Intracellular fluorogenic supramolecular assemblies for self-report bioorthogonal prodrug activation

Yan Zhao,[a,b] Qingxin Yao,[a,b] Jiali Chen,[a,b] Ruijia Zhang,[a,b] Jialei Song,[a,b] Yuan Gao*[a,b]

[a] CAS Center for Excellence in Nanoscience, CAS Key Laboratory of Biomedical Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology, Beijing 100190, China.

[b] University of Chinese Academy of Sciences, Beijing 100190, China.

*Email: gaoy@nanoctr.cn

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1. General methods

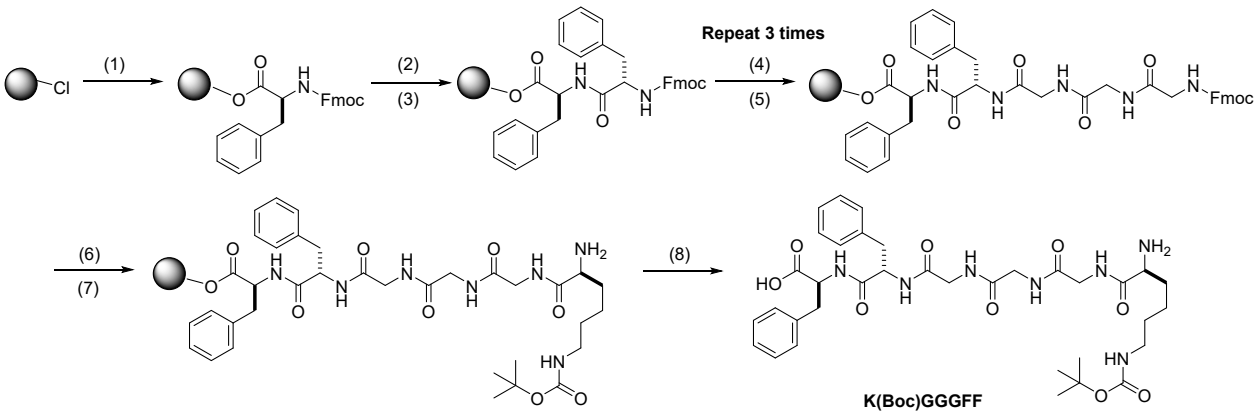
All chemicals were purchased from commercial sources without further purification unless otherwise stated. TCO-OH (CAS: 1414375-00-0) was purchased from Beijing Zhongkeliyan Biotechnology company. 6-well plates and 96-well plates were purchased from Corning. Mito-Tracker Red, Lyso-Tracker Red, BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 647, Calcein/PI were purchased from Beyotime.

NMR spectra were recorded on a Bruker 400 MHz Fourier transform spectrometer. TEM images were obtained on a Tecnai G2 20 S-TWIN transmission electron microscope. The confocal images were

gained by confocal microscope (Zeiss 710 and Ultraview Vox). The fluorescence spectra were recorded on a F98 fluorometer. Flow cytometry analysis was conducted by Attune NxT and BD FACS C6. Oscillatory rheological test was performed by Kinexus Pro^{+} rheometer.

2. Chemical Synthesis

2.1 The synthesis of peptides



(1) Fmoc-L-Phe-OH, DIPEA, DMF; (2) 20% piperidine / DMF; (3) Fmoc-L-Phe-OH, HBTU, DIPEA, DMF;

(4) Fmoc-L-Gly-OH, HBTU, DIPEA, DMF; (5) 20% piperidine / DMF;

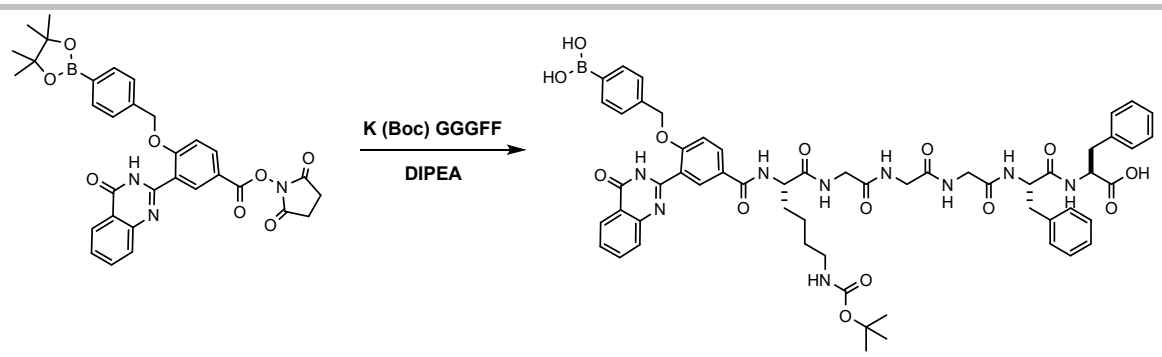
(6) Fmoc-L-Lys(Boc)-OH, HBTU, DIPEA, DMF; (7) 20% piperidine / DMF; (8) 2%TFA

The synthesis of peptides followed the standard solid phase peptide synthesis (SPPS) using 2-chlorotrity resin and the corresponding Fmoc-protected amino acids. The synthetic route of K (Boc) GGGFF and molecular structures were shown above. Peptides K (Boc) GGGFF and K (Boc) GGGGF were purified by semi preparative HPLC to give white powder and confirmed by MOLDI-TOF MS and ^{1}H-NMR.

K (Boc) GGGFF (67.2%) ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 8.68 (t, 1H), 8.40 (d, 1H), 8.25 (t, 1H), 8.13 - 8.01 (m, 4H), 7.34 - 7.12 (m, 10H), 6.77 (t, 1H), 4.54 (td, 1H), 4.43 (td, 1H), 3.87 - 3.65 (m, 6H), 3.58 (d, 1H), 3.07 (dd, 1H), 3.01 - 2.84 (m, 4H), 2.73 - 2.65 (m, 1H), 1.68 (q, 2H), 1.37 (s, 12H). MOLDI-TOF MS (m/z): calcd. 711.359; found [M+ Na] $^{+}$, 734.452.

K (Boc) GGGGF (62.4%) ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 8.70 (t, 1H), 8.26 (t, 1H), 8.18 (t, 2H), 8.12 - 8.05 (m, 3H), 7.33 - 7.16 (m, 5H), 6.77 (t, 1H), 4.42 (td, 1H), 3.87- 3.70 (m, 7H), 3.64 (dd, 1H), 3.04 (dd, 1H), 2.93 - 2.81 (m, 3H), 1.79 - 1.60 (m, 2H), 1.37 (s, 12H). MOLDI-TOF MS (m/z): calcd. 621.312; found [M+ Na] $^{+}$, 644.234.

2.2 The synthesis of BQA-peptides



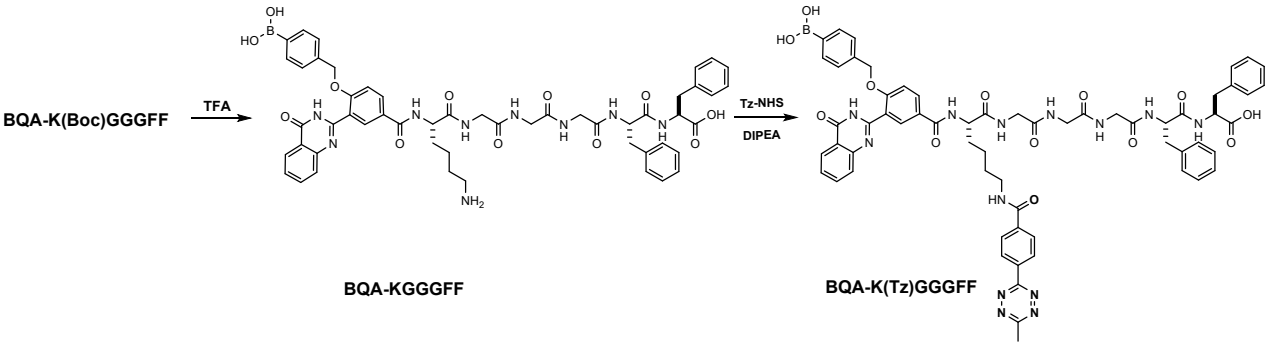
BQA-NHS **BQA-K(Boc)GGGFF**

The synthesis of BQA-NHS was referred to the published methods.[1] BQA-NHS (51.2 mg, 0.1 mmol) was dissolved in 0.5 mL DMF, then DIPEA (32 μL, 0.2 mmol) was added to obtain a well-dispersed solution. Peptides (0.1 mmol) were suspended in the solution. The mixture was stirred at 30 ℃ for 2 h. The product was purified by semi preparative HPLC to give white powder.

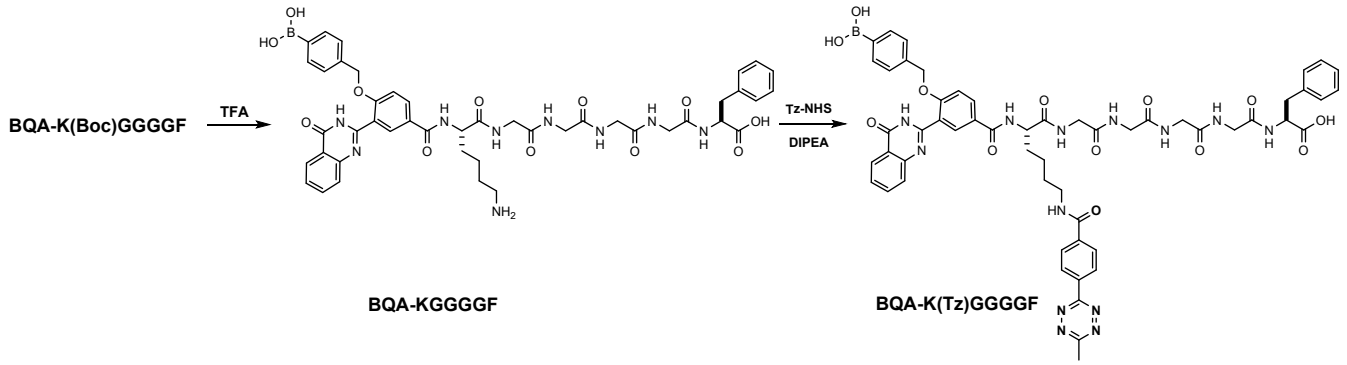
BQA-K (Boc) GGGFF (92.5 mg, 83.4%) ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 8.53 (d, 1H), 8.41 - 8.19 (m, 5H), 8.16 (d, 1H), 8.14 - 7.93 (m, 6H), 7.87 - 7.82 (m, 1H), 7.76 (d, 3H), 7.59 - 7.49 (m, 2H), 7.44 (d, 2H), 7.35 (d, 1H), 7.30 - 7.16 (m, 10H), 5.28 (s, 2H), 4.60 - 4.36 (m, 5H), 3.71 (m, 6H), 3.10 - 2.83 (m, 8H), 1.83 - 1.60 (m, 4H), 1.33 (s, 12H).

BQA-K (Boc) GGGGF (83.9 mg, 82.3%) ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 8.54 (d, 1H), 8.29 - 8.21 (m, 2H), 8.20 - 8.07 (m, 5H), 8.03 (t, 1H), 7.85 (t, 1H), 7.75 (s, 2H), 7.55 (t, 1H), 7.44 (d, 2H), 7.36 (d, 1H), 7.23 (m, 5H), 6.75 (t, 1H), 5.28 (s, 2H), 4.42 (m, 2H), 3.74 (m, 8H), 3.64 (m, 2H), 3.04 (m, 1H), 2.93 - 2.84 (m, 3H), 1.81 - 1.64 (m, 2H), 1.39 - 1.21 (m, 12H).

2.3 The synthesis of BQA-peptides-Tz



BQA-KGGGFF **BQA-K(Tz)GGGFF**

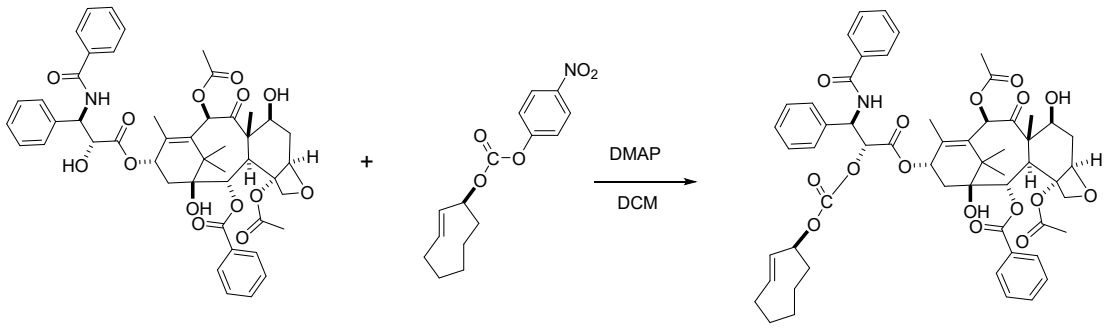


BQA-peptides were added to TFA to remove Boc, and the products were precipitated with ether. Then, peptide KGGGFF (50.5 mg, 0.05 mmol) / KGGGGF (46.0 mg, 0.05 mmol) was dissolved in a mixed solution of 0.2 mL water and 0.2 mL acetonitrile, DIPEA (16 μL, 0.1 mmol) was added to obtain a well-dispersed solution. After dissolving tetrazine (32.7 mg, 0.1 mmol) in 0.2 mL acetonitrile, the solution was added to mixed solution and stirred at 30 ℃ for 2 h. The product was purified by semi preparative HPLC to give dark-pink powder.

BQA-K (Tz) GGGFF (**1**) (96.3mg, 78.9%) ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 8.55 (d, 1H), 8.40 - 8.20 (m, 5H), 8.17 - 7.96 (m, 6H), 7.87 - 7.80 (m, 1H), 7.75 (t, 3H), 7.50 (td, 5H), 7.36 (d, 1H), 7.32 - 7.05 (m, 9H), 5.28 (s, 2H), 4.49 (m, 4H), 3.73 (m, 5H), 3.57 (s, 3H), 3.11 - 2.88 (m, 8H), 2.71 (m, 1H), 1.83 - 1.71 (m, 2H), 1.50 - 1.14 (m, 6H). MOLDI-TOF MS (m/z): calcd. 1193.472; found [M+ H] $^{+}$, 1194.629.

BQA-K (Tz) GGGGF (**2**) (90.2mg, 79.8%) ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 8.55 (d, 1H), 8.37 (d, 2H), 8.29 (d, 1H), 8.26 (d, 1H), 8.13 (dt, 5H), 8.04 (d, 1H), 7.86 - 7.81 (m, 1H), 7.75 (d, 2H), 7.62 - 7.40 (m, 5H), 7.35 (d, 1H), 7.29 - 7.16 (m, 4H), 5.27 (s, 2H), 4.49 - 4.38 (m, 2H), 3.71 (m, 7H), 3.15 - 2.79 (m, 7H), 1.83 - 1.68 (m, 2H), 1.51 - 1.19 (m, 6H). MOLDI-TOF MS (m/z): calcd. 1103.425; found [M+ H] $^{+}$, 1104.654.

2.4 Synthesis of axial-(E)-cyclooct-2-en-1-yl paclitaxel carbamate (TCO- PTX)



(E)-cyclooct-2-en-1-yl (4-nitrophenyl) carbonate (TCO-NO_2) (20.0 mg, 68.7 μmol), PTX (117.3 mg, 137.4 μmol) and DMAP (1.0 mg, 8.2 μmol) were suspended in 1 mL anhydrous DCM, then the mixture was stirred in the dark at 30 ℃ for 12 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (Hexane: EtOAc = 1:1) to give TCO-PTX (54.6 mg, 79.1%) ^{1}H NMR (400 MHz, Chloroform-d) δ (ppm) 8.15 (d, 2H), 7.76 (d, 2H), 7.61 (t, 1H), 7.55 - 7.33 (m, 11H), 6.96 (q, 1H), 6.28 (d , 2H), 5.99 (m, 1H), 5.68 (d, 1H), 5.54 - 5.43 (m, 2H), 4.97 (d, 1H), 4.32 (d, 1H), 4.20 (d, 1H), 3.81 (d, 1H), 2.54 (m, 2H), 2.45 (d, 3H), 2.39 (m, 2H), 2.23 (s, 3H), 2.20 - 1.96 (m, 5H), 1.91 (d, 3H), 1.68 (s, 3H), 1.66 - 1.40 (m, 4H), 1.24 (s, 3H), 1.13 (s, 3H), 1.08 (m, 1H), 0.84 - 0.75 (m, 1H).

3. Supplementary Methods

3.1 Preparation of hydrogel

Assembly precursors **1** was dissolved in 10 mM fructose solution at 5 mM. 1 mM NaOH was used to adjust the pH of the solution. Then, 100 mM H_2O_2 was added to form a hydrogel.

3.2 TEM

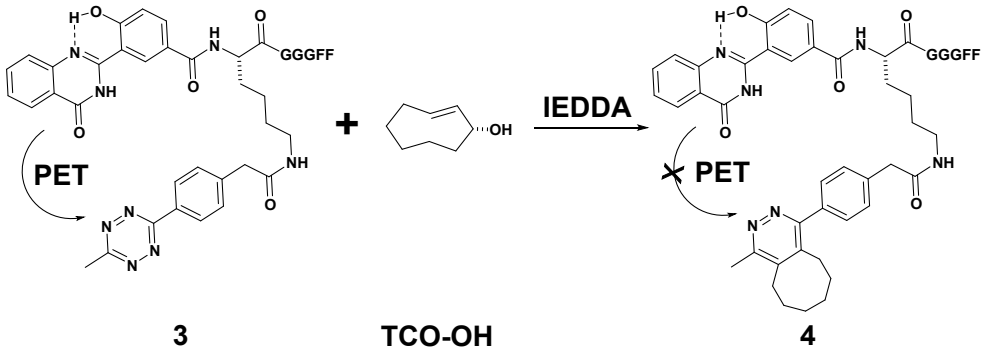
TEM samples of assemblies: 8 μL assemblies were placed on a 300 meshes copper coated with carbon film and stained with uranium acetate for thrice. The samples were set to dry overnight. TEM images were obtained by Tecnai G2 20 S-TWIN at 200 kV.

TEM samples of cell fractions: Cells were pre-incubated with **1** and then collected for cell lysis and fractionation.[2] Firstly, the nuclei fragments were obtained by 600 g centrifugation for 10 min. Then, the supernatant was continued to centrifuge at 15000 g for 5 min and the precipitate were cell fragments include mitochondria, lysosomes and peroxisomes. The TEM samples of cell fractions were prepared using uranium acetate staining.

3.3 Oscillatory rheological test

The samples were measured on Malvern Kinexus Pro$^+$ at 25 °C, with 0.5 mm gap, 1 Hz frequency, and 0.5% strain. Each sample was repeatedly measured at least 3 times.

3.4 Fluorescence liberation of hydrogel by bioorthogonal reaction



3 TCO-OH 4

5 mm hydrogel was prepared and 10 eq. TCO-OH was added for bioorthogonal reaction. Images of hydrogel before and after adding TCO-OH were captured under 365 nm light. Fluorescence emission spectra were recorded on fluorometer with an excitation peak at 330 nm.

3.5 Analytical HPLC

Oxidization by H_2O_2: **1** was dissolved in the mixture of acetonitrile and H_2O (1:1) at 1 mM. HPLC traces before and after adding H_2O_2 were gained. The detected wavelength was 254 nm.

Liberation of PTX was tested by analytical HPLC.

3.6 Cell culture conditions

MCF-7 human mammary adenocarcinoma cell line and HeLa human cervical adenocarcinoma cell line were cultured in Dulbecco's Modified Eagle Medium (Gibco). The culture medium was supplemented with 10% fetal bovine serum (Gibco) and 1% pen strep (Gibco). MCF-10A human mammary epithelial cell line was cultured in specific medium (Procell) (DMEM＋5% HS＋20 ng/ml EGF＋0.5 μg/ml Hydrocortisone＋10 μg/ml Insulin＋1% NEAA＋1% P/S). The cultures were kept at 37 ℃, 5% of CO_2, and 90% humidity.

3.7 BQH fluorescence and colocalization

Cells were seeded in a confocal dish with glass substrate at an appropriate density. Cells were cultured with 1 mL 100 μM **1** for 8 h. Then, the culture medium was removed and cells were washed with PBS. Next, cells were incubated with pre-warmed 1 μM Mito-Tracker Red and Lyso-Tracker Red staining solution for 30 min at 37 ℃. Last, the cells were rinsed with PBS. 20 μM TCO-PTX PBS solution was added to activate fluorescence. Fluorescent images were captured by CLSM (Zeiss 710).

3.8 Cell viability

Cells were seeded in 96-well plates at a density of 5×10^3 cells per well. After attachment incubation for 12 h, the culture medium was removed and replaced by 200 μL different concentrations of **1**. After 24 h incubation, cell viability was determined by MTT assay (Solarbio). After removing the culture medium, 100 μL fresh medium containing 10 μL thiazolyl blue tetrazolium bromide (MTT) and 90 μL medium was added to each well. After an incubation for 4 h, the media was gently removed. Last, 110 μL DMSO was added and the absorbance was detected at 490 nm on a plate reader (PerkinElmer).

To test the efficacy of prodrug activation, cells were seeded in 96-well plates at a density of 5×10^3 cells per well. After 12 h incubation for cell attachment, the culture medium was replaced with 200 μL 100 μM **1**. After 8 h incubation, cells were washed with PBS and cultured with 200 μL different concentrations of TCO-PTX for 72 h.

To test the efficacy of TCO-PTX or PTX, cells were incubated with each compound for 72 h after cell attachment.

3.9 Microtubule morphology staining

Cells were plated in a confocal dish with glass substrate. Cells were cultured with 1 mL 100 μM **1** for 8 h. Then, the culture medium was replaced by 1 mL 20 μM TCO-PTX. For positive control, cells were cultured with 1 mL 20 μM PTX. After 24 h incubation, cells were washed three times and stained with 1 μM Tubulin-Tracker Red (Beyotime) for 30 min. Fluorescent images were captured by CLSM (Ultraview Vox).

3.10 Intracellular BQH and EdU fluorescence analysis

Cells were seeded in 6-well plates at a density of 2×10^5 cells per well. After 12 h incubation for cell attachment, the culture medium was replaced by 1 mL 100 μM **1**. After 8 h incubation, cells were treated with 1 mL 0.5 μM TCO-PTX for 72 h. Culture medium was added as negative group. Then, cell samples were prepared according to BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 647. All samples were analyzed with flow cytometry (Attune NxT).

3.11 Cell cycle analysis

Cells were seeded in 6-well plates at a density of 2×10^5 cells per well. After 12 h incubation for cell attachment, cells were cultured with 1 mL 100 μM **1** for 8 h. Then, the culture medium was replaced by 1 mL 20 μM TCO-PTX. 1 mL 0.5 μM PTX was added as positive control. Culture medium was added

as negative control. After 72 incubation, cell samples were prepared according to Cell Cycle and Apoptosis Analysis Kit (Beyotime). All samples were analyzed for the DNA content with flow cytometry (BD FACS C6).

3.12 Preparation of 3D breast cancer spheroids

3D breast cancer spheroids were generated using the liquid overlay technique (LOT). Cells were plated in 96-well plates coated with 1% agarose at a density of 2×10^4 cells per well and cultured for 7 days. MCF-7 spheroids were collected and filtered by 70 μm cell filters (Solarbio) for further examinations.

3.13 BQH and live/dead staining on 3D breast cancer spheroids

3D spheroids were cultured with 100 μM **1** for 8 h, then samples were washed with PBS for three times and cultured with 20 μM TCO-PTX for 72 h. The samples were washed to remove culture medium and stained with 1 μM Calcein/PI solution for 20 min. Last, all the samples were washed with PBS for three times and removed to confocal dishes. Fluorescent images were captured by CLSM (Zeiss 710).

4. Supplementary Results

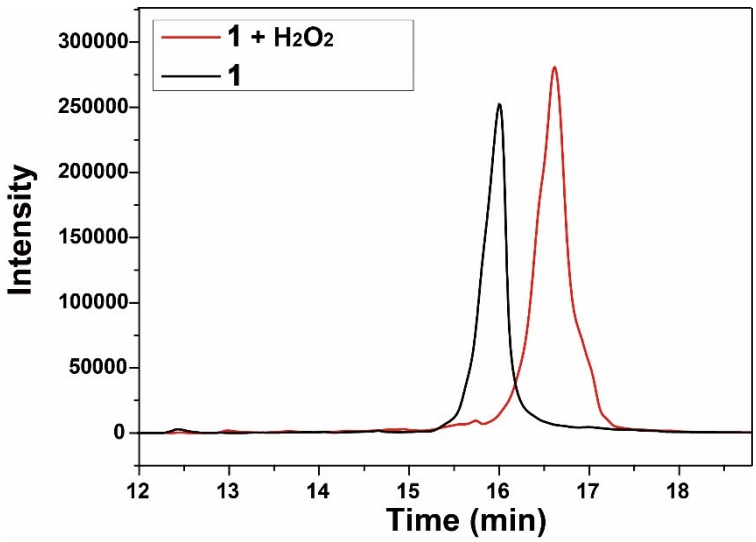


Figure S1. HPLC traces of **1** before and after adding H_2O_2.

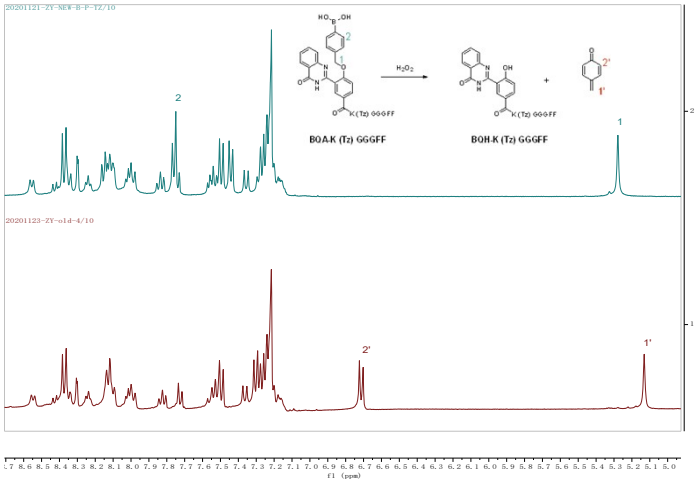


Figure S2. Comparison of ^{1}H NMR of **1** and the corresponding oxidation products **3** before and after adding H_2O_2.

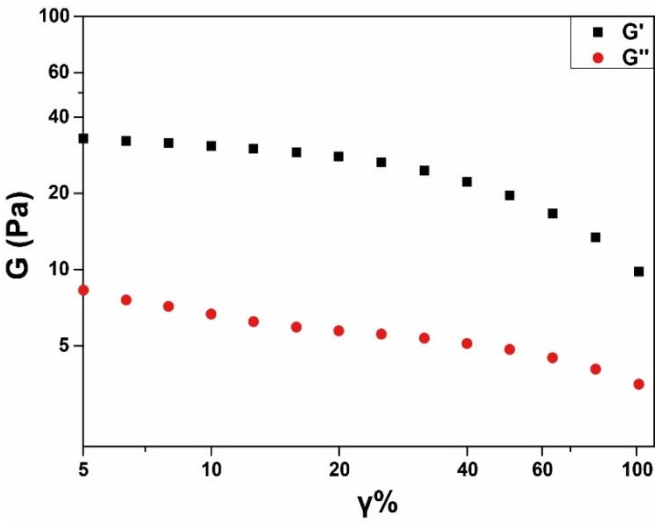


Figure S3. Strain sweep of the dynamic storage modulus (G′) and loss modulus (G″) of 5 mM hydrogel.

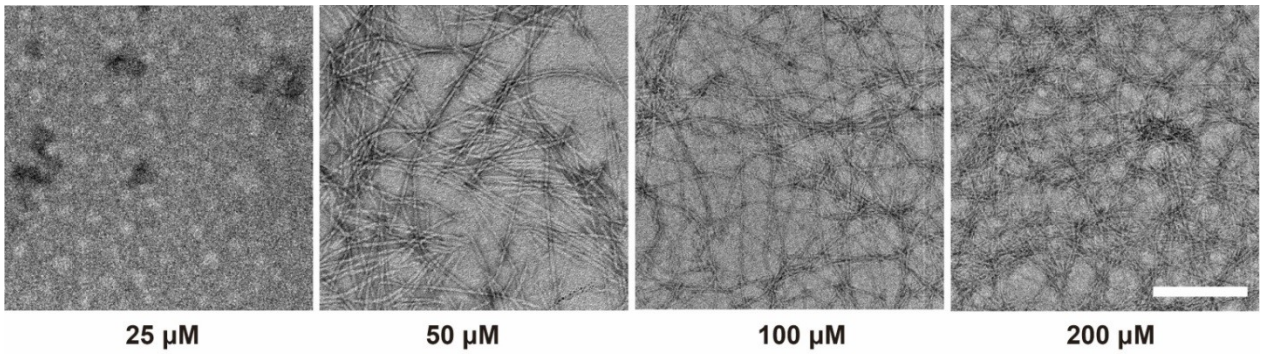


Figure S4. TEM images of the assembly morphology of **3** at different concentrations. Scale bar, 200 nm.

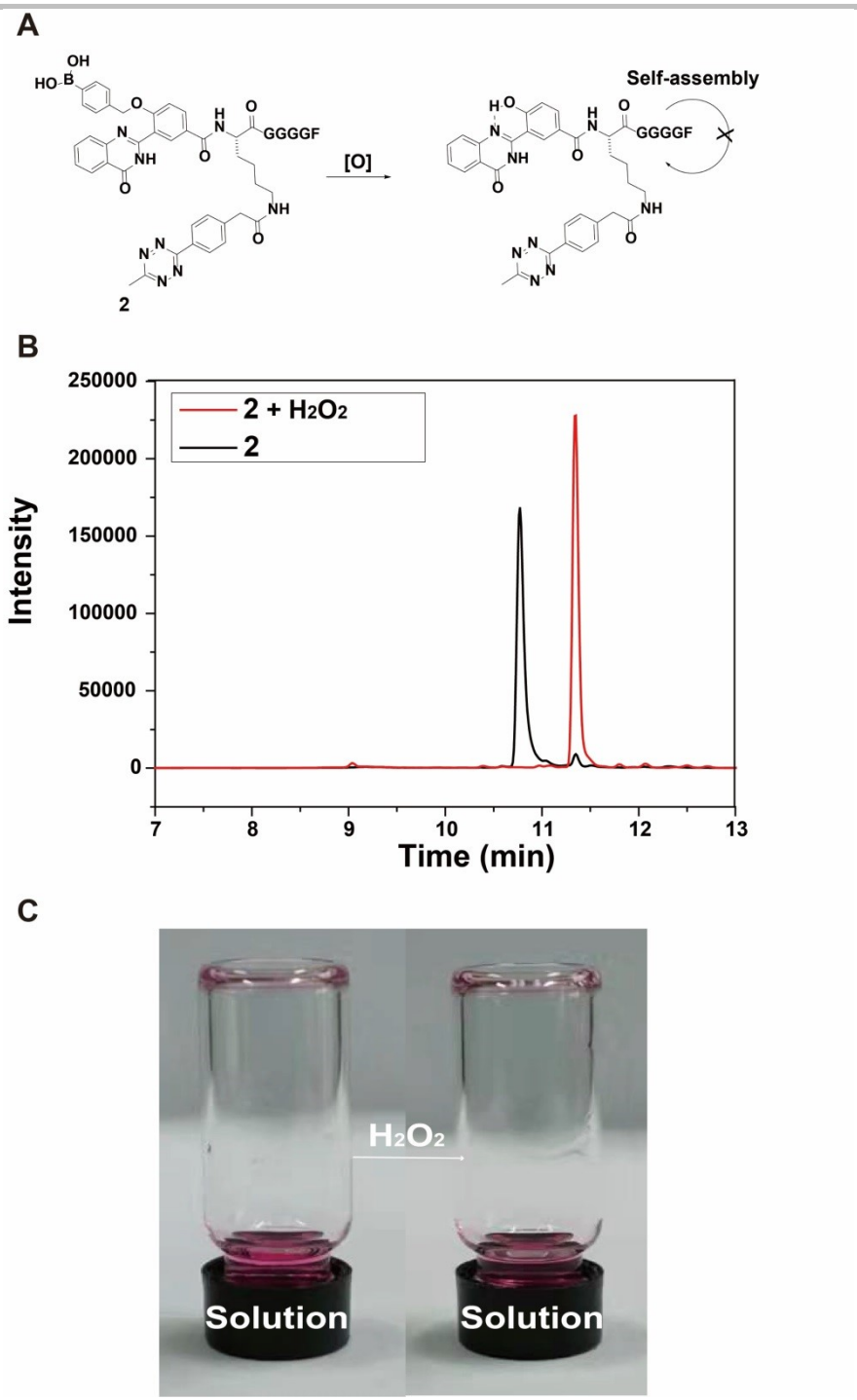


Figure S5. (A) Oxidation of **2** by H_2O_2. (B) HPLC traces before and after adding H_2O_2 and (C) optical images of 5 mM **2** solution which failed to form a hydrogel upon oxidation.

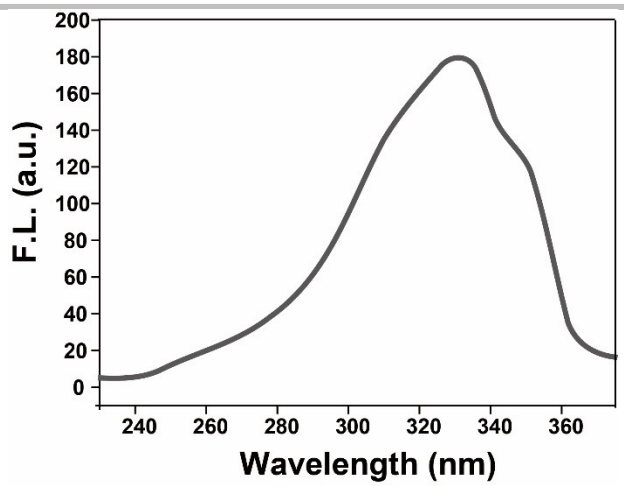


Figure S6. Fluorescence emission spectrum of 5 mM **3** with activation of TCO-OH.

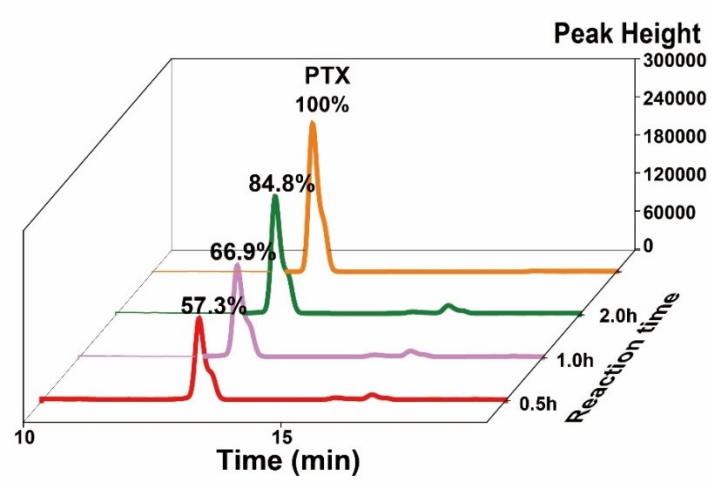


Figure S7. Time dependent reaction efficiency between **3** and TCO-PTX to liberate PTX.

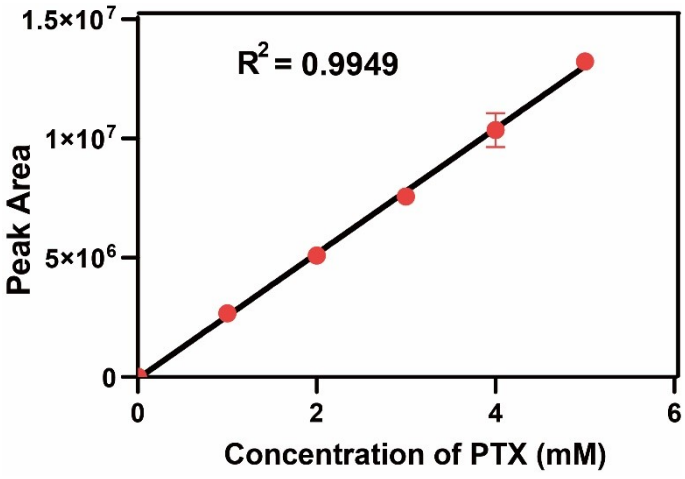


Figure S8. Standard curve of PTX.

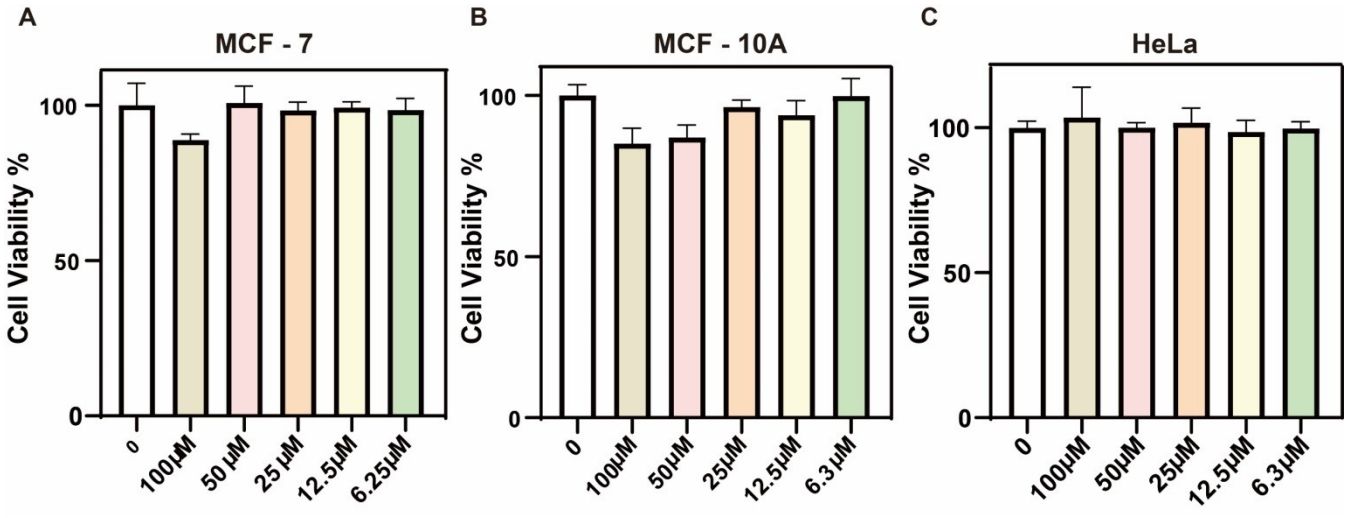


Figure S9. Cytotoxicity of assembly against (A) MCF-7 (B) MCF-10A (C) HeLa.

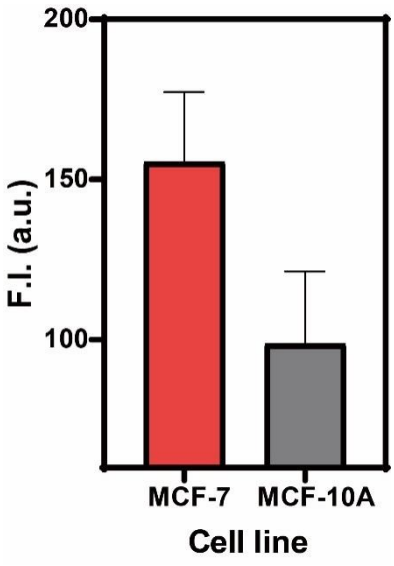


Figure S10. ROS level of MCF-7 and MCF-10A.

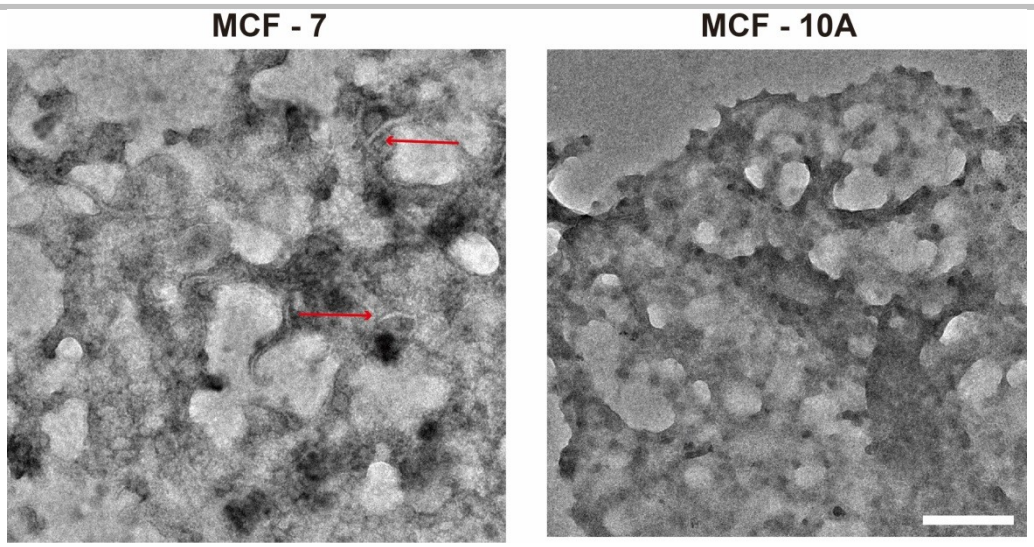


Figure S11. TEM images of cellular fractions. Scale bar, 200 nm.

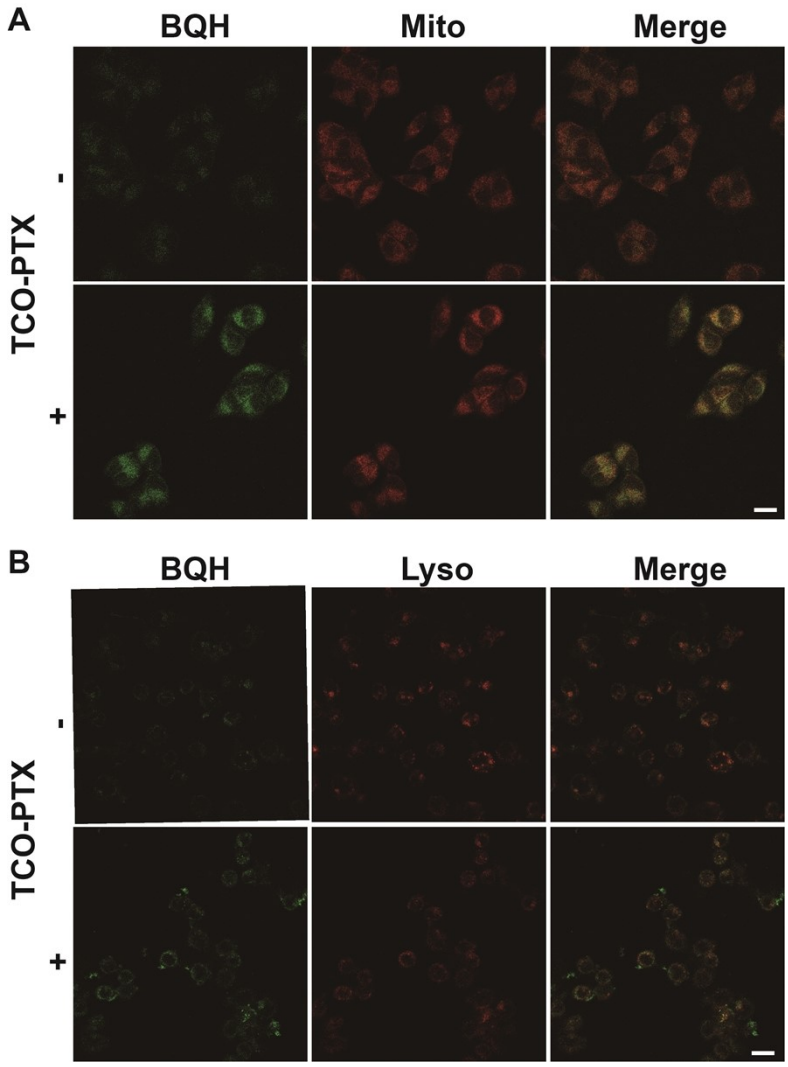


Figure S12. CLSM images of HeLa cells. Colocalization of **1**/TCO- PTX with (A) Mito tracker, (B) Lyso tracker. Scale bars, 20 μm.

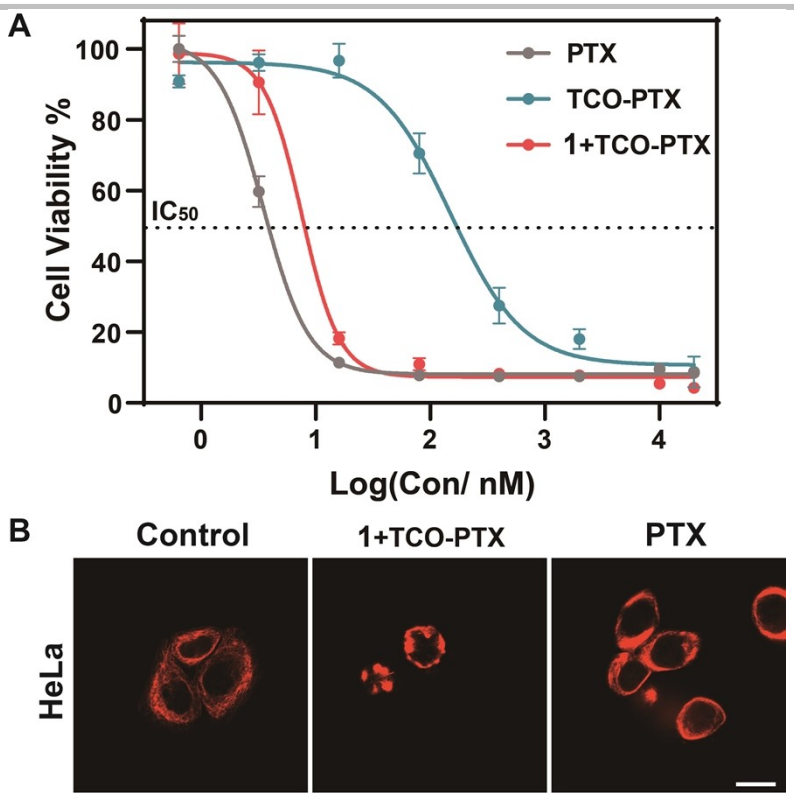


Figure S13. (A) Cytotoxicity tests and (B) microtubules morphology of prodrug activation against HeLa cells. Scale bar, 20 μm.

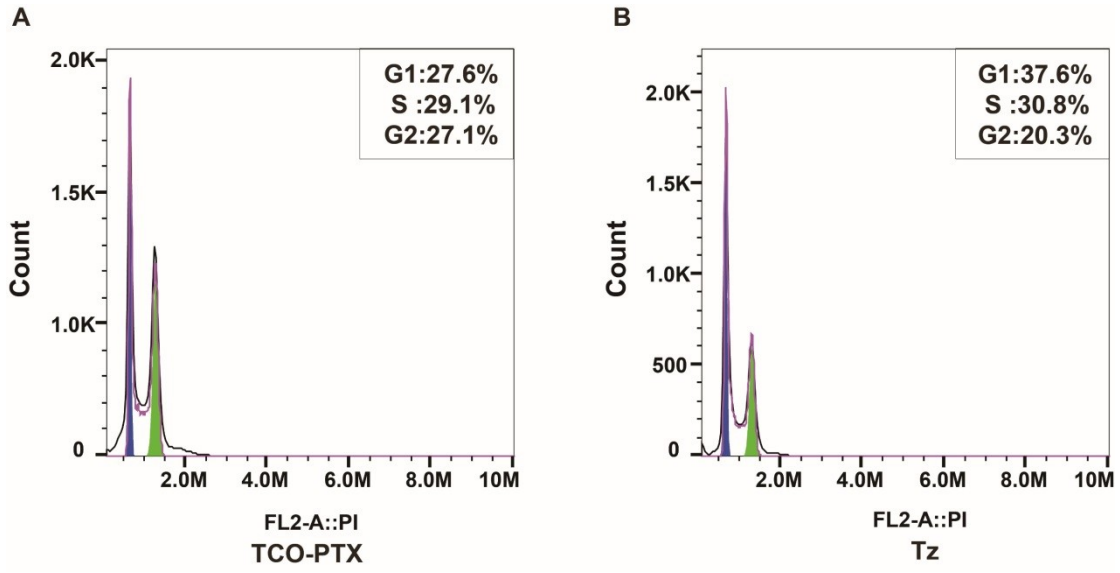


Figure S14. Cell cycle analysis of MCF-7 cells incubated with (A) TCO-PTX and (B) Tz for 24 h.

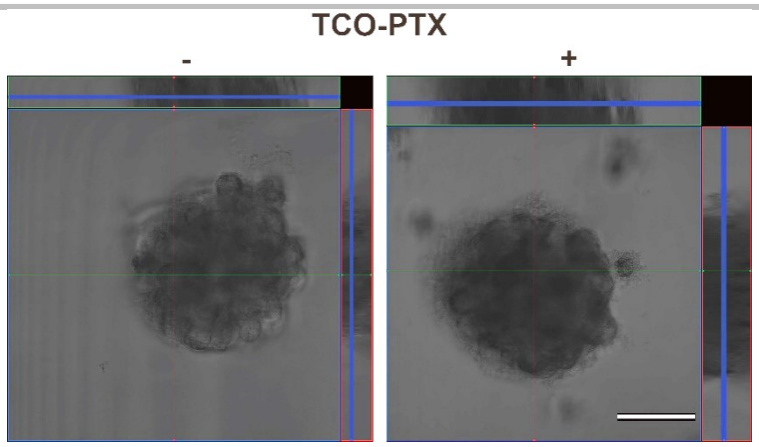


Figure S15. Bright field images of cell spheroids. Scale bar, 50 μm.

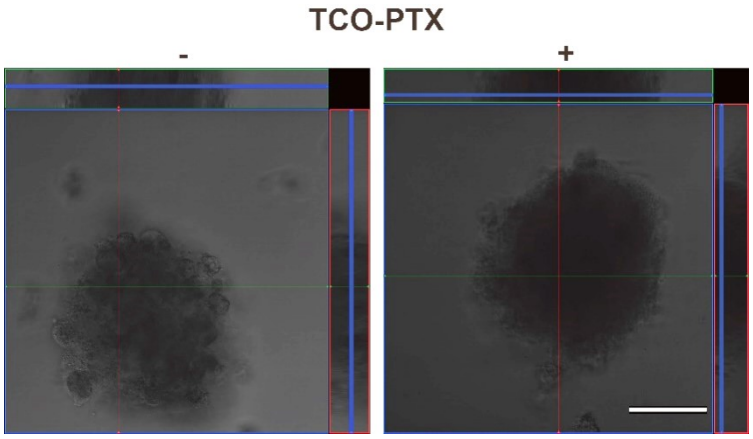


Figure S16. Bright field images of cell spheroids. Scale bar, 50 μm

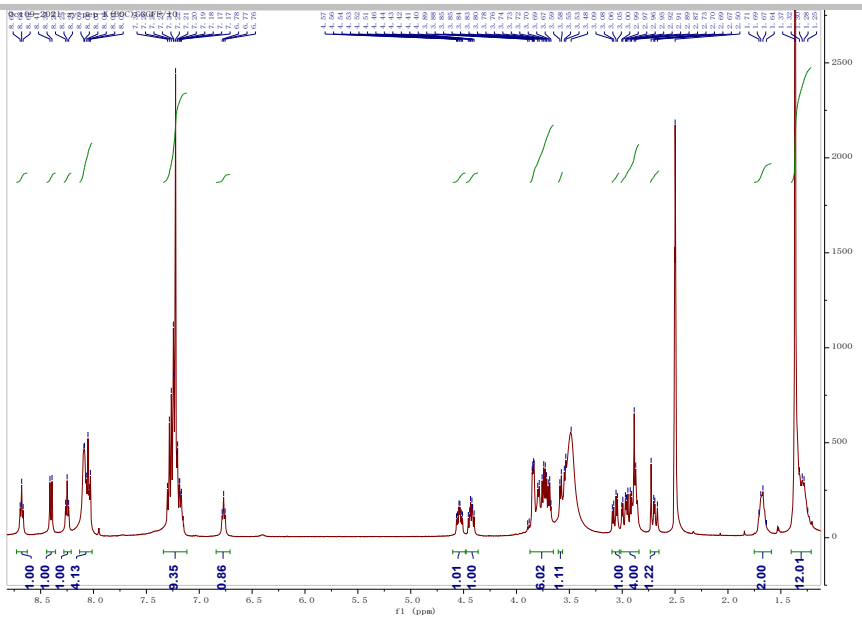


Figure S17. The ^{1}H NMR of K (Boc) GGGFF.

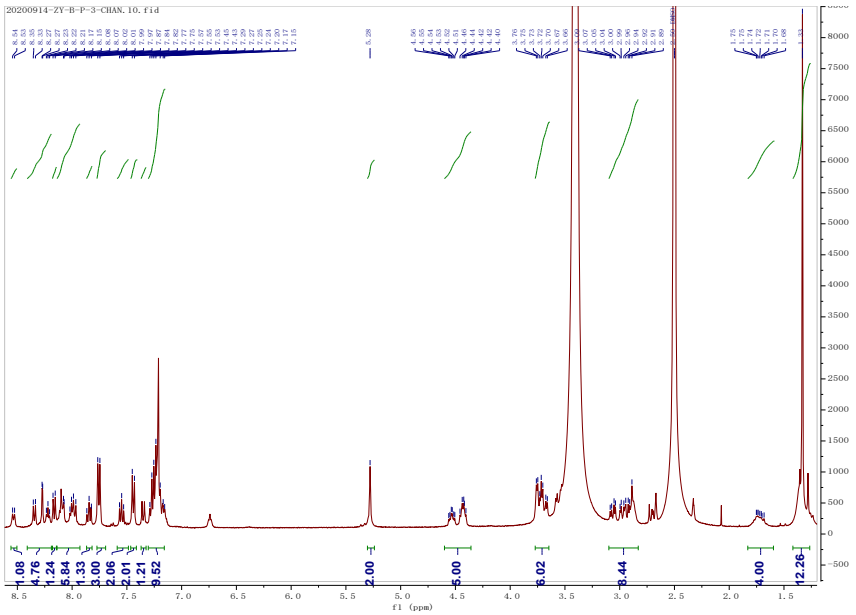


Figure S18. The ^{1}H NMR of BQA- K (Boc) GGGFF.

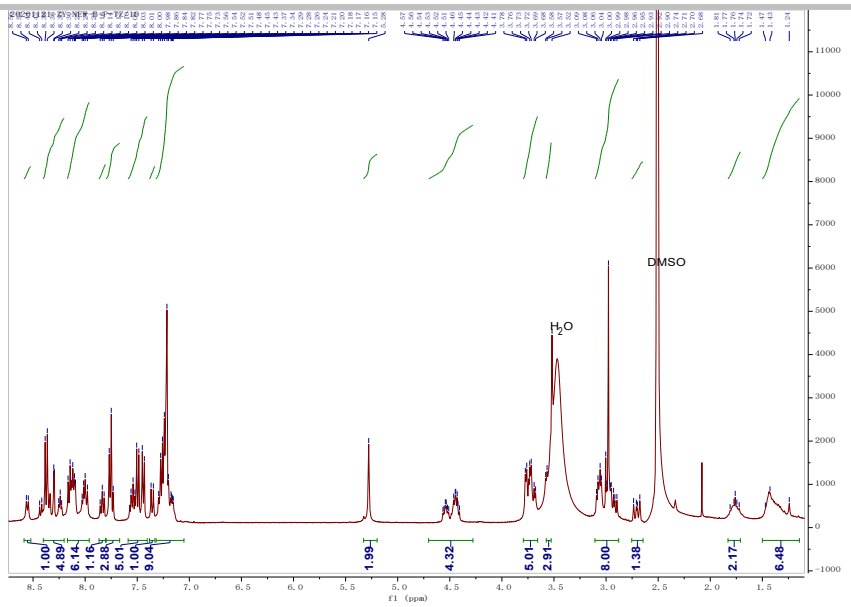


Figure S19. The ^{1}H NMR of BQA- K (Tz) GGGFF.

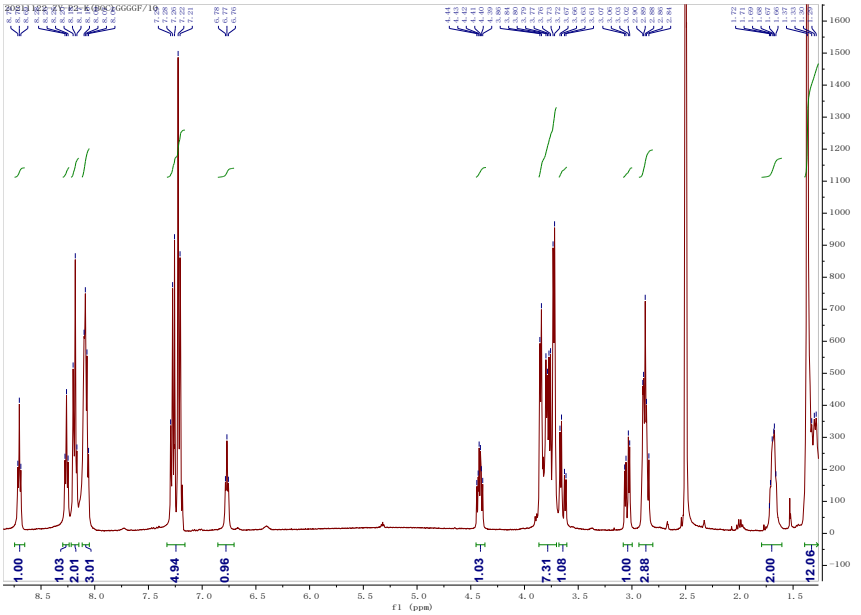


Figure S20. The ^{1}H NMR of K (Boc) GGGGF.

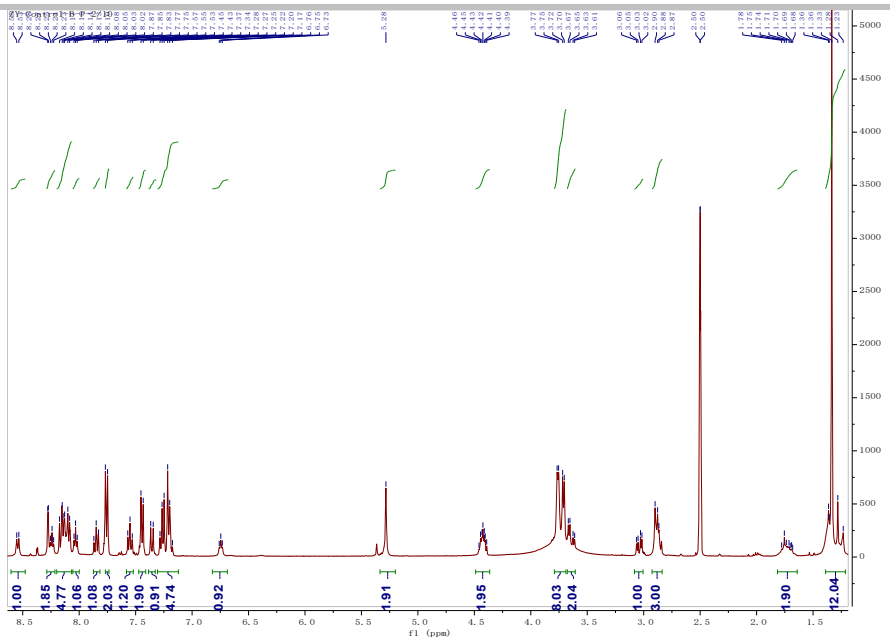


Figure S21. The ^{1}H NMR of BQA-K (Boc) GGGGF.

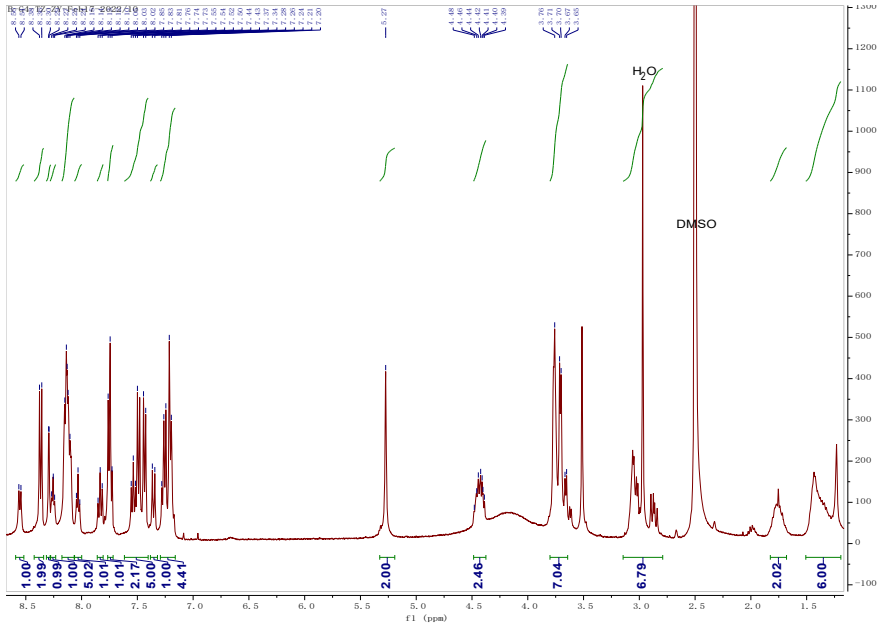


Figure S22. The ^{1}H NMR of BQA-K (Tz) -GGGGF.

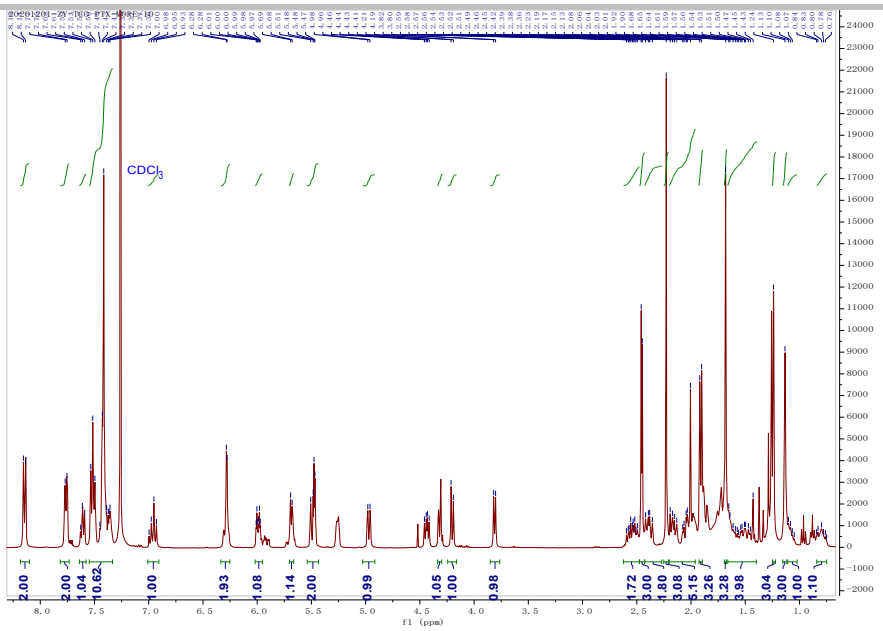


Figure S23. The ^{1}H NMR of TCO- PTX.

3. References

1 Z. Huang, Q. Yao, J. Chen and Y. Gao, *Chem. Commun.*, 2018, **54**, 5385-5388.

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