

## GREEN PREPARATION OF FLUORESCENT CARBON DOTS FROM WATER CHESTNUT AND ITS APPLICATION FOR MULTICOLOR IMAGING IN LIVING CELLS

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A green approach was developed from the preparation of fluorescent carbon dots (CDs) by using water chestnut as precursor. The preparation of CDs was performed by simple pyrolysis. The as-prepared CDs are uniform in size, and the quantum yield was 12.6% by using quinine sulfate as the reference. Significantly, not only the precursor of CDs and whole synthesis procedure was green, but also the CDs obtained here exhibited various advantages including high fluorescent quantum yield, excellent photo stability, non-toxicity and satisfactory stability. Additionally, the CDs were employed as cell-imaging agents. The inherent cytotoxicity of CDs was evaluated using HepG2 cell, and the cell viabilities were estimated to be greater than 90% upon addition of the CDs over a wide concentration range of 0-200 $\mu\text{g mL}^{-1}$ . It was then successfully applied for the fluorescence imaging of HepG2 cells. Thus, the as-prepared CDs hold great promise to broaden applications in fluorescence probe and bioimaging.

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### 1. Introduction

Emerging as the newcomers to the carbon nanomaterials, carbon dots (CDs) are generally composed of  $\text{sp}^2$  hybridized carbon atoms with abundant oxygenous and hydrogenous residues with the sizes below 10 nm [1, 2]. Owing to outstanding optical properties, low toxicity, high sensitivity, good biocompatibility, resistance to environmental change and robust chemical inertness, CDs are attracting considerable attention in bioimaging [3, 4], photocatalysis [5, 6] and light-emitting devices [7]. Various methods have been reported in preparation of fluorescent CDs, such as chemical oxidation methods [3, 8-11], electrochemical oxidation processes [12-14], carbonizing organics routes [15-18] and hydrothermal cutting strategies [19,20]. Nevertheless, these approaches usually involve complex post-treatment processes, expensive equipment, and high cost and severe synthetic conditions. Therefore, exploring new methods for synthesizing CDs is still desired. In recent years, several researchers have devoted their time to the preparation of fluorescent CDs emitters without using organic chemicals in every single step, which is called green chemistry concept. And some green synthetic approaches have been developed for the preparation of CDs by using inexpensive renewable resources as precursors, such as soybeans [21], orange juice [22], gas soot [23], grass [24], watermelon peel [25], cornflour [26], carnation [27], ginger [28] and honey [29]. From the point of view of material synthesis, the exploration of new carbon source for simple, economical, and green synthesis of CDs is also highly desirable.

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In this work, we demonstrate the first use of low cost wastes of water chestnut as a carbon source for green preparation of water-soluble fluorescent CDs with a quantum yield (QY) of approximately 12.6%. We further demonstrate that the as-prepared CD is an excellent probe in cellular imaging.

## 2. Experimental

### 2.1. Materials

Freshwater chestnut was purchased from a local market. Its skin was peeled. HepG2 cells (hepatocellular carcinoma cell) were obtained from the American Type Culture Collection (Rockville, MD, USA). Dimethyl sulfoxide (DMSO, >98.0%) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT,  $\geq 98\%$ ) were purchased from Sigma-Aldrich. MB,N, N-dimethyl formamide (DMF), NaOH, H<sub>2</sub>SO<sub>4</sub>, NaCl, and HCl are analytical grade and were used without further purification. Water was purified with a Milli-Q plus 185 equip from Millipore (Bedford, MA, USA) and used throughout the work.

### 2.2. Instruments

Transmission electron microscopy (TEM) images were taken on a Philips Tecnai G2 F20 electron microscope operating at 200 kV (Philips, Netherlands). The X-ray photoelectron spectroscopy (XPS) of the sample was measured on an ESCALAB 250Xi X-ray Photoelectron Spectroscopy (Thermo Elemental). Fourier transform infrared spectroscopy (FT-IR) study was conducted from KBr pellets on a PerkinElmer FT-IR spectrophotometer (PerkinElmer, USA). Fluorescence lifetime experiments were performed by a FL3-P-TCSPC time-resolved fluorescence spectrometer (Horiba Jobin Jvon, France). Raman spectra were obtained on a Raman Microscope (Renishaw, UK). All fluorescence spectra were obtained on a LS-55 fluorescence spectrometer (Perkin Elmer, USA). The UV-Vis absorption spectra were characterized by a Cary UV-Vis spectrophotometer (Agilent Technologies). Cyclic voltammograms experiments were performed by a CHI760 electrochemical workstation (CH Instruments, Shanghai). Cell imaging was examined by confocal laser scanning microscopy (CLSM, Olympus FluoView FV1000). Cytotoxicity evaluation was tested by enzymelinked immunosorbent assay (ELISA) reader (Multiskan Mk3, Thermofisher).

### 2.3. Preparation of CDs

CDs were prepared by pyrolysis of water chestnut juice. Water chestnut juice was put into a typical PTFE reactor, and carbonized at the temperature of 180 °C for 3 h with a heating rate at 10 °C · min<sup>-1</sup>. The dark black products were mechanically ground to fine powders after cooling down to room temperature. 0.1 g of resultant sample was added into 10 mL purified water, and then ultrasound dispersed to form a black solution. The CDs were collected by removing larger particles through filtration with a filtration membrane that the pore size was 0.22 μm. The obtained CDs solution was kept at 4 °C for further characterization and use. The mechanism for the formation of carbon dots involves hydrothermal carbonization of the major constituents of water chestnut juice such as sucrose, glucose, fructose, amino acid, carotenes and ascorbic acid. The preparation and application procedure has been illustrated in Fig.1.



Fig.1. Schematic illustration of the synthesizing and application of fluorescent CDs from water chestnut juice

#### 2.4. Measurement of fluorescence quantum yields

Quinine sulfate (0.1M H<sub>2</sub>SO<sub>4</sub> as solvent; QY=0.54) were chosen as standards. The fluorescence quantum yields (QYs) of CDs (in water) were calculated by following methods. Absolute values were calculated according to the following equation:

$$\varphi_x = \varphi_{st} \times (I_x / I_{st}) \times (\eta_x^2 / \eta_{st}^2) \times (A_{st} / A_x)$$

Where  $\varphi$  is the QY,  $I$  is the measured integrated emission intensity,  $\eta$  is the refractive index of the solvent, and  $A$  is the optical density. The subscript "st" refers to standard with known QY and "x" refers to the unknown samples. For aqueous solution,  $\eta_x / \eta_{st} = 1$ .

#### 2.5. Cell incubation

HepG2 Cells were grown in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, invitrogen), 100 μg mL<sup>-1</sup> streptomycin and 100 units mL<sup>-1</sup> penicillin.

#### 2.6. Cytotoxicity evaluation

The cytotoxicity of CDs was evaluated by a MTT assay. HepG2 cells were seeded and incubated in a 96-well plate overnight at a density of 1 × 10<sup>4</sup> cells per well for 24 h. The culture medium was then removed, and the CDs solution were added into each well with increasing concentrations from 0 to 200 μg mL<sup>-1</sup> and incubated for 24 h before replacing the medium with 200 μL fresh complete medium containing 20 μL MTT (5 mg mL<sup>-1</sup> in PBS). The plate was incubated for another 4 h before all medium was removed and added 150 μL DMSO, and then follow shaking for 15 min. The absorbance of each well was measured at 570 nm using an Enzyme linked Immunosorbent Assay (ELISA) reader with pure DMSO as a blank. Non-treated cell was used as a control and the relative cell viability (mean% ± SD, n = 3) was expressed as Abs<sub>sample</sub>/Abs<sub>control</sub> × 100%.

#### 2.7. Cell imaging

HepG2 cells were seeded on the bottom of 35 mm glass culture dishes 24 h before CLSM detection. The culture medium was then replaced by 2.5 mL fresh medium containing 200 μg mL<sup>-1</sup> CDs, and the cells were incubated for another 12 h and 24 h at 37°C under 5% CO<sub>2</sub>, respectively. Subsequently, the cells were washed with isotonic PBS (pH=7.5) three times. The CDs labeled samples were examined by CLSM with a fixed excitation wavelength at 405 nm and the corresponding emission wavelength ranged from 500-530 nm.

### 3. Results and discussion

#### 3.1. Characterization of CDs

The morphology and structure of CDs were confirmed by TEM image analysis. Fig. 2 shows TEM image of the as-prepared CDs, which appear as spherical particles with diameters of 2-7 nm and an average size of 3.9 nm. Raman spectra further confirm the quality of the as-prepared CDs.

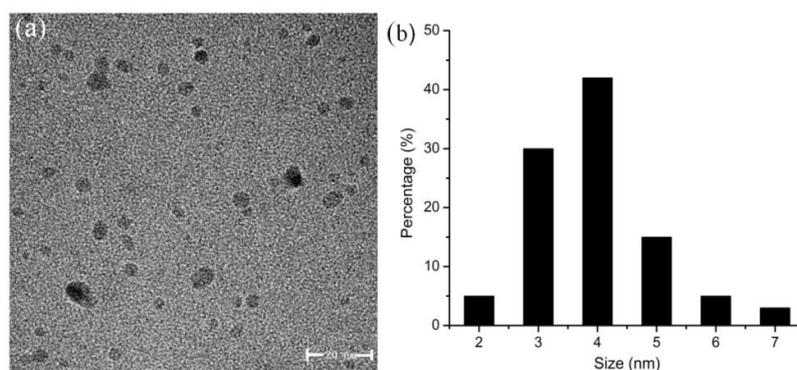


Fig.2. TEM images of the CDs (a) and Size distributions of the CDs (b)

As shown in Fig.3, the functional groups existing in the as-prepared CDs were characterized by Fourier transform infrared spectrometry. The peaks at around 3440, 2980, 1630, and 1060  $\text{cm}^{-1}$  are separately vested in the vibrations of O–H/N–H, C–H, C=O, and C–O bonds, and the peak at around 1390  $\text{cm}^{-1}$  is assigned to  $\text{sp}^2$ -CH in olefinic configuration. The good water dispersibility of CDs for bioapplications attributed to these hydrophilic groups. Moreover, the surface groups were also investigated by X-ray photoelectron spectroscopy (XPS, Fig.4a). The high resolution spectrum of C1s exhibits three main peaks (Fig.4b). The binding energy peak at 284.7 eV confirms the graphitic structure ( $\text{sp}^2$  C–C) of the CDs. The peak at about 285.9 eV suggests the presence of C–O and C–N, and the peak around 287.7 eV could be vested in C=O. These have also confirmed that a large number of hydrophilic groups on the surface of the CDs, resulting in the good water solubility. The high resolution spectra of N1s (Fig.4d) reveal the presence of N (399.9 eV) atoms.

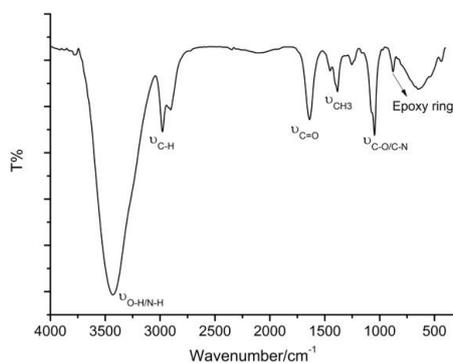


Fig. 3. FTIR spectrum of the as-prepared CDs

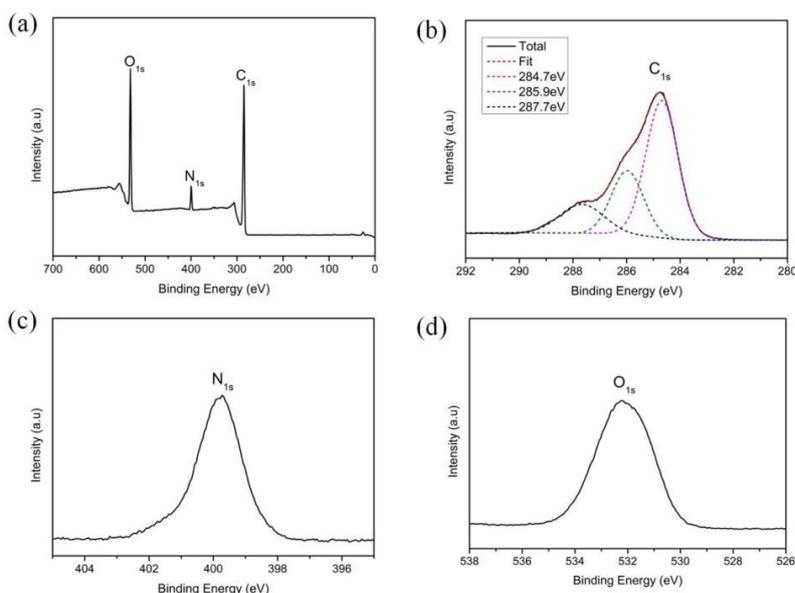


Fig.4. XPS spectra (a), high-resolution C1s peaks (b), high-resolution N1s peaks(c) and high-resolution O1s peaks (d) of the as-prepared CDs

### 3.2. Optical properties of CDs

The luminescent properties of as-prepared CDs were investigated. The aqueous solution of CDs indicates strong blue fluorescence when the sample is irradiated by UV light at 365 nm, revealing strong fluorescence of the CDs. In addition to this, the sample solution is highly transparent under ambient conditions as Fig. 5 showing, suggesting the excellent aqueous dispersibility of the CDs owing to a large number of surface-attached hydrophilic amino groups. Fig.6 shows the typical absorption and fluorescence spectra of the CDs. No obvious absorption peak but a wide absorption band was observed in absorption spectrum, which is possibly due to the relatively broad size distribution of CDs [12, 30]. It is worthwhile to note that the fluorescence emission spectrum of the as-prepared CDs shows a strong peak at 522 nm when excited at 440 nm. The fluorescence quantum yield measured against an aqueous solution of quinine sulfate in 0.1 M  $\text{H}_2\text{SO}_4$  ( $\lambda_{\text{ex}}=345$  nm) was about 10.6%. The average fluorescence lifetime of CDs is 3.90 ns, and the life time in the magnitude of nanosecond suggests that the as-prepared CDs are suitable for optoelectronic and biological application [36].

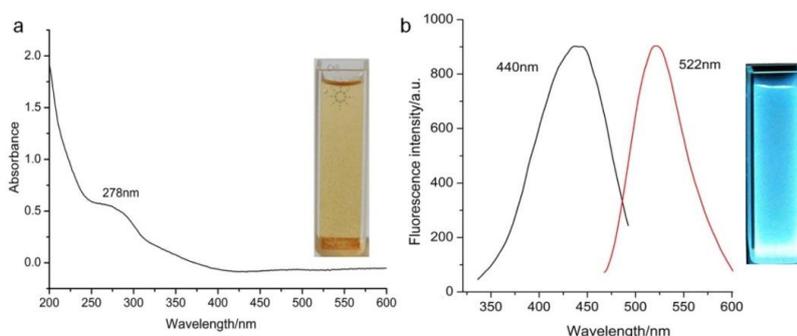


Fig.5. The optical properties of CDs aqueous solutions. (a) UV-Vis absorption spectra (inset: photographs taken under visible light). (b) Optimal excitation and emission FL spectra (inset: photographs taken under 365 nm UV light)

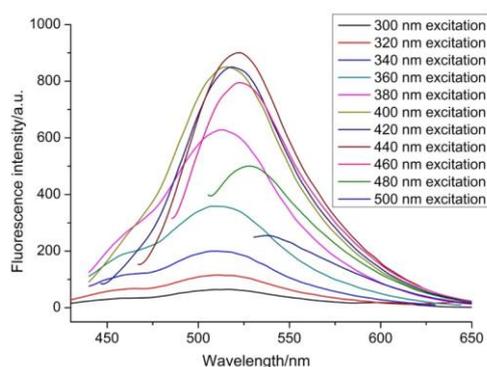


Fig. 6. The excitation dependent Fluorescence behaviors of the as-prepared CDs

The photoluminescence performance of the as-prepared CDs in different pH solutions was measured. Fig.7 shows the fluorescence intensity of CDs at different pH values. As can be seen, the CDs emit strong fluorescence from pH 7 to 8. The stable fluorescence intensity could be understood in terms of the change in surface charge owing to protonation–deprotonation [32]. Under strong acidic (pH < 7) or strong alkaline (pH > 8) conditions, the fluorescence intensity was decreased due to the damage of crucial fluorescence group structures. The effect of ionic strength was also investigated on the stability of CDs in a solution of 10 mM PB (pH7.5) containing different concentrations of NaCl and KCl (from 0.25M to 2.5M). As shown in Fig.8, the fluorescence intensity kept constant in a wide range (from 0.25M-2.5M) of NaCl concentrations, and it did not have an obvious change after continuous excitation at 365 nm UV light for 3 h (Fig.9a), or being stored at room temperature for 60days (Fig.9b). The CDs solution exhibits a long-term homogeneous phase without any noticeable precipitation at room temperature. This suggested the excellent photostability of the as-prepared CDs, and its wide range of applications [33].

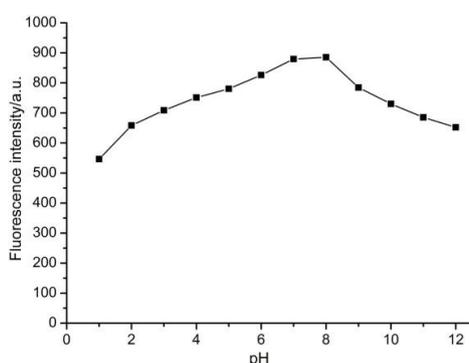


Fig. 7. Effect of pH value on the fluorescence intensity of the as-prepared CDs

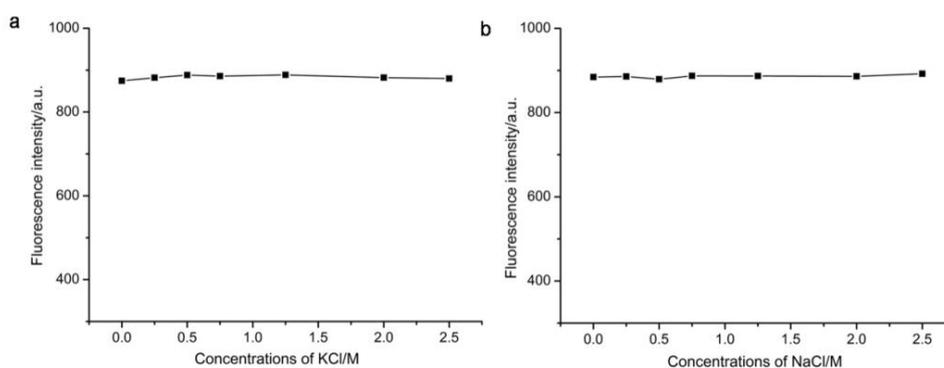


Fig.8. Fluorescence intensity of as-prepared CDs in pH 8 PB solution after adding various concentrations of KCl(a) or NaCl(b) solutions. (the concentrations of KCl or NaCl solutions: 0, 0.25, 0.5, 0.75, 1.25, 2.0 and 2.5 M)

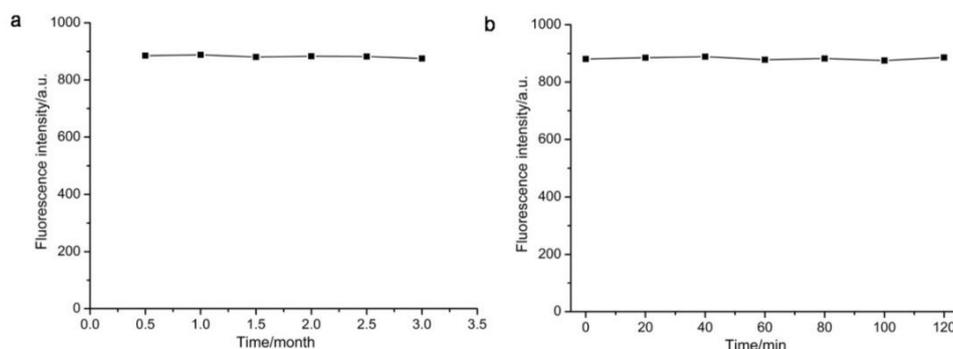


Fig. 9. The fluorescence intensity variation of the CDs with storage time (a) and 365 nm UV light illumination (b)

### 3.3. Cytotoxicity and application in living cell imaging

For further biological applications, MTT assays were carried out to evaluate the cytotoxicity of the as-prepared CDs to HepG2 cells. As expected, the cell viabilities were estimated to be greater than 90% upon addition of the CDs over a wide concentration range of 0–200  $\mu\text{g}/\text{mL}$  (Fig.10). High cell viabilities confirmed the low toxicity, excellent biocompatibility, and great potential of the as-prepared CDs for imaging in living cells. These indicate that the as-prepared CDs can be safe for in vitro and in vivo applications.

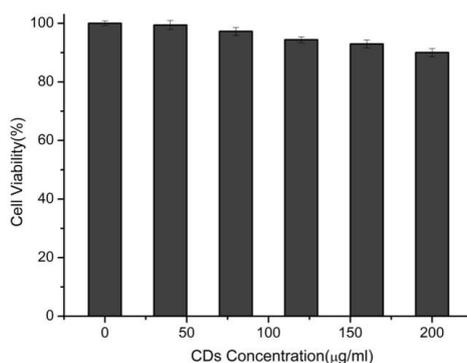
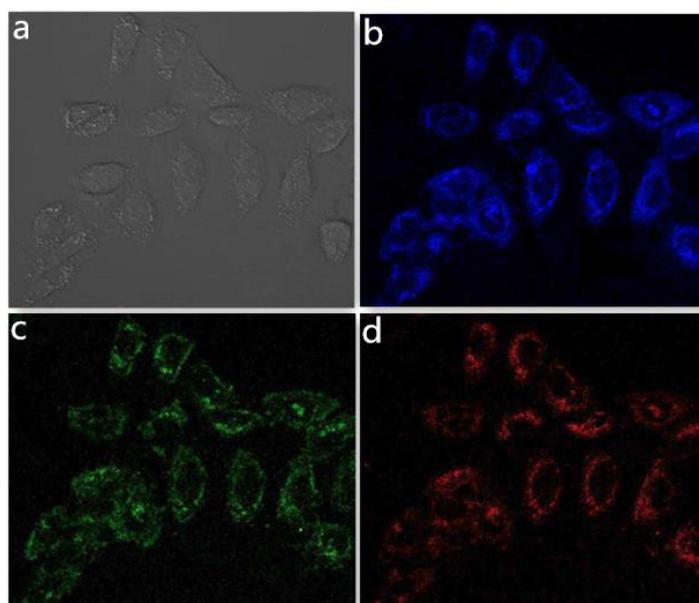


Fig. 10. Cell viability of HepG2 cells in the presence of different concentrations of CDs.

The CDs gave the advantages of small size, high luminescence efficiency, good photostability, and especially biocompatibility, which make them superior in potential bioimaging applications as an ideal fluorescent probe. Based on these excellent fluorescence properties, experiments were carried out to further demonstrate the availability of the as-prepared CDs for imaging in HepG2 cells. Fig. 11 shows CLSM images of HepG2 cells incubated with CDs for 12 h and 24 h at 37 °C. Under the 405 nm excitation, a significant blue emission from the intracellular region could be observed. The strong fluorescence on the HepG2 cells incubated with CDs suggested that CDs had penetrated the cells and were able to label both the cell membrane and the cytoplasm of the HepG2 cells. The results indicate that the CDs have a very good biocompatibility as bioimaging agents, and could be used as an excellent optical imaging probe.



*Fig.11. CLSM images of HepG2 cells incubated with CDs ( $100 \mu\text{g mL}^{-1}$ ) at 37 °C for 12 h after washing with PBS(pH=7.5). (a) Bright field, (b)fluorescent image excited with a 405 nm laser, (c) fluorescent image excited with a 488 nm laser, (d) fluorescent image excited with a 514 nm laser.*

#### **4. Conclusions**

In summary, we have first successfully established a strategy for synthesizing fluorescent CDs origination from water chestnut via a simple, green and low cost one-step pyrolysis. The preparation method offers several advantages over current synthetic techniques. Firstly, the process is facile without any complex or post-treatment procedures. Secondly, the starting material is green, economical and eco-friendly. Thirdly, the as-prepared CDs possess good water-solubility, strong blue luminescence, and can serve as high-performance optical imaging probes. Particularly, this synthetic method is of great potential for large-scale synthesis of fluorescent CDs. What's more, the as-prepared CDs show the low cytotoxicity and excellent biocompatibility, and the as-prepared CDs were successfully applied for imaging of HepG2 cells. Thus it can be used as an eco-friendly material for in vitro and in vivo bioimaging applications in cell biology and other sensing applications.

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