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Highly Photoluminescent Carbon Dots Derived from Linseed and Their Applications in Cellular Imaging and Sensing

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Abstract

Carbon dots (CDs) synthesized from natural organics precursors (such as glucose, citric acid, glycerol, and chitosan, et al) have attracted great interests since the natural organic precursors provide abundant carbon sources, varieties of heteroatom doping (such as N, S, and P) and good biocompatibility. However, previous approaches utilized organic solvents during the synthesis procedures, which limited their widespread development in biomedical applications. Here, facile synthesis of a new kind of bright CDs has been reported through an eco-friendly method that employs the linseed as a natural precursor. The obtained CDs possessed a high quantum yield of 14.2 %, excellent solubility and photostability as well as excitation-dependent photoluminescence (PL). In addition, the as-prepared CDs exhibited great potential in cell imaging, thanks to negligible cytotoxicity, as well as excellent biocompatibility and great resistance to photobleaching. Subsequently, the as-prepared CDs were also applied in the fabrication of biosensor for sensitive detection of butyrylcholinesterase (BChE) based on the fluorescence quenching mechanism, which can be used as an indicator for detecting pesticides and nerve gases. By monitoring the change in the fluorescent intensity of the CDs, the activity of BChE was sensitively analyzed. The limit of detection (LOD) of BChE was 0.035 mU mL^{-1}. The prepared CDs have potential applications in both biosensors and bio-imaging.

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1. Introduction

Recently, carbon dots (CDs) have gained increasing attention because of their superior properties of high water solubility[1-3], surface chemistry[4], good biocompatibility[5, 6] good photo-stability[7] and unique photoluminescence (PL) properties[8-20]. Unlike semiconductor quantum dots, which have limited applications because of their significant cytotoxicity, CDs are considered to be potential candidates as imaging agents for *in vivo* analysis due to their outstanding biocompatibility. Meanwhile, CDs demonstrate adjustable emission properties, which make them suitable for sensitive biochemical detection and photovoltaic devices[21-36]. Besides, the PL quantum yield of CDs has been improved through surface passivation and heteroatom doping methods to induce better charge/carrier transport[37-40]. Currently, various CDs preparation approaches have been reported. Among these reported technologies, the hydrothermal approach exhibits high efficiency that allows it to be scale-up for industrial production.

Varieties of organic precursors have been utilized to synthesize CDs, including glucose[25], citric acid[26], glycerol[27], and chitosan[28]. Recently, the CDs synthesized from natural organics have attracted great interests since the natural organic precursors provide abundant carbon sources, varieties of heteroatom doping (such as N, S, and P) and good biocompatibility[41]. In particular, cabbage[41], juice[42], and soy milk[43] have been used as precursors to synthesize CDs. The synthesis of homogeneous water-soluble CDs with unique surface properties and aqueous stability from natural materials is important in biomedical applications because it is environment-friendly, less toxic, and compatible with mass production[44-46]. Inspired by the capability of providing inexpensive natural carbon sources, we use linseed to synthesize highly photoluminescent CDs through an environmentally friendly hydrothermal method (Scheme 1). Linseed is a non-toxic and highly nutritional biomaterial[47] and is less expensive than other reported organic carbon

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precursors, including juice, eggs and soy milk. The as-prepared CDs exhibit excellent PL properties and functional surface chemistry. Utilizing these properties, we explored the applications of the synthesized CDs in both bio-imaging and bio-sensing. The as-prepared CDs with functional groups formed on the surface through the synthesis process were successfully used for imaging MCF-7 cells. Meanwhile, the CDs were also applied in the development of biosensor for quantitative determination of butyrylcholinesterase (BChE) enzyme. The fluorescence of the CDs can be efficiently quenched by the enzymatic hydrolysis reaction product of BChE. By monitoring the change in the fluorescent intensity of the CDs, the activity of BChE was sensitively analyzed.

2. Materials and methods

2.1 Materials

Linseed powder was purchased from local market. Hydrochloric acid (HCl, 39%) and sodium hydroxide (NaOH) were of analytical grade and purchased from local suppliers. Fetal bovine serum (FBS), RPMI-1640 Media was supplied by Gibco Invitrogen. The MCF-7 cell line was ordered from ATCC. For cell cultures, collagen G, Eagle's medium, L-glutamine, gentamycin, penicillin and streptomycin were purchased from ATCC. Other reagents related to cell experiments were purified and sterilized. Millipore purified water was used to synthesize the CDs.

2.2 Characterization

Transmission electron microscopy (TEM) images were obtained by Philips CM200 UT (Field Emission Instruments, USA). The tube was operated at 40 KV accelerating voltage and 15 mA current. X-ray Diffraction (XRD) characterization was carried out by Rigaku Miniflex 600. UV-vis spectra were measured by UV-2450 spectrometer. Dynamic Light Scattering (DLS)

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measurements were recorded by Nano ZS equipped with a solid-state He-Ne laser (λ=633 nm). The fluorescence spectra were measured by a Cary Eclipse fluorimeter. Cellular imaging was performed with a confocal laser fluorescence microscope (Leica SP8 Point Scanning Confocal).

2.3 Synthesis of CDs

Linseed powder (0.2 g) purchased from the local grocery store was washed with purified water for three times, and then dispersed in 20 mL water. The linseed/water mixture was sealed in a Teflon container, which was then autoclaved at 180 °C for 12 h, followed by cooling down to room temperature. The dark brown solution was obtained after the autoclave. This solution was purified with a dialysis bag (MWCO ~ 3.0 KDa) and dialyzed against water for 1 day to remove the reaction residue and by-products. During the dialysis, the water was replaced every 6 h. Finally, CDs were freeze-dried to solid (~15.6 mg total).

2.4 Cell Imaging

The resulting CDs powder was re-dispersed into DMEM medium, then filtered and sterilized stocking solutions containing CDs (0.2 μg μL^{-1}) were added to the 6-well plate containing MCF-7 cultured by culture medium (2 mL), respectively. After incubation of cells with CDs for 24 hours in an incubator at 37˚C with CO_2 (5%), the culture medium of cells was washed out by 1× PBS for the cell imaging. The bright and fluorescence fields of as-treated MCF-7 were observed with the 40× objective of confocal laser fluorescence microscopy.

2.5 Cytotoxicity assay

The cytotoxicity of CDs for MCF-7 cells in vitro was performed by the standard MTT assay. MCF-7 cells were incubated in the 96-well plate with the cell density of $1.0x10^5$ per mL for 24

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hrs. Then the medium was discarded and treated with DMEM medium containing the CDs with various concentrations for 24 hrs. After that, the cells were incubated with 20 µL of MTT (5 mg mL^{-1} in PBS) for 4 hrs. After incubation process, 150 µL DMSO was added in each well. The optical density was recorded by microplate reader at the wavelength of 490 nm.

3. Results and discussion

3.1 Synthesis of CDs from linseed

The CDs were prepared by a simple hydrothermal process. Linseed powder (0.2 g) purchased from the local grocery store was washed with purified water for three times, and then dispersed in 20 mL water. The linseed/water mixture was sealed in a Teflon container, which was then autoclaved at 180 °C for 12 h, followed by cooling down to room temperature. The dark brown solution was obtained after the autoclave. This solution was purified with a dialysis bag (MWCO ~ 3.0 KDa) and dialyzed against water for 1 day to remove the reaction residue and by-products. During the dialysis, the water was replaced every 6 h. Finally, the obtained CDs were freeze-dried to form brown powder. The resulting CDs could be homogeneously dispersed into various solvents. The whole synthesis process does not require any organic solvent, which is extremely environment-friendly. In this work, CDs were dispersed in aqueous solution.

3.2 Characterization of the prepared CDs

The morphology of as-prepared CDs was characterized by transmission electron microscopy (TEM, Philips CM200 UT). The TEM images showed in Figure 1a reveals that the as-prepared CDs exhibit spherical morphology with the particle size in a range of 4-8 nm (Figure S1). The high-resolution TEM image shows that the CDs have a high crystalline structure with well-

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resolved lattice fringes (Figure 1a, inset). The inter-planar distance of the lattice fringes is measured to be 0.21 nm, corresponding to the spacing between the (100) plane of the graphite carbon. The typical XRD pattern of the as-prepared CDs shows a broad diffraction peak centered at approximately 22.0 degrees (Figure 1b), which is attributed to the highly disordered structure and the small size of the CDs[13].

As shown in Scheme 1, the obtained CDs exhibit yellow color under sunlight, while they emit bright green color under UV light with the excitation wavelength of 365 nm. As shown in Figure 2a, the UV-vis absorption spectrum of the CDs displays absorption bands in the UV region at about 242 and 324 nm, corresponding to π-π and n-π transition of carbonyl groups, respectively[48]. The excitation spectrum of the CDs reveals an optimal excitation wavelength of 395nm (Figure S2). Figure 2b presents the emission spectra of the obtained CDs at different excitation wavelengths. When excited at different wavelengths, the emission peak wavelengths of the CDs are kept almost constant at 503 nm. This PL emission behavior can be attributed to the surface state affecting the band gap of the CDs (Figure 2b)[48]. In addition, the PL quenching was observed in the solid form of CDs, which is attributed to an electronic quenching effect on the excited state. The quantum yield (QY) of CDs is approximately 14.2% with the quinine sulphate as the reference fluorescence material, which is comparable to some of the previously reported CDs [49-52].

The surface states of the as-prepared CDs were investigated using both Fourier-transform infrared (FT-IR) spectroscopy and X-ray photoelectron spectroscopy (XPS). The FT-IR spectrum of the as-prepared CDs shown in Figure 1c reveals that the CDs possess a variety of hydrophilic groups, including -OH (3418 cm^{-1}), C-H (2879 cm^{-1}), -COOH (1712 cm^{-1}), and C-O from epoxy (1209 cm^{-1}). Moreover, stretching vibrations of C=C (1524 cm^{-1}) and C-N= (1384

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cm^{-1}) are observed, indicating the existence of amino bending and aromatic ring structures on the surface of the CDs. The CDs with profuse surface hydrophilic groups are endowed with promising PL behavior, along with good polarity and aqueous dispensability. The full-scan XPS spectrum of the obtained CDs is presented in Figure 3a, in which three peaks at 284.0, 398.0, and 530.6 eV are observed, corresponding to C1s, N1s, and O1s regions, respectively. The atomic ratio of C1s, N1s and O1s was 65.28%, 4.47% and 29.13%, respectively, which indicates N atoms doping. The deconvolution of the high-resolution C1s region (Figure 3b), N1s region (Figure 3c) and O1s region (Figure 3d) XPS spectra are also presented. The detailed XPS spectrum of C1s region further demonstrates the presence of C-C/C=C bond (284.4 eV), C-OH bond (285.5 eV), C-N bond (286.96 eV), and O-C=O bond (288.18 eV) on the CDs surface. Figure 3c reveals that the XPS spectrum of N1s region consists of three bond peaks located at 398.2 eV, 399.1 eV and 400.2 eV, which correspond to pyridine-like nitrogen, amino nitrogen and pyrrolic nitrogen, respectively. In addition, C-OH (533 eV) and C=O (531.6 eV) groups are observed on the CDs surface (Figure 3d). These results (FT-IR and XPS) indicate that the surface of the as-prepared CDs possesses different functional groups, including π-conjugated domains and abundant residual amino groups, as well as a high degree of oxidation. This large amount of functional groups should be generated from the carbonization and polymerization reactions with carbonaceous materials, including fiber, sugar, vitamins, and amino acid in the linseeds during the hydrothermal process. Therefore, the one-step synthesis of CDs from linseeds is a promising method to obtain CDs with rich surface chemistry. Based on the fluorescence mechanisms of CDs, the surface chemical structures of CDs play a critical role in determining their PL properties[53-55]. Previous results confirmed that the carboxyl groups and high oxidation on the CD surface could cause surface defects, which could serve as capture centers for electrons, resulting

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in surface-state fluorescence. In addition, nitrogen doping could induce significant local distortion, which causes upshifting of the Fermi level and excitation of electrons into the conduction band, enhancing the QY of CDs significantly. Thus, we hypothesize that the good PL properties of the obtained CDs are attributed to their surface conditions. To further confirm that the high PL intensity is originated from the surface states of the CDs, the pH-dependence of the PL properties were investigated. The results show that considerable decrease in the PL intensity can be observed as the CDs are dispersed in aqueous solutions with high acidity (pH < 5) or high alkalinity (pH > 9) (Figure S3a), while the PL intensity shows no obvious decay in the pH range from 5 to 9 (Figure S3b). In addition, the emission wavelength of the CDs shows significantly red-shift as the pH value decreased (Figure S3c), which may be due to the surface change induced by protonation-deprotonation as in the previous reports[41].

The PL stability of the as-prepared CDs in biological buffers (PBS) with different ion concentrations was investigated. The results show that negligible changes in the PL intensity were observed as the ion concentrations of the PBS buffers increase up to 20 mM, indicating great potential of applying the CDs in various biological environments (Figure S4a). Furthermore, the resistance of the CDs to photobleaching was tested by exposing the CDs under long-term UV irradiation. Under continuous UV excitation for four hours, only slight PL decay (~ 10.2 %) was observed (Figure S4b). These results indicate that the CDs derived from linseed possess high PL stability and strong photo-bleaching resistance.

3.3 Bio-imaging using the prepared CDs

For biomedical applications, the prepared CDs are expected to have excellent biocompatibility in cellular environments. The cytotoxicity of the CDs was first evaluated using a standard MTT

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assay with MCF-7 cells. As shown in Figure 4, the CDs exerted negligible cytotoxicity on MCF-7 cells after the cells were incubated with the CDs with increasing concentrations up to 200 μg mL^{-1} for 24 h. The cell viability decreased to 76.3% as the concentration of the CDs reached 250 μg mL^{-1}. Thus, the administered concentration of the CDs for cellular imaging is chosen as 200 μg mL^{-1}, which achieves more than 90% cell viability after incubation with the obtained CDs. Compared with other reported work[13], the as-prepared CDs exhibits broader administered concentration range for cellular imaging, indicating that the CDs derived from linseed can serve as safe imaging agents for *in vivo* imaging applications.

In order to investigate the potential of CDs for cellular imaging, MCF-7 cells were incubated with the as-prepared CDs (200 μg mL^{-1}) for 4 h, followed by imaging using a confocal fluorescence microscope. The MCF-7 cells emitted green fluorescence under 405 nm laser excitation, respectively. The green emissions were obviously observed due to the excitation-dependent PL properties of CDs. To further confirm the uptake of the CDs in MCF-7 cells, the cytoskeleton β-Actin was stained for fluorescence imaging. The merged images revealed that the fluorescence emission of the CDs was predominantly from the cytoplasmic area, demonstrating that the CDs successfully penetrated the membrane of MCF-7 cells and diffused into cytoplasm, but did not enter the cell nuclei.

3.4 Detection of BChE

Bestowed with superior optical properties, the as-prepared CDs encouraged us to further investigate their potential sensing applications. Through broadly screening[56-60], the capability of the obtained CDs for biosensing is demonstrated by detecting butyrylcholinesterase (BChE). The sensing mechanism is based on the hydrolysis reaction induced fluorescence quenching. The as-

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prepared CDs without any modification were directly employed as a fluorophore for biosensing. During the detection, the CDs were mixed with the solution of acetylthiocholine (ATCl, 1.0 mmol L^{-1}) and 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB, 50 μmol L^{-1}). When BChE is introduced into ATCl/DTNB solution, the ATCl will be hydrolyzed to thiocholine (TCl), which will trigger the decomposition of DTNB, generating 2-Nitro-5-thiobenzoic acid (NTB). The absorption band (centered at ~ 412 nm) of the NTB overlaps with the excitation band of the prepared CDs, resulting in PL quenching of the CDs. As shown in Figure S5, when 150 mU mL^{-1} BChE is introduced into the CD/ATCl/DTNB solution, the PL intensity of the CDs at 510 nm is significantly reduced about 50 %. The fluorescence spectra of CDs in the presence of BChE with different concentrations are shown in Figure 6. The PL intensity of the CDs at 510 nm is continuously decreasing as the BChE concentration increases. There is a good linear relationship between the PL intensity ratio $(F_0\text{-}F)/F_0$ and the logarithm of BChE concentration in the range of 0.06−300 mU mL^{-1} (Figure 6 Inset). F_0 and F was the PL intensity of the CD probe in the absence and presence of BChE, respectively. The regression equation was $(F_0\text{-}F)/F_0$ = 0.168+0.147 Log[BChE], mU mL^{-1}. The limit of detection (LOD) for BChE was 0.035 mU mL^{-1}. The detection range and the LOD of the obtained CDs were compared with other reported methods of BChE detection (Table S1). Our method showed a comparable BChE detection range and LOD. The as-prepared CDs can be used as a sensitive probe for detecting BChE.

Selective recognition capability is an essential parameter to evaluate the performance of a sensing probe, especially for a nanosensor with potential applications in complex biological environments. Therefore, the selectivity of the CDs for BChE detection was further investigated with different interfering substances, including metal cations, glucose, amino acids, and various other nonspecific proteins. It is evident in Figure S6 that even in the presence of interfering

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substances (500 μg mL^{-1} for protein and 0.5 mmol L^{-1} for other substances), the CDs-based system still works the same for 150 mU mL^{-1} of BChE (~5 μg mL^{-1}), indicating that the developed BChE sensing system could provide excellent ability for resisting interference from common substances. Thus, the established system is suitable for selective detection of BChE.

4. Conclusion

In summary, we developed a facile and green method for synthesis of high-quality CDs using linseed as a natural precursor. Rich functional groups are formed on the CDs surface through the synthesis process. The CDs exhibit excellent PL properties, thus displayed immense potential for cellular imaging. Bio-imaging of MCF-7 cells using the as-prepared CDs was demonstrated. Furthermore, the as-prepared CDs were used for detecting enzyme BChE based on fluorescence quenching. Combined with the DTNB-assisted target recognition, the obtained CDs shows good sensitivity and specificity for BChE detection. The prepared CDs could serve as a promising candidate for both biosensors and bio-imaging.

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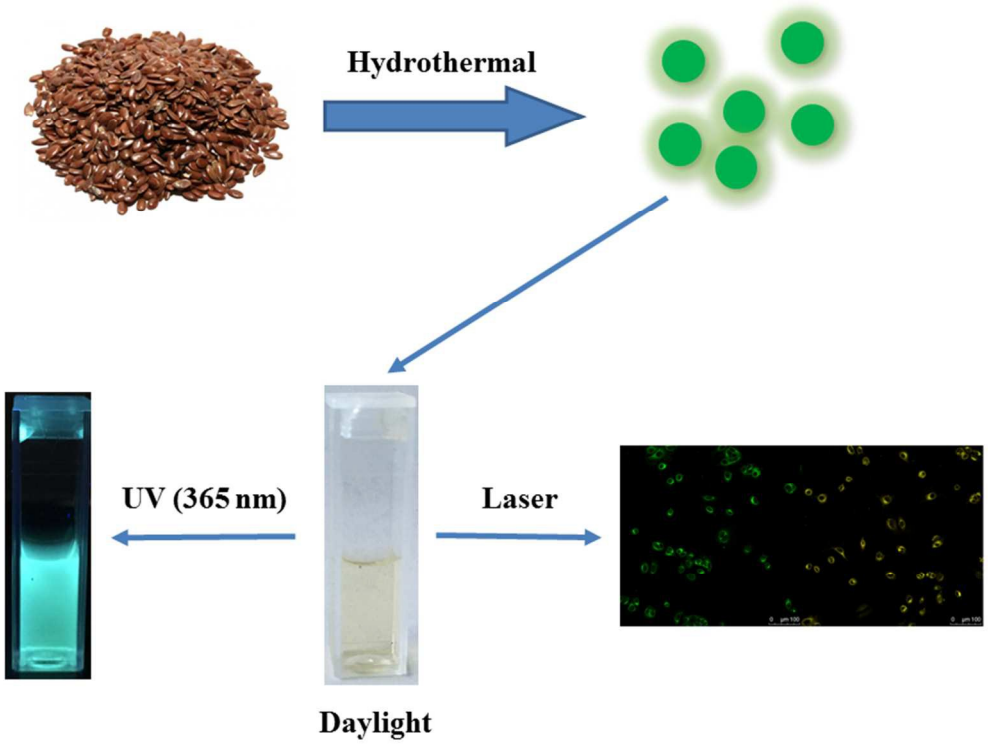

Scheme 1. Schematic presentation of synthesis of CDs from linseed with hydrothermal treatment.

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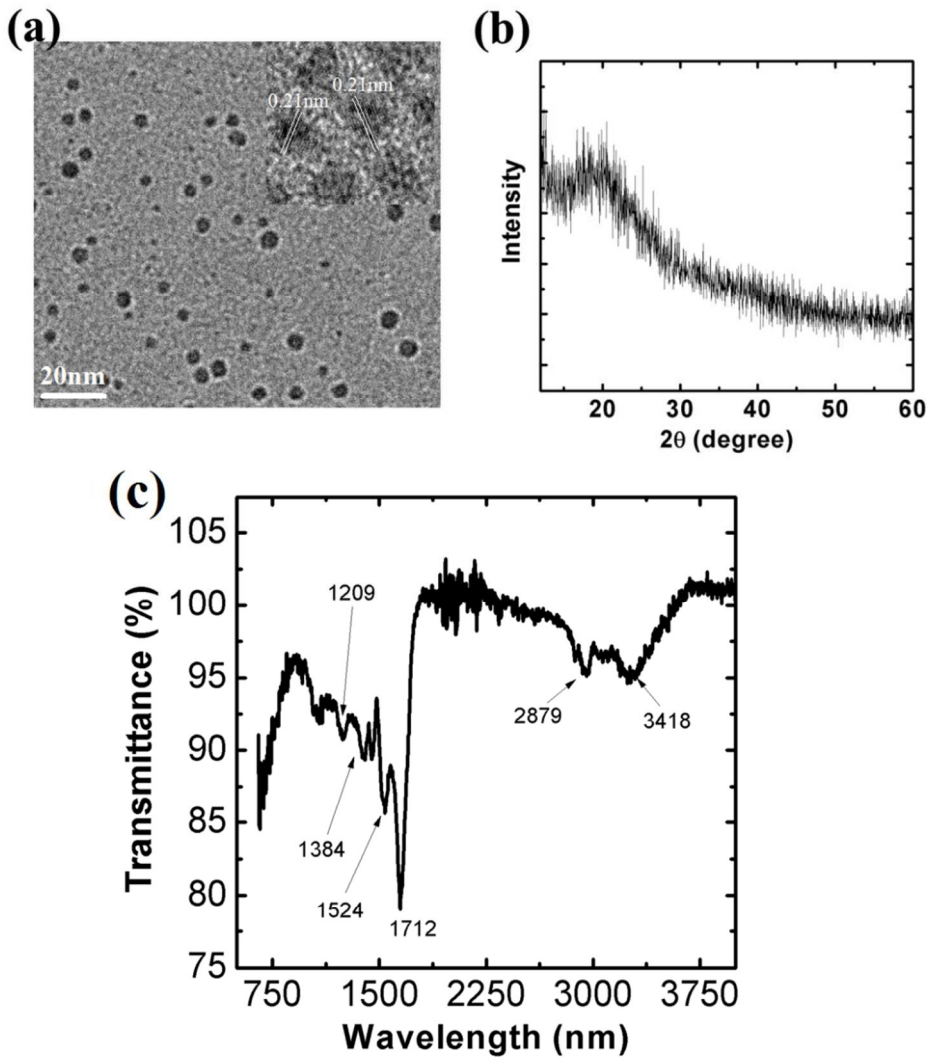

Figure 1. TEM images (a), XRD (b) and FT-IR patterns (c) of the as-obtained CDs.

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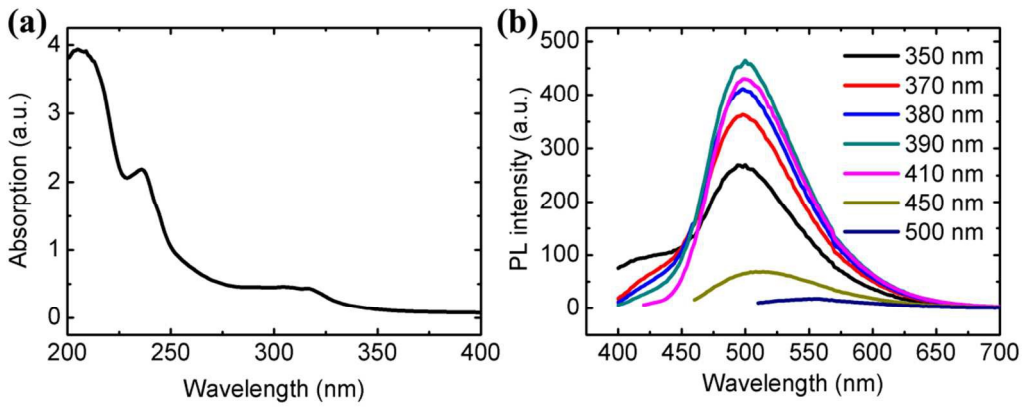

Figure 2. UV-visible absorption of CDs (a) and emission spectra of CDs (b).

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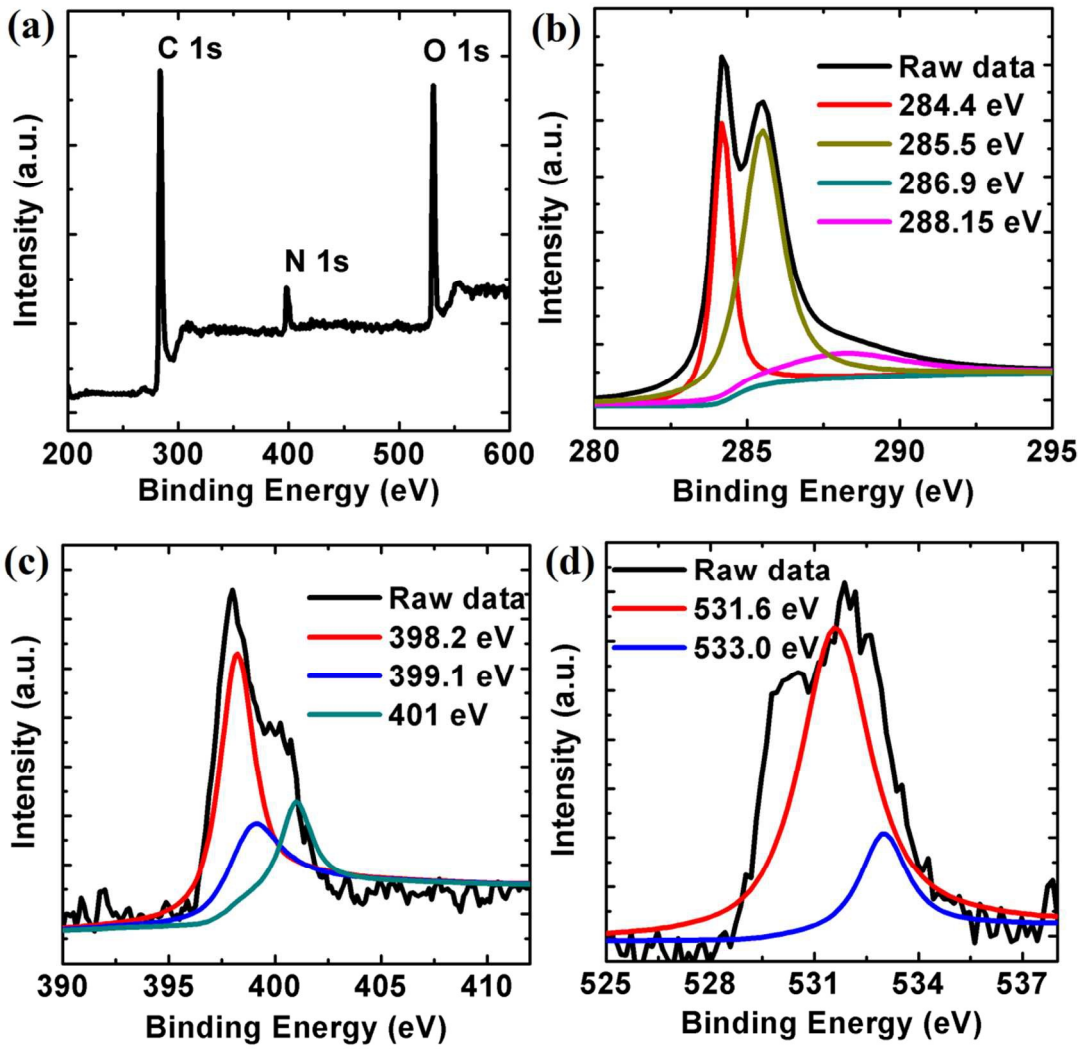

Figure 3. XPS spectra of CDs (a), high-resolution XPS spectra of C1s (b), N1s (c), and O1s (d).

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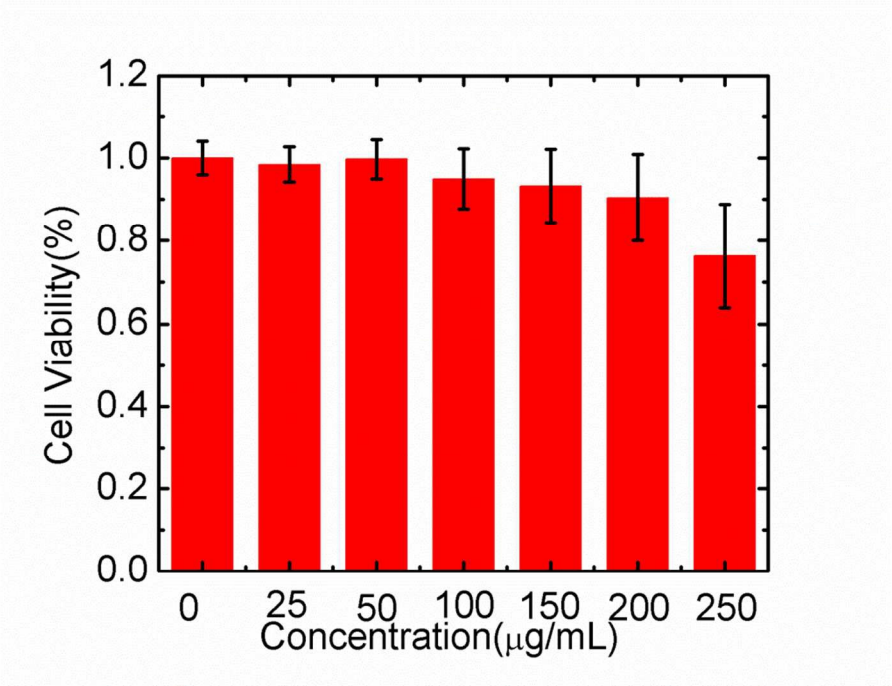

Figure 4. Cytotoxicity of CDs incubated with MCF-7 cells at increasing concentrations from 0 to 250 μg mL^{-1}.

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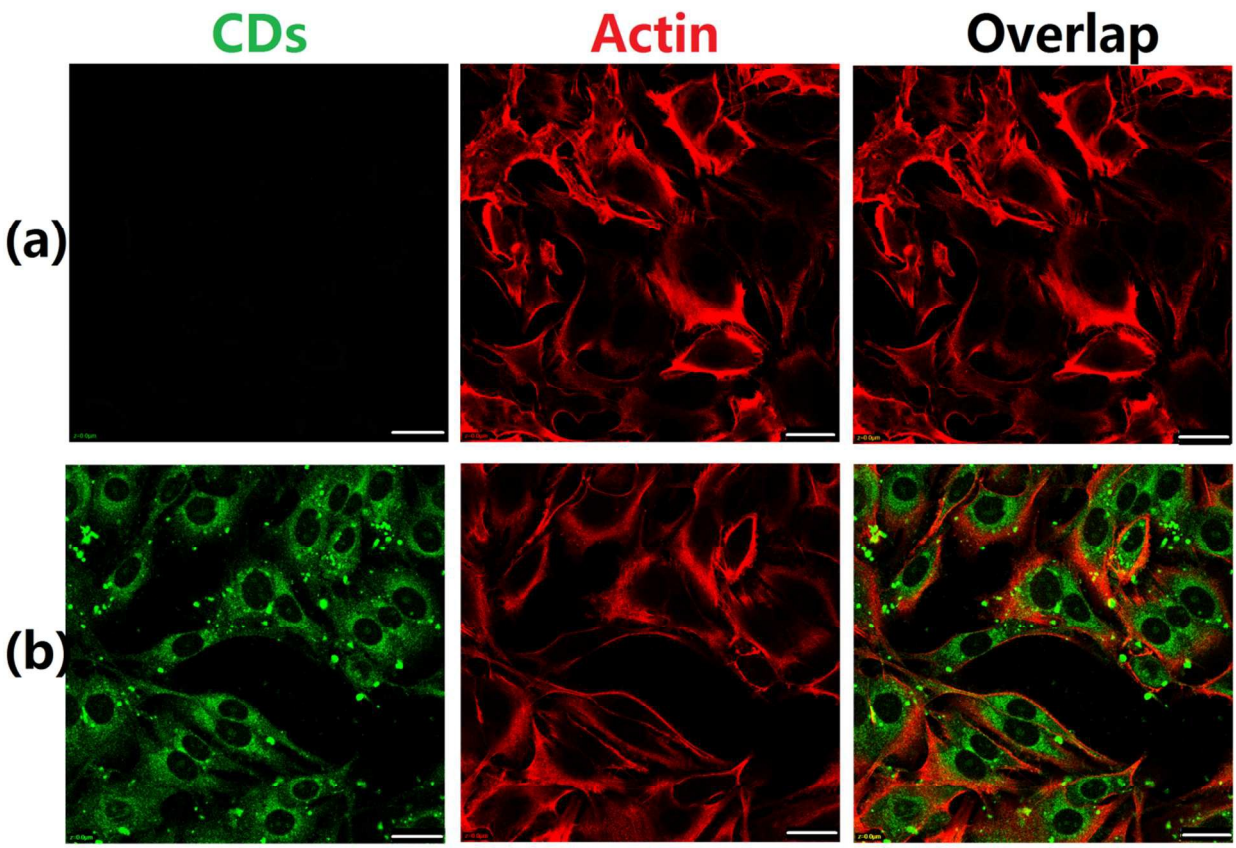

Figure 5. Confocal fluorescence images of MCF-7 cells excited under a 405 nm laser for CDs (green) and 543 nm laser for F-Actin (Red). (a) Without CDs incubation as a control. (b) Incubated with CDs for 24 h. Scale bars: 20 μm.

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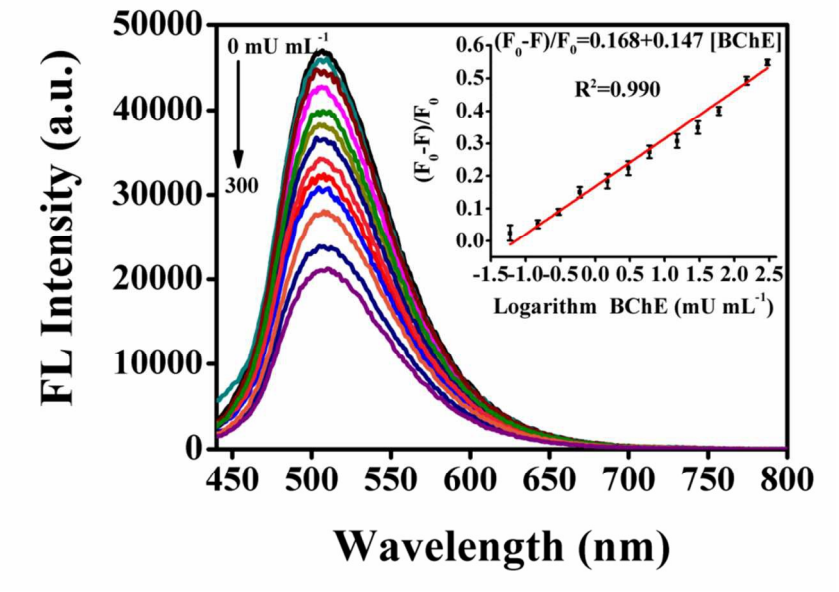

Figure 6. The fluorescence spectra of CDs with different concentrations of BChE (from 0 to 300 mU mL^{-1}). Inset was the linear plot of fluorescence intensity ratio $(F_0\text{-}F)/F_0$ versus the logarithm concentration of BChE (0.06, 0.15, 0.3, 0.6, 1.5, 3.0, 6.0, 15, 30, 60, 150 and 300 mU mL^{-1}). The concentration of ATCl and DTNB was 1 mmol L^{-1} and 50 μmol L^{-1}, respectively. F and F_0 were the FL intensity of the CD/ATCl/DTNB system in the presence and absence of BChE, respectively.

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