Effect of precursor molecular structure on the facile synthesis of efficient red-emissive two-photon carbon dots: real-time cellular imaging and high-resolution deep-tissue imaging

Pooria Lesani (pooria.lesani@sydney.edu.au)
THE UNIVERSITY OF SYDNEY

Aina Hazeera Mohamad Hadi
The University of Sydney

Mansi Khetarpaul
The University of Sydney

Zufu Lu
The University of Sydney

Hala Zreiqat
The University of Sydney

Article

Keywords:

Posted Date: December 29th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2403572/v1

License: ☑️ This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

In recent years, the development of carbon dot-based fluorescent nanoparticles for bioimaging applications has attracted the attention of scientific community. However, the functionality of the majority of these developed fluorescent systems is confined in to the blue-to-green region of the light spectrum, limiting their application as bioimaging agents. Here, we report the facile design and development of highly efficient two-photon excitable red-emissive carbon dots (D-CD) and demonstrate their high performance in bioimaging applications. Our comprehensive study on the effects of synthesis parameters on the photophysical and physicochemical properties of carbon dots demonstrated the importance of precursor selection and consideration of its molecular structure in developing red-emissive carbon dots. The developed D-CD is highly biocompatible and non-toxic, with remarkable photostability in cells under two-photon near-infrared excitation. The present study point to the great potential of D-CD as an efficient bioimaging agent for cellular biolabeling, long-term and real-time cellular imaging, and high-resolution deep-tissue imaging in complex biological systems.

Introduction

Carbon dots (CDs) are widely investigated for various bioimaging applications due to their superior physicochemical and biological properties \(^1\); compared to the traditional inorganic and organic materials. For instance, semiconductor quantum dots and organic dyes, suffer from cytotoxicity and photobleaching, respectively \(^2\). Besides their good biocompatibility and high photostability, CDs have excellent water solubility, ease of synthesis and surface functionalisation, excellent two-photon excitation capability, and unique optical tunability \(^3\). However, the problem remains their confined emission in the blue-green region of the light spectrum, limiting their wide biological applications. In attempt to address this issue, research has been directed to altering the optical properties of CDs through varying the synthesis methods and/or synthesis parameters, broadening their use in a wide range of applications \(^4\).

A number of synthesis routes have been utilised for the development of CDs, including microwave irradiation, chemical ablation, and electrochemical carbonisation. However, hydrothermal/solvothermal is the most common method due to its low energy consumption and simple processing \(^2,5,6\). Several parameters are involved in this method, including precursor and dopant quantity, solvent, heating time, heating temperature, and particle size distribution range \(^4\). Varying these synthesis parameters can significantly affect the physicochemical and photophysical properties of the CDs, allowing for different potential applications. We previously, we demonstrated the effects of solvent, heating time, dopant quantity, and particle size distribution on the properties of CDs, where the solvent showed the most significant impact on the CDs optical properties \(^4\). However, the optical characteristics were only redshifted from the ultraviolet to the green region of visible spectrum. These blue-green emissive CDs require high-energy excitation resulting in photobleaching, photodamage, and limited penetration depth due to the light absorption and scattering by biological tissues \(^2\). This can damage the surrounding cells;
and paired with the strong blue-green autofluorescence of biological tissue, limits the application and effectiveness of CDs as bioimaging agents.

To overcome these limitations, significant interest has been garnered in developing two-photon excitable CDs with excitation and emission wavelengths within the red-to-near infrared (NIR) region. Red-shifting the optical properties of CDs to longer wavelengths minimises the issues associated with blue-green emissive CDs and allows for their use as a bioimaging agent within different spectral ranges for cells and tissues. Two-photon excitable CDs can be excited by the simultaneous absorption of two lower energy photons, reducing the reliance on high energy photons. This increases their tissue penetration depth whilst eliminating issues associated with photodamage, photobleaching, and autofluorescence.

Currently, a few studies have reported on the development of two-photon excitable red-emissive CDs for biolabeling and bioimaging applications. While some promising results were obtained, there are still some drawbacks. Specifically, the CDs suffer from low fluorescence quantum yield, relative high toxicity, low photo- and chemical-stability, and insufficient two-photon absorption. In addition, the development of these CDs tends to be a fortunate outcome of reported synthesis methods, as they lacked a systematic study on how various synthesis parameters would affect physicochemical and biological properties of as-synthesised CDs.

In this study, we developed the next generation of two-photon excitable red-emissive CDs (D-CD) through our novel synthesis method and a rationalised selection of the starting precursors. A comprehensive study was conducted to investigate the effects of the precursor's molecular structure on the CDs optical properties. D-CD was found to have a high quantum yield (QY), outstanding photostability in complex biological environments, excellent biocompatibility, two-photon excitation ability, and rapid cellular uptake and localisation within specific cellular compartments. We then examined the effectiveness of D-CD as a high-resolution bioimaging agent for both cellular and deep-tissue applications within complex biological systems.

Results

Effect of precursor molecular structure on CD optical properties

The hydrothermal/solvothermal process has been widely used to fabricate CDs due to its cost-efficient and eco-friendly nature. Lan et al. hydrothermally synthesised two-photon excitable NIR-emissive CDs at 180°C for 24 h using polythiophene and diphenyl diselenide as the precursors, and sodium hydroxide aqueous solution as the solvent. The CDs had a QY of 0.2% and were further used for in vitro and in vivo imaging and photothermal therapy. In another study, the red emissive two-photon CDs with QY of 26.28% were fabricated through hydrothermal treatment of dopamine and o-phenylenediamine at 200°C for 8 h. The synthesised CDs were utilised for in vivo bioimaging in nude mice. The citric acid and urea...
were also used as ingredients for solvothermal synthesis of red-emissive two-photon CDs, using dimethylformamide (DMF) as the solvent at 160°C for 6 h (QY = 26% in DMSO) \(^{13}\). These CDs were further applied for \textit{in vivo} NIR fluorescence imaging in mice.

The previous studies established that red-emissive two-photon CDs can be synthesised under a range of different synthesis conditions, with each possessing distinctive photophysical properties \(^{9}\). Therefore, optimising the synthesis parameters is an important step to ensure the reproducible preparation of CDs with optimal optical properties, safety, and biocompatibility for a range of biological applications. Our recently published study \(^{4}\), involved optimising the different synthesis and post-synthesis parameters (solvent, heating time, dopant quantity, and particle size distribution range) and investigating the effects on the photophysical and biological properties of CDs. In that study \(^{4}\), we demonstrated that CDs synthesised for 8 h in deionised (DI) water, with a nitrogen doping ratio of ascorbic acid : arginine mass = 1:1.5, and with largest particle size distribution of 30–100 KDa, demonstrated optimal photophysical and biological properties, aptly named 30–100 KDa-CD. However, the variation in these parameters can only alter the excitation and emission wavelengths of 30–100 KDa-CD from the ultraviolet to green region of the visible spectrum, limiting its wider application as bioimaging agent.

We have recently noticed that the precursor molecular structure may have a direct impact on the photophysical and physicochemical properties of the CDs. This is because the starting material breaks down only partially during the synthesis process, resulting in the retention of some aspects of the initial structure in the synthesised CDs \(^{9}\). Therefore, this study involved synthesising CDs using the optimised synthesis conditions obtained from our previous study, with the inclusion of 1,4,5,8-tetraaminoantraquinone as an additional precursor with aromatic structure to ascorbic acid and arginine with aliphatic structures. The chemical, structural, and photophysical properties of the 30–100 KDa-CD were then compared with the new synthesised CDs.

The CDs were separately synthesised in the presence of water (to give W-CD), ethanol (E-CD) and DMSO (D-CD). These solvents are commonly used when synthesising CDs via the hydrothermal/solvothermal method, due to their different physiochemical properties \([11]\). The as-synthesised CDs were filtered using centrifugal filters with molecular weight cut-offs (MWCO) of 30–100 kDa to obtain their optimum size distribution range \([4]\). The comparison between the three CDs shows that using DMSO as the solvent results in an efficient hydrothermal synthesis reaction with a high degree of carbonisation. We found that D-CD is a more suitable bioimaging agent compared to W-CD and E-CD, due to its promising QY value and optimal excitation and emission wavelengths (Table 1, Figure S1). TEM images also show a narrow size distribution and spherical morphology of D-CD with an average size of 9.03 ± 1.30 nm (Fig. 1A, B).
Table 1
Summary of optical properties of synthesised CD samples

<table>
<thead>
<tr>
<th>sample</th>
<th>optimal $\lambda_{\text{ex}}$ (nm)</th>
<th>optimal $\lambda_{\text{em}}$ (nm)</th>
<th>QY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W-CD</td>
<td>580</td>
<td>680</td>
<td>24</td>
</tr>
<tr>
<td>E-CD</td>
<td>590</td>
<td>690</td>
<td>16</td>
</tr>
<tr>
<td>D-CD</td>
<td>595</td>
<td>700</td>
<td>33</td>
</tr>
</tbody>
</table>

$\lambda_{\text{ex}}$, excitation wavelength; $\lambda_{\text{em}}$, emission wavelength; QY, quantum yield; FWHM, full width at half maximum.

To determine the effects of the precursor molecular structure on the CDs physicochemical properties, the structural and optical properties of the developed D-CD were investigated and compared with 30–100 kDa-CD. The surface chemistry and chemical functionality of these CDs were analysed using FTIR and XPS techniques, respectively. The FTIR spectra of 30–100 kDa-CD (Fig. 1C) displayed peaks corresponding to N–H (3991 cm$^{-1}$), O–H (2951 cm$^{-1}$, 1399 cm$^{-1}$, 1356 cm$^{-1}$), C–H (1770 cm$^{-1}$, 1459 cm$^{-1}$), C = N (1684 cm$^{-1}$), C = C / N–H (1597 cm$^{-1}$), and C–N (1175 cm$^{-1}$, 1102 cm$^{-1}$, 1050 cm$^{-1}$). The FTIR spectra of D-CD show the formation of O–H / N–H (3260 cm$^{-1}$), C–H (2920 cm$^{-1}$, 2850 cm$^{-1}$, 1470 cm$^{-1}$), C = N (1774 cm$^{-1}$), C = C/N–H (1651 cm$^{-1}$), C = O / S = O (1575 cm$^{-1}$), C–C (1415 cm$^{-1}$), C–N (1320 cm$^{-1}$), C–S (1178 cm$^{-1}$), N–O (1106 cm$^{-1}$), C–O (1015 cm$^{-1}$), and C = S (950 cm$^{-1}$).

The comparison between the FTIR spectra of both CDs demonstrates a blue shift and reduced intensity in most D-CD bands, along with the presence of new functional groups such as C = O, C = S, C–S, N–O, C–O, and S = O. These differences account for the major chemical and/or structural changes that may have occurred due to the interactions between the aromatic and aliphatic structures present in the precursors. In addition, the presence of a strong stretching vibration band (O–H / N–H), the introduction of a large number of oxygenated and nitro- functional groups (carbonyl (C = O), ether (C–O), nitro (N–O)), and the formation of new bands at 1415 cm$^{-1}$ (C–C) and 1470 cm$^{-1}$ (C–H), indicate the development and growth of new polyaromatic structures and carbon domains in D-CD compared to 30–100 KDa-CD$^{14,15}$. The formation of sulfur-functional groups (C–S, C = S, S = O) can be attributed to using DMSO as the solvent.

XPS survey scans were acquired for 30–100 KDa-CD and D-CD to further confirm FTIR results and identify the CDs’ chemical composition. These scans show three and four dominant peaks in 30–100 KDa-CD (corresponding to C 1s, N 1s, O 1s) and D-CD (corresponding to C 1s, N 1s, O 1s, S 2p), respectively (Fig. 2, S4, Table 2). The atomic percentage (At%) of these two CDs is reported in Table 2, and demonstrates the presence of more significant amounts of nitrogen and oxygen within the structure of D-CD, compared to 30–100 KDa-CD. Analysing the high-resolution spectra (C 1s, N 1s, O 1s, S 2p) of both samples indicates the introduction of a large number of oxygenated and nitrogen functional groups into the structure of D-CD as well as the presence of new functional groups (C-H, Graphitic N, C-OH, COOH,
C-S, S-H, S-C, S = O) (Table S1, S2). These results support the findings obtained from the FTIR results and reveal the formation of new polyaromatic structures and carbon domains within D-CD due to the integration of an additional aromatic precursor (1,4,5,8-tetraaminoantraquinone).

Table 2
Atomic percentage of CDs from XPS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>%C 1s</th>
<th>%N 1s</th>
<th>%O 1s</th>
<th>%S 2p</th>
</tr>
</thead>
<tbody>
<tr>
<td>30–100 KDa-CD</td>
<td>60.47</td>
<td>22.5</td>
<td>17.0</td>
<td>n/a</td>
</tr>
<tr>
<td>D-CD</td>
<td>43.76</td>
<td>25.57</td>
<td>25.30</td>
<td>5.37</td>
</tr>
</tbody>
</table>

The optical characteristics of 30–100 KDa-CD and D-CD were studied using ultraviolet-visible (UV-Vis) absorbance and fluorescence (FL) spectroscopies (Fig. 3, S2). 30–100 KDa-CD possess an absorption peak at 336 nm, ascribed to the transition of lone pair electrons from n-nonbonding electron bonds to π*-antibonding electron bonds due to the presence of C = N bonds. The emission peak observed at 450 nm, when excited at the maximum excitation wavelength (λ_{ex} = 360 nm), is likely due to the electron relaxation in C = C and graphitic C-N bonds in the nitrogen-doped graphitic sp2 hybridised cores. As compared to 30–100 KDa-CD, the absorption spectrum of D-CD demonstrates a shoulder peak at 320 nm and a main peak at 595 nm, confirming the contribution of π-π* transition of the aromatic C = C bond, and π-π* and n-π* transition of the aromatic π system due to the presence of C = N, C = O, and C = S bonds. The FL spectrum of D-CD shows a sharp peak at 700 nm upon excitation at the maximum excitation wavelength (λ_{ex} = 595 nm). The overlap between the absorption and FL spectra of D-CD at ~ 595 nm further indicates the presence of newly formed aromatic structures, resulting in the observed NIR emission (Figure S3). Our results demonstrate that introducing a precursor with an aromatic molecular structure and high-density sp2 content can significantly change the chemical, structural, and photophysical characteristics of CDs. This is due to the higher degree of carbonisation of the carbon cores, higher oxidation, aromaticity, and greater conjugation of sp2 carbons in the newly formed CDs structure. Increasing the rate of formation of aromatic rings, increases the size of conjugated-π-systems within the structure of D-CD, leading to a shift in valence electrons which reduces the HOMO-LUMO energy gaps and causes a red-shift in emission wavelengths. Additionally, the greater density of carboxyl and hydroxyl groups correlates to a lower band gap energy (E_{bg}) and greater electron donating behaviour of D-CD, respectively, which further shifts the emission wavelengths into the NIR region.

D-CD displayed a significantly higher QY (0.33), compared to 30–100 KDa-CD (0.2), which can be attributed to the greater concentration of the nitrogen dopant and the introduction of a sulfur dopant in D-CD. According to FTIR/XPS results, these dopants resulted in the formation of new sulfur groups and introduced more nitrogen groups, altering the surface structure and functional groups distribution of D-CD. These functional groups have a low E_{bg} and high electron donating behaviour and would act as excitation energy traps with a concomitant effect of enhancing the fluorescence. Hence, we have
clearly demonstrated the importance of precursor selection and consideration of its molecular structure on the optical properties of D-CD.

D-cd For Cellular Bioimaging

Currently, most CDs and fluorescent molecules only have excitation and emission wavelengths within the blue to green region of the visible light spectrum with deficient photostability and biocompatibility, compromising their in bioimaging applications. Therefore, the development of CDs with longer wavelengths towards the red-to-NIR region, high photostability, and low toxicity is essential for long-term and real-time monitoring of biological processes.

In recent years, the development of two-photon excitable fluorescent probes has received significant attention owing to their ability to be excited through simultaneous absorption of two near-to-mid-NIR photons. This minimises the interference from background species, lowers the risk of photodamage to biological tissues, and in-turn longer observation time, and increases the penetration depth in tissue. We demonstrated that through single-photon excitation (SPE), D-CD can be excited and emit light in the orange (595 nm) and red (700 nm) regions of the light spectrum, respectively. Next, we examined the two-photon excitation (TPE) ability of D-CD and observed an excitation peak at 945 nm, and an emission peak at 580 nm upon single excitation at 945 nm (Figure S5). We used MTS assay to determine the cytotoxicity of fibroblasts cultured in the presence of various concentrations of D-CD. Our results showed that fibroblast remained viable when cultured for 72 h in the presence of D-CD at concentrations ranging from 0 to 800 µg/mL (Figure S6). These results indicate the potential use of D-CD as a labeling and imaging agent for \textit{in vitro} imaging of biological cells.

The application of D-CD as a labeling agent for fluorescent cellular imaging was also investigated. This was done by performing SPE and TPE fluorescence imaging of live fibroblasts incubated with 200 µg/mL D-CD for 24 h. The overlay (Fig. 4C, E) of the fluorescence images (Fig. 4B, D) and differential interference contrast images (Fig. 4A) show simultaneous cytoplasm and nucleolus labeling of D-CD in live cells. The localisation of D-CD in different cellular organelles may be due to the size and surface charge heterogeneity of D-CD, as well as its interactions with ribosomal RNA, present in both the cytoplasm and nucleolus. Additionally, the higher resolution and stronger fluorescence of the image obtained from TPE (Fig. 4D), compared to SPE (Fig. 4B), demonstrates that D-CD has a great TPE ability in living cells.

The Z-stack images were also analysed to determine the precise location of D-CD along the Z-axis of the fibroblasts. From Figure S7, cellular uptake and localisation of D-CD occurred in the cytoplasm and nucleolus rather than solely adsorbing onto the surface of fibroblasts, indicating that D-CD was able to penetrate through the cell membrane.

Photostability is essential to ensure the use of D-CD for long-term and real-time bioimaging. The application of commercially available fluorescent labeling agents, such as Alexa 488-streptavidin with ~180 s photostability, is limited due to their poor photostability resulting in lower imaging resolution,
signal intensity, and reduced working life. Here, we assessed the photostability of D-CD through continuous monitoring of fibroblasts incubated with D-CD (200 µg/mL) using a two-photon confocal fluorescence microscope equipped with a benchtop incubator at 37°C with 5% CO₂ for 25 min (λ_ex = 945 nm). The fluorescence intensity of D-CD was reduced by 19% in the first 15 minutes; however, it remained highly stable with negligible changes when continuously imaged for up to 25 minutes (Fig. 5A-G). The exceptional photostability of D-CD is significantly higher than previously reported fluorescent nanomaterials and commercially available cell agents. Therefore, the superior properties of D-CD, such as excellent biocompatibility, low toxicity, two-photon excitation ability, rapid cellular uptake and localisation within cellular compartments, and high photostability in complex biological environments make them an ideal candidate for fluorescence cellular labeling and bioimaging.

**D-cd For Deep Pigskin Tissue Imaging**

The application of already developed fluorescent nanomaterials and molecules for deep-tissue imaging has been limited due to their excitation and emission wavelength lying within the 300–400 nm range. This causes strong tissue autofluorescence from biological tissue, which can significantly decrease the signal-to-noise ratio and reduce contrast, affecting the sensitivity and accuracy of tissue imaging. Using single-photon ultraviolet-visible light as the excitation source causes strong scattering and refraction of the light, limiting the penetration depth. Further, the risk of photodamage to the cells and tissues is increased, and the observation time is shortened. In this study, the deep-tissue penetration depth of D-CD was examined by injecting it into pigskin tissue through the transdermal route and washing it with PBS buffer five times prior to imaging (Fig. 6A). Two-photon excitation fluorescence imaging was performed to investigate the capability of D-CD for deep-tissue imaging (λ_ex = 945 nm, λ_em = 400–800 nm). D-CD demonstrated excellent penetration and distribution within pigskin tissue in a short period, which may be due to their small size (9.03 nm, Fig. 1A, B). Analysing the Z-stack fluorescence images found that D-CD were able to image the tissue for a penetration depth of up to 455 µm. Compared to recently developed two-photon excitable blue-green emissive CDs, D-CD showed remarkably higher tissue penetration depth, spatiotemporal resolution, and signal-to-noise ratio as well as minimal autofluorescence, tissue light scattering, and tissue damage (Fig. 6B). These results indicate the potential use of D-CD as a labeling and imaging agent for in vivo monitoring of biological tissues.

Thus, this study successfully demonstrated the promising physicochemical and biological properties of D-CD and their application in cell labeling, long-term and real-time cellular imaging, and high resolution deep-tissue imaging. D-CD has the potential to replace commercially available fluorescent nanoparticles and bioimaging agents due to its superior photophysical and physicochemical properties.

**Discussion And Conclusion**

In summary, we have successfully developed novel two-photon excitable red-emissive carbon dots (D-CD) by rationally selecting appropriate precursors via a systematically optimised hydrothermal synthesis
method. Compared with previously synthesised CDs which only used aliphatic structured precursors (30–100 KDa-CD), the integration of a precursor with an aromatic structure resulted in a greater degree of carbonisation, higher oxidation and aromaticity, and greater size of conjugated-π-systems within the structure of the D-CD. These chemical and/or structural changes resulted in a significant red-shift in the excitation and emission wavelengths of D-CD. Our findings indicate that D-CD possesses optimal optical properties and can be excited using both single-photon ($\lambda_{ex} = 595$ nm) and two-photon ($\lambda_{ex} = 945$ nm) excitation with emission peaks at 700 and 580 nm, respectively. Further, D-CD possesses a high quantum yield (0.33), negligible cytotoxicity (~100% cell viability after 3 days of treatment), excellent photostability and real-time monitoring ability of cellular compartments (up to 25 min). We showed that D-CD can be utilised as a high-performance agent for simultaneous cytoplasm and nucleolus labeling in live fibroblasts. D-CD can also be used for high resolution deep-tissue imaging applications, as a tissue penetration depth of up to 455 µm was obtained in pigskin tissue, as well as minimal autofluorescence and light scattering. Therefore, this study indicates the significant potential of D-CD as an agent for biolabeling, and long-term and real-time monitoring of biological events in vitro and deep in vivo.

Materials And Methods

Materials

All chemicals used were of analytical grade and required no purification prior to usage. L-Ascorbic acid, L-arginine, ethanol, Dimethyl sulfoxide (DMSO), 1,4,5,8-tetraamionanthraquinone (Disperse Blue 1) were purchased from Sigma-Aldrich (Australia). Dulbecco's Modified Eagle's Medium (DMEM), TrypsinLE Express and phosphate-buffered saline (PBS) were purchased from Gibco Life Technologies (USA). Fetal calf serum (FCS), penicillin and streptomycin were purchased from ThermoFisher Scientific (USA). Primary Human Dermal Fibroblasts from the foreskin, herein referred to as fibroblast, were kindly donated by Prof. Rebecca Mason's group at the University of Sydney. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)−2H-tetrazolium) (MTS) was purchased from Progema (USA). Deionised water was used throughout.

Preparation Of Cds

In this study, CDs were prepared using a one-step hydrothermal/solvothermal route, adapted from our previous study 4. L-Ascorbic acid (0.5 g), L-arginine (0.75 g), and Disperse Blue 1 (0.5 mg) were mixed in 50 mL DMSO followed by ultrasonication to achieve a homogeneous mixture. The solution was transferred into a 100 mL Teflon-lined stainless-steel autoclave chamber with a 50% filling ratio and heated at 180°C for 8 hours, and a dark blue solution was obtained (Fig. 7). The initial purification was performed by passing the solution through a 0.22 µm cellulose acetate Millipore filter (Millipore, USA) to remove the large or agglomerated particles. Next, the CD were separated by size by centrifugation through dialysis tubes with different molecular weight cut-offs (MWCO) to obtain CD with particle size distribution in the range of 30–100 kDa, herein referred to as D-CD. The sample was dried using the rotary evaporator.
(Heidolph, Germany) to produce a 2 mg/mL D-CD particle solution by adding water and kept refrigerated at 4°C for further use. A similar process was used for the synthesis of other samples, except that the solvent was replaced with water and ethanol to prepare W-CD and E-CD, respectively.

**Characterisation**

Transmission electron microscopy (TEM) images were captured by JEOL JEM-2100, operating at 200 kV. Fourier transform infrared (FT-IR) spectra were obtained on a Bruker Tensor 27 FT-IR spectrometer (USA). X-ray photoelectron spectroscopy (XPS) data were obtained using a Kratos Axis Nova spectrometer (Kratos Analytical, UK), equipped with a monochromatised aluminium X-ray source (Alkα 1486.6 eV) operating at 10 mA and 15 kV (150 kW). Survey and high-resolution spectra were acquired at detector pass energies of 160 and 20 eV, respectively. The XPS data were analysed with Thermo Avantage processing software (v5. 9902). Ultraviolet-visible (UV-Vis) absorption and fluorescence spectroscopy were performed using a Varioskan Lux spectrometer (Thermo Fisher Scientific, Singapore). A Leica SP5 II multiphoton confocal microscope (USA) equipped with femtosecond pulsed infrared laser and benchtop incubator (5%, 37°C) was used for investigating the two-photon absorption, emission behaviour of synthesised CD as well as live imaging of fibroblasts and deep pigskin tissue imaging.

**Quantum Yield Measurement**

Fluorescence quantum yield (QY) was determined by plotting the absorbance values and integrated fluorescence intensities of quinine sulfate (QS) as the standard (QY of 54% in 0.1 M H₂SO₄) and the samples at the same wavelength. The gradients of the linear fits of these lines were input into the following formula:

\[
QY = QY_R \frac{m}{m_R} \frac{n^2}{n_R^2}
\]

Where the subscript “R” refers to the reference QS, whilst no subscript refers to the CD sample; “m” is the gradient of the linear fit of absorbance against integrated fluorescence intensity; “n” is the refractive index of the solvent which for Q was ethanol and for carbon dots in water.

**Cell Culture, Viability, And Bioimaging**

Fibroblasts (passage 10) were cultured in ventilated T-175 tissue culture flasks in a complete medium containing DMEM, supplemented with 10% (v/v) FCS, 100 U/mL penicillin and 100 µg/mL streptomycin, herein referred to as supplemented DMEM, and incubated at 37°C in a 5% CO₂ environment. The medium was refreshed every 3 days until 80–90% confluence was reached.
MTS assay was used to determine cell viability. Fibroblasts were incubated with increasing concentrations of D-CD. Fibroblasts were seeded in 96 well plates at 10,000 cells/well density and incubated at 37°C in a 5% CO₂ for 24 h. Next, the medium was removed and replaced with 200 µL of DMEM medium containing CDs of different concentrations (100, 200, 400, and 800 µg/mL). The control cells were supplemented with DMEM only. Fibroblasts viability was assessed using CellTiter 96® AQueous One Solution Reagent (Promega) according to the manufacturer’s instructions. Briefly, 100 µL of MTS solution was added to each well and incubated for 4 h at 37°C under 5% CO₂. The absorbance of the plate was then measured at 490 nm using a Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific, USA). The cell viability (%) was calculated relative to the control averages as per the formula:

\[
\text{Cell viability} = \frac{[A]}{[A]_{control}} \times 100
\]

Where \([A]\) and \([A]_{control}\) is the average absorbance of the control and sample wells, respectively. The mean and standard error of the mean of these cell viability percentages were calculated in Origin® 2018, and SEM was plotted on column graphs as error bars.

The application of D-CD as a labeling agent for fluorescent cellular imaging and deep pigskin tissue imaging was investigated.

Fluorescence live-cell imaging of the fibroblasts incubated with D-CD was performed to investigate its ability as a labeling agent for cellular imaging. Fibroblasts were seeded onto 35 mm glass-bottom dishes at a density of 100,000 cells per dish and incubated for 24 h at 37°C in 5% CO₂. After 24 h, supplemented DMEM was removed from dishes and replaced with medium containing CDs (200 µg/mL) and left to incubate for 24 h at 37°C in 5% CO₂ to allow for the uptake of D-CD by fibroblasts. After incubation, cells were washed three times with 1.0 mL of PBS (10 mM, pH 7.4). The incubated cells with D-CD were immediately observed by a two-photon confocal fluorescence microscope equipped with a benchtop incubator (Leica TCS SP5 II, USA) at 37°C in 5% CO₂.

For tissue imaging, first we cut pigskin with the thickness of ~ 4000 µm. Next, D-CD (200 µg/mL) was transdermally injected into pigskin tissue and washed with PBS buffer five times prior to imaging. Two-photon confocal fluorescence microscope (Leica TCS SP5 II, USA) was used to perform deep pigskin tissue imaging.

**Declarations**

**Acknowledgements**

The National Health and Medical Council (Grant No, APP1107470), The Australian centre for Microscopy and Microanalysis facilities for transmission electron microscopy, and two-photon confocal microscopy,
The Sydney Analytical for spectroscopic measurements.

**Author Contributions**

The experiments were designed and conceived by P.L and H.Z. The carbon dot synthesis, characterisation was conducted by P.L and A.H.M.H. Live cellular imaging and deep pigskin tissue imaging were performed by P.L. Cell culture was performed by Z.L. The manuscript was written by P.L and revised by M.K and H.Z.

**Conflict of Interest statement**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**References**


Figures

**Figure 1**

(A, B) Transmission electron microscopy (TEM) image and particle size distribution of D-CD, respectively (scale bar represents 100 nm). (C) Fourier-transform infrared (FT-IR) spectra of 30-100 KDa-CD and D-CD.
Figure 2

(A, B) XPS full scan spectrum, (C, D) C 1s spectra, (E, F) N 1s spectra, (G, H) O 1s spectra of 30-100 KDa-CD and D-CD, respectively.
Figure 3

Absorption and fluorescence emission spectra of (A) 30-100 KDa-CD, (B) D-CD. ABS stands for absorption, and FL for fluorescence emission. (C-E) Photograph of 30-100 KDa-CD aqueous solution in daylight, under $\lambda_{ex} = 405$ nm and $\lambda_{ex} = 680$ nm, respectively. (F-H) Photograph of D-CD aqueous solution in daylight, under $\lambda_{ex} = 405$ nm and $\lambda_{ex} = 680$ nm, respectively.
Figure 4

(A) Differential interference contrast (DIC) image, (B, D) single-photon excitation (SPE, $\lambda_{ex}=458$ nm) two-photon excitation (TPE, $\lambda_{ex}=945$ nm) fluorescence images, (C, E) and overlay of DIC and fluorescence images of fibroblast incubated with 200 µg/mL D-CD for 24 h. Fluorescence images were taken under consistent microscopy conditions using SPE and TPE (fixed gain, laser power, objective, xyz position). The images are false colour (scale: 15 µm).
Figure 5

Real-time two-photon fluorescence images of fibroblast incubated with 200 µg/mL D-CD. (A-F) Time-lapse confocal fluorescence images of D-CD in fibroblasts were acquired every 1 min ($\lambda_{ex}=945$ nm) under continuous laser irradiation for 25 min. The images are false colour (scale bar: 45 µm). (G) Average corrected cell fluorescence of fibroblast obtained from (A-F).
Figure 6

(A) Schematic of the setup to examine the D-CD penetration depth in pigskin tissue. (B) Two-photon-excited Z-stack fluorescence images of pigskin tissue incubated with D-CD (200 µg/mL) from 0 to 455 µm; Δz = 35 µm, λ_{ex} = 945 nm, λ_{em} = 400–800 nm. Scale bar: 200 µm.
Figure 7

Hydrothermal/solvothermal synthesis diagram of D-CD. The solvent was replaced with water and ethanol to prepare W-CD and E-CD, respectively.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFile.docx](#)