Supplementary Information

Bioinspired *Camellia japonica* carbon dots with high near-infrared absorbance for efficient photothermal cancer therapy

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Experimental Section

Synthesis of S-CDs. The naturally fallen *Camellia Japonica* flowers were collected from the local *Camellia japonica* trees grove in Chonnam National University (Gwangju, South Korea). The collected *Camellia Japonica* flowers were sun-dried for 2 days before smashed into powders by a mixer grinder. The Grinding of the dried *Camellia Japonica* flowers was performed on high-energy ball mill (Fritsch Pulverisette 6, Germany). The grinded biomass powders were obtained after 24 h continuous ball-milling at a rotation speed of 200 rpm. The milled powders (2 g) were dispersed in 20 mL of deionized (DI) water and placed in the Teflon-linded autoclave and maintained at 180 ℃ for 5 h. After cooling to room temperature, the obtained mixtures were washed and filtered by a membrane (0.22 μm pore). The resulting dark brown solution was dialyzed to obtain S-CDs with a uniform particle size distribution. The synthesized S-CDs are stored into a glass vial at 4 ℃ for further use.

Characterization of S-CDs. The elemental features and morphology of S-CDs were evaluated *via* transmission electron microscopy (TEM: Tecnai F20 ST, FEI, USA) with an energy-dispersive X-ray spectroscopy (EDS) module and atomic force microscope (AFM: XE-100, Park Systems, South Korea). The phase formation and structural analysis of S-CDs were estimated using powder X-ray diffraction (XRD: Rint-1000, Rigaku Corp., Japan), laser Raman spectrophotometry (NRS-5100, Jasco Inc., USA), and Fourier transform infrared spectroscopy (FT-IR: IRPresitge-21, Shimadzu Corp., Japan). The chemical bonding between the elements of S-CDs was measured by X-ray photoelectron spectroscopy (XPS: Multilab 2000, Thermo Fisher Scientific, UK). The amount of sulfur was examined by CHNS elemental analysis (vario MICRO cube, Elementar, Germany).

Optical property analysis. All optical measurements were performed in phosphate-buffered saline (PBS) at pH 7.4. Absorption spectra of S-CDs were measured using a fiber optic FLAME spectrometer (Ocean Optics, Dunedin, USA). The fluorescence emission spectra of S-CDs were analyzed using a SPARK® 10M microplate reader (Tecan, Männedorf, Switzerland) at an excitation wavelength of 360 nm and emission wavelengths ranging from 400 to 700 nm.

Photothermal conversion efficiency of S-CDs. Photothermal conversion efficiency was evaluated by recording the temperature change of the S-CDs (45 μg/mL) as a function of time under 808 nm laser irradiation at a power density of 1.1 W/cm^2 in a quartz cuvette. A FLIR® thermal imager (FLIR Systems, Wilsonville, USA) was used to record the temperature changes of the S-CD solution. The S-CD solution was continuously irradiated with a 808 nm laser until a steady temperature was reached. After the irradiation was switched off, the temperature decrease was monitored to determine the rate of heat transfer from the system. The photothermal conversion efficiency (η) of the S-CDs was calculated using the following equation as reported previously:[1,2]

$$\eta = \frac{hA\Delta T_{max} - Q_s}{I(1 - 10^{-A\lambda})}$$

where h is the heat transfer coefficient, A is the surface area of the container. ΔT_{max} is the temperature change of the sample solution from the ambient temperature (T_{surr}, 24.8 °C) to the equilibrium temperature (T_{max}, 71.5 °C). Q_s is the heat dissipation from the light absorbed by the quartz sample cell, which is 37.8 mW. I is the laser power density (1.1 W/cm^2), and A_λ is the absorbance of S-CDs at 808 nm (0.1134). The value of hA can be calculated using the following equation:

$$\tau_s = \frac{m_D C_D}{hA}$$

where τ_s is the sample system time constant, m_D and C_D are the mass (0.2 g) and heat capacity (4.2 J/g) of DI water used as a solvent, respectively. To calculate τ_s, a dimensionless parameter (θ) should be acquired by following equation:

$$\theta = \frac{\Delta T}{\Delta T_{max}}$$

where ΔT is the temperature change of a temperature difference between the solution temperature and ambient temperature. ΔT_{max} is the temperature change at the maximum steady-state temperature. Thus, τ can be determined by applying the linear time data from the cooling period versus ln(θ) by following equation:

$$t = -\tau (\ln \theta)$$

Therefore, *hA* can be calculated.

***In vitro* photothermal cytotoxicity.** HT-29 human colorectal adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC® HTB-38™, Manassas, USA). The cancer cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS: Gibco BRL, Paisley, UK) and an antibiotic-antimycotic solution (100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B; Welgene, Daegu, South Korea) in a humidified 5% CO_2 atmosphere at 37 °C. The final concentration of S-CDs (45 μg/mL) was added when the cells attained 50~60% confluence. To visualize the photothermal therapy (PTT)-induced cell death in S-CD treated cells, PTT was performed under 808 nm laser at 1.1 W/cm^2 for 2 min. After PTT, the cells were allowed to incubate for another 3 h and then stained with propidium iodide (PI, a red fluorescent dye to detect dead cells) for 30 min. After washing twice with PBS, the stained cells were imaged using a Nikon Eclipse Ti-U inverted microscope system (Nikon, Seoul, South Korea). Image acquisition and

analysis were performed using NIS-Elements Basic Research software (Nikon). All fluorescence images were acquired at identical exposure times and normalized.

Cell viability assay. Cell toxicity and proliferation were evaluated using the alamarBlue™ (Thermo Scientific, Waltham, USA) assay. The HT-29 cells were seeded onto 96-well plates (1×10^4 cells per well). To determine cytotoxicity depending on the concentration, the cancer cells were treated with the S-CDs (30, 60, 100, and 200 μg/mL) for 1 h and cultured for 24 h after treatment. At each assay time point, the incubation cell medium was replaced with 100 μL of fresh medium, and 10 μL of the alamarBlue solution was directly added to each 100 μL well; the plates were then incubated for 4 h at 37 °C in a humidified 5% CO_2 incubator. Finally, the 96-well plates were placed in a microplate reader (SPARK® 10M, Tecan) to measure the absorption intensity at 570 nm and the fluorescence intensity at 590 nm. Cell viability was calculated using the following formula (A is the average absorbance): cell viability (%) = $(A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}}) \times 100$.

HT-29 xenograft mouse model. Animal care, experiments, and subsequent euthanasia were performed in accordance with protocols approved by Chonnam National University Animal Research Committee (CNU IACUC-H-2017-64). Six-week old adult male NCRNU nude mice weighing approximately 25 g (N = 3 independent experiments) were purchased from Orient Bio Inc. (Seongnam, South Korea). HT-29 cancer cells (1×10^6 cells per mouse) were harvested and suspended in 100 μL of PBS followed by subcutaneous injection into the right flank of each mouse. When the tumors reached a size of ~1 cm in diameter, PBS and the S-CDs were administered intratumorally. Animals were sacrificed and imaged over a certain period of time.

***In vivo* tumor imaging.** *In vivo* fluorescence imaging was performed using a FOBI fluorescence imaging system (NeoScience, Suwon, South Korea). The fluorescence signal of S-CDs injected in

tumors was monitored at 525 nm. All fluorescence images were normalized identically for all conditions. To confirm the *in vivo* antitumor effect, the macroscopic features of each group were observed at fixed time intervals for a week. The tumor volumes were calculated using the following formula: $V = 0.5 \times \text{longest diameter} \times (\text{shortest diameter})^2$.

Assessment of *in vivo* photothermal effect. HT-29 tumor-bearing mice were intratumorally administered with PBS and the S-CDs. After injection, the mice were anesthetized, and their tumor sites were laser-irradiated (1.1 W/cm^2, λ = 808 nm) for 5 min. Temperature changes in the tumor sites were monitored using a FLIR® thermal imager (FLIR Systems) and data were recorded from the beginning of the laser irradiation at a step-size of 1 min during the entire laser irradiation period. At 24 h post-irradiation, tumors were excised from the treated mice for subsequent histological analysis using hematoxylin and eosin (H&E) staining.

Histological analysis. Resected tumors were preserved for H&E staining and microscopic assessment. Samples were fixed in 4% paraformaldehyde and flash frozen in optimal cutting temperature (OCT) compound using liquid nitrogen. Frozen samples were cryosectioned (10 μm in thickness per slide), stained with H&E, and observed by microscopy. Histological imaging was performed on a Nikon Eclipse Ti-U inverted microscope system. Image acquisition and analysis was performed using NIS-Elements Basic Research software (Nikon).

TUNEL assay. Tumor tissues were collected from the S-CD and laser-treated group to confirm an observation of apoptotic cell death. They were fixed in 4% paraformaldehyde at -20 °C for 30 min and cryosectioned (10 μm in thickness per slide). Then, the samples were washed with PBS and incubated with a terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) reagent containing terminal deoxynucleotidyl transferase and fluorescent isothiocyanate

dUTP using a DeadEnd™ Fluorometric TUNEL System (Promega, Madison, USA). After incubation, they were stained with DAPI (1 μg/mL) for 30 min to investigate the cell nucleus by UV light microscopic observations (blue). Fluorescence imaging was performed on a Nikon Eclipse Ti-U inverted microscope system. Image acquisition and analysis was performed using NIS-Elements Basic Research software (Nikon). All fluorescence images were acquired at identical exposure times and normalized.

Statistical analysis. Statistical analysis was carried out using a one-way ANOVA followed by Tukey's multiple comparisons test. Differences were considered to be statistically significant at a level of $p < 0.05$. Results were presented as mean ± S.D. and curve fitting was performed using Prism version 4.0a software (GraphPad, San Diego, USA).

Table S1 Comparison of the PTT conditions and photothermal conversion efficiency between S-CDs and previously reported CDs.

Reference	Carbon source	Dose (μg/mL)	Laser power (W/cm^2)	Laser wavelength (nm)	Irradiation time (min)	Conversion efficiency (%)
3	Citric & nitric acid solutions	20,000	2	808	10	3.77
4	Watermelon juices	20,000	1.4	808	10	30.6
5	*Hypocrella bambusae* fungi	2,000	0.8	635	10	27.6
6	Cyanine dye & poly(ethylene glycol)	800	2	808	10	38.7
7	Ayurvedic plants	500	0.5	750	10	-
This study	***Camellia japonica* flowers**	**45**	**1.1**	**808**	**5**	**55.4**

Table S2 The CHNS elemental analysis of S-CDs.

CD component	S (wt%)	C (wt%)	N (wt%)	H (wt%)
Camellia japonica flowers	4.915	64.734	0.327	4.848

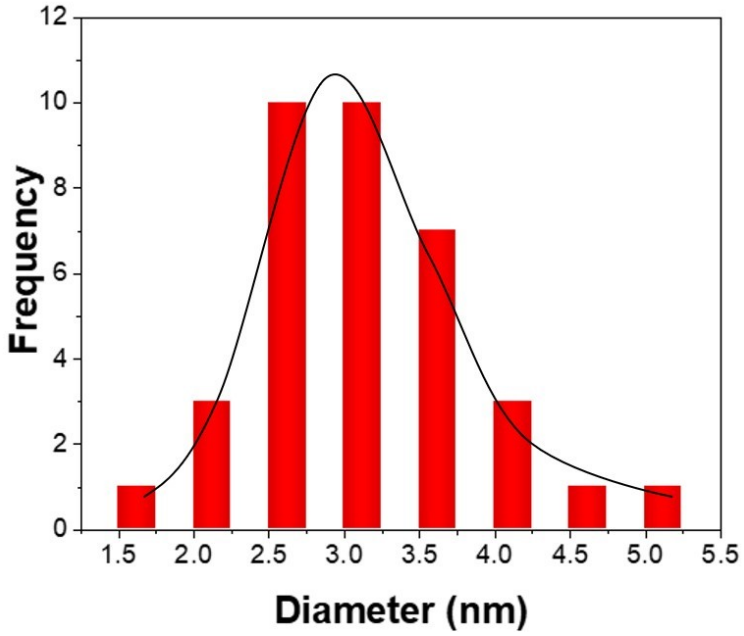

Fig. S1 Average size distribution of S-CDs by the TEM images.

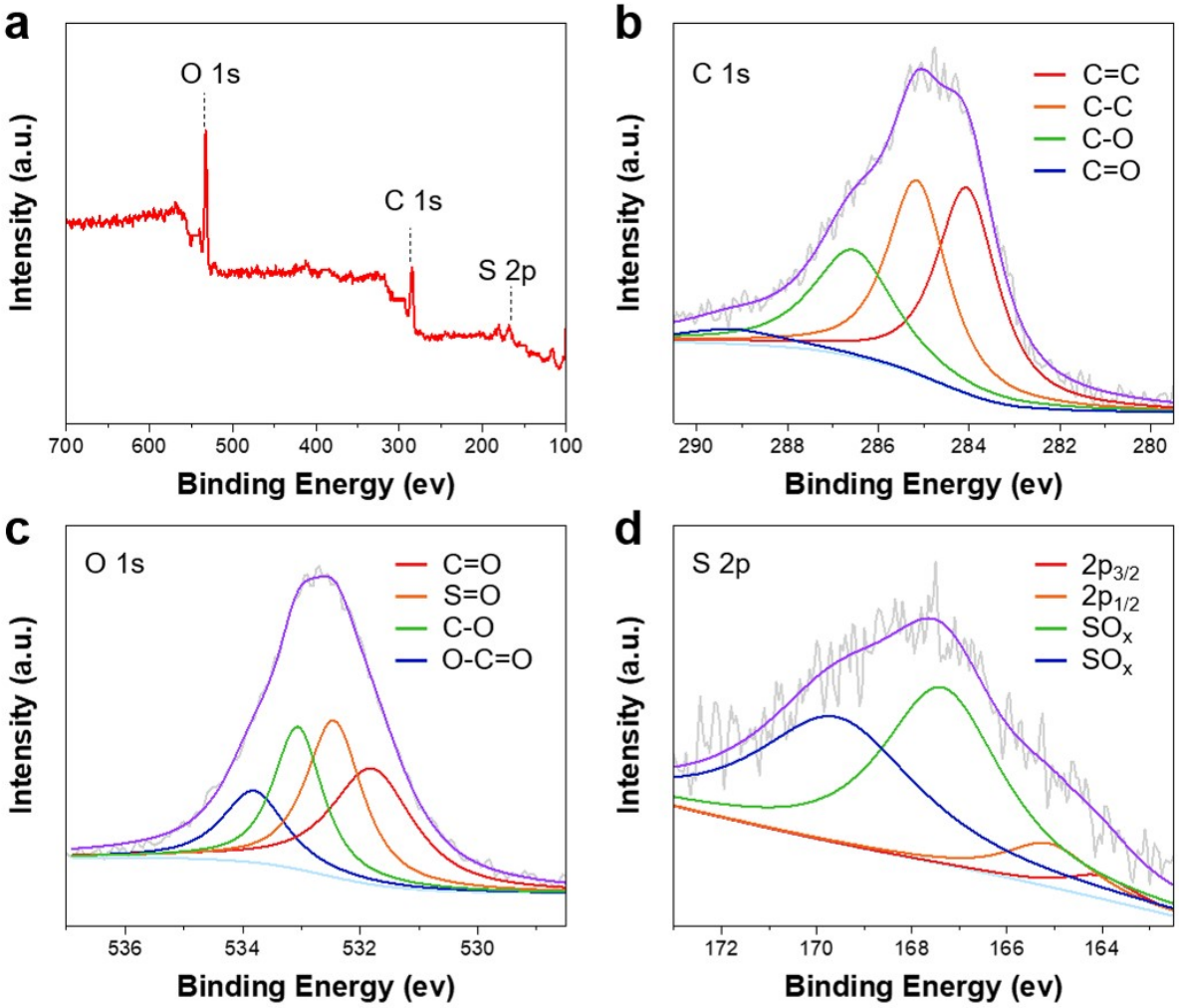

Fig. S2 (a) Full survey XPS spectra of the S-CDs. (b) C 1s, (c) O 1s, and (d) S 2p XPS spectra of the S-CDs.

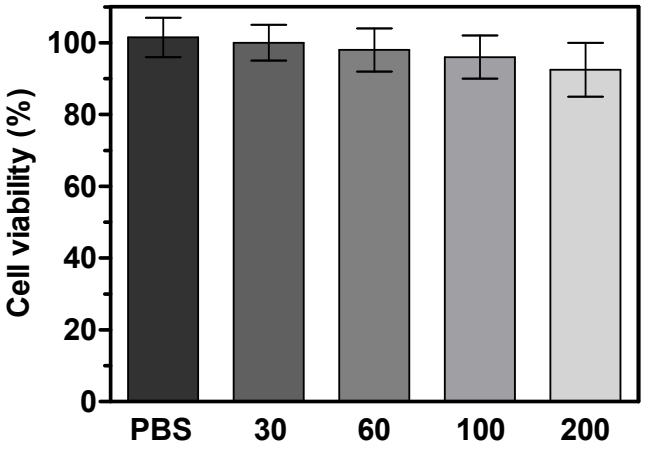

Fig. S3 Cell viability assay of the S-CDs using HT-29 cancer cells. The percentage cytotoxicity was determined after 24 h of treatment with various concentrations of the S-CDs.

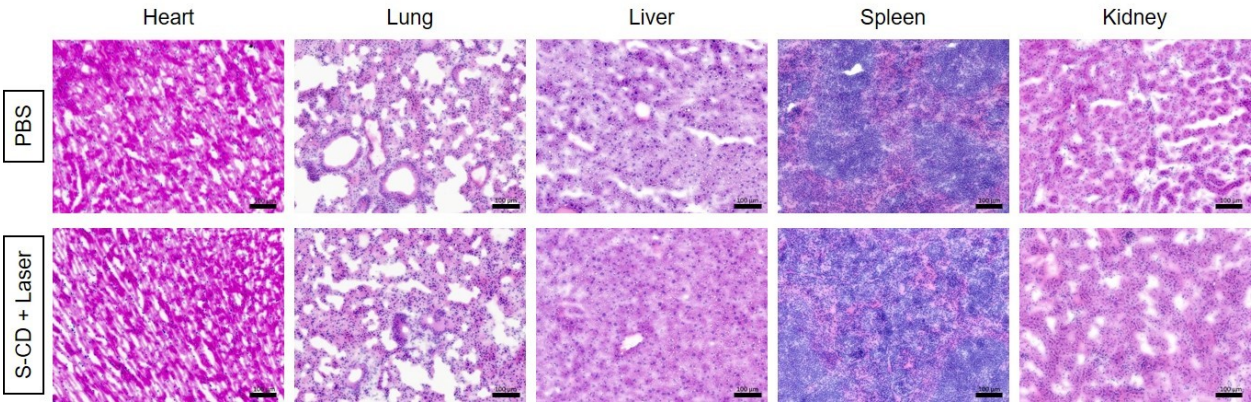

Fig. S4 H&E stained images of major organs including heart, lung, liver, spleen, and kidney tissues after PBS and PTT treatments. Images are representative of three independent experiments. Scale bars = 100 μm.

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