Electronic Supplementary Information

Two-photon active nucleus-targeting carbon dots: Enhanced ROS generation & photodynamic therapy for oral cancer

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1. Materials & Methodology

1.1 Materials
Curcumin and folic acid were purchased from Sigma-Aldrich (Sydney, Australia). Dialysis membranes were purchased from Spectra/Pro Biotech (Australia), fetal bovine serum (FBS) and dimethyl sulfoxide (DMSO) were purchased from Roswell Park Memorial Institute (RPMI-1640). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Thermo Fisher Scientific Inc., oral cancer cells (H413) were provided by the Institute of Dental Research, Westmead Hospital, Australia.

1.2 Synthesis of CDs
The CDs were synthesized using a bottom-up hydrothermal method. For this purpose, 500 mg of curcumin and 500 mg of folic acid powder were used as the source of carbon. The mixture was dissolved in a beaker containing 25 mL of water and sonicated for 5 min. This solution was transferred into a 100 mL Teflon-lined stainless-steel autoclave and heated at 2400 °C for 4 h. The resulting yellowish suspensions were separated by centrifugation at 10000 rpm for 30 min. The supernatant was further processed by ultrafiltration via dialysis (MWCO 30 kDa) for 30 min at 5000 rpm. The stock solution with a quantitative yield of 72% was lyophilized and stored by the name CDcf for characterization. For comparison, 1 gm curcumin was also used to prepare carbon dot denoted as CDc following the same protocol stated above.

1.3 Characterization
1.3.1 Instruments
To examine the size and size distribution of carbon nanomaterials, a transmission electron microscope (JEOL 2100, USA) operating at 200 kV was used. The synthesized carbon nanomaterials were deposited on Cu grids, coated with a carbon film (200 mesh) and dried in
a vacuum desiccator for two days. The Nicolet 6700 FTIR spectrometer (ThermoFisher, USA) was used to obtain the percent transmission and surface state data for the samples. The Cary60 UV–Vis Spectrophotometer (Agilent Technologies, USA) was used to record UV–visible absorption spectra. Fluorescence spectra with variable excitation wavelengths were obtained by Fluoromax-4 spectrophotometer (Horiba scientific, USA). To investigate the chemical composition of the CDs, X-ray photoelectron spectroscopy (XPS) measurements were performed (Thermo Scientific ESCALAB 250Xi USA) using a monochromatic Al-K X-ray source (h = 1486.68 eV). The confocal microscopic images were obtained using a confocal multiphoton laser scanning microscope (Leica TCS SP 5). Flow cytometry (Guava analyser) was used to measure the intensity of cells.

1.3.2 Cell Culture, staining & fluorescence imaging
Human oral cancer line H413 cells were incubated at 37°C in DMEM containing 10 vol% FBS, 2 mM glutamine, 20 mM HEPES for three days. The cells were seeded on an eight-chamber glass slide at a density of 10^5 per well and incubated for a day. Then, 50 μg of both CDs per ml of DMEM was added to the wells. After 2 hours incubation, the cells were washed with PBS twice and fixed with 4% paraformaldehyde solution for 20 minutes on imaging slides. The multispectral one photon confocal imaging was captured using 405, 480, 555 and 615 nm wavelength excitation laser. The multiphoton image was also taken by the excitation of 780 nm two photon laser.

1.3.3 Detection of reactive oxygen species
The fluorescence intensity of 2’,7’-dichlorofluorescein (DCFH), which is released from non-fluorescent DCFH-DA was employed as a probe for ROS measurement. Firstly, CDc and CDcf dissolved in ethanol (50 μg/ml) were blended with the stock solution of DCFH-DA (10 μM). Then, the solution was placed under 780 nm multiphoton laser with 35 mW for 0 to 10 mins. Immediately after the irradiation, the solutions were centrifuged at 12,000 rpm and the fluorescence of the supernatants were measured for the estimation of the produced ROS.
EPR characterization was conducted (Bruker EMXnano EPR Spectrometer) using 2,2,6,6-Tetramethylpiperidine (TEMP) and 5,5-Dimethyl-1-pyrrolineN-oxide (DMPO) (100 mM), as the spin traps for ^1O_2 and · O_2• / ·OH, respectively. The EPR spectra were collected on 5 min irradiated (780 nm, 2p laser) samples of CD.
1.3.4 Determination of ROS generation in vitro

H413 cells were first seeded in 6-well plates in DMEM for 24 h before further manipulation. Cells were then incubated with 200 μg/ml each of CDc and CDCF for 2 h. The treated cells were washed with PBS twice and incubated with 10 μM of the fluorescent probe DCFH-DA for 30 min. After the unloaded probe has been removed using PBS, the indicative cells were irradiated with 780 nm multiphoton laser from 0 to 10 min. Finally, the fluorescence intensity of the cells was monitored by flow cytometry (excitation at 488 nm, emission at 530 nm).

1.3.5 Cell Cytotoxicity

To evaluate the ROS mediated cytotoxicity of H413 cell line, the two-photon irradiated CDc and CDCF samples were tested using the standard MTT assay. The samples were seeded, incubated with carbon dots and irradiated as per the protocol described in section 3.3.4. The media for the control and irradiated samples were removed, and the cells were washed with PBS twice. Subsequently, 10 μL of MTT solution (5 mg/mL) was added to each well to a final volume of 100 μL. The plates were then placed in the CO₂ incubator for an additional 12 h. The medium was removed and 100 μL DMSO was added into each well. The plate was then gently shaken for 2 min at room temperature under dark condition to dissolve the formed precipitate. Absorbance values were determined with Bio-Rad model-680 microplate reader at 490 nm (corrected for background absorbance at 630 nm). The cell viability was estimated according to the following equation: Cell Viability (%) = (OD_{Treated}/OD_{Control}) × 100%, where OD_{Control} was obtained in the absence of carbon dots, and OD_{Treated} was obtained in the presence of carbon dots.

1.3.6 DNA damage analysis

DNA damage was evaluated by determining the breaks in DNA strands through single cell gel electrophoresis (SCGE) or “comet” assay. Firstly, H413 cells (1 × 10⁵) were seeded into 24-well culture plates and mixed with 200 μg /ml each of CDc and CDCF for 2 h followed by 780nm two photon irradiation for 10 min. About 200 of H413 cells for each tested group were collected and suspended in 400 mL PBS. Then, 70 μL low-melting agarose was mixed with 30 μL of cell suspension followed by spreading upon microscopic slides coated with normal melting agarose. The slides were next soaked in lysis buffer (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, 1% Triton X-100, 10% dimethyl sulfoxide at pH 10) and kept in icebox for 30 min. Electrophoresis of the slides was conducted for 20 min at 300 mA, 25 V. After washing the slides twice in PBS, Syber green (20 μg mL⁻¹) was used to stain and visualize under the
fluorescent microscope. The percentage of damaged DNA was quantified using the Comet Score software. The analytical parameter tail length (in μm) was chosen as the unit of DNA damage, corresponding to the head diameter of the comet subtracted from the total length. Thus, the occurrence of DNA damage was identified by the formation of stained comets, unlike intact DNA that presented circular morphology [1].

1- TEM of CDc

![Fig. S1](image_url): Morphological characteristics of carbon quantum dots: (a) TEM image of CDc; (b) Particle size distribution histogram of CDc; (c) HRTEM image of CDc.
2- FTIR spectra of CDc

Fig. S2 FTIR spectra of curcumin derived carbon dots (CDc).

3- XPS full scan spectrum of CDc

Fig. S3 XPS full scan spectrum for curcumin derived carbon dots (CDc).
4-PL spectra of CDc

![4-PL spectra of CDc graph]

**Fig. S4** PL spectra of CDc as a function of excitation wavelength.

5-Multiphoton PL spectra of CDc

![Multiphoton PL spectra of CDc graph]

**Fig. S5** Multiphoton PL spectra of CDc as a function of excitation wavelength.
**Fig. S6** Mechanism of CDcf migration to the nucleus (FR: folate receptor; GPI: glycosyl-phosphatidyl-inositol).

**Fig. S7** Deconvolution of Z-stack images of whole cells with nucleus at variable depths of 0 to 3.5 μm. (scale=15 μm).
**Fig. S8** Confocal images of oral cancer cells at 2 µm depth for staining with CDcf (a) and with CDc (b); corresponding fluorescence intensity profiles of CDs as a function of Z-stack depth (c).

**Fig. S9** ESR signatures for photo irradiated (780 nm 2p laser, 5 min) ROS of CDcf, singlet oxygen $^1\text{O}_2$ (red), $\text{O}_2^-$ (green) and $\cdot\text{OH}$ (black).
Reference