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SHORT COMMUNICATION

Imaging of Bacterial and Fungal Cells Using Fluorescent Carbon Dots Prepared from *Carica papaya* Juice

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Abstract In this paper, we have described a simple hydrothermal method for preparation of fluorescent carbon dots (C-dots) using *Carica papaya* juice as a precursor. The synthesized C-dots show emission peak at 461 nm with a quantum yield of 7.0 %. The biocompatible nature of C-dots was confirmed by a cytotoxicity assay on *E. coli.* The C-dots were used as fluorescent probes for imaging of bacterial (*Bacillus subtilis*) and fungal (*Aspergillus aculeatus*) cells and emitted green and red colors under different excitation wavelengths, which indicates that the C-dots can be used as a promising material for cell imaging.

Keywords C-dots · *Carica papaya* juice · Fluorescence · XRD · Bacterial and fungal cells

Introduction

Fluorescent nanoparticles have attracted a lot of attentions in the past few decades due to their unique optical properties and applications, which endow them as good probes in imaging of various inorganic (metal and anions), organic (drugs) and cells [1]. As compared to organic dyes and semiconductor quantum dots, C-dots exhibit tremendous advantages for in vitro and in vivo imaging of target analytes, which is due to multifunctional groups, ease of preparation, and biocompatible nature [2]. To date, various synthetic routes such as carbonization, chemical oxidation, hydrothermal, electrochemical, microwave, pyrolysis, ultra-sonication and solvo-thermal methods have been developed for the preparation of C-dots and their applications in sensing and cell imaging [3]. Among these approaches, hydrothermal method proved as one of the simple and green synthetic route for the preparation of water dispersible C-dots using natural resources as precursors.

In this connection, various natural resources including orange juice [4], gelatin [5], low-cost organic chemicals [6], sugar cane juice [7], chitosan [8], coffee grounds [9], candle soot [10], watermelon peel [11], sweet pepper [12], pomelo peel [13], *Trapa bispinosa* peel [14], konjac flour [15], bamboo leaves [16], hair [17] and milk [18] have been used as precursors for the preparation of fluorescent C-dots. The prepared C-dots were successfully used as probes for visualization of various species from complex matrices. Similarly, Li's and Zhang's groups prepared two types of C-dots with either excitation-independent blue emission or distinctive excitation-dependent full-color emissions using chloroform and diethylamine as raw chemicals [19]. Lee and co-workers developed a simple and green synthetic approach for the large-scale preparation of water-soluble C-dots using many kinds of food wastes as sources [20]. Dong's group prepared multifunctional fluorescent C-dots using citric acid and ethylenediamine as precursors [21]. Shao's group developed a simple hydrothermal route for the preparation of nitrogen-doped C-dots and used as fluorescent probes for cell imaging [22]. Bendicho et al. developed in situ ultrasound-

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assisted synthetic approach for the preparation of C-dots using fructose as a carbon source and used as a probe for sensing and imaging of methylmercury [23]. Chang and coworkers prepared florescent C-dots from fresh tender ginger juice and studied their biocompatible nature on human hepatocellular carcinoma cells (HepG2), normal mammary epithelial cells (MCF-10A) and normal liver cells (FL83B) [24]. One-pot green synthetic approach was developed for the preparation of carbon nanoparticles by using food grade honey as a raw material [25]. Prasannan and Imae described a simple one-pot hydrothermal route for the preparation of fluorescent C-dots using orange waste peels as raw materials [26]. Huo and coworkers prepared biocompatible multicolor photoluminescent C-dots by using activated carbon as a precursor [27]. The C-dots acted as biocompatible fluorescent probes for imaging of cells with multicolor emission and localization of cytoplasmic molecules within cells. Although these fluorescent C-dots are prepared from various raw materials, these C-dots are used as a single cell imaging probe either bacteria or cells. Therefore, the exploration of a new carbon source for green synthesis of fluorescent C-dots and their applications for imaging of multiple species (bacteria and fungus) are highly desirable.

In this work, we have developed a single-step hydrothermal route for the preparation of fluorescent C-dots using *Carica papaya* juice as a carbon source (Scheme 1). We studied the effect of temperature for carbonization of *Carica papaya* juice. It is green chemistry approach for the preparation of large-scale fluorescent C-dots without addition of any additive. The prepared C-dots are well dispersed in water with an average size of 3.0 nm and exhibited strong emission peak at 461 nm when excited at 383 nm. The prepared C-dots acted as probes for imaging of bacterial (*Bacillus subtilis*) and fungal (*Aspergillus aculeatus*) cells.

Experimental

Materials and Reagents

All reagents were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Ultrapure water (18.2 MΩ/cm) from a Milli-Q ultrapure system was used in this study. *Carica papaya* was purchased from local vegetable market, Surat, Gujarat, Inida.

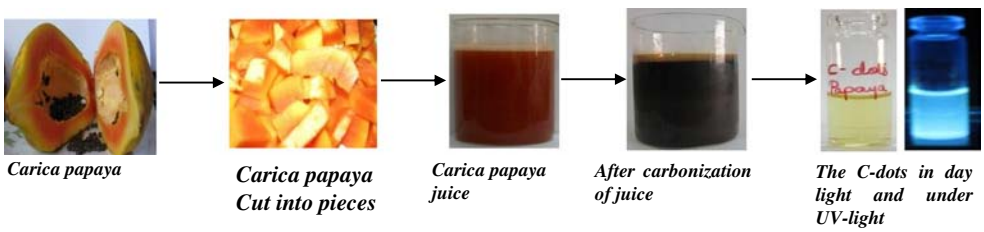
Preparation of *Carica Papaya* Juice

Fresh *Carica papaya* was purchased from a local market. After removal of its upper layer (skin), the obtained *Carica papaya* pulp was grounded in a mixer grinder. The extracted *Carica papaya* juice (500 mL) was transferred to autoclave reactor and then treated hydrothermally at different temperatures (125, 150 and 170 °C) for 12 h. The reaction solution was filtered through 0.45 μm filter paper to remove large particles and then washed with dichloromethane to remove unreacted organic moieties. The filtrate was centrifuged (15,000 rpm, 20 min), and the brownish yellow supernatant (20 mL) was dialyzed in ultrapure water (80 mL) through a dialysis membrane (MWCO 12,000-14,000 Da) for 12 h. The pH of the C-dots was found to be 6.0 and the solution was stored at 4 °C and used as a stock solution for further use.

C-dots as Probes for Cell Imaging

Bacterial cultures of *Bacillus subtilis* were prepared by transferring an aliquot of starter culture to a solution of working broth (Luria– Bertani, LB, Fisher Scientific, Fair Lawn, NJ) [28]. The diploid strain (*Aspergillus aculeatus*) was grown in yeast extract-peptone-dextrose (YPD) medium (yeast extract - 10 mg/mL, bactopeptone - 20 mg/mL, glucose - 20 mg/mL, pH 6.5±0.2) at 30 °C and then the cultures were inoculated for 4–16 h at 37 °C. Intermittently, aliquots of bacteria and fungus were removed from the batch culture with a sterile pipette to monitor growth, until the optical density (600 nm) was reached that correlated to a region previously established to be in the log phase of growth.

A 10-fold dilution of the mother growth medium was diluted with cell suspension and used for cell imaging. All solutions and materials were prepared in sterilized tubes. To prepare bacteria- and fungus- C-dots conjugates, the cells were fixed by using 70 % (*v/v*) ethanol at 4 °C for 5 min and the fixed cells were resuspended in 100 mM of phosphate buffer containing C-dots (40 μg/mL) for 10 min at 35 °C. The cell imaging was performed by using excitation wavelengths at 488 (green), and at 561 (red) nm.



Scheme 1 Single-step hydrothermal method of fluorescent C-dots using *Carica papaya* juice as a precursor

Minimum Inhibitory Concentration (MIC)

The synthesized C-dots were examined for antimicrobial activity against *E. coli* using paper disc diffusion technique. The Mueller–Hinton agar media were sterilized (autoclaved at 120 8C for 30 min), poured at uniform depth of 5 mm and allowed to solidify. The microbial suspension (10^5 CFU/mL) (0.5 McFarland Nephelometery Standards) was streaked over the surface of media using a sterile cotton swab to ensure even growth of the organisms. The tested compounds were dissolved in dimethyl sulfoxide (3.12– 100 mg/mL). Sterile filter paper discs (Whatman No. 1) were soaked with known concentration of the respective test compound and then placed on the solidified nutrient agar medium that inoculated with the microorganism. The plates were incubated for 24 h at 37 °C. A control disc impregnated with an equivalent amount of dimethyl sulfoxide without any sample was also used and did not produce any inhibition. Ciprofloxacin was used as a control drug for antibacterial activity.

The MIC of C-dots was determined by agar streak dilution method. A stock solution of the C-dots (100 mg/mL) in dimethyl sulfoxide was prepared and graded quantities of the test compounds were incorporated in a specified quantity of molten sterile agar (nutrient agar for evaluation of antibacterial and sabouraud dextrose agar for antifungal activity). The medium containing the test compound was poured into a Petri dish at a depth of 4–5 mm and allowed to solidify under aseptic conditions. A suspension of the respective microorganism (10^5 CFU/mL) was prepared and applied on plates with serially diluted compounds in the range of 3.12–100 mg/mL in dimethyl sulfoxide and incubated for 24 h (bacteria) and 48 h (fungi) at room temperature.

Measurement of Fluorescence Quantum Yield (Φ)

Fluorescence quantum yield (Φ) of the C-dots was measured by using following equation [29]:

$$\Phi_1 = \Phi_2 I_1 A_2 \eta_1^{\,2} / I_2 A_1 \eta_2^{\,2}$$

Where I_1 and I_2 are the fluorescence intensities of the C-dots and the standard. A_1 and A_2 are the optical densities of the

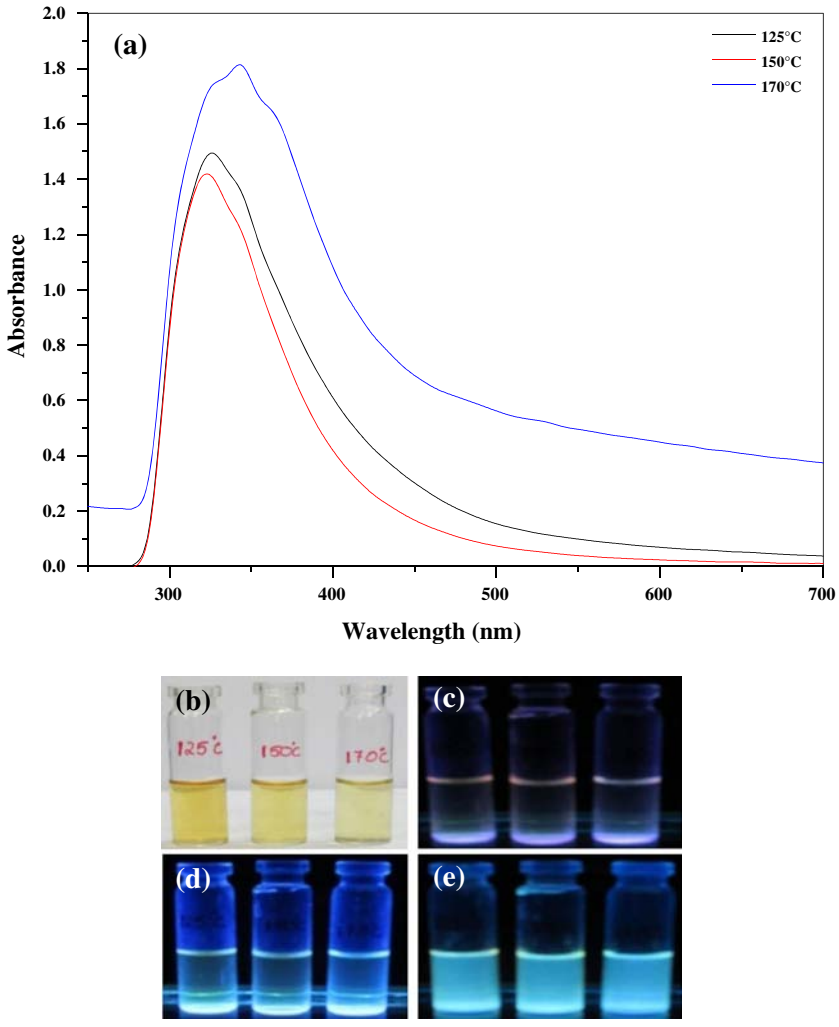


Fig. 1 **a** UV-visible absorption spectra of C-dots at different temperatures. Photographic images of C-dots at different temperatures in **b** day light and their fluorescence properties under UV light excitation wavelengths **c** 254, **d** 302, and **e** 365 nm

C-dots and the standard. The η_1 and η_2 are the refractive indices of the C-dots and the standard, respectively. The standard quantum yield of Φ_2 was 0. 54 for quinine sulfate at 360 nm in 0.1 M H_2SO_4 (refractive index: 1.33) and the C-dots were dissolved in water (refractive index: 1.33). The quantum yield of the C-dots is 7.0 % at excitation wavelength 383 nm.

Instrumentation

UV-visible spectra were measured by using Maya Pro 2000 spectrophotometer (Ocean Optics, USA) at room temperature. The fluorescence spectra were recorded using an RF-5301 PC Shimadzu spectroflourometer. Fourier transforminfrared (FT-IR) spectra were recorded on a Perkin Elmer (FT-IR spectrumBX, Germany). The morphology and the microstructure of the C-dots were examined by HR-TEM on a JEOL 3010 with an accelerating voltage of 200 kV. The X-ray diffraction (XRD) profiles of the C-dots were recorded on a Rigaku diffractometer (Rigaku, Japan) equipped with graphite monochromatized CuKα (λ=0.15405 nm) radiation in the range from 10 to 70°.

Results and Discussion

Characterization of C-dots

The preparation of C-dots involves only by using *Carica papaya* juice as a precursor, which can be referred as "green chemistry". The prepared C-dots were well dispersed in water, yellowish, and stable for months. To prepare high fluorescent C-dots, we studied the effect of heating on the formation of fluorescent C-dots by UV-visible spectrometry and UV light (Fig. 1). It can be observed that all C-dots showed absorbance at 343 nm with slight variation in intensities. The dispersed C-dots samples exhibited strong blue emission under UV illumination at 365 nm, indicating that the formation of C-dots at a high heating temperature (170 °C).

Furthermore, we also investigated the effect of hydrothermal heating time for the preparation of fluorescent C-dots (Fig. 2). The UV-visible spectra and photographs of C-dots under UV lights showed that the C-dots are formed at all reaction times (3, 6, 9, and 12 h). However, the maximum absorbance and strong blue emission were observed at heating time 12 h. Therefore, we selected 12 h and 170 °C as the best heating time and temperature for the preparation of strong fluorescent C-dots using *Carica papaya* as a raw material.

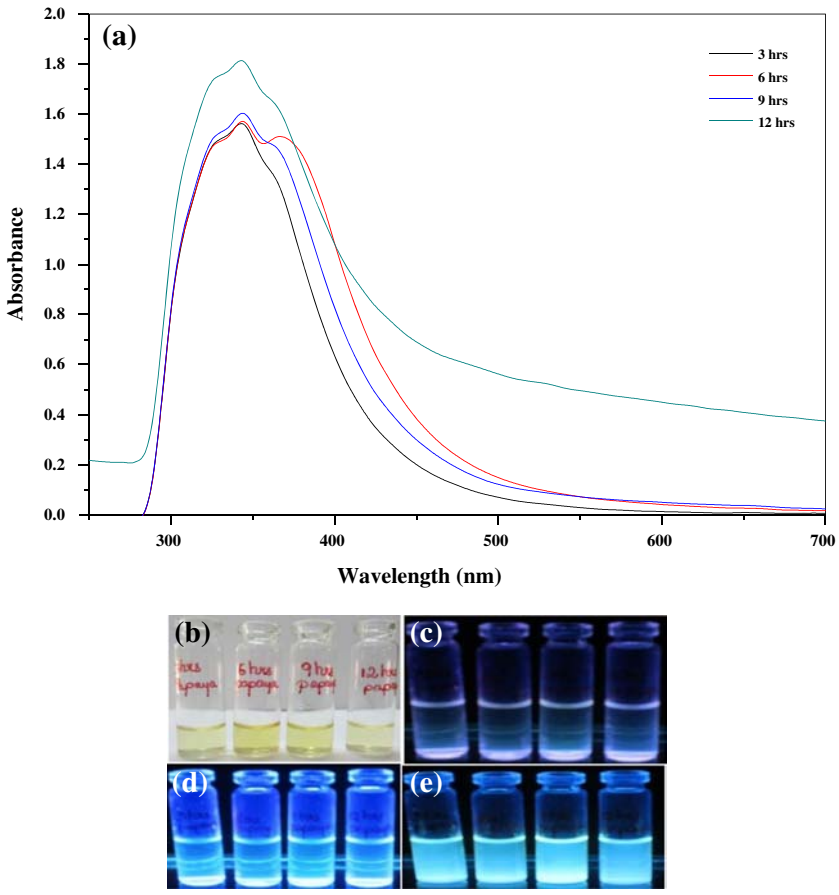


Fig. 2 **a** UV-visible absorption spectra of C-dots at different heating times. Photographic images of C-dots at different heating times in **b** day light and their fluorescence properties under UV light excitation wavelengths **c** 254, **d** 302, and **e** 365 nm

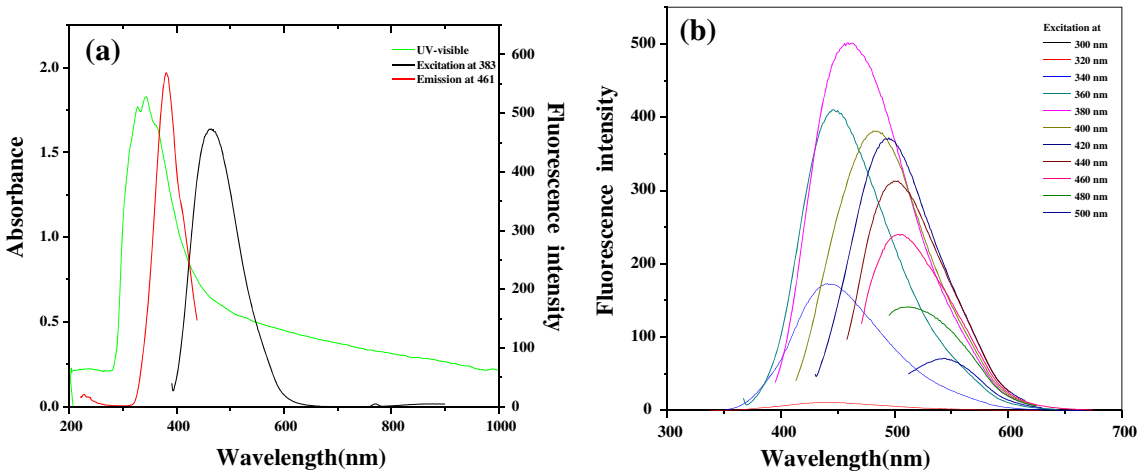


Fig. 3 **a** UV-visible absorption and fluorescence emission spectra (λ_{ex}=383 nm) of C-dots at 170 °C **b** spectra at different excitation wavelengths from 300 to 500 nm

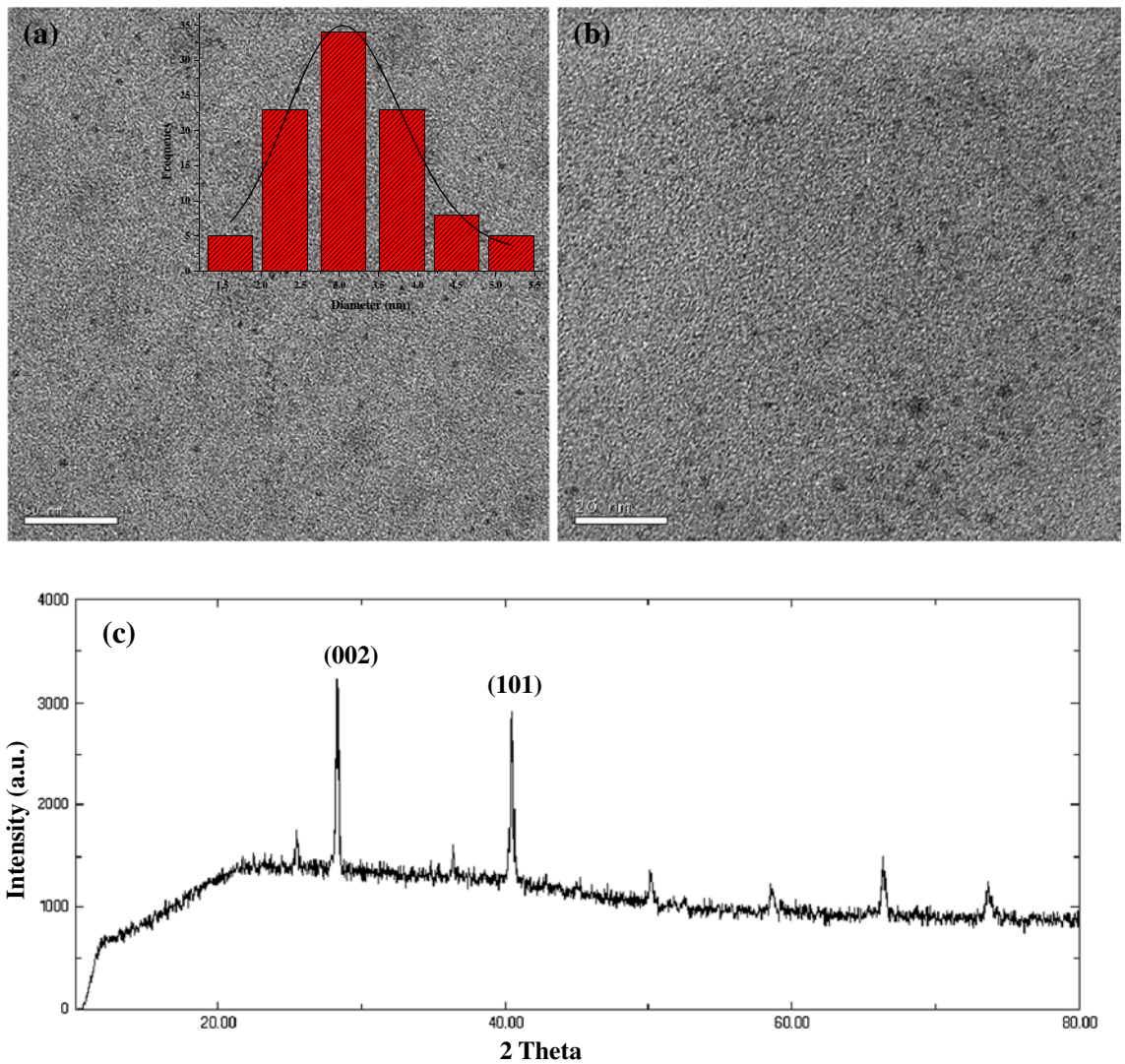


Fig. 4 HR-TEM images of C-dots at (**a**) 50 and (**b**) 20 nm. Inset image of size distribution pattern; and (**c**) XRD pattern of C-dots

Figure 2 shows the typical UV-visible absorption spectrum of C-dots. These results indicated that the C-dots exhibit an absorption band around at 250–350 nm, indicating the typical absorption of the polycyclic aromatic π orbitals in the C-dots. The prepared C-dots displays the light yellow color, which exhibits bright blue fluorescence under UV light at an excitation of 365 nm (Fig. 2e).

Furthermore, the C-dots show the maximum emission peak centered at 461 nm, when excited at 383 nm (Fig. 3a), with a full width at half maximum (FWHM) of 102 nm, which is similar to that of the carbon particles obtained from potato (*Solanum tuberosum*) β-cyclodextrin and alloyed quantum dots [30–32]. The FWHM of the C-dots is due to the narrower size distribution of the C-dots and the limited processes of radiative recombination of photo-generated carriers. The featureless absorption characteristics indicates that the prepared C-dots can belong to the indirect bandgap semiconductor nanoparticles, suggesting that the C-dots act as an excellent candidates for the effective light-emitting and targeted tracing [33]. To address whether the C-dots showed excitation-dependent emission character or not, we studied the emission spectra of C-dots at different excitation wavelengths from 300 to 500 nm (Fig. 3b). When an excitation wavelength was higher than 400 nm, the emission intensities of C-dots are decreased remarkably and exhibited red shift with the excitation to the longer wavelength. Similar phenomenon is often observed in the other carbon nanoparticles, indicating the excitation-dependent emissions are unique feature of C-dots, which also confirms the presence of heteroatoms in the prepared C-dots.

To further investigate the morphology and size of the C-dots, we studied HR-TEM for the estimation of C-dots size (Fig. 4a and b). The HR-TEM image clearly reveals that the prepared C-dots are well dispersed in water with a spherical morphology and a narrow size distribution of 3.0 nm in diameter (Inset of Fig. 4a). The XRD pattern of prepared C-dots was shown in Fig. 4c. It can be observed that the C-dots exhibit two sharp diffraction peaks (2θ=28° and 40°) and also show small diffraction peaks (2θ=50° and 58°), which is attributed to carbon composed in a considerably random fashion. These results indicate that the carbon atoms exist as densely packed alkyl chains with turbostratic and graphitic carbons [34], revealing that the poor crystalline nature of C-dots, which is due to more number of oxygen containing groups [34].

Furthermore, we also studied the FT-IR spectrum of the C-dots (Supporting Information of Figure S1). The absorption bands at 3264 and 1674 cm^{-1} are corresponded to the vibrations of O–H and C=O bonds, and the asymmetric and symmetric stretching vibrations of the carboxylate groups are also observed at 1398 and 1065 cm^{-1}. The peaks around 1606 cm^{-1} corresponded to C=C stretching of C-dots, which are consistent with the UV-visible absorption spectra (Fig. 1). These results indicated that the presence of aromatic

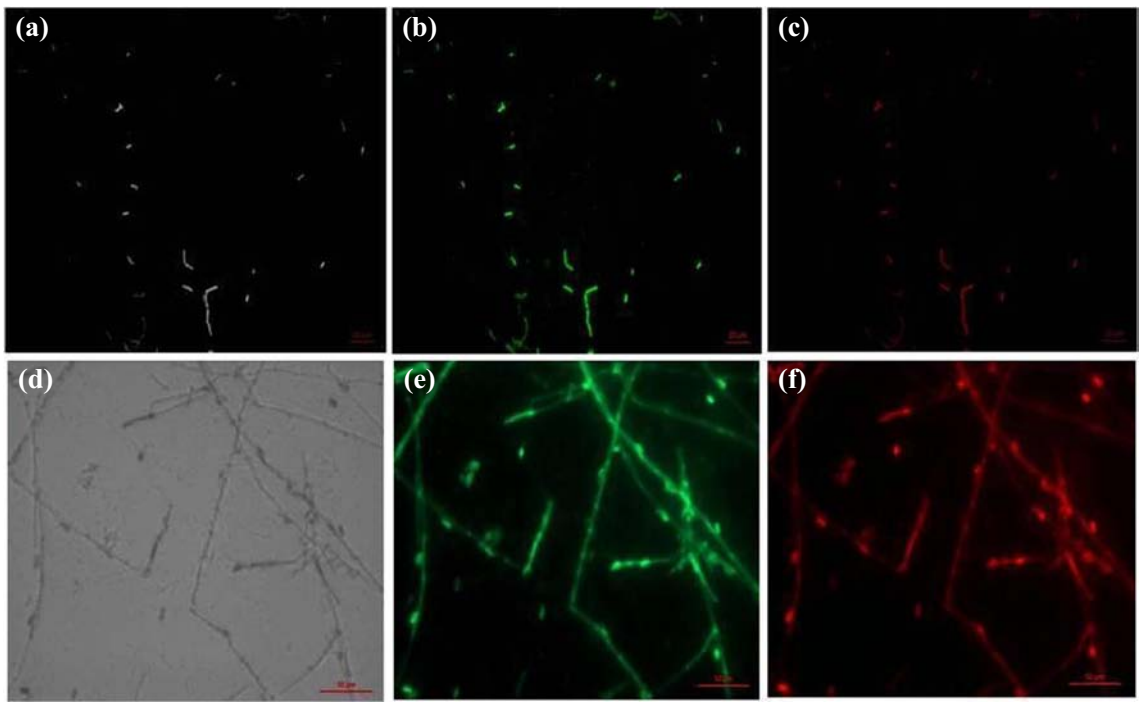


Fig. 5 Confocal laser microscopic images of *Bacillus subtilis* cells after incubation at 37 °C for 1–6 h (**a**) phase contrast without C-dots and fluorescence mode at excitation wavelengths (**b**) 488 (*green*), and (**c**) 561 (*red*) nm without bright field. Confocal laser microscopic images of and *Aspergillus aculeatus* cells after incubation at 37 °C for 1–6 h (**d**) bright field without C-dots and fluorescence mode at excitation wavelengths (**e**) 488 (*green*), and (**f**) 561 (*red*) nm

compounds in *Carica papaya*. The peaks in the range of 1000–1300 cm^{-1} are assigned to the C–OH stretching and OH bending vibrations, implying the existence of large numbers of residual hydroxyl groups. These results raveled that the surfaces of C-dots are fully functionalized with hydrophilic groups (hydroxy, carboxylic and carbonyl (−CHO and C=O) groups), which can improve the hydrophilicity and stability of the nanoparticles in aqueous solutions. The quantum yield of C-dots derived from *Carica papaya* juice is 7.0 %, which exhibit lower quantum yield than the reported methods [4, 35, 36]. This is due to the presence of carboxylic acids (citric acid and ascorbic acids), which plays a key role in the generation of C-dots with high quantum yield.

Imaging of Bacterial and Fungal Cells Using C-dots as Probes

The C-dots were incubated with *Bacillus subtilis* and *Aspergillus aculeatus* and studied their cellular up takes at room temperature, as shown by confocal fluorescence images (Fig. 5); no fluorescence was observed from the control samples without C-dots treatment. However, the strong green and red fluorescence were obtained when excited at 488 (green) and 561 nm (red) from C-dots-labeled *Bacillus subtilis* cells, which indicates that the prepared C-dots were effectively up taken to the cells. Similarly, green and red fluorescence images were observed from C-dots-labeled *Aspergillus aculeatus* when excited at 488 and 561 nm. It can be observed that the shape and the length scales of *Bacillus subtilis* cells were in rod-like images, which adopt a wide range of morphologies dependent on the growth conditions [36]. The confocal fluorescence microscopy images illustrate that *Bacillus subtilis* and *Aspergillus aculeatus* clearly internalize water-dispersed C-dots. Furthermore, these images revealed that the homogeneous distribution of C-dots within bacterial and fungal cells. The bacterial and fungal cells are able to internalize the C-dots, and are not degraded because C-dots retain their distinct wavelength-specific emission properties inside the cells. Furthermore, the images from those cells showed the occupancy of C-dots in inner region of the cell cytoplasm. Based on these results, we can assume that the C-dots can effectively accumulate near the cell's nuclei, that is, the end of the probe migration inside the live cell internalization pathway [37].

In order to prove that the prepared C-dots are biocompatible nature or not, we studied the MIC values of C-dots (16.2 – 500 μg/mL) against *E. coli* strains (Supporting Information of Figure S2). These results revealed that the prepared C-dots did not behave as bacteriostatic and bactericidal agents. Therefore, the prepared C-dots are effectively localized with nucleoids in bacterial and fungal cells, which indicates that the prepared C-dots can be used as fluorescent probes for cellular imaging in prokaryotes that address many issues in different fields of research, such as drug delivery and cell labeling.

Conclusions

In conclusion, we have described the use of hydrothermal method for green synthesis of fluorescent C-dots using *Carica papaya* juice as a precursor. The fluorescent C-dots are prepared in single-step without any chemical modifications. The prepared C-dots exhibited multicolor emissions (green and red) when excited at different wavelengths. The prepared C-dots were used as fluorescent probes for imaging of bacteria and fungus cells, suggesting that the C-dots are nontoxic and good biocompatibility, which demonstrates their potentials for both in vitro and in vivo applications.

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