Enhanced photochemical therapy of cancer cells using carbon dots integrated with black phosphorus nanosheets

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1. Materials and methods

1.1. Synthesis and characterization of PCDs and BPNSs

Aqueous solutions of D-glucose and L-aspartic acid (Macklin, China) were prepared at various molar ratios.[1] The mixtures were sonicated for dissolution, and the pH was adjusted to a desired value using 1 M NaOH or HCl solutions. The reaction was performed in a polytetrafluoroethylene-lined reactor at 125 °C for 30 min, followed by 200 °C for 20 min. After the reaction, the solution was cooled to room temperature. Subsequently, 20 mL of ultrapure water was added, and the mixture was centrifuged at 13,000 rpm for 30 min. The supernatant was then dialyzed using a 3000 Da dialysis bag for 24 h. The dialyzed solution was lyophilized to obtain PCD powder, and the concentration was determined linearly using UV-Vis absorption of CDs for subsequent quantification. For PCDs, there is a strong linear relationship between concentration and absorbance at 360 nm (R^2 = 0.999), with the linear equation fitting being $A_{360\ nm}/l$ = 15.858 C_{PCDs} − 0.008 (Figs. S2A and S2B). Different proportions of D-glucose and L-aspartate were synthesized to obtain various products: CD28, CD37, CD46, CD55, CD64, CD73, and CD82 (the numbers represent the ratio of D-glucose to L-aspartate).

For the preparation of BPNSs, 5 mg of black phosphorus crystal powder and 1 mL of NMP were added to a 100 mL beaker, and then ground with a glass rod until the solution turned black. Next, 100 mg of NaOH was added to the beaker, followed by 49 mL of NMP. The solution was transferred to a reaction flask and sonicated at room temperature for 12 h. The resulting solution was centrifuged at 4000 rpm for 10 min, the supernatant was extracted and centrifuged again at 12,000 rpm for 15 min. The supernatant was discarded, and the precipitate of BPNSs was obtained. This precipitate was washed twice with ultrapure water (12,000 rpm, 5 min), re-dispersed in ultrapure water, and stored in a refrigerator at 4 °C. The powder was obtained by lyophilization, and its concentration was linearized using the UV-Vis absorbance of BPNSs for subsequent quantification. For BPNSs, there is a strong linear relationship

between concentration and absorbance at 464 nm (R^2 = 0.999), with the fitting equation being $A_{464\ nm}/l$ =8.719 C_{BPNSs} − 0.002 (Figs. S2C and S2D).

For the preparation of CD-BPNSs, the quantified PCDs and BPNSs were mixed at a mass ratio of 2:1, sonicated for 5 min, and stored in a refrigerator at 4 °C for subsequent experiments.

The morphology and structure of PCDs and BPNSs were analyzed using transmission electron microscopy (TEM, JEM-2100F, JEOL, Japan). UV-Vis absorption spectra were captured using a Genesys 150 spectrophotometer (Thermo Fisher Scientific, USA). Fluorescence spectra were obtained using an F96PRO spectrofluorometer (Lengguang, China). The structures of PCDs were characterized by Fourier transform infrared spectroscopy (FTIR, Nicolet iS50 infrared spectrometer, USA). X-ray photoelectron spectroscopy (XPS, Thermo Scientific K-Alpha, USA) was used to analyze the surface structure and elemental state of the prepared samples. Cyclic voltammetry (CV) was carried out using an electrochemical workstation (CHI 660E, CH Instruments, China), with the modified electrode as the working electrode, a platinum wire auxiliary electrode, and Ag/AgCl electrode as the reference electrode. Atomic force microscope (AFM) images were recorded by an Agilent 5500 (USA).

1.2. Cell culture

Mouse microglia cells (GL261), human cervical cancer cells (HeLa), mouse breast cancer cells (4T1), and human non-small cell lung cancer cells (A549) were procured from the Cell Bank of the Chinese Academy of Sciences. The GL261 cells and HeLa cells were cultured in DMEM (Gibco, USA) media supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (Hyclone, USA). Meanwhile, the 4T1 and A549 cells were maintained in 1640 (Gibco, USA) media, also containing 10% fetal bovine serum and 1% penicillin-streptomycin. All cell lines were incubated in a humidified environment with 5% CO_2 at 37 °C.

1.3. Cytotoxicity assays of PCDs, BPNSs, and CD-BPNSs complexes

The cytotoxicity in HeLa cells, GL261 cells, A549 cells, and 4T1 cells was assessed using an MTT assay. Cells in good growth condition were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated in a 5% CO_2 environment at 37 °C for

24 h. Afterward, the culture media were removed, and 100 μL of different concentrations of PCDs, BPNSs, and CD-BPNSs prepared in DMEM or 1640 culture media were added for incubation. Following a 12-hour incubation period, the culture media were aspirated and the cells were washed once with PBS (HyClone, USA). Next, 100 μL of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, Solarbio, China) prepared in DMEM or 1640 media (1 mg/mL) were added to each well and incubated for 4 h. After discarding the MTT solution, 150 μL of dimethyl sulfoxide (Macklin, China) were added to each well. Absorbance at 490 nm was measured using a BioTek microplate reader, and cell viability was calculated based on the absorbance readings.

1.4. Assessment of photothermal characteristics

Photothermal performance test in an aqueous solution: Different concentrations of PCDs, BPNSs, and CD-BPNSs dispersions were prepared with ultrapure water. The samples were irradiated with an 808 nm laser at a power of 1 W/cm^2 for 5 min, and the temperature was recorded every 30 seconds using a thermal infrared imager. The horizontal axis represented time, while the vertical axis represented temperature.

Photothermal treatment of cancer cells: Initially, the respective cell types were counted and seeded into 96-well plates at a density of 1×10^4 cells per well. The plates were incubated in a 5% CO_2 incubator at 37 °C for 12 h to allow cell attachment. PCDs, BPNSs, and CD-BPNSs were diluted into different concentration solutions using DMEM or 1640 culture medium. Subsequently, 100 μL of the solution was added to each well and incubated for 12 h. After incubation, the solution was removed, and the wells were washed with PBS. Then, 200 μL of culture medium was added to each well. The cells were irradiated with an 808 nm laser at a power of 1 W/cm^2 for 5 min per well, followed by further incubation in the incubator for 4 h. After incubation, the culture medium was removed, and 100 μL of MTT solution (1 mg/mL) prepared with culture medium was added to each well and incubated for an additional 4 h. The MTT solution was then removed, and 150 μL of DMSO was added and shaken for 5 min. Finally, the absorbance at a wavelength of 490 nm was measured using an enzyme-labeled instrument, and the cell survival rate was calculated.

1.5. ROS detection

The cells were first counted and seeded into 24-well plates at a density of 5×10^4 cells per well. The plates were incubated at 37 °C in a 5% CO_2 incubator for 24 h to allow for adherent growth. CDs, BPNSs, and CD-BPNSs were diluted to different concentrations with DMEM or 1640 culture media, and 500 μL of each solution was added to the respective wells and incubated for 12 h. After incubation, the solutions were removed and the wells were washed once with PBS. Each well was then filled with 500 μL of culture solution and illuminated with an incandescent lamp (power 6.4 mW/cm^2) for 10 min. Subsequently, DCFH-DA solution, diluted to a concentration of 10 μM with serum-free culture solution, was added and incubated for 25 min, followed by two washes with PBS. Finally, an appropriate amount of PBS was added, and the cells were observed using a fluorescent inverted microscope (Leica, German).

1.6. Theory calculations

All density functional theory (DFT) calculations were carried out utilizing the Gaussian 16 package.[2] Geometric optimization and frequency calculations were conducted at the SMD-B3LYP/6-31G(d) level by considering the solvent effect of water via the solvation model based on density (SMD).[3] The binding energy (E_b) of complex was calculated at the SMD-B3LYP-D3(BJ)/6-31G+(d) level by considering the dispersion correction of D3(BJ).[4] The E_b value of complex was calculated by the following equation,

$$E_b = E(\mathrm{A}) + E(\mathrm{B}) - E(\mathrm{Complex})$$

Reference

1 M. Zheng, S. Ruan, S. Liu, T. Sun, D. Qu, H. Zhao, Z. Xie, H. Gao, X. Jing and Z. Sun, Self-targeting fluorescent carbon dots for diagnosis of brain cancer cells, *ACS Nano*, 2015, **9**, 11455-11461.

2 M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, G.A. Petersson, H. Nakatsuji, X. Li, M. Caricato, A.V. Marenich, J. Bloino, B.G. Janesko, R. Gomperts, B. Mennucci, H.P. Hratchian, J.V. Ortiz, A.F. Izmaylov, J.L. Sonnenberg, Williams, F. Ding, F.

Lipparini, F. Egidi, J. Goings, B. Peng, A. Petrone, T. Henderson, D. Ranasinghe, V.G. Zakrzewski, J. Gao, N. Rega, G. Zheng, W. Liang, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, K. Throssell, J.A. Montgomery Jr, J.E. Peralta, F. Ogliaro, M.J. Bearpark, J.J. Heyd, E.N. Brothers, K.N. Kudin, V.N. Staroverov, T.A. Keith, R. Kobayashi, J. Normand, K. Raghavachari, A.P. Rendell, J.C. Burant, S.S. Iyengar, J. Tomasi, M. Cossi, J.M. Millam, M. Klene, C. Adamo, R. Cammi, J.W. Ochterski, R.L. Martin, K. Morokuma, O. Farkas, J.B. Foresman and D.J. Fox, 2016 Gaussian 16 Rev. C.01, Wallingford, CT, Gaussian 16.

3 V.S. Bernales, A.V. Marenich, R. Contreras, C.J. Cramer and D.G. Truhlar, Quantum mechanical continuum solvation models for ionic liquids, *J. Phys. Chem. B*, 2012, **116**, 9122-9129.

4 Grimme S, Ehrlich S and Goerigk L. Effect of the damping function in dispersion corrected density functional theory, *J. Comput. Chem.*, 2011, **32**, 1456-1465.

2. Results

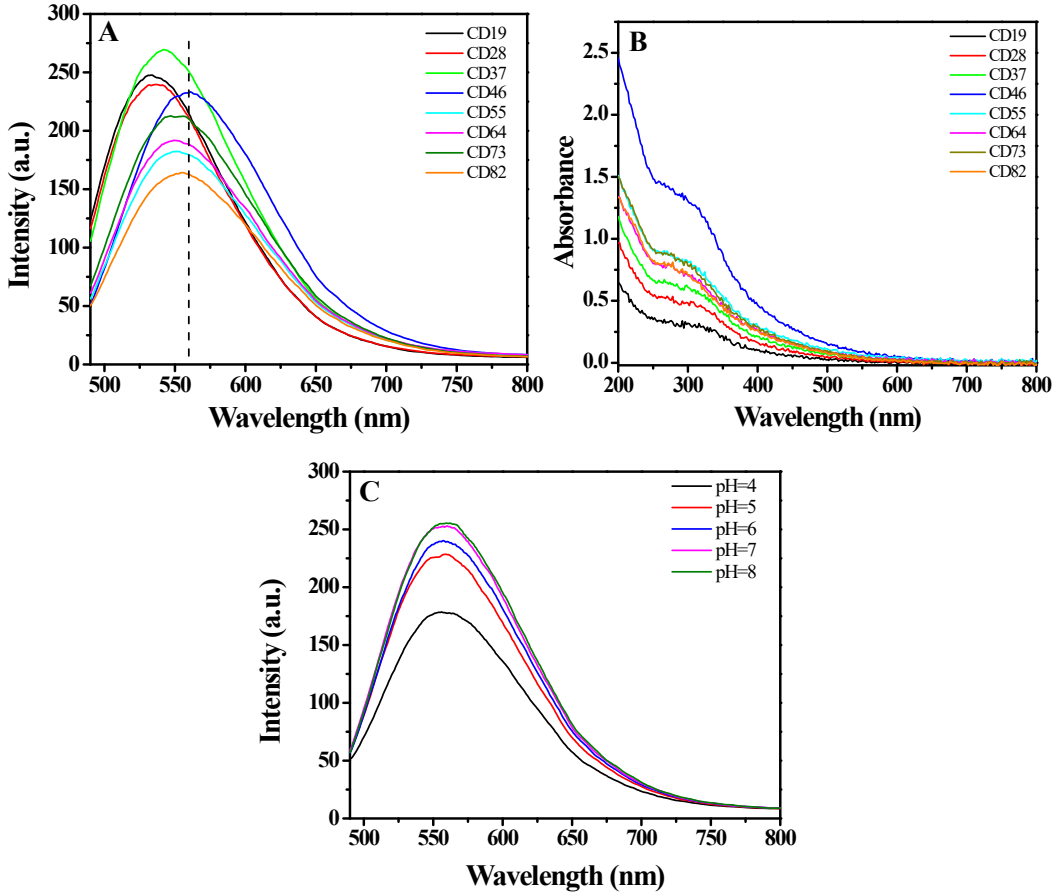


Fig. S1 (A) Fluorescence spectra and (B) UV-Vis absorption spectra of PCDs with different feedstock ratios, (C) fluorescence spectra of CD46 at different pH

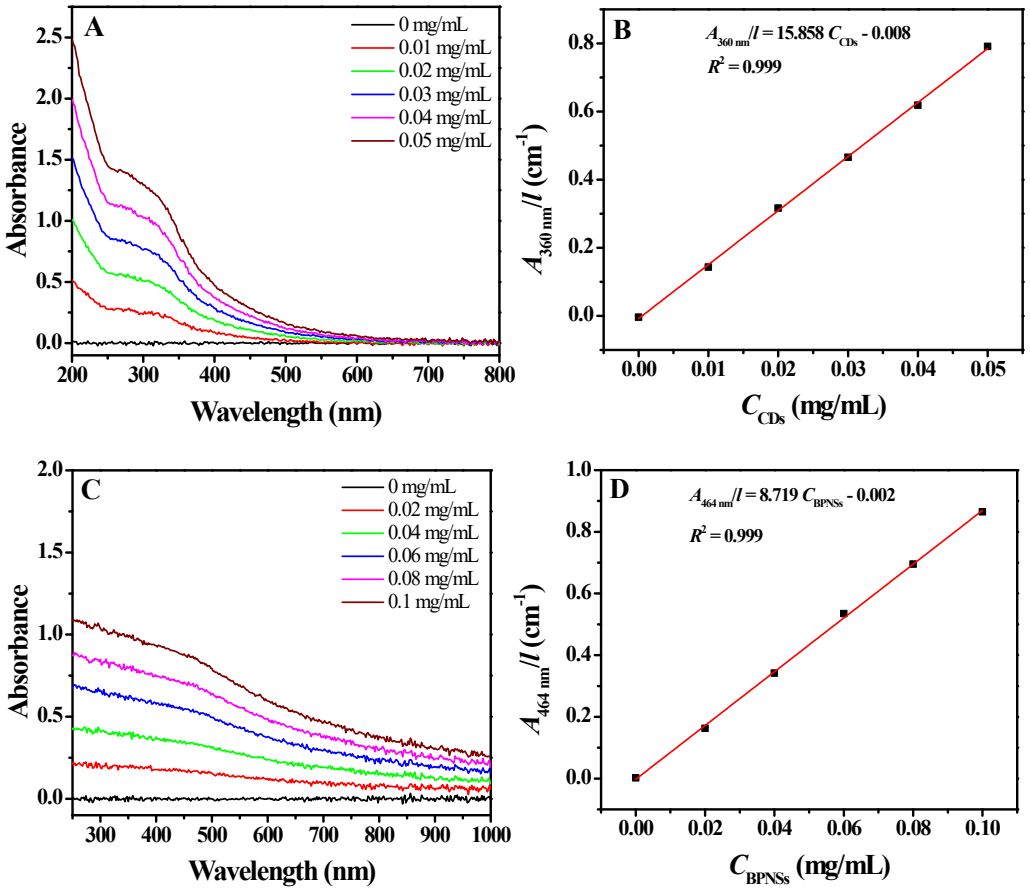


Fig. S2 (A) UV-Vis absorption spectra of CDs at different concentrations. (B) Linear plot of CDs concentration and absorbance at 360 nm. (C) UV-Vis absorption spectra of BPNSs at different concentrations. (D) Linear plot of BPNSs concentration and absorbance at 464 nm

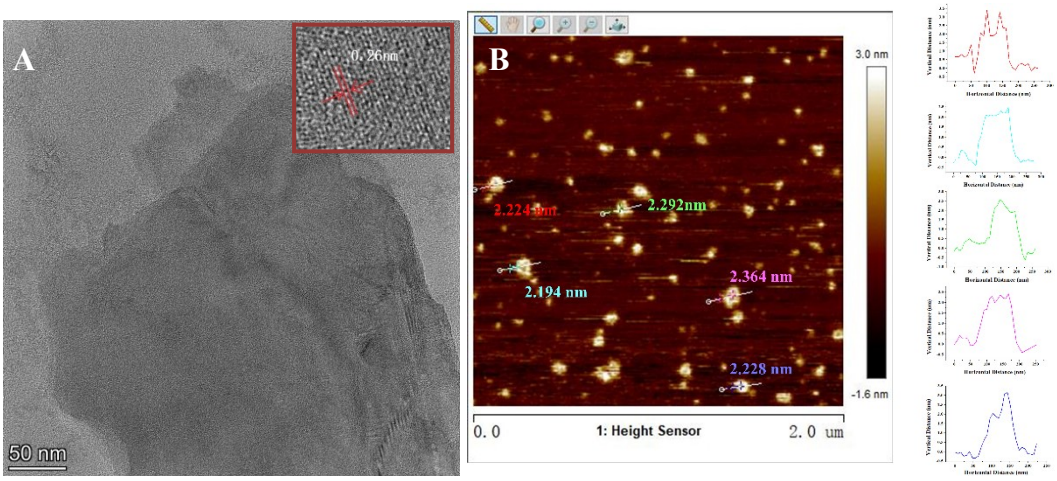


Fig. S3 (A) TEM and (B) AFM images of BPNSs. The inset of (A) is HRTEM image

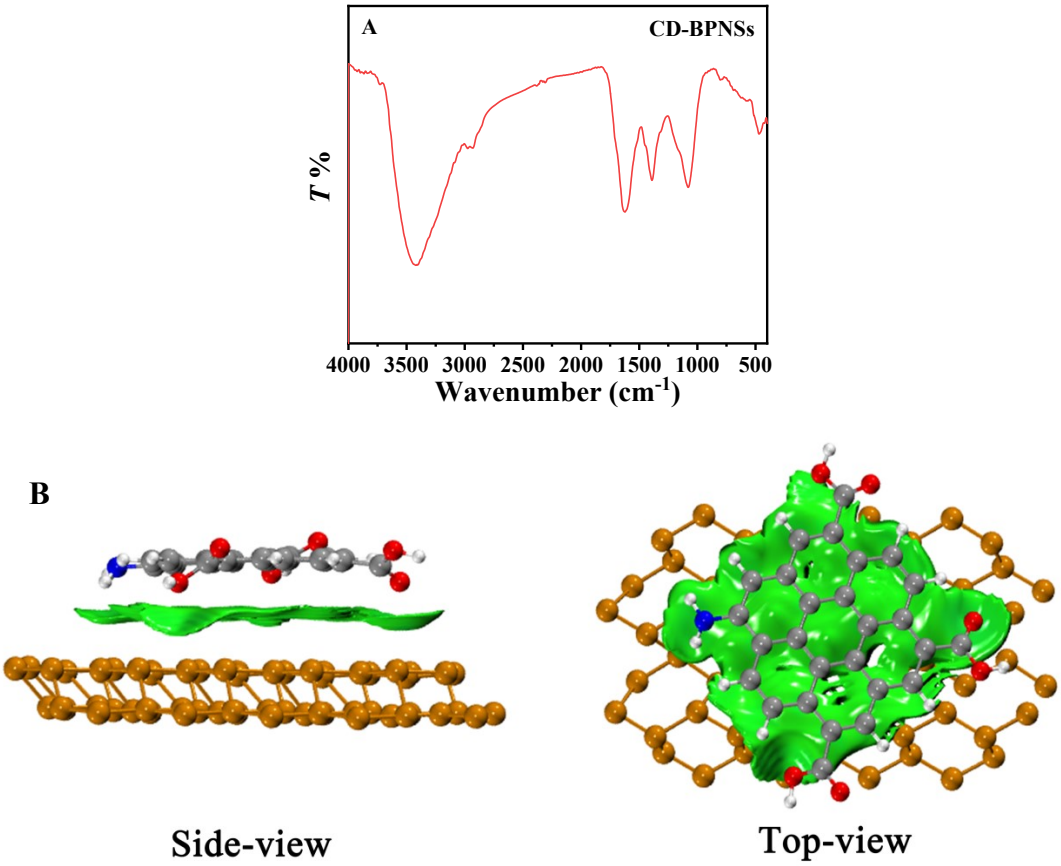


Fig. S4 (A) FTIR spectra of different samples, (B) The IGMH colored isosurface diagram of the complex (isosurface is 0.001 a.u.)

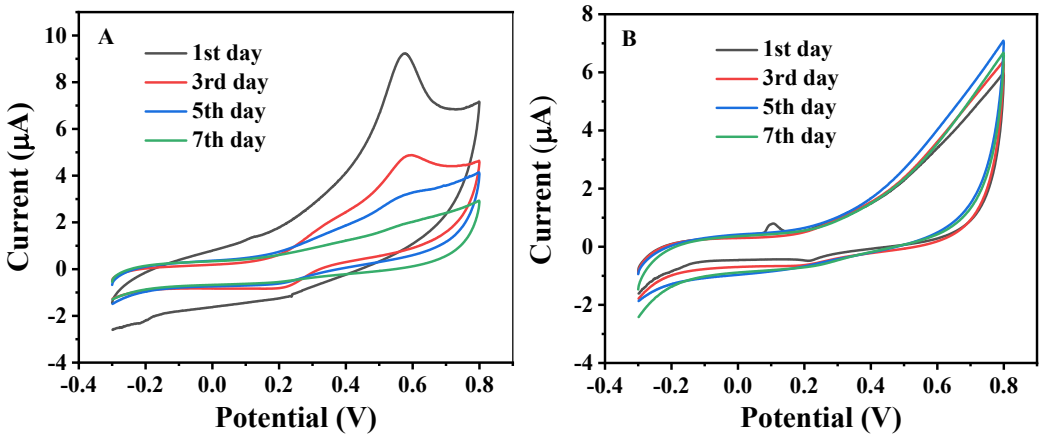


Fig. S5 CV curves of (A) BPNSs and (B) CD-BPNSs for different days

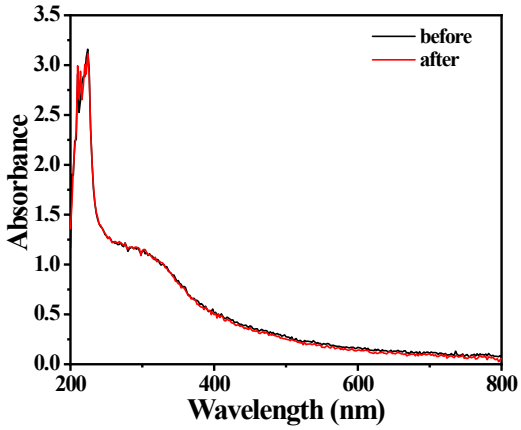


Fig. S6 UV-Vis absorption spectra of CD-BPNSs before and after irradiation

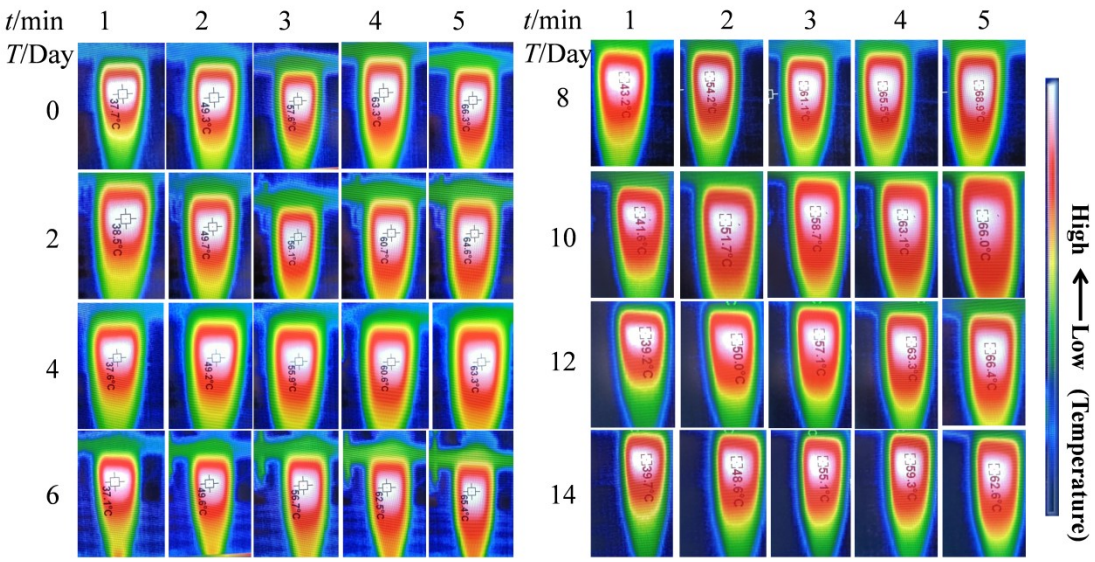


Fig. S7 The actual picture of the photothermal effect for different days

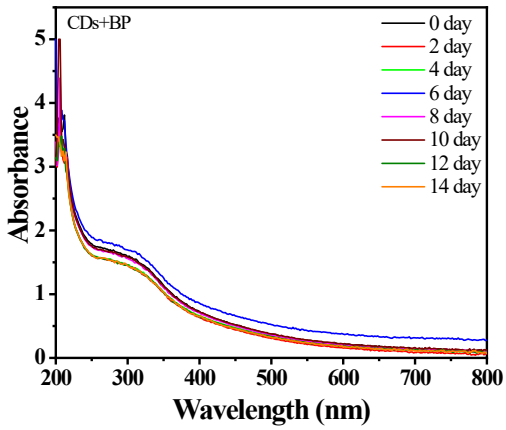


Fig. S8 UV-Vis spectra of CDs-BPNSs for 14 days

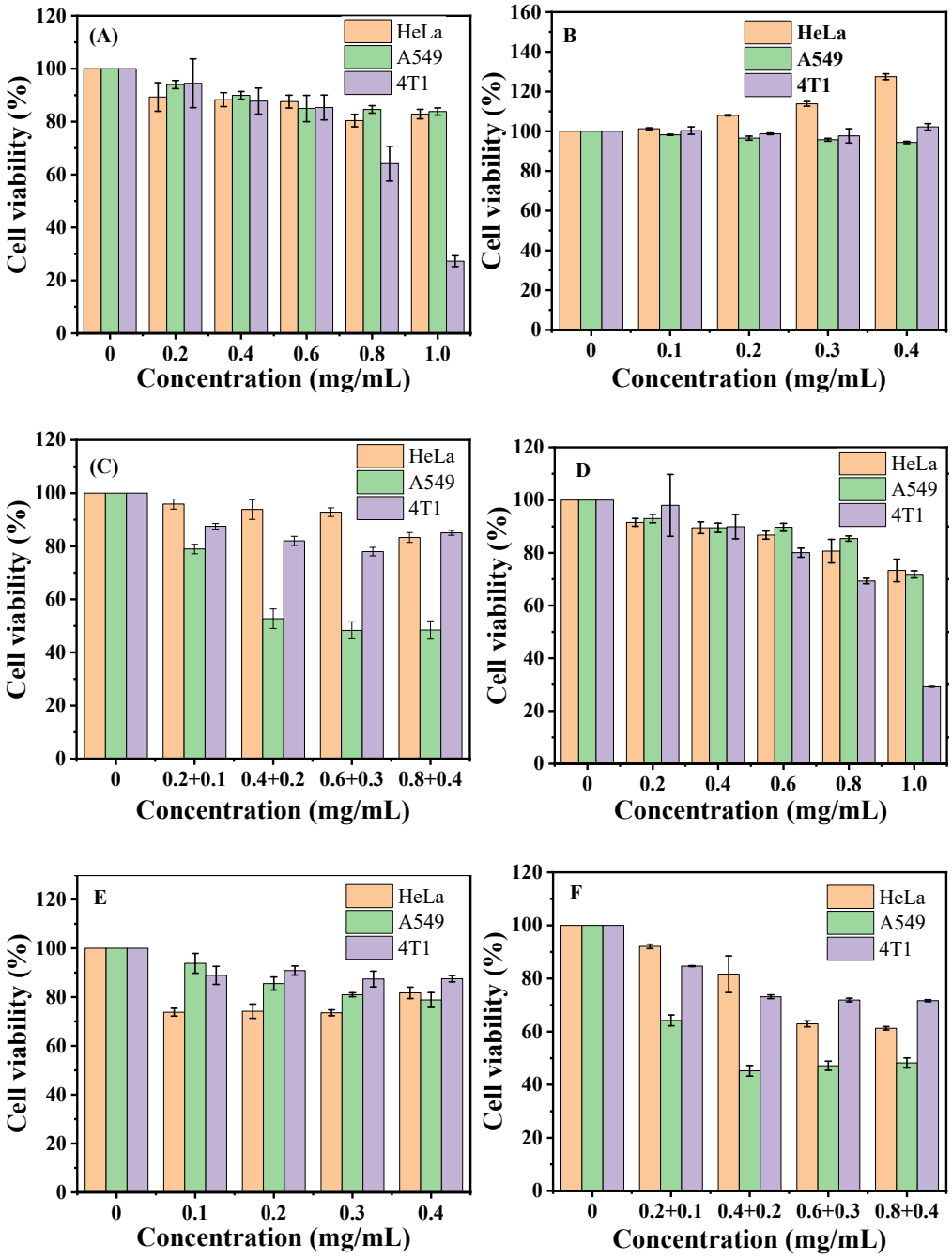
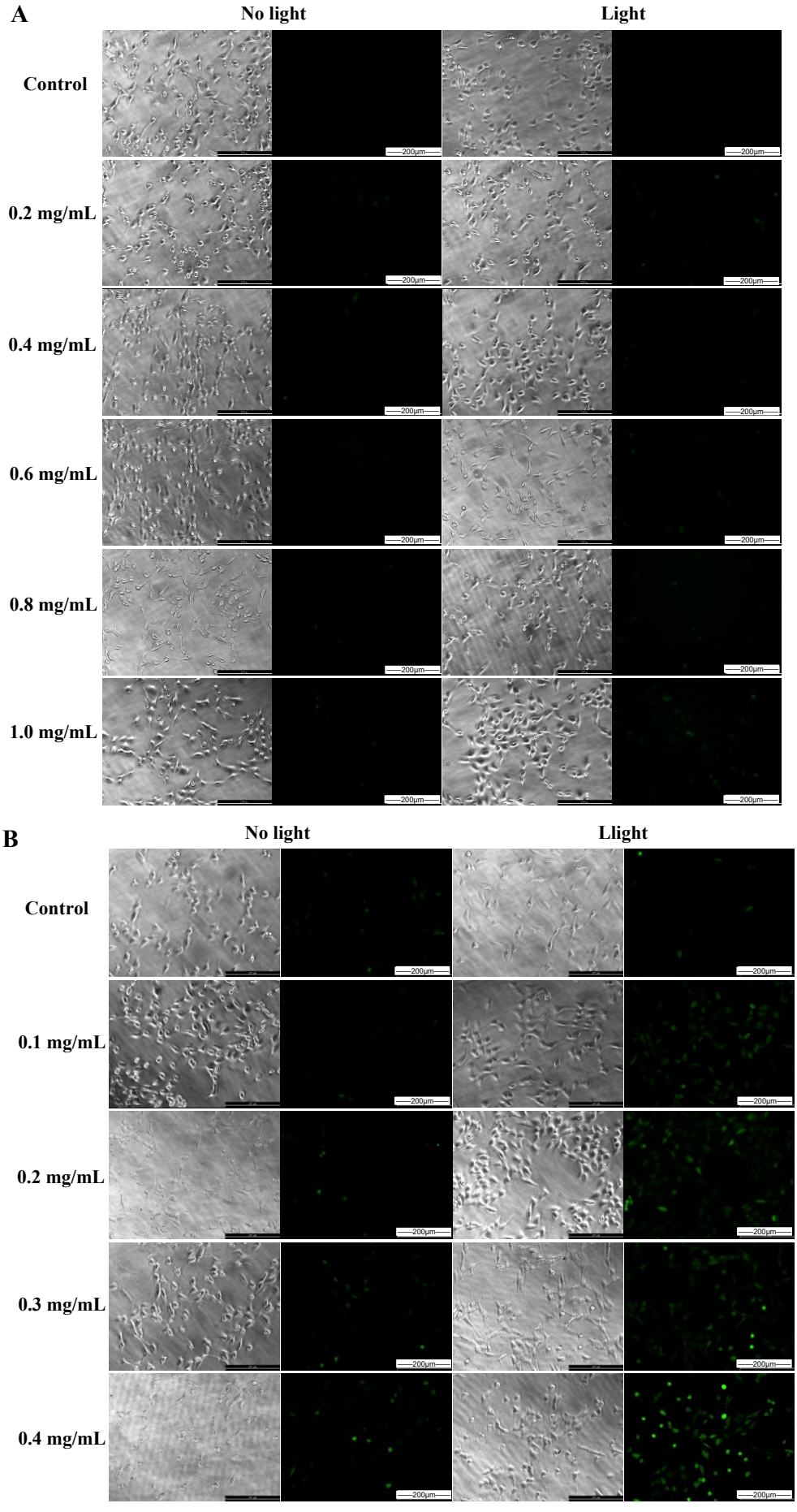


Fig. S9 MTT results treated with (A) PCDs, (B) BPNSs and (C) CD-BPNSs (n =3), MTT results after photothermal treatment with (D) PCDs, (E) BPNSs and (F) CD-BPNSs (n =3)


A
No light
Light
Control
0.2 mg/mL
0.4 mg/mL
0.6 mg/mL
0.8 mg/mL
1.0 mg/mL
B
No light
Llight
Control
0.1 mg/mL
0.2 mg/mL
0.3 mg/mL
0.4 mg/mL

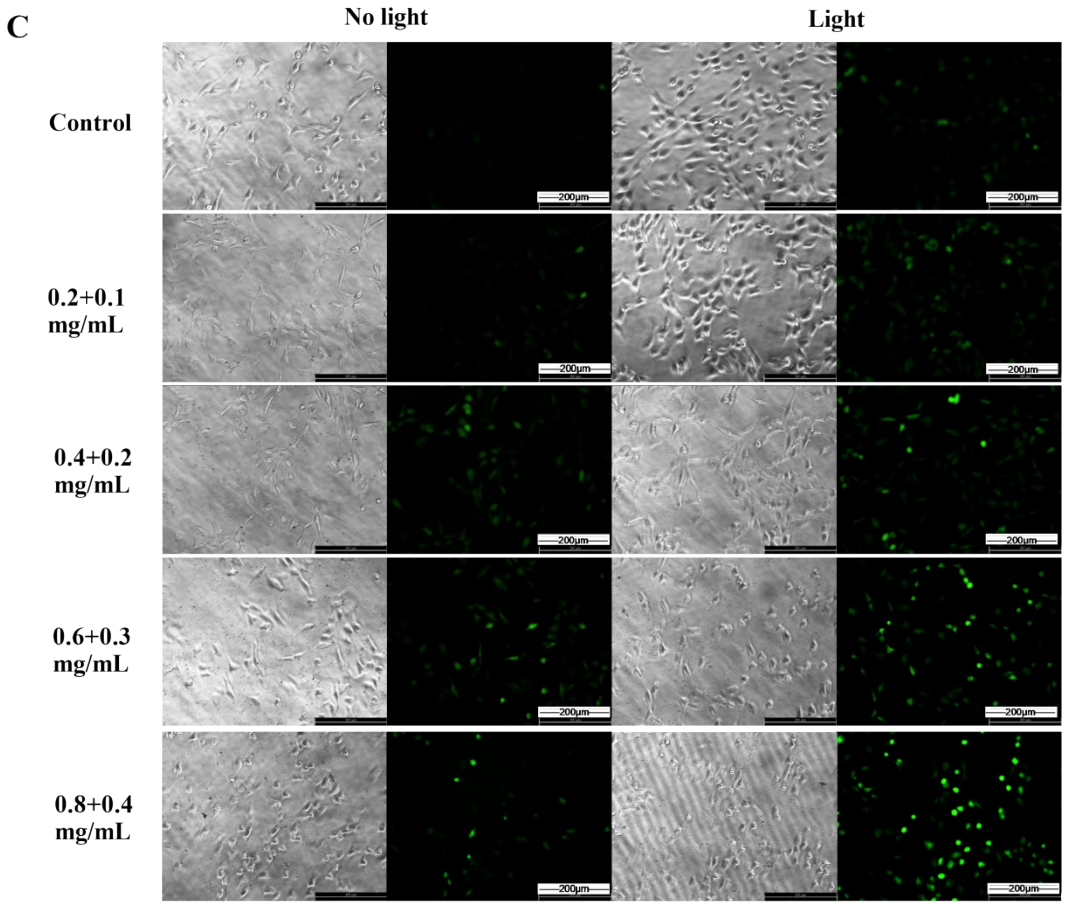


Fig. S10 Bright and fluorescence images of GL261 cells after treated with (A) PCDs, (B) BPNSs and (C) CD-BPNSs with or without light. The scale of the drawing is 200 μm.

Table S1 The binding energy (E_b) value of complex.

Complex	E_b (kcal/mol)
I	52.41