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One-step hydrothermal approach to fabricate carbon dots from apple juice for imaging of mycobacterium and fungal cells☆

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ABSTRACT

A facile green hydrothermal method was developed for the preparation of fluorescent carbon dots (CDs) using apple juice as a raw material. The synthesized fluorescent CDs were characterized by UV–vis, fluorescence, Fourier transform infrared (FT-IR), dynamic light scattering (DLS), high resolution transmission electron microscopic (HR-TEM), life-time measurement and laser scanning confocal microscopic techniques. The CDs showed bright blue emission under UV-light (λ_{ex} = 365 nm). The CDs were used as alternative biocompatible fluorescent probes for imaging of bacteria (*Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*) and fungal (*Magnaporthe oryzae*). It was shown that the prepared CDs had no toxic effect on the both cells lines (bacteria and fungal), indicating that the CDs did not inhibit growth of bacterial and fungal cells, which confirms that the CDs exhibit good biocompatibility.

1. Introduction

In the ever-expanding field of nanomaterial research, the CDs have attracted much interest over the last decade due to their remarkable novel properties such as biocompatibility, low toxicity, easy functionalization, chemical inertness, photostability, and valuable photoluminescence [1,2]. In recent years, the CDs have proved to be as potential eco-friendly fluorescent probes for visualizing structural or functional images of living systems down to the molecular level. The advent of new optical properties of CDs has made the research on the development of various "smart" nanosystems such as bioimaging [3–6], biochemical assays [7,8], drug delivery [9], photocatalysis [10] and light-emitting devices [11,12]. As a result, much efforts have been focused on the development of various synthetic approaches for the preparation of fluorescent CDs including laser ablation [13,14], arc discharge [15], pyrolysis [16], oxidation [17], electrochemical exfoliation [18], hydrothermal treatment [19], plasma treatment [20], microwave irradiation [21–24] and microwave/ultrasonic passivation [25,26]. On the other hand, even though the above methods were successfully applied to prepare CDs, unfortunately they require tedious process, sophisticated instrumental set-up, limited spectral efficiency, and low product yield. In view of green chemistry approach, there is an urgent need to establish a new green synthetic approach for the preparation of fluorescent CDs and their applications in bioimaging.

Over the past few years, hydrothermal carbonization has proved as an eco-friendly, traditional and soft chemical route for the preparation of CDs in aqueous media, which produces highly efficient fluorescent probes for recognizing various chemical species and cells *in vitro* and *in vivo* [27]. Hydrothermal synthesis of CDs using various renewal carbon sources has provided great advancement over existing physical methods (laser ablation, arc discharge and plasma treatment), which is due to its simplicity and production of CDs with good quantum yield. In hydrothermal method, CDs were prepared by using either low-cost or biowaste materials as raw materials. For example, CDs were successfully prepared by using various renewable resources as precursors including chitosan [28], coffee grounds [29], watermelon peel [30], pomelo peel [31], *Trapa bispinosa* peel [32], orange juice [33], gelatin [34], and low-cost organic chemicals [35,36]. It can be noticed that the CDs were successfully generated using bio-waste/plant materials as raw materials, which avoids the use of costly/toxic chemicals or large amounts of chemicals and complicated post-treatment processes.

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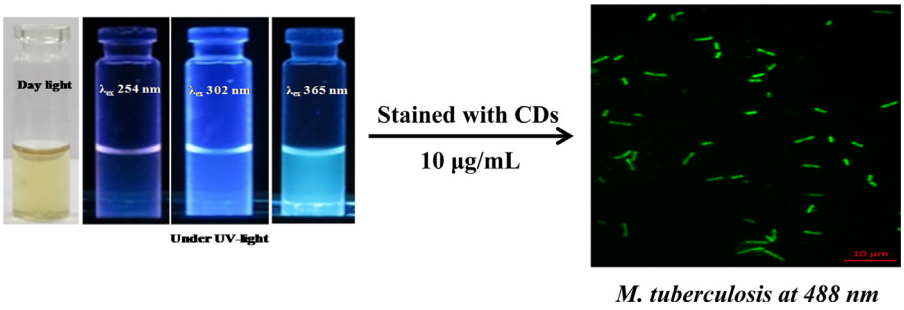

Scheme 1. Fluorescent nature of CDs under UV light illumination at different excitation wavelengths (254, 302 and 365 nm) and its application as a fluorescent probe for the imaging of *M. tuberculosis*.

Furthermore, Li's and Zhang's groups [37] developed a new strategy for the preparation of two types of CDs with either excitation-independent blue emission or distinctive excitation-dependent full-color emissions using chloroform and diethylamine as precursors. Tian and co-workers [38] developed a CDs-based fluorescence detection method for sensing of reactive oxygen species in biological tissues. A novel and large-scale strategy was developed for the preparation of N-doped CDs with high yield by pyrolyzing ethanolamine as a precursor [39]. Dong et al. [40] described one-step microwave method for large-scale fabrication of water dispersible fluorescent CDs using citric acid and ethylenediamine as precursors for bioimaging of cells and sensing of Hg^{2+} ion. Shao's group [41] prepared N-doped graphite-like phase CDs for multicolor real-time imaging of cancer cells. Wang's and Chen's teams [42] reported a simple green synthetic method for the fabrication of fluorescent CDs using hair as a raw material. Bendicho et al. [43] developed *in situ* ultrasound-assisted synthetic method for preparation of CDs using fructose as a carbon source for the detection of methylmercury. Recently, various natural precursors such as konjac flour [44], bamboo leaves [45], sugar cane juice [46] and milk [47] have been used as carbon sources for the preparation of fluorescent CDs and acted as probes for imaging of cells and sensing of inorganic species. Furthermore, Jun et al. [48] illustrated the use of apple juice as a carbon source for the preparation of fluorescent CDs and used as probes for sensing of Hg^{2+} ion in environmental water samples. Abdollahi's group [49] also synthesized CDs from fruit juice and used as a fluorescent probe for the sensitive and selective detection of Hg^{2+} ion with a detection limit of 14 nM. These reports illustrated that the selection of precursors play a key role to modify the surface chemistry of CDs with multiple functional groups for sensing and cellular imaging of target analytes in biocomplex samples. To date, no reports are available for imaging of bacteria (*Mycobacterium tuberculosis, Pseudomonas aeruginosa*) and fungal (*Magnaporthe oryzae*) cells using apple juice derived CDs as imaging probes.

Herein, we report a simple hydrothermal method for the preparation of fluorescent CDs using apple juice as a raw material. The prepared CDs are highly water-soluble, nano-sized (4.5 ± 1.0 nm) and emitted bright blue fluorescence. The surface chemistry, size, and morphology of CDs were characterized by FT-IR, DLS and HR-TEM. The multicolor CDs are quite stable against photo-bleaching as compared with organic dyes and used as fluorescent probes for imaging of bacteria (*P. aeruginosa* and *M. tuberculosis*) and fungal (*M. oryzae*) cells (Scheme 1).

2. Materials and methods

2.1. Chemicals

Malus domestica (apple) was purchased (Rupees 50/- for 500 g) from the local market of Surat, India and used as a precursor. Dichloromethane, sodium chloride, glucose, glycerol and dimethyl sulfoxide were obtained from Merck Ltd., India. All chemicals were of analytical grade and used without further purification. Milli-Q-purified water was used for sample preparations.

2.2. Synthesis of carbon dots from apple juice

The fluorescent CDs were synthesized *via* hydrothermal carbonization method using apple juice as a carbon source [48]. Briefly, 500 g of apples was chopped into small pieces and converted into liquid by adding 500 mL of water. Vacuum filtration was carried out in order to obtain the pulp free juice for the carbonization. The resulting mixture was transferred into a 700 mL stainless-steel autoclave, heated at constant temperature of 150 °C for 12 h, and then allowed to cool down naturally. The obtained dark brown solution was filtered through 0.45 μm filter to remove large particles and then washed with dichloromethane to remove unreacted organic moieties. The aqueous solution was collected and centrifuged at 15,000 rpm for 20 min to remove solid residues and further dialyzed for 24 h against double distilled water. The obtained CDs were stored at 4 °C for further characterization and cell labeling.

2.3. Confocal microscopic imaging of cells using CDs as probes

P. aeruginosa colonies were grown on a Luria-Bertani (LB) medium (10 mg/mL Tryptone, 5 mg/mL Yeast Extract and 10 mg/mL NaCl pH 7.0) at 37 °C, 180 rpm. The colonies were used to inoculate with 10 mL of fresh LB medium and grown overnight at 37 °C. The Lowenstein and Jensen (L.J.) medium was harvested in 0.85% saline in bijou bottles and used as a medium for the culture of *M. tuberculosis* H37Rv. The above culture tubes were incubated at 37 ± 1 °C for 24 h followed by streaking of *M. tuberculosis* H37Rv (5×10^4 bacilli per tube). The growth of bacteria was seen after 12, 22 and 28 days of incubation. *M. oryzae* was cultured on complete medium of 5 × YEG (0.5% yeast extract and 2% glucose) and oatmeal agar (OA) Plates 25 °C [50]. To enhance the culture growth, plates were grown under constant fluorescence light at room temperature. The cultures were vortexed at 150 rpm, and their growth was monitored by measuring the absorbance at 600 nm. Bacteria (*P. aeruginosa* and *M. tuberculosis*) and fungal (*M. Oryzae*) cells were collected from the middle of exponential growth phase and used for confocal microscopic studies. Briefly, 70% (*v*/*v*) ethanol was used for the internalization of CDs by both bacterial and fungal cells at 4 °C for 5 min. The staining of cells were carried out by re-suspending of cells in 100 mM of phosphate buffer that contained 10 μg/mL of CDs. The cells-CDs-conjugates were washed thrice with double distilled water and their images were measured by using a Carl Zeiss 510 LSM laser scanning confocal microscope in blue, green, and red regions with laser excitations at 405, 488, and 561 nm, respectively.