Copper-coordinated dendritic photosensitizer for spatiotemporally synergistic PDT-CDT cascade antitumor therapy

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1 Experimental section

1.1 Materials and solvents

The chemicals for organic synthesis, including 4-(diphenylamino)benzaldehyde, 4-hydroxyphenylacetonitrile, glycidol, sodium hydride, 18-crown-6, sodium hydroxide, potassium carbonate, anhydrous ethanol, and anhydrous magnesium sulfate were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). The chemicals for organic synthesis, including Tetrafluoroterephthalonitrile, 4,7-dihydroxy-1,10-phenanthroline, dichloromethane, petroleum ether, and N,N-dimethylformamide (DMF) were obtained from Adamas. The reagents for detecting various ROS, including 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA), dihydroethidium (DHE), and dihydrorhodamine 123 (DHR 123), were sourced from Aladdin Reagent Co., Ltd. (Shanghai, China). All cell lines used in this study were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences.

1.2 Analytical instrument

^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded on a Bruker Avance-400 spectrometer (400 MHz, Bruker Co., Germany) using DMSO-d_6 as solvents. The UV-Vis absorption spectra were obtained using a PerkinElmer Lambda 35 UV/Vis system (Perkin Elmer Life and Analytical Sciences, Shelton, USA) using quartz cuvettes. The fluorescence spectra were recorded on a PerkinElmer LS55 spectrofluorometer (Perkin Elmer Life and Analytical Sciences, Shelton, USA). The DLS measurements were performed by using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, UK). The size and morphology of those as-prepared samples were characterized by Scanning electron microscope (SEM, FEI 123 Quanta250). Cell imaging was conducted on a Confocal laser scanning microscope (CLSM, Zeiss 710 3-channel, Germany) and an Olympus inverted fluorescence microscope (Olympus, Japan).

1.3 Synthesis and characterization of TPA-OH

A mixture of 4-(diphenylamino)benzaldehyde (1.36 g, 5 mmol) and 4-hydroxyphenylacetonitrile (0.75 g, 5.6 mmol) was dissolved in ethanol. Sodium hydroxide (0.24 g, 5 mmol) was added to the solution, which was then heated under reflux at 80 °C for 4 h. After completion of the reaction, the mixture was cooled to room temperature. The pH was adjusted to slightly alkaline using hydrochloric acid solution. The mixture was extracted with dichloromethane (3 × 20 mL). The combined organic layers were dried over anhydrous magnesium sulfate and filtered to afford the crude product. The crude

product was purified by column chromatography on silica gel using petroleum ether/dichloromethane (5:1, v/v) as the eluent to obtain compound TPA-OH as orange solid (1.2 g, 63% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.84 (s, 1H), 7.79 (d, J = 8.7 Hz, 1H), 7.66 (s, 1H), 7.59 – 7.43 (m, 2H), 7.34 (dt, J = 15.7, 7.7 Hz, 4H), 7.22 – 7.00 (m, 6H), 6.91 (dd, J = 30.7, 8.7 Hz, 4H), 6.69 (d, J = 8.7 Hz,1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 158.17 (s), 148.85 (s), 146.21 (s), 139.01 (s), 130.28 (s), 129.74 (d, J = 9.9 Hz), 126.89 (s), 125.29 (s), 124.94 (d, J = 14.7 Hz), 124.36 (s), 120.51 (s), 118.66 (s), 116.10 (s), 115.89 (s), 106.85 (s), 40.15 (s), 39.94 (s), 39.73 (s), 39.55 (s), 39.42 (d, J = 21.0 Hz), 39.10 (s), 38.90 (s). ESI-MS (ESI) m/z calculated for $C_{27}H_{20}N_2O$ $[M]^+$ 388.10, found $[M+H]^+$ 389.16.

1.4 Synthesis and characterization of TPAGP and TPAGP@Cu

A mixture of compound TPA-OH (0.5 g), 18-crown-6 (0.4 g), and sodium hydride (0.2 g) was dissolved in 30 mL of anhydrous DMF under a nitrogen atmosphere. The mixture was stirred at room temperature for 1 h, then heated to 80°C. Glycidol (2 mL) was added dropwise to the reaction mixture. After complete addition, the reaction was maintained at 80°C for 18 h. After completion of the reaction and cooling to room temperature, the mixture was dialyzed using a 1000 Da dialysis bag. The dialyzed product was then dried in a vacuum oven at 50℃ for 24 h to afford compound TPAGC. A mixture of compound TPAGC (0.3 g) and crosslinker tetrafluoroterephthalonitrile (0.2 g) was dissolved in 30 mL of DMF. Potassium carbonate (K_2CO_3, 0.8 g) was added and the mixture was heated to 60°C. After reacting for 1 hour, 4,7-dihydroxy-1,10-phenanthroline (0.21 g) was added, and the reaction was continued for a further 12 hours. Upon completion and cooling to room temperature, the mixture was dialyzed using a 1000 Da dialysis bag. The dialyzed product was then dried under vacuum at 50°C for 24 h to afford compound TPAGP. Finally, TPAGP@Cu nanoparticles were formed by complexing TPAGP with Cu^{2+} at a mass ratio of 10:1. The crude reaction mixture was transferred into a dialysis bag (MWCO 1000 Da) and dialyzed against PBS buffer (pH 7.4) containing 10 mM EDTA-2Na for 12 h with three changes of the external buffer. EDTA efficiently chelates and competitively removes physically adsorbed or weakly bound Cu^{2+}, allowing free copper ions to diffuse out. After dialysis, the solution was ultracentrifuged to collect the pellet, and the supernatant was discarded. The pellet was resuspended in PBS buffer and centrifuged again; this washing step was repeated three times. The final product, TPAGP@Cu, was obtained by freeze-drying.

1.5 Assessment of ROS production by TPA-OH and TPAGP

DCFH-DA was employed as a fluorescent indicator to assess the generation of ROS and detailed

experimental procedure was listed below. 4 mL of 0.01 M NaOH solution was added to 1 mL of 1 mM DCFH-DA solution. The mixture was magnetically stirred for 30 minutes under light-protected conditions to achieve deacetylation. 20 mL of phosphate-buffered saline (PBS, pH 7.4) was added to the deacetylated product with continued light protection. A working DCFH stock solution (blank control) was prepared by mixing 1 mL of the above solution with 39 mL PBS. The experimental group consisted of the following two mixtures: 4 mL of the working stock solution with 20 μL of a 10 mg/mL TPA-OH photosensitizer solution; and 4 mL of the working stock solution with 20 μL of a 20 μg/mL TPAGP photosensitizer solution. After thorough mixing, the solution was subjected to white light exposure 20 mW/cm^2) in 30-second or 10-second intervals to facilitate the monitoring of total ROS production, which was quantified by measuring the incremental fluorescence emission at 525 nm, with excitation occurring at a wavelength of 488 nm. ABDA was utilized as an indicator for singlet oxygen ($^1O_2$). Stock solutions were prepared containing 4 mM ABDA with either 1 mg/mL TPA-OH or TPAGP, utilizing DMSO as the solvent. Subsequently, a mixture of 60 μM ABDA and 10 μg/mL of the respective photosensitizer (TPA-OH or TPAGP) was created in a DMSO/PBS solution at a ratio of 1:99 (v/v). The resultant solution was then subjected to white light exposure at an intensity of 20 mW/cm^2, and the alterations in the ultraviolet absorption spectrum within the wavelength range of 340-460 nm were meticulously recorded. Concurrently, the decrease in absorbance at 400 nm or 401 nm was monitored over varying exposure durations to assess the production efficiency of $^1O_2$. DHR 123 was employed as a fluorescent probe for the detection of free radicals. Solutions of DHR 123, TPA-OH and TPAGP were prepared in DMSO at concentrations of 0.2 mM, 1 mg/mL and 0.1 mg/mL. A mixture was then created by combining 1 μM DHR 123 with either 5 μM TPA-OH or 0.5 μM TPAGP photosensitizer in a DMSO/PBS solution at a ratio of 1:99 (v/v). Following exposure to white light at an intensity of 20 mW/cm^2 for varying durations, the alterations in fluorescence intensity within the wavelength range of 500-650 nm were recorded, using an excitation wavelength of 488 nm. The production of free radical ROS was assessed by plotting the fluorescence intensity change curve at 527 nm. The method for ROS detection in pure organic solvents by TPA-OH was identical to the procedure described above, except for the substitution of the aqueous PBS buffer with DMSO as the organic solvent.

1.6 Evaluation of cellular uptake and imaging capabilities of TPAGP@Cu

MDA-MB-231 cells were seeded at a density of 1×10^5 cells per well in 6-well plates and incubated

for a duration of 24 h. The cells were then co-incubated with 20 μg/mL TPAGP@Cu nanocomposite for 4 h. Following incubation, cellular monolayers were rinsed three times with PBS to remove non-internalized TPAGP@Cu. Subsequently, cells were fixed with 4% (w/v) paraformaldehyde at room temperature for 10 min. Fluorescence imaging was ultimately performed using an inverted fluorescence microscope.

1.7 Cytotoxicity evaluation of TPAGP and TPAGP@Cu

MDA-MB-231 cells were seeded in 96-well plates at a density of 5,000 cells/well and incubated for 24 h. Cells were treated with serially diluted solutions of TPAGP and TPAGP@Cu (0, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40 μg/mL) for 24 h. Residual compounds were removed by triple-rinsing with PBS. 100 μL of CCK-8 solution was added to each well, followed by 2 h incubation. The absorbance at 450 nm for each well was measured using a microplate reader. The phototoxicity assay was performed with reference to the dark toxicity assay. Cells were co-incubated with photosensitizers TPAGP and TPAGP@Cu under dark conditions for 6 h, followed by irradiation with a 450 nm laser at power densities of 5 mW/cm^2 and 3 mW/cm^2 for 3 min respectively. Following irradiation, the cells were returned to the incubator for an additional 18 h of culture. Finally, the absorbance of each well at 450 nm was measured using a microplate reader.

1.8 Hemolysis assay of TPAGP@Cu

First, 0.5 mL of venous blood was collected from healthy rats and centrifuged at 5000 rpm for 10 min. The supernatant (containing leukocytes and plasma) was discarded. The red blood cells (RBCs) were washed five times with physiological saline and then resuspended in physiological saline to prepare a 2% RBC suspension. A fixed volume of the RBC suspension was added to different concentrations of TPAGP@Cu solutions (5, 10, 20, 40, 80, 160 μg/mL) as the experimental groups. An equal volume of RBC suspension was added to PBS and deionized water to serve as the negative control and positive control groups, respectively. All tubes were gently mixed and incubated at a constant temperature of 37 °C for three hours. After incubation, each sample was centrifuged at 5000 rpm for 10 minutes. The supernatant was transferred to a 96-well plate, and its absorbance at 450 nm was measured using a microplate reader.

1.9 Detection of intracellular ROS generation by TPAGP@Cu

To assess intracellular ROS production upon light exposure, the DCFH-DA indicator was utilized. MDA-MB-231 cells were seeded in 6-well plates at a density of 2×10^5 cells/well and incubated with 40

μg/mL TPAGP@Cu for 6 h. Unbound compounds were removed by gentle PBS washing. Cells were subsequently loaded with 10 μM DCFH-DA for 30 minutes, followed by three PBS washes. Photostimulation was performed using a 450 nm laser at 5 mW/cm^2 irradiance for 3 minutes. Fluorescence imaging was conducted immediately using an inverted fluorescence microscope.

1.10 Staining of live and dead cells

The photodynamic eradication of cancer cells utilizing TPAGP@Cu was further substantiated through live and dead cell staining assays. Following the inoculation of MDA-MB-231 cells in 6-well plates and a 24 h incubation period, TPAGP@Cu (80 μg/mL) was introduced, and the incubation continued for an additional 6 h. Subsequently, the samples were irradiated with a 450 nm laser at a power density of 5 mW/cm^2 for 3 minutes, followed by further incubation in the dark for 18 h. After treatment, the cells were washed three times with PBS to remove residual compounds. Then, Calcein-AM stock solution was added and the cells were incubated at 37 °C in the dark for 20-25 minutes. Next, 4 μM propidium iodide (PI) stock solution was introduced, and incubation was continued for 5 minutes under light-protected conditions to facilitate adequate dye penetration and binding to cellular targets. Thereafter, the cell culture dishes were rinsed three times with PBS to eliminate unbound dye and reduce background interference. Finally, cell viability was assessed using an inverted fluorescence microscope.

1.11 Determination of GSH depletion by Cu^{2+} and concomitant generation of Cu^{+}

The TPAGP@Cu NPs (20 μg/mL) was incubated with 1 mM GSH. At different time (0, 1, 3, 6, 12 h), 300 μL of the mixture was added into 1.68 ml PBS solution (pH = 7.4). Subsequently, 20 μL DTNB (0.5 mg/ml, ethanol) was added. The peaks at 412 nm were observed by UV-vis spectroscopy.

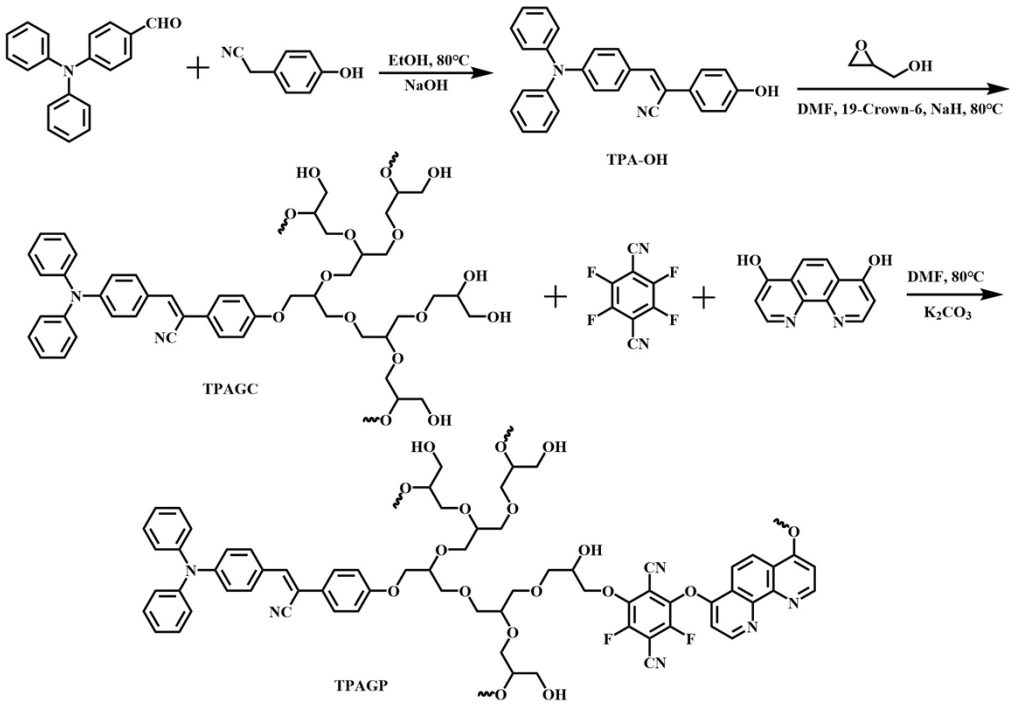
According to the Bicinchoninic Acid (BCA) kit instructions, reagent A and reagent B were mixed at a ratio of 50:1 (v/v) to prepare the BCA working solution. Two solutions were separately prepared: 0.1 mM GSH solution, and a mixed solution containing 0.1 mM GSH and 300 μg/mL TPAGP@Cu. Into two centrifuge tubes, 2.0 mL of BCA working solution was added, followed by the addition of 1.0 mL of each of the above two solutions (the GSH solution and the GSH + TPAGP@Cu mixed solution), respectively. After mixing, the mixtures were incubated at 37 ℃ for 2 h. After the reaction, the absorbance at 562 nm was measured using a UV-Vis spectrophotometer.

1.12 Verification of hydroxyl radical (•OH) generation

To evaluate the Fenton effect of TPAGP@Cu, TMB was used to detect ·OH generation. In a PBS

buffer (pH 5.0, total volume 45 mL), TPAGP@Cu (final concentration 100 μM, 0.45 mL of 10 mM stock solution), H_2O_2 (final concentration 100 μM, 4.5 mL of 1 mM working solution), and TMB (final concentration 40 μg/mL, 1.8 mL of 1 mg/mL stock solution) were added sequentially, with TMB added last to initiate the reaction. A control system without the catalyst was also set up. Each reaction mixture was incubated at 37 °C in the dark with shaking for 10, 20, 40, and 60 min. The absorbance at 652 nm was measured, and the amount of TMB oxidation product was used to evaluate the ability of TPAGP@Cu to catalyze H_2O_2 to generate ·OH.

2 Results



Scheme S1 The synthetic routes of the compounds TPA-OH, TPAGC, and TPAGP.

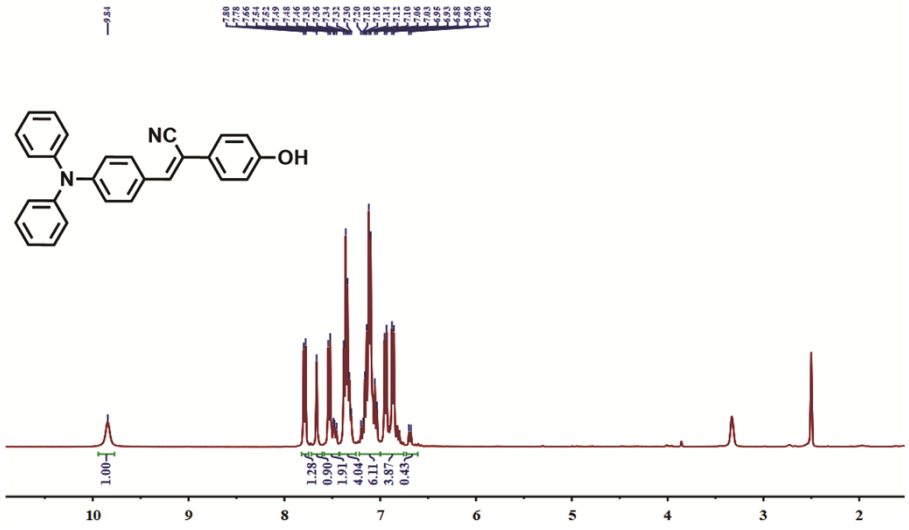


Fig. S1 ^{1}H NMR spectrum of TPA-OH in DMSO-d_6.

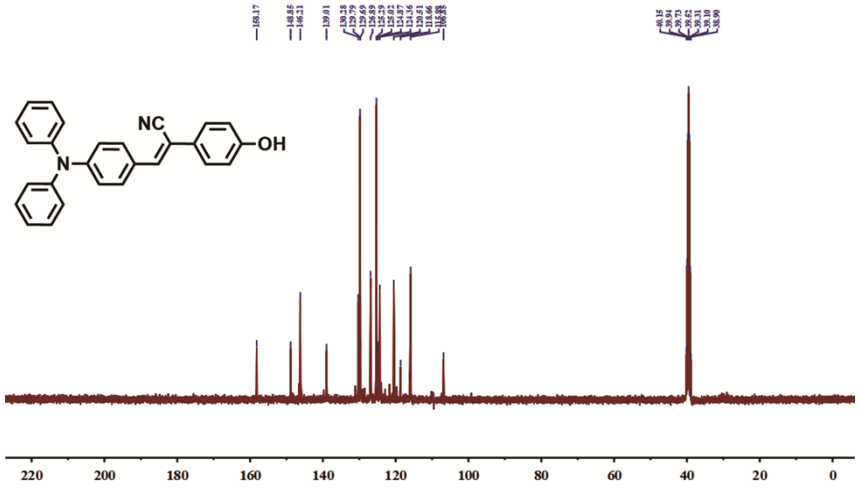


Fig. S2 ^{13}C-NMR spectrum of TPA-OH in DMSO-d_6.

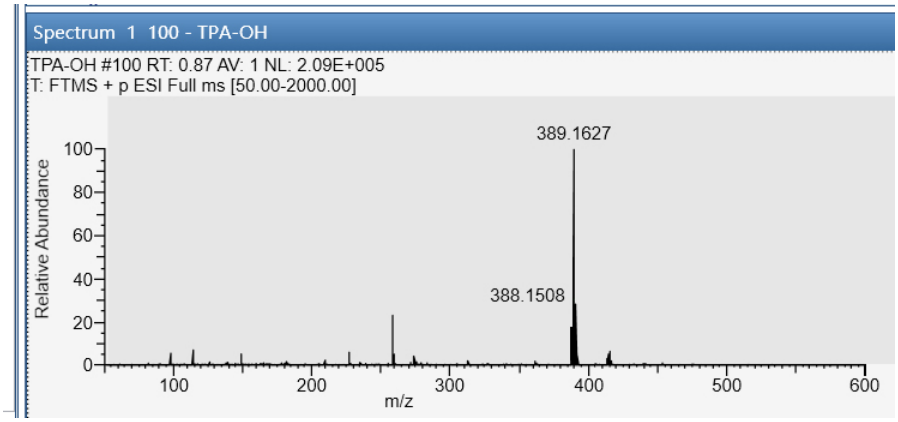


Fig. S3 ESI-MS date of TPA-OH.

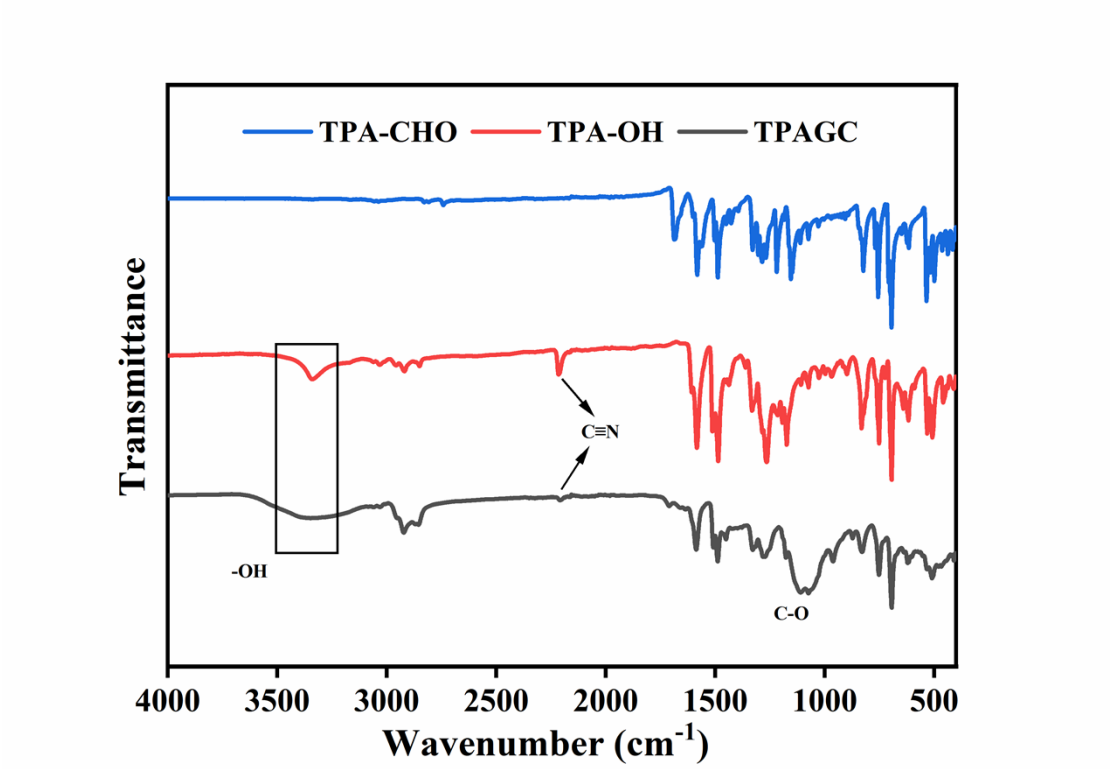


Fig. S4 FT-IR spectra of TPA-CHO, TPA-OH, and TPAGC.

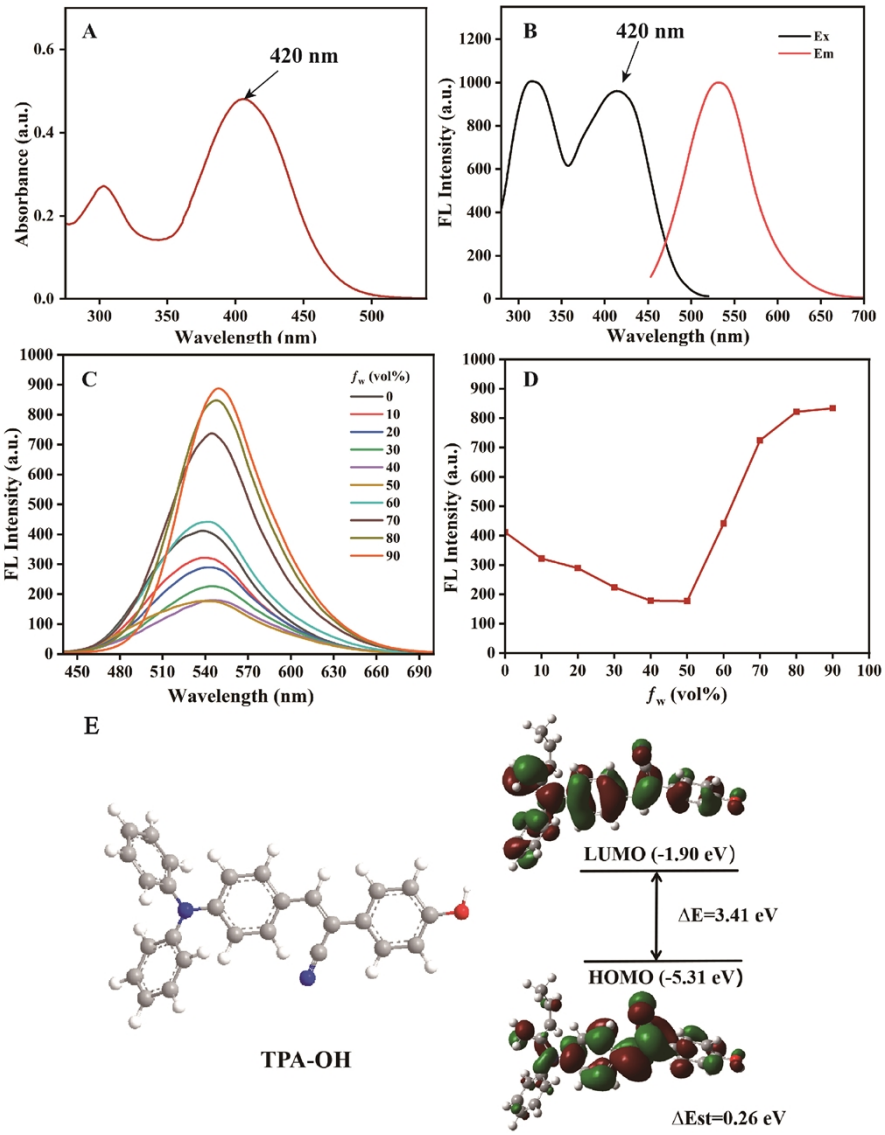


Fig. S5 (**A**) UV absorption spectrum of TPA-OH; (**B**) the excitation and emission spectra of TPA-OH in DMSO/H_2O mixture; (**C**) fluorescence emission spectrum of TPA-OH in DMSO/H_2O mixtures with different water volume fractions (f_w); (**D**) the relationship of fluorescence intensities of TPA-OH versus water fraction in DMSO/H_2O solution. (**E**) 3D molecular architecture of TPA-OH with frontier orbital visualization, including HOMO-LUMO spatial distribution, energy gap (ΔE), and singlet-triplet gap.

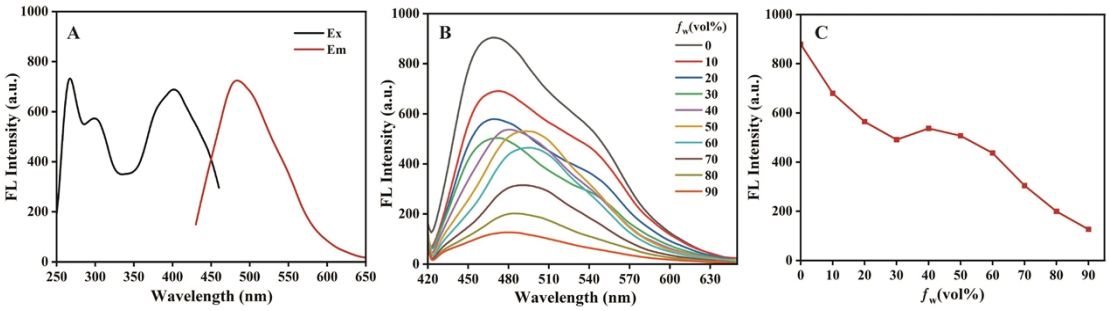


Fig. S6 (**A**) The excitation and emission spectra of TPAGP in DMSO/H_2O mixture; (**B**) Fluorescence emission spectrum of TPAGP in DMSO/H_2O mixtures with different water volume fractions (f_w); (**C**) The relationship of fluorescence intensities of TPAGP versus water fraction in DMSO/H_2O solution.

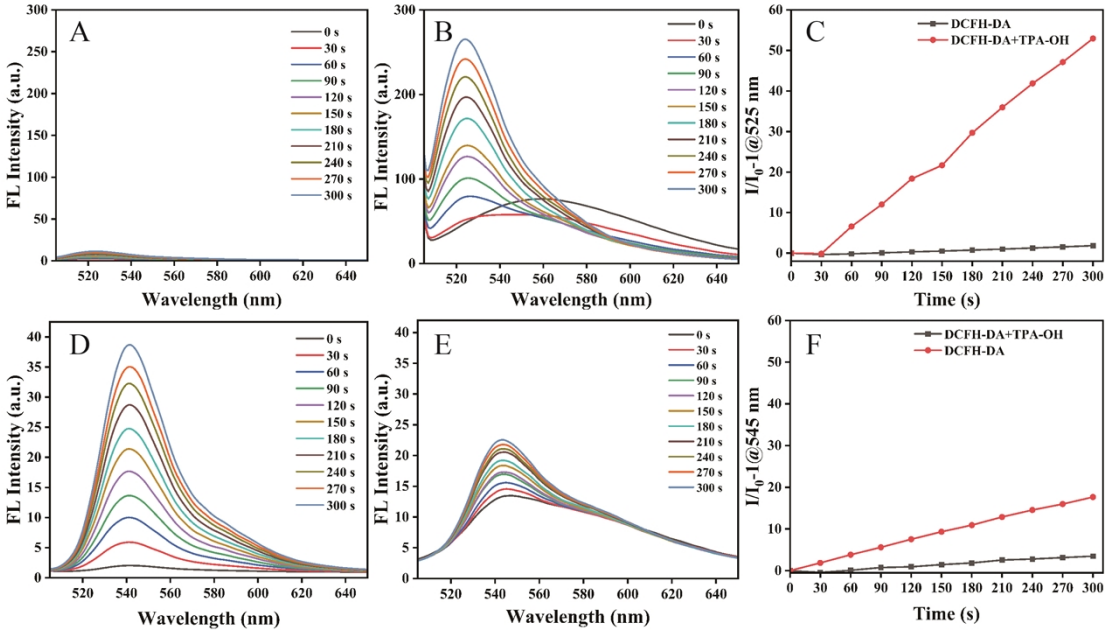


Fig. S7 Fluorescence spectra of DCFH-DA in DMSO/PBS (1:99, v/v), **(A)** Blank, **(B)** TPA-OH, **(C)** Plot of I/I_0-1 at 525 nm with light irradiation for different time. Fluorescence spectra of DCFH-DA in DMSO, **(D)** Blank, **(E)** TPA-OH, **(F)** Plot of I/I_0-1 at 545 nm with light irradiation for different time.

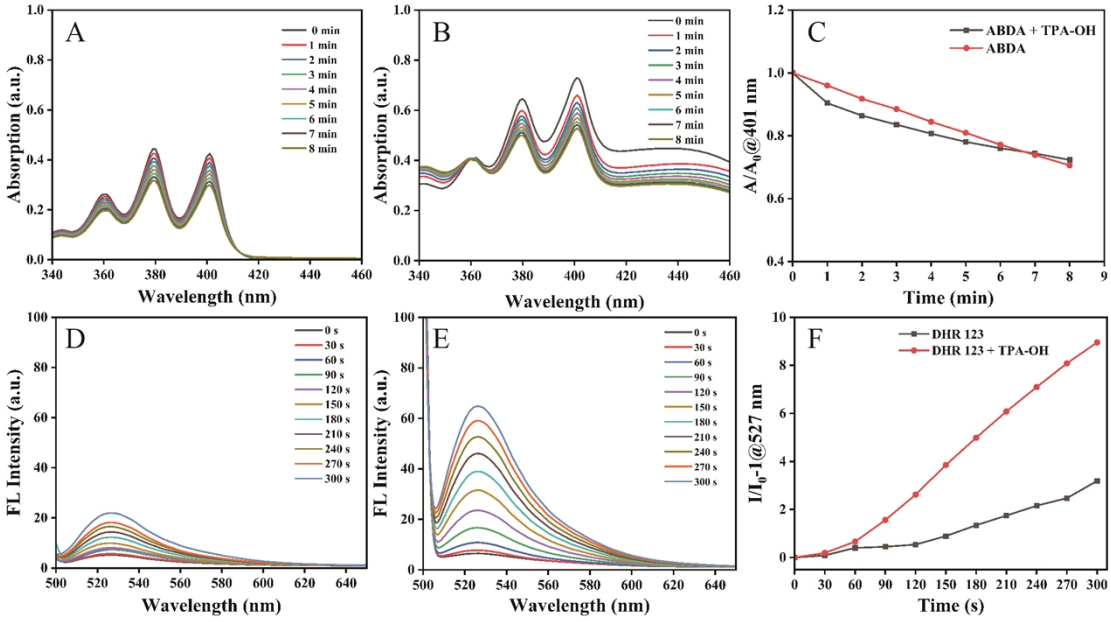


Fig. S8 UV absorption spectra of ABDA, **(A)** Blank, **(B)** TPA-OH, and **(C)** Plot of A/A_0 at 401 nm. FL

spectra of DHR 123, **(D)** Blank, **(E)** TPA-OH, and **(F)** Plot of I/I_0-1 at 527 nm.

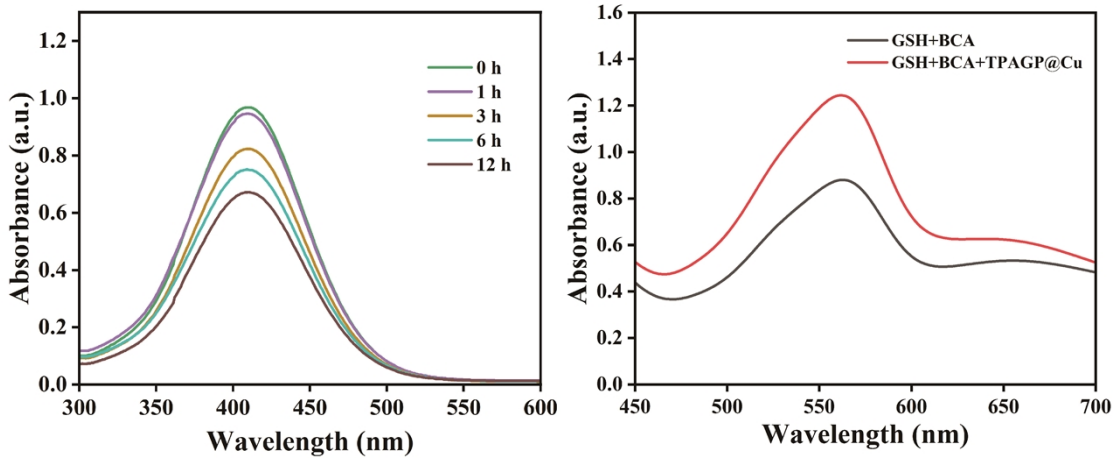


Fig. S9 (A) UV absorption spectra of DTNB solutions incubated with TPAGP@Cu and GSH for different time, **(B)** UV absorption spectra of GSH+BCA and GSH+BCA+TPAGP@Cu systems after 2 h of incubation.

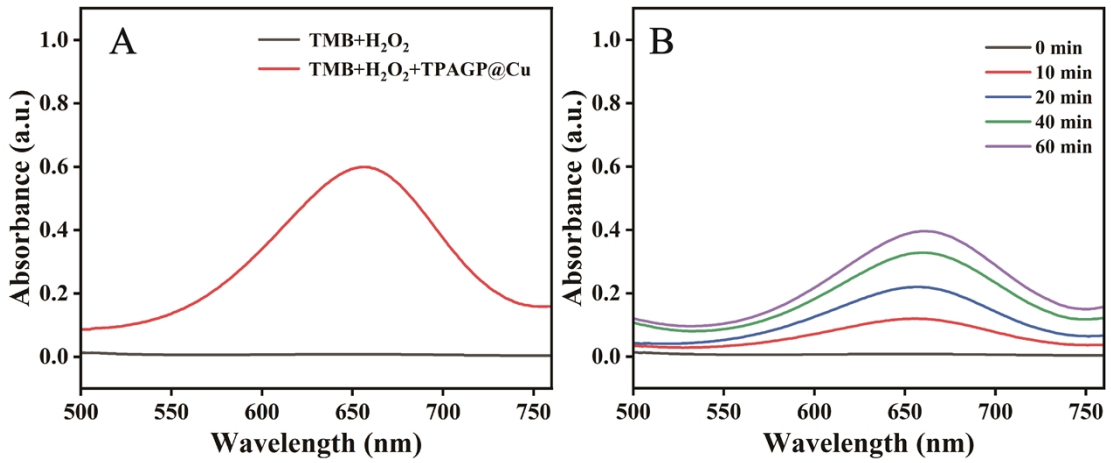


Fig. S10 (A) UV absorption spectra of mixture solution of TMB + H_2O_2 and TMB + H_2O_2 + TPAGP@Cu, **(B)** UV absorption spectra of mixture solution of TMB + H_2O_2 + TPAGP@Cu incubated at 37°C for different time.

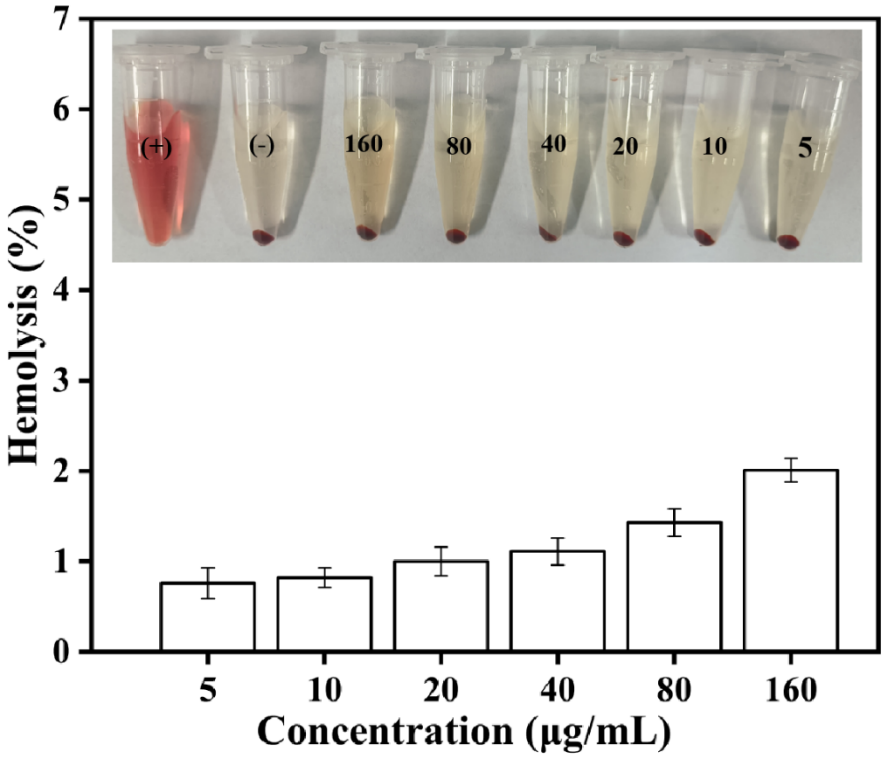


Fig. S11 *In vitro* hemolysis assay results for TPAGP@Cu nanoparticles at various concentrations. The hemolysis percentage is plotted against the concentration of nanoparticles ranging from 5 to 160 μg/mL. The positive control (+) shows complete hemolysis, while the negative control (-) indicates no hemolysis. Error bars represent standard deviation from three independent measurements. The photographic inset displays the visual comparison of hemolysis across different concentrations, where the intensity of color corresponds to the degree of hemolysis. Results demonstrate that TPAGP@Cu nanoparticles exhibit low hemolytic activity, suggesting good biocompatibility at the tested concentrations.

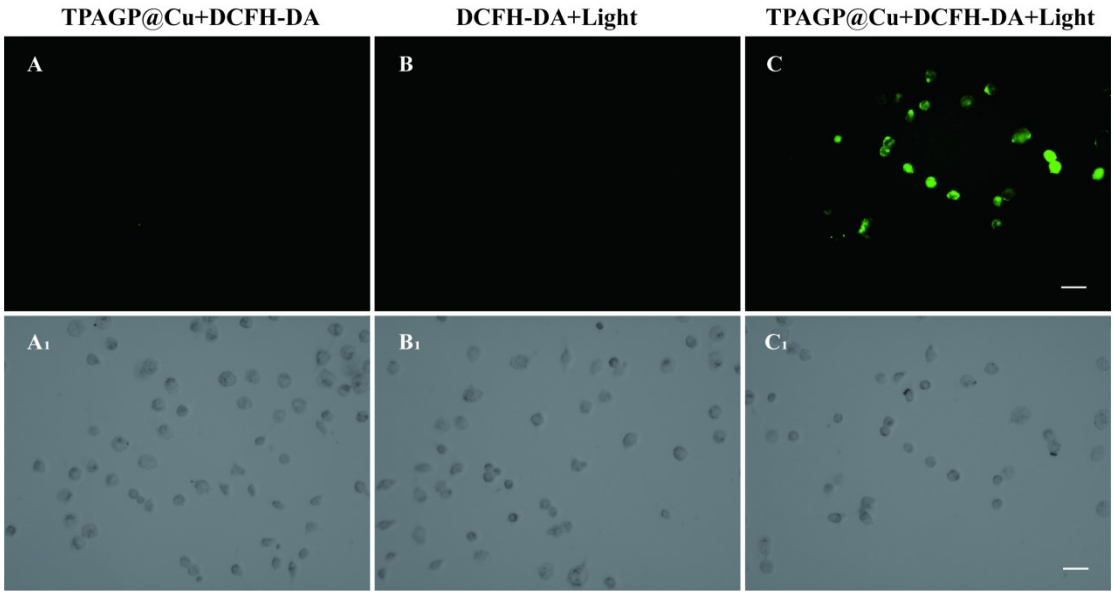


Fig. S12 Intracellular total ROS detection using DCFH-DA fluorescence. The images depict the fluorescence microscopy results for cells treated with DCFH-DA under different conditions: (**A**) TPAGP@Cu nanoparticles plus DCFH-DA without light exposure; (**B**) DCFH-DA with light exposure alone; (**C**) TPAGP@Cu nanoparticles plus DCFH-DA with light exposure. The corresponding bright-field images ($\mathbf{A_1}$, $\mathbf{B_1}$, $\mathbf{C_1}$) show the cell morphology under each condition. Scale bar represents 50 μm.

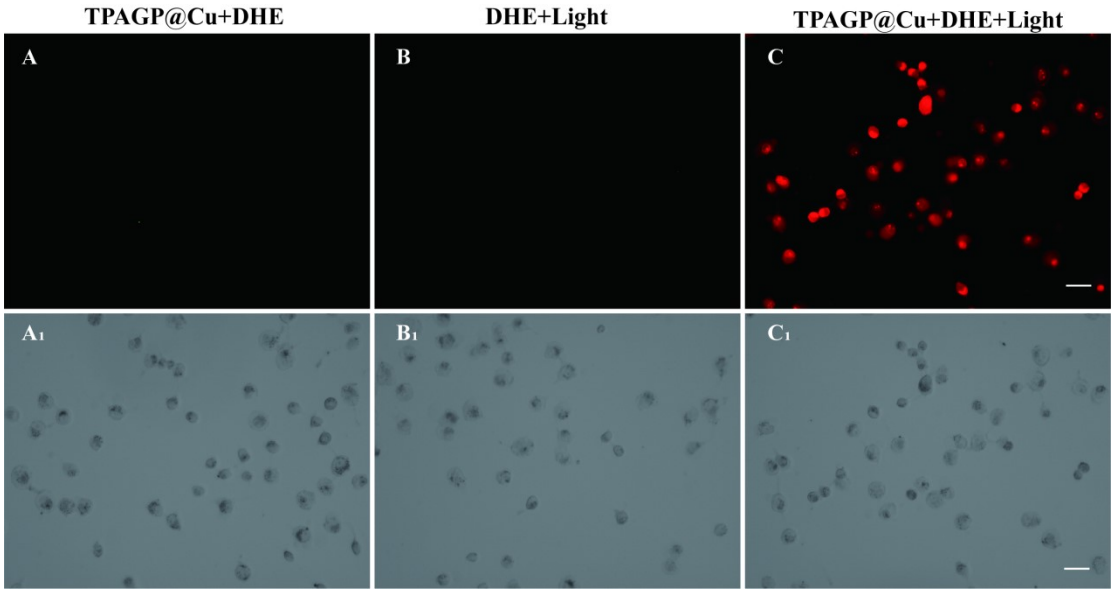


Fig. S13 Fluorescence imaging of cells stained with DHE for ROS detection. The cells were treated with TPAGP@Cu + DCFH-DA, DCFH-DA + Light and TPAGP@Cu + DCFH-DA + Light, respectively. Scale bar = 50 μm.

Table. S1 ICP-OES data for the determination of copper content in TPAGP@Cu.

Parameter	Value
Sample mass m_0 (g)	0.12
Final volume V_0 (mL)	10
Test element	Cu
Element concentration in test solution C_0 (mg/L)	2.3684
Dilution factor f	100
Element concentration in digestion solution C_1 (mg/L)	536.838
Element content in sample C_x (mg/kg)	44736.5
Element content in sample W (%)	4.4736