Development of nitrogen and sulfur-doped carbon dots for cellular imaging

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A B S T R A C T
Heteroatom-doped carbon dots (CDs) have attracted extensive interest because of their improved electronic and fluorescence properties with heteroatom doping. In this study, a new synthetic method for nitrogen (N) and sulfur (S)-doped CDs was developed via a hydrothermal method using methionine and citric acid as raw materials. The as-prepared CDs exhibit excellent optical properties and good biocompatibility. The spherical N,S-doped CDs have an average diameter of 5 nm. They consist of C, O, N, and S, and take on excellent water solubility due to the hydroxyl and carboxyl, amino groups on the surface. The CDs have a photoluminescence quantum yield of 13.8% using quinine sulfate as a reference; the average fluorescence lifetime of the CDs was 3.67 ns. The CDs with good photoluminescence properties and the maximum excitation wavelength and emission wavelength locate at 330 nm and 405 nm, respectively. In addition, their fluorescence intensity almost does not change under the conditions of acid, alkali, and high salt, which indicated their anti-photobleaching property and good light stability. Based on the good biocompatibility and strong fluorescence emission of the CDs, they can be used as fluorescent imaging reagents.

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1. Introduction

Carbon dots (CDs) are a new type of fluorescent carbon nano-material that have attracted significant attention due to their useful qualities such as good water solubility, excellent photoluminescence properties, environmental friendliness, and good biocompatibility [1–4]. They have great potential application in the fields of cell imaging, nanomedicine, and drug analysis [5–11]. Raw materials for the synthesis of CDs are developed not only from the carbon-containing materials [12,13], but also from the natural, green, environmentally friendly substances [14,15]. Currently, the use of easily accessible and natural precursors as starting materials is preferred for the synthesis of CDs. Preparation methods have also evolved from the initial cumbersome and complicated processes to the present one-step synthesis. Fluorescent imaging reagents with strong fluorescence signals greatly improve the imaging results. Therefore, the application of CDs in bioimaging can be extended through improving the luminescence efficiency by element doping and surface modification [16–20]. For example, functional CDs can be obtained by surface passivation using terminal amino group-containing materials [19]. However, the cumbersome procedures limit functional CDs application in bioimaging.

In this study, a new method involving hydrothermal synthesis of nitrogen and sulfur (N, S)-doped CDs was developed using citric acid and methionine (2-amino-4-methyl-thiobutyric acid) as raw materials. Methionine is one of the essential amino acids which make up the human body. In order to improve the luminescence properties and the biocompatibility, N, S-doped CDs were synthesized using citric acid as the carbon source and methionine as the source of nitrogen and sulfur. This method has many advantages, including short reaction time, low cost, and excellent light-emitting performance of the CDs obtained by doping. The CDs with good fluorescence properties and excellent biocompatibility were eventually applied to cell imaging. The experimental process is shown in Scheme 1.
2. Experimental

2.1. Chemicals and apparatus

All reagents were of analytical grade. Methionine was purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). Citric acid was purchased from Chongqing Chuandong Chemical Reagent Factory, China. Britton-Robinson (BR) buffer solution was used to adjust the acidity of the CDs. All the solutions used in the experiment were prepared using ultrapure water (18.2 MΩ).

A Teflon autoclave (25 mL, Jinan Henghua Technology Co., Ltd., China) and a blast drying oven (Shanghai Yiheng Scientific Instrument Co., Ltd., China) were used to prepare the CDs. Mixer (QD-9011, Jiangsu Linqi Bell Instrument Manufacturing Co., Ltd., China) was used for mixing the solutions of raw materials. A hydrothermal method was used to determine the absorption spectrum of the CD solution. Field emission transmission electron microscopy (FETEM, Tecnai G2 F20 S-TWIN200 KV, FEI Corporation, UK) was used to characterize the morphology and particle size of the CDs. The hydrated particle size and surface charge of the CDs were determined using a zeta potentiometer (Malvern Instruments Co., Ltd., UK). A Fourier transform IR spectrometer (FTIR-8400S, Shimadzu Corporation, Japan), X-ray photoelectron energy dispersive spectrometer (ESCALAB 250, Thermo, USA), and laser confocal Raman spectrometer (Lab-RAM HR 800) were used to characterize the functional groups and structural features on the surface of the CDs. A fluorescence spectrophotometer (F-2500, Hitachi, Japan) was used to determine the excitation and emission spectra of the CDs. A ultraviolet-visible-near infrared (UV–Vis–NIR) spectrophotometer (UV-3600, Shimadzu Corporation, Japan) was used to determine the absorption spectrum of the CD solution. Field emission transmission electron microscopy (FETEM, Tecnai G2 F20 S-TWIN200 KV, FEI Corporation, UK) was used to characterize the morphology and particle size of the CDs. The hydrated particle size and surface charge of the CDs were determined using a zeta potentiometer (Malvern Instruments Co., Ltd., UK). A Fourier transform IR spectrometer (FTIR-8400S, Shimadzu Corporation, Japan), X-ray photoelectron energy dispersive spectrometer (ESCALAB 250, Thermo, USA), and laser confocal Raman spectrometer (Lab-RAM HR 800) were used to characterize the functional groups and structural features on the surface of the CDs. The fluorescence lifetime was measured using a fluorescence spectrometer (FL-TCSPC, Jobin Yvon Co., France). Cytotoxicity was measured using a microplate reader (Mode 680, BIO-RAD, USA). A fluorescent confocal microscope (IX-81, Olympus, Japan) was used for cell imaging.

2.2. Preparation of the fluorescent CDs

Methionine (0.8 g) and citric acid (1.0 g) were added into a 20 mL small beaker, and dissolved by slow addition of 5 mL pure water. Then the above-mentioned solution was transferred into a Teflon-lined stainless steel autoclave (internal volume of 20 mL), sealed with a steel sleeve, and then heated in a blast drying oven at 200 °C for 3 h. After the completion of the reaction, the reactor was cooled down to room temperature. The reaction product was centrifuged for 15 min at 12,000 r/min to remove the large particulate material, and thus the CD supernatant was obtained. Then, the residual starting material molecules were removed through dialysis for 12 h using a dialysis bag with a cut-off molecular weight of 500, and water was changed every 2 h. Solid powder was obtained through freezing.

2.3. Calculation of the fluorescence quantum yield

The relative fluorescence quantum yield was determined based on the method reported in literature [18]: quinine sulfate solution (0.1 M) was used as a reference (quantum yield was 54%). The emission and absorption spectra of the CDs with different concentrations and also of quinine sulfate were measured under the irradiation light of 330 nm wavelength. The relative fluorescence quantum yield was obtained by calculating the ratio of the peak area integral of the fluorescence emission peak to the absorption value obtained at the same concentration under the excitation of 330 nm wavelength.

2.4. Investigation of the cytotoxicity

Cell proliferation was measured by cell counting kit-8 (CCK-8) assay. In this experiment, human epidermal type 2 (HEp-2) cancer cells were selected to examine the toxicity of the CDs.

First, HEp-2 cells were digested with 0.05% trypsin, and then a cell suspension (2 × 10 cells/mL) was prepared using complete medium (RPMI 1640 medium with 10% fetal bovine serum (FBS, v/v)). Finally, the cell suspension was inoculated into a 96-well cell culture plate with a capacity of 100 μL per well and cultured in a cell incubator under the condition of 37 °C and 5% CO2 for 24 h. The culture plate was then taken out and the supernatants were removed from each well. The cells were washed once with phosphate buffer saline (PBS) and complete culture medium was added. For the experimental group, cell solution (90 μL) and CD solutions (10 μL) with different concentrations (0, 0.01, 0.05, 0.1, 0.2, 0.5, and 1 mg/mL) were added into each well, while for the control group, cell solution (100 μL) was added into each well. After further incubation for 24 h, the cell culture plate was taken out. The supernatants of each well were discarded and the cells were rinsed twice with PBS solution. Then PBS solution (90 μL) and CCK-8 solution (10 μL) were added into each well and incubated in a cell incubator for 1 h. Finally, the absorbance value of each well at a wavelength of 405 nm was measured using a microplate reader, and the experimental results were recorded and analyzed.

2.5. Cellular imaging experiment

HEp-2 cells in complete medium supplemented with 2% FBS were initially seeded in 35 mm glass-bottom dishes with a density of 1 × 105 cells per dish and incubated for 24 h at 37 °C and 5% CO2. Then, 100 μL, 1 mg/mL of the synthesized CD solution and 900 μL medium were added and incubated for 12 h. After that, the

![Scheme 1](image_url) Scheme 1. Scheme of the synthesis of nitrogen and sulfur doped CDs with blue luminescence using hydrothermal method and their application in cell imaging.
cells were washed three times with PBS buffer and then another 100 μL fresh medium was added for confocal imaging. Cell imaging was performed using an Olympus IX-81 fluorescence confocal microscope at different time.

3. Results and discussion

3.1. Characterization of the CDs

The particle size and morphology of the as-prepared CDs were characterized by TEM. As Fig. 1A shows, the CDs present a monodispersed spherical or nearly spherical morphology. The statistical chart in Fig. 1B, which was obtained from TEM image, shows that the sizes of the CDs were in the range of 3–6 nm with an average particle size of 5 nm.

In order to investigate the elements and functional groups existing on the surface of the CDs, the CDs were characterized by X-ray photoelectron spectroscopy (XPS) and IR spectroscopy. The absorption peak of the CDs at 3442 cm$^{-1}$ of the IR spectrum of the CDs belongs to the stretching vibration of O-H and N-H groups [20]. The peaks at 2923 and 2847 cm$^{-1}$ are attributed to the stretching vibration peak of C-H. The stretching vibration peak of C-O is located at 1338 cm$^{-1}$. The peak at 1169 cm$^{-1}$ can be attributed to the bending vibration of C-H (Fig. 2A) [20]. It’s shown that elements...
such as C, N, O, and S exit in the CDs (Fig. 2B), indicating the successful doping of N and S into the CDs, which is similar to the literature report on the synthesis of CDs using L-cysteine [20]. The XPS result also exhibited the existence of a small amount of Si, which might be due to the utilization of silicon pellets as the carrier during the measurement with the introduction of Si. Fig. 2C displays the C 1s spectra of the CDs, exhibiting obvious absorption peaks at 284.6, 285.5, and 288.6 eV, indicating that the carbon elements belonged to three different compositions corresponding to C-C, C-N, and C=O [20], respectively.

In order to further explore the structure of the CDs, the CDs were placed on a gold substrate and then characterized by Raman spectroscopy with laser excitation at 532 nm. Fig. 2D shows that the synthesized CDs have obvious Raman characteristic peaks, and a strong G band peak appears at 1600 cm$^{-1}$, which belongs to the sp$^2$ hybridized carbon. The results indicated that the obtained CDs were mostly composed of sp$^2$ hybridized carbon. A weaker D band peak at 1374 cm$^{-1}$ belonged to sp$^3$ hybridized carbon. The Raman spectra of the bare CDs were obscure during the experimental process, which may be due to the strong background signal caused by the strong fluorescence intensity. This phenomenon has also been reported in literature [20]. The above-mentioned experimental results indicated that the CDs contained abundant functional groups such as carboxyl groups, hydroxyl groups, nitrogen-containing groups, and a small amount of sulfur-containing groups. These functional groups led to the excellent water solubility of the CDs.

3.2. Spectral characteristics of the CDs

The UV–vis absorption spectra and fluorescence spectra of the aqueous solution of the CDs are shown in Fig. 3A. The CDs exhibit an absorption band at 305 nm in the UV region and show a shoulder absorption peak at 310 nm. The appearance of the shoulder peak was caused by a π-π$^*$ or n-π$^*$ transition of carbon nanomaterial [21]. This characteristic of the CDs is consistent with that of the intercalary semiconductor nano materials [22]. Therefore, to a certain extent, the CDs can be classified as semiconductor materials. Under exposure to a UV hand-lamp with a 365 nm wavelength, the CD solution showed a blue-green fluorescence. When the CDs solution was irradiated with a light of 330 nm, a strong emission peak was observed at 405 nm, and there was mirror symmetry between the maximum excitation peak and the maximum emission peak. Fig. 3B shows that when the wavelength of the excitation light gradually increases from 330 to 500 nm, the emission peak of CDs also gets red-shifted from 405 to 480 nm, accompanied by a gradual decrease of the fluorescence intensity of the CDs. This phenomenon indicates that the CDs exhibit the characteristics of stimulus-dependent emission. It is possibly due
to the uneven particle size of the CDs or different emission sites on the surface, which is similar to the conclusions reported in previous literature [22].

Moreover, the relative quantum yield of the CDs obtained by the relative method was 13.8%, which was higher than that of the CDs without doping as reported in the previous literature [23]. The increase of quantum yield may be related to the N-containing chromophore on the surface of the CDs, because the introduction of N atoms in the CDs can deactivate the active sites on the surface [13]. Therefore, N doping is considered to be an extremely effective method to increase the quantum yield of the CDs [24,25]. Furthermore, in order to explore the luminescence mechanism of the CDs, the decay trend of emission intensity at 330 nm wavelength along with time was investigated. The result indicated that the fluorescence lifetime of the CDs was only 3.67 ns. The CDs had a short fluorescence lifetime, and the luminescence mechanism is likely to be the radiative recombinations of excitons [26].

3.3. Stability of the CDs

The fluorescence intensity of the aqueous solution of the CDs was almost constant after irradiation with 330 nm excitation light for 30 min successively without a light blinking phenomenon (Fig. 4A). Thus, it was proved that the CDs had excellent anti-photobleaching properties. Furthermore, the aqueous solution of the CDs also exhibited better salt resistance. Sodium chloride (NaCl) solutions with different concentrations in the range of 0–2.5 mol/L were added to the CD solution, and the result showed that the fluorescence intensity of the CDs remained nearly consistent (Fig. 4B). Moreover, the fluorescence intensity of the CDs was hardly affected by the pH of the solution. Fig. 4C demonstrates that the fluorescence intensity of the CDs shows a relatively stable state with the adjustment of the pH of the CD solution in the range of 2.56–11.92 using BR buffer. The excellent stability discussed above would be beneficial for the successful application of the CDs as fluorescent probes in target tracing, cell imaging, and so on.

3.4. Cellular cytotoxicity and cellular imaging

From the above experimental results, it can be found that the prepared CDs have extremely good stability. These excellent properties make the CDs have a great potential for application in the field of cell imaging. In order to further study the biocompatibility, the cytotoxicity of the prepared CDs toward HEP-2 cells was evaluated using CCK-8 assay. Fig. 5 shows that the viability of the cells was still around 90%, even under a higher concentration of CDs (1 mg/mL) with a long incubation time (24 h), which indicated less toxicity of the CDs. Therefore, the CDs can be safely applied to cell imaging.

The CDs showed relatively obvious blue fluorescence after incubation with the cells, which indicated that the fluorescence of the CDs was still maintained in the cells. It is worth noting that the fluorescence intensity of the CDs in HEP-2 cells was constant for almost 2.5 h (Fig. 6), which was consistent with the result of the light stability of the CDs examined in solution. The results showed that compared to the quantum dots with relatively strong toxicity and easily photobleached fluorescent dye, the CDs synthesized using methionine as a raw material show an incomparable advantage and can be effectively utilized as a fluorescent probe for a wide range of applications in biological imaging.

4. Conclusions

In summary, methionine and citric acid were selected as raw materials for the synthesis of N, S-doped CDs by a one-step hydrothermal method. The synthesized CDs exhibited an excellent fluorescence performance and photobleaching resistance ability. They showed excellent resistance ability against the influence of strong acid and base, high ionic strength and extremely excellent stability. Moreover, the good biocompatibility of the CDs makes them potential candidates for cell imaging analysis.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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