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BACTERIAL BIOIMAGING APPLICATIONS OF CARBON DOTS DERIVED FROM HMTA: CHARACTERIZATION AND EFFICACY IN CELLULAR IMAGING

S.Muthamil Selvan[1], K.Vijai Anand[1,✉], K.Govindaraju[2] and T. Mahalakshmi[3]

[1]Department of Physics, Sathyabama Institute of Science and Technology, Chennai-600119, India

[2]Centre for Ocean Research, Sathyabama Institute of Science and Technology, Chennai-600119, India

[3]Department of Biotechnology, University of Madras, Chennai-600 025, India

✉Corresponding authors: anandkvijai78@gmail.com

ABSTRACT

Fluorescent carbon dots (CDs) with superior optical properties were synthesized from Hexamethylenetetramine (HMTA) using a simple one-step hydrothermal process. The CDs exhibit strong blue fluorescence under UV light and were characterized by powder X-ray diffraction (XRD) and high-resolution transmission electron microscopy (HRTEM). These CDs were evaluated as fluorescent probes for imaging *Escherichia coli* and *Staphylococcus aureus* cells, demonstrating their potential in cell imaging applications.

Keywords: HMTA, Carbon Dots, Cell Imaging, *Escherichia coli, Staphylococcus aureus*, Fluorescent Probes.

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INTRODUCTION

Carbon nanomaterials demonstrate unique functional behaviors and characteristics influenced by their size, attributed to their remarkable surface reactivity and distinctive electrical, magnetic, and optical features. These unique properties paved the way for the progress of new synthetic methods to control nanoparticle's shape and size which are used to tune their absorption and emission properties.[1] Nanoparticle-based imaging is a promising new technology for disease detection. Molecular probes can visualize cellular function and measure molecular processes in living organisms. However, further improvements are needed in specificity, stability, and circulation half-life.[2] Carbon dots (CDs) are a novel category of tiny oxygenated carbon nanoparticles measuring <10 nm, which have photoluminescence capabilities that rely on the excitation wavelength and their size.[3,4] Various methodologies have been employed to synthesize Carbon Quantum Dots (CDs), which are primarily divided into two main pathways: (i) top-down methods, and (ii) bottom-up methods.[5,6] (i) Top-down pathways associated with the transformation of larger carbon materials into CDs. A few of the common methods in this classification include laser ablation, electrochemical exfoliation (ECE), and chemical oxidation methods.[7,8,9] These methods essentially break down larger carbon structures into smaller CDs. (ii) Bottom-up pathways, on the other hand, start with smaller precursor materials and build up CDs from them. Approaches in this category include microwave-assisted synthesis, plasma processing, as well as hydrothermal and solvothermal methods.[10-13] These techniques enable the controlled assembly of CDs from smaller carbon-rich precursors. The ease of preparation and modification of CDs offers several advantages, including the ability to fine-tune their electronic and optical properties. Additionally, CDs can mediate interactions with analytes, leading to either fluorescence quenching or enhancement. This property makes CDs valuable in various applications. In recent years, significant improvements in the synthesis techniques and raw materials have led to remarkable increases in the Photoluminescence Quantum Yield (PL QY) of Carbon Dots (CDs). These advancements have resulted in CDs exhibiting bright PL emissions that are comparable to those of molecular fluorophores.[14] This molecular-like brightness is often linked to the existence of molecular fluorophores on the CD surface or floating freely in the dispersion due to inadequate purification processes.

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These fluorescent carbon materials are extensively used in cellular imaging, chemical sensing, and biosensing, even within living cells and organisms. Their small size, similar to biological macromolecules, allows for functionalization and entry into cells, providing valuable insights into cellular interactions through fluorescent emissions.[15] For biological applications, carbon dots are preferred as they don't cause cellular abnormalities. In the present work, the preparation and structural characterization of CDs using Hexamethylenetetramine (HMTA) explores their potential for bioimaging applications towards *S. aureus* and *E. coli* cells.

EXPERIMENTAL

Materials and Methods

In this study, Hexamethylenetetramine (HMTA) with a purity of ≥ 99.0 % and deionized water were used as key materials. Both were procured from Sigma-Aldrich, India, and as obtained, in preparing the study samples.

Hydrothermal Synthesis of HMTA-Derived CDs

CDs derived from HMTA were synthesized via a hydrothermal method. Initially, 4g of HMTA was dissolved in deionized water of 50 mL. This solution was subsequently placed in a 100 mL Teflon-packed stainless steel (SS) autoclave. The autoclave was carefully sealed and heated at 180°C for 8 h. After the heating process, the resulting solution was cooled to room temperature and centrifuged for 10 minutes at 7000 rpm to separate out the larger particles. The precipitate and supernatant were collected. The complete procedure is illustrated in the scheme in Fig.-1.

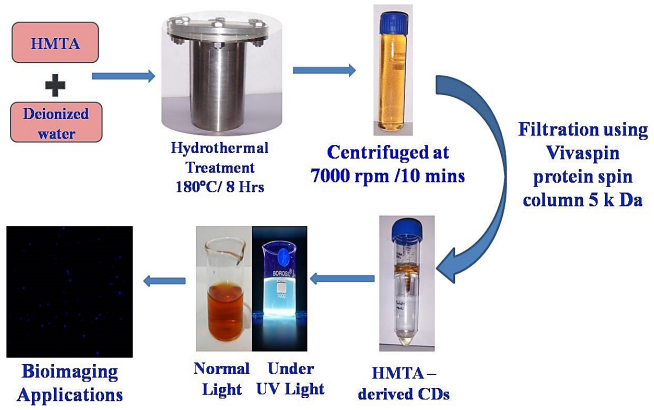

Fig.-1: Schematic Representation for the Preparation of HMTA – Derived Carbon Dots

Instrumentation

To examine the internal structure and shape of the CDs, a TALOS F200S G2 High-Resolution Scanning Transmission Electron Microscope (HR-STEM) was employed at an accelerating voltage of 200 kV. For HR-STEM analysis, the samples were prepared by depositing an aqueous suspension onto a copper grid of 300 mesh covered with a skinny lacey carbon layer. In addition, XRD patterns of the synthesized CDs were analyzed using an ARL EQUINOX 3000 X-ray Diffractometer with a CPS120 detector. The analysis employed using CuKα (λ= 0.15405 nm) and was conducted over an angle range of 10° to 70°.

CDs as Probe for Cell Imaging

HMTA-derived CDs were used to enhance fluorescence visualization, demonstrating their effectiveness in real-time imaging of bacterial cells. This approach highlights the potential of CDs for detailed cellular imaging applications.[16,17] Bacterial strains *Staphylococcus aureus* and *Escherichia coli* were transferred to a primary culture in nutrient broth. The strains were grown at 37°C for 24 h. After incubation, aliquots of the bacterial culture were taken to record the growth till the optical density at 600 nm (OD_{600}) reached a value corresponding to the log phase. The mother culture was diluted with the bacterial suspension and used with HMTA-derived CDs as a probe for cell imaging. The prepared bacterial samples were fixed

with 70% ethanol, resuspended with CDs further incubated for 15 minutes at 30°C. The prepared slides were then examined under a phase-contrast and fluorescence microscope.

RESULTS AND DISCUSSION

XRD Analysis

Figure--2 exhibits the XRD pattern of HMTA-derived CDs. The X-ray Diffraction peak at 22.38°corresponds to the (002) hkl values. The XRD pattern of HMTA-derived carbon dots is characterized by a broad peak, which is indicative of a disordered carbon structure.[18] The disordered structure of HMTA-derived carbon dots exhibits distinctive properties, which include high surface area and their ability to emit light.

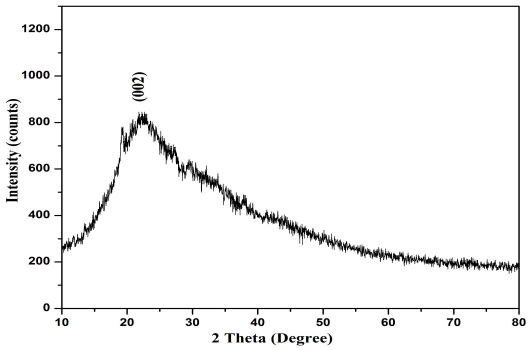

Fig.-2: XRD Pattern of HMTA-Derived CDs

HR-TEM Studies

To explore the average size and morphology of the synthesized HMTA-derived CDs, HR-TEM characterization was performed.[19] Figure-3 represents the typical HR-TEM results of HMTA-derived CDs, From Fig.-3 (a b) the majority of CDs exhibit nearly elongated rod-like shapes.

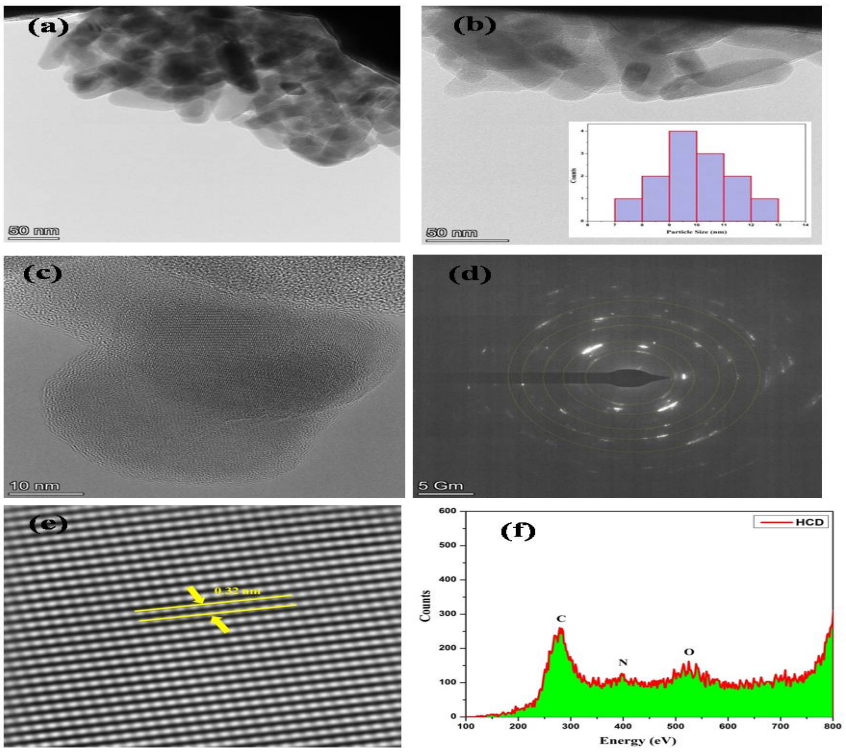

Fig.-3: (a, b) TEM images and Inset Histogram. (c) HR-TEM (d) SAED pattern of CDs. (e) d Spacing and (f) EDAX Patterns of HMTA-derived CDs

The TEM histogram provides further evidence of the distribution of particles concerning size and is shown in Fig.-3b Inset. Figure-3c represents the HR-TEM Fig. of HMTA–derived CDs. The pattern of selected area electron diffraction (SAED) shown in Fig 3d matches with the XRD pattern. Figure-3e exhibits the d spacing (0.32 nm) of HMTA derived – CDs. Figure-3f presents the EDAX spectrum of the HMTA-derived CDs, confirming the presence of C, O, and N with the CDs. The SAED pattern shows well-defined diffraction spots, indicating a high degree of ordering and regularity in the crystal lattice.

Bacterial Cellular Uptake Studies Using CDs

Due to their photo-stability, high water solubility, and low toxicity, CDs represent a compelling option for bio-imaging. We employed fluorescence imaging on bacterial cells (*S. aureus* and *E. coli*) to see if CDs might be applied in biological systems. Figure-4a&b presents bright-field images of *S. aureus* and *E. coli* cells with and without CDs, while Fig.-4 (c, d) displays fluorescence microscopic images of cells treated with HMTA-derived CDs. The scale bar in each image represents 5 µm Fig.-5 provides confocal laser microscopy images of *S. aureus* and *E. coli* after incubation. In Fig.-5 (a), bright-field images without HMTA-derived CDs, as well as images combing bright-field and fluorescence modes are displayed. The wavelength of excitation was used to capture the fluorescence signal. The images were captured in the excitation wavelength at 405 nm (blue). Figure-5 (b) highlights the fluorescence microscopic images of HMTA-derived CDs interacting with *S. aureus* and *E. coli* at the same excitation wavelength, without bright-field images. The scale bar for these images also indicates 5 µm.

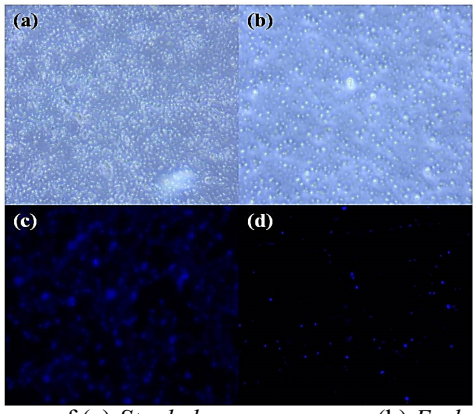

Fig.-4: Phase Contrast Microscopic Image of (a) *Staphylococcus aureus,* (b) *Escherichia coli*, (c & d) Fluorescence Microscopic Image of Carbon Dots Treated *Staphylococcus aureus* and *Escherichia Coli* Cells

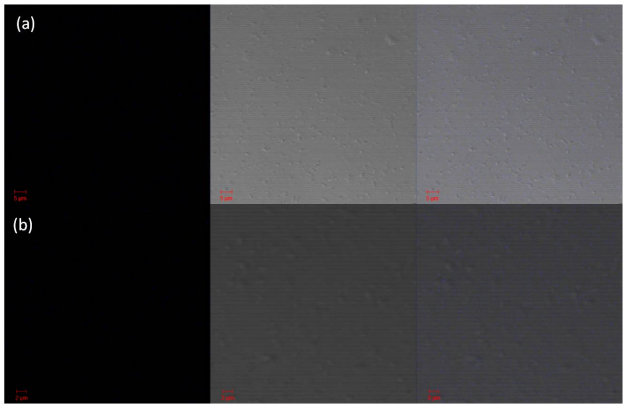

Fig.-5: Confocal Laser Microscopic Images of *Escherichia coli* and *Staphylococcus aureus* after 1–3 h Incubation at 37°C. (a) Bright-Field With and without CDs, and Fluorescence Mode at 405 nm (blue). (b) Confocal Images of CDs with *E. coli* and *S. aureus* at 405 nm (blue), Scale Bar: 5 µm

These images, obtained using HMTA-derived CDs and various imaging modes, help demonstrate the interactions and dynamics of *S. aureus* and *E. coli* during incubation. Our study successfully applied HMTA-derived CDs in real-time imaging of *S. aureus* and *E. coli* cells, suggesting their potential for various scientific and medical applications in cellular visualization.[20,21] Confocal laser microscopy was

employed to image *S. aureus* and *E. coli* cells later incubated for 1 to 3 h at 37°C. Figure-5a shows bright-field images with and without CDs. CDs-treated cell images are shown in Fig.-5b and were analyzed using Laser scanning confocal microscopy. The scale bar is 5 μm. The microscopic images obtained using carbon dots and various imaging modes provide insights into the interactions and dynamics of *S. aureus* and *E. coli* cells during incubation.[22,23] These findings show, that the synthesized CDs were taken up by bacterial cells, and their uniform distribution inside the cells suggests a potential mechanism for endocytosis.[24,25] This research offers valuable insights into the potential applications of carbon dots in cellular imaging, holding promise for a wide range of scientific and medical fields.

CONCLUSION

In conclusion, CDs were successfully synthesized using the non-toxic surfactant HMTA via a simple hydrothermal process. These CDs were physicochemical and characterized by XRD and HR-TEM. The surface-attached amino groups on HMTA-derived CDs notably enhanced their fluorescence, which enables selective detection. CDs with stronger fluorescence were effective for bacterial imaging. For bio-imaging purposes in both *in vitro* and *in vivo* cell biology as well as various sensing applications, the current technology enables upward scalability of the one-pot green chemistry method for the production of CDs with biocompatible and fluorescent, which may be utilized as fluorescent imaging probes.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest regarding the publication of this manuscript.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

S. Muthamil Selvan https://orcid.org/0000-0002-2148-6463
K. Vijai Anand https://orcid.org/0000-0003-0770-5479
K. Govindaraju https://orcid.org/0000-0002-9687-2174
T. Mahalakshmi https://orcid.org/0009-0007-3246-2524

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