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Electronic Supplementary Information for

Rational design of gemcitabine prodrug with AIE-based intracellular light-up characteristics for selective suppression of pancreatic cancer cells

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Experimental Section

Materials

2,2'-Dithiodiethanol, N,N-Diisopropylethylamine (DIPEA), 4-nitrophenyl chloroformate, tert-Butylbromoacetate, cathepsin B from bovine spleen, glutathione monoester (GSH-OEt), Buthionine-sulfoximine (BSO), and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Benzopheone, 4-hydrobenzopheone, and acryloyl chloride were purchased from Energy Chemical. Zinc powder and pyridine were obtained from Sinopharm Chemical Reagent Co., Ltd. Gemcitabine hydrochloride was purchased from Dalian Meilun Biology Technology Co., Ltd. CA-074-Me was purchased from ApexBio. The polypeptides TPE-GFLGDDDDDGKGDGR and TPE-GFLGDDDDDGKGGDR were synthesized by ChinaPeptides Co., Ltd (>95%). Human pancreatic cancer cell line BxPC-3 was obtained from KeyGEN BioTECH.

Characterization

^{1}H NMR spectra were recorded on a Bruker DMX500 instrument. Mass spectra were recorded on Bruker Esquire 3000 plus. A 0.1% trifluoroacetic acid solution in H_2O and acetonitrile was used as the eluent for HPLC experiments (Agilent 1200). UV-vis spectra were carried out with a UV-vis Shimadzu UV-2505 spectrometer using 1-cm-path length quartz cuvettes. Fluorescence emission spectra were recorded from PerkineElmer LS 55 fluorescence spectrometer with the excitation wavelength of 320 nm. Dynamic light scattering (DLS) measurements were performed using Zetasizer Nano-ZS from Malvern Instruments equipped with a He-Ne laser at wavelength of 633 nm at 25 °C. Intensity-average hydrodynamic diameter (D_h) was adopted in this research.

Synthesis of 2-((2-hydroxyethyl)disulfanyl)ethyl acrylate (HSEA)

HSEA was synthesized according to a published reference [S1]. 15 mL of 2,2′-Dithiodiethanol (0.12 mol) was dissolved in anhydrous N,N-dimethylformamide (DMF) (250 mL) in a 500 mL round-bottomed flask containing 20 mL of triethylamine (TEA) (0.14 mol) under an Argon atmosphere.11 mL of acryloyl chloride (0.13 mol) was added dropwise and allowed to react overnight. Crude product was extracted into CH_2Cl_2 and purified via silica gel chromatography (EtOAc/hexanes) to provide monoacrylate-substituted 2,2′-dithiodiethanol HSEA. The ^{1}H NMR of HSEA was shown in Figure S1.

Synthesis of reduction-responsive and clickable gemcitabine derivative 2-((2-hydroxyethyl) disulfanyl) ethyl acrylate (HSEA-GEM)

200 mg of HSEA (0.962 mmol), 497 mg of DIPEA (3.846 mmol) and a catalytic amount of pyridine was dissolved in 5 mL of dry CH_2Cl_2 in a flask. 582 mg of 4-nitrophenyl chloroformate (2.886 mmol) in 5 mL of dry CH_2Cl_2 was added dropwise at 0 °C and stirred for 4 h at room temperature. The reaction mixture was concentrated in vacuum and then dissolved in 3 mL DMF. 760 mg of gemcitabine hydrochloride (2.889 mmol) in 3 mL of DMF and 0.8 mL of TEA were added and continued to stir for 24 h. The reaction mixture was diluted in 10 mL of water. The compound was extracted with CH_2Cl_2 and the organic layer was dried over anhydrous sodium sulfate. Finally, the crude product was purified by column chromatography on silica gel using CH_2Cl_2/Methanol (15:1) as eluent to afford a yellow solid. The ^{1}H NMR of HSEA-GEM was shown in Figure S2. LC/MS further confirmed the

chemical structure of HSEA-GEM (calculated value for [M]+:497.07, obtained value for [M+H]+: 498.1).

Synthesis of carboxylated tetraphenylene (TPE-COOH)

In order to synthesize TPE conjugated polypeptide, TPE-COOH was first synthesized as previously reported with slight modification[S2]. The synthetic route was shown in Scheme S1. Benzopheone (36.4 g, 0.2 mol), 4-hydrobenzopheone (38 g, 0.2 mol), zinc powder (32 g, 0.48 mol) and 600 mL of THF were added into a three-necked flask under stirring at 0 ºC. 26 mL of $TiCl_4$ (0.24 mol) was then slowly added. The mixture was stirred at room temperature for 0.5 h and then refluxed overnight. After cooled to room temperature, 100 mL of dilute hydrochloric acid (1mol/L) was added and extracted with dichloromethane (CH_2Cl_2). The crude product was purified by a silica gel column and hydroxylated TPE (TPE-OH) was obtained as a white solid. 2 g of tert-Butylbromoacetate (20 mmol), 7 g of TPE-OH (20 mmol), 4 g of K_2CO_3 (30 mmol) and 100 mL of acetonitrile were added into a flask. The mixture was refluxed over night at 100 ºC. The resulting mixture solution was separated by filtration. The crude was purified through silica gel column. This product was added into a solution (CH_2Cl_2:TFA=1:1) and stirred vigorously. After 3 h, the mixture solution was poured into water and extracted with CH_2Cl_2 for three times. The collected organic layer was concentrated under reduced pressure. TPE-COOH was obtained as white powder. The ^{1}H NMR and mass spectrum of TPE-COOH were shown in Figure S3 and Figure S4.

Synthesis of TPE-GEM-RGD and TPE-GEM-RDG.

TPE-GEM-RGD was synthesis by Michael addition reaction of TPE conjugated polypeptide TPE-GFLGDDDDDGKGDGR and HSEA-GEM. Typically, 50 mg of TPE-GFLGDDDDDGKGDGR (28 μmol) and 41.7 mg of HSEA-GEM (84 μmol)

were added into 5 mL of DMF. 10 μL of TEA was then added. The reaction was performed at 50 ºC for 48 h. The solution was dialyzed against DMF for 48 h and then water for 24 h (MWCO 1000). The solution was then lyophilized to obtain white powder. TPE-GEM-RDG was synthesized by the same procedure unless GFLGDDDDDGKGGDR was used. The ^{1}H NMR of TPE-GEM-RGD and TPE-GEM-RDG were shown in Figure S6 and Figure S7.

Cathepsin B-responsive fluorescence light up.

PBS (10 mM, pH 5.0) and TPE-GEM-RGD solution (1.0 mM) were mixed with cathepsin B (10 UN mL^{-1} stock solution) to achieve the final concentrations of 10 μM TPE-GEM-RGD and 0.5 UN mL^{-1} Cathepsin B. The mixed solution was incubated at 37 °C and the fluorescence spectra were recorded at designated time with the excitation wavelength of 320 nm.

In vitro release of GEM from TPE-GEM-RGD

In vitro drug release behavior of TPE-GEM-RGD was investigated in 10 mM PBS at pH 7.4. DTT was added into TPE-GEM-RGD solution to achieve the final concentrations of 20 mg mL^{-1} TPE-GEM-RGD and 10 mM of DTT. After incubation of the solution at 37 °C for 5 h and 10 h, 0.2 mL of the solution was taken out. HPLC was employed to determine the release of GEM. Free GEM was used as control.

Cell Culture.

BxPC-3 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) in 5% CO_2 at 37 °C.

Fluorescence microscope imaging

BxPC-3 cells were seeded in a 24-well plate at 5×10^4 cells per well and incubated for 12 h at 37 °C. Then, the medium was replaced with 10 μM TPE-GEM-RGD in fresh culture medium. In some experiments, the cells were pretreated with (20 μM) cathepsin B inhibitor CA-074-Me for 2 h. After incubation with TPE-GEM-RGD for 3 h, the medium was removed and the cells were washed with PBS for three times. The fluorescence images of TPE-GEM-RGD in cells were observed by fluorescence microscope. Meanwhile, 10 μM TPE-GEM-RDG prodrug without RGD targeting was incubated with BxPC-3 cells by the same procedure, which was used as a negative control.

Endocytosis mechanism investigation

In order to investigate the cellular internalization pathway of TPE-GEM-RGD, cell culture was carried out at 4 ºC or pretreated with NaN_3 in parallel with regular incubation conditions.

Low temperature incubation at 4 °C: BxPC-3 cells were seeded in a confocal dish at 3×10^4 cells per well and incubated for 12 h at 37 °C. The medium was replaced with 10 μM TPE-GEM-RGD in fresh culture medium. BxPC-3 cells were then incubated at 4 °C, instead of the regular 37 °C condition.

Incubation of cells under ATP depletion: For the ATP-depletion studies, BxPC-3 cells were seeded in a confocal dish at 3×10^4 cells per well and incubated for 12 h at 37 °C. The cells were pretreated with 10 mM NaN_3 and 50 mM 2-deoxy-D-glucose for 30 min at 37 °C. Then, the medium was replaced with 10 μM TPE-GEM-RGD in fresh culture medium. After incubation with TPE-GEM-RGD for 3 h, the medium was removed and the cells were washed with PBS for three times. The fluorescence images of TPE-GEM-RGD in cells were observed by fluorescence microscope.

In vitro cell cytotoxicity assay

The cytotoxicities of GEM prodrug against BxPC-3 cells were evaluated in vitro by MTT assays.

In order to prove the intracellular reduction-responsive release of GEM from TPE-GEM-RGD prodrug, cells were seeded in 96-well plates at 6×10^3 cells per well in 200 μL of culture medium for 12 h. Cells were then treated with 0.5 mM GSH inhibitor BSO for 12 h or 10.0 mM GSH promoter GSH-OEt for 2 h. Cells without pretreatment were used as a control. After washing cells with PBS, TPE-GEM-RGD prodrug (final concentration from 0.2 to 10 μg mL^{-1} equivalent to gemcitabine) were added and incubated for another 48 h. Then, 20 μL of MTT (5 mg mL^{-1}) was added. After 4 h, the culture medium was replaced with 150 μL DMSO to dissolve the obtained crystals. The absorbance was measured at a wavelength of 570 nm using a microplate Bio-Rad reader (Thermo Fisher Scientific, Waltham, MA). Data were expressed as average ± SD (n = 3).

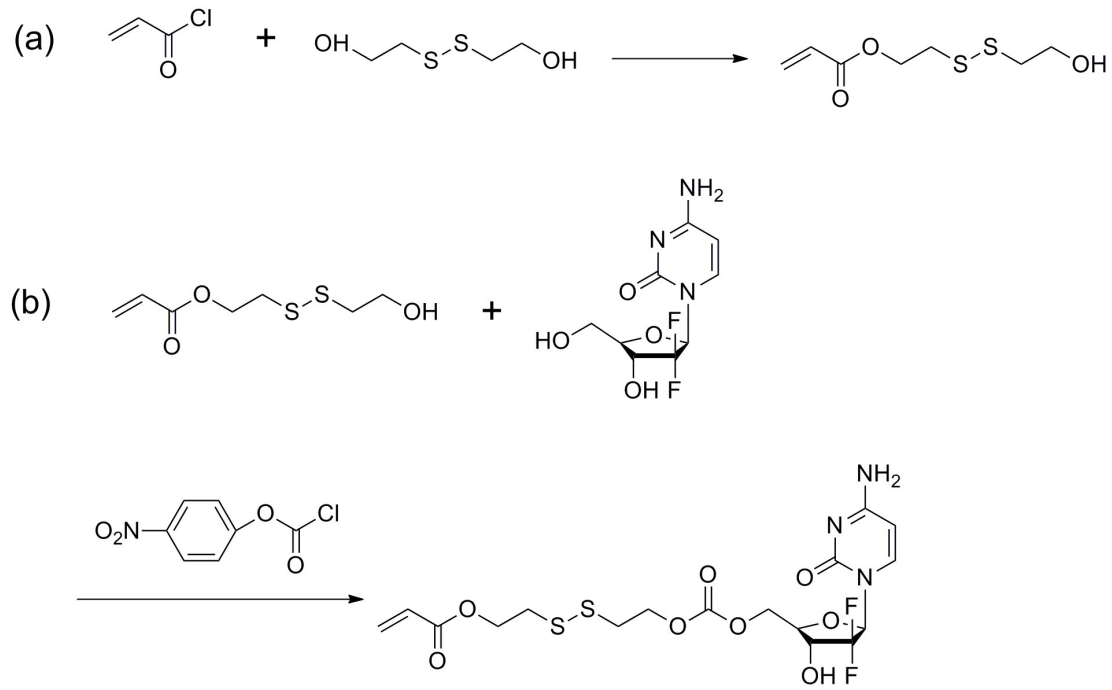
In order to evaluate the feasibility of using RGD-targeted GEM prodrug to suppress pancreatic cancer cells, the cell viabilities of free GEM, nontargeted TPE-GEM-RDG prodrug and targeted TPE-GEM-RGD prodrug were studied. Briefly, cells were seeded in 96-well plates at 6×10^3 cells per well in 200 μL of culture medium for 12 h. After washing cells with PBS, free GEM, TPE-GEM-RDG prodrug, and TPE-GEM-RGD prodrug (final concentration from 0.2 to 10 μg mL^{-1} equivalent to gemcitabine) were added and incubated for another 72 h. Then, 20 μL of MTT (5 mg mL^{-1}) was added. After 4 h, the culture medium was replaced with 150 μL DMSO to dissolve the obtained crystals. The absorbance was measured at a wavelength of 570 nm using a

microplate Bio-Rad reader (Thermo Fisher Scientific, Waltham, MA). Data were expressed as average ± SD (n = 3).

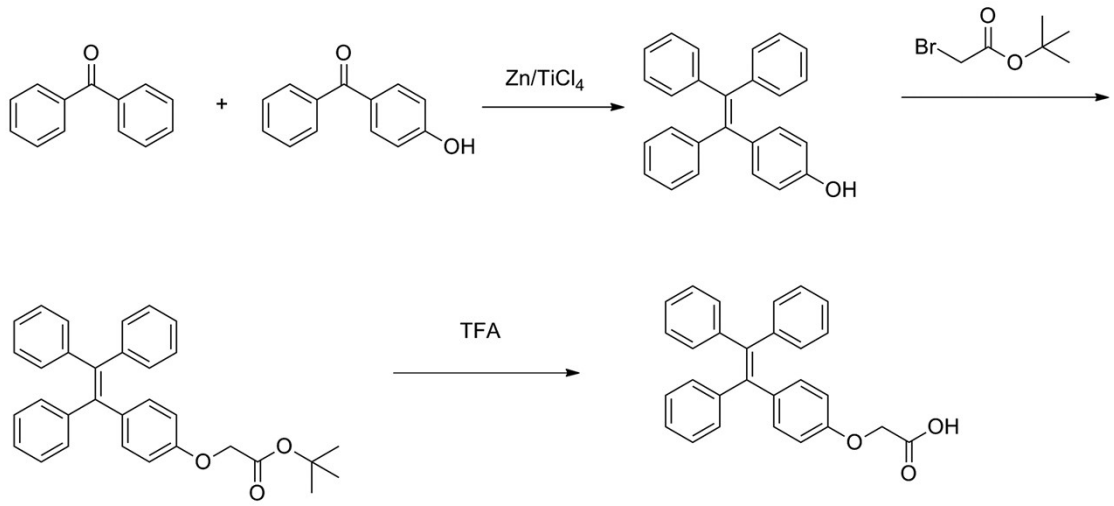
References

[S1] S. S. Dunn, S. Tian, S. Blake, J. Wang, A. L. Galloway, A. Murphy, P. D. Pohlhaus, J. P. Rolland, M. E. Napier and J. M. DeSimone, *J. Am. Chem. Soc.,* 2012, **134**, 7423-7430.

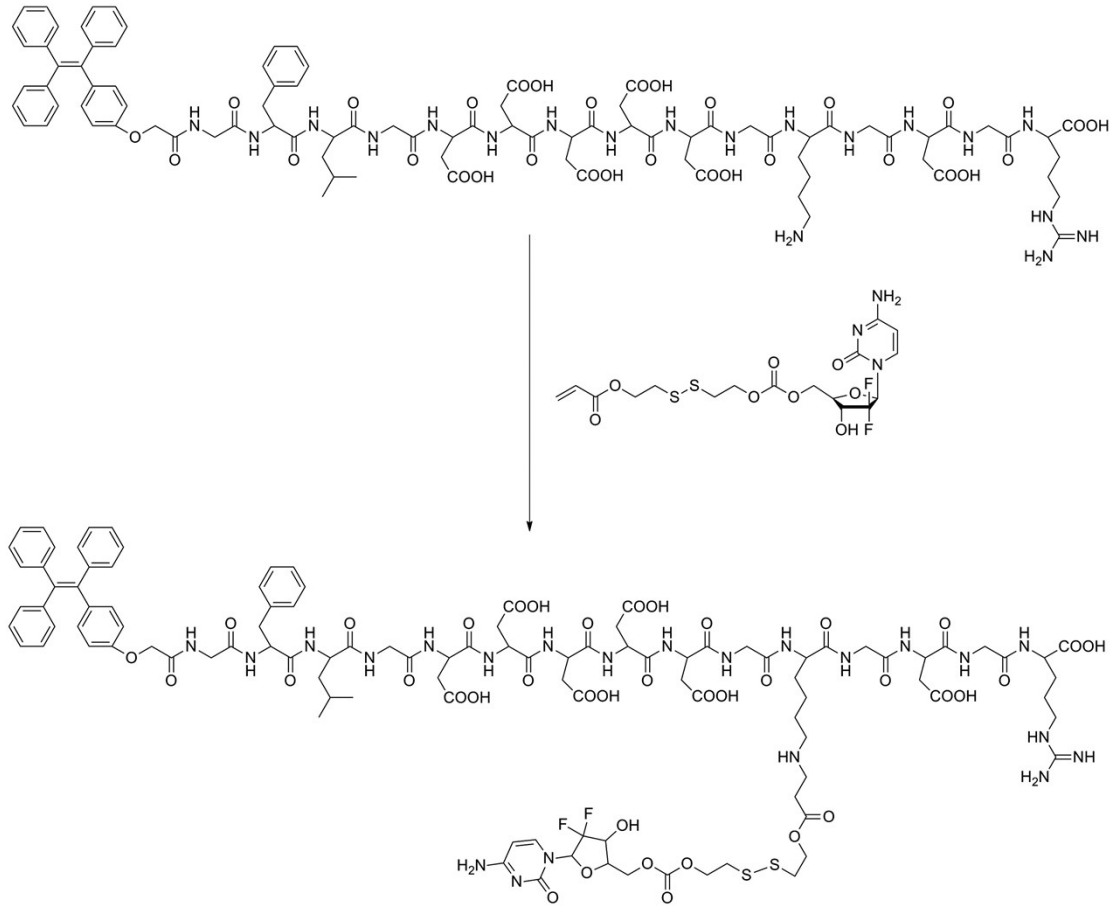
[S2] H. Wang, Y. Huang, X. Zhao, W. Gong, Y. Wang, Y. Cheng, *Chem. Commun.,* 2014, **50**, 15075-15078.



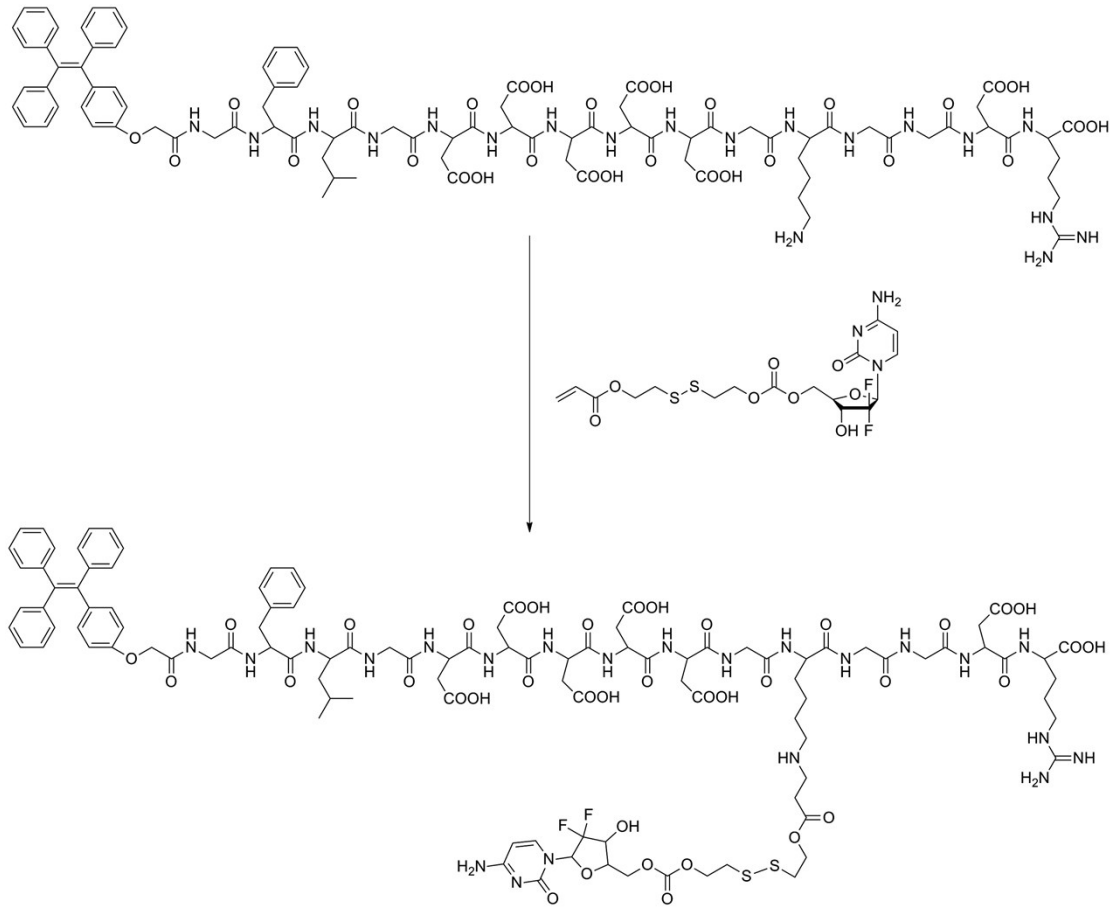
Scheme S1. Schematic illustration of the synthesis of (a) HSEA and (b) HSEA-GEM.



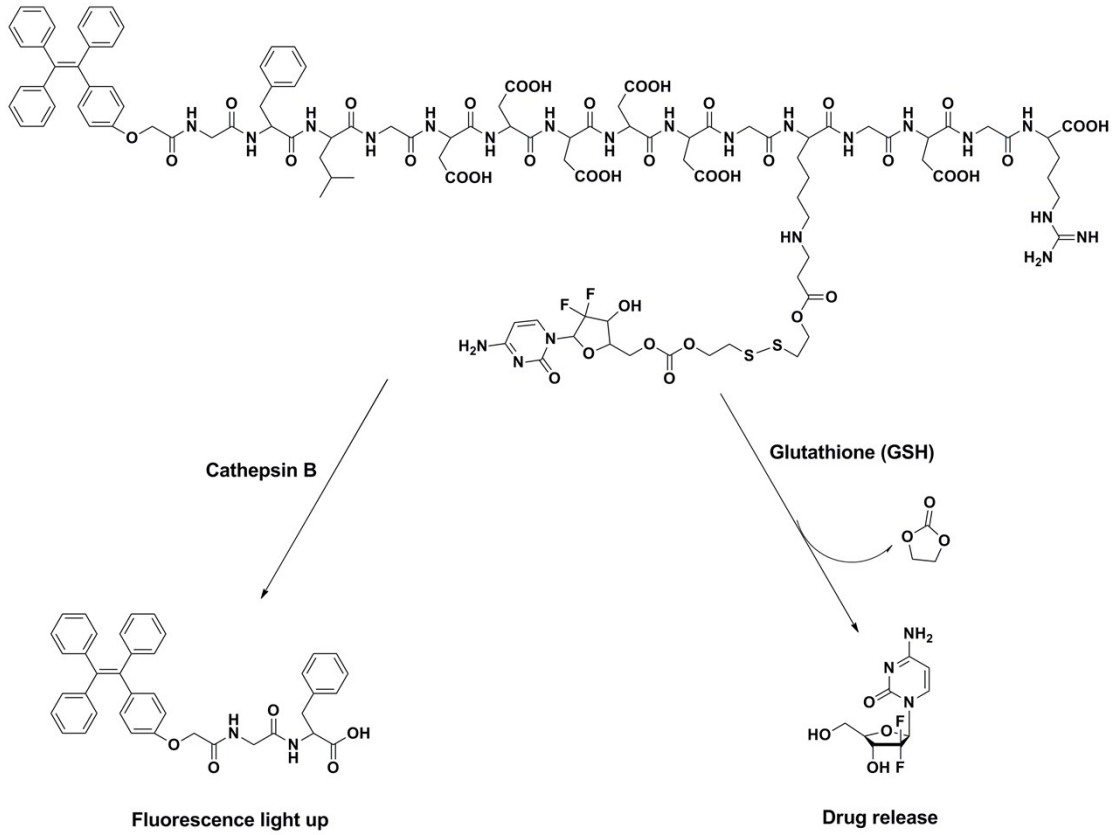
Scheme S2. Schematic illustration of the synthesis of TPE-COOH.



Scheme S3. Schematic illustration of the synthesis of TPE-GEM-RGD.



Scheme S4. Schematic illustration of the synthesis of TPE-GEM-RDG.



Scheme S5. Schematic illustration of TPE-GEM-RGD for cathepsin B-responsive fluorescence light up and GSH-responsive GEM release.

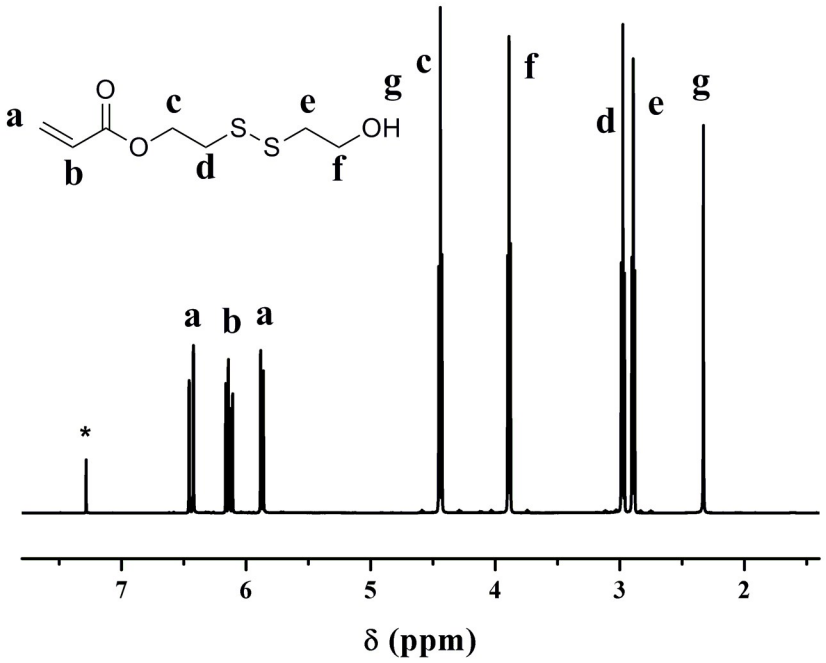


Figure S1. ^{1}H NMR spectra of HSEA. * Indicates residual NMR solvent.

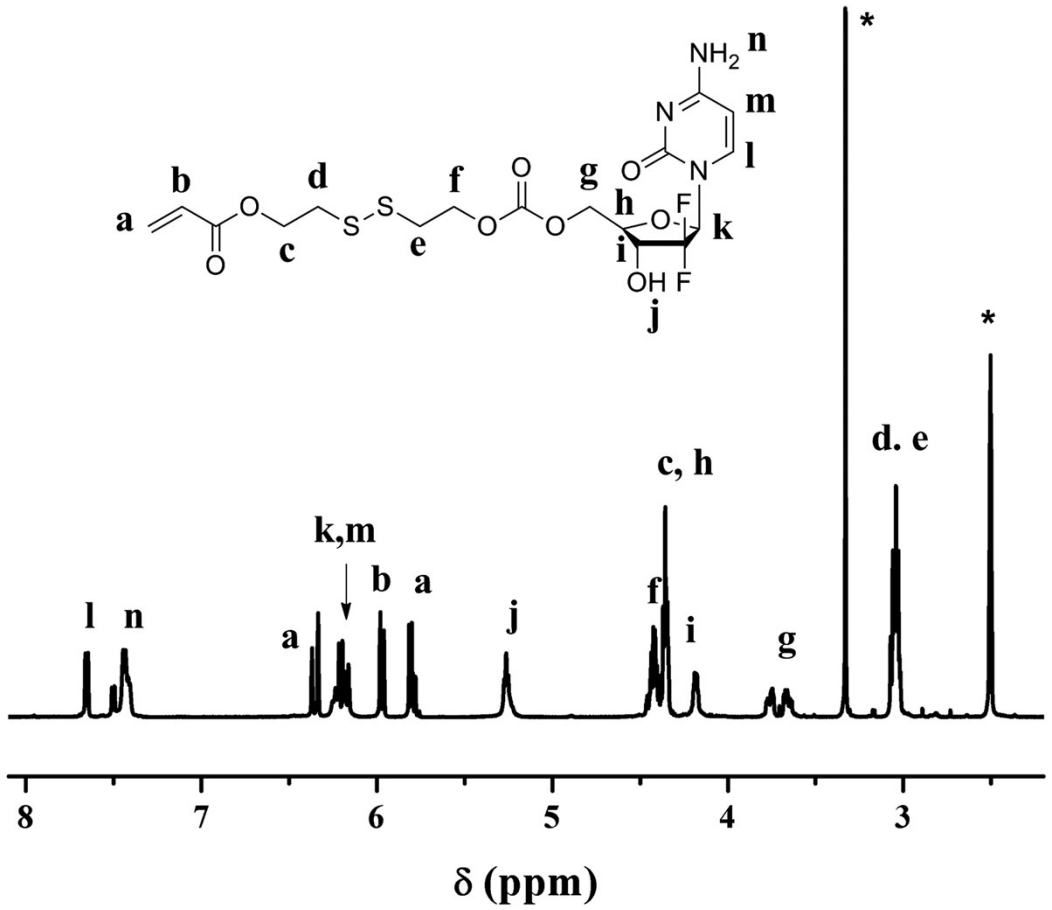


Figure S2. ^{1}H NMR spectra of HSEA-GEM. * Indicates residual NMR solvent.

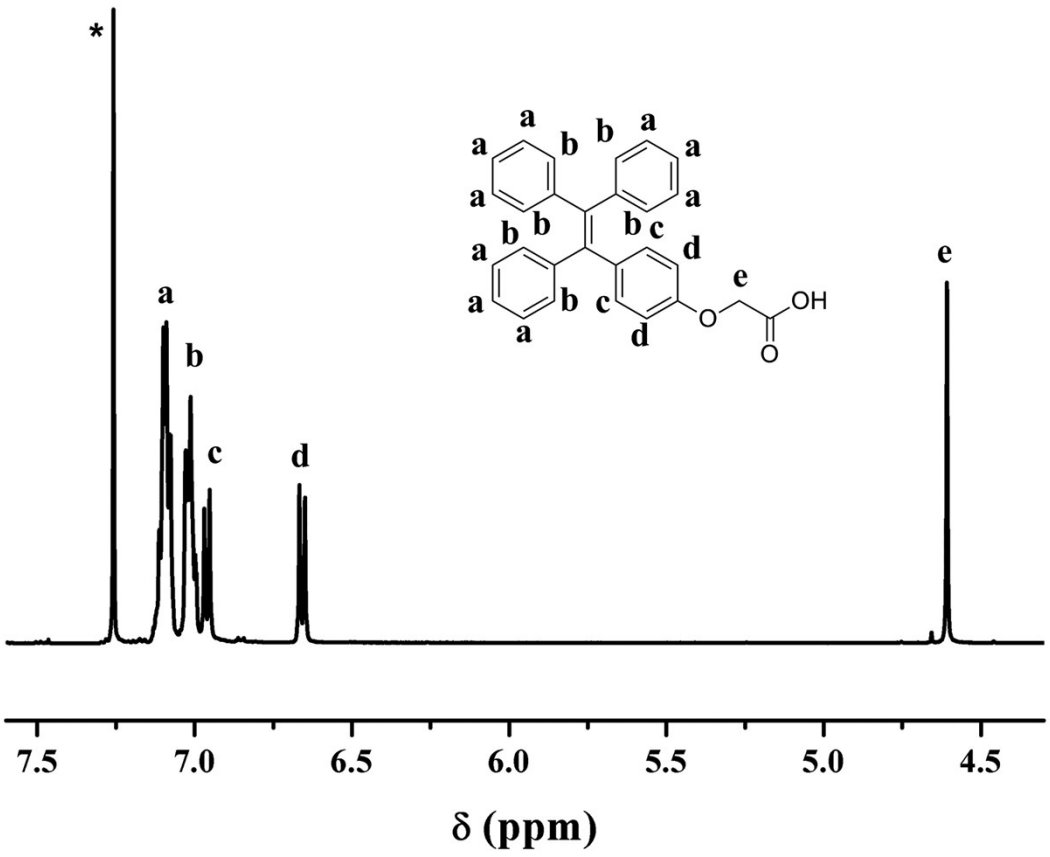


Figure S3. ^{1}H NMR spectrum of TPE-COOH. * Indicates residual NMR solvent.

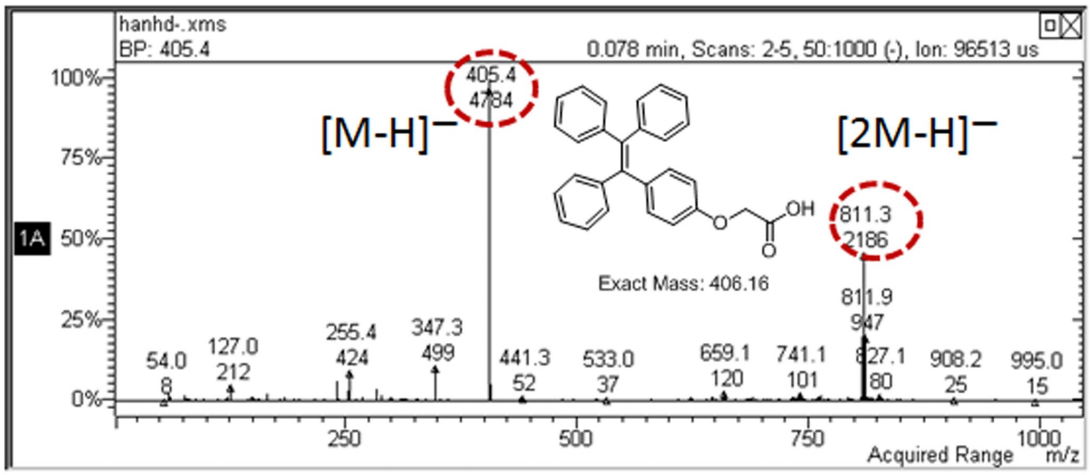


Figure S4. The mass spectrum of TPE-COOH.

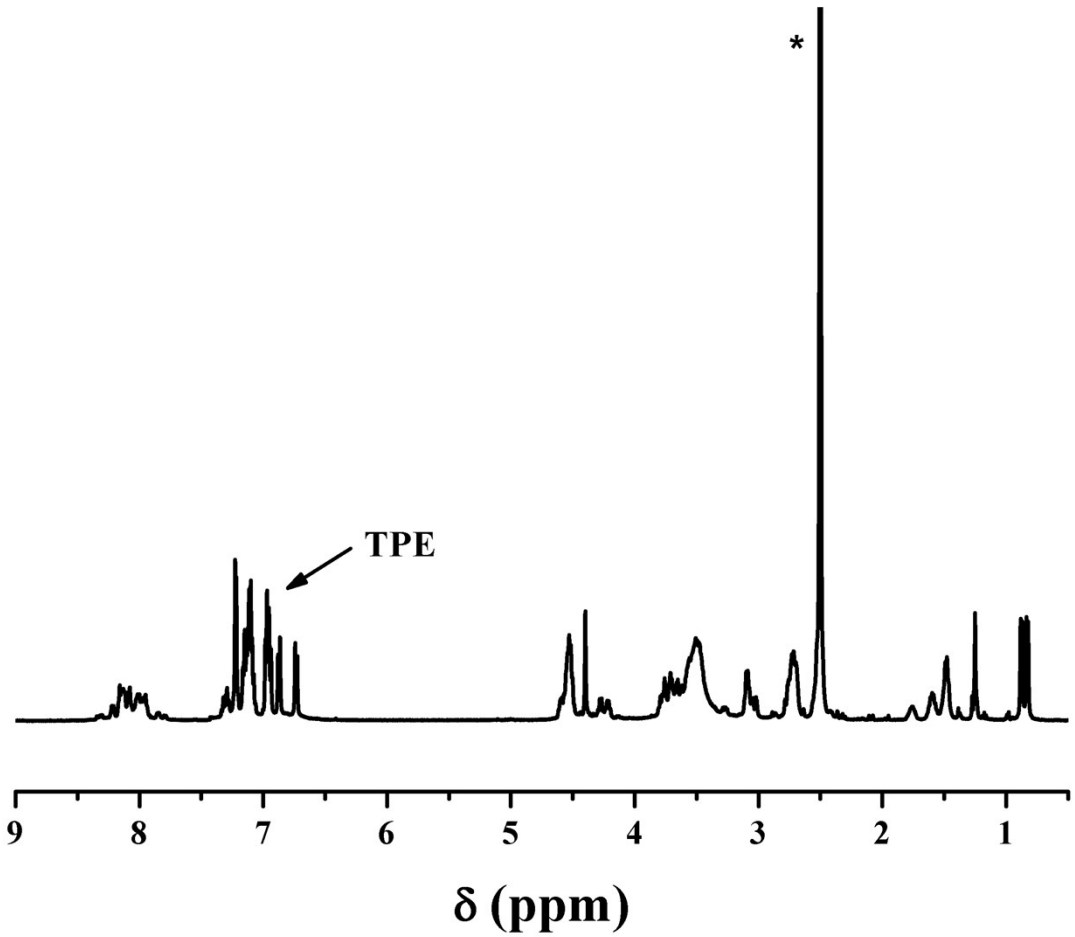


Figure S5. ^{1}H NMR spectra of TPE-GFLGDDDDDGKGDGR. * Indicates residual NMR solvent.

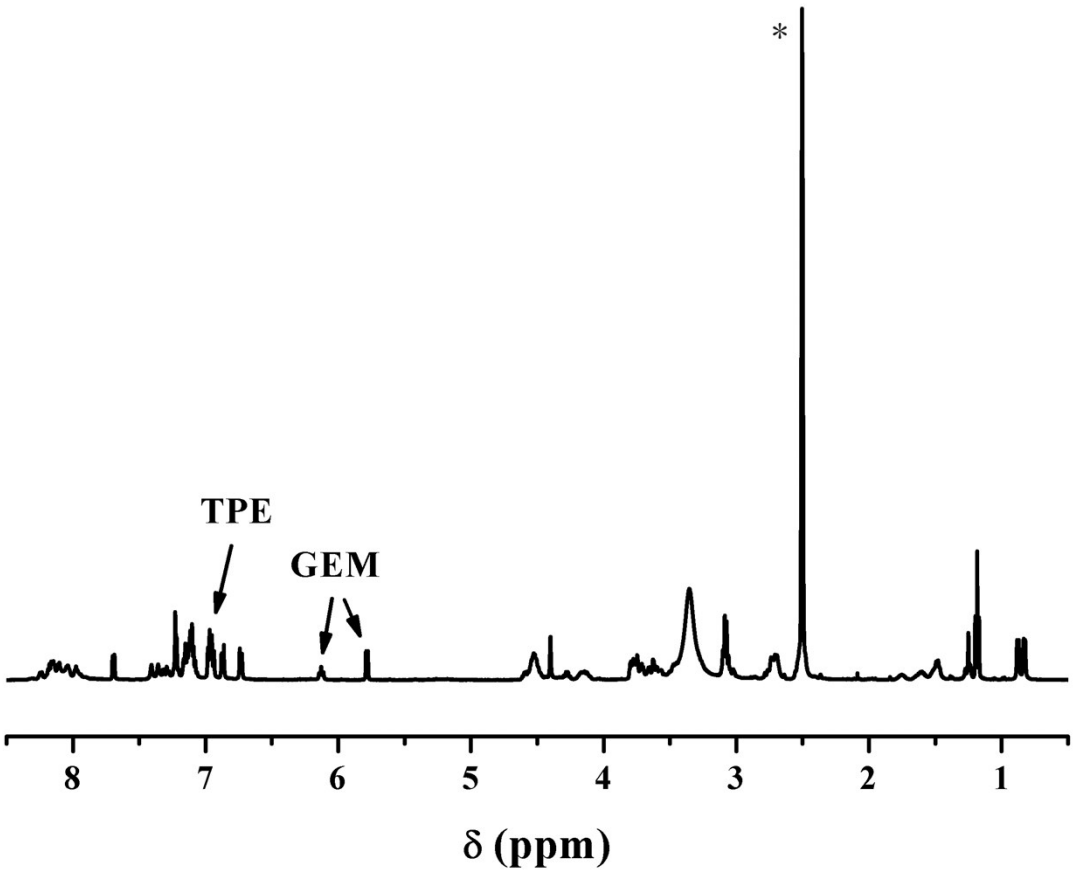


Figure S6. ^{1}H NMR spectra of TPE-GEM-RGD. * Indicates residual NMR solvent.

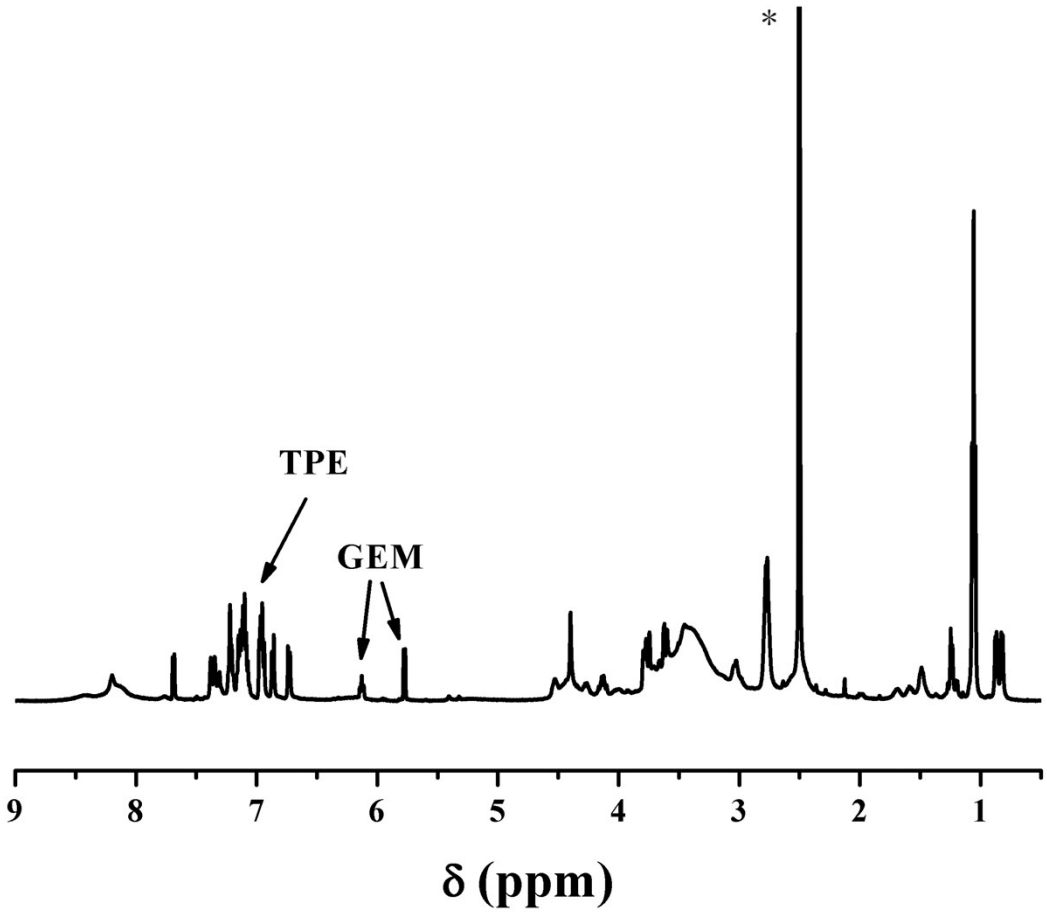


Figure S7. ^{1}H NMR spectra of TPE-GEM-RDG. * Indicates residual NMR solvent.

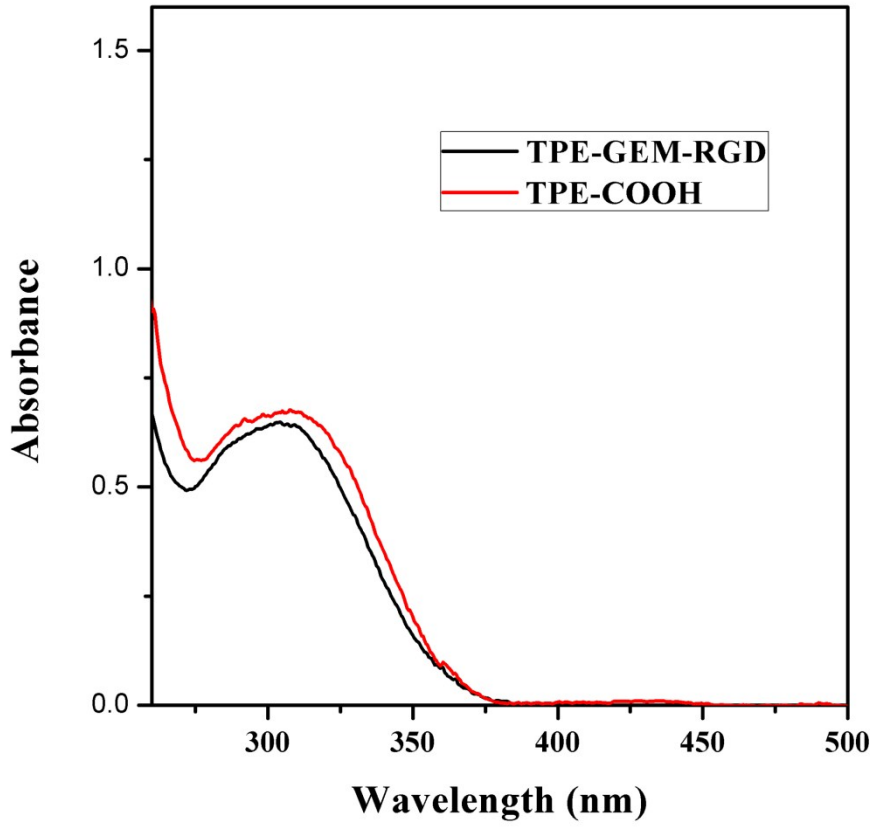


Figure S8. UV-vis spectra of TPE-COOH and TPE-GEM-RGD in DMSO/PBS mixture (1/199, v/v).

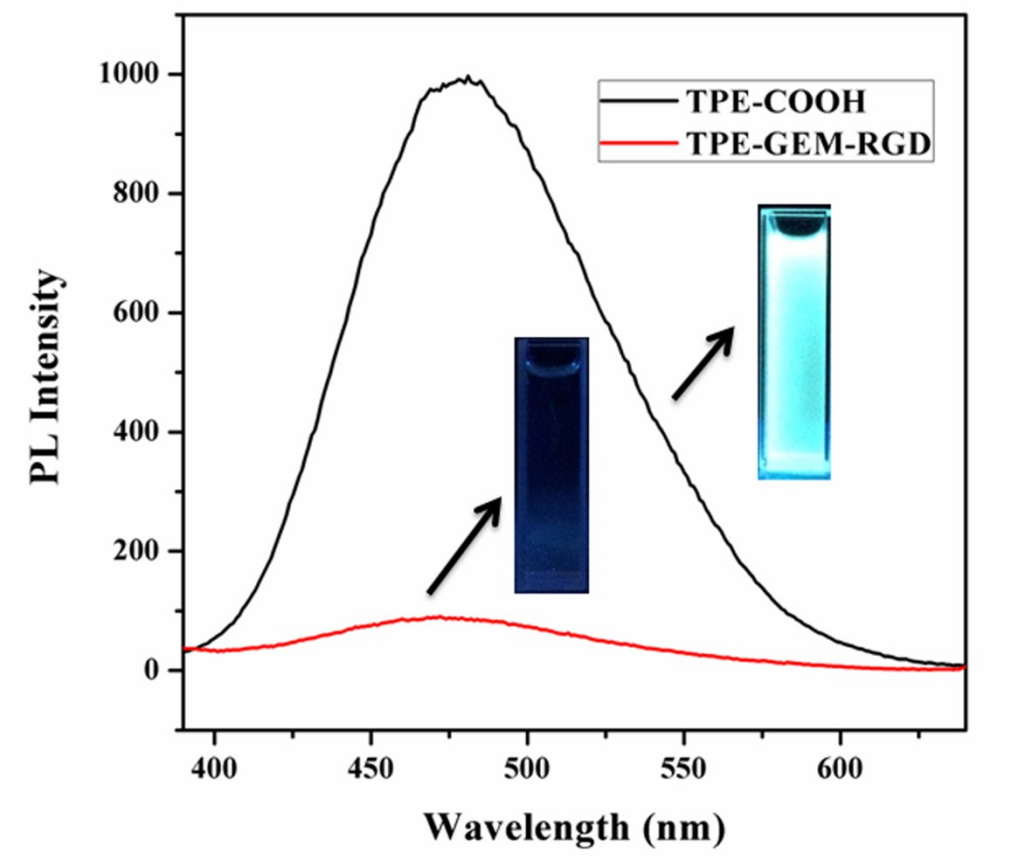


Figure S9. Fluorescent emission spectra of TPE-COOH and TPE-GEM-RGD in DMSO/PBS mixture (1/199, v/v). Inset: Corresponding digital photos taken under irradiation by a UV lamp at 365 nm.

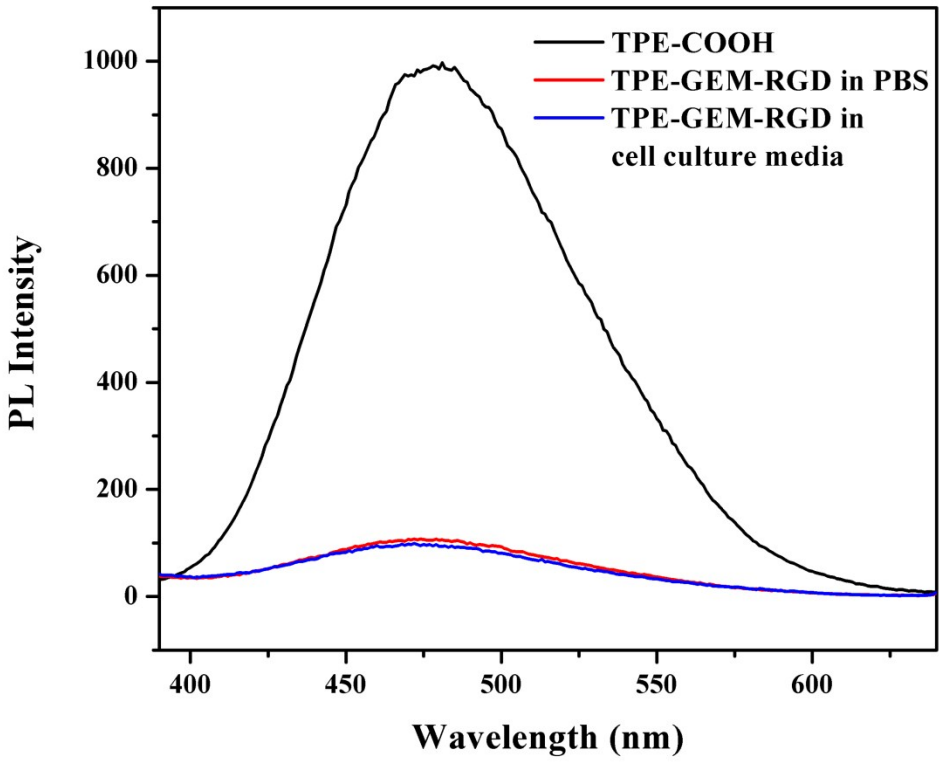


Figure S10. Fluorescent emission spectra of TPE-GEM-RGD in PBS and cell culture media. The fluorescent emission spectra of TPE-COOH in DMSO/PBS mixture (1/199, v/v) was shown for comparison.

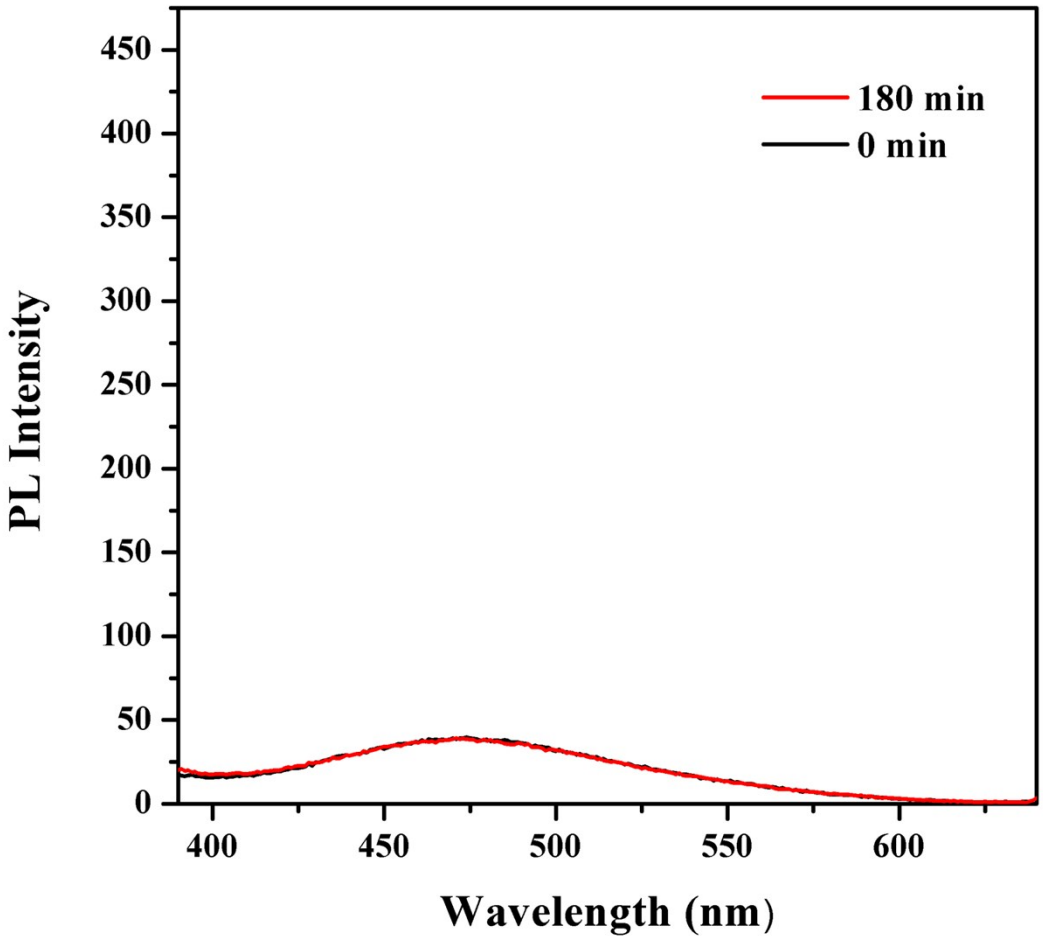


Figure S11. Time-dependent fluorescent emission spectra of TPE-GEM-RGD (10 μM) in the absence of cathepsin B in PBS.

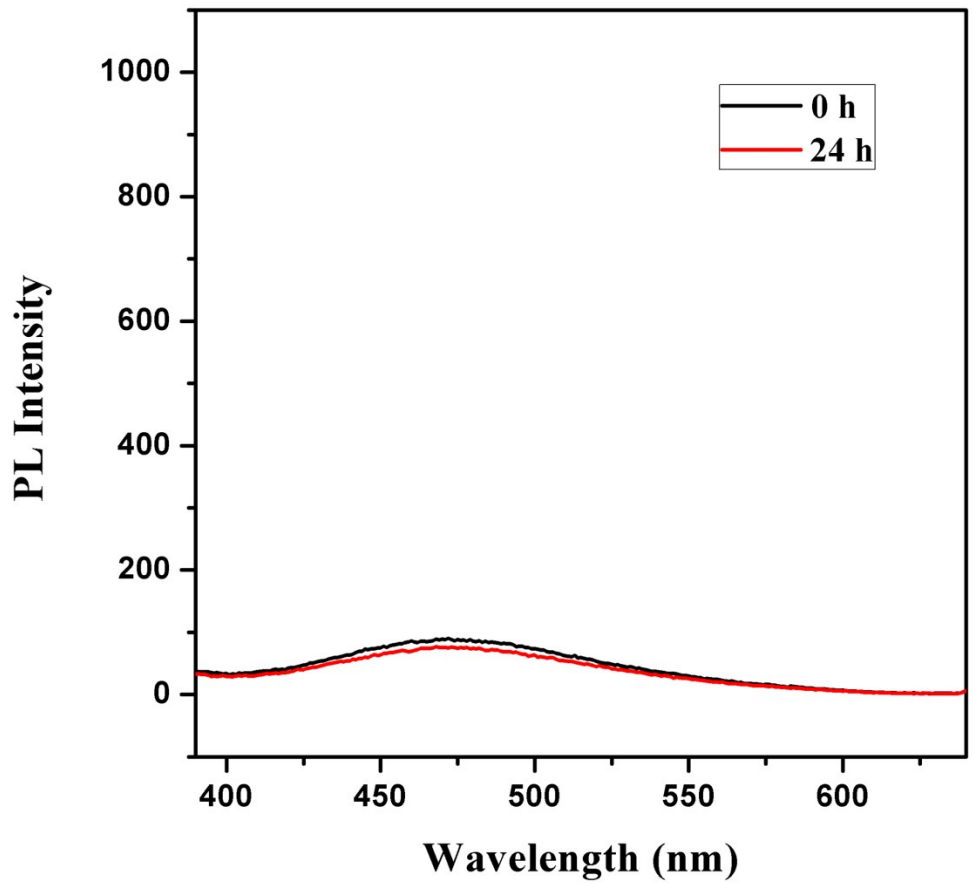
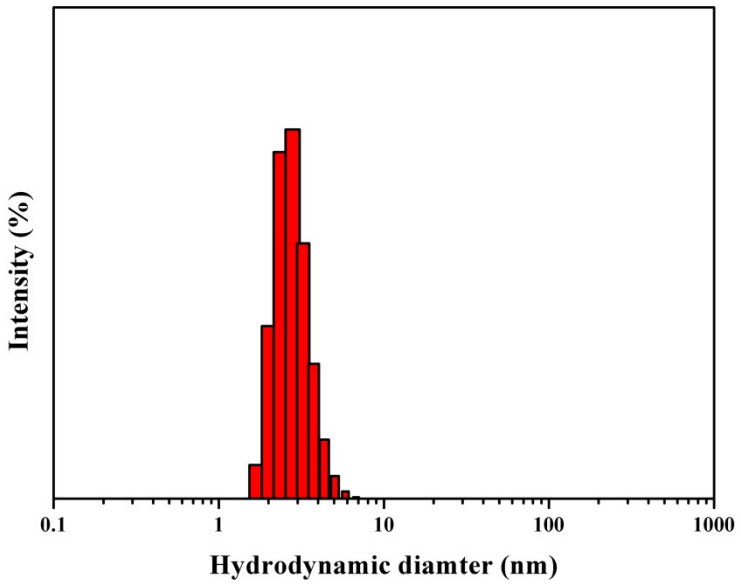
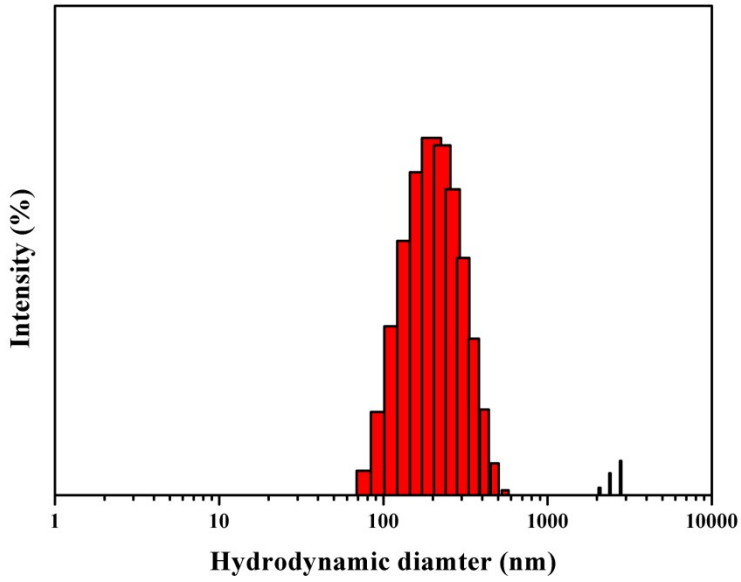


Figure S12. Time-dependent fluorescent emission spectra of TPE-GEM-RGD in the presence of GSH.



(a)



(b)

Figure S13. Intensity-average hydrodynamic diameter of TPE-GEM-RGD (a) before and (b) after incubation with cathepsin B in PBS.

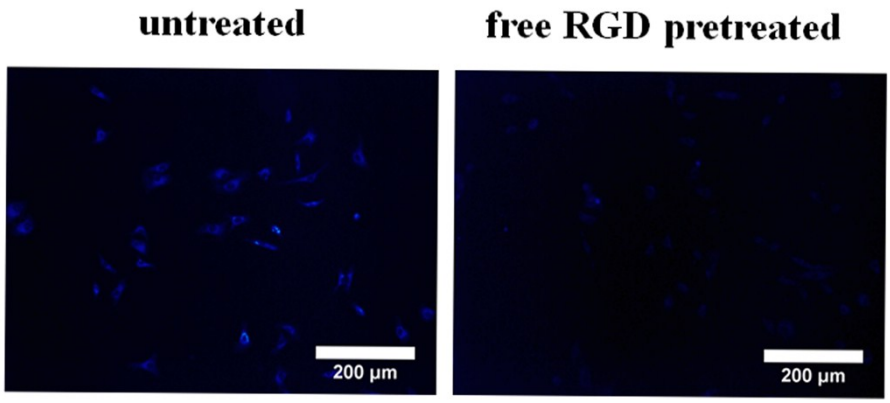


Figure S14. Fluorescent microscopy images of (a) BxPC-3 cells incubated with TPE-GEM-RGD for 3 h (left); (b) BxPC-3 cells pretreated with free RGD for 3 h and incubated with TPE-GEM-RGD for 3 h.(right)

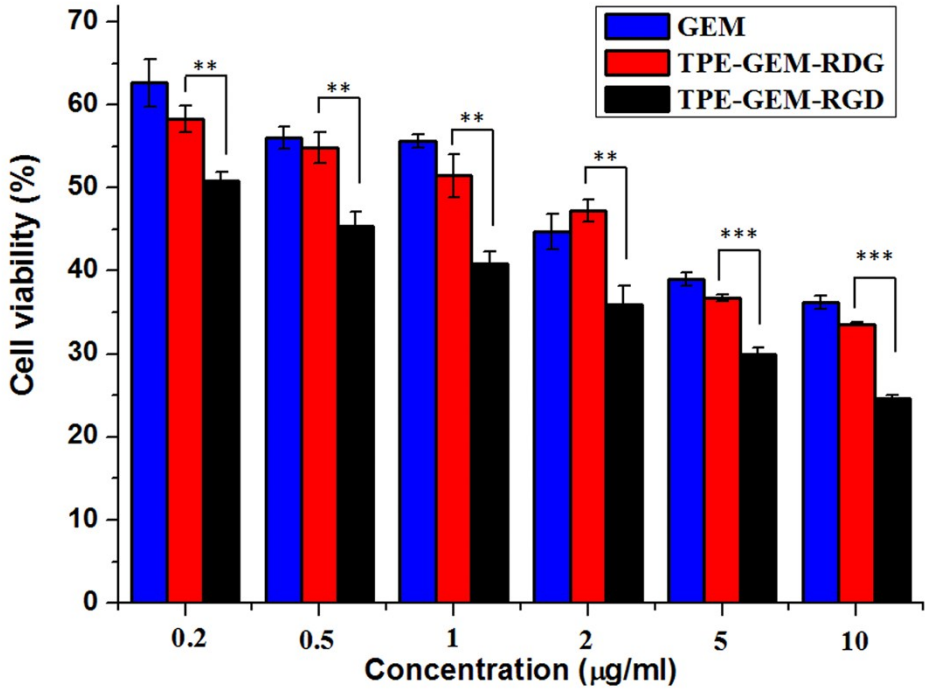


Figure S15. Cell viability of BxPC-3 cells incubated with various concentrations of TPE-GEM-RGD or TPE-GEM-RDG prodrug for 72 h. (Asterisk indicate statistically significant differences (**p <0.01; ***p <0.001))