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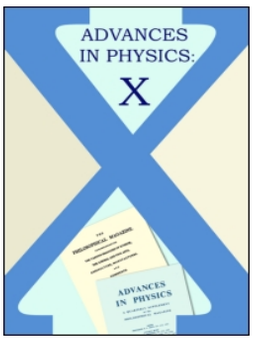

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REVIEW ARTICLE

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Advances in fluorescent carbon dots for biomedical applications

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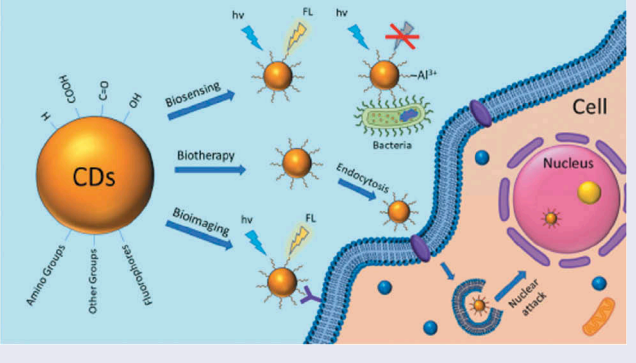
ABSTRACT

Carbon Dots are an emerging class of carbon-based nanoparticles, which since their discovery have attracted tremendous attention because of their exceptional fluorescent, chemical and mechanical properties as well as high photostability and biocompatibility. This unique combination of outstanding characteristics, together with the ease with which they can be synthesized, qualify carbon dots as highly promising materials for applications in electronics and biology, in particular, for biosensing, bioimaging, biotherapy and drug delivery. In this review, we present some of the most recent applications of carbon dots in biology and medicine, concentrating on their fluorescence properties, biocompatibility and efficiency; we also discuss how improvements could prompt their use in human studies. We illustrate how carbon dots, prepared through several facile and cost-effective methods by either the bottom-up or the top-down route, can be used for imaging cells and bacteria and as sensing probes of metal cations. Moreover, we explain how their astonishing versatility has given rise to new biotherapy methods especially in the field of cancer theranostics.



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1. Introduction

Carbon Dots (CDs) are novel carbon nanoparticles, which were first discovered in 2004 by Xu et al. [1]. during the purification of single-walled carbon nanotubes. Since then, CDs have found application in research areas such as optoelectronics [2] and biology [3]. Their intriguing fluorescent properties have triggered new fields of studies in sensing, bioimaging, optoelectronics, biotherapy, catalysis and medicine [4,5]. CDs exhibit high photostability, good biosensing, and excellent biocompatibility with low cytotoxicity. Such properties make them a better candidate than the well-established semiconductor quantum dots (QDs) for many applications [5,6]. Moreover, their facile and cheap preparation methods offer a unique advantage in nanotechnology in terms of fast and cost effective production [7].

In recent years, many efforts have been devoted to the preparation of biocompatible CDs with a high quantum yield (QY), comprising values higher than 20%, which offer better bioimaging and biosensing capabilities [8]. However, the biocompatibility and high QYs are not only difficult to be individually regulated, but they also compete with each other. Trying to improve the biocompatibility of CDs Zheng and coworkers [9] found that the cytotoxic effects decay with size. Enhancing the surface passivation for higher biocompatibility may yield lower photoluminescence (PL) intensity and *vice versa*. However, although it is not yet clear to what extent surface passivation disturbs the biocompatibility, CDs are emerging as superior fluorophores for bioapplications [10]. Additionally, a great challenge is the preparation of red emissive CDs. In fact, the latter do not induce photobleaching since the red-light beam can effectively penetrate tissue [8]. Passivation of CDs with the appropriate functional groups and/or molecules can assist the efficacy of biotherapy, as the former can serve as nanocarrier of therapeutic agents [11] or as medication itself [12]. In general, passivation, as well as doping, can enhance the bioimaging capabilities of CDs. However, exploration of modified CDs with high QY and low cytotoxicity is still in its infancy [13,14]. To deal with the above mentioned issues, new methods and techniques of preparation are needed. Efficiency and biocompatibility of the CDs must be improved. As an essential starting point, the principle of CDs must be understood thoroughly [15].

The purpose of this review is to give a complete synopsis on recent work in the fields of bioimaging, biosensing and biotherapy, and propose alternative methods that could advance existing techniques. In general, in bioimaging and biosensing, CDs can replace the conventional quantum dots as probes for diagnostics for early disease detection and cellular imaging [9]. On the other hand, in biotherapy, CDs can perform as cancer treatment agents as well as delivery transporters for other diseases. Drug delivery [16], photodynamic therapy [3] and photothermal therapy [17] are

some of the prevailing applications that exploit the unique properties of CDs, surpassing the established methods, which are responsible for a high degree of fatalities [18]. Therefore, a comprehensive demonstration of some of the highlighted features of these methods is essential, as they offer new capabilities in the fields of biology and medicine.

2. Carbon dots

The abundance of new preparation methods for CDs makes their use convenient and effective [5]. CDs are formed either by a top-down method, which relies on the fragmentation of carbon allotropes such as nanotubes, graphene and fullerenes, or by a bottom-up method where carbon hydrates are introduced as a carbon source to react with solvents in special synthesis conditions [19]. Preparation methods such as hydrothermal/solvothermal treatment [5] and microwave irradiation [20] are the most common and straightforward ones, which comprise fewer or no additional chemicals. Other non-common methods, which show reasonable results, are laser ablation, chemical ablation and electrochemical carbonization [5]. These techniques are shown in Figure 1 along with various pathways, which lead to different applications, thus illustrating the versatility of CDs [7].

Depending on their structure, CDs are classified into three categories as illustrated in Figure 2 [19]. These categories are graphene quantum dots (GQDs), carbon nanodots (CNDs), and polymer dots (PDs) [9,19]. All types of CDs exhibit comparable PL properties despite their dissimilar structure, size and surface functional groups. GQDs consist of few graphene layers, comprising chemical groups on the edges, which derive from reactants originating from carbon sources or from subsequent reactions [19]. On the other hand, CNDs possess a spherical shape and can have a crystalline graphite-like lattice in carbon quantum dots (CQDs), or an amorphous structure in carbon nanoparticles (CNPs) [19]. PDs, on the other hand, can be either cross-linked/aggregated linear polymers or polymer chains aggregated around a spherical carbon core [19]. As a result, the PL of these nanomaterials displays different lifetimes, intensities as well as different emission wavelengths depending on their structure, size and surface groups. However, different types of CDs cannot be distinguished by looking at their photoluminescence properties, since they all lie between similar ranges, which the former cannot exceed [21].

The relationship between properties and morphology was thoroughly studied by Ananthanarayanan and coworkers [21], who calculated the emission wavelength of GQDs with different sizes and shapes, as well as of GQDs with different surface groups and doping. Their study showed an increase in wavelength with size as well as with the number of surface groups [21]. Interestingly, the authors also demonstrated that different

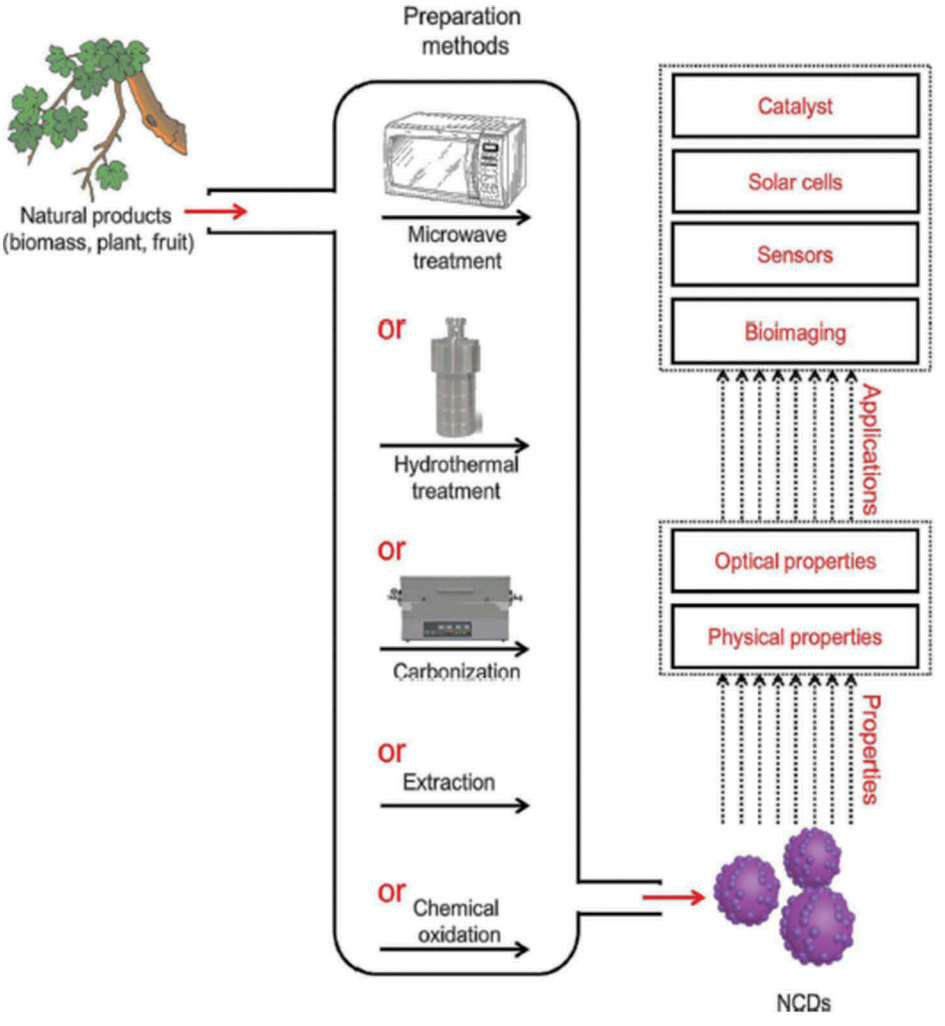

Figure 1. Illustration of the different pathways of preparation and applications of CDs. [Ref. 7; reproduced with permission].

types of doping with heteroatoms can induce either a red-shift or a blue-shift on the emission spectrum. Most importantly, depending on the type of ligands used for passivation, CDs can exhibit negligible cytotoxicity, thus becoming appropriate for *in vivo* experiments [22].

Hitherto, different types of CDs have been presented, most of which exhibit broad absorption peaks in the UV-vis spectrum and PL emission peaks in the visual spectrum from blue to green [8]. For *in vivo* studies the challenge is to create CDs emitting with a high QY in the red (635–700 nm) [8], and to accomplish that, the mechanism behind the intriguing PL properties of CDs must be better understood.

Even though many studies have proposed the exact mechanism behind the marvellous properties of CDs, none of these mechanisms is confirmed to

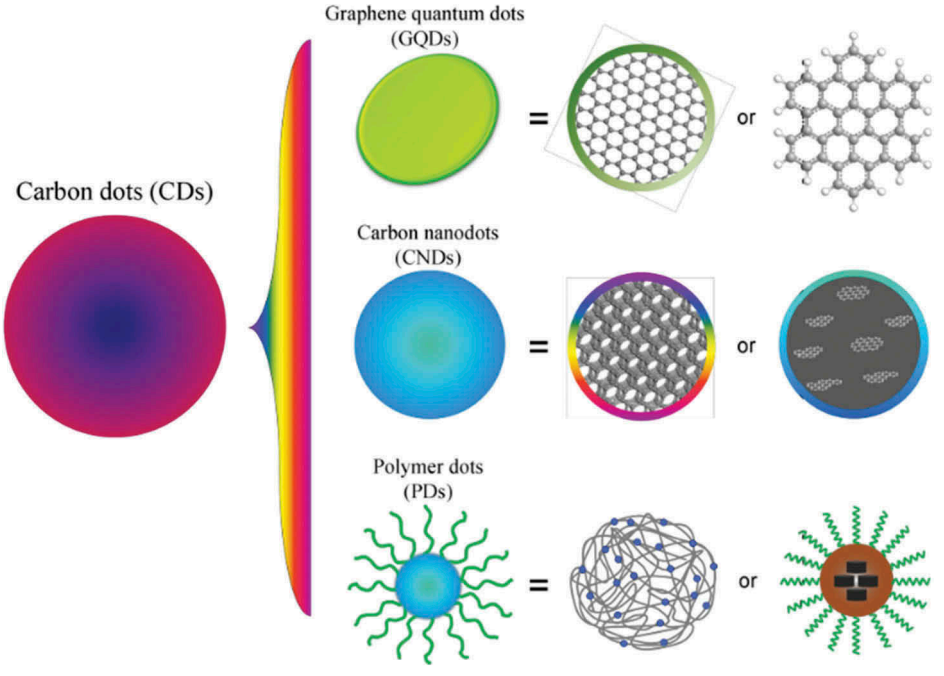

Figure 2. The three classes of Carbon Dots (CDs). a) Graphene quantum dots consist of one or 2–3 graphene layers, b) Carbon nanodots possess graphite or amorphous-like structure and c) Polymer dots consist of aggregated linear polymers or polymer chains around or inside a carbon core. [Ref. 19; reproduced with permission].

date. The most accepted mechanism so far suggests that the PL emission derives from the surface groups on the CDs edges and not from their size, as QDs [6]. Specifically the CDs' absorption in UV-vis spectrum, exhibits a peak at 230–280 nm, which is ascribed to the π-π^* transition of aromatic sp^2C-C bonds. A shoulder between 300 and 550 nm is credited to the n-π^* transition of C=O/C=N and C-O/C-N bonds or other connected groups is reported in Zheng et al.'s review paper [9], where GQDs with adjusted size are linked to shifts in the maximum of the emission spectrum. Based on these results, we can conclude that even though the quantum confinement is not the only cause for the PL properties of CDs, it plays a critical role in the emission and absorption spectra and has to be taken into account when describing the properties of these novel materials.

Since the theory behind the absorption and emission spectra of CDs is still incomplete, it is difficult to establish the correlation between the maximum emission and the QY with the size of the CDs, their population and their surface functionality. However, some simple rules have been established; for instance, nitrogen doping was found to increase [21] the QY of the CDs and many studies have been based on this finding [14]. Carbon dots with considerably high QY have been under investigation for the last decade

since they could be beneficial for bioimaging. In the next paragraphs we report some of the most interesting studies in the field of bioimaging concentrating on the QY of CDs.

3. Carbon dots for bioimaging

Since 2006, when Sun et al. [23]. passivated CDs with PEG_{1500N} and PPEI-EI for cell labelling, CDs have replaced semiconductor quantum dots in cellular imaging *in vitro* and *in vivo*. Because all three types of CDs exhibit low cytotoxicity [9] combined with outstanding fluorescent properties [19], many applications have been proposed, exploiting the surface passivation [24] of CDs, which usually leads to better results than those for non-passivated CDs [25].

Ten years later, in 2016, a new facile method for imaging cancer cells, expressing an expanded level of folate receptor [26] on their surface, was demonstrated by Bhunia and coworkers [27]. CDs were prepared with folic acid as carbon source, mixed with NaOH and heated up to 90°C for 1.5 h to induce carbonization. The resulting CDs had diameters of approximately 3.5 ± 0.6 nm and showed a graphitic crystalline structure [27]. Thanks to their uniform surface functionalization, these CDs exhibited an excitation-independent spectrum peaked at ~450 nm and a maximum QY of 9% [27]. Significantly, when tested with different cancer cell lines, they showed intense labelling of HeLa and SKOV3 cells, which expressed increased levels of folate receptor, while lower fluorescence intensities were observed for HepG2 and MCF7 cell lines, indicating low folate receptor expression [27]. A549 and CHO cells presented negligible intensity because of their insignificant folate receptors levels [27]. Bhunia et al. [27]. also measured the cell viability when treated with CDs *via* the MTT assay, and showed that for a concentration of 1.2 mg/mL, it ranges between 80 and 50% for the various cell lines and increases with decreasing concentration. Unquestionably, this study succeeded in demonstrating a new method for labelling HeLa and SKOV3 cancer cell lines even though the cytotoxicity remains considerably high for *in vivo* studies.

A step forward on the issue of viability was made by Li et al. [28], who demonstrated a new type of PD in 2016. The authors prepared hydrophobic CDs by thermal oxidation. These CDs were transformed to fluorescent CD monomers via amidation of methacryloylchloride. After copolymerization with N-isopropylacrylamide (NIPAM), hydrophobic CDs grafted with poly-NIPAM (PNIPAM) (CDs-g-PNIPAM) were synthesized. This study [28] showed that CDs-g-PNIPAM exhibit thermoresponsive fluorescent properties because their fluorescence intensity decayed when the temperature was increased from 30 to 75 °C. Specifically, consequent to the temperature change, the polymer chains undergo a phase transition causing the

aggregation of the hydrophobic CDs, thus reducing their fluorescent emission. Importantly, a test on HaCaT normal cells showed 85% viability even at high concentration (2 mg/mL) [28]. However, the small QY (3.5%) called for further improvement.

An exciting study by Liu et al. [29]. presented CDs with high cell viability and an intriguing, never observed before, selectivity of stem cells towards different CDs. The authors prepared CDs hydrothermally with PEG_{1500N} and EDA as functional groups. The pegylated PEG_{1500N}-CDs were used for cell imaging of adenocarcinoma HT-29 cells and breast cancer MCF-7 exhibited a much improved QY of 40% [29]. On the other hand, the EDA-CDs used for stem cell imaging showed a relatively high QY of 30 %, but lower than that of the PEG_{1500N}-CDs [29]. Moreover, the study demonstrated that PEG_{1500N}-CDs bind more effectively to Sprague-Dawley rat mesenchymal stem cells than EDA-CDs, but that under anti-cationization treatment EDA-CDs can be extraordinarily efficient and preferable because of their lower cytotoxicity owing to their short ligand length [30].

A study with further improvement on the QY was presented by Feng et al. [31]., who proposed a facile green method for nitrogen-doped CDs (N-CDs) without the need to employ synthetic chemicals. Specifically, using silkworm chrysalis as carbon and protein raw material source, they observed relatively large CDs with an average size of 19 nm and an increased number of nitrogen and oxygen groups, which contribute to a high QY of 46% at 350 nm excitation. However, despite the high QY, the excitation (300–-400 nm) and emission (350–550 nm) spectra of these N-CDs make them impractical for *in vivo* studies.

Following up on this finding, a work published by the Sun group [32] demonstrated that surface passivation of CDs with inorganic salts such as ZnO, TiO_2 or ZnS, along with organic functionalization can improve the QY. Compounds used as ligands for passivation are NH_2–polyethylene glycol (PEG) [40] and bare polyethyleneimine (PEI) [41], which demonstrated enhanced QY properties. Indeed, the measured emission QY from such CDs was about 45% and 50% for ZnO and ZnS, respectively, and the authors ascribe this surge to the high surface passivation by succeeding organic functionalization [42]. Such a high QY was demonstrated in few studies such as those of Table 1, which reported CDs with a QY higher than 36%. Consistently, the presented CDs possess an increased number of doubly bonded oxygen (=O) and hydrogen surface groups. Specifically, the CDs of Zhu et al. [4]., characterized by a large number of =O, -OH and =NH groups, exhibit the highest QY value recorded for fluorescent carbon-based materials.

As previously stated, red emissive CDs (R-CDs) are desirable for *in vivo* studies due to the deeper penetration of the light in tumors and tissue, and their lack of photobleaching. Sun et al. [8]. demonstrated efficient R-CDs with

Table 1. CDs reported high QY.

a/a	Carbon source	QY (%)
1	Cocoon silk [14]	38
2	Dried Shrimp [33]	54
3	Gelatin [34]	31.6
4	Cow manure [35]	65
5	Citric acid and ethylenediamine [4]	80
6	Jinhua Bergamot [36]	50.78
7	Overcooked BBQ [37]	40
8	Orange waste peel[38]	36
9	Branched polyethyleneimine [83]	54.3
10	Succinic acid and tris (2-aminoethyl) amine [39]	49.9
11	Diammonium hydrogen citrate and urea [84]	46.4

an adequate QY of 22.9% at 540 nm excitation, which is ascribed to the n-π^* transitions of the C = N/C-N and C-O functional groups on the surface. An essential property of these CDs is the two-photon excited fluorescence (TPEF), which makes them almost perfect for *in vivo* studies as it eliminates the autofluorescence from the biological matrix and permits high penetration depths [8]. Interestingly, the R-CDs prepared by microwave assisted heating, displayed a high selectivity towards the RNA-rich nucleolus of MCF-7 cells. Sun and coworkers [8] stained living and fixed cells with fluorescent dyes as shown in Figure 3, and demonstrated successful labelling of HeLa and MCF-7 cells. Interestingly, the merged image of MCF-7 cells allows to distinguish the nucleoli from the nucleus as well as the plasma membrane from the cytoplasm. This study manages to show that simply prepared and modified CDs are powerful enough to glow in an intracellular environment and can illuminate small organelles.

Recently, Zhan et al. [43]. demonstrated a new method for creating not only highly emissive red dots but also CQDs, which emit in the different ranges with relatively high QYs, namely 40% for emission in the red, 71.7 % for emission in the yellow, 26.7% for emission in the green and 27.5 % for emission in the blue. The authors reported a new method for the manipulation of the band gap of CQDs using controlled fusion to manipulate the composition, the functional groups as well as the size of the CQDs [43]. More specifically, they prepared CQDs dispersed in 1,3,6-Trinitropyrene (TNP), to different solvents to create differently coloured CQDs as shown in Figure 4 [43].

The different band gap of the various CQDs is primarily a result of the different sizes since it decreases with increasing CQD size, as expected for quantum confinement; however, the surface groups on the CQDs cannot be neglected since this study concluded that the surface states constitute a 'synergistic factor' in controlling the PL properties of CQDs [43]. CDs with an excitation-dependent photoresponse are advantageous for bioimaging because they can be exploited as multicolour probes.

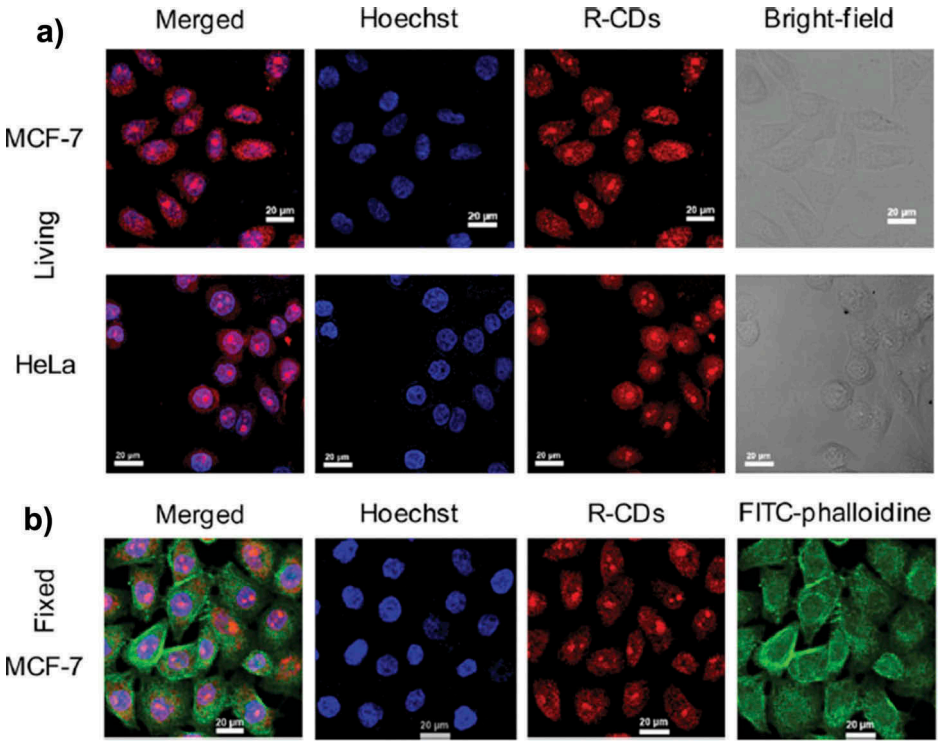

Figure 3. Staining capabilities of red carbon dots (R-CDs) and their compatibility. Different organelles exhibit selectivity toward miscellaneous dyes and CDs. (Hoechst: Nucleus selective, R-CDs: Nucleolus selective, FITC-phalloidine: Cell membrane selective) (a) Fluorescent images of HeLa and MCF-7 living cells stained with Hoechst and R-CDs. (b) Fluorescent images of MCF-7 fixed cells stained with Hoechst R-CDs and FITC-phalloidine. [Ref. 8; reproduced with permission].

Even though excitation-dependent fluorescence was observed in many studies [44,45], the QY, or else the PL intensity, significantly quenches upon changing the excitation wavelength. Usually, when CDs show an excitation-dependent response, the latter has the form of one peak with a maximum intensity at a specific excitation and emission wavelength [46]. Upon divergence from the optimum excitation wavelength, the emission wavelength also diverges, while the emission intensity decays fast [47].

Pan et al. [47]. made notable progress on this aspect by preparing so-called full-colour CDs, using microwave heating of a citric acid and formamide solution at 160 °C. These CDs showed excitation-dependent fluorescence with a satisfactory intensity across the entire spectrum. The excitation spectra (Figure 5(c)) ranged between 330 nm and 600 nm, while the emission region extended between 400 nm and 800 nm, displaying three different dominating maxima at 466, 555 and 637 nm, as depicted in Figure 5(b) [47]. PL lifetime measurements revealed the different nature of the emissions; namely blue emission attributed to transitions of the aromatic π system and green to red

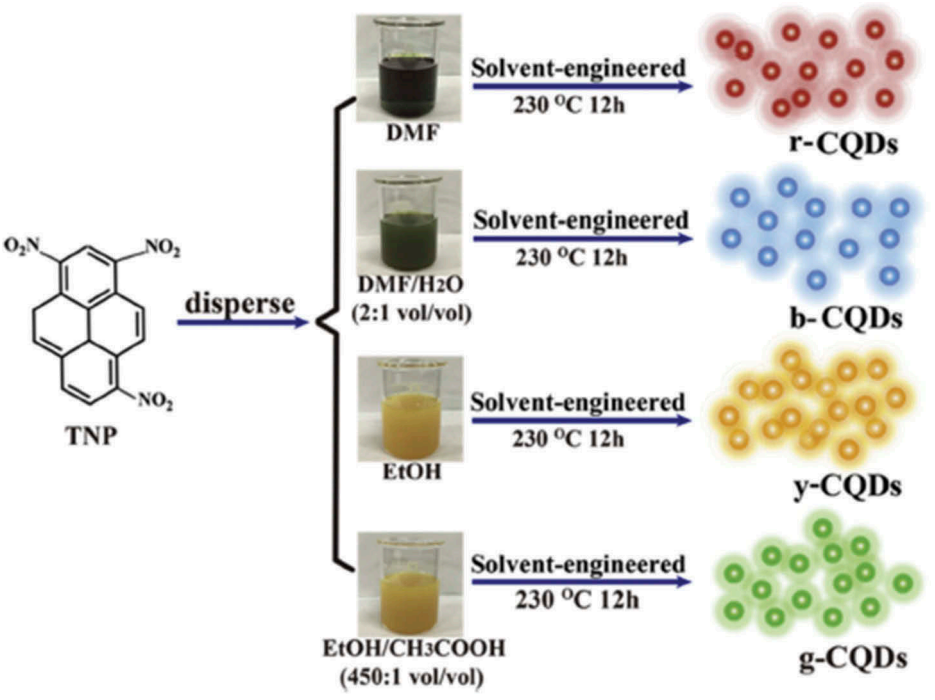

Figure 4. A schematic illustration of four different pathways for the preparation of multicolour fluorescent CDs. [Ref. 43; reproduced with permission].

emission assigned to n-π^* transitions of the C=O/C=N and C-N surface bonds [47]. The authors demonstrated these CDs have potential for multimodal sensing of metal ions and *in vitro* labelling agents with a substantial cell viability of 85%.

Consequently, from the viability outcome, another perspective emerges: the high immortality rate of CDs indicates their potential as bioimaging and biosensing agents for *in vitro* and *in vivo* studies. Although CDs are the best candidates for replacing semiconductor quantum dots in bioimaging, the average QY presented in the literature is still lower than that of semiconductor QDs. Consequently, the exploration of methods to enhance the CDs' QY is essential. However, when it comes to biosensing, biocompatibility, broad linear detection range as well as low detection limits seem to be equally important as a high QY.

4. Carbon dots for biosensing

The use of CDs as biosensing probes for bio-applications originates from their physical, electronic and electrochemical properties, which make them sensitive in different environments [9]. Owing to their small size, their sensitivity aptitudes increase, and furthermore, smaller samples can be

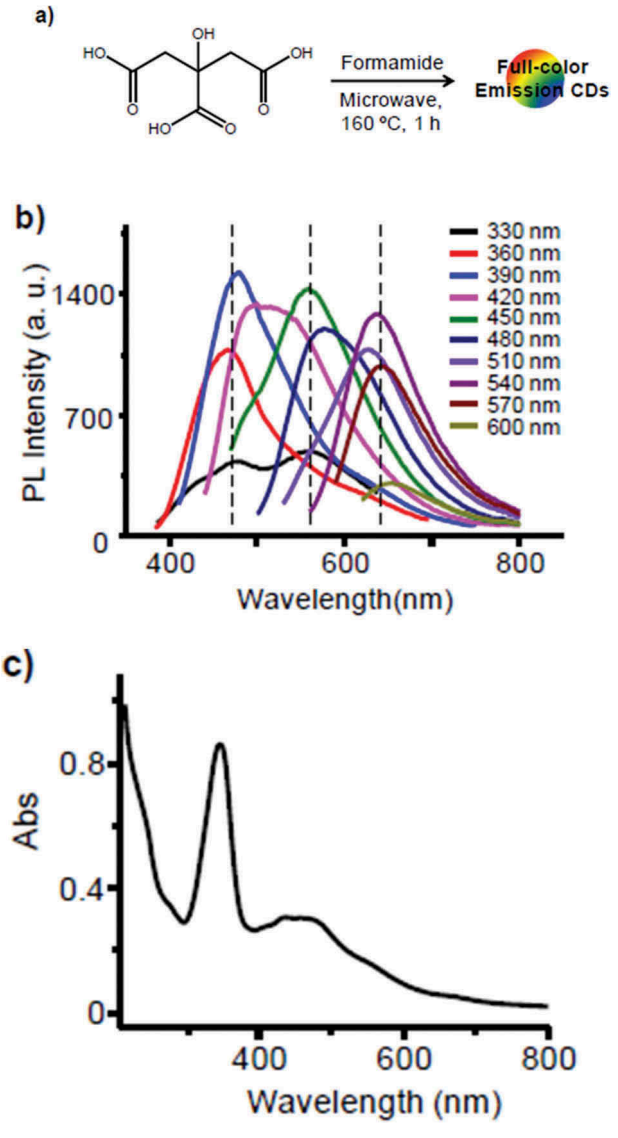

Figure 5. (a) A schematic illustration of the preparation of the CDs. (b) PL spectra of the CDs under different excitation wavelengths. (c) UV-vis absorption spectra of the CDs. [Ref. 47; reproduced with permission].

used in order to lower the cost [48]. Additionally, the turn to nanotechnology reduces the detection limits of biosensors, which need to be as low as ng/ml or even fg/ml [48]. In the past few years, it has been accepted that many degenerative disorders are not isolated from the host, but on the contrary, are completely correlated [18]. Nowadays, with CDs as biosensing probes, the presence of metal ions as warning signs for diseases in a human body can be easily detected.

4.1. Metal ion sensing

Transition, heavy and rare-earth metal ions in the human body can be related to many common diseases but also indicative of illnesses or long-term degenerative disorders [48–50]. Although numerous studies have been performed regarding application of CDs in biosensing, few reports manage to present a clear overview for different metals ions or to demonstrate superior sensing capabilities based on CDs. In the following paragraphs, we review some of the most recent studies, which have a pioneering character because they demonstrate unique results for a range of different metal ions.

The most common method for sensing metal ions is based on a fluorescent assay in order to evaluate the PL intensity changes in different concentration ranges [50]. Explicitly, the PL intensity of CDs exhibits good linearity in a broad concentration range of ions (linear detection region) and a low detection limit, meaning that ion concentrations of the order of 10^{-7} and lower can be detected. Undoubtedly, one of the most interesting metal ions is Al^{3+} because it is related to ageing [51], reduction of neural stem cells [51], high-risk factor for kidney damage [50,51], Alzheimer's [49,50] and Parkinson's diseases [49,50].

In 2017, Shi et al. [50]. showed that yellow emissive CDs with a QY of 11% could be obtained through a one-step hydrothermal treatment at 180 °C using 4-aminosalicylic acid as a carbon source. These yellow emissive CDs were used for Al^{3+} detection in aqueous solution with paper-based sensing, which is very convenient for application. A cytotoxicity assay revealed their high biocompatibility (85% viability with an MTT assay). Paper-based sensing of Al^{3+} in aqueous solution revealed two linear detection regions in the concentration ranges of 10–200 μM and 225–400 μM, as well as a detection limit of 1.69 μM [50]. Remarkably, the detection capability of these CDs is also specific since other examined ions showed negligible effects on the PL intensity.

Another crucial ion to detect in the human body is Fe^{3+} since it is a crucial co-factor of several proteins important in the prognosis of diseases such as Parkinson [52]. Additionally, Fe^{3+} has an essential role in the human metabolism and acts as an Escherichia coli metabolite [53,54]. Paau et al. [52]. presented a new method for CDs synthesis using chitosan as a carbon source, acetic acid as the condensation agent, and 1,2-ethylenediamine as the N-dopant. The N-CDs, which were prepared by microwave-assisted pyrolysis, showed QYs reliant on the N doping. CDs with low doping showed a QY as low as 6.8 % and highly doped CDs a moderate QY of 20.1% [52]. Furthermore, the linear detection region of CDs' PL was estimated for a concentration range of 0.28–50.7 μM, with 0.28 μM being the lower detection limit. Although these CDs showed a higher QY than other studies, recently more sensitive CDs with a lower detection limit and a broader linear

detection region such as those prepared with garlic, and trypsin and dopamine were demonstrated [55]. One such study is the work by Diao et al. [56], who prepared blue and green CDs using a one-pot hydrothermal treatment using syringa obtata Lindl as a carbon source. The cells incubated with blue and green emissive CDs showed 85 and 80% viability respectively, thus confirming their high biocompatibility. Although the QY for both types of CDs remained low (12.4 and 6.5% for blue and green CDs, respectively) interestingly, these CDs exhibited not only higher sensitivity to Fe^{3+}, but also sensitivity to H+ [56]. The linear Fe^{3+} detection was observed in a concentration range of 0.5–80 μM, with a lower detection limit at 0.11 μM. Additionally, the green CDs showed a linear enhancement of the PL intensity upon pH increase from 1.98 to 8.95 when applied for pH sensing [56]. These results indicate the potential of CDs for multi-sensing or sensing in various pH environments.

Another unique research was carried out by Sun et al. [55]., who demonstrated one of the lowest detection limits along with a broad linear detection region for the detection of Fe^{3+}. In their study, CDs, prepared by hydrothermal treatment of lycii fructus, showed a remarkably high viability of 90% combined with a moderate QY yield of 17.2%, a linear detection region for concentrations in the range of 0–30 μM and a detection limit as low as 21 nM [55]. To put this detection limit in perspective, Table 2 compares the sensing performance of different fluorescent probes for Fe^{3+} detection [55].

Importantly, this work presents CDs with very high sensitivity and a reasonable linear detection region and tests their versatility in sensing applications, including river water, urine and Hela cell line [55].

Recently, Liu et al. [57]. developed two types of CD nanohybrids, synthesized by solvothermal treatment using bamboo leaves, for radiometric fluorescent sensing of Pb^{2+} and Hg^{2+}. Detection of such heavy meal ions is vitally important because of their carcinogenic and toxic effects when entering the human body. With these new nanohybrids Liu et al. [57]. demonstrated detection limits of about 0.14 and 0.22 nM for Pb^{2+} and Hg^{2+}, respectively, which are very competitive values in comparison with currently used sensing probes.

Table 2. Comparison of the sensing performance of different fluorescent probes for Fe^{3+} detection. [Ref. 57; reproduced with permission].

Fluorescence probes	Material	Detection limit (μM)	Linear range (μM)
N,S-CDs	Blueberry	4	25–500
GQDs/PS-AER	Polystyrenicanion/exchange resin	0.65	1–7
DTPDA	4-(diphenylamino) benzaldehyde, etc.	0.38	–
GQDs	Ordered C-SiO2	0.3	3–60
CDs	Garlic	0.2	0–500
NDI-A	Mono boc-ethylene diamine, etc.	0.10	–
CNPs	Trypsin and dopamine	0.03	0.1–500
CDs	Cotton	0.027	0.05–10
S-GQDs	Graphite/sodium	0.0042	0–0.7
CDs	Lycii Fructus	0.021	0–30

Going one step further, work by Pan et al. [47]., already cited in section 3 above as an example of multidimensional sensing, demonstrated an appealing way for the detection of thirteen different metal ions relying on the fluorescence intensity of excitation-dependent CDs where the various metal ions anchor differently. The CDs were prepared as shown in Figure 5(a) and present three maxima, which have a crucial role in multidimensional sensing.

These maxima exhibit different PL intensity decay ratios upon different metal ion anchoring. Thus, they can be used in multidimensional sensing since they are able to detect a wide range of metal ions. Figure 6, which shows the fluorescence responses of the CDs, evidences that the emission ratio between the three maxima can be utilized as a highly accurate indication of the presence of a specific metal ion. This approach to metal ion sensing is suitable for simultaneous detection and discrimination of various ions as well as being very precise and diverse.

Likewise, Chen et al. [58]. synthesized by oxidative polymerization carbon-based nanoparticles (CNPs) for the detection of different metal ions considered xenobiotic and associated with various diseases.

Notably, the signal of eight different metal ions, Cr^{3+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, Cd^{2+}, Pb^{2+}, Co^{2+}, and $Ni^{2+,}$ could be identified for concentrations as low as 0.5 μM. The 'fingerprint' of the different metal ions was detected using double and triple sensing modes where mixtures of various ions at different concentrations were discriminated with very high accuracy.

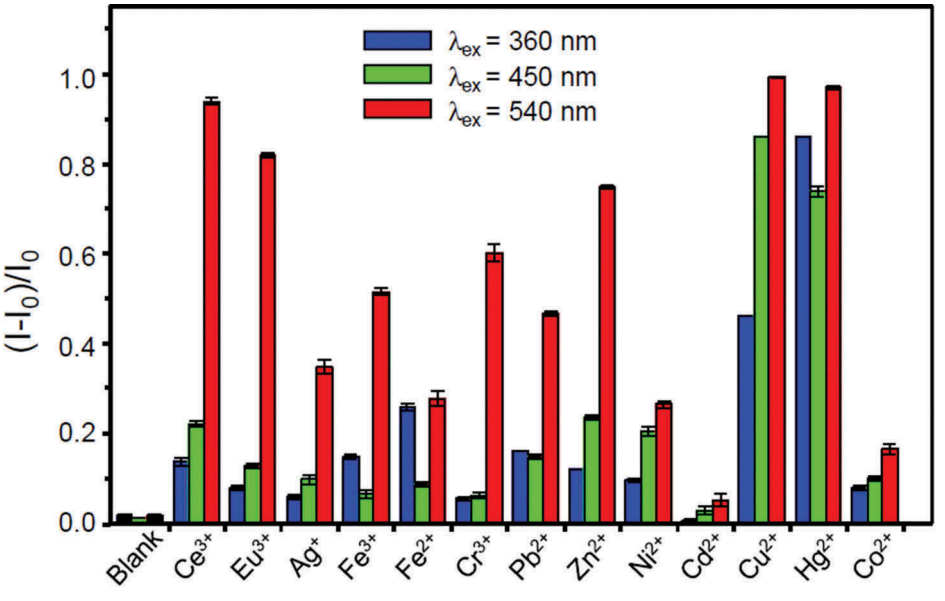

Figure 6. The fluorescence responses of the CDs as probes for 13 metal cations. The three maxima exhibit a different response in the presence of specific metal ions, thus determining which ion is detected. [Ref. 47; reproduced with permission].

However, although CDs are capable of sensing metal ions with very low detection limits, it is still a challenge to develop multidimensional sensors and improve the detection limits within one solution.

In conclusion, CDs have been shown beyond any doubt to be suitable for sensing and metal ion determination in different environments. Beyond that they also be used in bacteria sensing, as discussed in the next section.

4.2. Bacteria sensing

CDs also find application in bacteria detection and can be used *e.g.* to detect food contamination in order to prevent epidemics and global pandemics [59]. The first publication of CDs as bacteria probes appeared in 2015 when Zhong et al. [60]. prepared an assay with modified CDs for detecting Staphylococcus aureus (S. aureus) bacteria. As illustrated in Figure 7, the authors functionalized CDs using vancomycin, a broad-spectrum antibiotic, which strongly binds to the surface of bacteria and is responsible for fluorescence quenching. The study demonstrated a linear detection range between 3.1810^5 and 1.5910^8 cfu/mL and a detection limit of 9.410^4 cfu/mL for S. aureus bacteria [60]. The same CDs were also employed for the detection of Gram-positive bacteria. This group of bacteria includes micro-organisms such as *Bacillus subtilis, Listeria monocytogenes, Salmonella, Pseudomonas aeruginosa* and *Escherichia coli* (E. coli). The detection limit was improved in 2016 by Lavkush et al. [61]., who employed magnetic CDs functionalized with chitosan and observed a detection limit of 310^2 and 3.510^2 cfu/mL for S. aureus and *E. coli*, respectively.

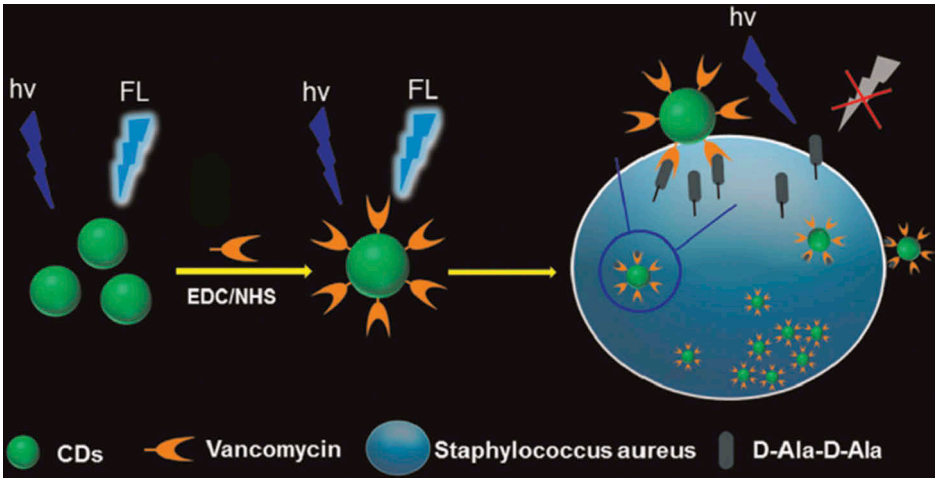

Figure 7. A schematic illustration of the preparation method of the CDs and the fluorescence signal derived from S. aureus bacteria detection. [Ref. 60; reproduced with permission].

The detection of *E, coli*, a species of bacteria usually found in the intestinal tract, was reported by Ahmadian et al. [62]. for CQDs prepared by one-pot hydrothermal treatment using lemon, grapefruit or turmeric extracts as a carbon source.

Notably, the as-prepared CQDs were combined with Fe_3O_4 nanoparticles, enabling them to be easily stimulated using magnetic fields. The authors observed PL intensity quenching as the population of the E. coli bacteria was growing, and implemented a quantitative detection of the latter using the Stern-Volmer relationship:

$$\frac{F_o}{F} 1 + K_{SV}[C] \tag{1}$$

where F_o and F are the PL intensities in absence and presence of E. coli, K_{sv} is the Stern-Volmer quenching constant and C is the bacteria concentration. The authors managed to prepare CQDs with a QY of ~20%, so as to estimate the population of bacteria after their addition into the CQDs solution [62]. Although the authors managed to prepare CDs with a considerable QY, the magnetic properties of the magnetic nanocomposites were not studied during their incubation with the bacteria.

Another interesting application is the bacteria detection via Foster Resonance Energy Transfer (FRET); this was implemented in 2018 by Choi and coworkers [63]. The authors prepared phenylboronic acid CDs, tested them in contaminated river water and observed a gradual increase of the PL signal after incubation with water bacteria for 60 min [63]. The most motivating part of this work is that these CDs permit detection with 100% performance for an extensive concentration range of bacteria [63].

Very recently, Zheng et al. [64]. created a fluorescent sensor array consisting of three different CDs functionalized with boronic acid (BA), vancomycin (Van) and polymyxin receptors (PM), respectively. These functionalized CDs could bind to various bacteria with different affinity, thus exhibiting different fluorescence intensities. Then, by using linear discrimi nation analysis, the authors demonstrated how to distinguish six different bacteria depending on their fluorescence response pattern [64]. The work of these authors can be characterized as exceptional since it managed to prove the existence of a specific bacterium amidst others, a breakthrough towards the realization of early diagnosis of bacterial infections. The approach recalls the one used by Pan et al. [47]., where one type of CDs with three different maxima were used to discriminate between metal ions. However, Zheng and coworkers [64] employed three kinds of CDs to obtain the different fluorescent intensities, which are dissimilar because of the various binding affinity of the bacteria to the different receptors. As shown in Figure 8, the fluorescence responses of the three CDs for different bacteria exhibit different ratios of intensities between them and can be used for detection and quantitative analysis of various bacteria colonies.

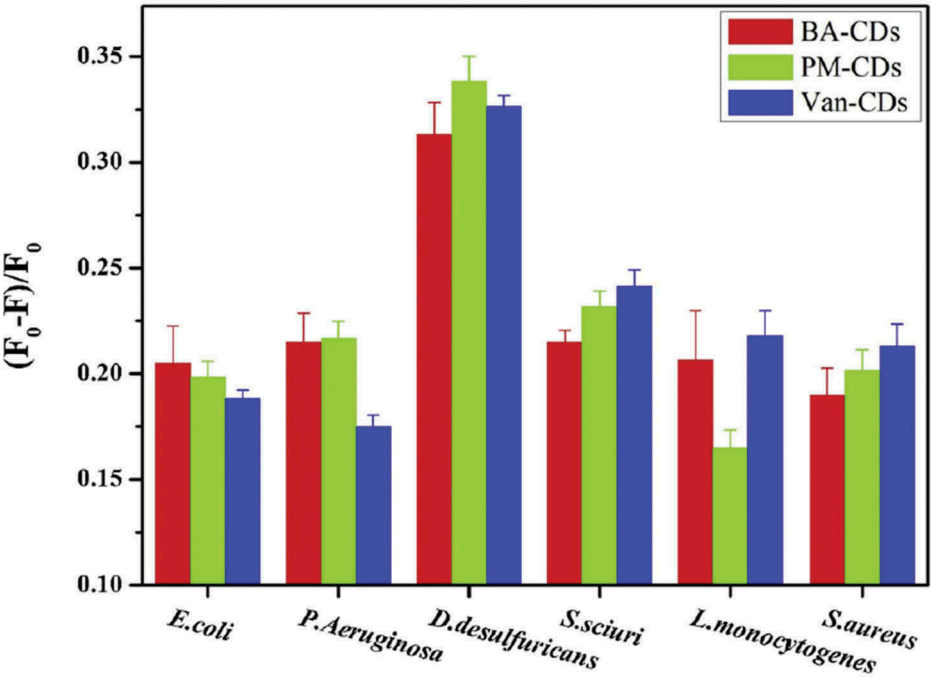

Figure 8. The fluorescence responses of the three different functionalized CDs in different bacterial environments. Different bacterial environments induce different fluorescence response and different ratio between the CDs. [Ref. 64; reproduced with permission].

All in all, this new study proves the high efficacy and versatility of CDs and gives a brief explanation of how CDs can be advantageous for the preparation of future simple and cost-effective sensor arrays for better bacteria sensing. Also, linear discrimination analysis seems promising for further development. Therefore, further functionalization studies of such CDs might lead to sensing an even broader range of targets.

4.3. pH sensing

pH-dependent behaviour of a probe cannot only be exploited for imaging and sensing of different environments, it can also utilized as a prognosis tool or even as a theranostic tool in biotherapy [46]. pH sensing is considered a highly beneficial property of CDs, which opens the way to *in vivo* early diagnosis for serious diseases such as cancer [46]. Detection of pH changes can in fact alert to pathological and physiological progressions such as cell cycle and apoptosis [65], receptor-mediated signal transduction, ion transport, muscle contraction, inflammation, and tumor growth [66]. Fluctuations of the PL intensity track the pH value after basic or acidic agents bind to or leave the surface of the CDs [66]. There are various

methods for pH sensing, all based on pH fluctuations, however, some of them lack photostability, others induce photobleaching, while many are very complicated and inconvenient. Some of these methods make use of small organic molecules and fluorescent proteins [66]. Nevertheless, their use has already started decreasing and they are being replaced by CDs, which have already displayed superior properties.

CDs prepared using p-phenylenediamine and ethanediamine as a carbon source by employing the microwave method were reported by Ding and coworkers [67]. The study showed bright orange fluorescent CDs comprising an emission at 592 nm with a QY of 14% and considerable viability of 91.5 % [67]. Particularly, these CDs showed enhanced fluorescent intensity as the pH declined from 9.45 to 2.45 and a linear detection region with gradual intensity change in a pH range of 4.45–7.00 [67]. Two months later, the same authors presented similar CDs, this time using the conventional one-pot hydrothermal preparation instead of the microwave technique [68]. The CDs were prepared using p-phenylenediamine as a carbon source and, as expected, they showed a fluorescent emission at 590 nm, having a QY of 11.5%, whilst the viability slightly dropped to 85% after 24 h of incubation with hepatic carcinoma cells (SMMC7721). In addition, these CDs exhibited two linear regions of pH in a range of 2.6–4.6 and 5–6.8 extending the linear range of the CDs of the previous work to more acidic environments [68]. Although these papers do not suggest substantially different results (single emission peak, small linear detection range), they illustrate similar and reproducible outcomes, and we consider them important because they confirm that dissimilar preparation methods result in CDs with comparable properties.

Up till now, many publications have proposed various types of CDs for pH sensing. However, CDs exhibiting only a single emission fluorescence maximum are influenced by factors such as the stability of the light source, the concentration of fluorescence probes, photobleaching, *etc*., which cannot be detected or recognized with a single fluorescence emission [66]. However, these restrictions can be overcome with ratiometric pH probes since the measured quantity is not the intensity of single fluorescence emission, but the ratio between the intensities of the two fluorescence maxima.

Apart from the ratiometric sensing, the biocompatibility of CDs is of equal importance, especially for *in vivo* studies. Interestingly, in 2017, Xiao et al. [69]. proposed a new green method for the synthesis of hydrophilic CDs that were prepared by direct addition of glucose powder into hot edible oil, and demonstrated that these CDs delivered intensity values in a broad pH range of 3.0–13.0. Remarkably, the fluorescent intensity showed a linear relationship with pH change. More importantly, the most distinctive characteristic of this work is the outstanding viability using the MTT assay. Specifically, the observed viability was higher than 98%, even after 24 h of incubation at

a high concentration of 1 mg/mL HeLa cells [69]. Additionally, these CDs showed a high affinity for Fe^{3+} metal ions because of the increased amount of -COOH surface groups [69]. Undoubtedly, CDs show potential for large scale applications because of their time- and cost-effective preparation. CDs for pH sensing seem very promising not only because of their low detection limits to different pH environments, but also their use in applications of vital importance, such as the early prognosis of tumors.

Kang et al. [70]. reported pH-responsive CDs for in vivo tumor-selective theranostics. In detail, the CDs were combined with zwitterionic molecules that possess a positive charge at acidic pH and a negative charge at basic pH. As a result, the CDs changed conformation in the acidic environment of the tumor cells (6.5–6.9 pH), releasing thus the therapeutic agents. The authors developed fluorescent assays to trace the acidic endosome/lysosome compartment inside MDAMB-231 tumor cells and perform photothermal therapy in balb/c mice. Their results showed good tumor cell uptake within 3 hours and negligible uptake by normal cells, an indication of their excellent suitability for in vivo applications. Last but not least, the complete ablation of the cancer cells and absence of noticeable organ damage elsewhere twelve days after treatment underlines the importance of pH-responsive CDs.

Recently, Gao and coworkers [71] developed pH-sensing probes with lysosome-targeting ability. For this, they developed CDs with excellent photostability, pH sensitivity and selectivity as well as low cytotoxicity, vital parameters for live imaging and sensing in living organisms. These CDs showed an increased fluorescent intensity in the acidic environment of lysosomes. However, the in vivo study showed CDs being metabolized by mice after 30 and 60 min, while the incubation time was estimated to 1 h for HeLa cells treated with CDs (100 μg mL^{-1}).

Consequently, it is still a challenge to develop pH-responsive sensors with great adaptability to different acidic environments. However, the excellent biocompatibility and selectivity of pH-sensing CDs make them outstanding candidates for concurrent diagnosis and treatment of tumors.

Another application of significant importance is DNA sensing. However, despite the fact that many studies have been performed involving nanoparticles, CDs as DNA probes have not yet been studied despite the fact that they exhibit excellent biocompatibility. In the next section, we present some recent studies in the field of DNA sensing that we find promising for further development.

4.4. DNA sensing

DNA sensors are of major importance since they can be extensively used in clinical, environmental and food analysis applications [72]. Although there are many reports for DNA sensors comprising QDs [73], there are only a few

involving CDs. Chai and coworkers [74] demonstrated GQDs with a QY of 20% which could serve as fluorescent probes for DNA after reduction with $NaBH_4$ (rGQDs). Exploiting the FRET between the rGQDs and carbon nanotubes (CNTs), the authors observed fluorescence intensity quenching upon π–π stacking interaction between the ssDNA-rGQDs and CNTs [74]. However, upon the addition of tDNA, ssDNA-rGQDs could dissociate from CNTs to produce dsDNA-rGQDs, thus recovering the fluorescence [74]. Subsequently, probing for complementary and mismatched nucleic acid sequences could be implemented with an ultra-low detection limit of the order of 10^{-10} M. Also, a biocompatibility assay showed that rGQDs, as well as CNTs, induce negligible effects on human cells.

GQDs with a QY of 15.5% have also been used for DNA damage monitoring in the report of Lu et al. [75]. In this work, cp53 ssDNA was directly grafted onto gold nanoparticles (AuNPs) instead of GQDs. The authors observed electrochemiluminescence signal quenching upon non-covalent binding between ssDNA-AuNPs and GQDs. Subsequent hybridization with p53 ssDNA was able to form dsDNA-AuNPs and weaken the non-covalent bond, inducing a gradual recovery of the electrochemiluminescence signal of GQDs [75]. The authors showed that this biosensor assembly could be used for the detection of DNA damage based on the different bonding ability to damaged p53 ssDNA and cp53 ssDNA-AuNPs. As a result, the difference between the quenching yields of AuNPs-ssDNA and AuNPs-dsDNA to GQDs created a sensing method for targeted DNA, with a detection limit of 13 nM. However, the authors indicate that the specific electrochemiluminescence biosensor requires further improvement for the quantification of nucleic acids and single nucleotide polymorphisms [75].

A lower detection limit of 0.45 nM was reported in 2018 by Zhang et al. [76]. who encapsulated CQDs in ultra-small platinum nanocrystals over nitrogen-doped graphene to form hybrid nanocomposites. The prepared nanocomposites were used for the detection of 8-OH-dG in human urine as well as damaged DNA and guanine, respectively, addressing the intensity change of the electrochemical signal of 8-OH-dG with DNA damage [76]. Despite the fact that the CDs have been used for DNA sensing, the work is in an early stage and complicated in terms of the preparation methods and number of materials used.

Conclusively, although CDs can effectively be used as DNA sensors, they do not show lower detection limits than 10^{-10} M, while other DNA biosensors have already surpassed that limit [72]. However, their detection limit is considerably low, and combined with their high biocompatibility makes them applicable *in vivo*. This calls for improvements in order to reduce the detection limits further and replace the existing DNA sensing agents.

4.5. Temperature sensing

Temperature sensing plays a vital role in nanomedicine as a novel diagnostic tool. Functionalized CDs provide precise, non-invasive temperature sensing for biomedical applications. Fundamental cell studies, as well as biomedical diagnosis, can be carried out by the development of novel CDs-based nano-temperature probes. Recently, Macairan et al. [77]. developed dual emission ratiometric temperature sensing probes via microwave synthesis. By monitoring the blue and red fluorescent spectra of CDs in living cells, they managed to detect temperature variation in the range of 5–60 °C with an absolute linear response ($R^2 = 0.999$). Interestingly the study reports a temperature sensitivity of 3.71% $^{o}C^{-1}$, which is comparable to polymer and quantum dot nanothermometers, while the thermal resolution was calculated to be as low as 0.048 K^{-1}. Similarly, Nguyen et al. [78]. developed multi-emission radiometric CDs with three distinct peaks at 400, 465, and 480 nm originating from different surface states. The temperature sensitivity of the dots was estimated to be 1.48% $^{o}C^{-1}$, slightly lower than the one reported by Macairan et al. [77]., but these CDs show an almost perfect linearity ($R^2 = 0.998$) over a wider temperature range. Additionally, the ratio of the peaks remained unchanged after a temperature reversibility test, conducted between 20 and 50 °C.

On the other hand, Liu et al. [79]. fabricated CDs with a wide temperature sensing range from −50 to 60 °C via a solvothermal crystallization process. These CDs promise well for low and high temperature in vitro application. They also showed an excitation-dependent photoluminescence. The capability to detect temperature fluctuations in such a wide temperature range will open the way to more fluorescence-based applications.

Multifunctional CDs capable of performing not only temperature sensing but also metal ion sensing and cellular imaging was reported in 2018 by Du et al. [80]. These N,P-dual-doped CDs, synthesized via acid-base neutralization using a blend of synthetic chemicals, were capable of temperature sensing in a wide range of temperatures from 25 to 80 °C, with a sensitivity of 1.79% per ^{o}C. In additions, their QY of about 15% is relatively high and they are suitable for Mn(VII) detection with a very low detection limit at 0.43 nM and a broad linear range from 10 μM to 200 μM.

In conclusion, the high-temperature sensitivity of CDs makes them promising for in vivo temperature sensing for biomedical prognosis. However, biocompatibility tests and advances towards a better QY are of vital importance in this context and should be addressed in the future. Additionally, multifunctional temperature sensing, along with cellular imaging makes these nanoprobes attractive for many other practical biological applications.

4.6. Multifunctional sensing

Recently, the advances in biology led to a breakthrough with multifunctional CDs, capable of detecting various types of compounds. These CDs can be applied for concurrent pH and metal ion sensing as well as imaging, a property that makes them promising for in vivo monitoring in the near future.

Moonrinta et al. [81]. expanded the conventional vapour sensing of CDs to multidimensional ion sensing. Specifically, the group prepared CDs from yogurt using a two-step pyrolysis/hydrothermal method. By monitoring the optical absorption of the CDs, they managed to detect formic acid vapour, while using a conventional photoluminescence technique they could sense metal ions with the same CDs. These authors also proved the interactions of the CDs and the ions at the binding sites, and they used component analysis to discriminate the ions. However, what is captivating in this story is the excellent photostability and the negligible mortality of the test cells, factors that confer to these particular CDs a high potential for biomedical applications.

Similarly, Li and coworkers [82] managed to integrate multidimensional sensing in a platform based on a single type of CDs, which could detect Fe3+, Cr(VI), and ascorbic acid via photo-induced electron transfer, inner filter effect (IFE) and elimination of IFE. Interestingly, in this work the CDs were tested on real samples: tap and lake water for the ion detection, as well as human urine for monitoring the ascorbic acid in real-time after swallowing ascorbic acid tablets.

However, while the papers discussed above focus on the multifunctionality of the CDs, the reports of Hu et al. [83]. and Khan et al. [84]. additionally consider the CDs' efficiency. In particular, Hu and coworkers fabricated CDs for imaging with very high quantum yield (54.3%), able to condense with DNA and showing a very high biocompatibility. The combination of these properties allowed the team to perform gene delivery [83]. Khan et al. [84]. demonstrated high QY CDs (46.4%), prepared from diammonium hydrogen citrate and urea, that could be applied in cellular imaging, Fe3+ sensing and, thanks to their biocompatibility, as fluorescent ink [84]. Both papers demonstrate CDs with more than one functionality, very high QYs and biocompatibilities, with top-class potential in view of future applicability.

Another interesting paper by Thongsai et al. [85]. extends the biomedical applications of their CDs with a different technological approach. These CDs prepared from tetraethylenepentamine and maleic anhydride, demonstrated a high quantum yield towards metal ion sensing, but could also be utilized to prepare fingerprint detectors and fluorescent plastics. Although the latter do not fall within the topic of biomedical applications, they are attractive in terms of potential use and versatility of CDs. All in all, multi-

sensing CDs constitute a powerful tool to overcome biological barriers, which otherwise up to now demand different devices for detection, involve complicated processes and require excessive time. Up to now CDs have generated new approaches to biosensing and bioimaging. However, the multifunctionality of CDs is not constrained to these fields as CDs can also be utilized for cancer treatment and other relevant theranostic applications, as discussed in the next section.

5. Carbon dots for biotherapy

Established techniques for biotherapy still need improvement since they are not always efficient in that they often prolong the life of patients only for days and not years or have very severe side effects. Invasive techniques, as well as chemotherapy, always induce irreversible damage to normal cells [86].

In the past decade, QDs were used as fluorescent agents for bioimaging and biotherapy since they are superior in terms of water dispersability and photostability [86]. CDs as a new class of nanoparticles, exhibit superior properties to their semiconductor forerunners regarding fluorescence intensity, photostability and photobleaching as well as cytotoxicity. More recently, CDs have also found their way into many biotherapy applications such as drug delivery [87], photodynamic [25] and photothermal therapy [8] as well as other more specialized methods. CDs are especially interesting in the field of drug delivery because of their biocompatibility and the fact that chemotherapy is one of the most well-established techniques for cancer therapy. [86,88]

5.1. Carbon dots as drug delivery agents

Although many chemotherapy drugs for cancer treatment such as doxorubicin (DOX) are approved for commercial use, they exhibit many drawbacks which should be considered for further improvement [87,89]. As an example, their relatively low permeation and retention (EPR) effect, low cell internalization and cytotoxicity to normal cells should be considered [90]. A reduction of these detrimental effects can be achieved by introducing CDs as theranostic agents for cancer treatment [46].

Such an approach was implemented by Yang et al. [87]., who grafted the common drug carrier β-cyclodextrin to CDs, to induce and also enhance controlled targeting. The CDs were prepared by one-pot hydrothermal treatment using a mixture

of citric acid, branched polyethyleneimine (BPEI) and folic acid (FA) at 200 °C. Following that, boric acid was introduced onto the CDs' surface, which then created a covalent bond with the drug carrier β-cyclodextrin. That bond dissociated in the acidic environment of tumor cells and thus β-

cyclodextrin could be released to expose the cell to the drug, as depicted in Figure 9. An advantage of this method is that the drug is not bound directly on the CD's surface but to β-cyclodextrin, thus inducing low cytotoxicity in normal cells and preventing the uncontrolled leakage [87].

Although this method showed increased biocompatibility for normal cells in comparison with the injection of free DOX, the viability was comparatively low [87]. While free DOX injection in normal and HeLa cells induced viability as low as 5% for both cell types, the induced viability using CD-β-cyclodextrin was 35 and 10% for normal and HeLa cells, respectively. These results demonstrate how promising the CDs could be as drug delivery agents.

In 2016, Feng et al. [90]. created pH/redox dual-responsive CDs to enhance anticancer drug delivery. The authors combined an ensemble of compounds, which were introduced on CDs prepared by citric acid and diethylenetriamine under a nitrogen atmosphere at 170 °C for 3 h. In detail, they developed CDs with Arg-Gly-Asp (RGD) peptide as targeting ligand, cisplatin (IV) as a prodrug and methoxy poly(ethylene glycol) (mPEG) as passivation for inhibition of the immune system, as shown in Figure 10(a)

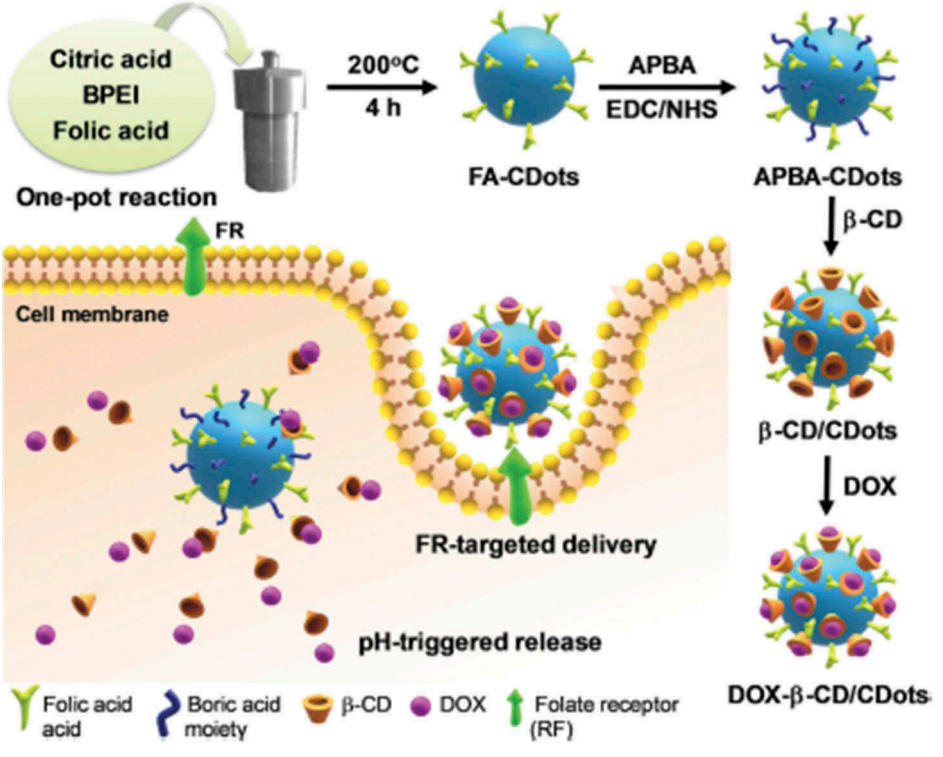

Figure 9. A schematic illustration of the preparation and the passivation of the CDs along with the internalization into the cell and the drug release mechanism. [Ref. 87; reproduced with permission].

[90]. In the tumor area, these CDs (CDs-RGD-Pt(IV)-PEG) undergo decomposition of PEG due to hydrolysis of the benzoic-imine bond at pH 6.8. Consequently, the RGD peptide is deshielded, inducing an enhanced affinity towards the integrin $\alpha_v\beta_3$ receptor, as sketched in Figure 10(b) [90]. After CDs-RGD-Pt(IV) enters the cell membrane, the cisplatin (IV) prodrug is converted to cisplatin, which binds to the nucleus' DNA and kills the cancer cell [90]. The authors observed promising results for the viability of human breast adeno-carcinoma epithelial cells (MDA-MB-231), which at a concentration of 200 μg/mL remained as high as 95% at pH 7.4, while it declined to 60% at pH 6.8 [90]. However, the same does not apply to MCF-7 cancer cells, which express a low concentration of the integrin $\alpha_v\beta_3$ receptor on their surface, thus demonstrating that CDs-RGD-Pt(IV)-PEG can only be applied to very specific cancer cells.

A more promising result concerning the viability was reported later by the same group [91]. They used cisplatin (IV) again as prodrug but did not employ RGD peptide as targeting ligand. Instead, they grafted ligands capable of alternating the charge character of the CD and inducing cell internalization at tumours. In particular, the CDs were passivated by PEG functionalized poly-(allyamine)/dimethylmaleic acid (DMMA) that induces a negative potential on the surface of CDs-Pt(IV)@PEG-(PAH/DMMA). Creating a negative charge on the CDs' surface resulted in an increase of the EPR effect and a prolonged circulation time since there is an induced screening between the cell membrane and the CDs' surface. On the other hand, at the extracellular environment of tumors, the CD's surface potential could alternate from negative to positive because PEG-(PAH/DMMA) is hydrolysed in more acidic pH, thus inducing cell internalization as a result of Coulombic forces. ζ potential experiments at 37 °C for 4 h showed that charge rises from −16.15 to +12.10 mV, causing uptake by a negative cell membrane at pH 6.8 [91]. Cytotoxicity measurements in A2780 cells showed a mortality of about 60% at pH 6.8, while the viability at pH 7.4 declined to 80% at a concentration of 11.44 μM [91].

Even though the work of Feng et al. [90,91] does not show extremely high cytotoxicity towards cancer cells, the results are of great importance, since they show very high internalization by tumor cells, fewer side effects and controlled release of cisplatin.

Another intriguing application of these novel materials was reported recently by Yang and coworkers [92], who demonstrated CDs capable of inducing osteoblastic differentiation for osteoporosis therapy and bone regeneration. The study reported a new kind of Mg^{2+}-doped CDs (Mg-CDs) prepared with a one-pot hydrothermal method using metal gluconate salts. Magnesium is an important element found in human bones and teeth, and known to affect cell proliferation and differentiation of osteoblast cells by improving alkaline phosphatase (ALP) activity and up-regulation related mRNA expression [92]. Treating mouse embryo osteoblast precursor cells (MC3T3-E1) with Mg-

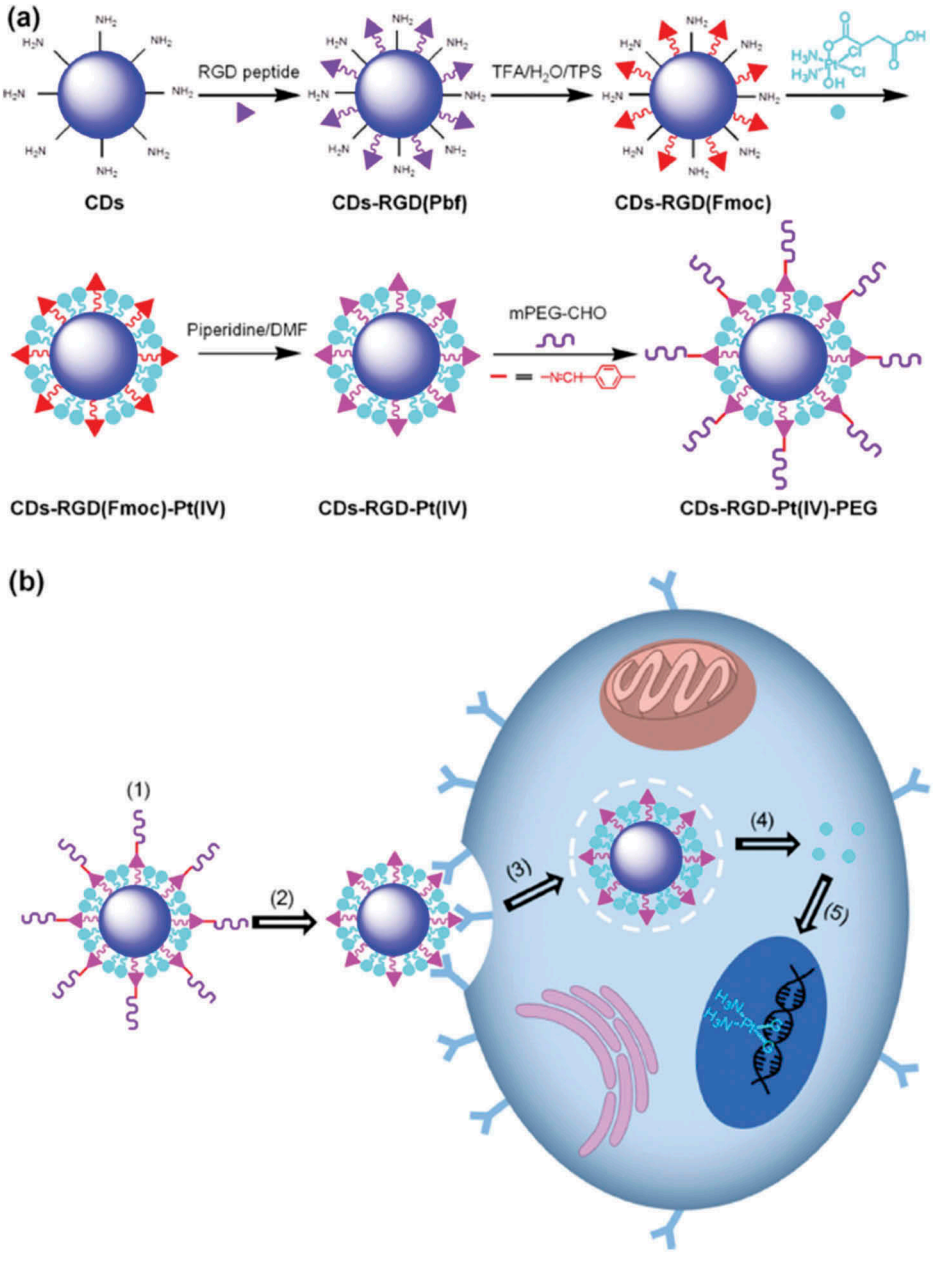

Figure 10. (a) The preparation pathway followed by Feng et al. [90] for the preparation of CDs-RGD-Pt(IV)-PEG; b) final process for cancer treatment. (1) fluorescent tracking of CDs-RGD-Pt(IV)-PEG before internalization, (2) tumor targeting by RGD peptide, (3) effective uptake by cancer cells through ligand-receptor interaction, (4) cisplatin(IV) prodrug is transformed to cisplatin under reductive cytosol, and (5) cisplatin attacks DNA in nucleus inducing cytotoxicity. [Ref. 90; reproduced with permission].

CDs, Mg^{2+} is delivered to the cells, which favours osteogenesis, and is attributed to the efficient uptake of CDs by the cells. Notably, although other metal doped

materials cause considerable cytotoxicity effects, metal doped CDs show negligible cytotoxicity, making them highly biocompatible [93].

5.2. Carbon dots as photodynamic agents

Photodynamic therapy (PDT) is one of the most promising non-invasive methods for cancer treatment since it results in highly efficient tumor killing with few side effects [94]. Moreover, PDT causes negligible drug resistance, less cytotoxicity and is thus preferable to traditional methods such as surgery, chemotherapy and radiotherapy [94]. However, the poor water solubility, photostability and absorption by nucleus are considerable downsides for the PDT agents [94].

Singlet oxygen, reactive oxygen species, as well as free radicals, are considered essential ingredients in PDT [25]. Nuclei readily react with oxygen species produced by photosensitizers or bare CDs under laser illumination in the presence of oxygen [25]. Preparation of GQDs with high singlet oxygen ($^1O_2$) generation *via* multistate sensitization was demonstrated in 2014 by Niu et al. [86]. as a solution to the issues mentioned above. GQDs were prepared by a one-pot hydrothermal method using polythiophene derivatives (PT2) as a carbon source and exhibited a preparation QY of ~130%, high water dispersibility and a strong emission peak at 680 nm. These results suggest that GQDs can be used for bioimaging and bio therapy *in vitro* and *in vivo* due to their high biocompatibility.

Additionally, a theoretical study convincingly demonstrated multistate sensitization as responsible mechanism for the high $^1O_2$ QY [86]. The authors showed that the excited triplet state of the GQDs is energetically lower than those of the traditional photosensitizers, as illustrated in Figure 11 [86]. As a consequence, the energy difference between the excited singlet and triplet state is energetically favourable for the formation of additional $^1O_2$ that has a formation energy of 22.5 kcal/mol. Viability measurements on HeLa cells showed that under irradiation the GQDs kill more than 80% of the cancer cells. For comparison, the concentration of protoporphyrin IX (PpIX) in HeLa cells should be ten times the concentration of GQDs mentioned above, to achieve the same mortality rate [86].

Although the work of Niu and coworkers [86] proved that GQDs induce higher cytotoxicity to cancer cells than PpIX, recent studies corroborated that traditional photosensitisers such as Ce6 are potentially good candidates for PDT when combined with CDs [10]. In 2018 Hua et al. [25]. demonstrated PpIX-based CDs, which induce a mortality rate of 92%. Using confocal microscopy, CDs fabricated by hydrothermal reaction of m-phenylediamine and L-cysteine, were observed to target the nucleolus that serves as cancer premonitory since it is responsible for ribosomal RNA production. Changes in nucleolus structure and population indicate

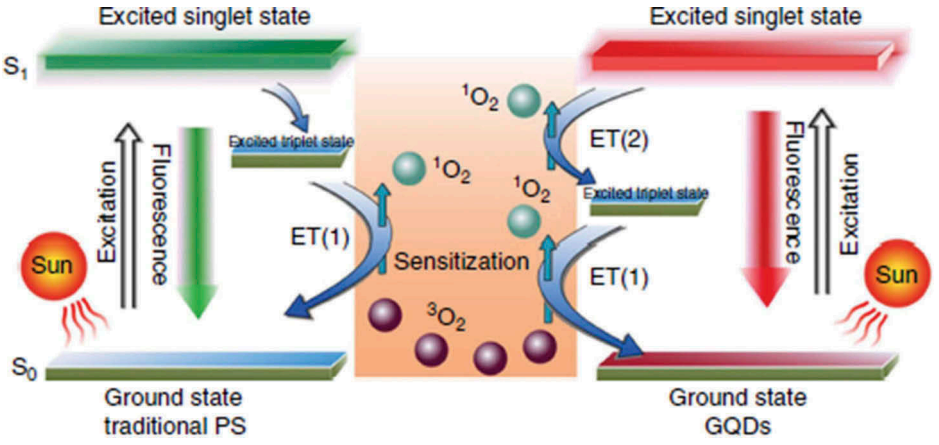

Figure 11. A schematic illustration of the energy diagram for the singlet oxygen generation by traditional photosensitizers (PS) and graphene quantum dots (GQDs). [Ref. 86; reproduced with permission].

a human disorder [25]. The authors ascribe the high cytotoxicity to the increased cell uptake, which was observed after binding of PpIX to the CDs. In addition, $^1O_2$ generation was estimated to 62.1% and found sufficient to induce high cytotoxicity to HeLa cancer cells after 4 h of incubation and 10 min laser irradiation at 635 nm (20 mW/cm^2) with a PpIX concentration of 4 μg/mL [25].

Regarding viability, Li et al. [10]. used chlorin Ce6-conjugated CDs (CDs-Ce6) for enhanced PDT as well as for bioimaging. Initially, CDs were considered for enhancing the fluorescence properties of the photosensitizer, which is normally excited by blue or UV light that has limited penetration and thus lower PL efficiency. Using fluorescent FRET excitable CDs, the fluorescence properties of the photosensitizer were enhanced, resulting in better fluorescence detection. Furthermore, after laser irradiation at 671 nm (30 mW/cm^2) with a concentration of 20 μM, MGC803 cells presented a mortality rate of about 90%. Without laser irradiation, the cell viability remained high at 90%, making this technique suitable for *in vivo* experiments [10].

Even though the previous papers mention high $^1O_2$ generation upon irradiation, solid tumors are recognised for their low oxygen environment, a feature commonly known as hypoxia. In order to deal with that issue, Zheng et al. [95]. demonstrated a new method to boost PDT *via* water splitting. Specifically, CDs were initially decorated with C_3N_4 to enhance their absorption in the red, a polymer containing protoporphyrin, a polyethylene glycol segment and a targeting RGD ligand. *In vivo* studies demonstrated that these CD-C_3N_4 nanoparticles had the ability to create a normoxic environment *via* water splitting, which is favourable for

enhanced PDT. Cell viability was tested for 4T1 cells and showed that under laser irradiation at 630 nm (50 mW/cm^2) for 5 min the new composite could achieve almost the same cell viability either in the hypoxic or normoxic environment at a concentration of 10 μg/mL. On the other hand, in a different experiment [95], PpIX treated 4T1 cells were proven incapable of inducing the same extent cytotoxicity in hypoxic and normoxic as CD-C_3N_4 nanoparticles. Therefore, the potential of CD-C_3N_4 nanoparticles in hypoxic environments can be of great importance, especially in combination with other methods such as photothermal therapy, which can have prominent results regardless of the low oxygen environment of tumors [95].

5.3. Carbon dots as photothermal agents

Not only photodynamic therapy, also photothermal therapy (PTT) has been proven to induce irreversible damage to many cancer cell types [96]. CDs can be used as potential photothermal agents since they can induce enormous temperature changes under irradiation [8].

In 2016, Sun et al. [8]. demonstrated CDs emitting in the red, which could be used for *in vivo* studies. In addition to their convincing bioimaging properties, these CDs were capable of drug delivery and PTT. The photothermal conversion efficiency was estimated to be 43.9% based on the fact that during laser irradiation at 671 nm (2.5 W cm^{-2}) for 3 min the water temperature increased from 19.5 to 60.4 °C with increasing CDs concentration (20–200 μg/mL) [8]. This high photothermal conversion efficiency could prompt cytotoxicity and efficiently kill ~90% of the HeLa and MCF-7 cancer cells at a concentration of 100 μg/mL [8]. Furthermore, at twice that concentration (200 μg/mL) the authors observed almost 100% mortality of these cancer cell[8].

Similarly, Wang et al. [97]. demonstrated near infrared-emissive CDs with a peak at 1000 nm. These CDs were doped with nitrogen and boron in order to achieve long wavelength emission and induce higher tissue penetration[97]. The as-prepared B-N-CDs showed a photothermal efficiency of 32.3%, which is lower than that of the red emissive dots of Sun and coworkers [8], but these CDs were slightly more cytotoxic for SF-763 cells. In fact, SF-763 cells treated with B-N-CDs at 100 μg/mL for 2 h, showed 96% mortality when irradiated with NIR light (808 nm, 1.5 W/cm^2) for 5 min [97]. Astonishingly, non-irradiated cells treated with B-N-CDs displayed viability of 90%. These promising results make CDs and PTT potential candidates for replacing conventional cancer treatment methods. However, progress is still needed in preparing CDs with lower cytotoxicity effects on normal cells.

Another approach proposed by Bao et al. [98]. showed the preparation of near infrared-emissive CDs with a maximum photothermal conversion

efficiency of 59.2%. The authors doped CDs with sulfur and nitrogen and tested them *in vivo* after intravenous injection. Unfortunately no information is given about the tumor mortality rate after laser treatment; however, viability was tested without laser treatment for three cell lines (4T1, HepG2, HeLa), and varied between 95–100% at the very high concentration of 1000 μg/mL, thus pointing to a very high biocompatibility of sulfur-nitrogen CDs [98].

In another pioneering work, GQDs with PDT and PTT properties were demonstrated by Ge and coworkers [12]. These authors used polythiophene benzoic acid as a carbon source to produce GQDs, which displayed a photothermal conversion efficiency of 36.2% when combined with polythiophene quaternary ammonium and a singlet oxygen efficiency of 27% when associated with polythiophene phenylpropionic acid as precursor. Using single red laser (635 nm, 2 W cm^{-2}), these GQDs induced a mortality rate of ~95% at a concentration of 200 μg/mL [12]. The authors showed that the high mortality rate derives from synergetic effects of PTT and PDT; in fact, their individual effects in separated experiments showed only limited results.

A recent study of Wu et al. [99]. demonstrated CDs with photodynamic and photothermal capabilities. The authors attached the Ce6 photosensitizer on the surface of CDs, which were proven earlier to display a high photothermal conversion efficiency (46%), in order to achieve higher cell mortality after irradiation via PDT. Low power NIR laser irradiation is desirable for clinical use since it offers less laser irradiation injuries, increases accuracy and causes less pain to patients. Interestingly, the photoacoustic, fluorescence and photothermal capabilities of the Ce6-functionalized CDs (Ce6-CDs) offer potential targeting, which results in better and painless phototherapy treatment with fewer damages [99]. The authors tested these Ce6-CDs on three cancer lines (4T1, HepG2, HeLa) and found no cytotoxic effects, while upon laser irradiation (671 nm, 500 mW cm^{-2}) for 20 min at a concentration of 200 μg/ml the mortality rate varied between 80 and 90% for the different cancer lines [99]. Despite the fact that the mortality rate is not the highest found in the literature [97], the authors managed to induce high mortality rates in cancer cells by using low irradiation power and a small amount of Ce6 [99]. These results indicate that not only the Ce6-CDs can be employed for *in vivo* studies, but also for clinical applications since they treat the tumor area in a more effective and precise way than other already reviewed studies.

All in all, we have seen that CDs can be the ultimate prognosis and treatment agents of many diseases, particularly of cancer. However, further investigation of their affinity to tumor binding sides is required to increase their biocompatibility and their effectiveness. CDs have undoubtedly proven their efficacy in theranostics and we expect them to be applicable in humans in the near future.

6. Conclusions

Carbon Dots have already shown that they can be potentially beneficial in many fields of biomedical research and applications and are expected to fully replace conventional semiconductor quantum dots in the coming years. *In vitro* and *in vivo* studies of CDs have demonstrated outstanding results regarding their cytotoxicity, biocompatibility and photostability. Moreover, their versatility makes them suitable for multimodal sensing and cancer treatment, increasing their efficacy in biotherapy. Additionally, their sensing properties have attracted tremendous interest since they can be used for various biomedical applications. More importantly, CDs can be prepared easily and cost-effectively by an immense number of methods and compounds, including almost every known organic complex as a carbon source. In this review, we presented CDs prepared with various techniques, showing an abundance of properties and different biocompatibilities. Now is the time to switch to a universal system for the preparation, which can also be scaled up to industrial production and opens the way to CD-based agents for biomedical applications that are better in sensing and treatment than currently used agents. Unquestionably, CDs constitute a promising material for theranostic and will play a vital role in many other applications in the near future.

Disclosure statement

No potential conflict of interest was reported by the authors.

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