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SHORT COMMUNICATION

A Green Route Towards Highly Photoluminescent and Cytocompatible Carbon dot Synthesis and its Separation Using Sucrose Density Gradient Centrifugation

Goldie Oza · Kusum Oza · Sunil Pandey · Sachin Shinde · Ashmi Mewada · Mukeshchand Thakur · Maheshwar Sharon · Madhuri Sharon

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Abstract An efficient, fast and green method for synthesis of Carbon dots (C-dots) using natural precursor *Citrus limone* under ultrasonic condition is demonstrated. Such as-synthesized C-dots were further purified using Sucrose density gradient centrifugation method (SDGC) which resulted in the separation of water-soluble, photo luminescent, monodispersed, highly photostable and chemically stable C-dot fractions (F1 and F2). They possess very small size (5–20 nm) as evidenced by High angle annular dark field-Scanning Transmission electron microscopy (HAADF-STEM) and very strong luminescence as shown by fluorescence spectroscopic studies. Cytocompatibility and bio imaging properties of both the fractions (F1 and F2) were then studied on Hep-2 cells. Quantum yield of F1 and F2 fraction was found to be 12.1 and 15 %, respectively.

Keywords Carbon nanostructures · Chemical synthesis · X-ray diffraction · Luminescence

Introduction

Photo luminescent carbon dots (C-dots) are a novel class of carbon nanostructures which possess unique optical, optoelectronic properties exhibiting size- and excitation wavelength-dependent luminescence properties [1]. The photoluminescence is dramatically demonstrated due to the quantum confinement effect as well as different emissive traps existing on C-dot surface [2]. Catastrophic leaching of quantum dots made of metals such as cadmium and selenium in the biological system has led to the appraisal of safety issues [3]. Thus, carbon dots are found to be attractive paraphernalia as compared to semi-conductors due to their low toxicity and cost-effectiveness [4]. There are plethora of approaches for synthesizing carbon dots, such as electrochemical synthesis of carbon dots from graphite electrodes [5], thermal oxidation of organic precursors [6], microwave assisted pyrolysis of molecular precursors [7]. But, the biggest flaw in all the above methods is their monodispersity, water solubility, biocompatibility and stability in physiological saline and the presence of obnoxious carbonaceous residues. To circumvent all such problems there is a need to develop facile, green synthetic approach which can produce high-quality C-dots, thus can be exploited in sensors, biological labelling and drug-delivery.

In this report, we present an ultrasonic method for the synthesis of C-dots using a natural precursor, *Citrus limone*

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G. Oza (✉) · K. Oza · S. Pandey · S. Shinde · A. Mewada · M. Thakur · M. Sharon · M. Sharon
Department of Electrical Engineering (SEES), CINVESTAV-IPN, Av Instituto Politecnico Nacional 2508, San Pedro Zacatenco, Gustavo A. Madero, 07360 Mexico City, DF, Mexico
e-mail: goldieoza@gmail.com

G. Oza · K. Oza · S. Pandey · S. Shinde · A. Mewada · M. Thakur · M. Sharon · M. Sharon
Nagarjuna University, Nagarjuna Nagar, Andhra Pradesh, India

G. Oza · K. Oza · S. Pandey · S. Shinde · A. Mewada · M. Thakur · M. Sharon · M. Sharon
National Sun Yat Sen University, 80424 Lotus Sea, Gushan District, Kaohsiung City, Taiwan

G. Oza · K. Oza · S. Pandey · S. Shinde · A. Mewada · M. Thakur · M. Sharon · M. Sharon
Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya, Aichi 466-8555, Japan

G. Oza · K. Oza · S. Pandey · S. Shinde · A. Mewada · M. Thakur · M. Sharon · M. Sharon
N. Shankaran Nair Research Center for Nanotechnology and Bionanotechnology, Jambhulphata, Ambernath, MS, India

extract and then treated by Sodium borohydride. Such as-synthesized C-dots were then separated by SDGC (Fig. 1). This is a novel report, in which a natural precursor has been carbonized to form highly luminescent Carbon nanoparticles which are uniquely water soluble and cytocompatible in nature. Further such particles can also be exploited as an efficient bio imaging agent.

Experimental

10 ml of *Citrus limone* juice was extracted, filtered and then treated with NaOH solution (5 M) under ultrasonication (20 kHz) for 1 h. When pale yellow coloured solution turned to brown, the reaction was checked for fluorescence under UV tube of 365 nm. Fluorescence was further enhanced on addition of strong reducing agent sodium borohydride (2 ml of 10 mM) in C-dots. 1 ml of such a solution was added in a sequentially layered 100–50 % (*w*/*v*) sucrose solutions from bottom to top in a 50 ml centrifuge tube for SDGC. This sample tube was then loaded in a fixed angle rotor and centrifuged at 5,000 rpm for 1 hr.

After separation of the two fractions, we have further centrifuged the samples at 12,000 rpm for 15 min using Universal 320R (Hettich, Germany) and then loaded into dialysis bags (molecular weight cut off=1,000, Thermo Scientific, USA). The samples were then dialyzed against ultrapure water for 48 h. The purified C-dots were then preserved at 4 °C.

On separation of the two fractions of different sizes, cytocompatibility of both the fractions were studied using Hep-2 cells. Confocal microscopy was performed for the comprehension of bio imaging characteristic of Carbon dots.

Results and Discussion

C-dot synthesis was efficiently performed using ultrasonication method in the presence of NaOH causing hydrolytic cleavage of carboxylic acids present in *Citrus limone* extract, leading to the formation of light brown coloured solution.

Further, on addition of sodium borohydride (2 ml of 10 mM solution), effervescence of hydrogen gas was seen with slight increase in the temperature. The mixture turned into dark brown colored solution which displayed blue fluorescence under UV light of 365 nm. Such a solution was then subjected to SDGC which separates carbon dots on the basis of hydrodynamic size, into two fractions which will be symbolized as F1 and F2 in future discussions (Table 1).

The fraction F1 displays blue-green coloured luminescence under UV light (Fig. 1i). The UV-Visible spectroscopic measurement of F1 shows a characteristic peak at 279 nm and a hump at 343 nm which is due to the σ-π* transition of the C=O band and the π-π* transition of the conjugated C=C respectively (Fig. 2ai). Quantum Yield of F1 fraction was 12.1 %. There was increment in the carbon content (49 %) due to pyrolysis as compared to citrus limone extract (44.5 %). Zeta potential (ζ) clearly indicated negative charge on the surface (Table 2). The F1 fraction in water at pH7.4 showed a potential of −37.5 mV. For the comprehension of its chemical stability, F1 fraction was added in physiological saline (Phosphate buffered saline, pH7.4) and its potential was −18.6 mV, whereas when added in Dulbecco's Modified Eagle medium having 10 % Fetal Bovine Serum (DMEM-FBS), it showed a potential of −12.5 mV. There is lowering of the as we go from water to saline to DMEM, which can be corroborated by agglomeration in saline and adsorption of proteins on to the C-dot in DMEM-FBS. However, there is no drastic change in the ζ values. Figure 3a provides HAADF-STEM of F1, from which we can observe that they are well-

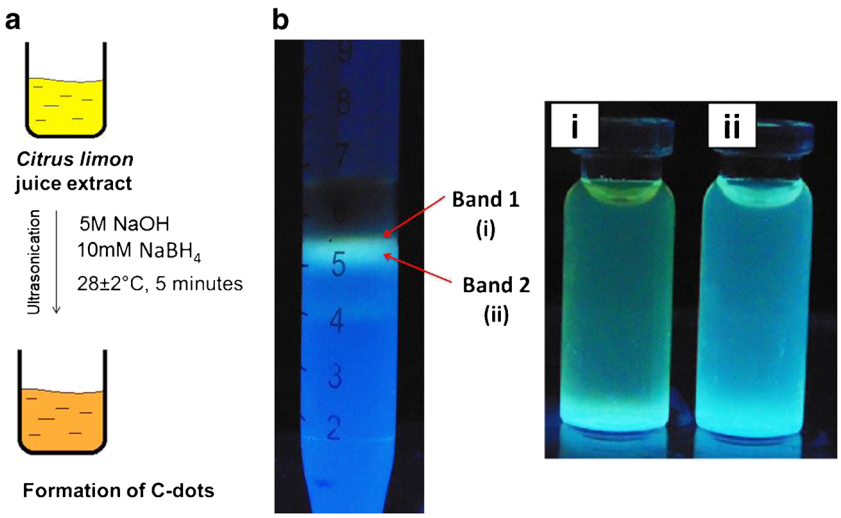

Fig. 1 **a** Schematic representation of C-dot synthesis from *Citrus limone* extract which was ultrasonicated in the presence of sodium hydroxide and then further reduced by Sodium borohydride at room temperature for 5 min **b** sucrose density gradient centrifugation of C-dots and separation of the two fractions; Band 1 corresponds to F1(*i*) while Band 2 corresponds to F2(*ii*)

Table 1 Percentage carbon and Quantum Yield of Citrus limone extract, F1 and F2 fractions of C-dot

	Percent carbon	Quantum yield
Citrus limone extract	44.5 %	–
C-dot Fraction 1	49 %	12.1 %
C-dot Fraction 2	55 %	15 %

dispersed with a diameter of 20 nm. Atomic resolution HAADF-STEM which is also termed as Z-contrast imaging is a promising and important tool for the comprehension of chemical information at the atomic scale. The HAADF-STEM imaging mode collects high-angle scattered electrons in contrast to Bragg scattered electrons, with an annular dark-field detector. The lattice spacing is in agreement with 002 facet of carbon fractions as can be depicted by X-ray diffraction pattern (ESI S2) i.e. 0.324 nm which is a signature marker of graphitic structure (sp^2). ESI S3 displays Raman spectrum of C-dots which exhibit two broad peaks at 1,353 and 1,583 cm^{-1} that can be considered as to be the signatures of D-band (sp^3) and G-band (sp^2), respectively for both the fractions. The presence of both the bands is a clear indication that C-dots are amorphous in nature [4]. D-band is symbolizing the vibration of carbon atoms with dangling bonds in the terminating plane of graphite having edge-defects while G-band denotes E_{2g} mode of graphite and is in correspondence with the vibration of sp^2-bonded carbon atoms in a hexagonal lattice structure [8]. FTIR spectra (Fig. 2da) depicts a sharp peak at 1,640 cm^{-1} (C=O vibrational stretch) and a broad peak at 3,300–3,500 cm-1 (O-H vibrational stretch) which displays the presence of carboxylate and hydroxyl groups on the surfaces of the C-dots of F1. The peak centered at 730 cm^{-1} exhibit C-H bending indicating the reduced form of C-dots.

The fraction F2 shows blue colored luminescence under UV light thus exhibiting smaller sized C-dots (Fig. 1ii). UV-Visible spectroscopy of F2 shows a characteristic peak at 244 nm and hump at 293 nm, but the intensities of the peak are much higher as compared to F1 (Fig. 2aii). Quantum Yield of F2 fraction was 15 %. The percentage carbon increased from 44.5 to 55 % due to pyrolysis, while Zeta potential (ζ) indicated negative charge (−42 mV) on the surface in the presence of water (pH 7.4) (Table 2). Higher ζ values can be correlated to higher stability. Furthermore, when F2 was added in Phosphate buffered saline having a pH of 7.4, ζ values were −22.3 mV. When F2 was added in DMEM-FBS, the ζ value was −19.1 mV. HAADF-STEM exhibits the size of C-dots in the range of 5–10 nm without any aggregation and lattice spacing around 0.324 nm (Fig. 2d). Thus the size of F2 fraction is less as compared to F1. FTIR measurements as depicted in (Fig. 2db) exhibit increased intensities of the peaks at 1,640 cm^{-1} (C=O vibrational stretch) for carboxyl group and a broad peak at 3,300–3,500 cm-1 (O-H vibrational stretch) for hydroxyl groups, akin to F1. The peaks detected at about 1,416 cm-1 and 1,021 cm-1 correspond to the symmetric and asymmetric stretching vibrations of C-O-C in the carboxylate groups, thus

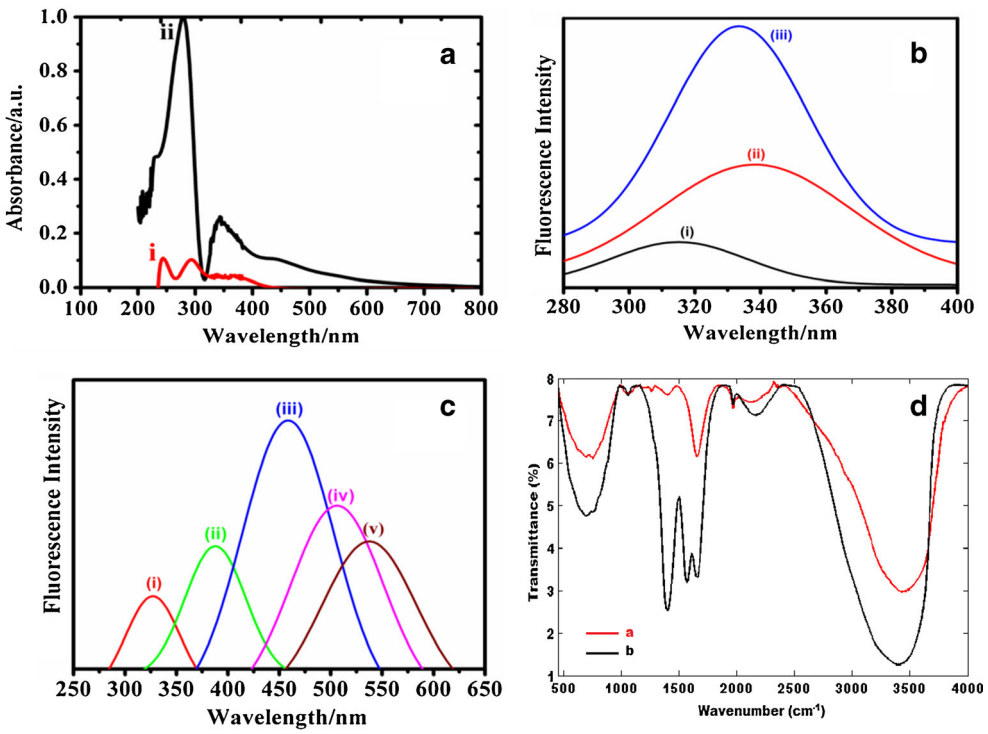

Fig. 2 **a** UV-Visible spectroscopy of Carbon dot fractions {F1 fraction is denoted as (*i*) and F2 is denoted as (*ii*)} derived from sucrose gradient centrifugation **b** photoluminescence intensity of F1 fraction *i*) 230 nm *ii*) 250 nm *iii*) 280 nm **c** photoluminescence intensity of F2 fraction *i*) 230 nm *ii*) 300 nm *iii*) 350 nm *iv*) 400 nm and *v*) 450 nm **d** FTIR of both the fractions (a corresponds to F1 and b corresponds to F2) after sucrose gradient centrifugation was performed to separate C-dots

Table 2 Chemical Stability of F1 and F2 fraction of C-dot

Fractions	Zeta potential
F1 (7.4) in water	−37.5 mV
F1 (7.4) in PBS	−18.6 mV
F1 (7.4) in DMEM-2%FBS	−12.5 mV
F2 (7.4) in water	−42 mV
F2 (7.4) in PBS	−22.3 mV
F2 (7.4) in DMEM-2%FBS	−19.1 mV

signifying that the surface of C-dots is highly hydrophilic in nature. Hence, SDGC has great impetus in separating the two fractions of different sized C-dots.

The Photoluminescence (PL) spectra of both F1 and F2 (Fig. 2b and c) demonstrate excitation wavelength-dependent mechanism when excitation wavelength is gradually increased from 230 to 280 nm for F1 and from 230 to 450 nm for F2. There was red-shift in the emission peaks for both the fractions. This PL spectrum can be attributed to excitation wavelength (λ_{ex})-dependent emission wavelength (λ_{em}). This (λ_{ex})-dependent (λ_{em}) and intensity is a consequence of quantum size effects and/or emissive traps [9] existing onto the surface of C-dots due to the presence of functional groups [10] or due to free zig-zag sites [11] or edge defects during the incorporation of functionality [10]. Fig. 2c and d shows photostability of the two fractions, F1 and F2 respectively. There was no photobleaching for 600 s in a fluorescence spectrophotometer when F1 and F2 fractions were excited by 280 nm and 350 nm wavelengths, respectively. This confirms very high photostability of both the fractions.

The detailed mechanism for the formation of C-dots in alkaline conditions under ultrasonication that can be contemplated as follows: *Citrus limone* extract can undergo hydrolytic fragmentation with the help of NaOH to form C-dots under irradiation of ultrasonic waves. Such waves are capable enough to generate alternating rarefactions and compressions in solution, thus causing implosive and violent collapse of small vacuum bubbles [12], leading to the formation of cavitations during compression. Such an implosion consequently leads to enhanced hydrodynamic shear stress in the liquid causing breakdown of the particles. The generated intermediates then undergo carbonization leading to the synthesis of Carbon dots. To enhance the photoluminescence property of such solutions, they were then subjected to sodium borohydride treatment which reduces carbonyl groups as well as introduce more surface defects in the sp^2 structure that leads to enhanced emissive traps, favouring augmentation of fluorescence [13].

To decipher the biological application of such Carbon dots as Fluorescent label, both F1 and F2 were subjected to Hep-2 cells and cytotoxicity assay was performed with a

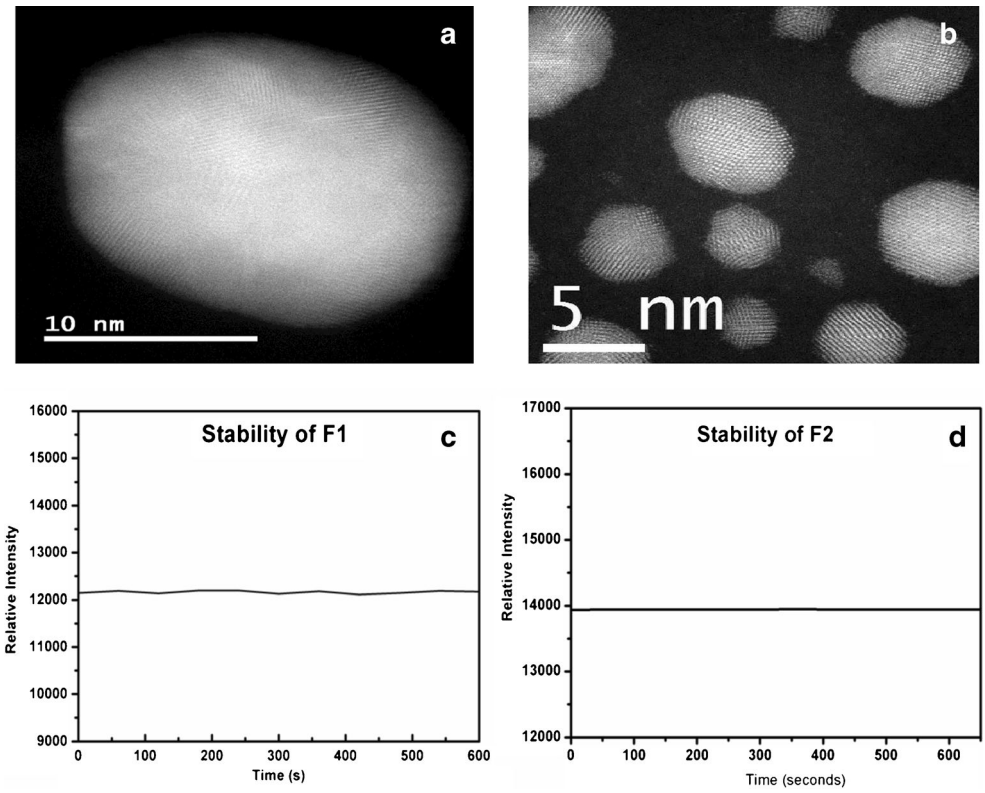

Fig. 3 **a** and **b** shows HAADF-STEM analysis of F1 and F2 Carbon dots exhibiting size of 20 and 5 nm respectively; **c** photostability test of F1 fraction in a fluorescence spectrophotometer using 280 nm excitation **d** photostability test of F2 fraction in a fluorescence spectrophotometer using 350 nm excitation

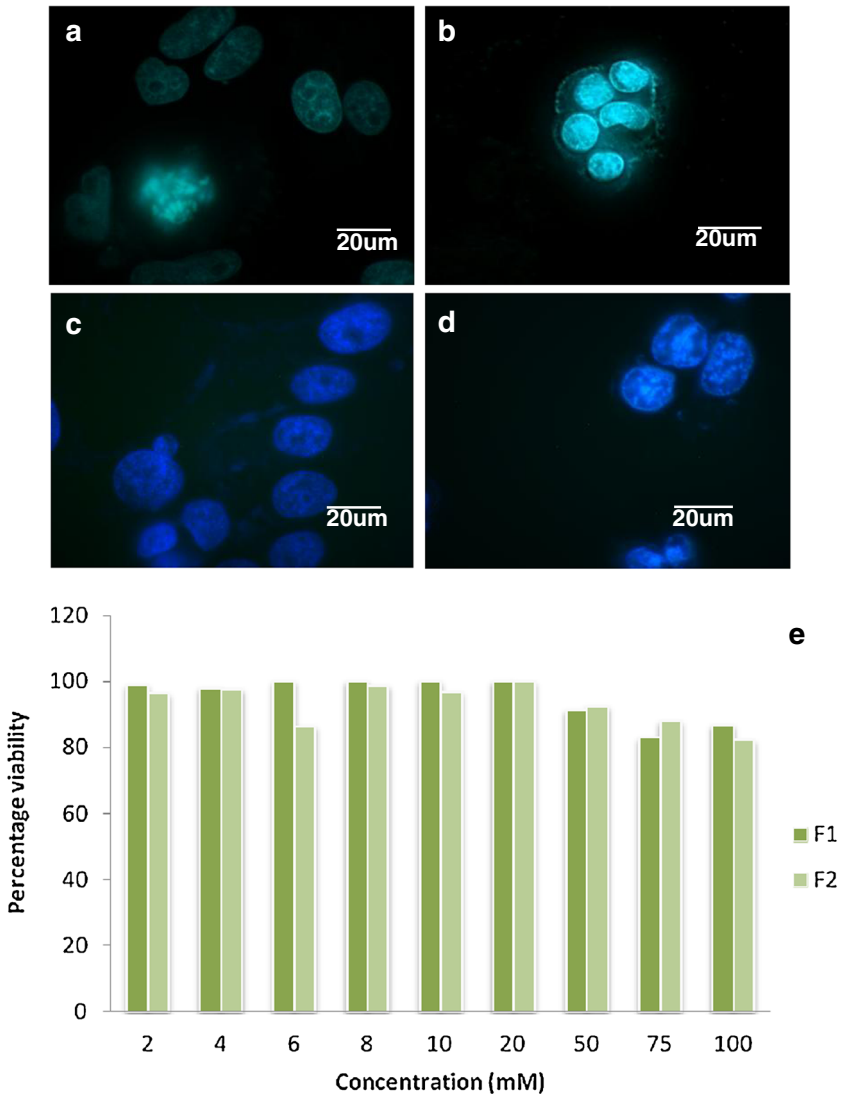

Fig. 4 Confocal microscopy images of Hep-2 cells labelled with 10 mM concentration of C-dots **a** and **b** λex=488 nm, detected with 530 nm long pass filter for F1 fraction **c** and **d** λex= 458 nm, detected with 475 nm long pass filter for F2 fraction; E) shows percentage cell viability of both the fractions of Carbon dots (F1 and F2)

conventional methylthiazolyldiphenyl-tetrazolium bromide-(MTT-) based study that is purely dependent on the colour change of MTT by mitochondrial succinate dehydrogenase (Fig. 4) [14]. This assay evidenced that both the fractions (F1 and F2) are non-toxic to Hep-2 cells at very high concentrations, thus can be used efficiently for cell-labelling studies. Confocal microscopy studies revealed that both F1 and F2 can be used as cell labelling agents especially nucleus since the hydrodynamic size of such particles are 5–20 nm and are small enough for easy uptake by the cells as well as sub-cellular structures. There is no morphological damage to the nuclei which can be corroborated by confocal microscopy studies. This proves that carbon dots can be exceptionally useful as a bio imaging agent for the detection of molecules in cells. Hence, these labelling applications are the indicators of carbon dots being a powerful, non-toxic alternative to semi-conductor nanocrystals. However, to enhance the Quantum yield of such nanostructures, there is a need to use different passivating agents so that they can be used at even lower concentrations for Bioimaging purpose.

Conclusion

To summarize, we developed a green synthetic route for the preparation of photoluminescent C-dots using a natural precursor, *Citrus limone* extract under ultrasonic condition. Such a Sodium borohydride reduced C-dot solution is then subjected to SDGC for the separation of different sized C-dots. To the best of our knowledge, this is the first report where ultrasonic method is used for synthesis of C-dots and then separation is done by density gradient centrifugation method. This method doesn't involve strong acids, very high temperatures

for carbonization step. The precursor used is also natural, green, economical and eco-friendly. Finally the separated C-dots exhibit good water solubility, strong photoluminescence and highly cytocompatible to be exploited as high-performance optical imaging probe without any inherent toxicity.

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