Opportunities and Challenges of Fluorescent Carbon Dots in Translational Optical Imaging

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Opportunities and Challenges of Fluorescent Carbon Dots in Translational Optical Imaging

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Abstract: The fluorescent carbon dot (C-dot) is a new class of carbon nanomaterials. It has a discrete or quasispherical structure, typically measures less than 10 nm and contains sp²/sp³ carbon, oxygen/nitrogen-based groups and surface-modified functional groups. Compared with semiconductor quantum dots (QDs), C-dots offer much lower toxicity and a better biocompatibility profile. Their other favorable features include easy and inexpensive synthesis and surface modification potential. C-dots can be morphologically classified into graphene-based quantum dots (GQDs) and amorphous carbon nanodots (ACNDs). Numerous methods have been developed to synthesize C-dots, and are mainly divided into ‘top-down’ and ‘bottom-up’ routes. In the top-down route, C-dots (mostly GQDs) is derived from the separation of large carbon precursors. The ‘bottom-up’ method primarily involves the dehydration, polymerization and carbonization of small molecules to form the GQDs and ACNDs through thermal/hydrothermal synthesis, microwave irradiation, and solution chemistry. Potential applications of C-dots have been explored in a number of cellular and in-vivo imaging approaches. However, some difficulties remain, including limited penetration depth and poorly controlled in-vivo pharmacokinetics, which depends on multiple factors such as the morphology, physicochemical properties, surface chemistry and formulation of C-dots. The exact mechanism of in-vivo biodistribution, cellular uptake and long-term toxicological effect of C-dots still need to be elucidated. An integrated multi-disciplinary approach involving chemists, pharmacologists, toxicologists, clinicians, and regulatory bodies at the early stage is essential to enable the clinical application of C-dots.

Keywords: Carbon Dots (C-dots), Photoluminescence (PL), Near-infrared (NIR), Quantum yield (QY), Optical Imaging, Iron oxide, Fe₃O₄.

1. INTRODUCTION

The rapid development of fluorescent nanomaterials over the past three decades has led to increasing biological and medical research into their potential applications [1]. Fluorescence nanoparticles are colloidal semiconductor quantum dots (QDs) measuring ~5-10 nm in size [2]. Compared with organic fluorophores, QDs are approximately 20 times brighter and 100 times more photostable than standard fluorescent dyes [3]. Due to their high photoluminescence quantum yield (PL QY) and the extent to which their optical properties can be modified by slightly altering their size, these nanoparticles have been explored as a platform for imaging applications [4-7]. However, metal elements, such as cadmium, are widely used in QDs (e.g. CdX, X= Te or Se or S), which makes clinical application difficult as these metal elements can be toxic [8, 9]. Although the toxicity of these QDs can be partially mitigated by embedding the toxic core with polymers or silica to form a core-shell structure [1, 10], such encapsulation methods result in larger hydrophilic QDs (~20 –30 nm). This would significantly limit in-tissue mobility and may hinder Förster resonance energy transfer (FRET), as well as induce undesirable in-vivo behaviors [11]. Compared with semiconductor QDs, fluorescent carbon dots (C-dots) is perceived as lower toxic alternatives in the development of optical imaging agents for various biomedical purposes [12-18].

In this review, we first present the composition and classification of C-dots, and compare the various synthetic approaches. Then, we provide an overview of optical performance, surface functionalization and nanocomposites of C-dots. In-vitro/in-vivo studies and toxicity profile of C-dots are then summarized, followed by a discussion of the requirements for clinical applications.

2. COMPOSITION AND CLASSIFICATION OF C-DOTS

C-dots commonly refers to a group of carbon fluorescent nanoparticles that have a discrete or quasispherical structure and typically measure less than 10 nm. They contain sp²/sp³ carbon, oxygen/nitrogen based groups and surface-modified functional groups [9]. They were discovered among the fragments of single-walled carbon nanotubes (SWCNTs) during purification [20]. Over the last decade, fluorescent C-dots have attracted much attention in bio-applications because of their small size, tunable fluorescence, inexpensive fabrication, and biocompatible features. Numerous important discoveries have been made, particularly with regard to improving their PL properties and methods to synthesis C-dots. To date, various types of C-dots have been synthesized and they can be morphologically classified into two major types, graphene-based quantum dots (GQDs) and amorphous carbon nanodots (ACNDs). In most cases, GQDs mainly consist of an sp² carbonic nanocrystalline structure, while ACNDs comprise a disordered carbon framework integrated by coexisting aspects of optical imaging agents for various biomedical purposes [12-18].

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In this review, we first present the composition and classification of C-dots, and compare the various synthetic approaches. Then, we provide an overview of optical performance, surface functionalization and nanocomposites of C-dots. In-vitro/in-vivo studies and toxicity profile of C-dots are then summarized, followed by a discussion of the requirements for clinical applications.
sp$^3$ and sp$^2$ hybridized carbon moieties with the possibility of surface functionalization. This irregular configuration is probably caused by relatively higher amounts of oxygen/nitrogen containing moieties that disrupt the systematic carbonic framework through covalent bonds [21]. In addition, both GQDs and ACNDs exhibit excellent fluorescent characteristics including broad emission wavelength ($\lambda_{em}$), tunable PL, excitation-dependent behavior and sometimes, multiphoton excitation properties. Their diversiform PL property has been a curiosity for many years. Several principles have been proposed including the possibility that the PL emissions of C-dots are generated by synergistic effects including a quantum confinement effect [22], the free zigzag sites with triplet carbone at particle edges [23], spontaneous emission of excitons in surface energy traps through passivation [24], and band gap alterations triggered by charge transfer effects [25].

3. COMPARISON OF APPROACHES TO SYNTHESIZE C-DOTS

3.1. Top-Down Approaches

To date, numerous methods have been developed to synthesize C-dots and these can be divided into ‘top-down’ and ‘bottom-up’ routes (Fig. 1). In the top-down route C-dots (mostly GQDs) is derived from the separation of large carbon precursors, and usually prepared from carbonic materials including carbon nanotubes [20], carbon fibers [26], graphite powders [27], carbon black [28], and even candle or tire soot [29, 30]. These carbonic materials with an sp$^2$ carbon structure are abundant, but have an infinite Bohr diameter and lack an effective bandgap to produce luminescence on excitation [31]. Thus, breaking down these large carbon sources into nano-scale particles is an essential step to endow them with PL through quantum confinement effects [22]. This can usually be achieved via arc-discharge [20, 32], combustion [30], electrochemical, or laser ablation [24, 27, 33].

The first C-dots was prepared using a top-down method by oxidizing arc-discharge soot with 3.3$_{8}$ HNO$_3$, followed by extraction of the sediment with NaOH solution, resulting in a black suspension [20]. A carboxyl-group-functionalized GQD was finally obtained by gel electrophoresis separation from the black suspension. These GQDs showed size-dependent PL properties (green-blue, yellow, and orange) when excited by 366 nm ultra-violet (UV) irradiation. Multicolor emission with increased water solubility of GQDs can be achieved by this method, but the limited output and the fact that the PL QY is less than 2% are major obstacles for its practical application.

In comparison with the arc-discharge method, electrochemical synthesis is an effective and scalable approach using various carbonic materials as precursors to synthesize C-dots without employing strong acid and purification procedures. It was initially performed by Zhou et al. in a degassed acetone nitrite solution with 0.1 M tetrabutylammonium perchlorate (TBAP) as the supporting electrolyte, and multi-walled carbon nanotubes (MWCNT) were used as the working electrode in an electrochemical cell consisting of a Pt wire counter electrode and an Ag/AgClO$_4$ reference electrode [34]. The GQDs was produced uniformly by cycling the applied potential with the color change of the electrolyte solution from colorless to dark brown, which emitted blue light when irradiated by a UV lamp. Other electrochemistry methods also involve fragmentation of MWCNT or graphite rod electrodes to form GQDs by using an electric field to peel off nanoribbons from the anode through electrochemical exfoliation of layers of graphite. The supporting electrolytes include TBAP [34, 35], ethanol [36], ionic liquid [37], NaH$_2$PO$_4$ [33], NaOH [38], and PBS/water [42,43], GQDs synthesized by electrochemical strategies generally range from 1–10 nm in size with blue/green/yellow PL emission reaching a PL QY of 14% [33, 38].

In contrast to the above described top-down methods, laser ablation pioneered by Sun et al. produces C-dots with multiple colors and surface passivation [24, 27, 39-42]. Sun et al. synthesized C-dots through laser ablation of a carbon target in the presence of water vapor with argon gas at 900 ℃ and 75 kPa, followed by refluxing in HNO$_3$ for 12 h and passivating the surface via heating mixture of acid-treated C-dots and simple organic species such as PEG$_{1500N}$ (amine-terminated polyethylene glycol) or PPEI-EI (poly(propionyl ethyleneimine-co-ethyleneimine)) at 120 ℃ for 72 h [24]. Finally the as-prepared C-dots gave tunable luminescence emission, which can be controlled by specific surface passivation (Fig. 2).

Recently, Hu et al. demonstrated a simple one-step laser ablation procedure that integrated synthesis and passivation. In this procedure, a pulsed Nd:YAG laser was used to irradiate graphite powders dispersed in three kinds of solvents (diamine hydrate, diethanolamine, and poly(ethylene glycol), i.e. PEG$_{1500N}$) for ultrasonication [27]. After 2 h laser irradiation, centrifugation was used.

Fig. (1). Schematic illustration of top-down and bottom-up approaches for C-dot preparation.
to separate the black carbon precipitate and a colorful supernatant containing C-dots. The as-synthesized C-dots measured 1–8 nm, and gave blue/green fluorescence with PL QY varying from 3% to 8% [27]. Li et al. reported a simpler procedure to produce C-dots [40]. Unlike earlier studies, they used nano-carbon materials (<50 nm) as the starting material and a simple solvent (such as ethanol, acetone, or water) as the liquid medium [40]. Typically, 0.02 g of nano-carbon material was dispersed in 50 mL of solvent for ultrasonication [40]. Subsequently, 4 mL of the suspension was dropped into a glass cell for laser irradiation, followed by centrifugation to obtain C-dots in the supernatant. The C-dots showed typical blue/green luminescence with excitation-dependent features.

In general, GQDs can be readily synthesized via top-down methods. This is because most carbon-based starting materials such as graphite, carbon fibers and carbon nanotubes possess high graphite crystallinity. During preparation, different cutting/passivation strategies may cause surface energy levels to change, thus resulting in a series of emissive traps. It is suggested that more effective surface oxidation or modification involving synergistic interactions between multi-chemical groups and the carbon backbone may lead to red-shifted PL emission of C-dots [19]. Nevertheless, there are few reports of near-infrared (NIR) fluorescent C-dots being synthesized using top-down methods. The relatively low PL QY (up to 20%) of C-dots with blue/green emission and synthesized using top-down methods is a critical shortcoming [24, 42].

### 3.2. Bottom-Up Approaches

The ‘bottom-up’ method primarily involves the dehydration, polymerization and carbonization of non-conjugated small molecules (e.g. citric acid, amino acids, carbohydrates etc.) to form the GQDs and ACNDs through thermal/hydrothermal synthesis, microwave irradiation, and solution chemistry. Typically, their size, optical properties and PL QY can be tuned by altering the reaction conditions, such as the composition of reagents, ratios, reaction time and temperature.

Thermal/hydrothermal synthesis is a highly effective and low-cost method as it directly leads to the formation and surface passivation of C-dots, whose surface chemical properties can be precisely designed by careful selection of the carbon source, solvent and passivating agents. This approach provides better control over the morphology and optical properties of C-dots. The first preparation of C-dots using thermal/hydrothermal synthesis was reported by Giannelis et al. [43]. In their synthesis, C-dots were prepared by single-step thermal carbonization of low-temperature-melting molecular precursors to form surface-passivated C-dots that were either hydrophilic or organophilic [43]. They used two different procedures, both yielding monodispersed C-dots with an average size of less than 10 nm. In the first procedure, organic ammonium citrate salts were thermally decomposed, with the citrate unit serving as the carbon source, and the organic ammonium moieties playing the role of the surface passivating agent [43]. Organophilic C-dots were prepared by directly pyrolyzing octadecylammonium citrate in air at 300°C for 2 h and washing with acetone and ethanol [43]. Hydrophilic C-dots were prepared by heating diethylene glycolammonium citrate hydrothermally in a teflon-lined stainless steel autoclave at 300°C for 2 h and washing with acetone [43]. The second procedure involved the pyrolysis of 4-aminoantipyrine (4AAP) in air at 300°C for 2 h. After pyrolysis, the raw product was dissolved in bis(trifluoromethyl)methanol and precipitated by the addition of water [43]. The first procedure provided a nearly uniform size (~7 nm) of C-dots, while 4AAP-derived C-dots were more variable in morphology with sizes ranging between 5 and 9 nm. X-ray powder diffraction (XRD) results revealed that all types of C-dots consisted of highly disordered carbon, which can be referred to as ACNDs. These as-prepared C-dots exhibited a wide fluorescence $\lambda_{ex}$ extending into the NIR range. In later research, many small organic molecules and polymers (citric acid, amino acids, carbohydrates and polyethylene amines etc.) were employed for C-dot synthesis using thermal/hydrothermal methods. These molecular precursors always contained -OH, -COOH, -C=O and -NH$_2$ groups, which can dehydrate at high temperatures. Although a variety of C-dot synthesis techniques based on thermal/hydrothermal methods have been reported, there are few reports for the preparation of C-dots with a high QY and high-output. Dong et al. reported the synthesis of strong blue fluorescent carbon quantum dots (CQDs) with 42.5% of QY produced using low temperature carbonization of branched polyethylenimine (BPEI) and citric acid (CA) [44]. Zhu et al. prepared highly luminescent (QY = 20.9%–80.6%) N-doped C-dots at large scale by using the hydrothermal reaction with ethylenediamine (EDA) and CA as starting materials [21]. More recently, and based on previous work reported by Zhu et al., Qu et al. improved...
Interestingly, increasing the diameter of C-dots can result in a green fluorescent C-dots (diameter, 3.78 nm) from leucine, and fluorescent C-dots (diameter, 2.88 nm) were prepared from tryptophan, of these C-dots depended on the type of amino acid used. Blue fluorescent hydrothermal reaction from the mixture of PEG 200 and a saccharide activated C-dots were synthesized by a simple microwave-assisted thermal/hydrothermal methods for C-dot synthesis [45-48]. In optimizing the reaction time, temperature and ratios between EDA and diethylene glycol (DEG) as the reaction medium [46]. These DEG-stabilized C-dots dispersed well in water and could be ingested by C6 glioma cells, suggesting their potential use for tumor cell labelling. Another study reported by Wei et al. demonstrated the fabrication of a series of multicolor N-doped C-dots with QY up to 69.1% through a microwave-assisted Maillard reaction within 35 minute from natural amino acids and glucose [45]. The size and \( \lambda_{\text{em}} \) of these C-dots depended on the type of amino acid used. Blue fluorescent C-dots (diameter, 2.88 nm) were prepared from tryptophan, green fluorescent C-dots (diameter, 3.78 nm) from leucine, and yellow fluorescent C-dots (diameter, 4.93 nm) from aspartic acid. Interestingly, increasing the diameter of the C-dots can result in a longer \( \lambda_{\text{em}} \).

Solution chemistry is another bottom-up synthetic strategy. It involves oxidative condensation of aryl groups to form the C-dots [22, 49-54]. Although it has been demonstrated that intramolecular oxidative cycloaddition was effective for the synthesis of GQDs from polyphenylene precursors, some GQDs may suffer from poor solubility with increasing size [22], and exhibit a tendency to aggregate due to strong intergraphene \( \pi-\pi \) interaction [50]. Li and Yan reported the synthesis of stabilized GQDs of uniform and tunable size by covalent binding of multiple 2,4,6-triaryl substituted phenyl moieties and edges of the graphene [22]. Three different-sized GQDs containing 168, 132 or 170 conjugated carbon atoms were obtained. More recently, Liu et al. prepared multicolor GQDs (PL QY, 3.8%) with a uniform size of ~60 nm diameter and 2–3 nm thickness using Hexa-peri-hexabenzocoronene (HBC) as the precursor through the process of pyrolysis and exfoliation, followed by refluxing with oligomeric PEG1500N and finally reduction with hydrazine [50]. It was noticed that the morphology of the GQDs was influenced by the pyrolysis temperature.

### 3.3. The Future of C-Dot Fabrication

Although the morphology and surface chemistry of C-dots are controllable during the preparation or post-modification phase in both top-down and bottom-up methods, several considerations in C-dot preparation need to be taken into account: 1) C-dot formation processes involving thermal/hydrothermal and microwave carbonization are usually uncontrollable due to the rapid and harsh reaction conditions, which result in irregular morphology with polydispersity, though this may be improved by carefully designing the molecular precursors and reaction conditions [45]. 2) Even though the solution chemistry method is a powerful approach to produce uniform GQDs and to study the origin of the PL mechanisms of fluorescent GQDs, these GQDs provide an extremely low PL QY as compared with C-dots fabricated using other bottom-up methods. 3) Most C-dots synthesized from top-down methods suffer from an insufficient PL QY. 4) Size control is important to obtain uniform properties and to carry out mechanistic studies. Typically, post-fabrication purification is required to separate different sized C-dots, usually by using centrifugation, dialysis, and electrophoresis. However, these procedures can be incomplete, time-consuming or largely reduce the final yield of products. 5) Surface chemistry properties are critical for solubility and in-vivo applications, and need to be well controlled during preparation or post-treatment. The features of representative fluorescent C-dots fabricated using the different methods and their optical properties are summarized in Table 1.

### 4. OPTICAL IMAGING PERFORMANCE

Despite their structure diversity, C-dots possess some common features for absorption and PL. C-dots generally show strong optical absorption in the UV region, with a descending tail extending into the visible range. C-dots without surface passivation, typically those synthesized from electrooxidation [34], laser ablation/irradiation [27], and top-down microwave methods [65], have an absorption band within the range of 260–320 nm. The absorption peak in this range is associated with the \( \pi-\pi^* \) transition of aromatic sp2 domains in the carbon core [66]. While the absorption band of C-dots lies between 350–550 nm after surface passivation [67], an absorption shoulder at around 300 nm, corresponding to \( \pi-\pi^* \) transition of C=O bonds or other connected organic groups, is observed [66]. Several attractive optical features of C-dots, both from fundamental and clinical application aspects, are their potential resistance to photobleaching [45, 68], excitation wavelength (\( \lambda_{\text{ex}} \))-dependent emission and broad \( \lambda_{\text{em}} \) [24, 69]. In one recent study, the emission of individual GQDs appeared to be free of the intermittency and bleaching effects that commonly appear in traditional organic dyes and semiconductor QDs [68]. The results indicate the superior potential of C-dots compared with traditional organic dyes and semiconductor QDs as ultra-compact fluorescent probes. As mentioned above, the \( \lambda_{\text{em}} \) of C-dots is usually wide with a larger Stokes shift than that of organic dyes, and the emission peak of C-dots can be tuned by changing \( \lambda_{\text{ex}} \). It has been speculated that the wide tunable emissions of surface-passivated C-dots may result from the broad size distribution of C-dots, their variable surface chemistry, or different edge defects/ emissive traps on the surface [19, 70, 71]. However, the exact mechanism for \( \lambda_{\text{em}} \)-dependent emission behavior still remains to be established, and the way to achieve optimal synergistic interaction between the carbon backbone and surface state so as to enhance PL emission is currently unknown. The wide \( \lambda_{\text{em}} \) and \( \lambda_{\text{ex}} \)-dependent PL properties can be used for multi-color fluorescence imaging, and their \( \lambda_{\text{em}} \) can be extended into the NIR region by raising the concentration of C-dots [52, 72]. This accessibility of C-dots is a primary requirement particularly for in vivo bioimaging applications because far-red and NIR light propagates for several millimeters into tissue. \( \lambda_{\text{em}} \) in this range can thus minimize tissue absorbance, scattering, autofluorescence and consequently optimize image quality (Fig. 3) [73-76].

![Light spectrum in nanometer](image_url)

**Fig. (3).** Penetration depth of light into tissue according to its wavelength.
<table>
<thead>
<tr>
<th>Classification</th>
<th>Method</th>
<th>Carbon Source</th>
<th>Passivating Agent</th>
<th>Reaction Conditions</th>
<th>Size</th>
<th>(\lambda_{ex})</th>
<th>Emission Color</th>
<th>PL QY (%)</th>
<th>Surface State</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GQDs</td>
<td>Arc-Discharge (TD)</td>
<td>Arc soot of SWNTs</td>
<td>HNO(_3)</td>
<td>Boiled for 48 h</td>
<td>~18 nm</td>
<td>366 nm</td>
<td>Green-blue, yellow, orange</td>
<td>1.6%</td>
<td>–COOH</td>
<td>[20]</td>
</tr>
<tr>
<td>GQDs</td>
<td>Laser irradiation</td>
<td>Graphite powders</td>
<td>Diamine hydrate, diethanolamine, PEG(_{200})N</td>
<td>Irradiate at 6.0 (\times 10^6) W cm(^{-2}) for 2 h</td>
<td>~1–8 nm</td>
<td>350, 420 nm</td>
<td>Blue, green</td>
<td>3–8%</td>
<td>–COOH, −OH, C–O–C</td>
<td>[27]</td>
</tr>
<tr>
<td>GQDs</td>
<td>Electrooxidation</td>
<td>Graphite</td>
<td>—</td>
<td>Electro-oxidized at 3 V</td>
<td>~1.9–3.2 nm</td>
<td>330, 370 nm</td>
<td>Blue, yellow</td>
<td>1.2%</td>
<td>−C=O</td>
<td>[33]</td>
</tr>
<tr>
<td>GQDs</td>
<td>Electrooxidation</td>
<td>Graphite</td>
<td>—</td>
<td>Electrolyzed at 80–200 mA cm(^{-2})</td>
<td>5–10 nm</td>
<td>340–410 nm</td>
<td>Green, yellow</td>
<td>14%</td>
<td>O=C–NH–NH(_2)</td>
<td>[38]</td>
</tr>
<tr>
<td>GQDs</td>
<td>Thermal oxidation</td>
<td>Citric acid</td>
<td>Sodium 11-amino-undecanoate</td>
<td>Heated at 300 °C in air for 2 h</td>
<td>~10–20 nm</td>
<td>340 nm</td>
<td>Full color</td>
<td>3%</td>
<td>−COO’ Na’</td>
<td>[55]</td>
</tr>
<tr>
<td>GQDs</td>
<td>Chemical oxidation</td>
<td>Carbon Fibers</td>
<td>H(_2)SO(_4), HNO(_3)</td>
<td>Heated at 120 °C for 24 h</td>
<td>1–4 nm</td>
<td>318, 331, 429 nm</td>
<td>Blue, green, yellow</td>
<td>—</td>
<td>C–O–C, C=O</td>
<td>[26]</td>
</tr>
<tr>
<td>GQDs</td>
<td>Chemical vapor deposition</td>
<td>Polycrystalline copper foils</td>
<td>—</td>
<td>Heated at 1000 °C for 40–50 min</td>
<td>5–15, 30–50 nm</td>
<td>320, 350, 380, 410 nm</td>
<td>Blue</td>
<td>—</td>
<td>C–O–C, C=O</td>
<td>[56]</td>
</tr>
<tr>
<td>GQDs</td>
<td>Microwave pyrolysis</td>
<td>Amino acids</td>
<td>Glucose</td>
<td>Two steps: heated at 125 °C for 30 min and 275 °C for 5 min</td>
<td>~2.2–5.1 nm</td>
<td>350, 430 nm</td>
<td>Blue, green yellow</td>
<td>30–69%</td>
<td>−COOH, −(C=O)N HR</td>
<td>[45]</td>
</tr>
<tr>
<td>GQDs</td>
<td>Hydrothermal synthesis</td>
<td>Citric acid</td>
<td>L-cysteine</td>
<td>Heated at 200 °C for 3 h</td>
<td>~5–9 nm</td>
<td>345 nm</td>
<td>Blue</td>
<td>73%</td>
<td>C–S–C, −NHR, −COO’</td>
<td>[57]</td>
</tr>
<tr>
<td>GQDs</td>
<td>Hydrothermal synthesis</td>
<td>Citric acid</td>
<td>Ethylenediamine (EDA)</td>
<td>Heated at 160 °C for 4 h</td>
<td>~2.3 nm</td>
<td>340–420 nm</td>
<td>Blue</td>
<td>94%</td>
<td>−(C=O)N HR, −COO’</td>
<td>[45]</td>
</tr>
<tr>
<td>GQDs</td>
<td>Hydrothermal synthesis</td>
<td>Fructose</td>
<td>Sulphuric acid</td>
<td>Heated at 170 °C for 4 h</td>
<td>~5.2 nm</td>
<td>300–57 nm</td>
<td>Full color</td>
<td>7.1%</td>
<td>C–S–C, −OH, −C=O</td>
<td>[58]</td>
</tr>
<tr>
<td>GQDs</td>
<td>Solution chemistry</td>
<td>3-iodo-4-bromobenzylamine</td>
<td>—</td>
<td>Oxidative condensation at 80°C</td>
<td>132–170 conjugated carbon atoms</td>
<td>390, 550, 605, 645 nm</td>
<td>Full color</td>
<td>—</td>
<td>−CH, C=C</td>
<td>[52]</td>
</tr>
<tr>
<td>GQDs</td>
<td>Solution chemistry</td>
<td>4-Bromo-phenyl moiety</td>
<td>1,3,5-trialkyl phenyl moieties</td>
<td>Oxidative condensation at different temperature</td>
<td>168 conjugated carbon atoms</td>
<td>390, 595, 740 nm</td>
<td>Full color</td>
<td>—</td>
<td>−CH, C=C</td>
<td>[53]</td>
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(Table 1) Contd….

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<tr>
<td>GQDs</td>
<td>Solution chemistry</td>
<td>Diphenyl-lacetylene, tetraphenyl-cyclopentadiene, triethynylbenzene</td>
<td>—</td>
<td>Heated at 170 °C for 18 h</td>
<td>~ 1.2, 1.9, 8–14 nm</td>
<td>320–44 nm</td>
<td>Blue, green yellow</td>
<td>3.6–8%</td>
<td>–CH, C=C</td>
<td>[54]</td>
</tr>
<tr>
<td>ACNDs</td>
<td>Laser ablation</td>
<td>Carbon soot</td>
<td>PEG$_{1500}$N</td>
<td>Heated at 900 °C and 75 kPa; 120 °C for 72 h</td>
<td>~ 5 nm</td>
<td>400 nm</td>
<td>Full color</td>
<td>4–10%</td>
<td>–OH, C–O–C</td>
<td>[24]</td>
</tr>
<tr>
<td>ACNDs</td>
<td>Candle Soot</td>
<td>HNO$_3$</td>
<td>Refluxed for 12 h</td>
<td>Combustion</td>
<td>~ 2 nm</td>
<td>315 nm</td>
<td>Blue, yellow, orange</td>
<td>0.8–1.9%</td>
<td>–OH, –COOH</td>
<td>[30]</td>
</tr>
<tr>
<td>ACNDs</td>
<td>Microwave pyrolysis</td>
<td>Saccharide</td>
<td>PEG-200</td>
<td>Heated in 500 W microwave oven for 2–10 min</td>
<td>~ 2.8–3.7 nm</td>
<td>330, 380 nm</td>
<td>Blue, green</td>
<td>3.1–3.6%</td>
<td>–COOH</td>
<td>[48]</td>
</tr>
<tr>
<td>ACNDs</td>
<td>Thermal pyrolysis</td>
<td>Citric acid 1-hexadecylamine (HDA)</td>
<td></td>
<td>Heated at 300 °C under argon flow in octadecene for 5 min–3 h</td>
<td>~ 4–7 nm</td>
<td>340–560 nm</td>
<td>Blue, green yellow</td>
<td>53%</td>
<td>–COOH, –C(=O)N HR</td>
<td>[59]</td>
</tr>
<tr>
<td>ACNDs</td>
<td>Thermal pyrolysis</td>
<td>Citric acid Ethanolamine (EA)</td>
<td></td>
<td>Heated at 180, 230, 300, 400 °C for 30 min</td>
<td>~ 8, 19 nm</td>
<td>275–600 nm</td>
<td>Full color</td>
<td>15–50%</td>
<td>–COOH, –C(=O)N HR, –OH</td>
<td>[60]</td>
</tr>
<tr>
<td>ACNDs</td>
<td>Thermal pyrolysis</td>
<td>Citric acid Diethylene-triamine (DETA)</td>
<td></td>
<td>Heated at 170 °C for 30 min</td>
<td>~ 3–5.5 nm</td>
<td>340–500 nm</td>
<td>Blue, green</td>
<td>88.6%</td>
<td>–C(=O)N HR, –OH</td>
<td>[61]</td>
</tr>
<tr>
<td>ACNDs</td>
<td>Hydrothermal synthesis</td>
<td>Citric acid Ethylenediamine (EDA)</td>
<td></td>
<td>Heated at 150, 200, 250 and 300 °C for 5 h</td>
<td>~ 2–6 nm</td>
<td>340–420 nm</td>
<td>Full color</td>
<td>20.9–80%</td>
<td>–COOH, –C(=O)N HR</td>
<td>[21]</td>
</tr>
<tr>
<td>ACNDs</td>
<td>Hydrothermal synthesis</td>
<td>Sodium citrate</td>
<td>Ammonium bicarbonate (NH$_4$HCO$_3$)</td>
<td>Heated at 180 °C for 4 h</td>
<td>~ 1.6 nm</td>
<td>340 nm</td>
<td>Blue</td>
<td>68%</td>
<td>–OH, –COO'</td>
<td>[62]</td>
</tr>
<tr>
<td>ACNDs</td>
<td>Low temperature self-polymerization</td>
<td>2-azidoimidazole</td>
<td>—</td>
<td>Heated at 50,70, 100 °C for 24 h</td>
<td>~ 2.1–5.4 nm</td>
<td>300–500 nm</td>
<td>Blue, cyan, green</td>
<td>9–14%</td>
<td>–OH, –NHR</td>
<td>[63]</td>
</tr>
<tr>
<td>ACNDs</td>
<td>Hydrothermal pyrolysis</td>
<td>Citric acid or ethylene glycol</td>
<td>Ethylenediamine end-capped polyethyleneimine PEI-EC</td>
<td>Heated at 180 °C until the color turned to orange.</td>
<td>~ 1–12 nm</td>
<td>240–695 nm</td>
<td>Full color</td>
<td>~6.5–19%</td>
<td>–NHR, –OH, C–O–C</td>
<td>[64]</td>
</tr>
</tbody>
</table>

The PL QY of C-dots is a key parameter for their potential bio-imaging application. The PL QY varies and depends on the synthesis methods and surface chemistry involved. For unpasivated/undoped C-dots, PL QYs range between 2% and 22.9% for
those prepared via stepwise solution chemistry and microwave-assisted acidic oxidation, respectively [52, 77]. Since GQDs commonly contain carboxylic and epoxide groups, which can act as non-radiative electron hole recombination centers, the removal of these oxygen-containing groups by either reduction or surface passivation may improve PL QY [78]. Shen et al. prepared GQDs-PEG with QY as high as 28%, which was twice that of GQDs (~13.1%) [79]. This improvement probably originates from the stabilization effect of excitons in the GQDs after passivation by PEG [70, 80]. Moreover, of PL QY can be dramatically enhanced by thermal or hydrothermal approaches, by choosing a specific carbon source and a doping/passivating agent [45].

In addition to Stokes shift type fluorescence, anti-Stokes type PL is another useful optical property of C-dots, or namely upconversion or multi/two-photon PL [39, 69, 81, 82]. Anti-Stokes type PL often refers to the non-linear sequential absorption of two or more photons and leads to the emission of light at a shorter wavelength than the excitation wavelength [83]. The upconversion PL of C-dots has received increasing attention in recent years. In 2008, Sun et al. reported that C-dots were strongly emissive in the visible region with excitation via either argon-ion laser (458 nm) or femtosecond pulsed two-photon laser in the NIR (800 nm) range [39]. Also, in-vitro evidence showed that C-dots could be potentially used for cell imaging with two-photon luminescence microscopy. Liu et al. demonstrated that biocompatible nitrogen-doped GQDs were efficient two-photon fluorescent probes for cellular and tissue imaging [82]. They prepared N-GQDs by a one-pot solvothermal approach using DMF as the solvent and nitrogen source. The N-GQD exhibited a two-photon absorption cross section as high as 48000 Göppert-Mayer (GM) units (1 GM is 10−24 cm4 s photon−1). The imaging penetration depth of 1800 μm achieved by N-GQD in tissue phantom significantly extended the imaging depth limit of two-photon microscopy (Fig. 4). It is anticipated that the upconversion PL of C-dots will provide new opportunities for two-photon luminescence imaging microscopy.

To conclude, in the current state of the art, the fact that C-dot emission wavelength is related to tissue penetration is one of the major weaknesses compared with QDs, fluorescent protein and indocyanine green (Table 2). GQDs shows relatively lower PL efficiency than ACNDs, and both C-dots exhibit high emission peaks ranging from the blue to green region of the spectrum. In addition, the PL QY of C-dots tends to fall in serum, and auto-fluorescence generates background noise that interferes with imaging quality.

5. C-DOT SURFACE FUNCTIONALIZATION AND NANO-COMPOSITES

Surface modification is an effective method to tune the surface properties of C-dots for selected applications. C-dots prepared from CNTs by the arc-discharge method lead to oxygen-containing groups at their surface [20]. Other methods include electrooxidation from graphite [84] and hydrothermal carbonization from organic molecules [44, 85-87], which showed similar results with surface functionalization using carboxyl groups or amines. The presence of these organic groups alters the physicochemical properties of C-dots including their water solubility, biostability, functionality and PL properties. Typically, the binding of amine-containing molecules (for example, ethanolamine, PEG1500), through the amide linkages is used for the surface passivation of C-dots for biological applications. As mentioned earlier, some single-step preparations of C-dots allow the direct incorporation of surface functionality by selecting appropriate starting materials [43, 44, 55, 85-87]. Chandra et al. synthesized green-PL-colored C-dots by microwave irradiation of sucrose with phosphoric acid [88]. Fluorescein, rhodamine B and α-naphthylamine were covalently functionalized onto the C-dots via EDC condensation, with improved fluorescence and reduced cytotoxicity [88]. In another study, folic acid was conjugated onto the C-dots, and such functionalization can be applied to target cancer cells [89].

More recently, attention has been drawn to the preparation of novel hybrids comprised of C-dots and inorganic nanoparticle cores (e.g., iron oxide [90, 91], gold [90], silica [92], and titanium [36, 91]). With C-dots based on organic compounds, surface functionalization, doping, or multifunctional nanocomposites for specific biomedical applications should select appropriate non-toxic elements or molecules for the configuration of C-dots, as their toxicity profile may influence the overall safety of C-dot nanocomplexes. Fe3O4@C-CDs nanocomposites were synthesized by a one-pot solvothermal method [91]. In this experiment, H2O2 is present as an oxidizing agent in the reaction medium, thus abundant small-sized fluorescent C-dots can be formed in situ in the porous carbon shell from the oxidation and deep decomposition of the precursor fer-

![Fig. (4). (a) Diagram showing the setup used for two-photon fluorescence imaging of N-GQDs (nitrogen doped GQDs) in tissue phantom with thickness ranging between 0 and 1800 μm. (b, c and d) shows upconversion cell imaging under a bright field, 800 nm excitation and the overlay image (Reproduced with permission from ref. [82]).](image-url)
rocene. In addition to the magnetic responsive properties and MRI ability ($r_2^* = 674.4 \text{ mM}^{-1} \text{s}^{-1}$) of the Fe$_3$O$_4$ nanocrystal core, the synthesized Fe$_3$O$_4$@C-CDs nanocomposites also exhibit attractive optical properties from CDs with strong and upconversion fluorescence (PL QY, ~6.8%), excellent photostability, and a NIR photothermal effect [91]. These results demonstrated that Fe$_3$O$_4$@C-CDs nanocomposites have combined the biocompatible iron oxide nanoparticles with carbon materials, thus providing potential for use in multimodality imaging and therapy. More recently, Zhou et al. developed multifunctional hybrid nanocomposites (Fe$_3$O$_4$@PC-CDs-Au) that integrated magnetic Fe$_3$O$_4$ nanocrystals, fluorescent C-dots and Au nanocrystals into a porous carbon matrix [90]. It was obtained via the synthesis of core-shell structured Fe$_3$O$_4$@C-CDs template nanoparticles, followed by loading and in situ reduction of Ag$^+$ ions, and a final replacement of Ag with Au nanocrystals via a galvanic reaction [90]. In vitro evaluation results indicated that the nanocomposites can enter intracellular regions and light-up mouse melanoma B16F10 cells with laser scanning confocal microscopy [90]. By taking the advantages of the combined photothermal effects of the carbon dots and the Au nanocrystals embedded in the carbon matrix, the NPs can not only serve as efficient NIR photothermal therapeutic agents to kill cancer cells, but also control the release rate of the loaded drug by NIR irradiation [90].

6. IN-VITRO AND IN-VIVO STUDIES

C-dots provide properties in photostability comparable to those in traditional semiconductor QDs, but with much lower cytotoxicity for long-term cell labeling and tracking. As an example, Xiong et al. reported GQDs derived from single-walled carbon nanotubes (SWNTs) which exhibited very low cytotoxicity towards HeLa cells with the LC50 over 5 mg mL$^{-1}$ for 24 h [93]. In comparison, semiconductor QDs exhibit cytotoxic effects in much lower concentrations varying from 62.5 μg mL$^{-1}$ to 400 μg mL$^{-1}$ depending on the size, number concentration or size and the type of cell lines tested [94]. In our recent studies, we prepared ACNDS by hydrothermal carbonization of citric acid and polyethylene amine (PEA) analogs (ethylenediamine (EDA), diethylenetriamine (DETA) and triethylenetetramine (TEPA)). These nitrogen-doped ACNDS demonstrated low cytotoxicity and bright fluorescence in HeLa cells. The results of the cell viability test demonstrated that these ACNDS did not exert any obvious cytotoxicity as cell viability was over 80% even at a relatively high concentration (800 μg mL$^{-1}$) of ACNDS (Fig. 5). The confocal fluorescence images of HeLa cells incubated with three types of ACNDS at a concentration of 200 μg mL$^{-1}$ and cell nucleuses stained with blue DAPI (4',6-diamidino-2-phenylindole) revealed high-contrast fluorescent signals form multi-color ACNDS around each nucleus (Fig. 6). The results obtained indicate that nitrogen-doped ACNDS can be used for high-contrast multicolor cell labeling.

![Fig. 5](image.png)

Fig. (5). In-vitro cytotoxicity testing results of three types of nitrogen doped ACNDS with increased concentrations against HeLa cells from an MTT assay. The cell viability steadily decreased to around 80% when concentrations reached 800 μg mL$^{-1}$. (Reproduced with permission from ref. [95]).

As compared with fluorescence cell labeling, in-vivo fluorescence imaging is more challenging for C-dots. As we mentioned in the sections above, a number of criteria for C-dots need to be considered, especially concerning the optical penetration in tissues and safety profiles. Yang et al. reported in-vivo optical imaging using C-dots [42]. In their experiment, PEGylated C-dots in an aqueous solution were injected subcutaneously into mice, and the fluorescence images at different excitation wavelengths were obtained with sufficient contrast in both green and red channel [42]. Moreover, they also performed sentinel lymph node imaging of ZnS salt doped C-dots following intradermal injection. The observation revealed that C-dot migration along the axillary lymph node was slower than that of semiconductor QDs. One possible reason could be the smaller sizes of the carbon dots (around 5 nm) and/or the surface PEGylation [42]. The longer time taken by C-dots to migrate to lymph nodes could result in a prolonged surgical procedure if they are used in fluorescence-guided surgery (Fig. 7).

Tao et al. applied GQDs with the same protocol to nude mice and obtained comparable results. In addition, they found the best fluorescence contrast with 595 nm excitation [96]. Recently, we studied three types of nitrogen-doped ACNDS for fluorescence imaging in mice following subcutaneous injection. The fluorescence imaging of the mice was captured with $k_0$ at 535 nm and 695–770 nm, respectively. Our results demonstrated that N fluorescence signals can be readily visualized following the injection of 50 μL of 0.05, 0.5 and 5 mg ACNDS into a nude mice (Fig. 8). The 5 mg dose of CD-EDA$_{2/1}$ showed the highest signal intensity, above $10^5$. The ROI fluorescence signals of both CD-DETA$_{2/1}$ and CD-TEPA$_{2/1}$ showed lower intensities than that of CD-EDA$_{2/1}$ at corresponding doses. It should be pointed out that autofluorescence was the major problem and significantly decreased the signal-noise ratios when the concentration of ACNDS was below 0.05 mg per 50 μL. Overall, the results suggested that non-labeled N-doped CQDs with sufficient contrast in both green and red channel can be used as optical contrast agents for near tissue fluorescence imaging with a concentration no less than 0.05 mg per 50 μL.

Yet another attractive biomedical application of C-dots is their ability to serve as a nanocarrier for drug delivery (such as siRNA/DNA and chemotherapy drugs) [18, 87, 97-99]. Sun et al. demonstrated in-vivo that C-dots was able to deliver anticancer drugs by using functionalized amino groups [87], which was synthesized via amide bonding between oxidized oxaliplatin (Oxa (IV)-COOH) and the surface group of C-dots. The in vivo results demonstrated that the distribution of oxaliplatin can be tracked by monitoring the fluorescence signal of CD-Oxa (C-dots-oxaliplatin nanocomplexes) [87]. Additionally, it has been reported that C-dots can be used as photodynamic therapy (PDT) agents by generating reactive oxygen species including singlet oxygen under absorption wavelengths, and kill cancer cells by inducing oxidative stress [17, 100].

7. TOXICITY PROFILE OF C-DOTS

A notable advantage of C-dots is their biocompatible elemental composition. The in-vitro cytotoxicity of C-dots has been studied by various research groups, which revealed that C-dots appear to have low toxicity to numerous cell lines [33, 85, 88, 101-107]. Zhang et al. investigated the cellular internalization, uptake mechanism, and cytotoxicity of GQDs in human gastric cancer (MGC-803) and breast cancer (MCF-7) cell lines [108]. The GQDs were synthesized via the photo-Fenton reaction of graphene oxide (GO) [109-111]. The cytotoxicity of GQDs was lower than that of graphene oxide sheets, which was proven by cell viability, internal cellular reactive oxygen species levels, damage to mitochondrial membrane potential, and cell cycle. In addition, they also found that the GQDs were internalized primarily through caveola-mediated endocytosis [108]. Wang et al. studied the cytotoxicity of C-dots synthesized by various combinations of passivation molecules on
C-dot precursors, and showed that the cytotoxicity of C-dots was dependent on the selection of surface passivation molecules [112]. In another study, Chandraet et al. evaluated the cytotoxicity of C-dots to healthy human blood cells by measuring the hemolysis rate [88]. C-dots with carboxyl groups at the surface exhibited some toxic effects, but the surface modifications by organic molecules through the amide bonds resulted in a significant reduction of cytotoxicity [88].

The in vivo evaluation of C-dots has also been studied in recent years. Sun et al. investigated in vivo safety and imaging optical performance of PEG1500N surface functionalized C-dots in reference to CdSe/ZnS QDs [113]. The quantification of C-dots in various organs in dissected mice was investigated via isotope-ratio mass spectroscopy, by using $^{13}\text{C}$-enriched PEGylated C-dots [113]. The results indicated that these C-dots had low levels of accumulation in the liver, spleen and kidneys according to the experimentally determined $^{13}\text{C}/^{12}\text{C}$ isotope-ratios. Liver and kidney functions were
also evaluated by serum biochemistry assays. Alanine amino trans-
ferase (ALT) and aspartate amino transferase (AST), uric acid
(UA), blood urea nitrogen (BUN), and creatinine (Cr) levels were
similar to those in control groups. Moreover, histopathological
examinations showed normal liver, spleen, and kidneys [113]. Fur-
ther toxicological studies according to regulatory guidelines will be
very useful.

Tao et al. investigated in vivo fluorescence imaging, biodis-
tribution and toxicity of carbon nanotube-derived C-dots (C-dots-
Ms) [96]. C-dots-Ms were radioactively labeled with 125I for phar-
macokinetic and biodistribution evaluations in mice. The blood
circulation of 125I-C-dots-Ms was fitted by a two-compartment
model, with first- and second-phase circulation half-lives of ap-
proximately 1 and 2 h, respectively [96]. The urine and feces of
mice were collected after injection, and high radioactivity was de-
tected in the urine and feces samples, suggesting that some of
the C-dots had possibly been eliminated through both renal and fe-
cal excretions. 125I-C-dots-Ms mainly accumulated in the reticuloen-
dothelial system (RES) organs, including the liver and spleen at post-
intravenous injection [96]. Systematic chemical analysis of the
blood over time, complete blood counts and histological studies
demonstrated the safety of C-dots in female Balb/c mice over 3
months following an injected dose of 20 mg kg⁻¹ [96]. Collectively,
in-vivo evidence has shown that C-dots are mainly taken up by
reticuloendothelial system (RES) organs, such as liver and spleen,
or eliminated by the renal excretion pathway after intravenous in-
jection [42, 96, 114, 115].
Even though the currently available in vitro/in vivo toxicity data shows an encouraging toxicity profile of C-dots, many safety considerations still need to be addressed before they can be used in clinical practice. For clinical biomedical imaging purposes, the best delivery route is intravenous injection, as this can ensure the distribution of C-dots throughout the body. The final hydrodynamic diameter (HD) of C-dots in the bloodstream is thus a critical factor for systemic clearance. Once the C-dots enter the bloodstream, various plasma proteins could non-specifically bind the C-dots due to its non-neutral surface charges, which consequently increases the final HD and obstructs the clearance pathway, since previous studies have shown that the major clearance of C-dots is through the renal and fecal pathways [96]. Renal clearance is mediated by the slit diaphragm of the glomerular basement membrane (GBM). The physiological pore size of the slit diaphragm is ~5 nm in diameter [116, 117]. The physicochemical properties of C-dots, including HD, dispersity, shape, flexibility, and surface charge will determine whether the C-dots can be filtered by the GBM [118-120]. In particular, C-dots that have an overall HD size exceeding 8 nm after interaction with plasma proteins, such as albumin, may never be filtered at all [118]. This circumstance would result in much longer exposure time to C-dots in the body. These C-dots would eventually be metabolized in the liver and/or be taken up by the reticuloendothelial system (RES) [121, 122]. In this regard, C-dots degenerated by the liver into clearable components and excreted into the bile and feces would be a desirable fate. However, C-dot uptake by the RES, involving phagocytic cells, primarily monocytes and macrophages, located in the reticular connective tissue of the liver, spleen, and bone marrow would be unclearable and result in long residence times in the body. In addition, surface modification of C-dots using biologically stable, hydrophilic, and neutral polymers, such as PEG can increase half-lives in the blood and uptake by the RES. Notably, large chemically stable or non-biodegradable nanoparticles could accumulate in these RES rich organs for long periods of time, and potentially cause immunotoxicity, reproductive risks, and carcinogenic effects [123]. It was also reported that nanoparticles may evade detection by the body’s immune system, and under rare circumstances cross the brain blood barrier (BBB) [124]. Moreover, even for C-dots that contain non-toxic elements, their heterogeneity with regard to size, agglomeration (π-π interaction) and sample purity is a major obstacle for standard evaluations. For clinical applications of C-dots, “Choi Criteria” were proposed as a guide for the use of nanoparticles for clinical biomedical imaging. These criteria include biodegradability, minimal non-specific tissue uptake and smaller than 5 nm with renal clearance [13].

### 8. DESIGN CONSIDERATIONS FOR THE CLINICAL APPLICATION OF C-DOTS

#### 8.1 Sensitivity: Signal to Background Ratios

Like any other optical contrast agents, C-dots must have the desired characteristics to be detected and visualized in target tissues. Therefore, as discussed in previous sections, optical imaging in the NIR window (700-900 nm) offers the greatest tissue penetration. Optical contrast agents for tissue imaging should be designed so that λex and λem are in the NIR window. NIR imaging can also minimize light scattering in tissue and signal interference from autofluorescence. In addition, to achieve a sufficient signal in target tissue, the following optical properties in a physiological environment must be considered: light absorptivity of contrast agents, PL QY, photostability, thermodynamic stability and fluorescence quenching [73]. Unfortunately, given the current research status of C-dots, only a few reports have shown success in preparing fluorescent C-dots that have λem extended into the NIR range, and these have a very limited PL QY [24, 64, 125].

#### 8.2. Stability and Safety: Biophysicochemical Interaction at the Nano-Physiological Interface

In designing contrast agents, one of the main considerations is the stability of nanoparticles in vivo and in vitro [12]. Biophysicochemical influences such as final HD, shape, surface area, surface charge, hydrophilicity/lipophilicity, surface functional groups/

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**Table 2.** The physicochemical properties of C-dots versus QDs, fluorescent protein (FP) and indocyanine green (ICG).

<table>
<thead>
<tr>
<th>Properties</th>
<th>C-dots</th>
<th>QDs</th>
<th>FP</th>
<th>ICG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical diameter</td>
<td>~1–10 nm</td>
<td>5–10 nm</td>
<td>~7 nm</td>
<td>~1 nm</td>
</tr>
<tr>
<td>Final HD (SF &amp; NSPB)</td>
<td>≤50 nm</td>
<td>≤50 nm</td>
<td>≤35 nm</td>
<td>≤20 nm</td>
</tr>
<tr>
<td>Stability in serum</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Morphological homogeneity</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Structural flexibility</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>λem range/TP</td>
<td>400–650 nm/+</td>
<td>450–1200 nm/+</td>
<td>470–650 nm/+</td>
<td>820 nm/+</td>
</tr>
<tr>
<td>Photostability</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PL QY</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Potential toxicity</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

SF = surface functionalization, NSPB = non-specific protein binding, HD = hydrodynamic diameter, λem = emission wavelength, TP = Tissue penetration, PL QY = photoluminescence quantum yield, ‘+’ = positive, ‘-’ = negative.
ligands are key factors to determine the interactions between nanoparticles and biological systems, which consequently affect the in vivo biocompatibility and toxicity of nanoparticles (Table 2) [126]. Most nanoparticles consisted of multiple organic and/or inorganic components, each of which has its own efficacy and toxicology profile, which requires individualized investigations [127]. Problems may arise if the nanoparticles are not readily excreted, biodegraded, or metabolized, or eliminated, and thus accumulate in organs/tissues for long periods. Additional investigations in a preclinical setting are required to clarify possible adverse outcomes. It is also necessary to fully evaluate the consequences of long-term retention of nanoparticles in vivo according to regulatory agency guidelines. Designing nanoparticles for rapid clearance is the one important prerequisite to avoid prolonged preclinical testing [13, 127]. A final HD less than 5.5 nm is the key criterion for the rapid elimination of nanoparticles via renal filtration after intravenous injection. In light of the “Choi Criteria” and current knowledge of C-dots, the safety profile of nanoparticles needs to be characterized before clinical translation. The profile will include morphology control (such as size/final HD, shape and uniformity), surface charge, solubility or dispersibility, biodegradability, oxidative stress or free radical generation, interactions between nanoparticles and serum protein, and short and long-term stability in various physiological environments. Finally, we would suggest that the main concerns for safety should focus on improvements in the homogeneity of the size and shape of C-dots, as well as their surface chemistry, because uneven surface chemistry may result in agglomeration or aggregation through π-π interactions, ionic bonding, hydrogen-bonding, and Van der Waals interactions between C-dots and C-dots/biomolecules, which can result in unpredictable biological fates. Moreover, product purity also needs to be addressed for standard evaluations and further applications.

8.3. Specificity: Passive and Active Targeting

The specificity of optical contrast agents is the ability to selectively accumulate or attach to target organ/tissue sites so as to improve imaging resolution by raising the signal to noise ratio [12]. Therefore, it is essential to design contrast agents with selective binding to the target sites, a reasonable half-life in the blood, and efficient elimination from the biological system before their application in clinical practice. Active targeting often refers to the conjugation of contrast agents with targeting ligands (such as small molecules, peptides, antibodies, and aptamers), which specifically interact with molecular targets, including receptors and enzymes [13, 128]. While passive targeting is associated with organ and tumor-specificity, and is governed by the physiochemical properties of contrast agents, or pathophysiological characteristics of irregular tumor vessels (EPR effects). To develop efficient targeted contrast agents, both the targeting ligands and physiochemical properties of contrast agents should be carefully designed to balance target and organ-specificity [129]. In the case of C-dots, some reports have demonstrated that they can be conjugated with various ligands mainly through the amide linkage for targeted imaging [18, 87, 130], or via surface passivation with neutral molecules for passive-targeted imaging [29, 42, 131]. Because of the limited in-vivo evidence of the efficacy of targeted imaging by C-dots, it is as yet difficult to judge the sensitivity and specificity of C-dots for biomedical imaging.

9. CLINICAL PERSPECTIVES

The field of biomedical optical imaging has been developing for over two decades and it already achieved some success in patient care, especially in image-guided surgery [132-134], which potentially provides an effective way to protect normal tissues (such as blood vessels and nerves) during surgical resection procedures (such as tumors and lymph nodes). In contrast to visible light, NIR fluorescence imaging has the advantage of penetrating relatively deeply into tissue to provide real-time, quantitative, and inexpensive imaging with a high signal/noise ratio at depths < 1 cm [135-137]. Since most biomolecules have minimal light absorption in the NIR window, and the naked human eyes is unable to detect light in the NIR range, a specific NIR contrast agent and specific imaging systems are required to facilitate NIR imaging in the field of surgical. Now, several NIR fluorescence image-guided surgery systems are commercially available. One organic-based NIR fluorescent agent, indocyanine green (ICG), has been approved by the FDA and European Medicines Agency (EMA) for surgical guidance of lymphatic mapping [138-140], hepatic tumors, metastases [141, and the fluorescence-aided endoscopic examination of superficial gastric tumors [142, 143]. Methylene blue can be used as a fluorescence agent in the 700 nm range and has been applied clinically in NIR fluorescence imaging [144-146]. Another small molecule, 5-aminolevulinic acid (5-ALA) is a non-fluorescent prodrug which induces the generation and accumulation of the fluorescent molecule protoporphyrin IX (PpIX) in epithelial and neoplastic tissues [147-149]. 5-ALA has been used for fluorescence cystoscopy in the detection of bladder cancer and in the removal of cancerous tissue from the bladder [150-152]. Although Methylene blue, ICG and 5-ALA-induced PpIX have been assessed in some clinical proof-of-principle studies in several types of surgery, they exhibit some shortcomings including poor photostability, non-specific uptake in normal tissues and organs which limited their uses. QDs process NIR absorption and emission properties with relatively high PL QY and better photostability, which result in high signal intensity, thus enabling long-term detection at lower concentrations compared with organic fluorophores, but their toxicity is a serious concern. In this regard, C-dots, given its good biocompatibility, may become a potential alternative to QDs. To achieve this goal, the early involvements of multi-disciplinary teams that combine chemists, pharmacologists, toxicologists, clinicians, pharmacists, and regulatory authorities, as well as imaging equipment manufacturers, is essential [153-156].

CONCLUSION

Given their organic and biocompatible nature, tunable PL, and versatile surface functionalization, C-dots is an attractive luminescent nanomaterial that may enable the development of a viable optical imaging platform. A major limitation of C-dots is tuning the PL into the NIR region and simultaneously achieving a high PL QY. Long-wavelength excitation is essential for NIR fluorescence imaging in order to obtain more effective tissue penetration and simultaneously increase the resolution. Thus a systematic investigation of PL mechanisms for different types of C-dots is required. Recent research in C-dots has been done in proof-of-concept experiments, and uncovered various physiochemical properties of C-dots. These properties are relevant for biological imaging [73, 153]. Although encouraging findings have been reported with regard to the applications of C-dots, their exact mechanism of cellular uptake and their long-term toxicological effects remain to be elucidated. This is a challenge because the pharmacokinetics and biodistribution of C-dots depend on many factors such as their morphology, physiochemical properties, surface chemistry and formulation. Further research is likely to continue, which will be beneficial for the overall development for C-dots, which will eventually become a more effective and less expensive alternative to conventional semiconductor QDs.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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