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Supplementary Information

A Disulfide-induced Supra-amphiphilic Co-assembly for Glycosylated Pro-drug-photosensitizer Nanoparticles in Combination Therapies

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Experimental section

Materials and instruments

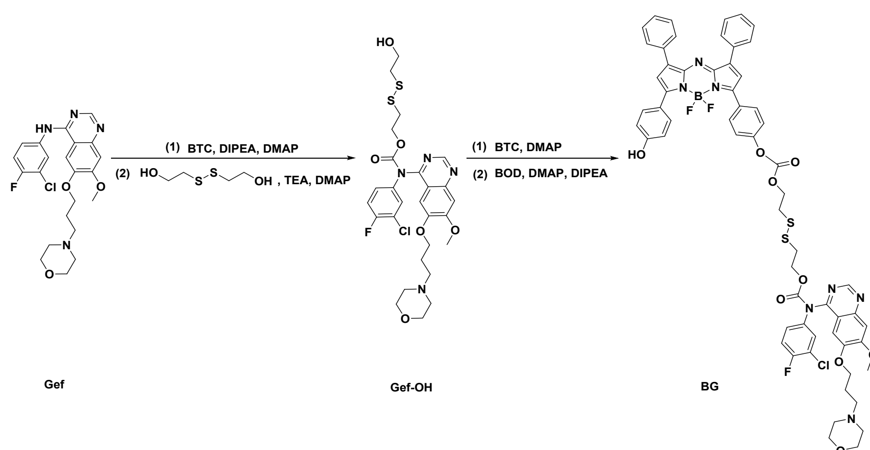
Materials. All reagents were purchased from commercial suppliers and used without further purification unless specified. Ammonium acetate, diethylamine, benzaldehyde, 4'-hydroxy acetophenone, nitromethane, boron trifluoride etherate and N,N-diisopropylethylamine (DIPEA) were purchased from Aladdin Chemical Reagent Co., 4-Dimethylaminopyridine (DMAP), triphosgene (BTC) 1,3-diphenylisobenzofuran (DPBF) were purchased from Energy Chemical Reagent Co., Gefitinib and lactose were purchased from Rhawn Chemical Reagent Co., 2-(2-(2-Chloroethoxy)ethoxy)ethanol was purchased from Tianjin Xiensi Biochemical Technology Co., Potassium thioacetate was purchased from Alfa Aesar (Shanghai, China) Chemical Reagent Co. Annexin V-FITC/PI apoptosis detection kit was purchased from Shanghai Yeasen Biotech Co., Ltd. (Shanghai, China). Human non-small cell lung cancer cells (A549), human hepatoma cells (HepG2), human cervical carcinoma cells (Hela), and human liver cells (HL7702) were purchased from KeyGEM BioTECH Co., Ltd. (Nanjing, China).

Instruments. High resolution ion mobility liquid chromatography-mass spectrometry (HRLCMS) (LC-30A+TripleTOF5600+, AB SCIEX). 400 MHz/ 500 MHz for ^{1}H, ^{13}C nuclear magnetic resonance (NMR) spectrometer (Bruker, German). The residual signals from DMSO-d_6 (^{1}H: δ 2.50 ppm), $CDCl_3$ (^{1}H: δ 7.26 ppm) or D_2O (^{1}H: δ 4.79 ppm) were used as internal standards. UV-Vis spectra were collected by Shimadzu 1750 UV-Visible spectrometer (Japan), and fluorescence spectra were collected by Hitachi F-7000 Fluorescence spectrophotometer (Japan). Dynamic light scattering (DLS) measurements were performed on a MALVERN Nano system (ZEN3600). Transmission electron microscopy (TEM) images were obtained from FEI TECNAI G2 SPIRIT BIO., Scanning electron microscope（SEM） images were obtained from FEI Nova Nano SEM-450. Confocal laser scanning microscopy (CLSM) data were obtained by CLSM (Andor REVOLUTION WD). Flow cytometry (FCM) data were obtained from BD FACSAria™ III. Liquid chromatography-mass spectrometry (LCMS) spectrums were obtained from High-resolution ion mobility liquid chromatography-mass spectrometry (HRLCMS LCMS, AB SCIEX, TripleTOF 5600+), High-performance liquid chromatography purity and drug release analysis were obtained from HPLC (Agilent, 1260).

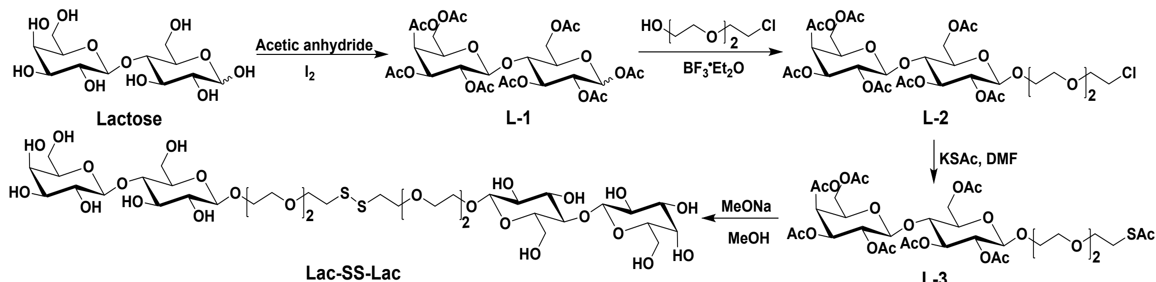
Synthesis of Compound BG

BG was synthesized referring to previously published papers[1-2]. In brief, Gef (0.116 g, 0.26 mmol), DIPEA (0.09 mL, 0.52 mmol), DMAP (0.016 g, 0.13 mmol), BTC (0.154 g, 0.52 mmol) were added into the anhydrous dichloromethane (DCM) in ice bath, under the argon protected, and reacted at room temperature for 6 h. Then, removed the solvent and dissolved in anhydrous tetrahydrofuran (THF), 2-hydroxyethyl disulfide (0.160 g, 1.04 mmol), triethylamine (0.210 g, 2.08 mmol) and DMAP (0.016 g, 0.13 mmol) were added dropwise to the THF solution, stirred under nitrogen protection at 0 °C for 72 h. The crude product was purified by silica gel column chromatography (EA: MeOH = 40:1, V:V), obtaining a white viscous solid, mark as **Gef-OH** (106 mg, yield 65%).^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 8.99 (s, 1H), 7.79 (s, 1H), 7.43 - 7.36 (m, 3H), 7.24 (s, 1H), 4.85 (s, 1H), 4.39 – 4.36 (t, J = 6.2 Hz,

2H), 4.17 – 4.15 (t, J = 6.4 Hz, 2H), 4.00 (s, 3H), 3.56 – 3.51 (m, 6H), 2.90 – 2.87 (t, J = 6.2 Hz, 2H), 2.70 – 2.67 (t, J = 6.3 Hz, 2H), 2.44 – 2.41 (t, J = 7.2 Hz, 2H), 2.35 (s, 4H), 1.93 – 1.90 (t, J = 6.8 Hz, 2H). Gef-OH (0.094 g, 0.15 mmol), DMAP (0.006 g, 0.045 mmol), BTC (0.090 g, 0.30 mmol) were added into the anhydrous DCM and stirred under nitrogen protection at 0 °C for 1 h. Then, added the DCM solution into the BOD[2] (0.106 g, 0.30 mmol), DIPEA (0.018 g, 0.15 mmol) and DMAP (0.006 g, 0.045 mmol) contained anhydrous THF solution. The mixed solution was stirred under nitrogen protection at room temperature for 16 h. The crude product was purified by silica gel column chromatography (EA: MeOH = 40:1, V: V) obtaining a blackish green solid, mark as **BG** (69 mg, yield 41%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 9.11 (s, 1H), 8.34-8.19 (m, 8H), 7.91 (s, 2H), 7.61-7.48 (m, 12H), 7.37 (s, 1H), 7.05-7.03 (d, J = 8.0 Hz, 2H), 4.53-4.49 (m, 4H), 4.29-4.26 (t, J = 8.0 Hz, 3H), 4.15-4.09 (m, 2H), 3.67-3.65 (t, J = 4.0 Hz, 5H), 3.10-3.06 (m, 4H), 2.61-2.55 (m, 4H), 2.09 (s, 3H).



Scheme 1. Synthetic route of **BG**.



Scheme 2. Synthetic route of **Lac-SS-Lac**

Synthesis of Compound L-1

The synthesis referred to the methods described in the literature[3]. I_2 (0.28 g, 1.11

mmol) was added to about 50 mL of acetic anhydride, stirred at room temperature until dissolved, then added the lactose (6.0 g, 16.65 mmol) and stirred at room temperature for 2 h. After the reaction, add about 50 mL of distilled water, and gradually add sodium thiosulfate to the solution under stirring until the solution is colorless. Then add dichloromethane (50 mL) and sodium bicarbonate in batches, stirring until there are no bubbles, extract with dichloromethane and distilled water, the organic phase dried with anhydrous sodium sulfate, remove the solvent under reduced pressure and obtain white crystalline powder **L-1** (8.67 g, 76.7%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 6.25-6.24 (d, J = 4.0 Hz, 1H), 5.48-5.43 (t, J = 10.4 Hz, 1H), 5.36-5.30 (d, J = 4.0 Hz, 1H), 5.14-5.10 (m, 1H), 5.02-4.94 (m, 2H), 4.48-4.43 (m, 2H), 4.17-4.06 (m, 3H), 4.02-3.98 (m, 1H), 3.89-3.79 (m, 2H), 2.18 (s, 3H), 2.16 (s, 3H), 2.13 (s, 3H), 2.06-2.05 (m, 9H), 2.01 (s, 3H), 1.96 (s, 3H).

Synthesis of Compound L-2

L-1 (1.320 g, 1.94 mmol) and 2-chloroethoxy-2-ethoxydiethanol (0.388 g, 2.30 mmol) were dissolved in 10 mL of dry dichloromethane, boron trifluoride ether solution (2.0 ml, 15.52 mmol) was added dropwise under the protection of N_2 and ice bath, stirred at room temperature for 24 h. Extracted with dichloromethane and sodium bicarbonate solution three times. The organic phase was dried with anhydrous sodium sulfate and purified by silica gel column chromatography (PE: EA = 1:1) to obtain the colorless oily compound **L-2** (397 mg, 26%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 5.40-5.35 (t, J = 9.6 Hz, 1H), 5.26 (s, 1H), 5.13-4.95 (m, 2H), 4.89-4.68 (m, 2H), 4.50-4.34 (m, 2H), 4.05-4.00 (m, 2H), 3.92-3.90 (d, J = 8.0 Hz, 1H), 3.82-3.79 (m, 1H), 3.68-3.64 (m, 4H), 3.60-3.55 (m, 10H), 2.07 (s, 3H), 2.04 (s, 3H), 1.97-1.96 (m, 12H), 1.87 (s, 3H).

Synthesis of Compound L-3

Compound **L-2** (0.280 g, 0.356 mmol) and potassium thioacetate (0.122 g, 1.068 mmol) were dissolved in 5 mL of dry DMF and stirred at room temperature under the protection of N_2 for 4 h. After the reaction, 20 mL of ethyl acetate was added and washed with distilled water, saturated sodium bicarbonate solution and saturated sodium chloride solution. The organic phase was dried with anhydrous sodium sulfate and purified by silica gel column chromatography (PE: EA = 1:1) to obtain the slight yellow oily compound **L-3** (210 mg, 70%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 5.49-5.45 (t, J = 9.6 Hz, 1H), 5.35-5.34 (m, 1H), 5.21-5.03 (m, 2H), 4.96-4.77 (m, 2H), 4.57-4.42 (m, 2H), 4.15-4.05 (m, 3H), 3.98-3.96 (m, 1H), 3.89-3.85 (m, 1H), 3.79-3.74 (m,

2H), 3.66-3.58 (m, 8H), 3.11-3.07 (m, 1H), 2.33 (s, 2H), 2.15 (s, 3H), 2.13 (s, 3H), 2.06-2.04 (m, 12H), 1.96 (s, 3H), 1.63 (s, 3H).

Synthesis of Compound Lac-SS-Lac

Put **L-3** (0.192 g, 0.228 mmol) and sodium methoxide (0.148 g, 2.741 mmol) in anhydrous methanol and stirred at room temperature for 12 h under the protection of N_2. Added cation exchange resin to the solution until t neutral, filter and remove the solvent under reduced pressure to obtain a nearly white compound **Lac-SS-Lac** (104 mg, 92%). ^{1}H NMR (D_2O, 400 MHz) δ (ppm): 4.49-4.47 (d, J = 8.0 Hz, 1H), 4.42-4.40 (d, J = 8.0 Hz, 2H), 4.05-4.01 (m, 1H), 3.95-3.48 (m, 45H), 2.93-2.90 (t, J = 6.0 Hz, 2H), 2.80-2.76 (t, J = 6.4 Hz, 1H). ^{13}C NMR (101 MHz, D_2O) δ (ppm) 102.90, 102.08, 98.09, 78.36, 75.36,74.77, 74.29, 72.83, 72.51, 71.72, 70.96, 70.47, 69.67, 68.57, 66.85, 61.45, 60.07, 43.67. HRLCMS calculated for $[M + Na]^+$ $C_{36}H_{66}O_{26}S_2$, 1001.3181. Found: 1001.3233.

Preparation of BG-L nano-scale particles (NPs)

BG (10 mg) was dissolved in 100 μL DMSO, and **Lac-SS-Lac** (160 mg) was dissolved in 200 μL DMSO, the two solutions were mixed under ultrasound. Then 25 mL water was added dropwise to DMSO solution and ultrasonic for 2 h. The precipitate (BG-L NPs) was collected by centrifugal separation and then dispersed in ultrapure water. The concentration of **BG-L NPs** was calculated by **BG**.

Characterization of BG-L NPs

The polydispersity index (PDI), zeta (ζ) potential, and hydrodynamic diameter of BG-L NPs were achieved by DLS and the morphology was analyzed by TEM, SEM. The BG loading ratio (weight ratio between BG and BG-L NPs) was calculated from the UV-Vis absorbance at 680 nm in EtOH.

GSH-responsive release and NPs disassembly

The disulfide bond would respond to GSH which is overexpressed in the tumor tissue microenvironment and released of Gef and BOD. BG-L NPs (0.14 mM) were dispersed in 2 mL GSH (10 mM) aqueous solution. The amount of released Gef was determined by UV-Vis of absorption in 320 nm. In addition, BG-L NPs (0.14 mM) was dispersed in 2 mL GSH (10 mM) DMSO solution and the release of BOD was shown by UV-Vis and fluorescence spectra.

As shown in the SEM and DLS analysis (Fig. S12), the sharpness of BG-L NPs in the GSH solution (10 mM) becomes irregular and the size significantly changes for the GSH-induced disulfide bond breaking which makes the nanoparticles unstable and then disassembled to form the larger-sized irregular aggregate.

HPLC and LCMS Analysis

The sample solutions were put into the HPLC (Agilent 1260) and separated on the chromatographic column C18 (4.6 mm × 150 mm, 5 μm). The mobile phase was methanol. The flow rate was 1 mL/min and the detection wavelength was 320 nm. The injection volume was 10 μL and the column temperature was maintained at 35°C. LCMS the mobile phase consisted of acetonitrile (A) / purified water (B) = 83:17 (v:v). The flow rate was 1 mL/min and the detection wavelength was 320 nm. The injection volume was 4 μL. As shown in Fig. S15, for the GSH (10 mM) induced disulfide bond breaking, the BG-L NPs disassembled and released Gef and BOD which was determined by the retention time of a single compound (BG, Gef, and BOD). As in Fig. S16, the standard curve of Gef was made by LCMS. Gef was completely released within 72 h. The 24 h and 48 h release rates were 21.0% and 52.3% respectively, which were consistent with the results of UV-Vis quantitative analysis.

Photothermal capability

The temperature of BG-L NPs aqueous solution with different concentrations was measured under the NIR light irradiation (160 mW/cm^2) of 685 nm for 10 min. The temperature of BG-L NPs aqueous solution (20 μM) with different light power was measured under the 685 nm NIR light irradiation for 10 min. Temperature and the photothermal conversion abilities of BOD (20 μM) and BG-L NPs (20 μM) aqueous solution were measured under the NIR light irradiation (160 mW/cm^2) of 685 nm. In addition, the temperature of BG-L NPs (20 μM) in 10 mM GSH aqueous solution was measured to investigate the photothermal effect of the photosensitizer BOD released from BG-L NPs.

Singlet oxygen generation

The $^1O_2$ production of BG-L NPs was evaluated using DPBF as a probe. BG-L NPs (20 μM), GSH (10 mM) and DPBF (0.2 mM) were mixed in 2 mL water, and the mixed aqueous solution was illuminated under the NIR irradiation light (80 mW/cm^2)

of 685 nm for 0, 3, 6, 9, 12, 15, 18 min respectively. The absorption intensity of DPBF at 422 nm was recorded by UV-Vis absorption spectra to investigate the $^1O_2$ production of the photosensitizer BOD which is released from BG-L NPs. The $^1O_2$ production of BG-L NPs (20 μM) and BOD (20 μM) aqueous solution was measured respectively by the same method.

Cell culture and Cytotoxicity Evaluation

Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Other cells were cultured in 1640 medium containing 10% FBS and 1% penicillin/streptomycin. All cells were incubated at 37 °C in a 5% CO_2 humid incubator., and harvested with trypsin, and then resuspended in a fresh medium.

Used HL7702 and A549, Hela and HepG2 cells as the control groups and experimental groups to evaluate the biocompatibility and cytotoxicity of BG-L NPs. The cells (5000 cells per well) were seeded in 100 μL of culture media in 96-well plates for 48 h. The medium was then replaced with a fresh medium that contained BOD, Gef, BG-L NPs at various concentrations, then continued to cultivate for 24 h. The lighting group cells were washed with PBS and replaced with fresh medium, exposed to the NIR irradiation light (80 mW/cm^2) of 685 nm for 20 min. Then, cultured with the non-light group for another 24 h. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) containing medium was added into each well and incubated for 4 h, removed the medium added dimethyl sulfoxide (100 μL) to each well to dissolve the formazan crystals. Finally, the plate was gently shaken for 10 min and the absorbance at 490 nm was recorded by a microplate reader to evaluate the cytotoxicity of the drug delivery system.

For cell apoptosis, Annexin V-FITC/PI was used to dye the cell. A549 cells were seed in 6-well plates and cultured for 24 h. Then BOD (5 μM), Gef (5 μM), and BG-L NPs (5 μM) were added respectively and incubated for 24 h. The cells were washed with PBS for three times and exposed to the irradiation of 685 nm LED light (80 mW/cm^2) for 20 min (except Gef group), continue to culture for 24 hours, and the cells were collected and stained by FITC/PI before FCM analysis.

Cell uptake and targeting ability of BG-L NPs

A549 cells, HepG2 cells, Hela cells and HL7702 cells (2×10^5) were seeded in 6-well plates and incubated for 24 h, BG-L NPs (5 μM) were added and incubated for 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h,16 h and 24 h respectively. Then, the cells were washed with PBS 3 times for CLSM analysis (sample of 0 h, 2 h, 4 h and 6 h) or the cells were collected for FCM.

A549 cells, HepG2 cells, Hela cells and HL7702 cells (2×10^5) were seeded in a 6-well plate and incubated for 24 h., BG-L NPs (5 μM) were added and incubated for 6 h, the target uptake of BG-L NPs was analyzed by CLSM and FCM.

Cellular reactive oxygen species analysis (ROS) detection

2′,7′ -Dichlorofluorescin diacetate (DCFH-DA) was chosen as an indicator to detect the intracellular generation of ROS via CLSM. Briefly, A549 cells (2×10^5) were incubated with BOD (5 μM) and BG-L NPs (5 μM) for 6 h, then, the cells were washed with PBS three times and exposed to the NIR irradiation light (80 mW/cm^2) of 685 nm for 20 min. stained with DCFH-DA (1 μM) for 30 min at 37 °C in the dark. The cellular fluorescence of the oxidized product DCF was analyzed by CLSM.

Supplementary Figures

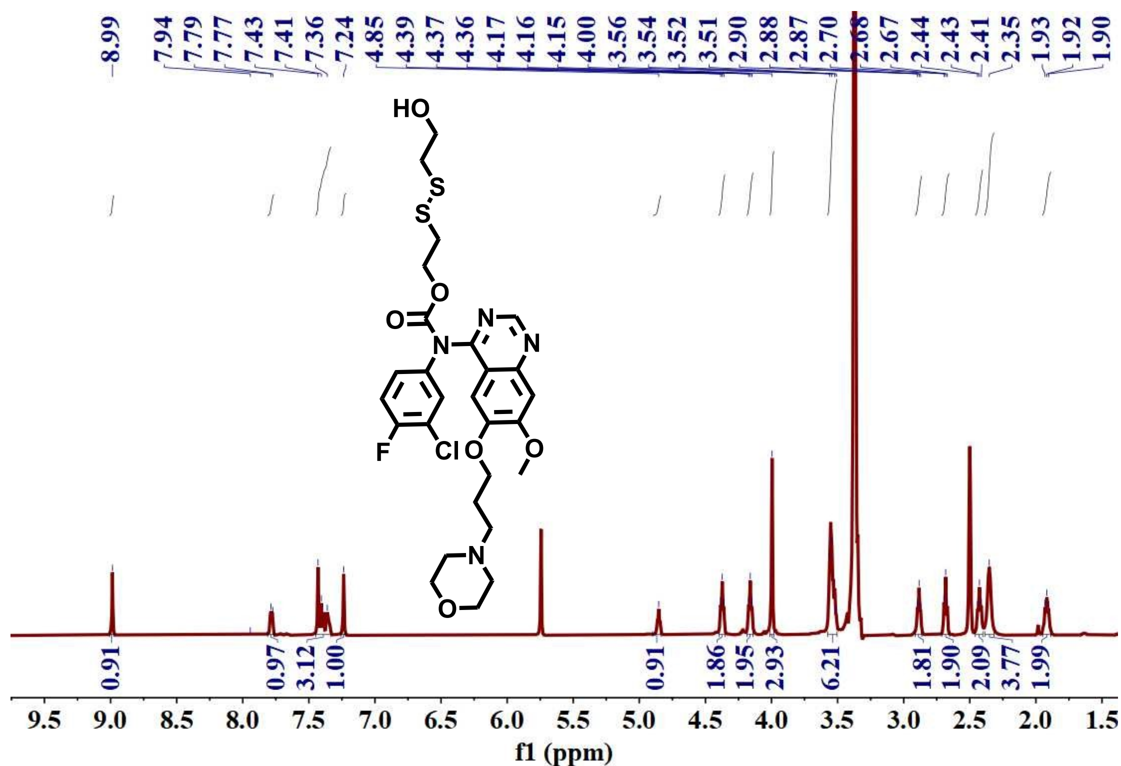


Fig. S1 ^{1}H NMR spectrum of Compound **Gef-OH** (DMSO-d_6)

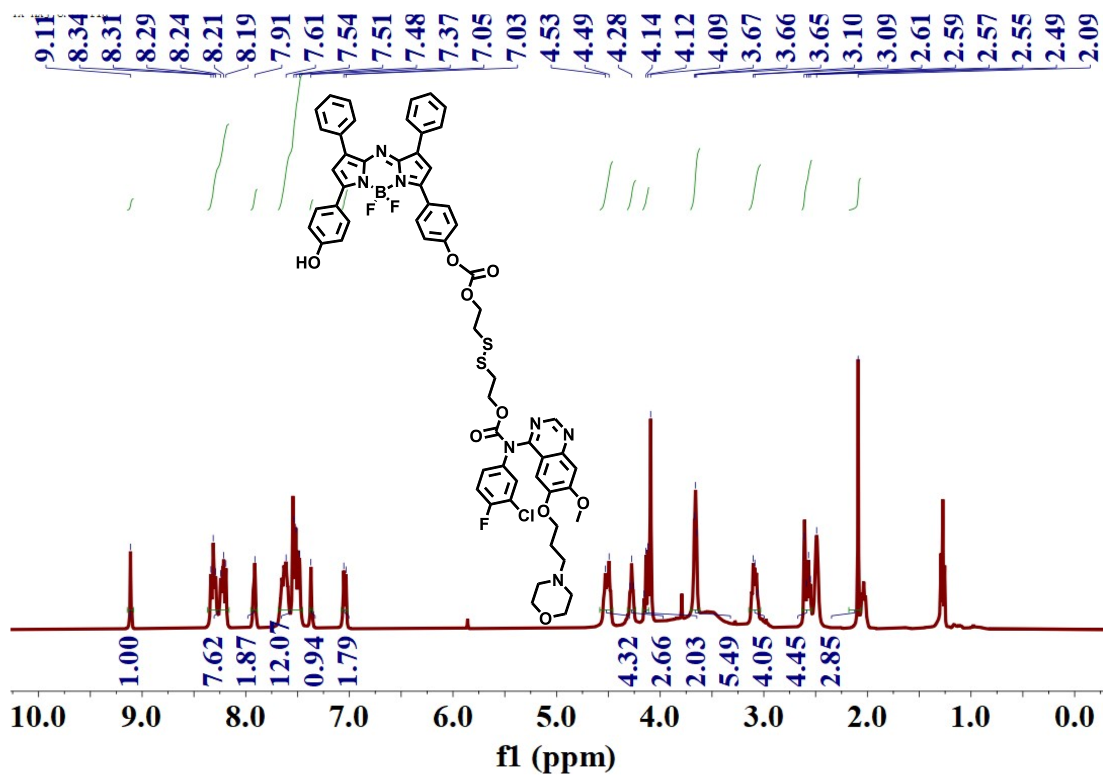
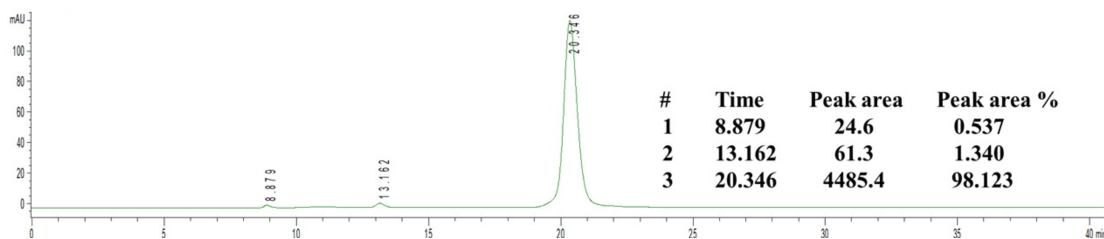


Fig. S2 ^{1}H NMR spectrum of Compound **BG** (DMSO-d_6)



#	Time	Peak area	Peak area %
1	8.879	24.6	0.537
2	13.162	61.3	1.340
3	20.346	4485.4	98.123

Fig. S3 HPLC spectrum of **BG**.

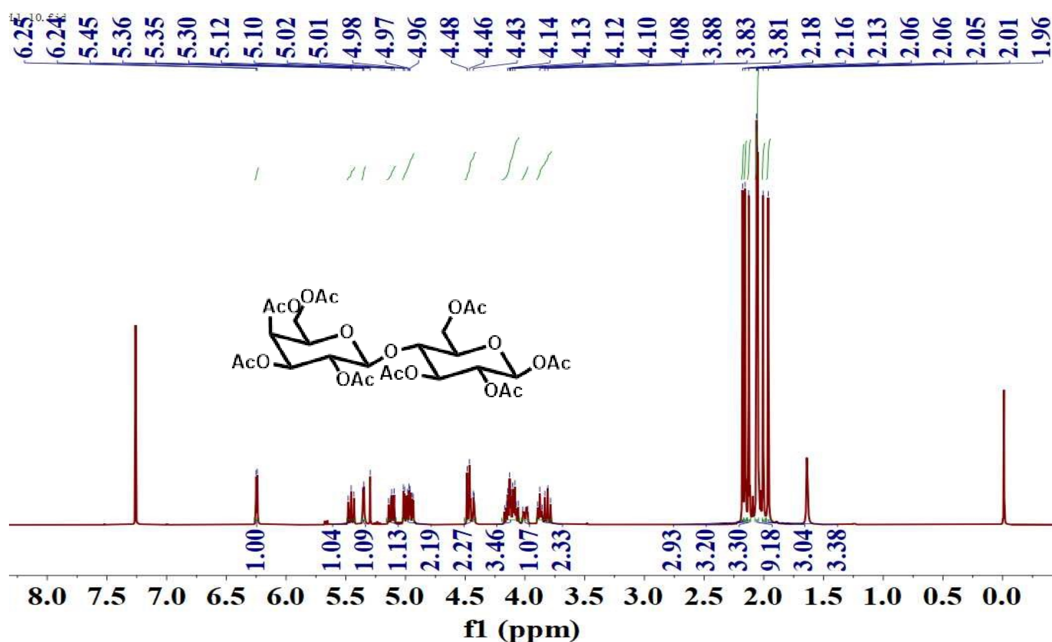


Fig. S4 ^{1}H NMR spectrum of Compound **L-1** ($CDCl_3$)

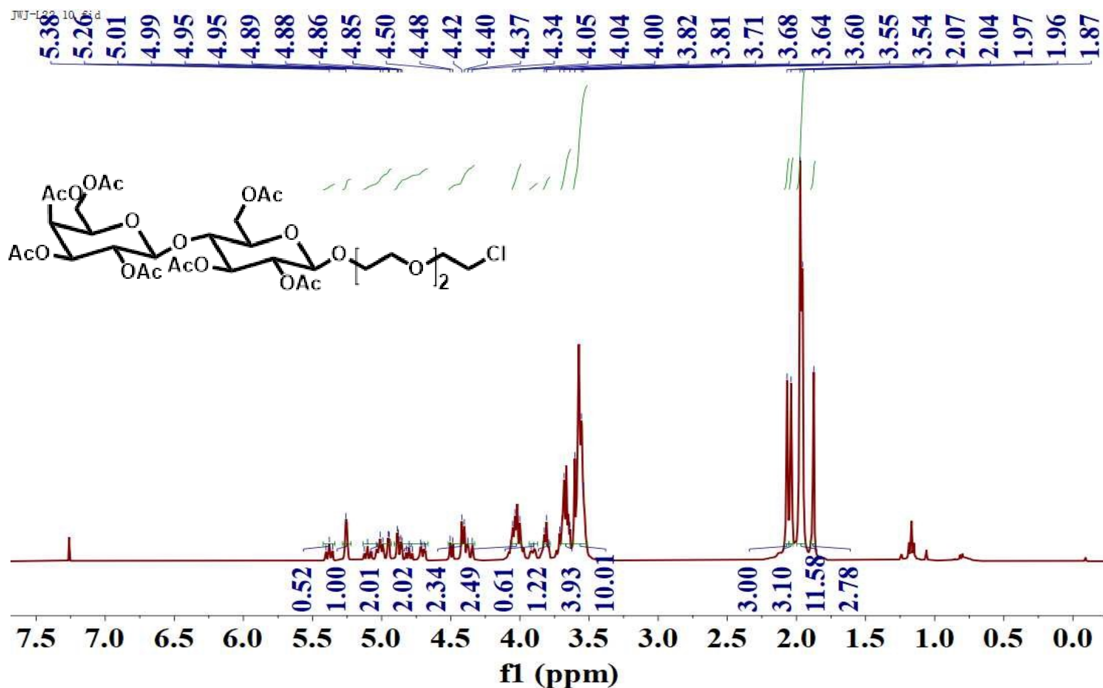


Fig. S5 ^{1}H NMR spectrum of Compound **L-2** ($CDCl_3$)

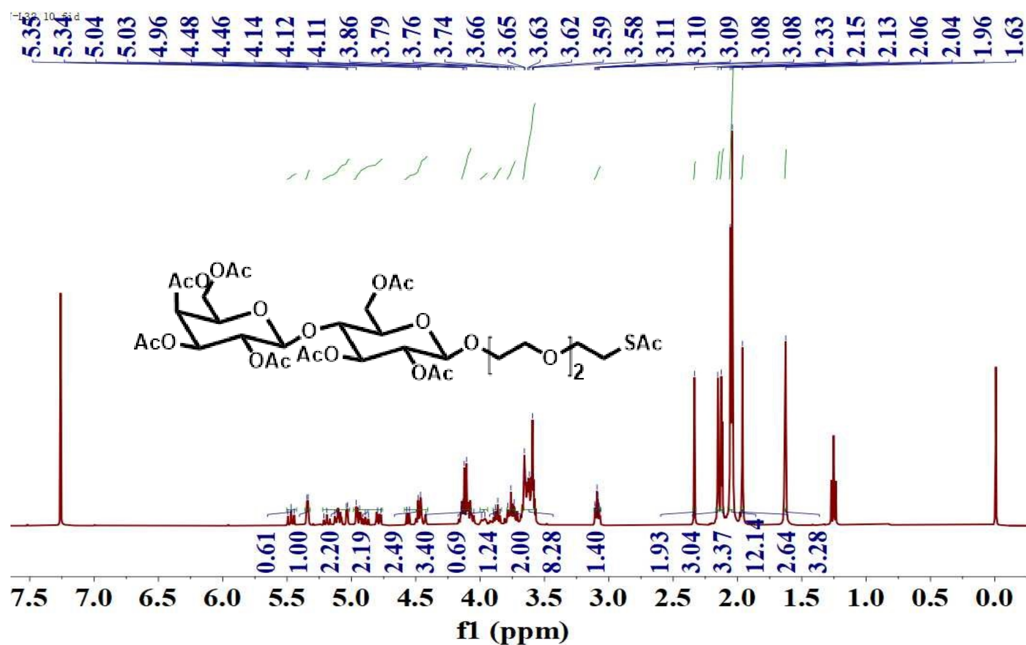


Fig. S6 ^{1}H NMR spectrum of Compound **L-3** ($CDCl_3$)

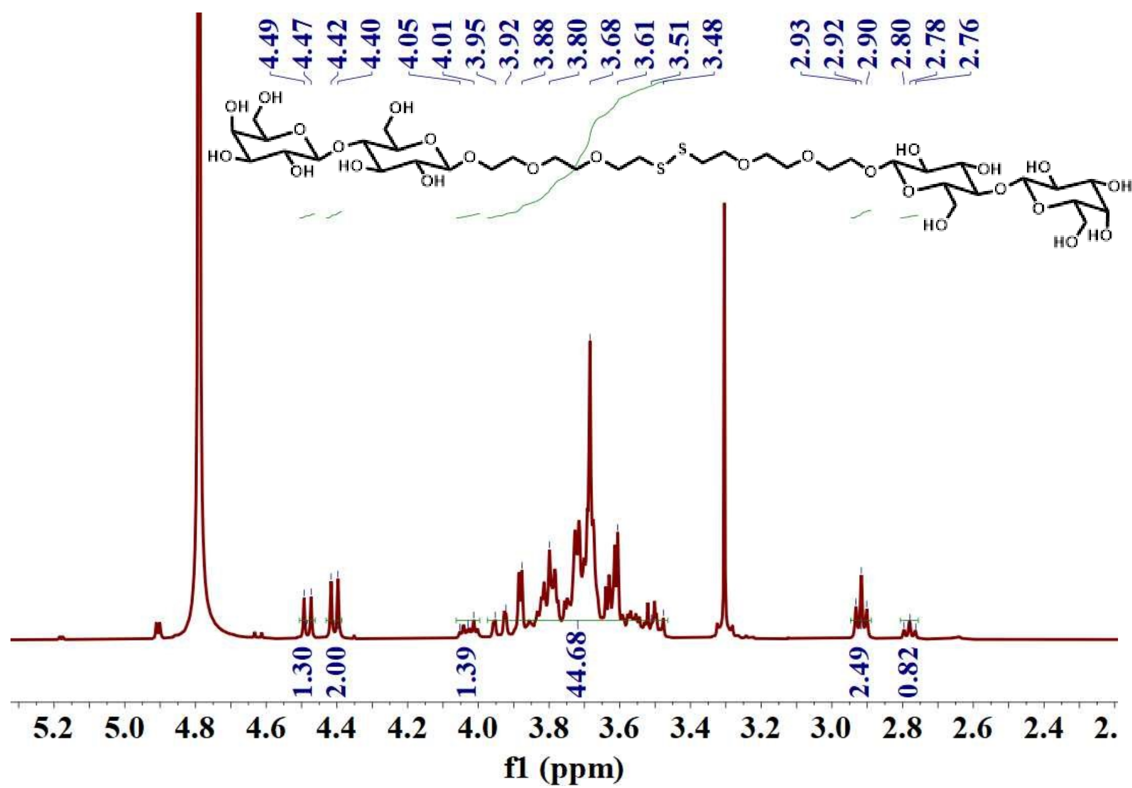


Fig. S7 ^{1}H NMR spectrum of Compound **Lac-SS-Lac** (D_2O)

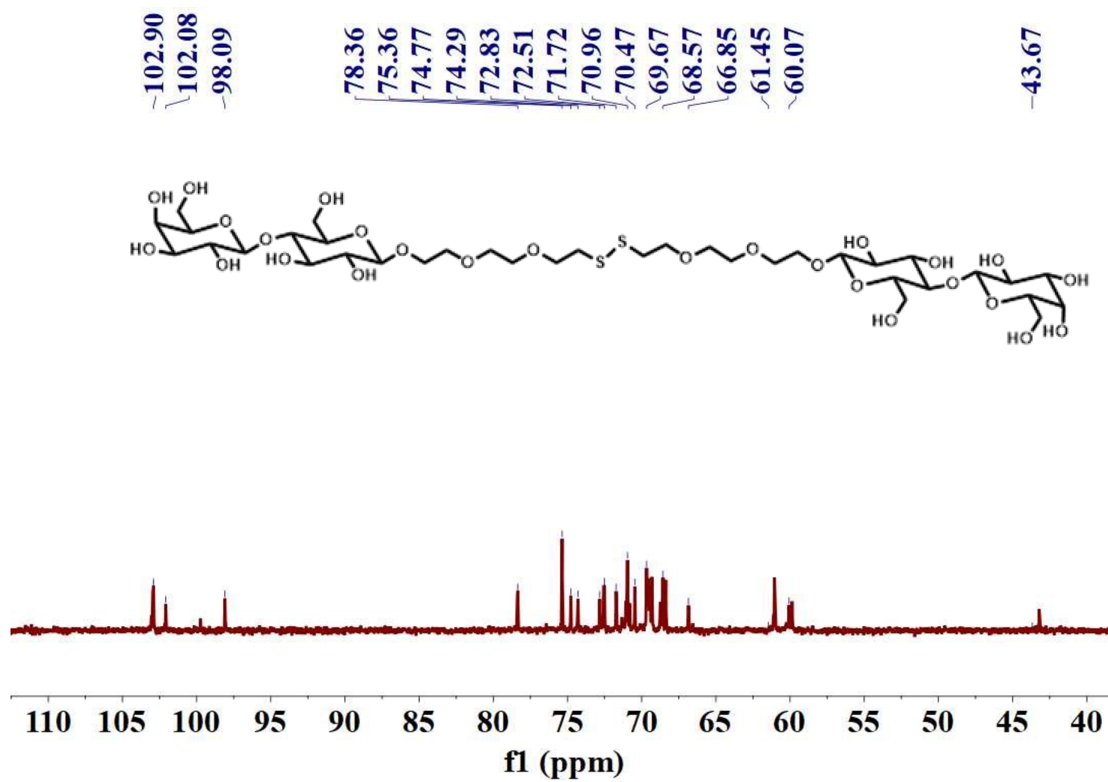


Fig. S8 ^{13}C NMR spectrum of Compound **Lac-SS-Lac** (D_2O)

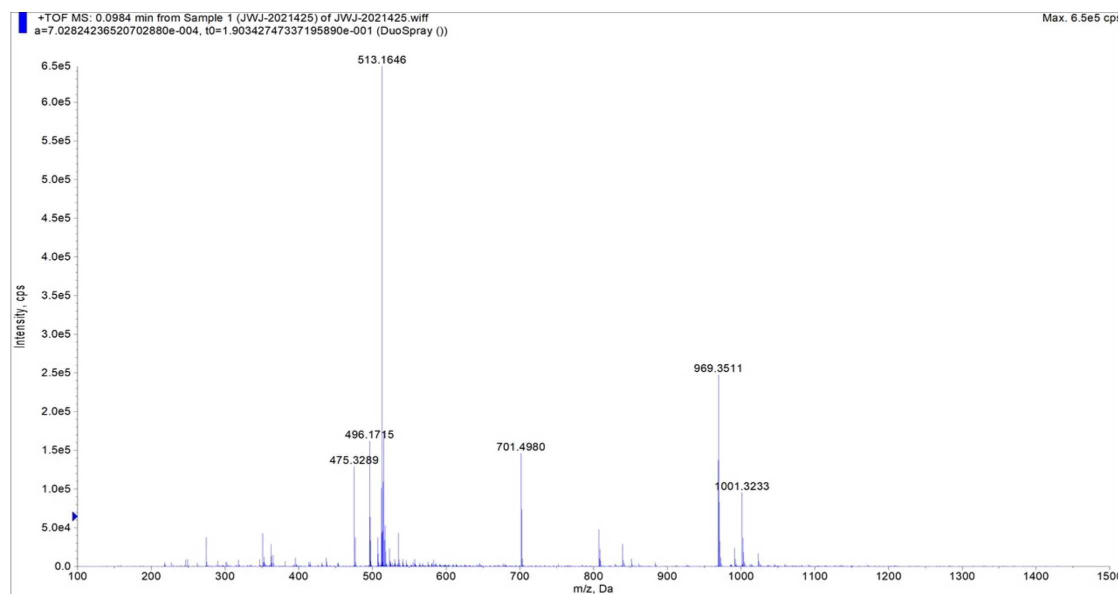


Fig. S9 HRMS spectrum of Compound **Lac-SS-Lac**

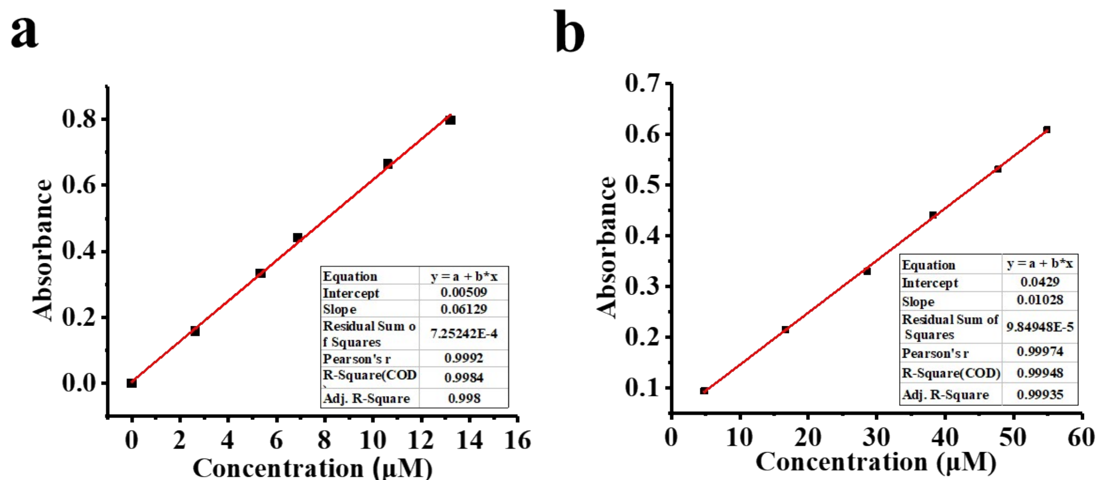


Fig. S10 (a) The absorbance standard curves of **BG**. (b) The absorbance standard curves of **Gef**.

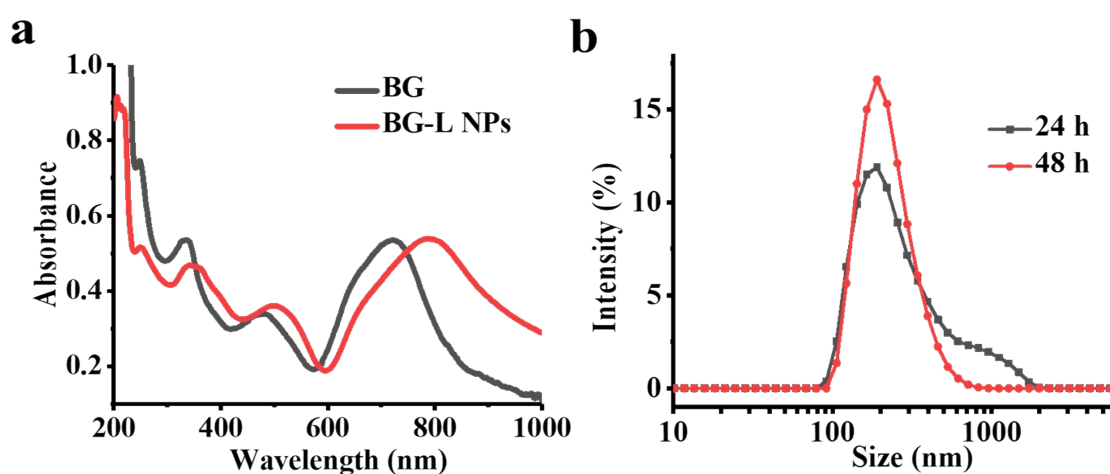


Fig. S11 (a) UV-Vis absorption spectrum of BG and BG-L NPs. (b) DLS analysis of **BG-L** NPs in serum contained 1640 medium for 24 h (black) and 48 h (red).

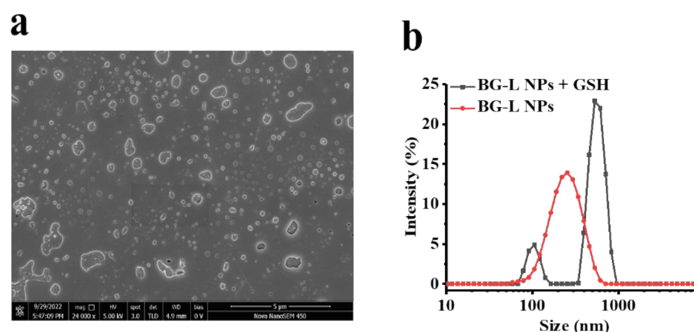


Fig. S12 (a) SEM of the BG-L NPs with 10 mM GSH for 48 h, scale bar: 5 μm; (b) DLS analysis of BG-L NPs (red) and BG-L NPs with 10 mM GSH for 48 h (black).

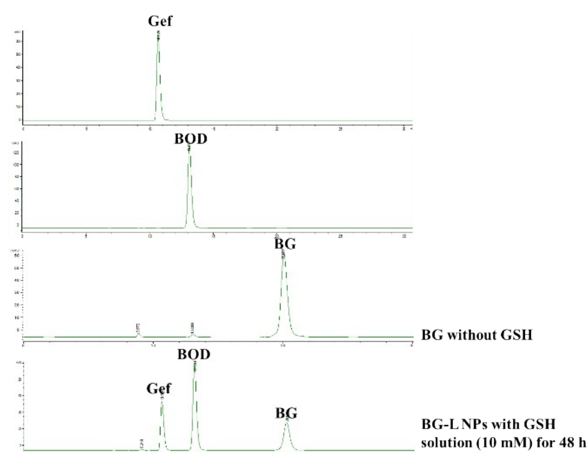


Fig. S13 The HPLC analysis of Gef (BOD) released from the BG-L NPs in GSH solution (10 mM).

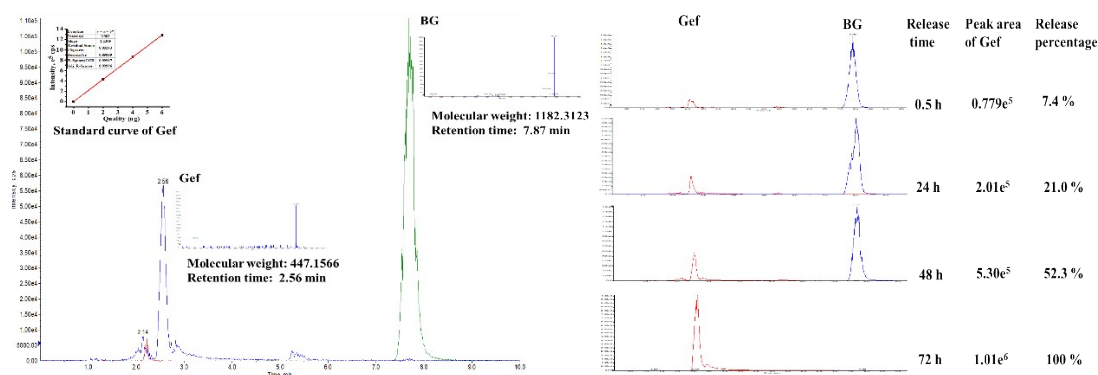


Fig. S14 The standard curve of Gef and the quantitative analysis of GSH-responded Gef release by LCMS.

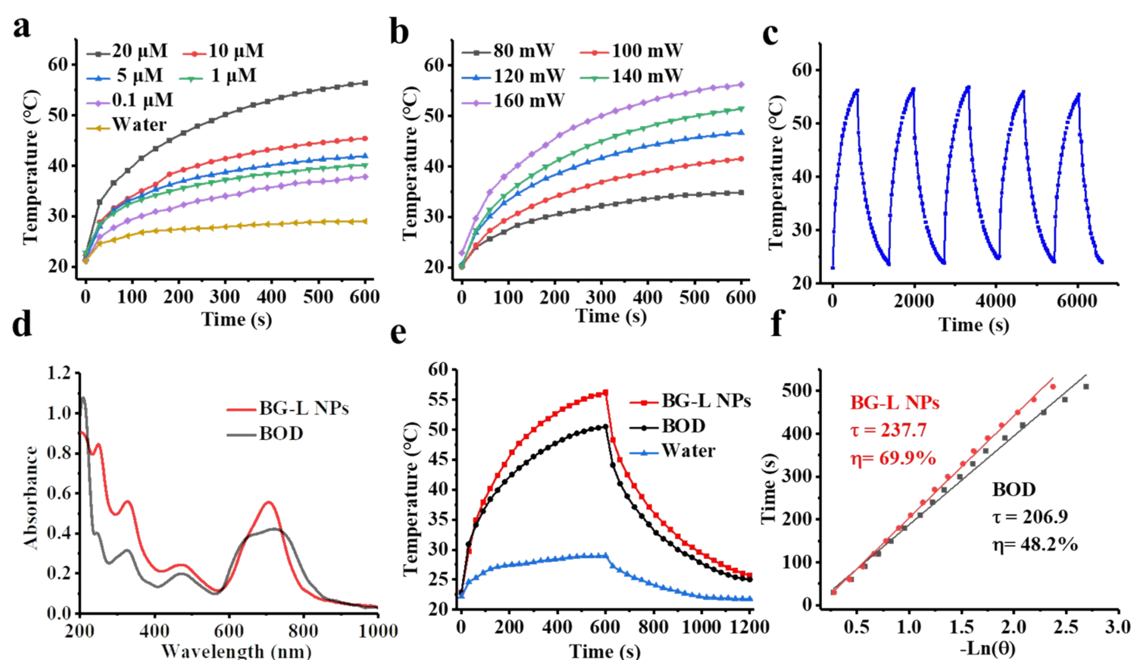


Fig. S15 (a) Photothermal heating curves of BG-L NPs aqueous solutions at different concentrations (0, 0.1, 1, 5, 10, 20 μM) under 685 nm light irradiation at a power of

160 mW/cm^2 for 10 min. (b) Photothermal heating curves of BG-L NPs aqueous solutions (20 μM) at different light power (80, 100, 120, 140, 160 mV) under 685 nm light irradiation for 10 min. (c) Photothermal stability test under 685 nm laser irradiation at 160 mW/cm^2 of BG-L NPs aqueous solutions (20 μM). (d) UV-Vis absorption spectrum of BG-L NPs (red) and BOD (black) aqueous solutions (20 μM) after light irradiation. (e) Photothermal transduction of BG-L NPs aqueous solution (red), BOD aqueous solution (black), and water (blue) under 685 nm light irradiation at a power density of 160 mW/cm^2. (f) The photothermal conversion efficiency (η) of BG-L NPs aqueous solution (red), BOD aqueous solution (black).

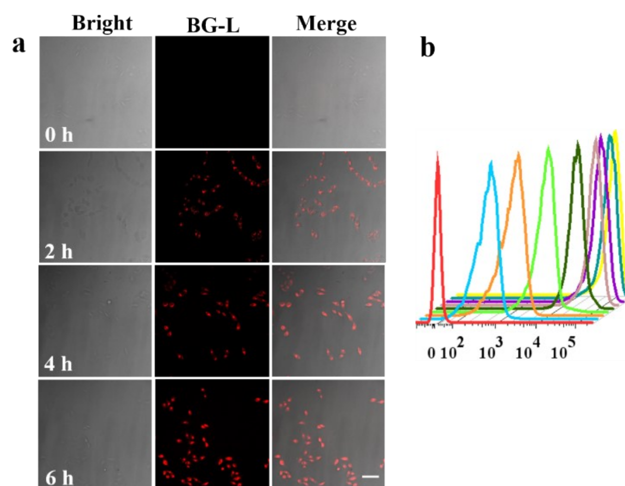


Fig. S16 Cellular uptake of BG-L NPS by A549 cells via CLSM (a) and FCM (b). Incubation time from left to right 0, 0.5, 1, 2, 4, 6, 8, 16 h and 24 h respectively. The concentration of BG-L NPs was 5 μM. Scale bars, 100 μm. For BG-L, Ex/Em: 637/730 nm.

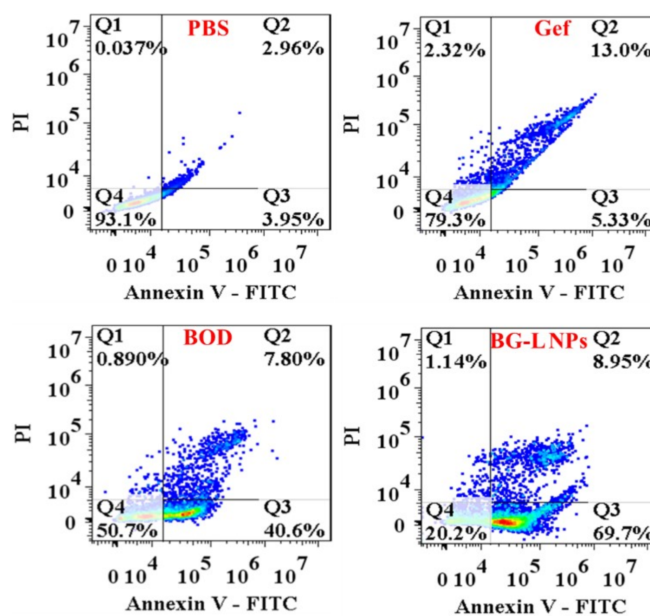


Fig. S17 Annexin V-FITC/PI apoptosis assay of A549 cells which was incubated with PBS, Gef, BOD and BG-L NPs respectively. The concentration was 5 μM. Irradiation of group BOD and BG-L NPs was performed with 685 nm light (0.08 W/cm^2, 20 min).

References

1 X. Song, X. Han, F. Yu, X. Zhang, L. Chen and C. Lv, *Theranostics*, 2018, **8**, 2217-2228.

2 M. Gao, F. Yu, H. Chen and L. *Chen, Anal. Chem.*, 2015, **87**, 3631-3638.

3 Y. Qu, X. Wang, Z. Pei and Y. Pei, *ChemMedChem*, 2022, **17**, e2021005.