ROS-accelerated quinone formation from gossypol in a dendritic AIE polymer enables synergistic Type I photodynamic therapy and chemotherapy

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1 Experiments section

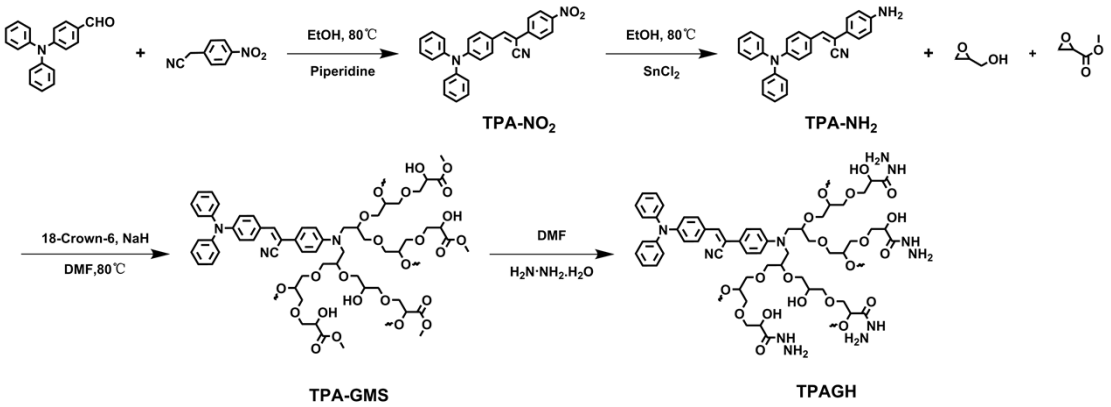
1.1 Reagents and equipment

The MDA-MB-231 cell line was obtained from the Cell Resource Center, Shanghai Institute of Life Sciences, Chinese Academy of Sciences. Calcein-AM/PI staining kit was purchased from Beijing Solarbio Science & Technology Co., Ltd. CCK-8 assay kit was acquired from Beyotime Biotechnology. Glycidol, methyl glycidyl, epichlorohydrin, piperidine, hydrazinium hydroxide, gossypol, sodium hydride (NaH), tin(II) chloride ($SnCl_2$), sodium bicarbonate ($NaHCO_3$), 18-Crown-6, 4-(diphenylamino)-benzaldehyde, 4-nitrophenylacetonitrile, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), hydrazine hydrate, 2,2'-(anthracene-9,10-diylbis(methylene))-dimalonic acid (ABDA), and dihydrorhodamine 123 (DHR 123) were purchased from Aladdin Chemical Reagent Co., Ltd (Shanghai, China). All other reagents and solvents were obtained from Sinopharm Group (Shanghai, China) and were of analytical grade, used without further purification. Absorption and emission spectra were recorded using a Purkinje General UV-1700 spectrophotometer and a PerkinElmer LS-55 fluorescence spectrophotometer, respectively. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were acquired on an Agilent DD2400-MR spectrometer. Infrared (IR) spectroscopy was performed on a Thermo Nicolet 380 spectrometer. Dynamic light scattering (DLS) and zeta potential measurements were conducted using a Malvern Zetasizer Nano ZS90.

1.2 Synthesis

The synthesis of TPA-NO_2, TPA-NH_2, TPA-GMS and TPAGH is illustrated in **Scheme S1**. TPA-NO_2 was prepared as follows: A mixture of 4-(diphenylamino)benzaldehyde (1.36 g, 5 mmol) and 4-nitrophenylacetonitrile (0.81 mL, 5 mmol) in ethanol (25 mL) was charged into a 50 mL round-bottom flask. Piperidine (0.05 mL, 0.05 mmol) was then added as a catalyst. The reaction was stirred at 80 °C for 4 h, cooled to room temperature, and the resulting solid was collected by filtration. The crude product was washed with ethanol (3 × 5 mL) and dried at 50 °C to afford TPA-NO_2 as a pure solid (1.58 g) in 76% yield. ^{1}H NMR (400 MHz, DMSO-6*d*) δ 8.28 (d, *J* = 8.6 Hz, 2H), 8.10 (s, 1H), 7.91 (dd, *J* = 23.4, 8.6 Hz, 4H), 7.39 (t, *J* = 7.6 Hz, 4H), 7.25 – 7.10 (m, 6H), 6.91 (d, *J* = 8.5 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-6*d*) δ 150.79, 147.13, 146.12, 145.90, 141.25, 131.95, 130.36, 126.69, 126.40, 125.59, 124.69, 119.61, 118.42, 104.10, 41.21, 40.63, 40.32, 40.01, 39.80, 39.59, 39.38. ESI-MS m/z cald $[M + H]^+$: 418.30, found : 418.15. Synthesis of TPA-NH_2: A mixture of TPA-NO_2 (835 mg, 2 mmol) and $SnCl_2$ (1.9 g, 10 mmol) in ethanol (30 mL) was stirred at 78 °C for 1 h in a 50 mL round-bottom flask. After cooling to room temperature, the reaction was quenched by adding saturated $NaHCO_3$ solution (100 mL) and

adjusting the pH to weak alkalinity. The resulting mixture was extracted with CH_2Cl_2 (3 × 20 mL), and the combined organic phases were concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using a mixture of petroleum ether and CH_2Cl_2 (1:3, v/v) as the eluent to afford TPA-NH_2 (0.775 g, 64% yield). ^{1}H NMR (400 MHz, DMSO-*d*6) δ 7.80 - 7.67 (m, 2H), 7.53 (d, *J* = 18.1 Hz, 1H), 7.42 - 7.22 (m, 5H), 7.21- 6.87 (m, 8H), 6.74 - 6.48 (m, 3H), 5.51 (d, *J* = 17.8 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$-*d*) δ 156.22, 146.82, 140.12, 130.47, 129.61, 127.37, 125.71, 125.27, 124.36, 121.23, 116.02, 84.30, 79.26, 75.16. ESI-MS m/z cald $[M + H]^+$: 388.20, found : 388.17. Synthesis of compound 3: Firstly, TPA-NH_2 (0.5 g, 1.3 mmol), 18-crown-6 (0.4 g, 1.5 mmol) and NaH (0.2 g, 8.3 mmol) were added to a 50 mL round-bottomed flask with 30 mL DMF. The mixture was stirred at room temperature for 1 h, then heated to 80 °C. Subsequently, 2 mL of epichlorohydrin was slowly added. After reacting for 1 h, 1 mL of epichlorohydrin was further added, and finally, the mixture was reacted at 80 °C for 18 h. The mixture was cooled to room temperature, and the product was dialyzed against deionized water and ethanol for 2 days using a dialysis bag (1000 Da). The final product was dried in the vacuum drying oven at 50 °C for 24 h to obtain TPA-GMS. Synthesis of TPAGH: TPA-GMS (0.3 g) was added to a 50 mL round-bottomed flask with 30 mL DMF, then 3 mL of hydrazine monohydrate was added to react at room temperature for 24 h. The mixture was dialyzed against deionized water and ethanol solution for 2 days using a dialysis bag (1000 Da). The dialyzed final product was dried in the vacuum drying oven at 50 °C for 24 h to obtain TPAGH.



Scheme S1 Schematic route for synthesis of TPA-NO_2, TPA-NH_2, TPA-GMS, and TPAGH.

1.3 Loading gossypol on TPAGH

The procedure for loading gossypol on TPAGH was described as follows: TPAGH (10 mg) was added to a 50 mL round-bottomed flask with 18 mL of buffer (PBS, pH = 7.2). Gossypol (5 mg) was added in

2 mL of ethanol, then, the mixture was added to the 50 mL round-bottomed flask. The reaction system was stirred for 24 h in dark. The mixture was carried out at a rotation speed of 9000 rpm for 30 min. The content of gossypol in TPAGH was determined by UV-Vis at 368 nm. The drug-loading capacity (DLC, wt%) and drug-encapsulation efficiency (DLE, wt%) of the TPAGH were calculated according to the following formulas:

$$DLC: W0/W1 \times 100\% \quad (1)$$

$$DLE: W0/W2 \times 100\% \quad (2)$$

Where W0 is the mass of encapsulated gossypol, W1 is the total mass of TPAGH and gossypol, W2 is the total mass of gossypol actually added.

1.4 Determination of drug release curves

The release curves of gossypol were determined by the dialysis method. The TPAGH-GS (5 mg) and 5 mL of PBS (pH = 7.2, pH = 5, respectively) were added to dialysis bags (1000 Da). Then, the dialysis bags were placed in beaker with PBS (pH = 7.2, pH = 5, respectively), and were added respectively for stirring (100 rpm). According to the standard curve of gossypol (368 nm), the mass of gossypol released was determined by UV-Vis in series of time interval (3 min). The cumulative release amount of gossypol was calculated according to the following formula.

$$Er: (Ve\Sigma_{1}^{n-1}Ci + \mathrm{V0Cn})/\mathrm{m} \quad (3)$$

Where Er is the cumulative release amount of gossypol (%), Ve is the volume for sample (mL), V0 is the total volume of gossypol released in the solution (mL), Ci is the concentration of gossypol measured (g/mL), m is the total drug content in sample (g), n is the number of samplings.

1.5 Determination of ROS generation in solution

The generation of ROS was assessed using specific fluorescent probes prepared according to established methods [1]. Briefly, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, 1 μM) was employed to detect total ROS; 2,2'-(anthracene-9,10-diylbis(methylene))-dimalonic acid (ABDA, 60 μM) was used for singlet oxygen ($^1O_2$) detection; and dihydrorhodamine 123 (DHR123, 1 μM) was applied for free radical measurement.

1.6 Cell viability assay

MDA-MB-231 cells were seeded into 96-well plates and incubated for 24 h under standard culture conditions. Subsequently, various concentrations of TPAGH and TPAGH-GS were introduced into the

wells, followed by further incubation at 37 °C for 24 h. After treatment, the cells were washed three times with PBS buffer. Then, 100 μL of CCK-8 solution (diluted to 10% in medium) was added to each well, and the plates were incubated at 37 °C for 2 h. The absorbance at 450 nm was measured using a Tecan GENios microplate reader. Cell viability was calculated by comparing the absorbance of treated wells with that of control wells. To evaluate phototoxicity, MDA-MB-231 cells were incubated with TPAGH for 6 h, exposed to 450 nm light for 3 min, and then further cultured for 18 h. Cell viability was assessed under the same CCK-8 protocol as described for dark conditions.

1.7 CLSM imaging

The cellular uptake behavior of TPAGH-GS was evaluated using CLSM, leveraging its intrinsic fluorescent properties. In details, MDA-MB-231 cells were first seeded and incubated under standard culture conditions for 24 h. The cells were then treated with TPAGH-GS at a concentration of 20 μg/mL and incubated for 4 h. Subsequently, the cells were washed three times with PBS to remove uninternalized nanoparticles and fixed with 4% paraformaldehyde at room temperature for 10 min. Cellular imaging was performed using a CLSM system with an excitation wavelength of 405 nm.

1.8 Staining of live/dead cells

MDA-MB-231 cells were seeded into 6-well plates and cultured under standard conditions for 24 h. Subsequently, the cells were treated with TPAGH/TPAGH-GS at a concentration of 80 μg/mL and further incubated for 6 h. Following this, the cells were exposed to 450 nm light for 3 min, washed three times with PBS (pH = 7.2), and then incubated for an additional 24 h. After the extended incubation, the cells were stained with 4 μM propidium iodide (PI) and 2 μM Calcein acetoxymethyl ester (Calcein-AM) for 30 min. Live and dead cells were distinguished based on their fluorescence: live cells were labeled with Calcein-AM with green fluorescence, while dead cells were stained with PI with red fluorescence. Cells that did not undergo light irradiation were used as the control group.

1.9 Detection of intracellular ROS

The intracellular generation of ROS was assessed using fluorescent probes DCFH-DA and dihydroethidium (DHE). DCFH-DA, a cell-permeable probe, is hydrolyzed by intracellular esterases to non-fluorescent DCFH, which is subsequently oxidized by various ROS (such as ·OH, H_2O_2, and $ONOO^-$) to yield highly fluorescent dichlorofluorescein (DCF), emitting green fluorescence. Meanwhile, DHE specifically reacts with superoxide anion ($O_2^{\bullet-}$) to form ethidium, which intercalates into DNA and produces bright red fluorescence. Together, these probes enable quantitative and qualitative evaluation of general

ROS and $O_2^{\bullet-}$ production, respectively, under light-induced photodynamic conditions. All experimental procedures were conducted in accordance with established methodologies described in references [27, 28].

1.10 Hemolysis assay

The hemocompatibility of TPAGH-GS was evaluated through a hemolysis assay to assess its potential to cause red blood cell (RBC) lysis. Fresh human whole blood (obtained from healthy volunteers with informed consent and ethical approval) was anticoagulated with heparin and centrifuged at 1500 rpm for 10 min to isolate RBCs. The RBCs were then washed three times with sterile phosphate-buffered saline (PBS, pH 7.4) and diluted to a 2% (v/v) suspension in PBS. Various concentrations of TPAGH (e.g., 5, 10, 20, 40, 80, and 160 μg/mL) were incubated with 500 μL of the 2% RBC suspension at 37 °C for 2 h. Positive control (100% hemolysis) was prepared by adding RBCs to distilled water, while negative control (0% hemolysis) consisted of RBCs incubated with PBS alone. After incubation, the samples were centrifuged at 3000 rpm for 10 min, and the absorbance of the supernatant was measured at 540 nm using a UV-Vis spectrometer. The hemolysis ratio was calculated using the following formula:

$$\text{Hemolysis (\%)} = (\text{As} - \text{An}) / (\text{Ap} - \text{An}) \times 100\%$$

where As, An, and Ap represent the absorbance values of the test sample, negative control, and positive control, respectively. A hemolysis ratio below 5% is generally considered indicative of good hemocompatibility for biomedical applications.

2 Results

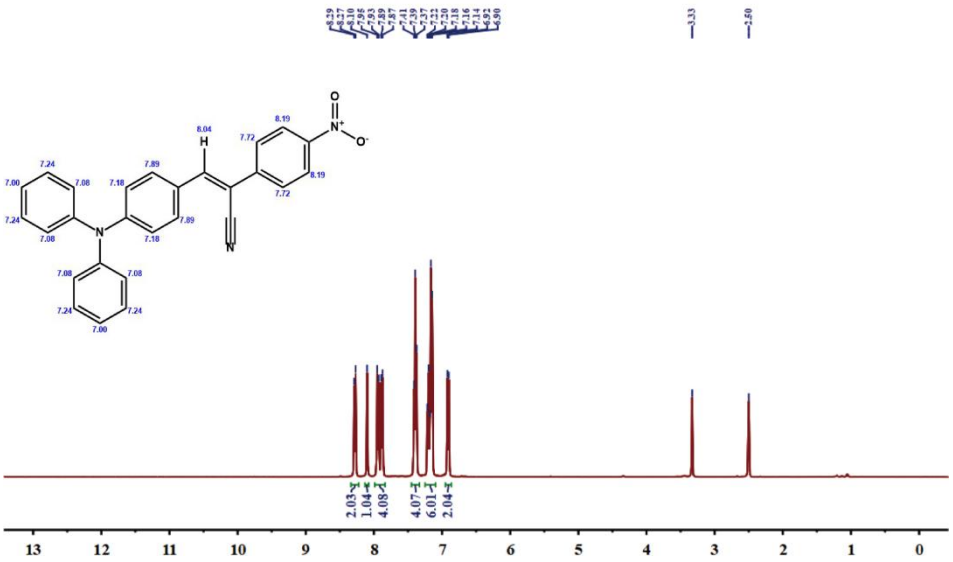


Fig. S1 ^{1}H NMR spectrum of TPA-NO_2 in DMSO-*d6*.

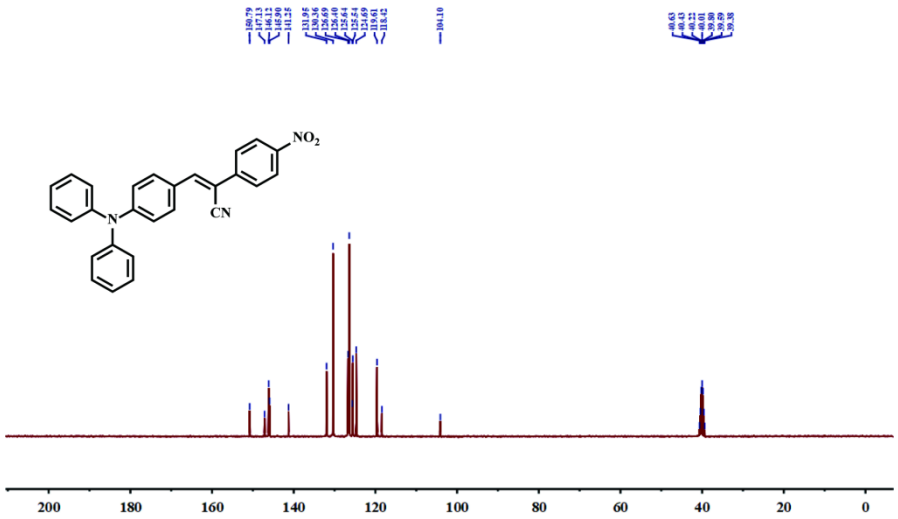


Fig. S2 ^{13}C NMR spectrum of TPA-NO_2 in DMSO-*d6*.

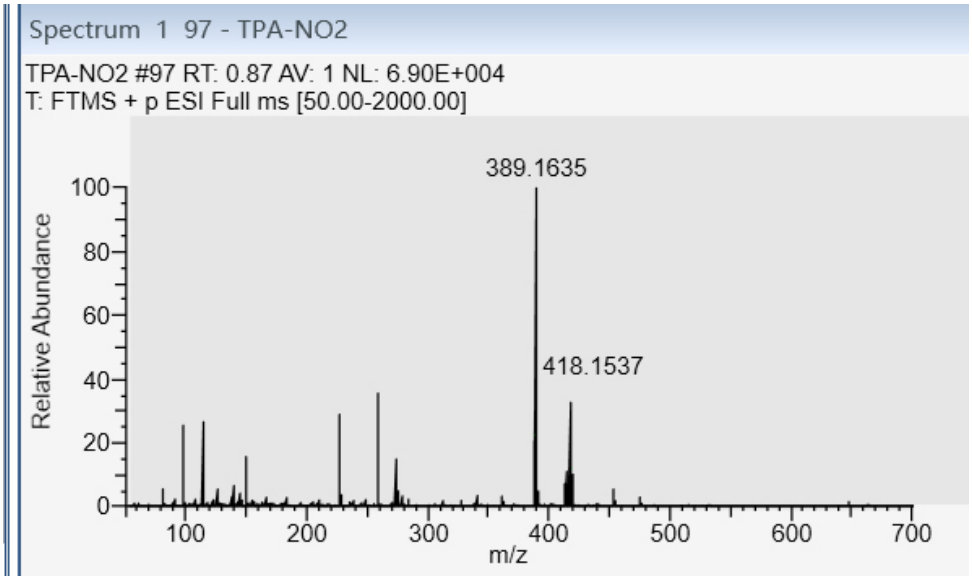


Fig. S3 The FT-MS of TPA-NO_2.

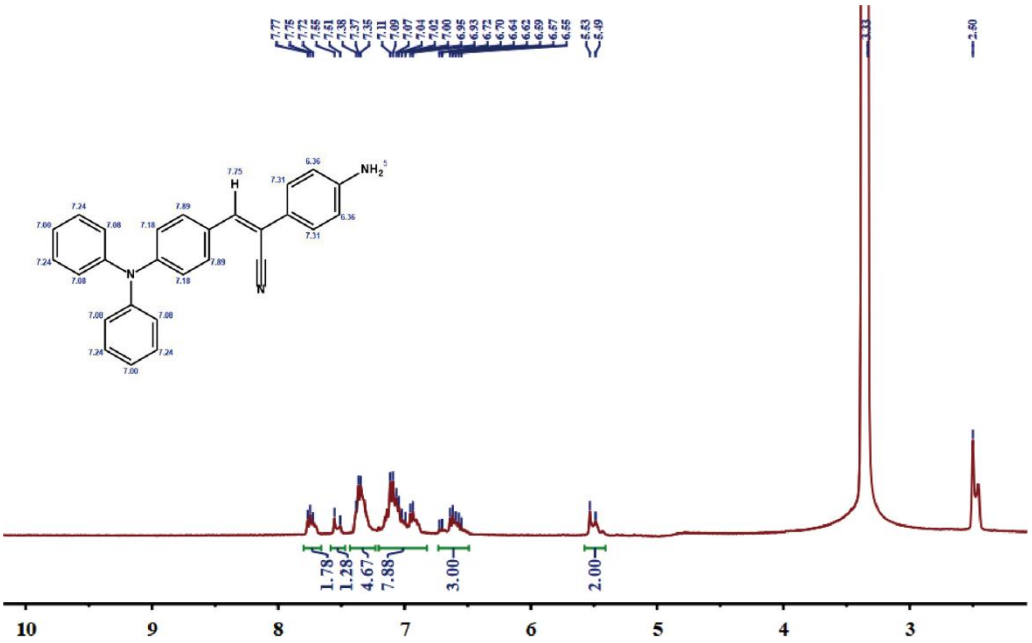


Fig. S4 ^{1}H NMR spectrum of TPA-NH_2 in DMSO-*d6*.

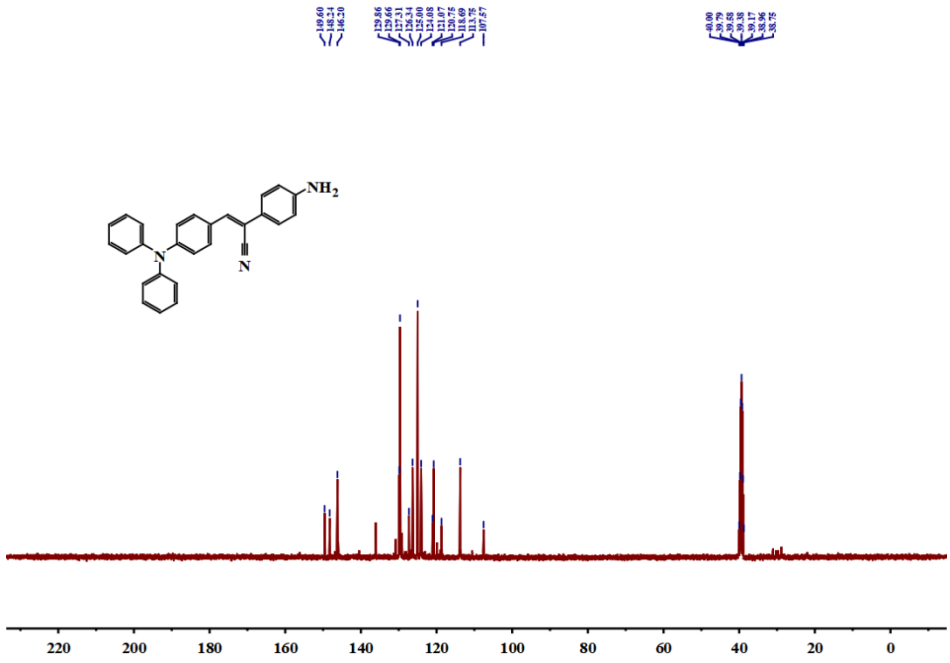


Fig. S5 ^{13}C NMR spectrum of TPA-NH_2 in DMSO-*d6*.

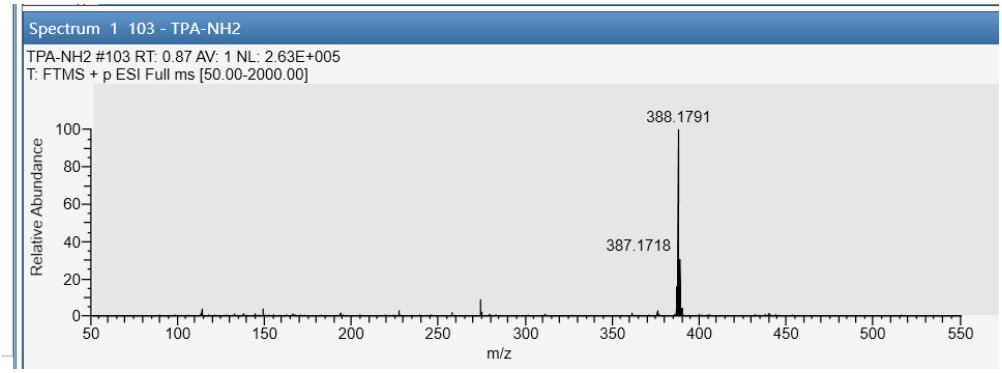


Fig. S6 The FT-MS of TPA-NH_2.

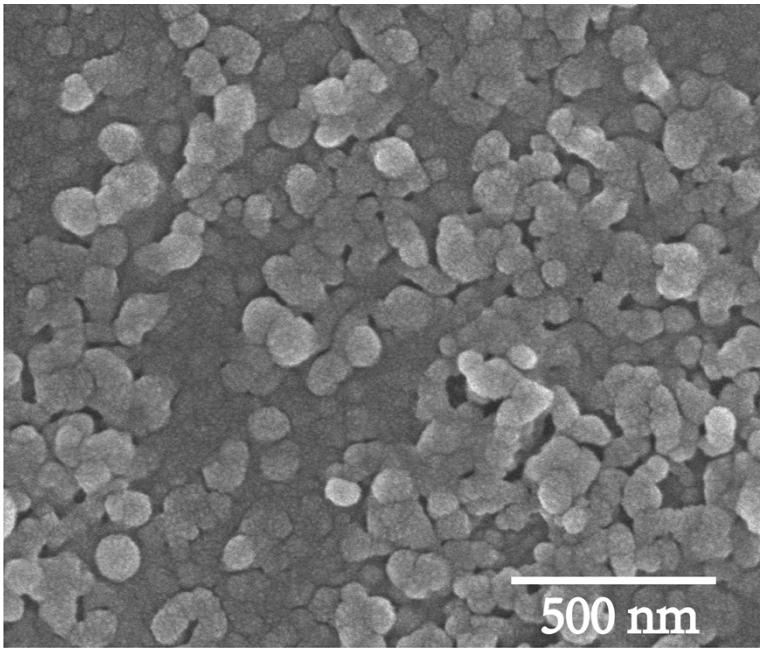


Fig. S7 The SEM of TPAGH.

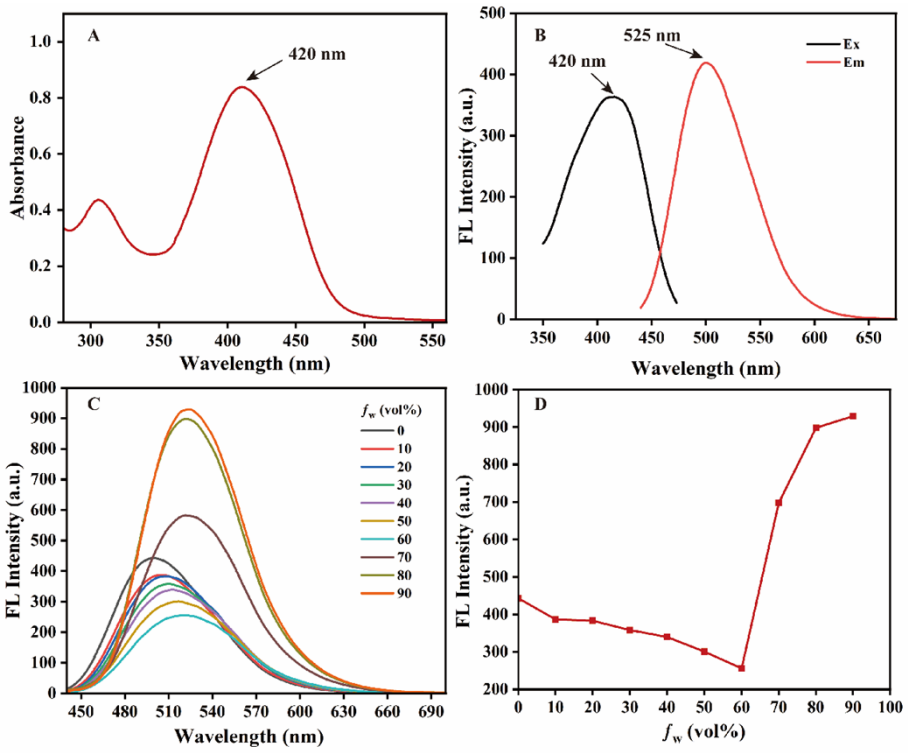


Fig. S8 (**A**) Normalized absorptions; (**B**) excitation and emission spectra of TPA-NH_2; (**C**) fluorescence emission spectra under different water fractions; (**D**) plot of fluorescence intensity versus water fraction (f_w).

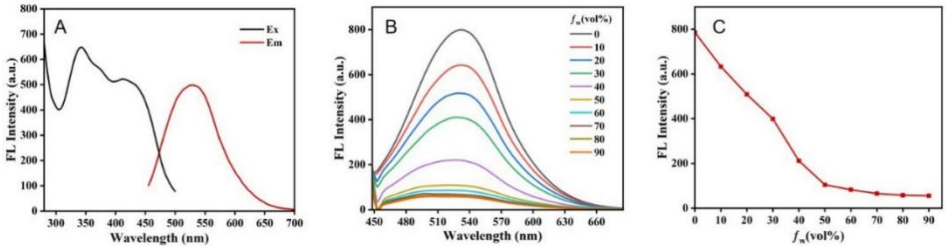


Fig. S9 (**A**) Excitation and emission profiles of TPAGH; (**B**) Fluorescence intensity of TPAGH at different water contents; (**C**) Variation of fluorescence intensity of TPAGH with respect to water content.

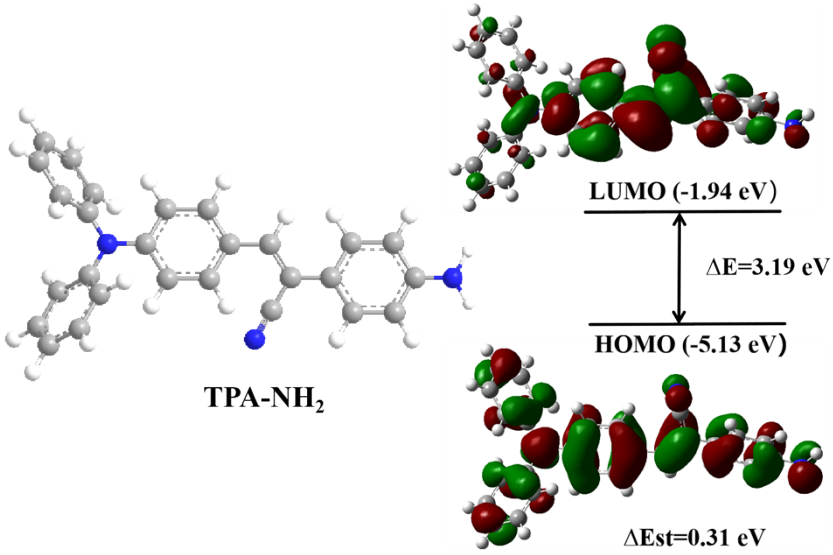


Fig. S10 (**A**) 3D structure of TPA-NH_2 (left) and (**B**) molecular orbital distribution of HOMO-LUMO and their energy gap (ΔE) and (ΔEst) values (right).

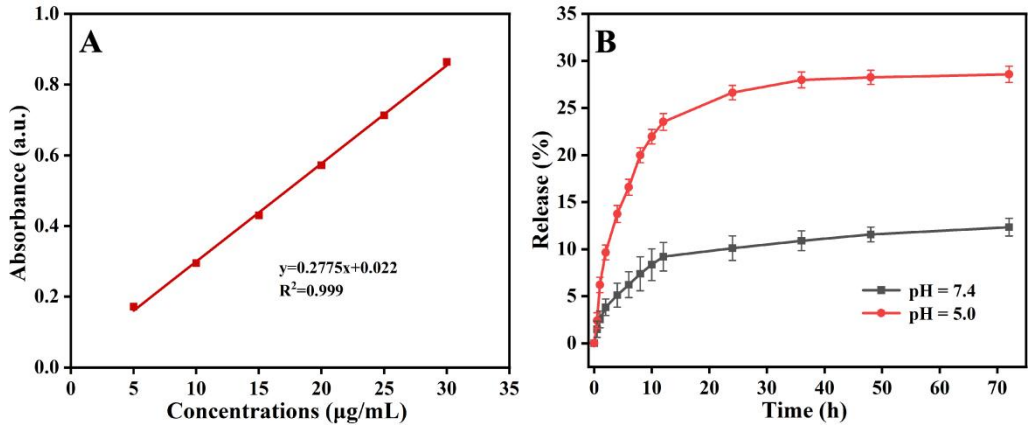


Fig. S11 (**A**) Absorption of different concentrations (5 μg/mL, 10 μg/mL, 15 μg/mL, 20 μg/mL, 25 μg/mL, 30 μg/mL) of gossypol at 368 nm. (**B**) Drug release capacity of TPAGH-GS in different pH (pH = 5, pH = 7).

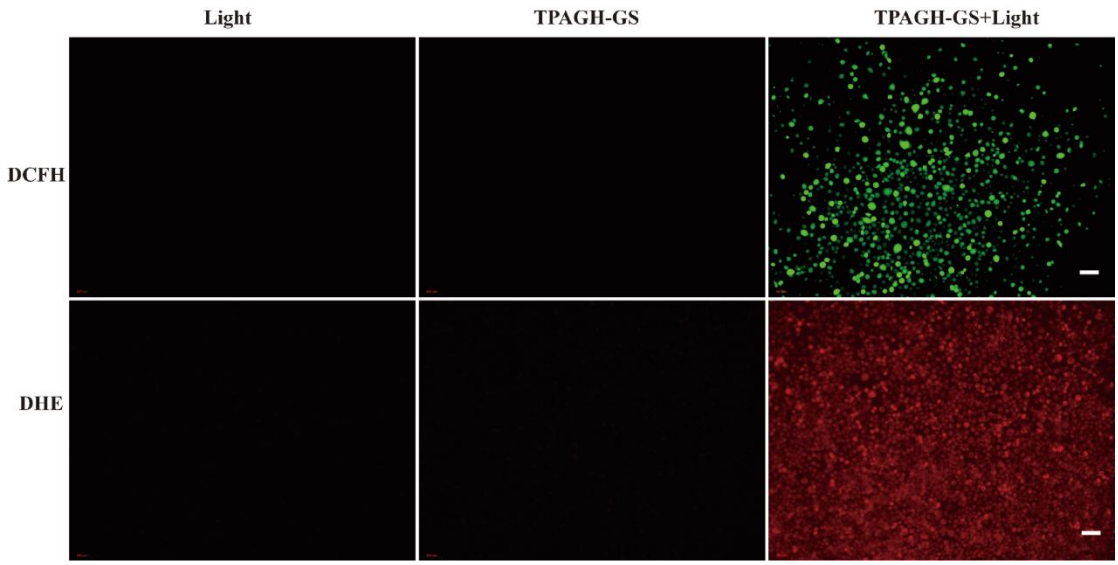


Fig. S12 Cells were fluorescently imaged with DCFH-DA (1×10^{-5} M) and DHE (1×10^{-5} M) as total cellular ROS and free radical-type ROS indicator, respectively. Concentration: TPAGH-GS (40 μg/mL); light irradiation at 450 nm (5 mW/cm^2) for 3 min, scale bar = 100 μm.

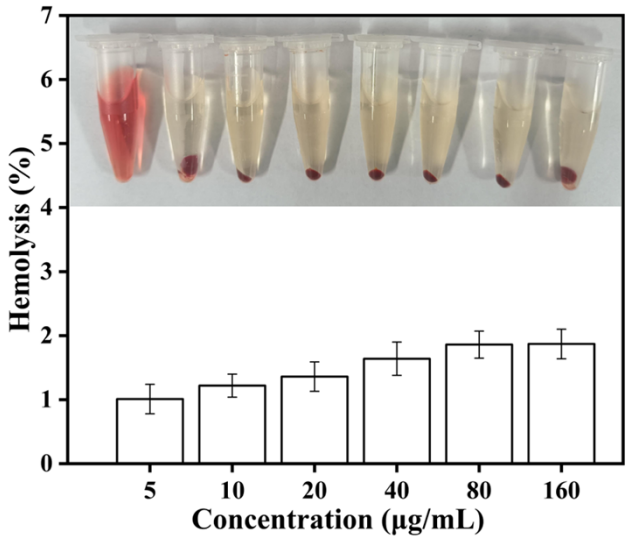


Fig. S13 Hemolysis rate of red blood cells after incubation with different concentrations of TPAGH-GS.

References

1. Z. Zou, D. Liu, J. Wan, K. Zhang, M. Liu, S. Huang, J. Tian, X. Zhang and Y. Wei, *Chem. Commun.*, 2025, **61**, 13189-13192; Z. Zou, Y. Xie, J. Wan, Q. Wan, J. Tian, X. Zhang and Y. Wei, *Chem. Commun.*, 2025, **61**, 500-503.