model than other surface charged Q-dots. The novel properties of CD such as their particle size and surface properties can circumvent rapid clearance by phagocytic cells, allowing both passive and active drug targeting. Moreover, CDs can reach the capillary vessels and to penetrate the tissues either through the para-cellular or the trans-cellular pathways. CDs conjugated Haloperidol [4-(4-(p-Chlorophenyl)-4-Hydroxypiperidino)-4'-fluorobutyrophenone] (an antipsychotic drug used for treatment of schizophrenia) has shown that a drug loading efficiency of 75.24% and *in-vitro* drug release efficiency at pH 7.2 in 72 h was >50% [175].

In comparison with conventional inorganic QD, CDs have many advantages, including low toxicity, chemical inertness, biocompatibility and no photobleaching. In addition, typical CDs contain a large amount of hydrophilic carboxyl and hydroxyl groups, imparting them with excellent water solubility and multi-functions. For enhancing the efficacies of CDs for various applications, CDs have been conjugated with inorganic, organic, biogenic and synthesized drugs and antibiotics. Though there has been a large amount of research work done on drug delivery using various C-dot conjugates, most of it is still at the bench scale and has not been commercialized.

5. CONCLUSION AND FUTURE PERSPECTIVES

In summary, this review provides a basic understanding of carbon quantum dots, its synthesis, properties, applications, as well as the present drawbacks in the use of CDs as therapeutic agent. Ever since the discovery of CDs they have attracted interest in widely studying their similarities as well as the surprising differences with respect to semiconductor QD. The overpowered properties in comparison to QD open up difficult as well as new fields of applications in health-care. CDs have been fabricated via different synthetic routes which are very facile and eco-friendly in contrast to that of QD. However, more and more work is still needed to acquire more stable and highly fluorescent carbon dots using the simplest techniques in order to make the procedure more cost effective. Various chemical strategies to enhance the fluorescence properties of carbon dots such as surface passivation with different molecules to make CDs stable and at the same time also helps in increasing the quantum yield, which is one of the most important aspects for biological applications; are being assessed. Many studies proposed that selected agents incorporated with CDs were conducted by the principle of covalent bonding, hydrogen bonding or electrostatic interaction through corresponding methods which were aforementioned.

A brief introduction to optical properties of CDs that has made it a suitable material for application in therapeutics such as photoluminescence, multiphoton excitation, upconversion photoluminescence, lack of blinking, resistance to photobleaching and its photocatalytic property, have been found to have a main role in bioimaging [184].

Table 2. *Some loaded CDs for targeting, imaging, and therapeutic functions tested against cancer cell lines as biosensing model.*

Loaded CDs	Optical emission wavelength	Therapeutic agents	Cell lines	Action of key
DOX/PEG-Chitosan@CDs	518 nm	DOX for ChT	Hela cells	Dox loaded onto the hybrid gels to give accuracy the release of DOX [176].
CDs-Pt(IV)-DOX	540 nm	Cisplatin and DOX for ChT	A2780 cancer cells	Weak acid condition Of CDs-Pt(IV)-DOX solution can internalize Cancer cells [177].
CDs-Oxa-Pt	550 nm	Oxaliplatin for ChT	HepG2 cells	The loaded drug was tracking and providing fluorescent signal to help injection time and dosage [178].
Nic-CB-CDs	450 m	Nic for ChT	MCF-7 cells	Loaded drugs enhanced IC50 of two-fold in human breast cancer cells [179].
CDs from CA Urea	650 nm	CDs for PTT	A549 cells	CDs showed NIR high fluorescence and a PTT conversion efficiency (54%) [180].
CD from CA in formamide	640 nm	CDs for PTT	MCF-7 and Hela cells	22% of QY and 43.9% of PT Efficiency [181].
CDs-Ce6	668 nm	Ce6 for PDT	Gastric cancer cells	Multi - functional Nanocarriers enhanced PDT on gastric cancer *in vivo* [182].
CDs-Porphyrin	670 m	TMPyP for PDT	Hela cells	CDs-TMPyP killed cancer cells via TPE of 700 nm fs laser [183].

Whereas, its chemical inertness, easy functionalization, water solubility, low toxicity and biocompatibility favour in its use in drug delivery. As far as it could be seen, their photoluminescence properties of agents-loaded CDs have not been adversely affected/decreased; in contrast with the use of CDs alone, the conjugates have provided a strong fluorescence inside the labelled cells for therapeutic efficacy. Agents-conjugated CDs potentially have shown many outstanding outcomes. The collected results in this review bring insight into the scientific useful answers of those selected agents to further improve nanotheranostics. Increasing awareness and research is being done in the area of CDs-based drug delivery system. It is

expected to continue to change the whole concept of medicines, including aspects such as product characteristics, bioavailability, pharmacokinetics, stability, drug use, and toxicity in humans. Use of CDs in biosensors and bioimaging, has been a great support to therapeutics. However, CDs prepared from diverse methods exhibit large size distribution and photoluminescence non-uniformity, and the complex and time-consuming separation and purification severely limit their further applications, impeding further bio applications of CDs. Moreover, the mechanism of photoluminescence is still unclear. This demands to develop an effective synthesis method with a high yield with a high quantum yield in a small size distribution; and also an understanding of CDs' photoluminescence phenomenon, especially their bright multiphoton emission, should be explored to facilitate their *in-vivo* applications.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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