Supporting Information

Self-assemblies with cascade effect to boost antitumor systemic immunotherapy

Ruipeng Li, ‡[a] Xiaoyu Zhao, ‡[a] Yunxia Wang,[a] Chenhao Guo,[a] Zhijun Wang,*[b] and Liheng Feng*[a]*

School of Chemistry and Chemical Engineering, Shanxi University, Taiyuan, 030006, P.R. China

Department of Chemistry, Changzhi University, Changzhi, 046011, P. R. China.

E-mail: wangyunxia@sxu.edu.cn (Wang, Y.); czxywzj@163.com (Wang Z.); lhfeng@sxu.edu.cn (Feng, L.)

Experimental Procedures

Chemicals and Reagents. All chemicals including 4,7-dibromo-2,1,3-benzothiadiazole, 2-(tributylstannyl)thiophene, $Pd(PPh_3)_4$, LDA Fmoc-Arg-OH, and C_3H_9ClSn were purchased from Macklin Biochemical Technology Co., Ltd. of China and Energy Chemical Commercial company (China) and used without further treatments. Nitric oxide (NO) kit was obtained from Jiancheng Bioengineering Institute of Nanjing (China). 3-Amino-4-aminomethyl-2',7'-difluorescein diacetate (DAF-FM DA) was obtained from Beyotime Biotechnology of China. Calcein-AM and propidium iodide (PI) were purchased from Solarbio Biotechnology Co., Ltd of China. Mouse breast tumor cells (4T1) and human breast tumor cells (MCF-7) were provided from Wuhan Procell Life Technology Co., Ltd of China. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) were obtained from Sino Biological Inc. Enzyme-Linked Immunosorbent Assay (ELISA) Kits for interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) were obtained from Bioswamp Biotechnology Co., Ltd of China. Hydrogen Peroxide (H_2O_2) Assay Kit, anti-CD3-PE, anti-CD4-FITC and anti-CD8a-FITC were obtained from Shanghai Maokang Biotechnology Co., Ltd (China). The male BALB/c mice (5-weed-old) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd (China).

Characterizations. ^1H NMR spectra of compounds were measured by Bruker AVANCE III HD 600 MHz spectrometer. Mass spectra (MS) were acquired on Thermo Scientific Q Exactive instrument (Thermo Fisher Scientific, USA), which equipped with an electroscope ionization (ESI) source. Gel permeation chromatography (GPC)

measurement was conducted by Agilent PL-GPC50. UV-vis absorption spectra were recorded on a UH5300 spectrophotometer and fluorescence emission was measured on a Hitachi F-4600 fluorescence spectrophotometer with a Xenon lamp as the excitation source. The morphology of the self-assemblies was determined by using scanning electron microscopy (SEM, JSM-7001F). Hydrodynamic sizes of self-assemblies were measured by photon-dependent particle size analyzer of Jnwinner802. A microplate reader (Bio-Rad) was employed to record cell viability. White light source (400–800 nm) with a xenon lamp (CXE-350) was provided by Beijing OPT Photoelectric Technology Co., Ltd (China). Fluorescence images were captured by a confocal laser scanning microscopy (Zeiss LSM 880). Flow cytometric analysis was measured by LSRFortessa X-20 BD Flow cytometer.

Synthesis of polymer PFLB. 4,7-Bis(5-trimethyltin thiophenoe-2-yl)-2,1,3-benzothiadiazole (monomer 1) was prepared according to the reported method.[1] (((2,7-Dibromo-9H-fluoren-9-yl)methoxy)carbonyl)-L-arginine (monomer 2) was synthesized as follows. A mixture of Fmoc-Arg-OH (500.0 mg, 1.26 mmol), iron powder (7.0 mg, 0.126 mmol), liquid bromine (1.0 g, 6.3 mmol), acetic acid (3 mL) and chloroform (15 mL) was stirred for 12 h at room temperature in the dark. After the reaction, 30 mL of distilled water was added and the mixture was extracted with ethyl acetate for 3 times. The organic phase was dried with anhydrous sodium sulfate. After removed the solvent, monomer 2 as the white solid (231.0 mg, yield: 33.1%) was obtained by column chromatography (methanol/dichloromethane = 1/7, v/v). ^{1}H NMR (600 MHz, *d*-$CHCl_3$) δ 7.87 (s, 1H), 7.83 (s, 1H), 7.74 (d, J = 7.9 Hz, 2H), 7.57 (d, J = 7.1 Hz, 2H), 4.43 (s,

1H), 4.40 (s, 1H), 4.29 (d, J = 5.9 Hz, 1H), 4.17 (d, J = 2.5 Hz, 1H), 3.20 (s, 2H), 1.92 (d, J = 8.3 Hz, 2H), 1.67 (s, 2H)；HRMS-ESI for $C_{21}H_{22}Br_2N_4O_4$ (m/z) 555.0060 $[M+H]^+$. Then, the polymer PFLB with fluorene, benzothiadiazole and thiophene groups in main chain and L-arginine in side chain was obtained by stille coupling reaction of monomer 1 and monomer 2 under palladium catalysis.[2] The specific synthesis route was as follows. A mixture of monomer 1 (113.0 mg, 0.18 mmol), monomer 2 (100.0 mg, 0.18 mmol), Tetrakis(triphenylphosphine)-palladium (20.0 mg, 0.018 mmol) and DMF (15 mL) was stirred at 100 ^oC for 48 h under nitrogen atmosphere. The reaction was poured into a large amount of distilled water, and a brown red solid was obtained by precipitation. The obtained solids were washed by toluene and dialyzed with acetone to obtain polymer PFLB (30.0 mg). ^1H NMR (600 MHz, *d*-DMSO) δ: 8.27-8.13 (m, 3H), 8.04 (s, 1H), 7.79 (d, J=2.2, 1H), 7.26 (d, J=47.9, 3H), 7.10 (s, 3H), 6.68 (s, 1H), 6.57 (s, 1H), 5.32 (s, 2H), 4.24 (dd, J=17.8, 7.9 Hz, 2H), 3.09 (d, J=6.0 Hz, 2H), 2.02-1.96 (m, 2H), 1.47 (dd, J=14.0, 4.8 Hz, 2H)；*Mn*=5036, *Mw*=7845, PDI=1.56.

Preparation of self-assemblies. Fmoc-Cys/Fe (FF) and Fmoc-Cys/Fe@PFLB (FFP) were prepared by mixing the solution of 10.0 μL Fmoc-Cys in DMSO (100.0 mM), 100.0 μL $FeCl_3$ in water (10.0 mM), with or without 10 μL PFLB in DMSO (10.0 mM for use *in vitro*, 20.0 mM for use *in vivo*) followed by 880.0 μL distilled water, respectively. The colloidal suspension was obtained under stirring or ultrasonic conditions. After centrifugation (14000 rpm, 10 min), the supernatant was discarded and the precipitations was washed three times with distilled water. Then, FF NVs and FFP

were obtained by dispersing the precipitations into 200 μL of water under ultrasound (10 min). Fmoc-Cys/Fe-GOx (FFG) and Fmoc-Cys/Fe@PFLB-GOx (FFPG) were prepared by adding 100.0 μL glucose oxidase (GOx) aqueous solution (0.25 mg/mL for use *in vitro*, 2.0 mg/mL for use *in vivo*) to the above formed FF and FFP NVs, respectively. Similarly, the suspensions were centrifuged (14000 rpm, 10 min) and washed with water for three times. These self-assemblies were resuspended in distilled water (200.0 μL). For all self-assemblies, the concentrations of Fe^{3+} and Fmoc-Cys were both 5.0 mM. The concentrations of PFLB and GOx were 0.5 mM (use *in vitro*) and 1.0 mM (use *in vivo*), 0.125 mg/mL (use *in vitro*) and 1.0 mg/mL (use *in vivo*), respectively, in corresponding self-assemblies. In all experiments, the concentrations of these self-assemblies were calibrated according to the amount of PFLB by measuring the absorption (500 nm) spectra and comparing the spectra with the pre-established calibration curves. For FF NVs, the concentration was calibrated according to the concentration of Fmoc-Cys that was determined by measuring the absorption (270 nm) spectra and comparing the spectra with the pre-established calibration curves. And other components were distributed according to the above proportion.

Detection of H_2O_2 generation. In order to eliminate the fluorescence interference of PFLB, FFG was constructed to evaluate the ability of GOx on self-assemblies to consume glucose and produce H_2O_2 by using fluorescence probe (Maokang Biotechnology, Shanghai). FFG ([GOx] = 50.0 μg/mL) were mixed with different concentrations of glucose (0.1 mg/mL, 0.5 mg/mL, and 1.0 mg/mL) and then added to the H_2O_2 probe solution (5.0 μM), respectively. The excitation and emission

wavelengths were 490 nm and 514 nm, respectively.

Quantitative determination of NO. The NO production ability of FFPG was quantitatively evaluated by nitrate reductase method (NO kit, Nanjing Jiancheng Bioengineering Institute). Glutathione (GSH) and white light was performed to verify the condition of self-assemblies decomposition and NO release. The concentrations of FFPG and GSH in the test solutions (2.0 mL) were 10.0 μM and 0.15 mM, respectively. For GSH response group, FFPG and GSH were pre-incubated for 1 h at 37 °C. The white light irradiation was performed at a density of 50.0 mW/cm^2 for 1 h. The determination of NO was performed every 10 min.

Qualitative determination of NO. Since GOx could consume glucose and generate H_2O_2, the effect of glucose on the NO generation of FFPG was investigated by using the reported fluorescence probe named Rhodamine B Spirolactam (RhBS).[3] The concentration of RhBS probe was 50.0 μM in a mixed solution of sodium phosphate buffer (0.1 M) and CH_3CN (v/v = 4/1). GSH was incubated with FFPG to ensure the self-assemblies decomposition and the final concentration was 0.3 mM. The final concentrations of RhBS, PFLB and GOx in the test solutions (1.0 mL) were 10.0 μM, 50.0 μM and 12.5 μg/mL, respectively. The light density was 50.0 mW/cm^2. The excitation and emission wavelengths were 540 nm and 580 nm, respectively. The fluorescence measurement interval was 30 min.

Cell viability *in vitro*. The viabilities of 4T1 cells and MCF-7 after treatment with self-assemblies were evaluated by a standard MTT assay. 4T1 cells were cultured in RPMI 1640 medium containing 10 % FBS and 1 % antibiotics (100 unit/mL penicillin and

100.0 μg/mL streptomycin) in an incubator with 5 % CO_2 at 37 °C. MCF-7 cells were cultured in DMEM containing 10 % FBS in an incubator with 5 % CO_2 at 37 °C. Both 4T1 cells and MCF-7 cells were inoculated in 96-well plates at a density of $7x10^3$ cells/well and divided to dark and light group. After incubation overnight, the cells were further incubated with fresh culture media containing various concentrations of FFPG ([PFLB] = 2.5 μM, 5.0 μM, 10.0 μM and 20.0 μM for 100.0 μL) for 4 h, respectively. For the light groups, each well was irradiated with the white light at light density of 50.0 mW/cm^2 for 30 min, and then incubated for 4 h. 10.0 μL of MTT (5.0 mg/mL) was added to each well, followed by incubation for 3 h. After removing the supernatant, 100.0 μL of DMSO was added to each well. The absorbance at 490 nm was measured and recorded using a microplate reader (Bio-Rad). The viabilities of 4T1 cells after treatment with FFG and FFP were also investigated, respectively. The concentration of PFLB was 20.0 μM, and the concentration of GOx was 5.0 μg/mL. All results were repeated three times and presented as mean ±SD.

Live/Dead cell staining Images of confocal laser scanning microscope (CLSM). After the proliferation of 4T1 tumor cells reached 70-80%, culture medium (1.0 mL) respectively containing FFG, FFP and FFPG was added. The concentration of PFLB in self-assemblies was 20.0 μM. The Light group was irradiated with white light at an intensity of 50.0 mW/cm^2 for 30 min. The cells were sequentially incubated for 4 h and washed three times with PBS, and then stained with Calcein AM/ PI for 15 min. After washed again with PBS, the fluorescence images were obtained by using CLSM with a 490 nm laser and a 545 nm laser for Calcein AM and PI, respectively. The color of

green and red represented the living cells and dead cells, respectively.

NO imaging *in vitro* by CLSM. The 4T1 cells were inoculated in 8-well plates at a density of $1x10^4$ cells/mL. After incubation for 48 h, the cells were further incubated with 1.0 mL fresh culture medium containing FFG, FFP and FFPG for 4 h, respectively. The concentration of PFLB in these self-assemblies was 10.0 μM. After removed old medium, the cells were incubated with 1.0 mL DAF-FM DA (5.0 μM) for another 0.5 h. The cells of light groups were irradiated with white light at an intensity of 50.0 mW/cm^2 for 20 min. Finally, the cells were washed three times with PBS and imaged by CLSM using a 495 nm laser, in which the presence of NO displayed the green fluorescence.

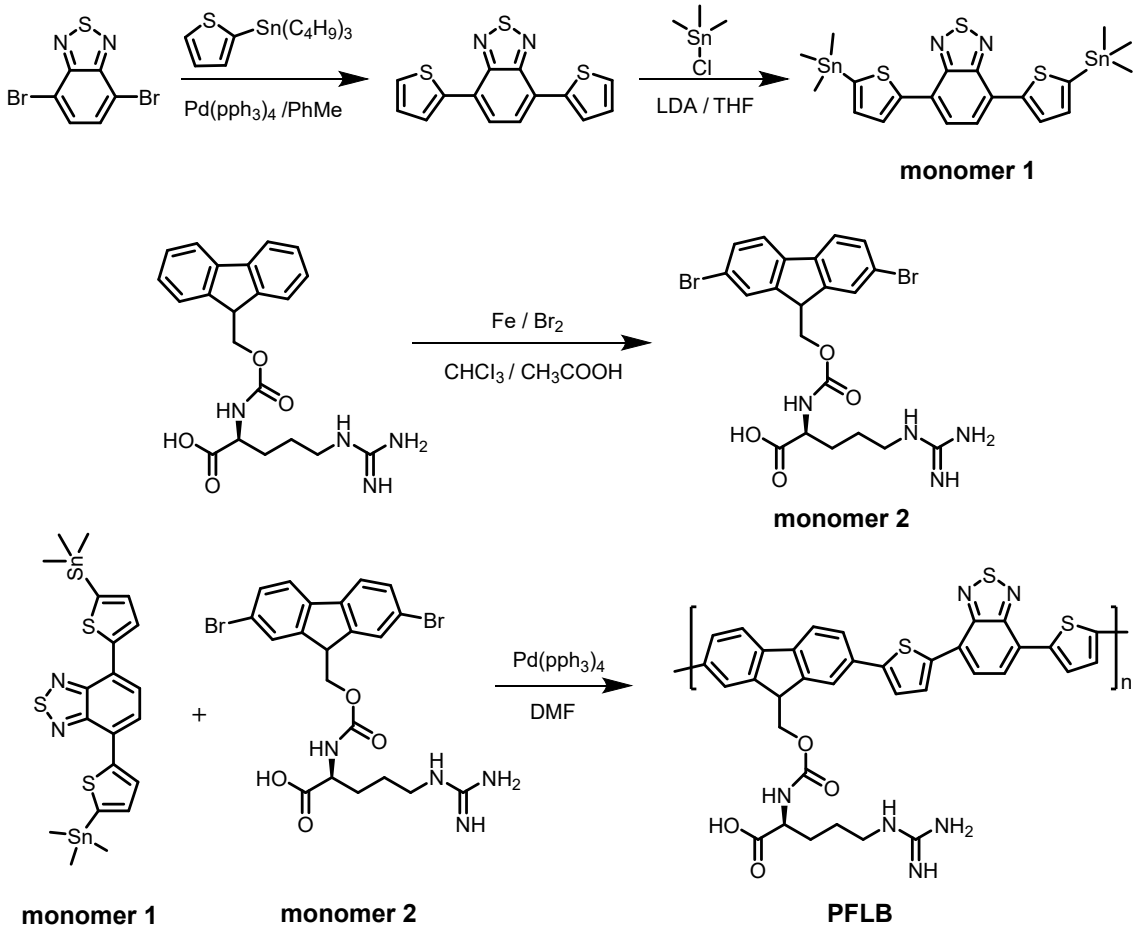
Dendritic cells activation *in vitro.* Dendritic cells (DCs) were obtained from 8-week-old BALB/c female mice. Briefly, mouse tibia and femur bones were removed and washed with sterile PBS to obtain mouse bone marrow cells. Subsequently, the cells were collected and cultured in RPMI 1640 culture medium containing 10 % FBS, GM-CSF (20.0 ng/mL) and IL-4 (10.0 ng/mL) for 6 days to obtain immature DCs. Then immature DCs were co-incubated with FFG (-/+Light), FFP (-/+Light) and FFPG (-/+Light) treated 4T1 cells for 24 h, respectively. The concentration of PFLB in different self-assemblies was 20.0 μM. The cells of light groups were irradiated with white light at an intensity of 50.0 mW/cm^2 for 30 min. The supernatant was collected and assayed for pro-inflammatory cytokines TNF-α using a standard ELISA.

***In vivo* assay of anti-tumor therapy.** All animal surgery was carried out in accordance with the relevant legislation and guidelines approved by the Animal Care and Use Committee of Shanxi University. Male BALB/c mice (5-week-old) were used to

construct 4T1 tumor models. 4T1 cells (1×10^6 cells) were injected subcutaneously into the right anterior axilla of mice as the primary tumors. After 6 days, 4T1 cells (5×10^5 cells) were injected subcutaneously into the left anterior axilla of mice as the distant tumors. Hence, bilateral 4T1 tumor models were established. After the diameter of primary tumors reached about 6 mm, 75.0 μL of the self-assemblies ([PFLB] = 1.0 mM, [GOx] = 1.0 mg/mL) were added by intratumoral injection. The blank control groups were injected with the same volume of normal saline. At 2 h after injection, the primary tumors of mice in light groups were irradiated by white light (200 mW/cm^2, 30 min) on day 0, 1 and 2, respectively. The distant tumors were without any treatment. Body weight and tumor volumes of mice were recorded continuously. All mice were executed after 24 days and tumors were collected and photographed.

***In vivo* immunotherapy assay.** After treatment for 3 days, the concentrations of TNF-α and IL-6 in serum of mice were detected by ELISA. After treatment for 10 days, primary tumors, distant tumors and spleens were removed from mice and prepared into a single cell suspension, and then stained with anti-CD3-PE, anti-CD8a-FITC and anti-CD4-FITC, respectively. Flow cytometry was used to determine the above immune cell populations.

Supporting Scheme and Figures



Scheme S1. Synthetic routes of monomers and polymer PFLB.

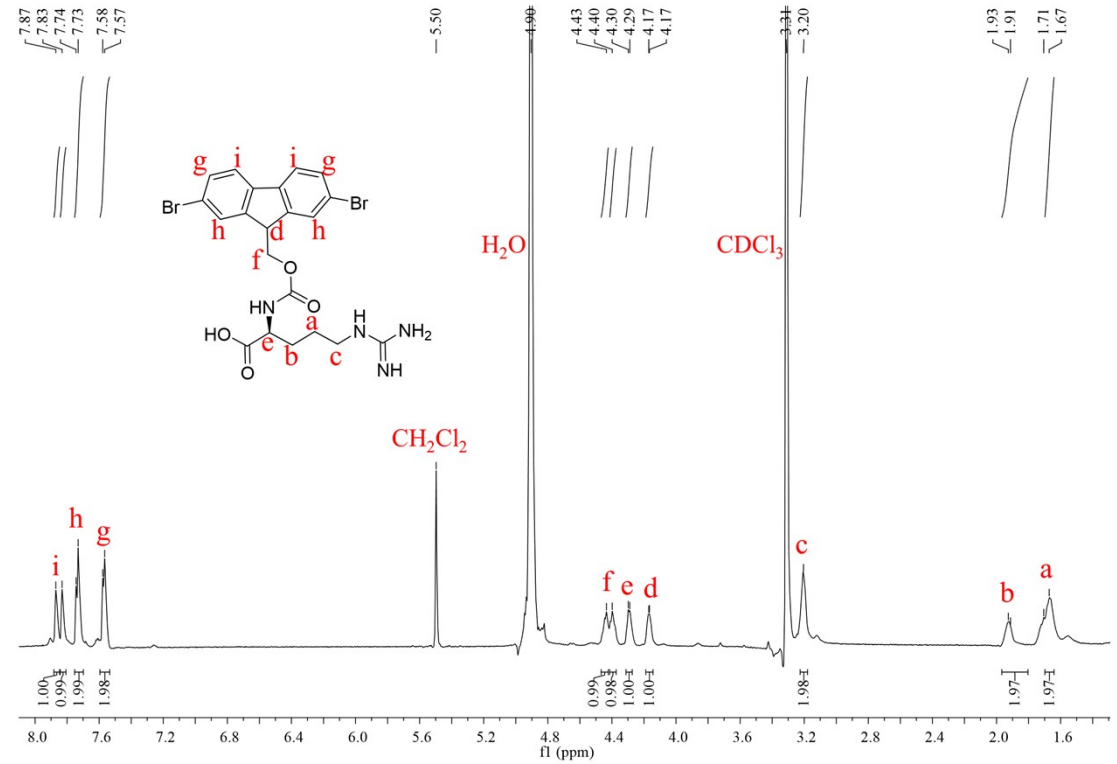


Fig. S1. The [1]HNMR image of monomer 2.

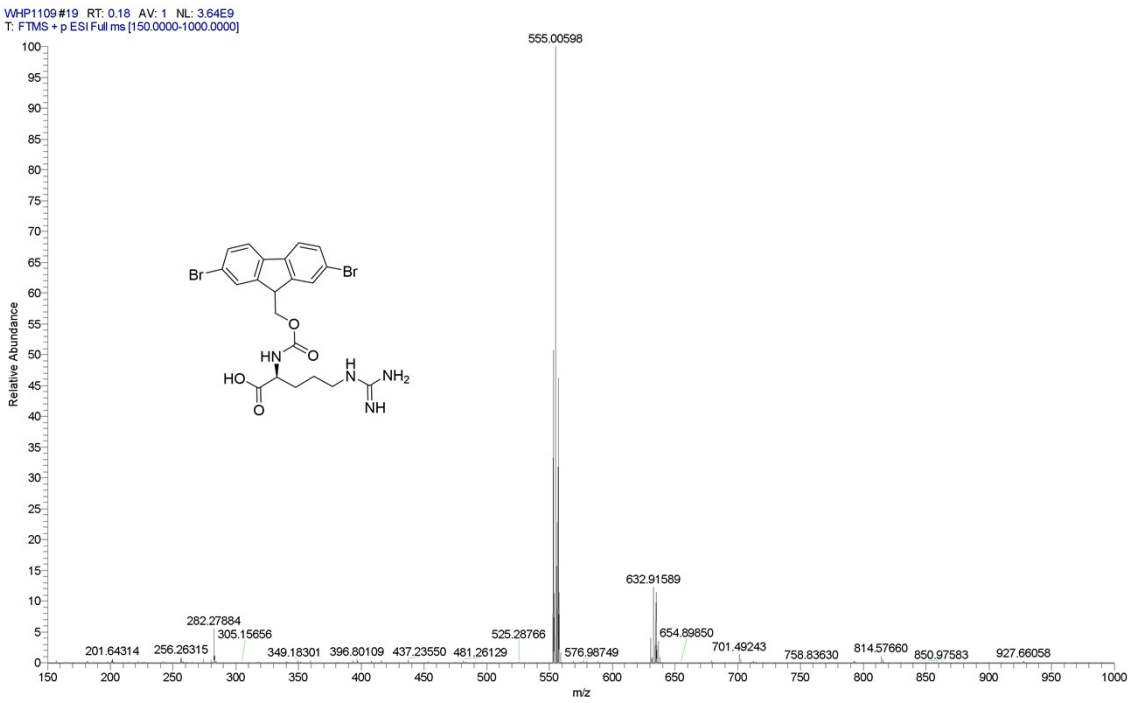


Fig. S2. High-resolution mass spectrum of monomer 2.

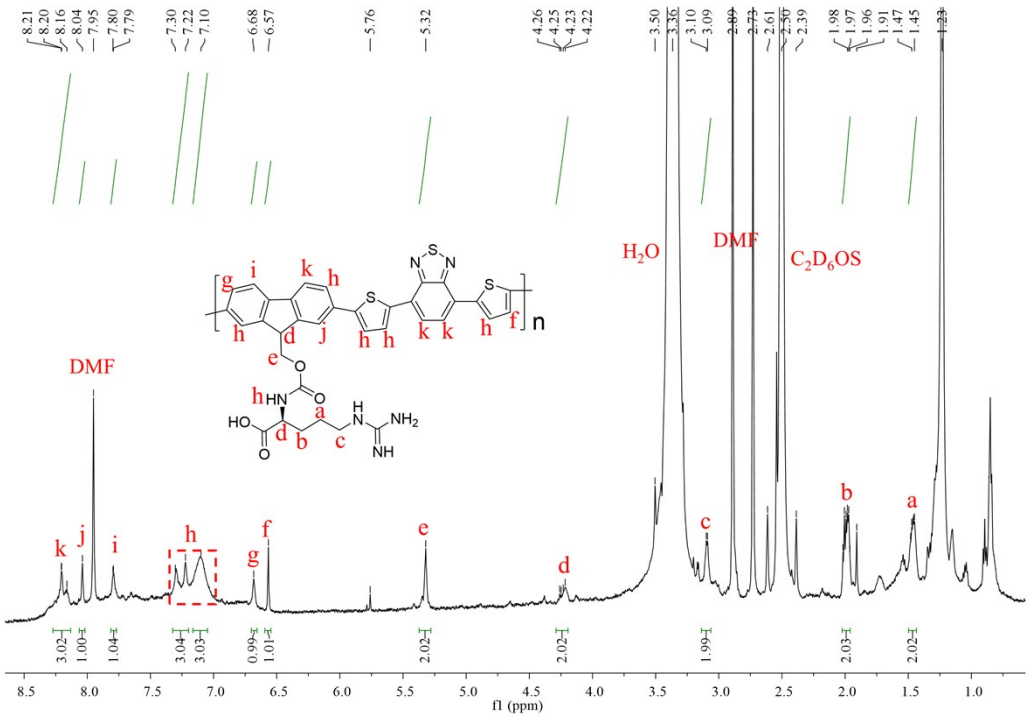
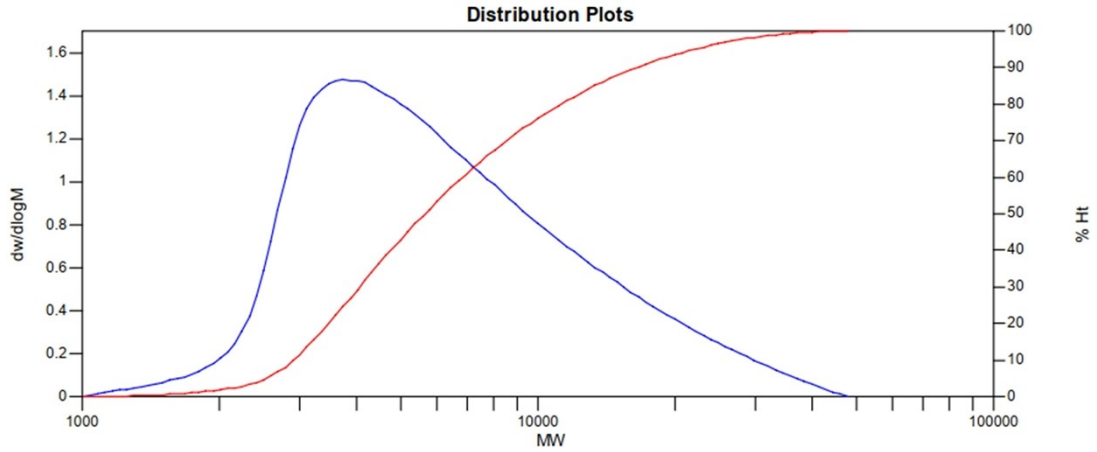


Fig. S3. The [1]HNMR image of PFLB.



MW Averages

Peak No	Mp	Mn	Mw	Mz	Mz+1	Mv	PD
1	3735	5036	7845	13084	19689	7264	1.55778

Fig. S4. The GPC analyses of PFLB.

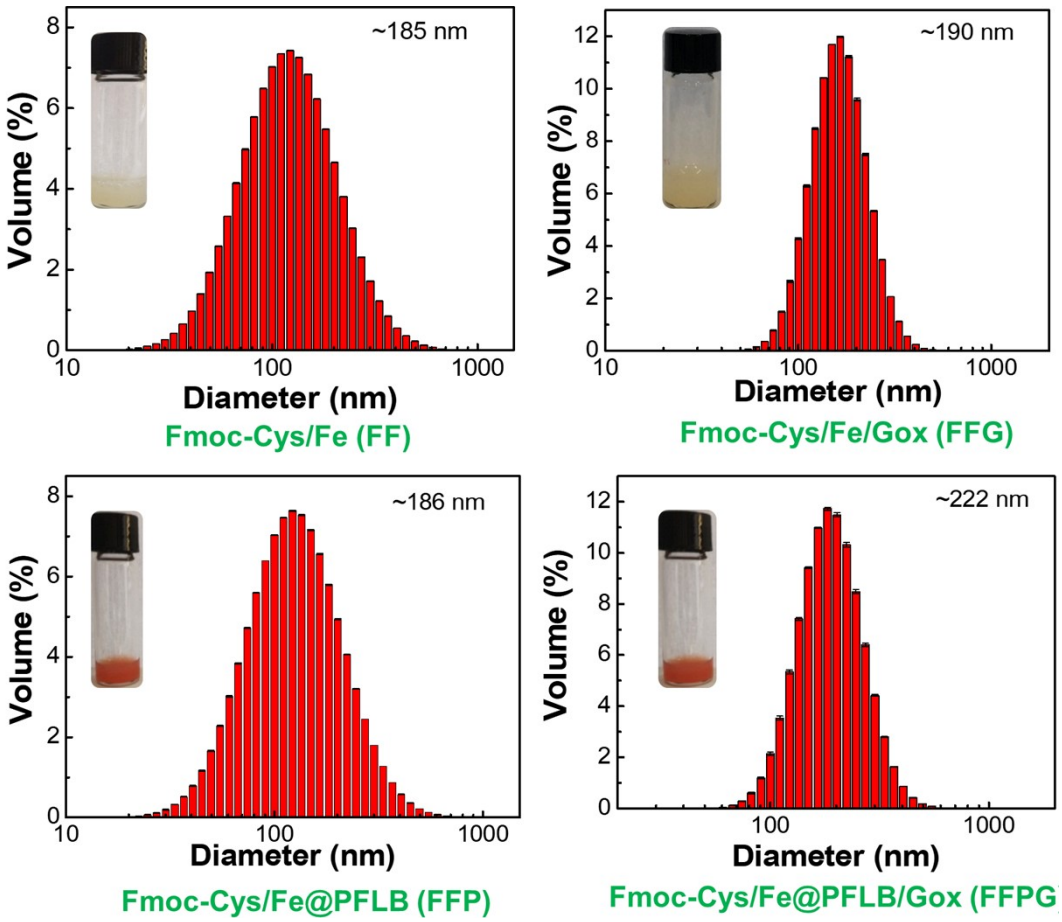


Fig. S5. The hydrodynamic diameters and corresponding aqueous solution photographs of different self-assemblies.

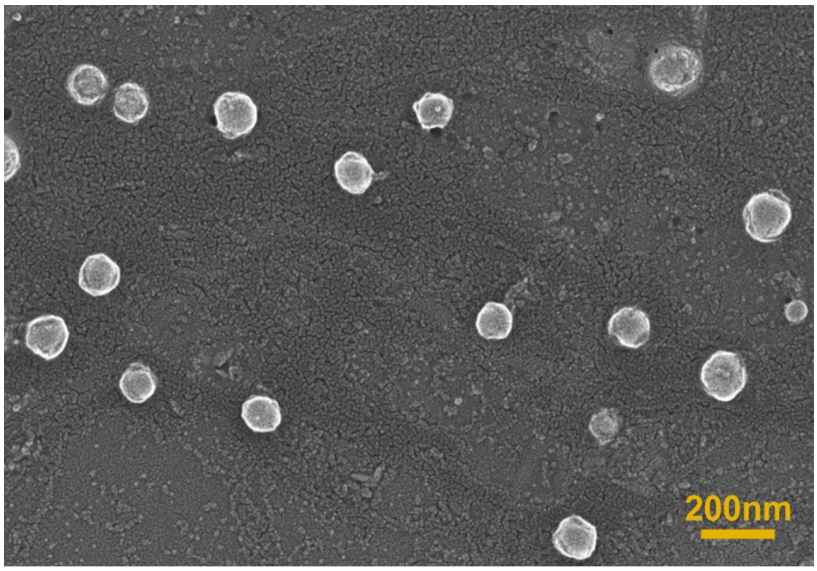


Fig. S6. SEM image of FFP. Scale bar: 200 nm.

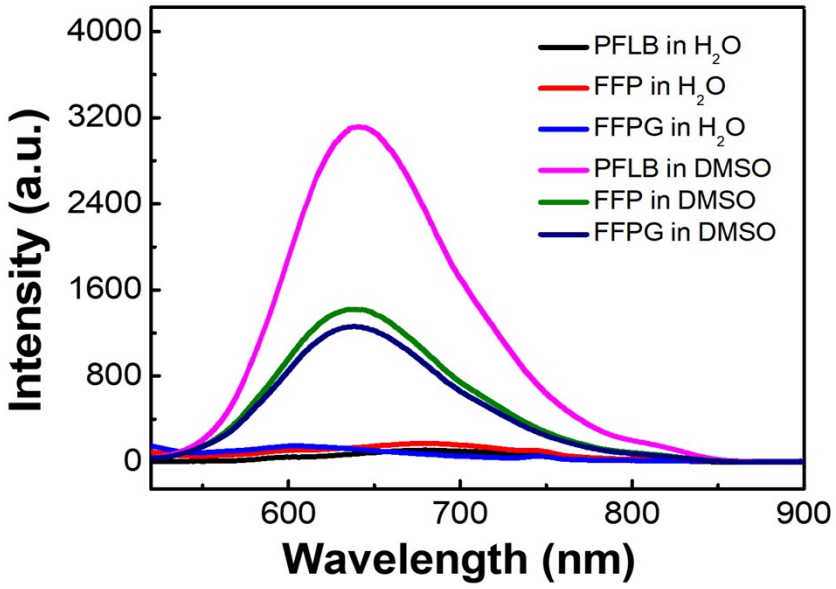


Fig. S7. Fluorescence emission spectra of PFLB, FF, FFP and FFPG in water or DMSO, respectively. λ_{ex} = 495 nm, [PFLB] = 2.0 μM.

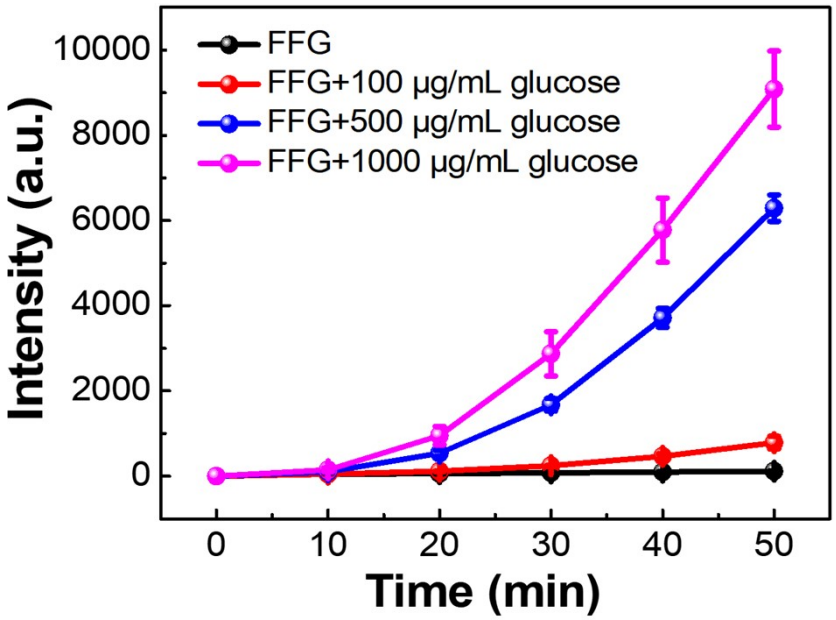


Fig. S8. Detection of H_2O_2 produced by FFG under different concentrations of glucose based on H_2O_2 probe ($\lambda_{ex}/\lambda_{em}$ = 490 nm/514 nm).

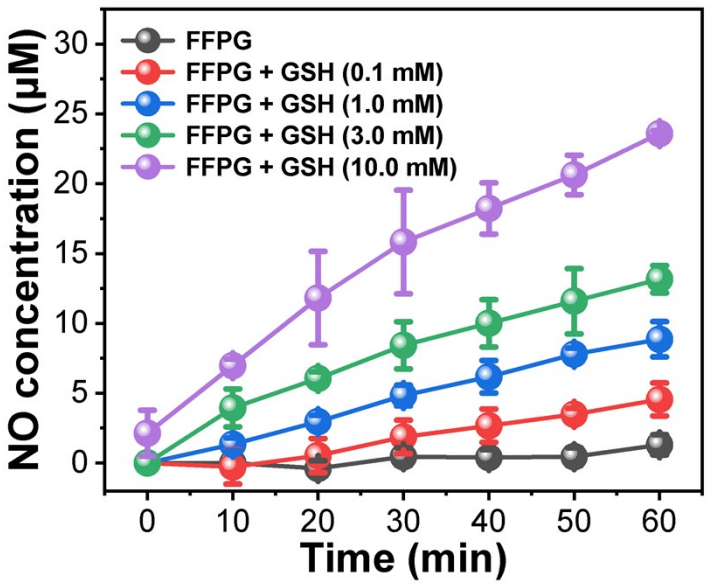


Fig. S9. NO produced by FFPG under different concentrations of GSH.

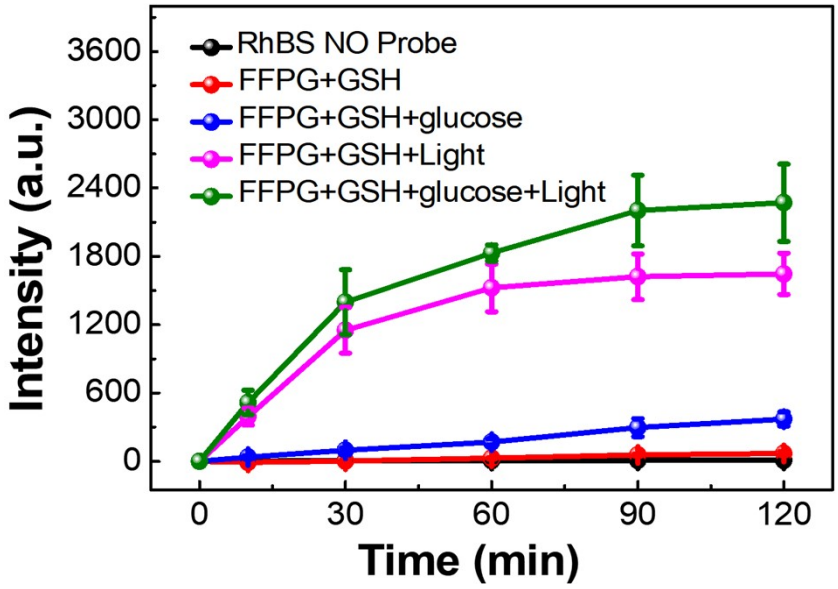


Fig. S10. NO generation detection of FFPG under different conditions based on NO RhBS probe. ($\lambda_{ex}/\lambda_{em}$ = 540/580 nm), (mean ± SD; n = 3).

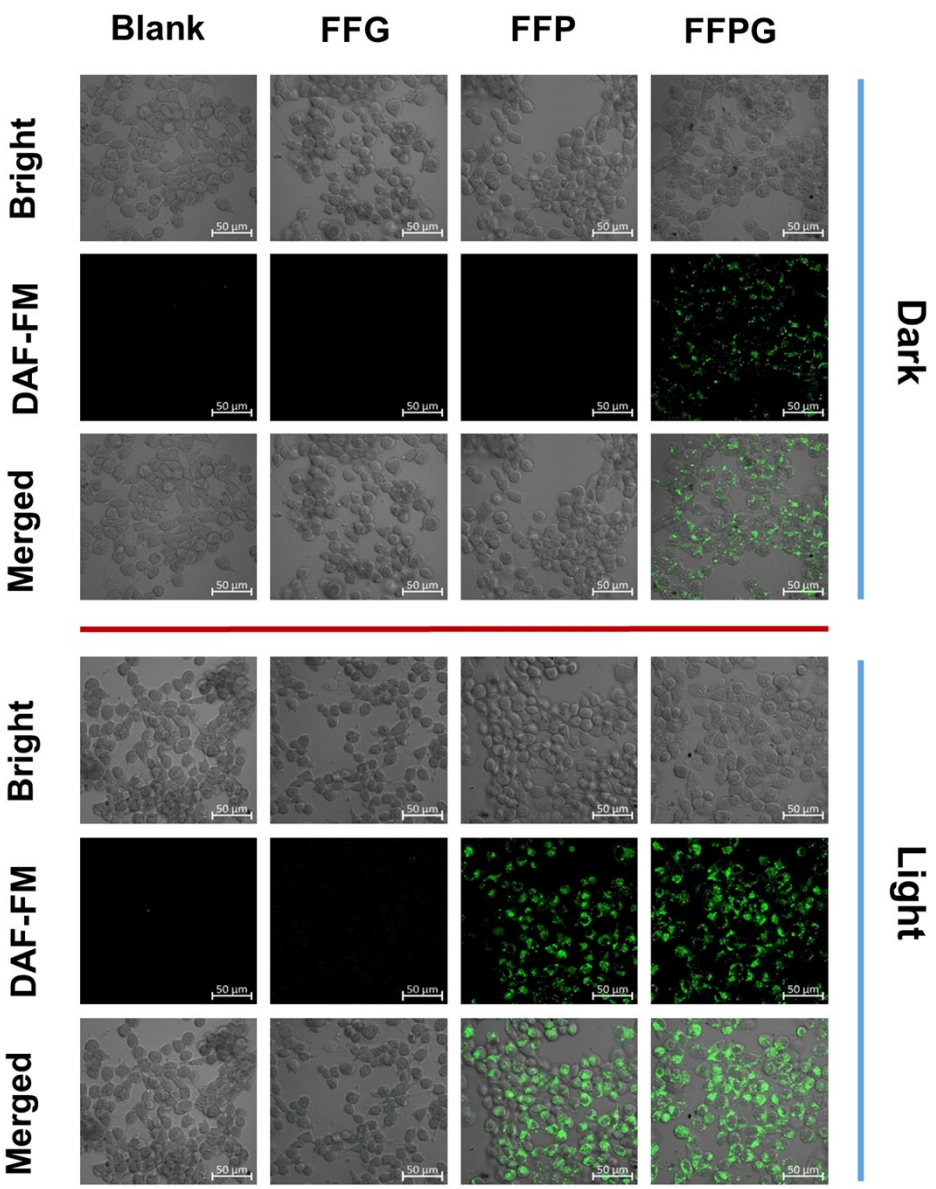


Fig. S11. The NO generation of various self-assemblies in 4T1 cells detected by DAF-FM DA fluorescence probe. The scale bar is 50 μm.

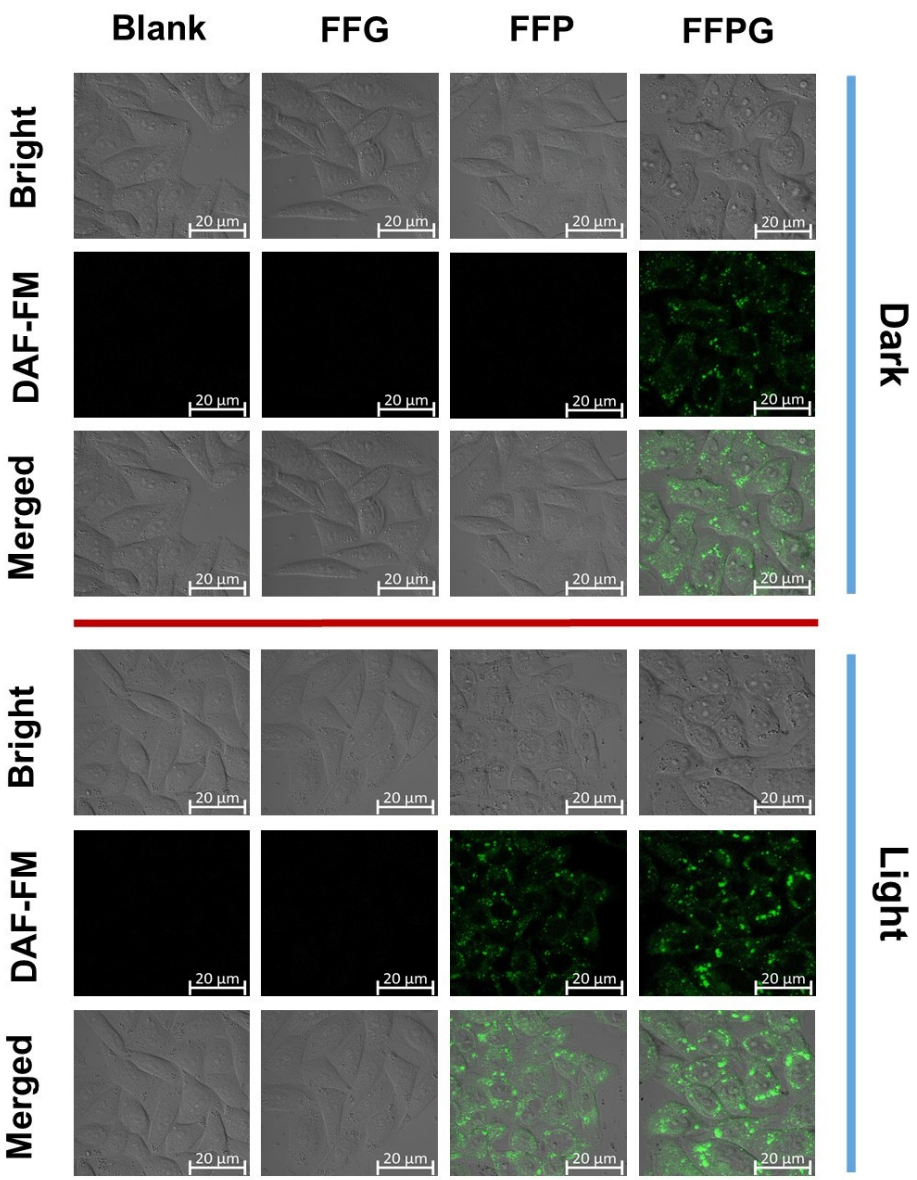


Fig. S12. The NO generation of various self-assemblies in MCF-7 cells detected by DAF-FM DA fluorescence probe. The scale bar is 20 μm.

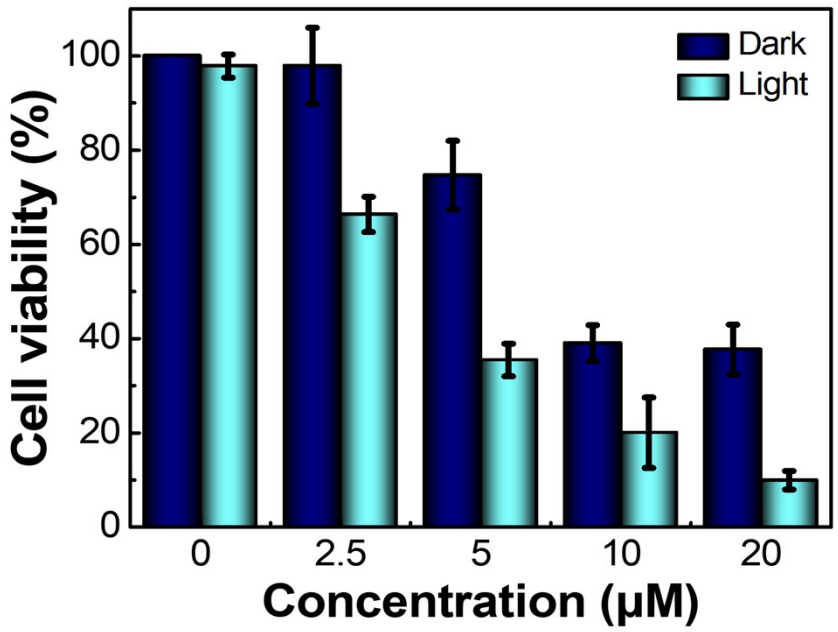


Fig. S13. Cell viability of MCF-7 cells treated with FFPG in dark and white light at different concentrations. Light intensity: 50.0 mW/cm^2, illumination time: 30 min.

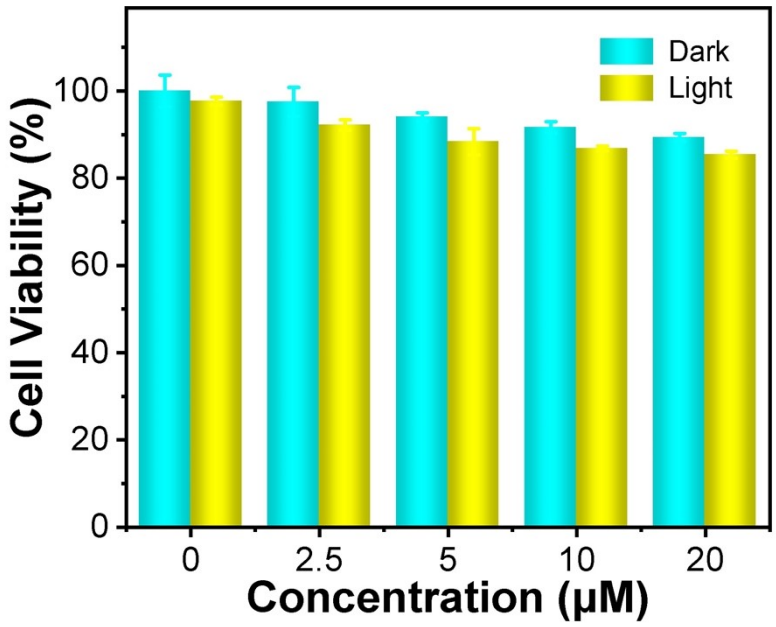


Fig. S14. Cell viability of L929 cells treated with FFPG in dark and white light at different concentrations. Light intensity: 50.0 mW/cm^2, illumination time: 30 min.

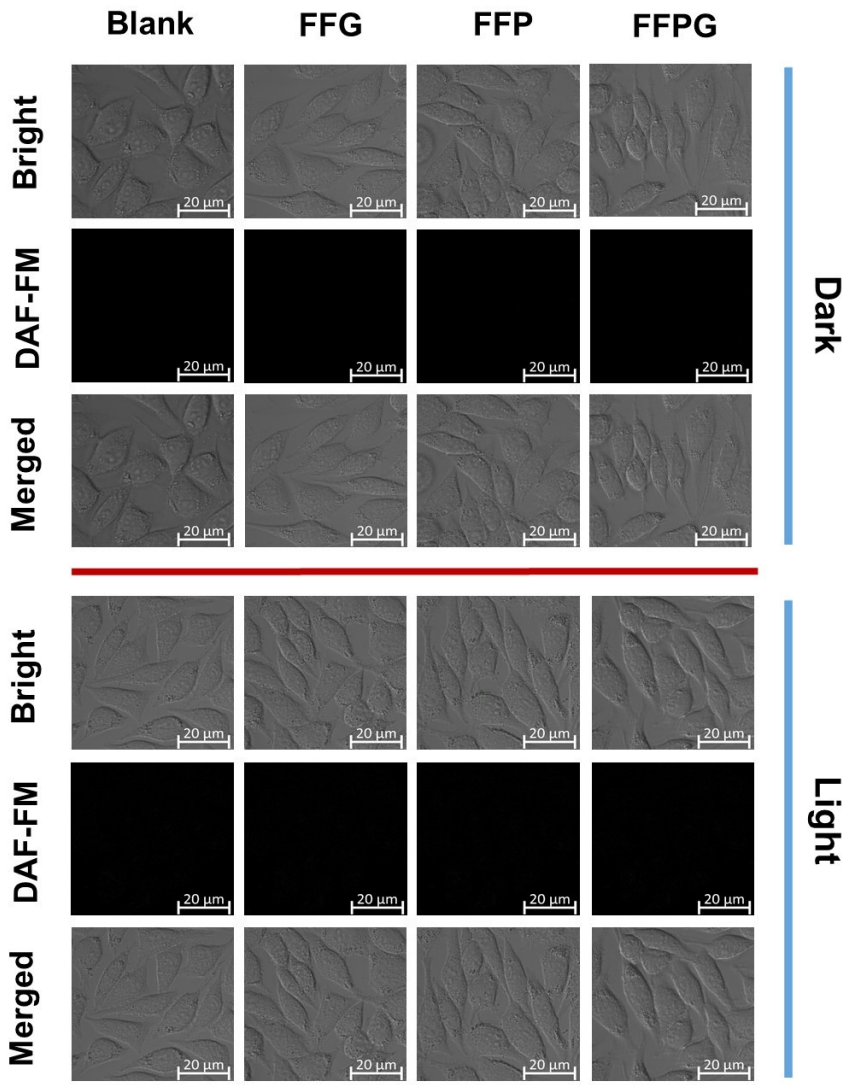


Fig. S15. The NO generation of various self-assemblies in L929 cells detected by DAF-FM DA fluorescence probe. The scale bar is 20 μm.

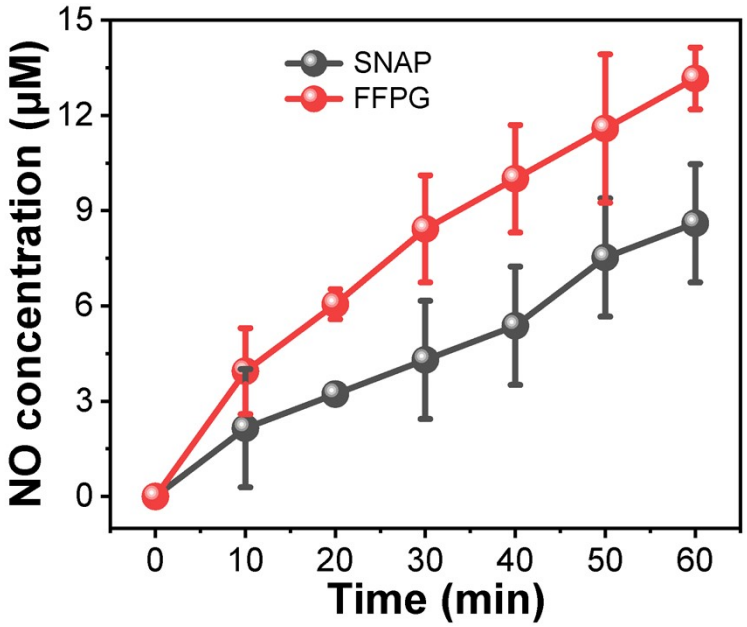


Fig. S16. NO generated by SNAP and FFPG under same concentration of GSH (3 mM).

Fig. S17. Photographs of bilateral tumor bearing mice during different treatments (including primary tumors and distal tumors).

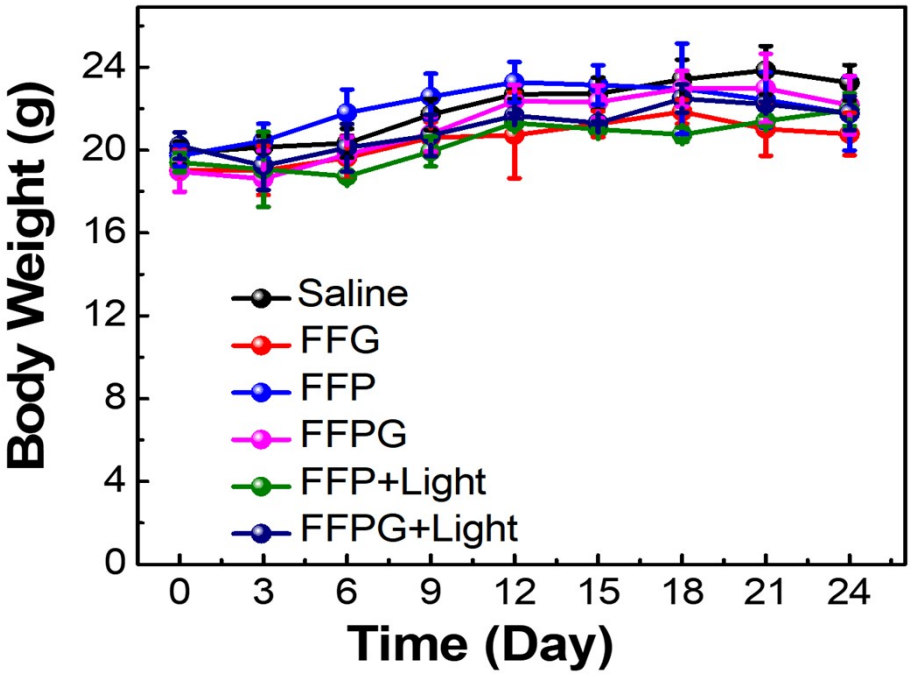


Fig. S18. Body weight changes of mice after different treatments in 24 days.

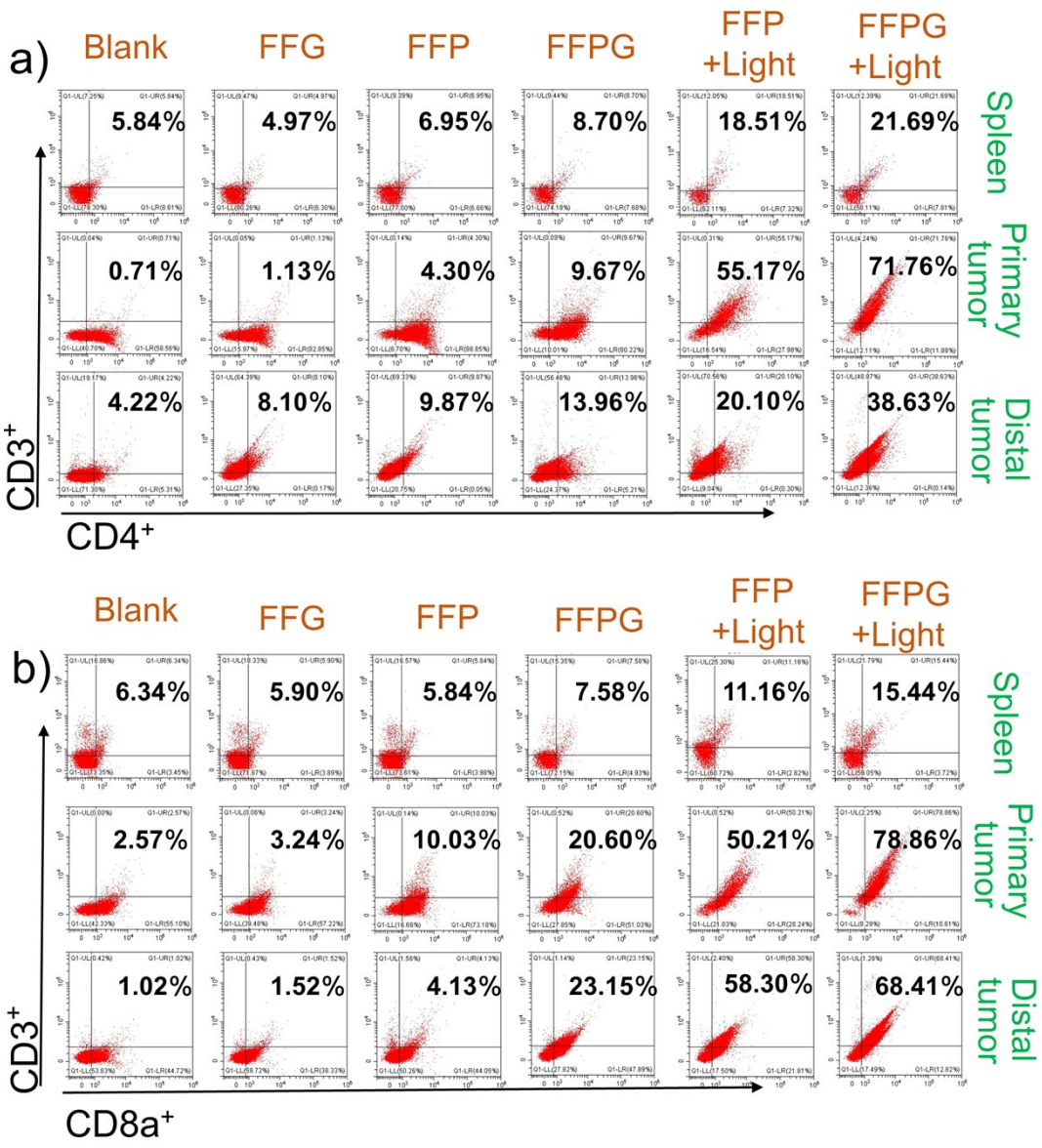


Fig. S19. (a) Flow cytometric analysis of $CD4^+$ T cells ($CD3^+$/ $CD4^+$) proliferation in mouse spleen, primary tumors and distant tumors after various treatments, respectively. (b) Flow cytometric analysis of $CD8^+$ T cells ($CD3^+$/$CD8a^+$) proliferation in mouse spleen, primary tumors and distant tumors after various treatments, respectively.

References

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