Electronic Supplementary Information (ESI)

A mitochondria-targeted fluorescent probe based on TPP-conjugated carbon dots for both one- and two-photon fluorescence cell imaging

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Experimental Section

Materials: Citric acid (>99.5%) was purchased from Damao Chemical Reagent Co. Ltd. (Tianjin, China). Urea (Electrophoresis Purity) was purchased from Bio-Rad Laboratories, Inc. (Hercules, California, U.S.) Triphenylphosphonium (TPP) were synthesised by our lab, according to the revised method.[1] 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) and N-hydroxysulfosuccinimide (NHS) were purchased from Medpep Company. Mitotracker Deep Red and Lysotracker Red were both purchased from Invitrogen (Carlsbad, CA, USA). Acetic acid (>99.5%) was purchased from Guanghua Chemical Reagent Co. Ltd. (Guangdong, China). Sephadex gel G-25 was obtained from GE Healthcare (Fairfield, San Diego, USA). Quinine sulfate was purchased from Aladdin. Ninhydrin was obtained from Ziyi Reagent Company (Shanghai, China). Sodium chloride (>99.5%) and sodium hydroxide (>96%) were purchased from Dalu Chemical Reagent Co. Ltd (Tianjin, China). Phosphate (H_3PO_4,>85%) was purchased from Hengxing Chemical Reagent Co. Ltd. (Tianjin, China). Boric acid (H_3BO_3, >99.5%) was purchased from Bodi Chemical Reagent Co. Ltd. (Tianjin, China).Water used throughout all experiments was purified with the Millipore system. All reagents were used as received.

Instruments and characterization

Transmission electron microscopy (TEM) was performed using a FEI Tecnai G^2 Spirit at an acceleration voltage of 120 kV. Fourier-transform infrared (FTIR)

spectra were recorded on a Burker Vector 22 spectrometer. X-ray diffraction (XRD) measurements was carried out using a PANalytical X'Pert PRO diffractometer (Almelo, Netherlands) in conjunction with Cu Kα radiation (λ = 0.15418 nm). All following measurements were performed in millipore water. Nano ZS90 Zetasizer (Malvern Instruments, Malvern, U.K.) was used to determine the Zeta potential and size distribution. Absorption and fluorescence spectra were recorded at room temperature with a UV-2550 UV-vis spectrophotometer (Shimadzu, Japan) and Luminescence Spectrometer 55 (Perkin-Elmer) respectively. Fluorescence quantum yields were determined by using quinine sulfate as a reference in 0.10 M H_2SO_4 solution (Φ = 0.54).

Synthesis of CDs and TPP-CDs

Carbon dots (CDs) were prepared by a hydrothermal oxidation method by using citric acid and urea as the carbon sources. Three types of CDs were prepared by adjusting the molar ratios of citric acid to urea, as shown in Table S1. Typically, urea (2.162 g, 36 mmol) was added to citric acid (1.152 g, 6 mmol) aqueous solution (in 20 mL of water). The mixture was heated hydrothermally in a stainless steel autoclave at 200 °C for 10 h. The obtaining brown solution was cooled down to room temperature, then filtered through a 0.22 μm membrane to remove large or agglomerated particles. Sephadex G-25 gel chromatographic column was used for the purification and the fluorescent fractions were collected, lyophilized and stored at 4 ^{o}C for further use.

Triphosphonium-conjugated CDs (TPP-CDs) were prepared via the

amidation of TPP-COOH to CDs-NH_2 using a modified EDC–NHS strategy. TPP-COOH (54 mg, 0.118 mmol) and 1.2-fold EDC·HCl and NHS were added in 1.5 mL DMF. The mixture was stirred for 60 minutes at room temperature, followed by adding 350 mg of CDs (Molar ratio of citric acid to urea = 1:6) in 1.5 ml of deionized water and the reactants were kept stirring for 48 hours at room temperature. After the water was removed by the lyophilizer, the raw products of TPP-CDs were further purified with SephadexG-25 gel column and the fluorescent fractions were lyophilized and kept in dark for further use.

Confocal fluorescence imaging

Hela human cervical cancer cells and TCA-8113 tongue squamous carcinoma cells were cultured in 1640 medium with 10% FBS. Two days before staining, cells were passaged and plated on Φ20mm cell culture dishes. The TPP-CDs and CDs were added to fresh culture medium and then filtered with 0.2 μm Acrodisc syringe filter before being added to cell dishes. The final concentrations were 2.5 mg/ml for both TPP-CDs and CDs. Cells without exposure to carbon dots were used as control. Half an hour prior to imaging, 100 nM Mitotracker Deep Red (Ex=644 nm, Em=665 nm) and 250nM Lysotracker Red (Ex=577 nm, Em=590 nm) were added to the cells. Before submitting for confocal fluorescence imaging, the cells were washed three times with PBS and kept in PBS for the observation.

Confocal fluorescence imaging studies were performed with an Olympus (Japan) laser based point scanning FV 1000 confocal microscope. Oil inserted 60x and 100x objective lens were used in xy and xyz mode with 800×800 pixel

resolution. 405 and 488 nm lasers were used for excitation of CDs and TPP-CDs with emitted light collected between 425-475 and 500-600 nm, respectively. MitoTracker Deep Red was excited with a 633 nm laser and emission was collected between 666-698 nm. LysoTracker Red was excited with a 543 nm laser and emission was collected between 580-644 nm. CDs and TPP-CDs were also excited by a MaiTai two-photon laser at 760 nm pulses (mode-locked Ti:sapphire laser, Tsunami Spectra Physics) and emission was collected between 495-540 nm. The images were analyzed with the Fluoview software.

To evaluate the photostability, 20% power of 488nm laser was used for excitation of the cells labelled with CDs and TPP-CDs for continuous 110s. Fluorescein was used as a negative control. 100 images were taken for each sample and their fluorescent intensities were analyzed with the Fluoview software.

For quantitative analysis of cellular uptake of TPP-CDs nanoparticles, different concentrations of TPP-CDs aqueous solutions from 2 to 0.125 mg/mL were prepared and taken fluorescent photos under excitation of 488 nm laser. The average fluorescent intensity was calculated with the Fluoview software. .The outlines of cells were drawn with the Fluoview software and the average fluorescent intensity in the area of each cell was calculated.

Cytotoxicity study

Hela cervical cancer cells were also used to assess the cytotoxicity of the TPP-CDs probes. The cell viability was measured using an MTT assay. Cells were cultured in 96-well plates (approximately 1×10^4 cells per well) with the medium

containing different concentrations of the TPP-CDs probes for 8 hours. Cells cultured in the free medium were taken as control. The number of viable cells was determined by MTT assay with 3-(4, 5-dimethylthiazole-2-yl)-2, 5-phenyltetrazolium bromide. 20ul of MTT solution (5 mg/ml MTT in phosphate buffer solution, pH 7.4) was added to each well and incubated for 4h at 37 ^{o}C. After removing the medium, 150 µl of DMSO was added to each well to dissolve the crystals absolutely. The absorbance of the cell lysate at 570 nm was measured through enzyme photometer (Bio-Rad). Cell viability was expressed as a percentage of the control. All the results are derived from the mean standard deviation (SD) of 4 samples.

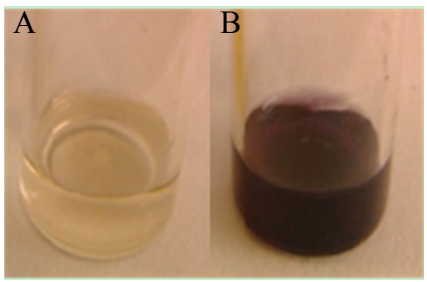

Fig. S1. Before (A) and after (B) ninhydrin reaction with –NH_2 groups on the surface of CDs. After the reaction with ninhydrin, the colour of CDs' aqueous solution changed to dark blue.

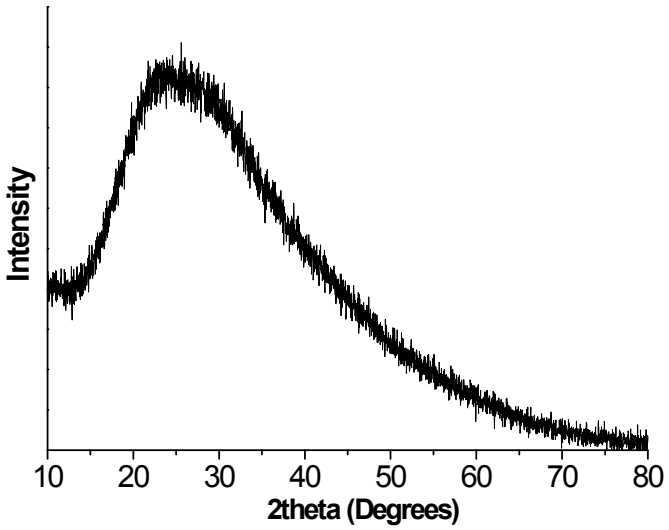

Fig. S2. XRD spectrum of CDs with one broad peak centered at 2θ=25°.

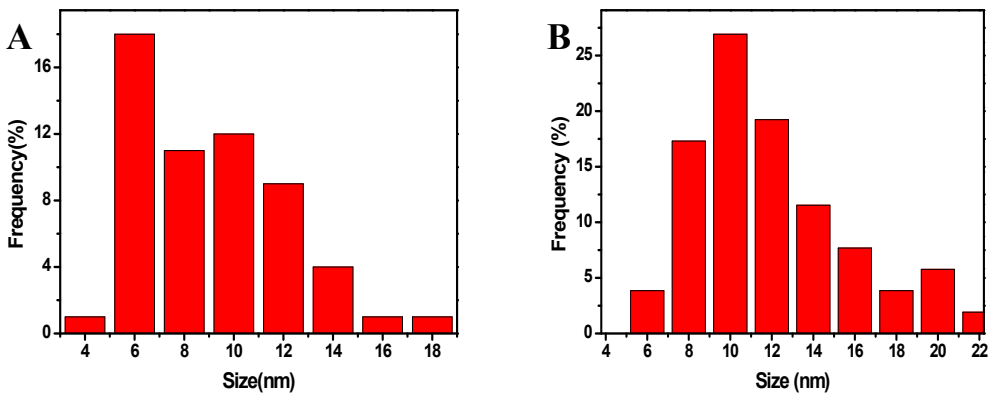

Fig. S3. Size distribution of CDs (A) and TPP-CDs (B)

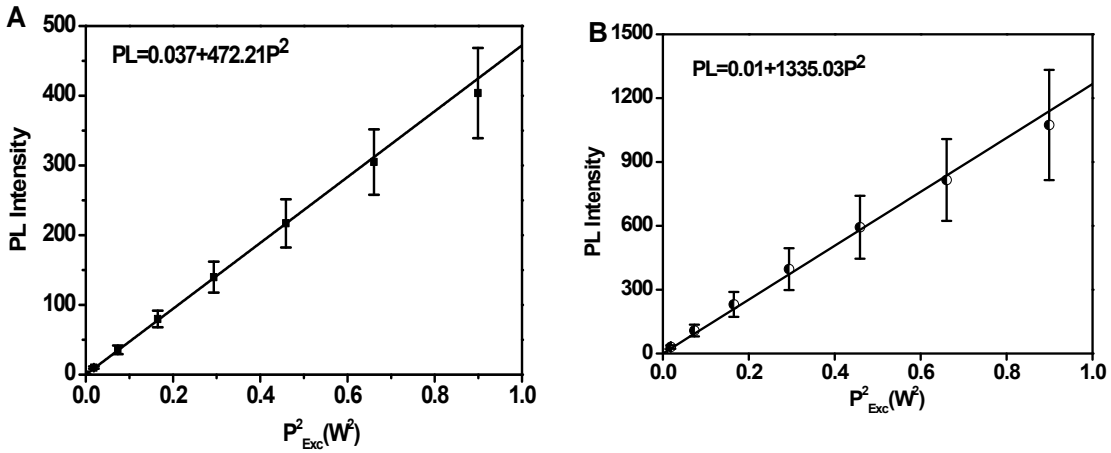

Fig. S4. Quadratic relationship of the photoluminescence (PL) intensity of the CDs (A) and TPP-CDs (B) powder with the different excitation laser powers at 760 nm (P_{Ex}, as measured at the focal plane).

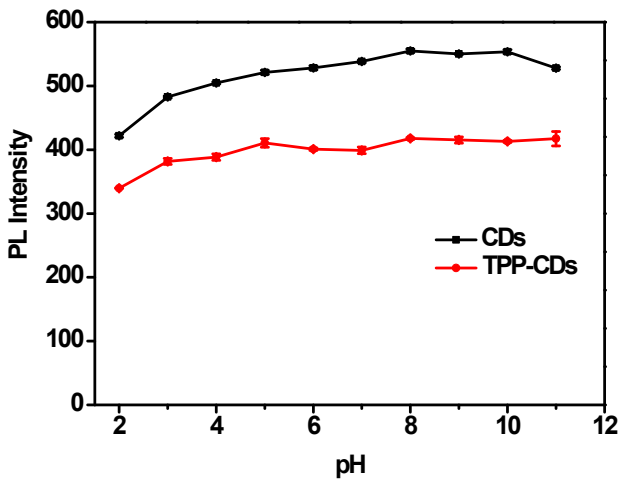

Fig. S5 Effect of pH values on photoluminescence (PL) intensity (λ_{ex}= 340 nm, λ_{em}=420 nm) of CDs and TPP-CDs.

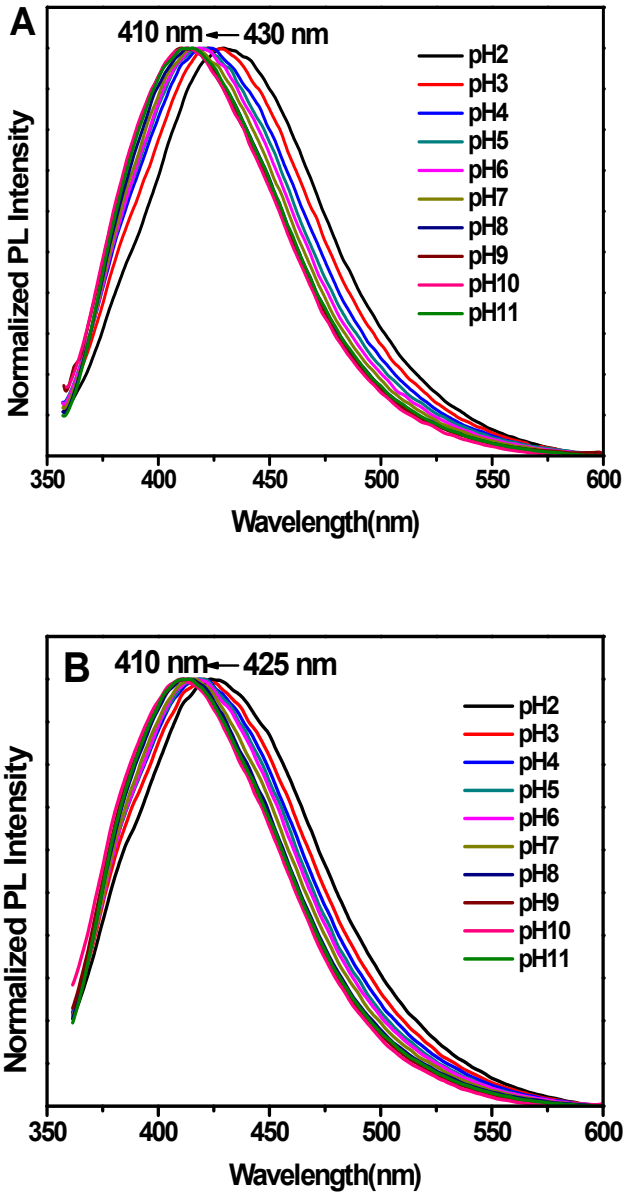

Fig.S6. Effect of pH values on photoluminescence (PL) (λ_{ex}=340 nm) emission wavelengths of CDs (A) and TPP-CDs (B).

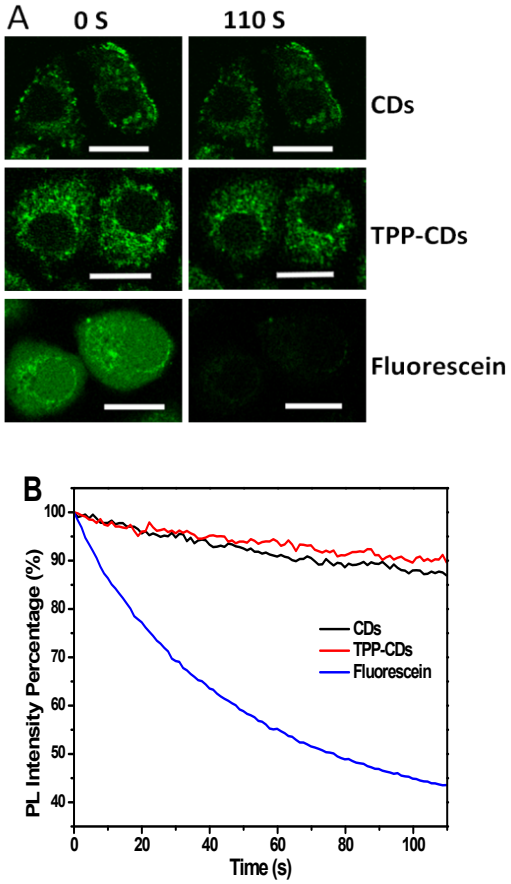

Fig. S7 Photostability study of CDs and TPP-CDs under continuous excitation of 488 nm laser (20% power) for 110s by using fluorescein as control.

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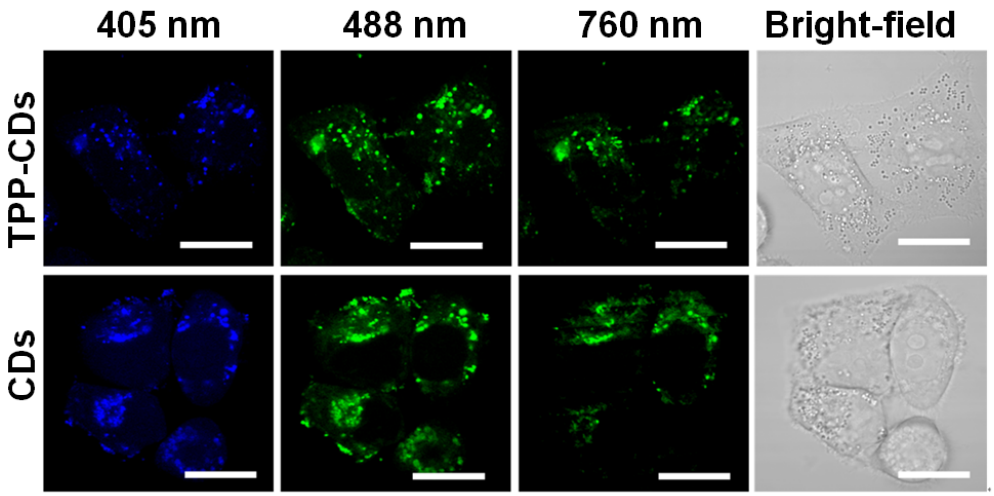


Fig. S8. One-(Ex: 405, 488 nm) and two-photon (Ex: 760 nm) fluorescence microscopy imaging of Hela cells treated with TPP-CDs and CDs. Scale bar = 20 μm.

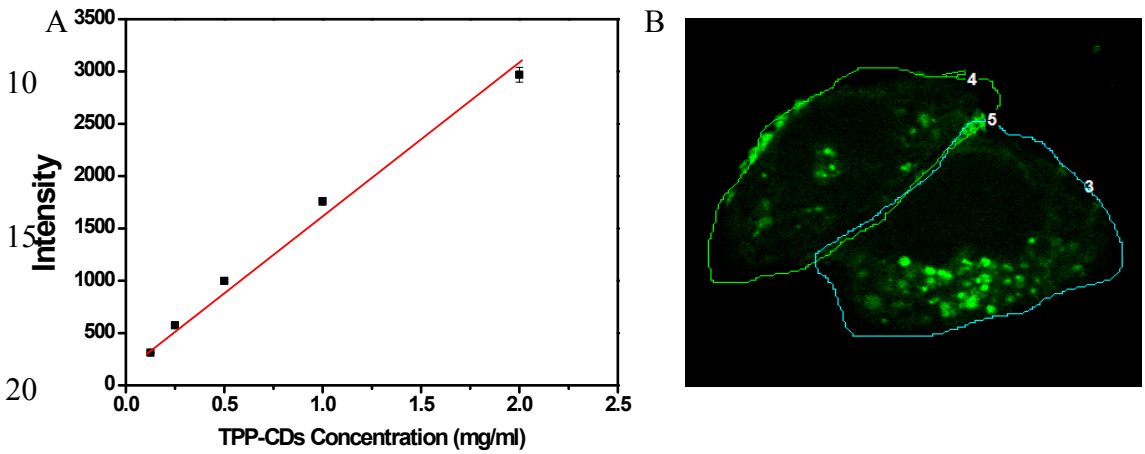
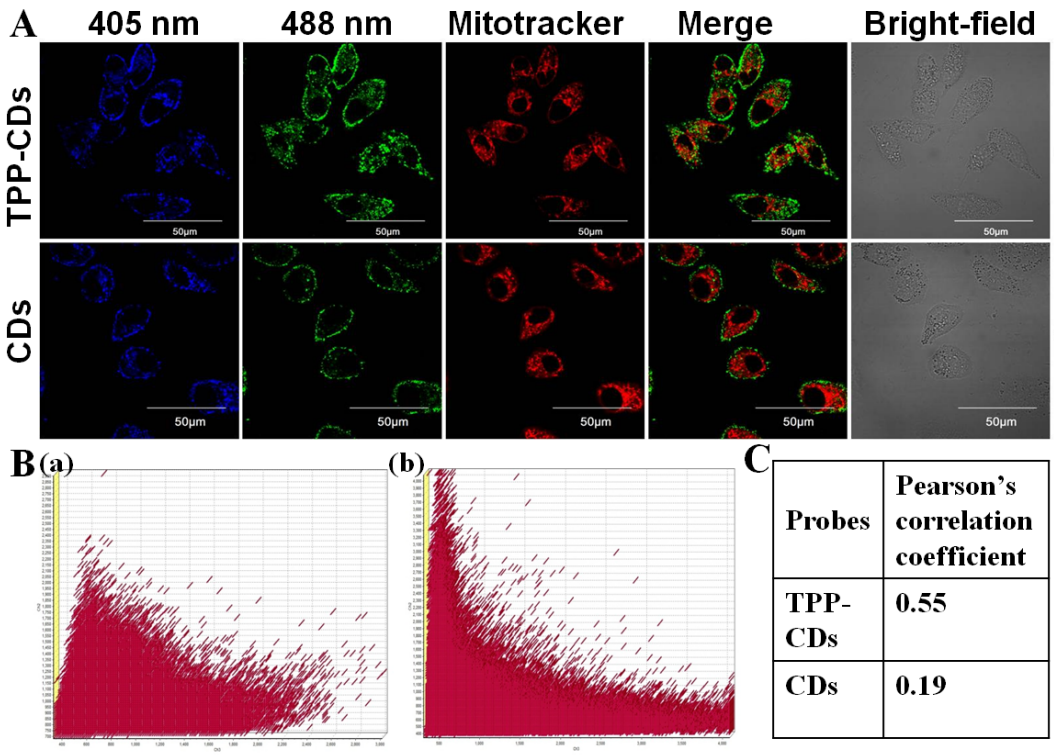


Fig. S9. Calibration curve for quantitative analysis of the cellular uptake of TPP-CDs, which was obtained by calculating the fluorescence intensity of different concentrations of TPP-CDs aqueous solutions under excitation of 488 nm laser (A). The outline of cells on the fluorescent images was drawn by using the Fluoview software and the average fluorescent intensity in the area of each cell was calculated (B). According to the calibration curve of the cellular uptake of TPP-CDs, the uptake of TPP-CDs of each cell is around 7.17×10^{-7} mg.



Probes	Pearson's correlation coefficient
TPP-CDs	0.55
CDs	0.19

Fig. S10. Subcellular localization of mitochondria targeted TPP-CDs and CDs in TCA-8113 cells. The cells were also stained with the mitochondrial marker MitoTracker Red Deep (Invitrogen) (red) to colocalize the TPP-CDs and CDs. (A) Confocal images show the overlap of mitochondrial staining (red) and targeted TPP-CDs (green). No significant overlap was observed with non-targeted CDs. Analysis of the merge effect between channel 488 nm and 635 nm for both TPP-CDs (B(a) and CDs (B(b) confirm the result. (C)Pearson's coefficients show the quantitative results.

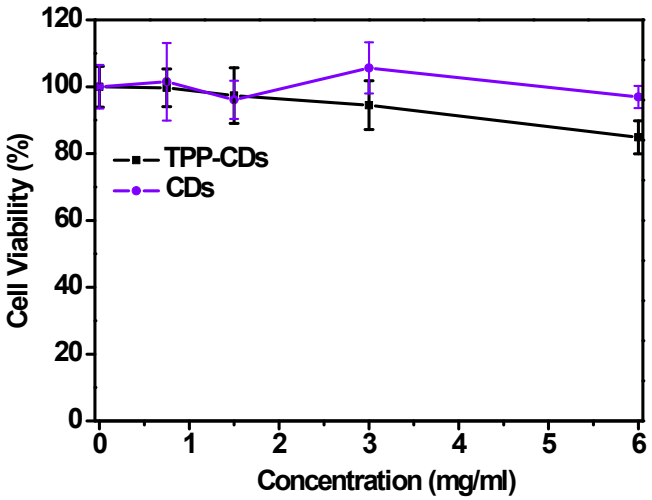


Figure S11. Cytotoxicity of both CDs and TPP-CDs incubated with Hela cells for 8 hours.

5 Table S1. Physicochemical parameters of the CDs

Molar ratios of citric acid to urea	Ex (nm)	Em (nm)	QY(%)	Colour reaction with Ninhydrin
6:1	330	420	26	Yellow
2:3	330	420	35	Light blue
1:6	330	420	29	Deep blue

Reference

1. F. D. Deroose and P. J. D. Clercq, *J Organ Chem*, 1995, **60**, 321-330.