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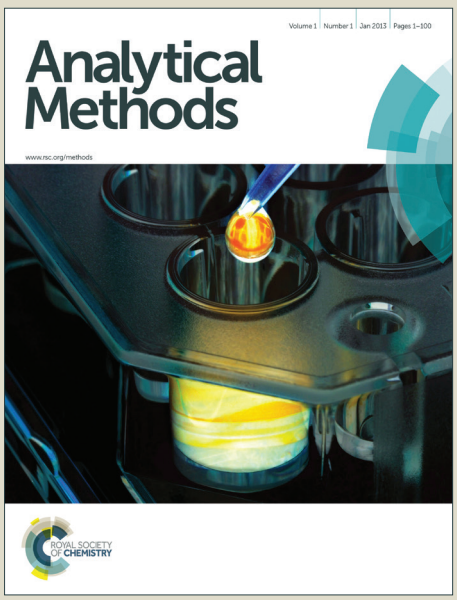

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One-step synthesis of fluorescent carbon dots for bacteria and fungus cells imaging

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Abstract

In this article, fluorescent carbon dots (C-dots) were synthesized by using *Punica granatum* (pomegranate) fruits as precursors in hydrothermal method and used as probes for imaging of bacteria (*Pseudomonas aeruginosa*) and fungus (*Fusarium avenaceum*) cells. The C-dots showed strong emission at 453 nm when excited at 383 nm. The C-dots were taken up by bacteria (*P. aeruginosa*) and fungus (*F. avenaceum*) cells, and two color (green and red) images were observed by confocal fluorescence microscopy, which confirms that the C-dots are rapidly internalized by bacteria and fungus cells.

Keywords: *Punica granatum* fruits, UV-visible, Fluorescence, HR-TEM, bacteria and fungus.

1. Introduction

Pseudomonas aeruginosa is a bacterium that can cause a wide variety of diseases to humans. *Fusarium avenaceum* is a fungi which can cause various diseases to plants. Therefore, a specific fluorescence imaging of bacteria and fungus cells is of great importance for the confirmation of their structures and functions.[1] In this connection, various organic molecules have been used as probes for *in vivo* and *in vitro* fluorescence imaging of wide variety cells in clinical samples.[2] However, they are not ideal agents for multiplexing, long-term, or real-time imaging of cells. Therefore, the development of bacteria- and fungus- targeted fluorescent probes with multicolor emission is very interesting for imaging of cells.

In recent years, the C-dots have proved as a new class of fluorescent nanoparticles and shown tremendous applications in multidisciplinary research.[3] Typically, C-dots are <10 nm size and exhibited strong intrinsic fluorescence. In this context, a wide variety of synthetic approaches such as laser ablation, arc discharge, combustion, thermal treatment, electrochemical oxidation, hydrothermal, ultrasonic treatment and microwave methods have been developed for the synthesis of fluorescent C-dots using various organic chemicals and natural resources as precursors.[4] Among these approaches, hydrothermal method is one of the best green chemistry approach for the preparation of large scale fluorescent C-dots using various waste and natural resource materials as carbon source. Therefore, considerable efforts have been focused on the development of biocompatible photoluminescent C-dots using various natural resources including orange,[5] banana,[6] watermelon peel,[7] *Trapa bispinosa* peel,[8] coffee grounds,[9] food waste,[10] milk,[11-12] sugar cane juice,[13] potato, [14] hair,[15] honey,[16] ginger juice,[17] orange waste peels,[18] and bread,

sugar, and jiggery[19] as precursors. The prepared C-dots acted as fluorescent imaging probes, and accumulated in the cell membranes, cytoplasm, and nucleus. In addition, the precursor selection plays a key role in the yields and properties of C-dots, which makes them as potential candidates for biomedical applications.

Herein, we report a one-pot hydrothermal synthesis of water- soluble C-dots using *Punica granatum* (pomegranate) fruit as a precursor. The synthesized C-dots are monodisperse with narrow size distribution, and showed high emission intensity without passivation. The C-dots were used as fluorescent probes for imaging of both bacteria (*P. aeruginosa*) and fungus (*F. avenaceum*) cells. Furthermore, we also studied the minimum inhibitory concentration of C-dots towards *Bacillus subtilis*.

2. Experimental Details

2.1. Chemicals

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. *Punica granatum* fruits were purchased from local vegetable market, Surat, Gujarat, India.

2.2. Synthesis of C-dots

Briefly, *Punica granatum* juice was obtained by grinding 400 g of *Punica granatum* fruits and 500 mL of juice was transferred into 750 mL teflon-lined stainless steel autoclave and then heated at 170 ^{o}C for 12 h. After heating, the product was left to cool down naturally, and a dark brown liquid was obtained, which indicates that the formation of C-dots. The effect of heating time (3, 6, 9, 12, 15 and 18 h) was investigated for confirmation of high yield C-dots. Finally, the C-dots were collected by dialysis against deionized water though a dialysis membrane for 12 h and stored at 4 ^{o}C for further use.

2.3. Bacteria and fungus culture and cells imaging

Single colony of *P. aeruginosa* was inoculated in 5 mL LB nutrient medium (Tryptone - 10 mg/mL, Yeast Extract - 5 mg/mL and NaCl - 10 mg/mL, pH 7.0), and cultured overnight at 37°C. Then, 50 µL of culture solution was transferred into a fresh nutrient medium (5 mL), stirred and cultured at 37 °C for 12 h. The entire sample was centrifuged at 8000 rpm for 5 min, and then the precipitate was suspended in saline. Similarly, *F. avenaceum* was cultured on complete medium of 5×YEG (0.5% yeast extract and 2% glucose) and oatmeal agar (OA) plates 25 °C. 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 absorbance at 600 nm. To prepare the bacteria- and fungus- C-dots conjugates, 70% (*v/v*) ethanol was used for the internalization of C-dots by cells at 4 ºC for 5 min. The cells staining were carried out by dispersing of cells in 100 mM of phosphate buffer containing C-dots (10 µg/mL) for 10 min at room temperature. Finally, the cells-C-dots conjugates were washed thrice with double distilled water and 20 µL of the above C-dots conjugates were put on a glass slide to measure fluorescence images. Fluorescence images of cells were recorded in green, and red region with a laser excitation of 488, and 561 nm, respectively. Minimum inhibitory concentration of the synthesized C-dots on *B. subtillis* was described in Supporting Information.

2.4. Instrumentation

UV–visible spectra were measured by using a May Pro 2000 spectrophotometer (Ocean Optics, USA). X-ray diffraction (XRD) patterns were carried out on a Rigaku diffractometer (Rigaku, Japan). Fourier transform infrared spectroscopy was performed

with a Perkin Elmer (FT-IR spectrum BX, Germany). The morphology and microstructure of CDs were examined by JEOL-HR-TEM 3010 with an accelerating voltage of 200 kV. The fluorescence spectra were recorded using a RF-5301 PC Shimadzu spectroflourometer. Cell images were measured using Carl Zeiss 510 LSM laser scanning confocal microscope. The fluorescence lifetime was measured using Horiba Jobin Yvon IBH Fluorocube instrument.

3. Results and discussion

3.1. Characterization of C-dots

Scheme 1 shows the schematic representation for the preparation of fluorescent C-dots using *Punica granatum* juice as a precursor. The prepared C-dots are transparent in daylight and exhibited blue luminescent under UV illumination at 365 nm. Figure 1 shows the UV-visible absorption spectra of C-dots at different hydrothermal heating time intervals (3, 6, 9, 12, 15 and 18 h). It can be noticed that the maximum absorbance of C-dots was observed at 12 h. To confirm this, we studied the fluorescence spectra of C-dots in the above hydrothermal heating time (3 – 18 h) under excitation wavelength 383 nm (Figure 2). As hydrothermal heating time increases from 3 to 12 h, the emission intensity of C-dots was gradually increased with increasing hydrothermal heating time and the highest fluorescence intensity was found at hydrothermal heating time 12 h. It can be noticed that emission intensity of C-dots was drastically decreased at hydrothermal heating time 15 and 18 h, which is due to the supramolecular self-organization of C-dots.[20] Furthermore, UV–visible spectra showed absorbance in the region of 250–400 nm and two peaks at 325 and 345 nm (Figure 3a), which are attributed to π–π^* transition of the C=O band and n–π^*

transitions of the conjugated C=C bands in polycyclic aromatic π orbitals. It should be noted that the absorption bands are slightly red-shifted, which may be attributed to the structure with larger conjugation.[21] The strong emission peak was observed at 453 nm with a full width at half maximum (FWHM) of 102 nm, when excited at 383 nm (Figure 3a), confirming the fluorescence of C-dots. Therefore, we selected 12 h as an optimum reaction time for the preparation of C-dots with good fluorescent yield. Furthermore, we also studied the emission characteristics of C-dots at different excitation wavelengths from 300 to 520 nm with 20 nm increment (Figure 3b). It can be observed that emission spectrum of C-dots was gradually red-shifted towards longer wavelengths with decreasing fluorescence intensity, which confirms that the bathochromic shift of emission from C-dots.[6,7,10] The quantum yield (QY) of C-dots was found to be 7.6%, which is comparable with the reported method.[11]

The surface chemistry of C-dots was characterized by FT-IR (Supporting Information of Figure S1a). It can be observed that the broad absorption bands around 3399 and 3444 cm^{-1} were assigned to O–H and N–H stretching vibrations, respectively. The peaks at 1415 and 1604 cm^{-1} can be ascribed to C–N and N–H bending vibrations, while the peaks at 1642 and 1470 cm^{-1} indicated the existence of carboxylate ion. The characteristic stretching vibration bands of C=O were observed at 1618 cm^{-1} and C=C group stretching vibration is attributed at 1457 cm^{-1}, indicating that the carbonization of organic compounds into the graphite-like nanostructures.[13-14,22-23] Furthermore, the peaks at around 3003 cm^{-1} and 1267 cm^{-1} were assigned to the C–H stretching vibration and C–H out-of-plane bending vibration. The sharp peak at 2359 cm^{-1} was assigned to intramolecular H-bridges in C-dots. These results strongly suggest that the prepared C-dots were

functionalized with various functional groups, and these surface groups provide an insight into the luminescence mechanisms, which allows us to use them as eco-friendly probes in biochemical investigations.

The HRTEM images of C-dots are shown in Figure 3c and d. As indicated, the diameter of C-dots is about 3.5 nm, and well dispersed from each other with the spherical shape. Supporting Information of Figure S1b shows the XRD pattern of C-dots. The intense peaks at 2θ = 28.42° and 40.56° are assigned to (002) and (101) diffraction patterns of graphitic carbon, respectively. The small diffraction peaks (2θ=50.30^{o}, 66.42^{o}) are corresponded to amorphous carbon in C-dots, revealing that the carbon nanostructures exist as densely packed alkyl chains with turbostratic and graphitic carbons. The lifetime decay curves of the fluorescence emission of C-dots were shown in Supporting Information of Figure S2. We found that the measured lifetimes for the different experiments are τ_1 = 2.14 ns, τ_2 = 4.28 ns, τ_3 = 8.5 ns and average life time is 4.97 ns. These results indicate that the fluorescence lifetime is sensitive to the nanostructured carbon particles, and similar values are obtained for C-dots those are prepared from various food materials.[7,14]

3.2. Fluorescence imaging

Figure 3 shows the fluorescence images of bacteria (*Pseudomonas aeruginosa*) and fungus (*Fusarium avenaceum*) cells by using C-dots as a probe. The cell internalized C-dots are excited by laser excitations at 405 and 458 nm, and emitted green and red fluorescence. The fluorescent areas were overlayed on the locations of cells, indicating that C-dots could be taken up by the cells and were accumulated in the cells. Due to their small size, surface functional groups and biocompatible nature, C-dots can be easily taken up by

the cells with high degree. As illustrated in Figure 4a and d, no fluorescence emission was observed in both bacteria and fungus without C-dots, which confirms that they could be easily enter into cell membranes and taken up by the cells. These results demonstrate that the prepared C-dots are successfully accumulated in the cell membranes and cytoplasm. In order to investigate their toxicity, we studied MIC of C-dots towards *B. substils* (Supporting Information of Figure S3). This result indicates that the prepared C-dots don't inhibit the growth of above bacteria, which is indicative of their biocompatible nature with cells. Therefore, the C-dots as alternative materials for conventional organic dyes and semiconductor quantum dots, and can be used as promising probes for imaging of various cells.

4. Conclusions

In conclusion, we have developed a one-step hydrothermal route for synthesis of C-dots using *Punica granatum* fruit as a precursor without further treatment or modification. The C-dots act as fluorescent probes for imaging of bacteria and fungus cells. They did not inhibit the growth of *B. substils*, which indicates that the C-dots are non-toxic. Therefore, the C-dots CNPs can be used as fluorescent probes for imaging of both animal and plant cells.

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Figure captions

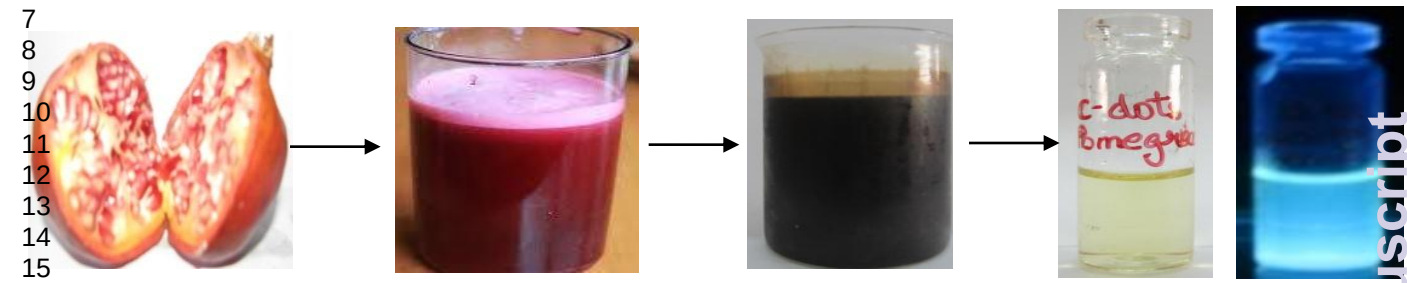
Scheme 1. Schematic procedure for preparation of fluorescent C-dots by one-step hydrothermal treatment using *Punica granatum* juice as a source.

Figure 1. (a) UV-visible absorption spectra of C-dots at different time intervals (3, 6, 9, 12, 15 and 18 h). Photographic images of synthesized C-dots in (b) day light and their fluorescence properties under UV light excitation wavelengths (c) 254, (d) 302, and (e) 365 nm.

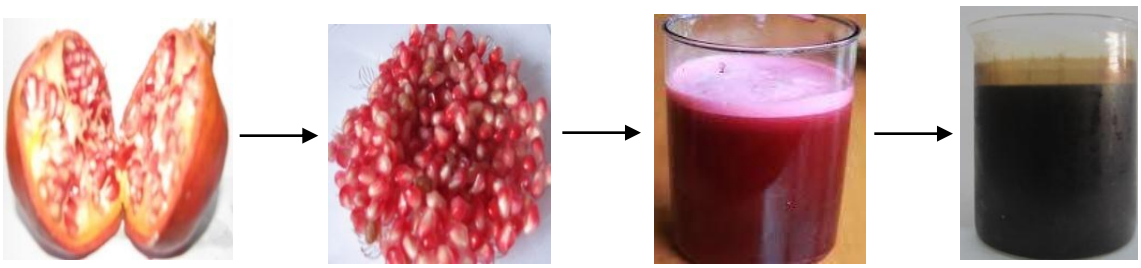
Figure 2. Fluorescence spectra of C-dots prepared at different heating time intervals from 3 – 18 h under excitation wavelength 383 nm.

Figure 3. (a) UV-visible absorption and emission spectra (λ_{ex} = 383 nm) of synthesized C-dots at 170°C for 12 h (b) emission spectra of the C-dots at different excitation wavelengths progressively increased from 300 nm to 520 nm with a 20 nm increment emission. HR-TEM images of C-dots at different magnifications (c) 100 nm and (d) 50 nm. Inset image of size distribution pattern.

Figure 4. Confocal laser microscopic images of *Pseudomonas aeruginosa* 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 *Fusarium avenaceum* 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 nm (red).



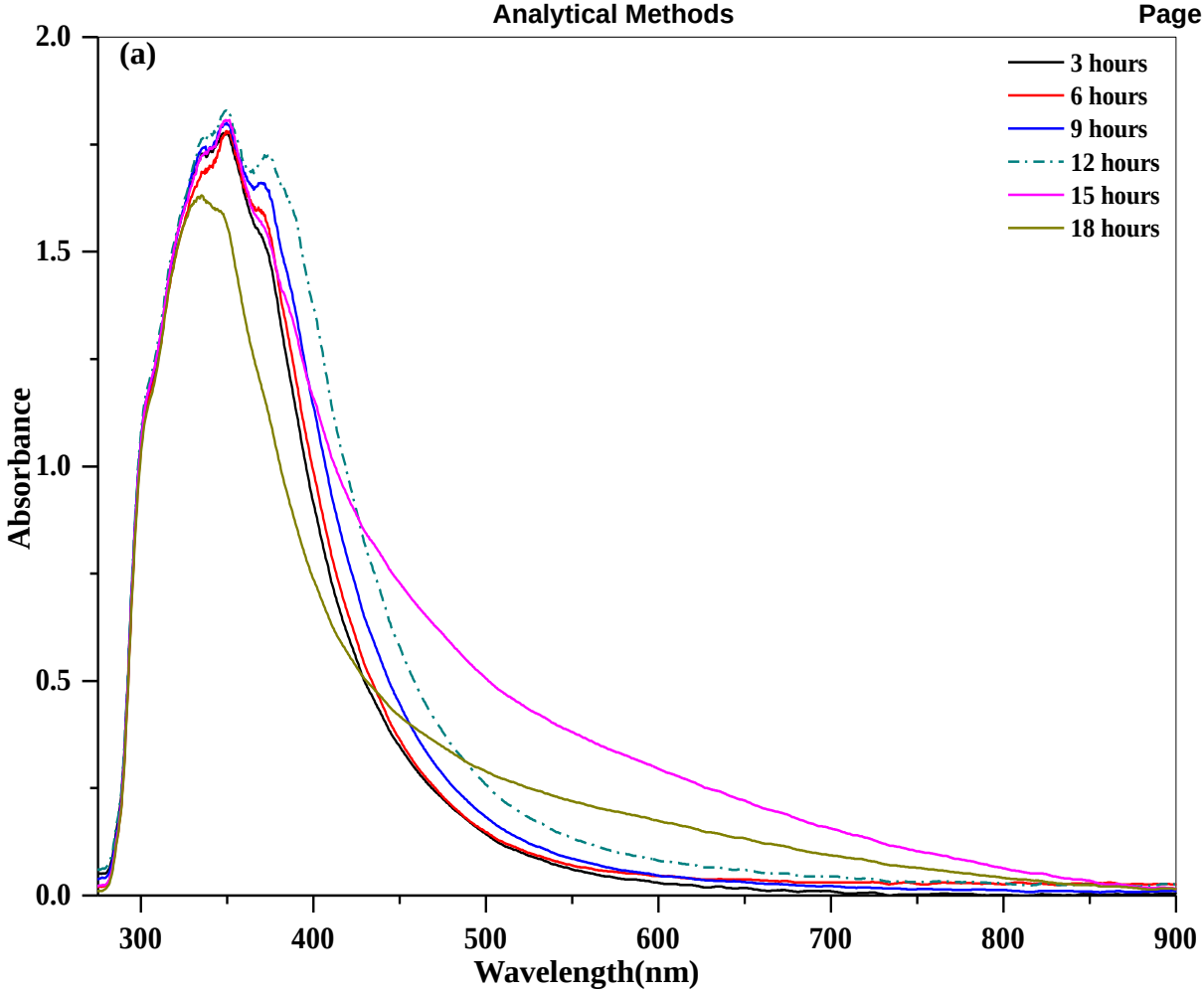
***Punica granatum* fruit** ***Punica granatum* juice** **After carbonization** **C-dots in day light and** **UV-light**

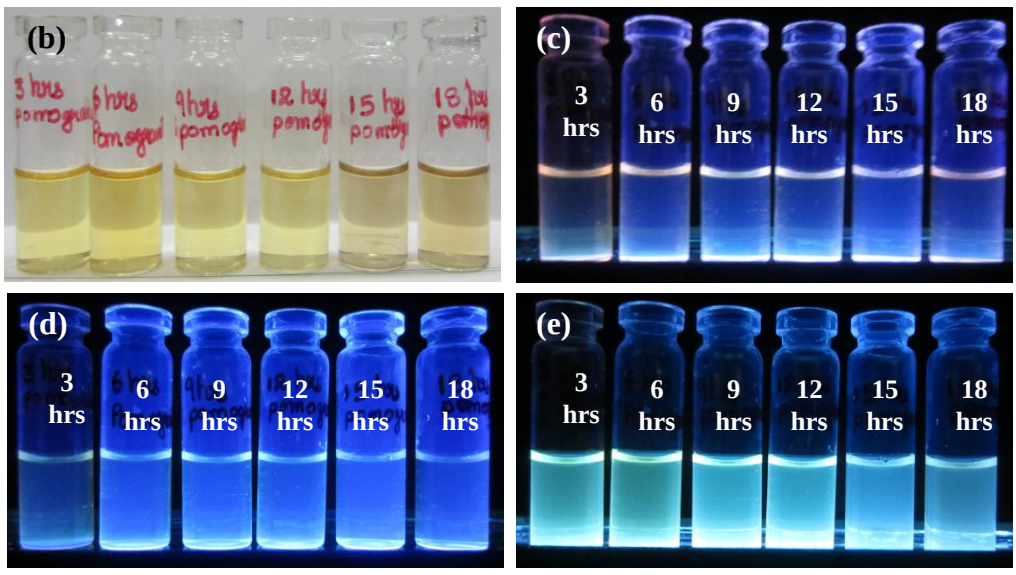


***Punica granatum* fruit** ***Punica granatum* juice** **After carbonization**

C-dots in day light and UV-light

Scheme 1.





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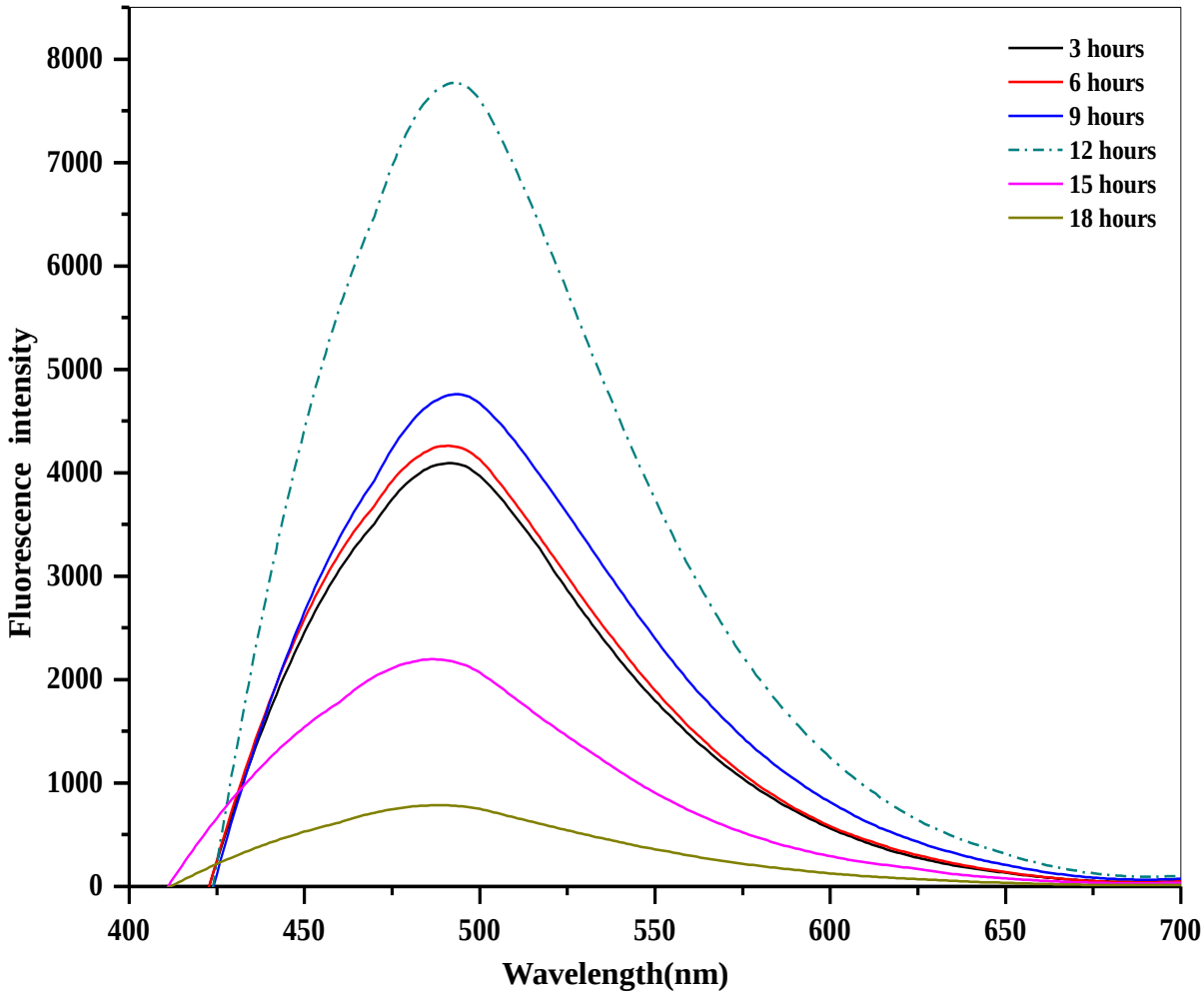
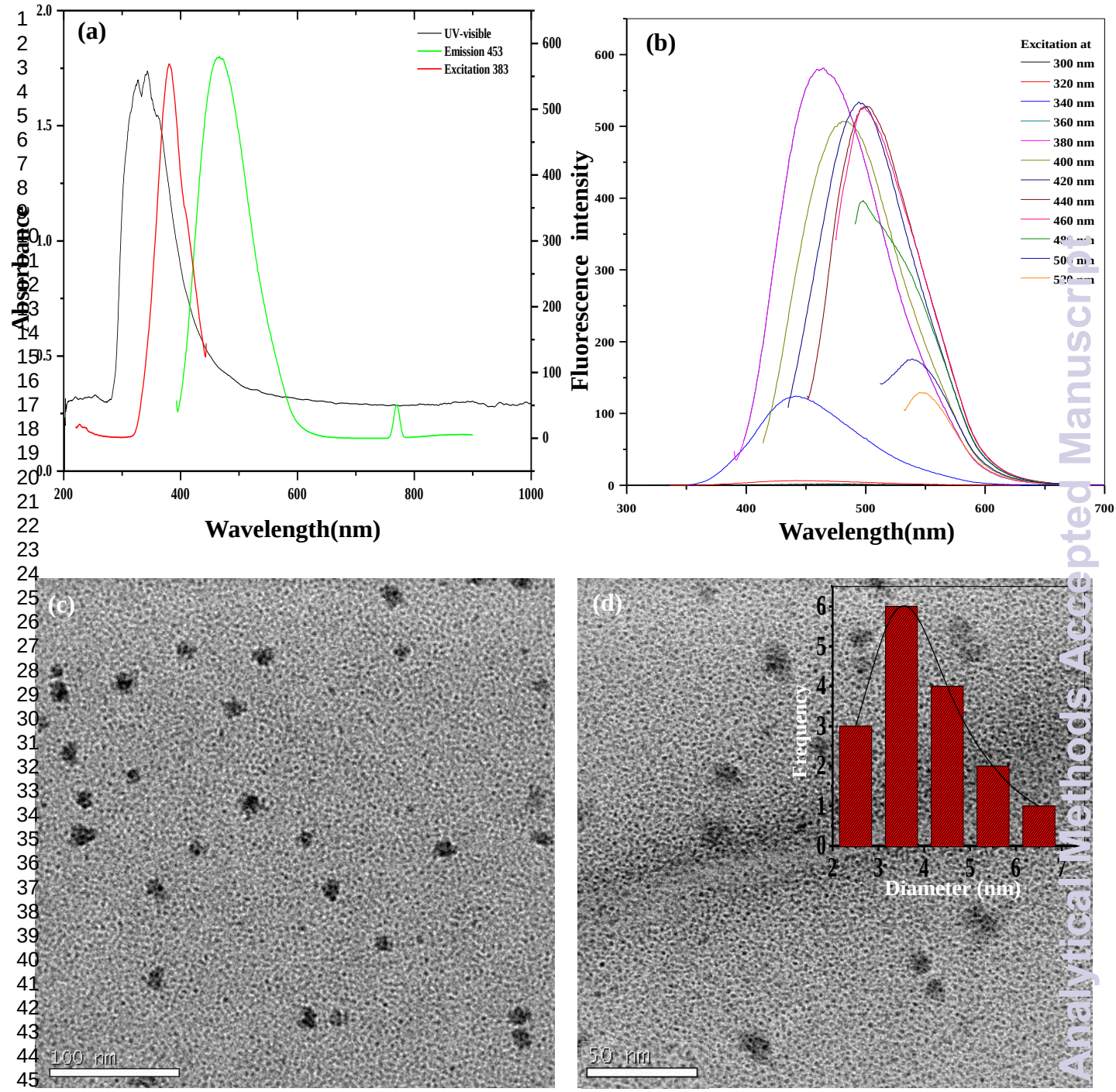


Figure 2.



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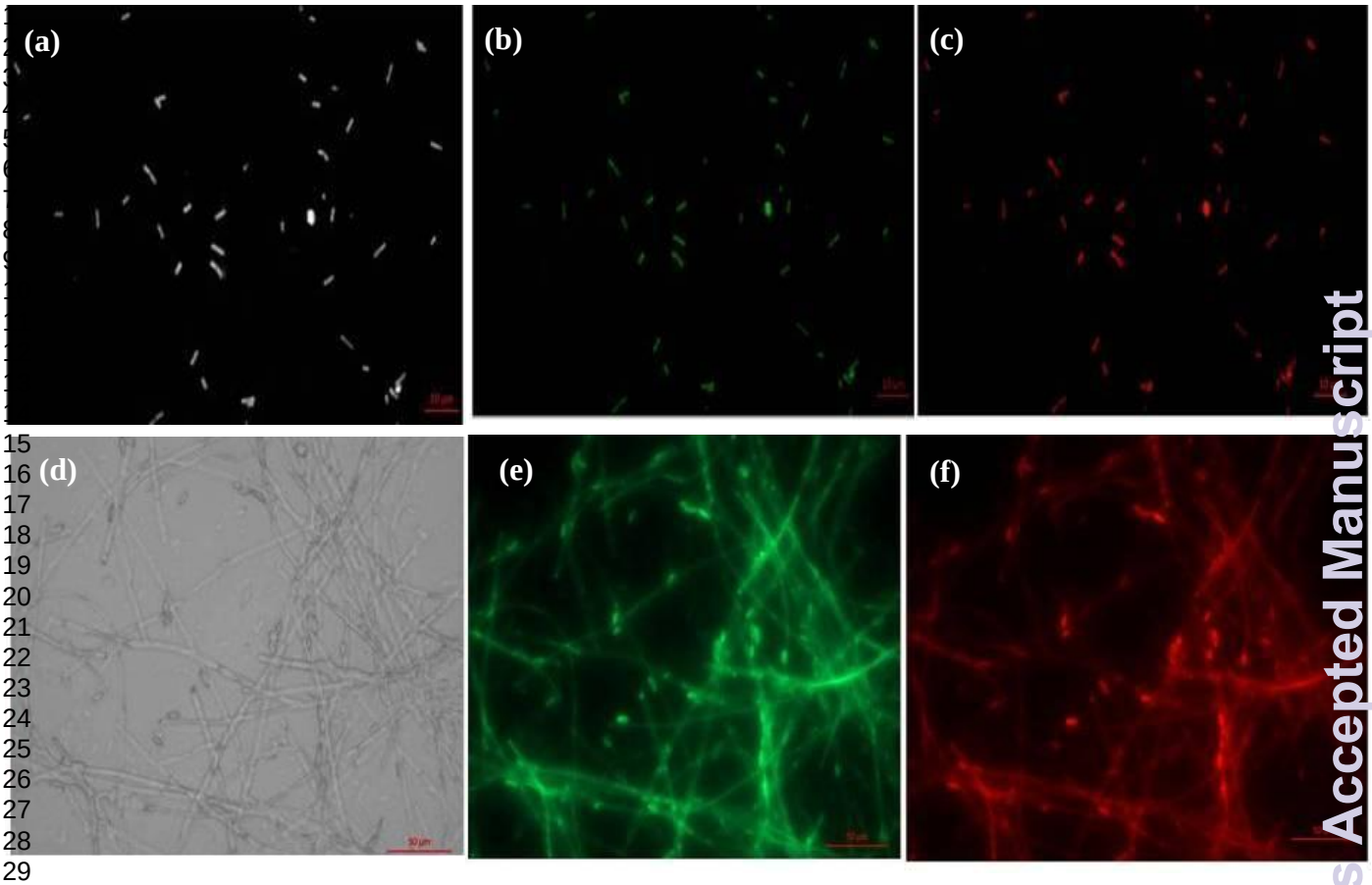

Figure 4.