Molecular Insights of Carbon Nanodots Formation and Their Two-Photon Emission Properties

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1. Introduction

Carbon nanodots (C-dots) are sub-10 nm fluorescent carbon nanoparticles synthesized from low-cost organic precursors that showcase exciting properties such as biocompatibility,[1] photostability,[2] easy surface functionalization,[3,4] or two-photon (TP) excited emissions.[5–8] These unique features have generated broad interest in sensing,[9] multimodal bioimaging,[10] photocatalysis,[11] drug delivery,[12] photonics,[13] and recently additive manufacturing.[14] C-dots are usually prepared by relatively simple synthetic procedures including top-down approaches such as laser ablation,[15] chemical,[16] and electrochemical oxidation[17] as well as bottom-up methods like microwave irradiation,[18] ultrasonication,[19] and hydrothermal[9]/solvothermal[20] approaches. However, the formation mechanism as well as the molecular structure of the C-dots, which can dictate their photoluminescence (PL) properties, is still barely understood. Several parameters such as the precursor molecules, the method of preparation, reaction time, and temperature could affect the optical properties of the as-synthesized C-dots.[21–26] A molecular understanding on how the precursor’s molecules affect the optical properties would be very useful to rationally design C-dots with the desired optical features.

The PL characteristics of C-dots originate from the bandgap transitions of conjugated π-electron rich domains, recombination of surface-trapped charges, electron–hole pairs inside small sp²-carbon clusters, or surface-trapped excitons.[24,27–31] The occurrence of the emission of PL states was explained in various studies by quantum size effects, surface states, and molecular states. For example, the inverse correlation between the highest occupied molecular orbital (HOMO)–lowest unoccupied molecular orbital (LUMO) energy gap and the size of the graphitic layers has been proposed to explain the observed size-dependent optical properties of different sized C-dots.[32–35] In addition, several other studies suggested that the presence of surface emissive trap states,[36–38] surface oxidation/reductions, and modifications[39,40] are responsible for the emissive behavior of C-dots. Their characteristic emission was suggested to originate from graphitic domains inside the carbonaceous core and extrinsically from surface states.[32–44] It was proposed previously that C-dots contain multiple chromophoric units connected to the carbonaceous core and that oxygenated surface defects form the
emission traps. In alternative studies, it was shown that the PL could be dominated by organic fluorophores associated with the carbonaceous core or its surface. The yield of these organic fluorophores varies with synthesis temperature, and it was postulated that at high temperatures, these fluorophores are dehydrated, polymerized, and then carbonized to produce highly photostable carbogenic cores of low quantum yield (QY). Thus, elucidating the molecular structures of these fluorophores within the C-dots could lead to a better understanding of their observed optical properties.

2. Results and Discussion

2.1. Synthesis and Characterization of the C-Dots

Various C-dots were synthesized based on published protocols with citric acid (CA) as the main carbon source in combination with passivating agents such as 2-aminoethylphosphonic acid (APE), polyvinylpyrrolidone (PVP), PEG-600 (PEG), PEG-diamine (PEGD), polyethyleneimine (PE), ethylenediamine (EDA), and p-phenylenediamine (PDA) (see structure in Figure 1). We employed microwave-assisted synthesis to prepare three different types of C-dots in aqueous solution based on three different precursors, aromatic PDA and aliphatic EDA, both containing amino groups, as well as aliphatic PEG without amino groups. The corresponding C-dots solution was termed CAPDA, CAEDA, and CAPEG, respectively. C-dot synthesis was performed for 20 min at 150 °C (8 bar, 70 W) in the microwave synthesizer. Then, the C-dots were purified through a 0.2 μm pore-sized cellulose membrane-based syringe filter to remove any larger aggregates present in solution. In addition, dialysis was employed to purify the C-dots from unreacted precursors and other byproducts. Polyacrylamide gel electrophoresis (Nu-PAGE) showed that CAEDA-p C-dots have larger dimensions than CAPDA-p C-dots as they diffused slower through the electrophoresis distance as shown in Figure S1a, Supporting Information. The average sizes of the C-dots were obtained using transmission electron microscopy (TEM) and narrowly dispersed particles of 2.3 ± 0.6 nm (CAPDA-p), 4.3 ± 1.4 nm (CAEDA-p), and 4.5 ± 2.1 nm (CAPEG-p) were found (Figure 1d–i) that could be dispersed in aqueous solution without the formation of larger aggregates. The C-dots were formed during microwave synthesis and no aggregate formation was observed.

The nanoparticles were well dispersed and remained colloidal stable in water and an average hydrodynamic diameter of about 5.6 nm (Figure S1b, Supporting Information) was calculated from dynamic light scattering data, which correlated well with the measured size determined from the TEM images of ~2.3 nm (Image J, v1.53c, W. Rasband, National Institute of Health, USA). The slight increase was attributed to the ligand or solvent shell around the nanoparticles. No aggregate formation was observed even after 1 year of storage indicating high colloidal stability (Figure S1c, Supporting Information).

C-dots were dispersed in water and CAPDA (greenish-yellow) and CAEDA (reddish-yellow color) revealed characteristic colors, whereas CAPEG appeared colorless. We supposed that these optical properties of the CAPDA and CAEDA nanoparticles could be related to the formation of carbonaceous cores and with the associated fluorophores (Figure 1a,b) and that in case of CAPEG, no associated fluorophores were formed (Figure 1c). In the following, we have first investigated the C-dot cores CAPDA-p, CAEDA-p, and CAPEG-p and then elucidated the molecular structures of the formed fluorophores to correlate the observed optical properties to the structural features of the fluorophore-associated C-dots.
2.2. Characterization of the Composition, Interlayer Spacing, and Energy Bandgap of the C-Dots Cores

X-ray photoelectron spectroscopy (XPS) characterization was performed to investigate the surface composition of the obtained C-dots. The deconvolution of high-resolution C 1s spectra revealed the presence of three peaks around 284.5, 285.8, and 288.5 eV for the C-dots CAPDA-p, CAEDA-p, and CAPEG-p. We attributed these peaks to C═C (sp²), C─C (sp³), C─H, C=O, or COOH surface functionalities of the C-dots.[63] Quantitative analysis revealed a sp², sp³-carbon ratio for C-dots CAPDA-p, CAEDA-p, and CAPEG-p of 1.28, 0.47, and 0.90, respectively (Figure 2a–c). A relatively higher content of sp²-hybridized carbon of the CAPDA-p C-dot surface could originate from the aromatic PDA precursor. X-ray diffraction (XRD) characterization was performed to investigate the crystallinity of the C-dots. A broad diffraction pattern indicating amorphous-like, rather distorted structures[63] consisting of a mixture of sp²- and sp³-hybridized carbon atoms was observed. CAPDA and CAEDA showed a broad characteristic peak angle at 19.50° and 17.64°, respectively, indicating the presence of a heterogeneous environment of the carbon atoms within the crystal structure (Figure S2, Supporting Information). In comparison, graphite, comprising pure sp²-hybridized domains, has a characteristic peak angle of 26.60°.[64]

Aberration-corrected high-resolution transmission electron microscopy (AC-HRTEM) (Figure 2d–f) was conducted to characterize the crystallinity as well as the structural variation in the C-dots derived by different precursors. The clearly lattice fringes in AC-HRTEM images demonstrate the high crystallinity of the carbonaceous cores, which was further evidenced by the sharp diffraction spots in the selected-area electron diffraction (SAED) patterns (Figure 2g–i). As the C-dots are randomly oriented on the TEM supporting film, we could only observe lattice fringes with different spacing. It is therefore significantly challenging to determine the exact atomic arrangement of the sp²/sp³-hybrid carbonaceous cores. However, we observed an increase in the largest lattice plane spacing, i.e., interlayer spacing, from CAPDA-p (0.34 nm), CAEDA-p (0.37 nm) to CAPEG-p (0.48 nm). Interestingly, the interlayer distance in graphite is 0.335 nm,[64] which is in a similar range as the spacing found in the CAPDA-derived C-dots. The high sp²-hybridization content from the aromatic PDA precursor in CAPDA could influence the interlayer spacing that possibly resulted in much smaller interplanar distances in C-dot CAPDA-p.

To assess the sp², sp³-hybridization content of the C-dot core, electron energy loss spectroscopy (EELS) was performed. The EELS spectra from the carbon K-edge of carbonaceous cores were deconvoluted using the "three-Gaussian" fitting method,[65] where a linear combination of three Gaussian functions was fitted in the π* and σ* energy regions. The first two functions were centered at 285.0 and 287.8 eV, which denotes the transition to the π*-state (sp²-bonding) and the third function was set at 292.2 eV that represented the transition to the σ*-state (sp³-bonding).[65–67] Quantitative analysis revealed sp², sp³-bonding...
ratios for C-dots CAPDA-p, CAEDA-p, and CAPEG-p as 0.61, 0.45, and 0.13, respectively (Figure S4 and Table S1, Supporting Information). This confirmed the presence of relatively larger $sp^2$-domains in CAPDA-p C-dots. Furthermore, Raman measurements were also carried out to investigate the carbon structure. The Raman spectra showed the presence of two clear signature peaks around 1358 cm$^{-1}$ (D-band) and 1586 cm$^{-1}$ (G-band) in the carbonaceous cores. The D-band corresponds to structural defects in graphitic $sp^3$-hybridized carbon and the G-band is represented by disordered $sp^2$-hybridized carbon clusters. The Raman spectra were deconvoluted and their corresponding areas were used to estimate the $sp^2$-to $sp^3$-carbon ratio ($I_G/I_D$ ratio) (Figure S5, Supporting Information). A reduction of the ratio from 1.4 for the CAPDA-p core to 0.8 for the CAEDA-p core indicated comparatively higher $sp^2$-content inside the CAPDA-p C-dots. Next, we investigated the formation of molecular fluorophores.

### 2.3. Characterizations of the Molecular Fluorophores Associated with C-Dots

The CA and the amine precursor react under microwave conditions and polymerization, dehydration, and continuous aromatization could occur that could lead to the formation of the C-dots as well as fluorophore molecules. Initially, the as-synthesized carbon nanodot solution was filtered through 0.2 μm pore-sized cellulose membrane-based syringe filter (CHROMAFILRC) to remove any possible aggregates before...
further purifications. In order to assess, whether C-dots and associated fluorophores were formed, we applied repeated dialysis purification to separate the carbonaceous C-dot cores from the free molecular fluorophores in solution. We envision that all the as-synthesized C-dots are associated with fluorophores similar structures that are either bound covalently or strongly physisorbed at their surface or mechanically trapped inside the C-dot structure. The CAPDA and CAEDA C-dots suspension turned colorless (as depicted Figure S6a, Supporting Information, digital image, schematic illustration in Figure S6b, Supporting Information) after purification by dialysis and HPLC (Figure S7a, Supporting Information), and the fluorescent solutions containing the fluorophores were isolated. In contrast, dispersed CAPEG as well as its surrounding solution appeared dispersed CAPEG as well as its surrounding solution appeared colorless, indicating that no fluorophores were formed (Figure S6c, Supporting Information). Purified fractions showed the presence of two chromophore molecules corresponding to fluorophore 1 that was formed in about 25% yield (structure 1, 264.07 g mol\(^{-1}\), \(\text{C}_{12}\text{H}_{12}\text{N}_{2}\text{O}_{4}\)) and fluorophore 2 that was formed in about 63% yield (structure 2, 246.06 g mol\(^{-1}\), \(\text{C}_{12}\text{H}_{10}\text{N}_{2}\text{O}_{4}\)) as depicted in Figure 3. The yield was estimated from NMR but inside the measured solution obtained from the fractions F1–F3 (Figure S7a, Supporting Information). The fractions F1–F3 showed strong electrospray ionization mass spectrometry (ESI-MS) signal at \(m/z\) 265.08 as compared to the fractions F4 and F5, indicating the presence of purified molecule in larger quantity in the fractions F1–F3 (Figure S7b, Supporting Information). Also, some impurities could not be removed and were detected in HPLC and in the NMR spectrum. The structures of the isolated fluorophores from CAPDA (1 and 2) and CAEDA 3 (216.07 g mol\(^{-1}\), \(\text{C}_{12}\text{H}_{12}\text{N}_{2}\text{O}_{4}\)) were analyzed by \(^1\)H-NMR as depicted in Figure 3a,b, S8, S15, Supporting Information, 13C-NMR (Figure S9, S16, Supporting Information), \(^1\)H-\(^{13}\)C HSQC (Figure S10, S17, Supporting Information), \(^1\)H-\(^{13}\)C HMBC (Figure S11, S18, Supporting Information), COSY (Figure S12, S19, Supporting Information), and DOSY (Figure S13, S20, Supporting Information). The shorter relaxation time due to the interaction of the connected quadrupole nuclei \(^{14}\text{N}\) to the neighboring dipole carbon atoms can be a plausible reason for broadening the \(^{13}\text{C}\)-NMR signal\(^{[68]}\) and resulting in the signal disappearance of carbon atom C8 in CAPDA fluorophore and C5 in CAEDA fluorophore (Figure S9, S14, S16, Supporting Information). The signals of two coupled AB systems were identified and assigned (CH3 groups C11, C13 in structure 1, CH2 groups C30, C33 in structure 2 of the CAPDA fluorophore and CH2 groups C1, C2 and C7, C10 in structure 3 of the CAEDA fluorophore) (Figure 3a,b, S8, S15, Supporting Information). The \(^1\)H-NMR signals of coupled hydrogen systems (H20, H21 and H22, H23) in CAEDA fluorophore were identified from \(^1\)H-\(^{13}\)C HSQC and \(^1\)H-\(^{13}\)C HMBC spectra (Figure S17, S18, Supporting Information). The proposed molecules and their elemental compositions were further confirmed by accurate mass and MS/MS analysis (Figure 3c,d, S21 and Table S2, Supporting Information). The highly symmetric structure 2 (\(\text{C}_{12}\text{H}_{10}\text{N}_{2}\text{O}_{4}\)) is a condensation product of structure 1 (\(\text{C}_{12}\text{H}_{12}\text{N}_{2}\text{O}_{4}\)). But the signals of structure 2 were not detectable in the mixture possibly because of ionization suppression effects. However, the fragmentation pattern of the CAPDA fluorophore molecular ion (\(m/z = 265.08\)) showed the presence of signals at \(m/z\) 247.06 and 229.05, suggesting that such cyclic molecules detected by NMR are very probable intermediates from one of the fluorophores in the mixture (Figure S22, Supporting Information). In general, the fragmentation pattern of the fluorophores shows multiple mass differences of \(m/z\) 18.01 for \(\text{H}_{2}\text{O}\) and 43.99 for \(\text{CO}_{2}\), indicating that the main decomposition pathway is dehydration and decarboxylation and thus confirming the presence of multiple number of –OH and –COOH functional groups in the precursor molecules. The reported structures were elucidated based on state-of-the-art \(^1\)H and \(^{13}\)C NMR spectra including 2D NMR correlation graphs (Figure S8–S13, S15–S20, Supporting Information) as well as ESI-MS spectra (Figure S32a, S33a, Supporting Information) and MS/MS spectra (Figure S21, Supporting Information) of the C-dot (a) CAPDA, (b) CAEDA molecular fluorophores and elemental composition analysis (Table S2, Supporting Information). Furthermore, similar ions with a molecular mass of 217.08 (MH\(^{+}\)) were found for fluorophores synthesized from CA and passivating agents ethylenediamine and 1,10-diaminocadene (DAD) under identical reaction conditions (Figure S23, Supporting Information). The MS/MS spectra together with the elemental compositions from accurate mass measurements and structural confirmation from NMR unambiguously prove the proposed structures. Formation of stable, low ring strain five- and six-membered structures during condensation and aromatization processes can be a probable reason for the formation of similar chemical structures from a longer alkyl chain containing precursor DAD molecule. Probable formation mechanism of the proposed fluorophores is illustrated in Figure 3e,f.\(^{[25]}\)

2.4. Optical Properties and TP Emission of the Dispersed C-Dots and the Associated Chromophores

All C-dots elicit a characteristic first absorbance peak in the ultraviolet region, i.e., between 350 and 400 nm (Figure 4a–c), and there was no appreciable absorption beyond 400 nm. C-dots obtained from CAPDA and CAEDA showed excitation-independent emission spectra (250–400 nm excitation range), whereas the emission of CAPEG was excitation dependent (300–400 nm excitation range). After excitation at \(\approx 350\) nm, fluorescent QYs of 28% (CAPDA), 72% (CAEDA), and 13% (CAPEG) were determined. A larger core size in CAEDA-p might be responsible for higher relative fluorescent QY compared to CAPDA.\(^{[24]}\) The presence of N-atoms in the precursors could lead to higher QY in CAPDA and CAEDA compared to CAPEG.\(^{[25,69]}\) Similar trends in QY were observed in the separated fluorophores. We also performed photostability experiments on the C-dot cores, and the isolated fluorophores are depicted in Figure 4d. The C-dot cores or the isolated fluorophore molecules were embedded in an agarose gel and continuously irradiated with one-photon laser excitation of wavelength 405 nm for about 6 min. The carbonaceous core CAPDA-p was significantly more photostable compared to the free molecular fluorophores 1 and 2 given in Figure 3.

Next, the CAPDA and CAEDA C-dots were subjected to an 810 nm laser excitation source to determine their TP absorption properties. Both CAPDA and CAEDA C-dots exhibited maximum TP emission at 810 nm excitation wavelength. Thus, we employed the same mass concentration, i.e., 1 mg mL\(^{-1}\), for further comparison. Although CAEDA showed higher one-photon
QY, we observed higher fluorescence intensity in the visible region for CAPDA-derived C-dots after 810 nm excitation (Figure 5a–d). In addition, CAPDA nanodots revealed a two-photon absorption cross section (TPACS) of 17.8 GM, whereas CAEDA nanodots have a considerably lower TPACS of 3.1 GM (Figure 5e). Both C-dots showed relatively low TPACS values when compared to values greater than 10^4 GM reported in the literature.\[70–72\] Nevertheless, our low TPACS values are in good agreement to the reported values in other reports.\[73,74\] However,

Figure 3. ^1^H-NMR characterizations of molecular fluorophore for the C-dots: a) CAPDA, two molecules were detected in the measured solution: 1 (264.07 g mol\(^{-1}\), C\(_{12}\)H\(_{12}\)N\(_{2}\)O\(_{5}\)) and 2 (246.06 g mol\(^{-1}\), C\(_{12}\)H\(_{10}\)N\(_{2}\)O\(_{4}\)) in 25% and 63% yield, respectively; b) CAEDA fluorophore 3 (216.07 g mol\(^{-1}\), C\(_{8}\)H\(_{12}\)N\(_{2}\)O\(_{5}\)). ESI-MS spectra showing the mass signals of the fluorophores associated to the C-dots: c) CAPDA (MH\(^+\) 265.08) and d) CAEDA (MH\(^+\) 217.08). Schematic diagram of the proposed formation mechanism for the synthesis of the molecular fluorophores associated with the C-dots e) CAPDA and f) CAEDA.\[25\]

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the facile synthesis, excellent photostability, and excellent biocompatibility make these TP active materials very attractive for various deep tissue biological applications.

Interestingly, if an excitation wavelength of 400 nm was applied, the one-photon emission intensity of an optically matched (i.e., of the same optical density) CAPDA C-dot solution was higher than that of CAEDA nanoparticles. In contrast, CAEDA C-dots emitted relatively higher PL at other excitations, namely, 300, 350, and 450 nm (Figure 5f). We believe that the higher one-photon emission of the CAPDA C-dot after 400 nm excitation corroborates with its larger TP absorption and emission properties at near 800 nm. We predict that the TP absorption and emission properties of CAPDA C-dots could be due to the higher π-electron content. In addition, we have performed the same TP excitation and compared the emission intensities from both carbonaceous cores CAPDA-p and CAEDA-p and their separated fluorophores 1 and 2. However, we did not find any significant change in their optical features implying a close association between the carbonaceous cores and the respective fluorophores.

2.5. Correlation of the Structure and the Optical Properties of the C-Dots and Their Associated Chromophores

The energy gap of the carbonaceous cores of CAPDA-p and CAEDA-p was measured as 1.91 and 1.56 eV, respectively, using cyclic voltammetry (CV). The observed bandgap decrease in CAEDA-p was further substantiated by optical bandgap calculations determined from the UV–vis absorption spectra, which gave a bandgap of 2.59 and 1.94 eV for CAPDA-p and CAEDA-p, respectively (Table 1 and Figure S26, S28, Supporting Information). Additionally, the energy bandgaps of the CAPDA- and CAEDA-derived fluorophores (1, 2, and 3, respectively) were simulated using TD-SCF DFT calculations implemented in the Gaussian package (Gaussian 09W, version 9.5), applying the B3LYP method, the DGTZVP basis set, and the CPCM solvation model of water. Interestingly, the energy bandgap showed an opposite trend for separated associated fluorophores. The HOMO–LUMO energy gaps were calculated to be 5.04 eV, 4.17 eV (both CAPDA-derived fluorophores 1, 2), and 5.40 eV (CAEDA-derived fluorophore 3) (Table 1 and Figure S29, Supporting Information). The observed energy gap trend in the molecular fluorophores could be attributed to the presence of π-electrons in the benzene ring in the CAPDA fluorophores with extended electron delocalization resulting in a lower bandgap. Table 1 summarizes the trend in the sp² and sp³-hybridization ratios and the HOMO–LUMO energy gap (eV) across various C-dots that were characterized by different techniques. We believe the presence of increased π-electrons in CAPDA C-dots may have a strong influence on their TP emission features.

Next, we have investigated the nature of the association between the fluorophores and the carbon nanodot by applying XPS, Fourier-transform infrared spectroscopy (FTIR), ESI-MS,
Figure 5. a,b) Scheme of two-photon and one-photon emissions of C-dots CAPDA and CAEDA that were excited at two different wavelengths, namely, 350 and 810 nm. c,d) Corresponding PL characteristic peaks of both C-dots (1 mg mL\(^{-1}\) each). After excitation at 350 nm, CAEDA revealed higher PL than CAPDA, whereas after excitation at 810 nm using an FL 6500 Pulse Fluorescence Spectrophotometer at 80 kW power, 400 V, CAPDA revealed higher PL instead. e) TPACS of C-dots CAPDA and CAEDA measured in water at an excitation wavelength of 810 nm using Rhodamine B as reference. f) PL behavior of as-prepared C-dots CAPDA and CAEDA under different one-photon excitation (300, 350, 400, and 450 nm) and same mass concentration 1 mg mL\(^{-1}\). Interestingly, the PL emission of C-dot CAPDA was significantly higher compared to C-dot CAEDA at 400 nm excitation (green dashed circle).

Table 1. Characterization of the carbonaceous cores CAPDA-p, CAEDA-p, and CAPEG-p and their associated molecular fluorophores 1, 2, and 3. The \(sp^2\)-and \(sp^3\)-hybridized carbon content of the C-dots was estimated from XPS, Raman and EELS measurements. The energy bandgaps of C-dots were evaluated from UV–vis, CV and Gaussian calculations.

| Characteristic properties | Method                  | Carbonaceous cores | Fluorophore |
|--------------------------|-------------------------|---------------------|-------------|
|                          |                         | CAPDA-p             | CAEDA-p     | CAPEG-p     | CAPDA | CAEDA |
| Ratio between \(sp^2\) and \(sp^3\)-hybridization | XPS binding energy     | 1.28                | 0.47        | 0.90        | 1.21  | 0.66  |
|                          | Raman peak              | 1.4                 | 0.8         | –           | –     | –     |
|                          | EELS                    | 0.61                | 0.45        | 0.13        | –     | –     |
| HOMO–LUMO energy gap [eV] | UV–vis (optical bandgap)| 2.59                | 1.94        | 3.23        | –     | –     |
|                          | CV                      | 1.91                | 1.56        | 1.67        | –     | –     |
|                          | Gaussian Calculations   | –                   | –           | –           | 5.04  | 4.17  | 5.40  |
and their optical characterizations. We found that the $sp^2$, $sp^3$-carbon ratio on the surface of the C-dot core CAPDA-p (1.28) appears in a similar range as the carbon ratio of its associated molecular fluorophore (1.21), determined by XPS characterizations (Figure 2a–c, S30 and Table S3–S5, Supporting Information). Also, the carbon ratio of the surface of the core CAEDA-p and the corresponding fluorophore 3 were similar with 0.47 and 0.66, respectively. These data could indicate remaining fluorophores associated with the C-dot core, which were challenging to separate by dialysis. FTIR studies of CAPDA-p C-dot core and the free fluorophores 1 and 2 were performed and many identical IR bands (Figure S31a, S31b, Supporting Information) such as the broad $O–H$ stretching frequency at $\approx 3050 \text{ cm}^{-1}$, the $C=O$ stretching of carboxylic acids at $1713 \text{ cm}^{-1}$, and prominent aromatic $–C=C$ stretching vibrations at 1578 and 1392 cm$^{-1}$ were found. ESI-MS data depicted in Figure S32, Supporting Information, indicated similar molecular ions (MH$^+$) of the CAPDA-derived fluorophores (265.08) with the C-dot core CAPDA-p (265.13). We believe that the observed fluorophores in the ESI-MS of the purified carbonaceous cores were generated during sample preparation. The slightly acidic conditions of the ESI solvent could cause a physical or chemical detachment of fluorophores from the core surface. Additionally, we uncovered that the C-dot CAPDA-p core as well as the separated fluorophores showed similar characteristic one-photon absorption and emission peaks at 450 nm under 400 nm excitation (Figure S34, Supporting Information, in CAPDA C-dots) as well as similar characteristic TP emission peaks after 810 nm excitation (Figure S25, Supporting Information). Therefore, even after several thorough washing steps and purification via dialysis, the presence of fluorophores in the carbonaceous cores could still be detected. Most likely, the fluorophores could be associated through covalent and noncovalent conjugation with the carbonaceous core.[77] In this case, the carbonaceous core could stabilize the organic fluorophore so that they do not bleach easily, whereas the fluorophore could serve as TP emission center.

2.6. Cytotoxicity of the Carbon Nanodots

C-dots have been used as drug delivery vehicles[78,79] as their surface could be functionalized easily through bioconjugation reactions[80,81] and they could be easily detected allowing their intracellular imaging and tracking.[10] In particular, their application as TP fluorescence tag for deep tissue imaging has been of great interest.[78,82] Therefore, we have studied the C-dots in vitro and cell viability tests (Tox-8 assays) have been performed with various weight concentrations using HeLa cells as cell model. Both the purified carbonaceous core CAPDA-p and the C-dot

![Figure 6](image-url)
solution reveal low cytotoxicity applying a HeLa cell line even at high concentrations 500 μg mL⁻¹ (Figure 6a).

The purified carbonaceous C-dot core CAPDA-p and CAEDA-p were studied by applying fluorescence lifetime imaging microscopy (FLIM). In aqueous solution, biexponential fluorescent decays of the carbonaceous cores CAPDA-p and CAEDA-p were observed after excitation at 810 nm and average lifetimes of 10.1 and 9.7 ns were determined, respectively (Figure 6b). Next, we recorded the FLIM signals inside living HeLa cell after 810 nm excitation. The average lifetimes of both C-dots decreased significantly inside cells compared to the bulk solvent, which was expected as many new nonradiative pathways could exist in this complex biological environment. A similar biexponential decay with an average lifetime of 1.2 and 3 ns was observed in FLIM for C-dot CAPDA-p and CAEDA-p, respectively (Figure 6c,d).

3. Conclusions

We have prepared three different C-dots based on aliphatic and aromatic molecular precursors and analyzed their structural and optical characteristics. During microwave synthesis, organic fluorophores associated with C-dot structures were formed mainly in the C-dots from CAPDA and CAEDA. CAPEG-derived C-dots did not show any associated molecular fluorophores and TP emission characteristics. We have separated the fluorophores from these C-dots and elucidated their molecular structure by NMR and mass spectrometry. Differences in the molecular structures of the formed fluorophore are responsible for the unique TP emission properties of the C-dots. The emergence of TP emission was observed and correlated to the hybridization state of the carbon atoms within the C-dot as well as the fluorophores associated to the C-dots. Higher TPACS was observed for CAPDA C-dots, which contained higher amounts of π-electrons in the fluorophores and carbonaceous core morphology. The fluorophores were bound to the C-dots via a noncovalent as well as covalent interaction and revealed higher photostability in the presence of the C-dots. The C-dots CAPDA and CAEDA were nontoxic in a cell model and fluorescence lifetime imaging was performed inside cells.

We believe that an improved understanding of the impact of the precursor molecules on the molecular structure of the C-dot core and the formed organic fluorophores would enable to rationally design C-dots with improved optical features, which would be of great relevance for their applications. We envision new avenues to design and tune tailor-made C-dots with improved QY, enhanced TP emission, and excellent photostability for bioimaging, therapeutic, and other relevant biomedical applications.

Research data are not shared.

Keywords

carbon nanodots, fluorescence lifetime imaging microscopy, formation mechanism, hybridization, molecular fluorophores, optical stability, two-photon emission

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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