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Angiogenic Profiling of Synthesized Carbon Quantum Dots

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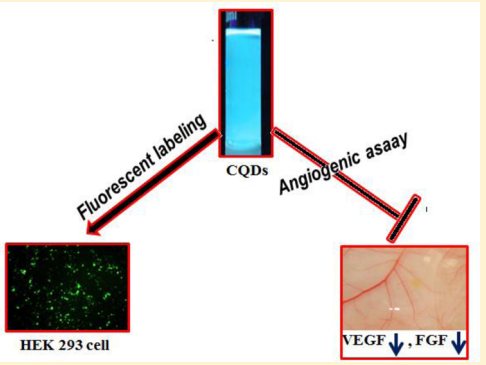
ABSTRACT: A simple method was employed for the synthesis of green luminescent carbon quantum dots (CQDs) from styrene soot. The CQDs were characterized by transmission electron microscopy, X-ray photoelectron spectroscopy, Fourier transform infrared, and Raman spectroscopy. The prepared carbon quantum dots did not show cellular toxicity and could successfully be used for labeling cells. We also evaluated the effects of carbon quantum dots on the process of angiogenesis. Results of a chorioallantoic membrane (CAM) assay revealed the significant decrease in the density of branched vessels after their treatment with CQDs. Further application of CQDs significantly downregulated the expression levels of pro-angiogenic growth factors like VEGF and FGF. Expression of VEGFR2 and levels of hemoglobin were also significantly lower in CAMs treated with CQDs, indicating that the CQDs inhibit angiogenesis. Data presented here also show that CQDs can selectively target cancer cells and therefore hold potential in the field of cancer therapy.



Quantum dots (QDs) <10 nm in size are emerging stars in the field of nanoscience. Among various dots, carbon quantum dots have received a remarkable level of attention because of their lower toxicity, high water solubility, surface modification flexibility, and unique photoluminescence.[1,2] Hence, carbon quantum dots (CQDs) would be a better choice in the field of biomedical, biosensing, catalysis, and solar cell applications.[3−6] Recently, CQDs could be produced from bulk graphite materials, active carbon, pyrolyzed polymers, or carbohydrates through various methods.[7−10] Several approaches, including arc discharge, laser ablation, electrochemical synthesis, and combustion/thermal/hydrothermal/acidic oxidation, have been proposed to produce fluorescent C-dots.[1,11−16] Herein, we present a facile method for synthesizing photoluminescent CQDs by NaOH passivation of styrene soot. The proposed method avoided strong acids and reduced the number of preparation steps. To the best of our knowledge, this is the first report demonstrating the anti-angiogenic property of CQDs. The anti-angiogenic properties of CQDs combined with nontoxicity to noncancerous cells will give new potential to carbon quantum dots.

Angiogenesis is the process of development of new blood capillaries from preexisting ones. It occurs by either of two main processes: (a) endothelial cell migration (sprouting) and (b) splitting of vessels (intussusceptions).[17] Some physiological events, including embryo development, wound healing, and ovulation, and several pathological conditions, including cancer, diabetic retinopathy, etc., rely on the process of angiogenesis.[18] VEGF and FGF are major pro-angiogenic factors, which are upregulated under pro-angiogenic conditions. In the case of tumors, angiogenesis plays an important role in the fact that tumors cannot grow beyond 1−2 mm^3 without inducing angiogenesis and the nutrients and oxygen cannot diffuse such long distances. The tumor responds to a hypoxic environment by secreting pro-angiogenic factors, triggering angiogenesis. The new vessels formed because of angiogenesis facilitate the supply of oxygen and nutrients to the growing tumor, thereby enhancing tumor survival and growth, so it has evolved as a therapeutic target in the case of cancer by inhibiting angiogenesis. In this study, we investigated the cytotoxicity and angiogenic activity of CQDs.

EXPERIMENTAL PROCEDURES

Meterials and Methods. Styrene, Drabkins Reagent, and XTT Reagent were purchased from Sigma-Aldrich (Milwaukee, WI). Dulbecco's modified Eagle's medium (DMEM) was purchased from Himedia. TRizol was purchased from Invitrogen. PMS from SRL, Fast Start Essential Master Mix were purchased from Roche. The High Capacity CDNA Reverse Transcription Kit was purchased from Applied Biosystems.

The size and morphology were studied through high-resolution transmission electron microscopy (HRTEM) on a

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FEI, TECNAI 30G2 S-TWIN microscope with an accelerating voltage of 300 kV. X-ray photoelectron spectroscopy (XPS) experiments were performed on an X-ray photoelectron spectroscope (model ESCALAB220IXL) equipped with an Axis Ultra, Kratos (1486.7 eV) monochromatic source for excitation. XPS spectra over a binding energy (BE) range of 0−1200 eV were obtained using an analyzer pass energy of 117.4 eV. UV−visible spectra were recorded with a Shimadzu computer-controlled double-beam UV−vis spectrophotometer (UV-2401PC, Shimadzu, Kyoto, Japan), The photoluminescence (PL) spectrum of the C-dot was recorded on a Spex-Fluorolog FL22 spectrofluorimeter equipped with a double-grating 0.22 m Spex 1680 monochromator and a 450 W Xe lamp as the excitation source. The IR spectra were recorded as KBr pellets on a PerkinElmer Spectrum One FT-IR spectrometer operating between 4500 and 400 cm^{-1}. Raman spectra were recorded with a confocal Raman microscope (WITec alpha 300R, WITec GmbH, Ulm, Germany). A laser beam with a wavelength of 633 nm from air-cooled argon was used for excitation. An inverted fluorescent microscope (Leica) was used for cell labeling studies. Real time polymerase chain reaction (PCR) was conducted on a Light Cycler 480 (Roche) machine.

Preparation of Carbon Quantum Dots. Carbon soot was collected by burning styrene in a limited supply of air. Thus, collected soot was stirred at 900 rpm for 25 min along with NaOH at pH 7.4. The solution was then sonicated and filtered through Whatman filter paper.

XTT Cell Viability Assay. HEK 293 and A549 cells were seeded in a 96-well plate at a cell density of 4000 cells/well. The plates were incubated overnight at 37 °C in a humidified incubator with 5% CO_2 for the cells to attach. CQDs at different concentrations were applied to the cells in triplicate wells and incubated for 24 h followed by an XTT assay. Briefly, the XTT assay was conducted by adding 25 μL of a working solution of XTT prepared in DMEM with 5 mM PMS to each well. After incubation for 4 h, the absorbance was read at 630 and 450 nm. The percentage viability was calculated by considering the viability of the untreated control as 100%.[19]

Cell Labeling Studies of Carbon Quantum Dots. Cell labeling studies were executed by fluorescence imaging of adherent cells, based on the intrinsic fluorescent property of CQDs. HEK 293 cells were seeded at a density of 10^5 cells/well in 96-well plates. After attachment, the cells were incubated with CQDs in DMEM for 1 h. Subsequently, the cells were washed thrice with PBS and subjected to imaging under a fluorescence microscope.

Chick Chorioallantoic Membrane (CAM) Assay for Angiogenesis. The CAM assay was performed on fertilized chick eggs, which were incubated for 4 days at 37 °C and at a relative humidity of 80%. During this period, the eggs were positioned with pointed end down and were spun several times. After being incubated, the eggs were opened on the air sac side, the shell was carefully removed with forceps, and the samples soaked in filter discs were applied to the CAM. The cavity was covered with parafilm, and the eggs were incubated at 37 °C at a relative humidity of 80% for 8 days. At the end of the incubation period, the CAMs were photographed and the level of hemoglobin in the CAM was estimated using Drabkin's reagent as a measure of vessel density. For this, the CAMs were homogenized in Drabkin's reagent and centrifuged and the absorbance of the clear supernatant was recorded at 546 nm. CAMs treated with filter discs soaked in PBS served as the vehicle control.[20,21] The levels of hemoglobin in the CAM were normalized to the total protein content of CAMs.

Quantitative Real-Time PCR. Total RNA was extracted from chick (*Gallus gallus*) chorioallantoic membrane with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of RNA using a high-capacity CDNA reverse transcription kit (Applied Bio systems), and quantitative real-time PCR was performed in a Light Cycler 480 machine. Actin served as the internal control. Nucleotide sequences of primer pairs used to determine the levels of chick VEGF, VEGFR2, and FGF mRNA were as follows: actin, forward primer (5′-GCT CTG ACT GAC CGC GTT A-3′) and reverse primer (5′-ACG AGC GCA GCA ATA TCA T-3′); VEGF, forward primer (5′-GGA GTT GTC GAA GGC TGC T-3′) and reverse primer (5′-TTG ATA ACT TCG TTG GGC TTC-3′); FGF, forward primer (5′-TTC TTC CTG CGC ATC AAC-3′) and reverse primer (5′-CGA TAG CTC GTCCAG-3′); VEGFR2, forward primer (5′-GGG GAAGAT GTA CTC GGT GA-3′) and reverse primer (5′-CAT CC A TGT TCA AAC ATC ACA A-3′).

RESULTS

Characterization of Prepared Carbon Quantum Dots. Dynamic light scattering (DLS) was conducted to analyze the aggregation tendency of prepared CQDs in solution. The DLS data (Figure 1a) revealed that CQDs possessed a particle size of

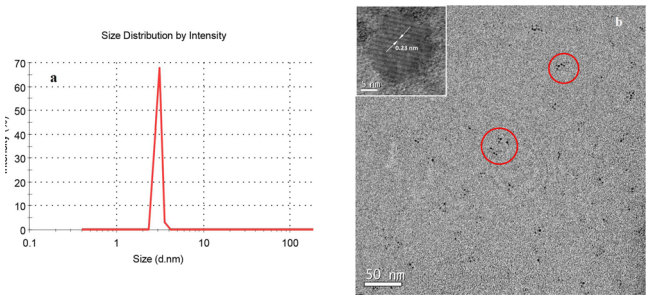

Figure 1. (a) DLS. (b) TEM and (inset) HRTEM image of CQDs.

3 nm, and it was also observed that CQDs do not exist in a large aggregated form in aqueous solution. The particle size of CQDs was further confirmed from TEM analysis (Figure 1b). The UV−visible spectrum showed two characteristic peaks: one at 290 nm due to the n−π^* transition of the C═O bond and another at 210 nm due to the $\pi-\pi^*$ transition of the C═C bond (Figure 2a). From the PL spectrum (Figure 2b), an

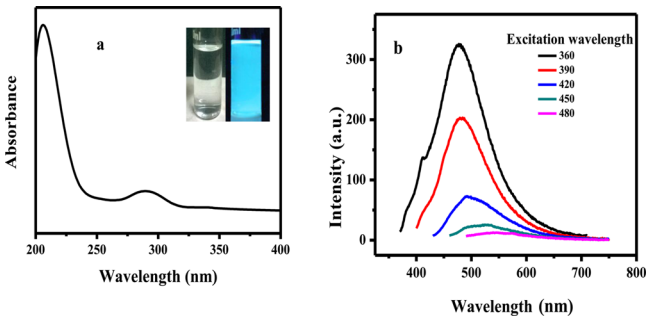

Figure 2. (a) UV−vis spectra. The inset shows the appearance of CQDs in the presence and absence of UV light. (b) PL spectra of the CQDs at different wavelengths.

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emission peak at 480 nm was observed under 360 nm excitation. Moreover, no bathochromic shift was observed under different excitation conditions (from 360 to 490 nm). FTIR analysis was conducted to identify the functional groups present on CQDs. A feeble absorption at 3452 cm^{-1} was due to the O−H stretching vibration of the hydroxyl group. Two bands at 2920 and 2880 cm^{-1} were caused by the stretching vibration of C−H in the CH_2 group. A peak corresponding to the C═O group at 1687 cm^{-1} and another peak corresponding to the aromatic C═C group (ring stretching) at 1628 cm^{-1} were depicted clearly by Figure 3a. A peak at 1032 cm^{-1} was

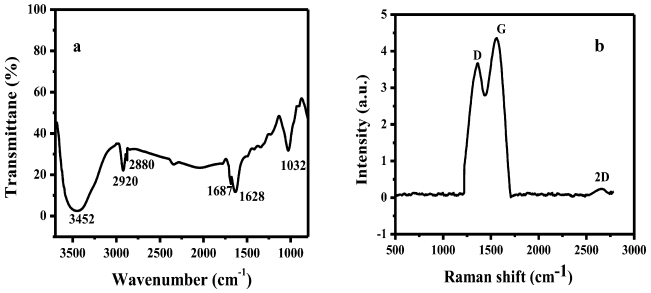

Figure 3. (a) FTIR and (b) Raman spectra of the CQDs.

also observed due to CO stretching of the alcoholic OH group. From the Raman spectrum (Figure 3b), two major peaks at 1578 and 1331 cm^{-1} corresponding to the G-band and D-band were observed. This confirmed the formation of the sp^2 nature of carbon quantum dots. Along with these two peaks, an additional peak at 2654 cm^{-1} due to a two-dimensional band was also observed. XPS measurement (Figure 4a) showed that

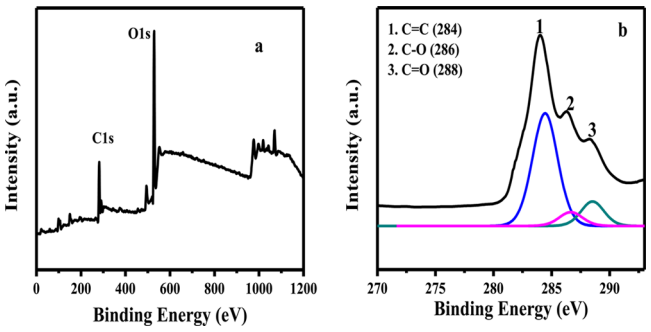

Figure 4. (a) XPS wide spectrum of CQDs. (b) C 1s spectrum of CQDs.

the CQDs contained mainly carbon (55 wt %) and oxygen (44 wt %). The C 1s spectrum (Figure 4b) revealed different types of carbon atoms at 284, 286, and 288 eV corresponding to C═C, C−O, and C═O groups, respectively.

Cell Viability Assay. CQDs were tested for their cytotoxic effects in cancer and non-cancer cell lines. Results from the XTT assay showed that carbon quantum dots at concentrations of 250, 125, 65, 31.25, and 15.62 μg/mL did not reduce the viability of HEK 293 cells (non-cancer cell line) beyond 20% (Figure 5a). However, in the case of the lung cancer cell line, a concentration of 15.625 μg/mL was sufficient for reducing cell viability beyond 50% (Figure 5b). Further, the viability of cancerous cells decreased beyond 80% with 250 μg/mL CQDs (16 times higher than that required for noncancerous cells).

Cell Labeling Studies of Carbon Dots by Fluorescence Imaging. Panels a and b of Figure 6 showed the transmitted

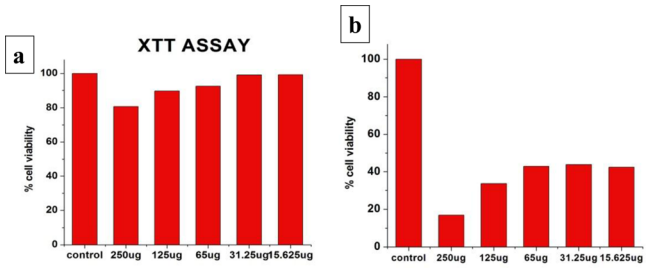

Figure 5. Percentage cell viability assay performed with the XTT reagent. Carbon quantum dots with serially decreasing concentrations (250, 125, 65, 31.25, and 15.62 μg/mL) were tested on (a) normal cells (HEK 293) and (b) a lung cancer cell line (A549).

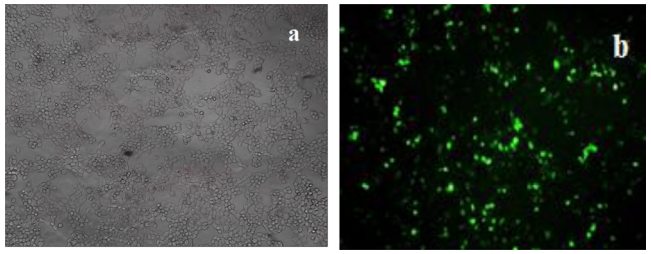

Figure 6. (a) Transmitted light image and (b) fluorescent image of HEK 293 cells after incubation with green CQDs for 1 h.

light image and fluorescent image of HEK 293 cells, respectively, after incubation with green CQDs for 1 h. HEK 293 cells were seeded in 96-well plates to study the cell labelling activity of fluorescent CQDs. After being incubated with dots for 1 h, cells were washed with PBS and the fluorescence inside the cells was visualized under a fluorescence microscope. Cell viability was found to be unaffected after incubation of cells with carbon quantum dots.

Anti-angiogenic Effects of Carbon Quantum Dots. A fertilized chick embryo was selected as the in vivo model system to study the angiogenic effect of carbon quantum dots; 100 μg of a carbon quantum dot solution was applied to the CAM of a 4-day-old chick embryo. The vascular densities in the CAMs were photographed on day 12 followed by estimation of hemoglobin as a measure of vascular density and angiogenesis. The results are presented in Figure 7c. Photomicrographic analysis revealed that, compared to the control (PBS) (Figure 7a), CAMs treated with carbon quantum dots exhibited a significantly lower blood vessel density (Figure 7b). Hemoglobin levels also suggested the anti-angiogenic nature of the carbon quantum dots.

Relative Expression Level of Angiogenic Markers. Further, to confirm the anti-angiogenic potential of CQDs, the expression levels of major angiogenic cytokines [VEGF (Figure 8a) and FGF (Figure 8b)] and markers [VEGFR2 (Figure 8c)] were studied. The results suggested that the expression levels of VEGF, FGF, and VEGFR2 were significantly lower in CAMs treated with CQDs than in the untreated controls.

CONCLUSIONS

Green luminescent carbon quantum dots with an average size of 3 nm were prepared, and their anti-angiogenic property was tested. In vitro cell culture showed that these CQDs could be useful in cell imaging by virtue of their fluorescent properties. The cell proliferation assay of the lung cancer cell line confirmed that CQDs have anticancer activity with almost no

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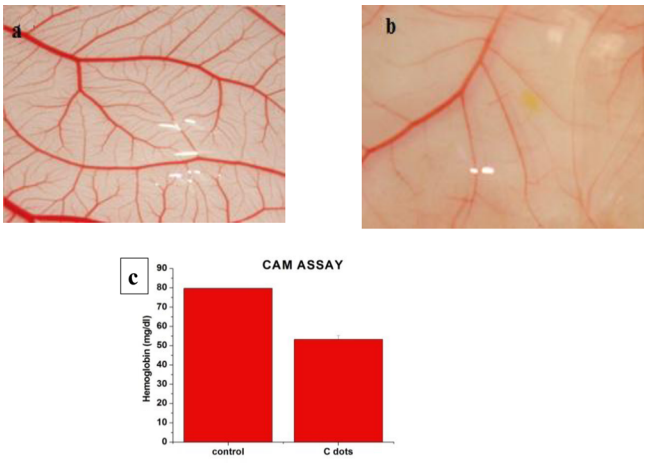

Figure 7. Angiogenic activity of carbon quantum dots. Photographs of chick embryo CAMs treated with (a) PBS and (b) CQDs. (c) Graphical representation of hemoglobin levels in control and treated CAMs.

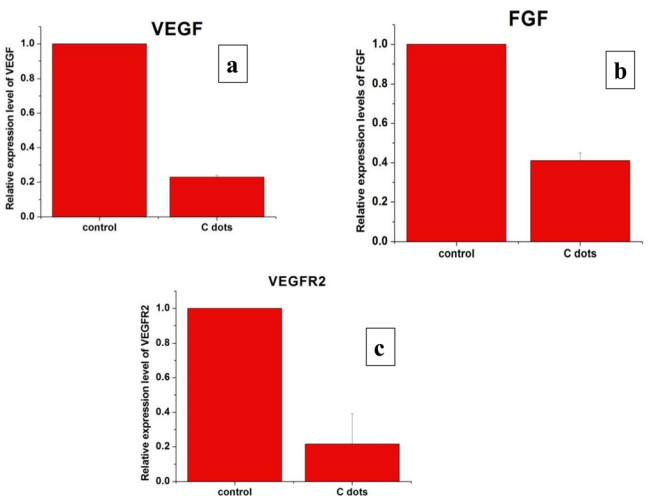

Figure 8. Expression level of angiogenic markers. Total RNA was isolated, and the expression levels of (a) VEGF, (b) FGF, and (c) VEGFR2 were analyzed by real-time PCR. Relative expression levels of the genes mentioned above were downregulated.

effect on normal cells. Angiogenesis is the formation of blood vessels from preexisting vasculature. In the case of tumors, the new vessels formed by angiogenesis supply nutrients and oxygen to the growing tumor. Therefore, inhibition of angiogenesis is a critical therapeutic target for restricting growth and metabolism of tumors. In this study, CQDs were shown to inhibit blood vessel formation by targeting VEGF, FGF, and VEGFR2 of the major angiogenic signalling pathway.

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Notes

The authors declare no competing financial interest.

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