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Electronic Supplementary Information for

Cascade enzymatic reaction activatable gemcitabine prodrug with AIE-based intracellular light-up apoptotic probe for *in situ* self-therapeutic monitoring

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Experimental Section

Materials

Benzophenone, 4-hydrobenzophenone and acryloyl chloride were purchased from Energy Chemical. Zinc powder and pyridine were obtained from Sinopharm Chemical Reagent Co., Ltd. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and cathepsin B were purchased from Sigma-Aldrich. Gemcitabine hydrochloride was received from Dalian Meilun Biology Technology Co., Ltd. Z-DEVD-FMK (caspase-3 inhibitor) was purchased from ApexBio. Bicinchoninic acid (BCA) and caspase-3 activity assay kit were provided by Thermo Fisher Scientific. The polypeptides TPE-GDEVDGE(GFLG-GEM)EKEKADGR (prodrug **1**), TPE-GDEVDGE(GAAG-GEM)EKEKADGR (prodrug **2**), and TPE-GDAADGE(GFLG-GEM)EKEKADGR (prodrug **3**) were synthesized by ChinaPeptides Co., Ltd (>95%). Human pancreatic cancer cell line BxPC-3 was obtained from KeyGEN BioTECH.

Characterization

Mass (MS) spectra and High Performance Liquid Chromatography (HPLC) spectra of the peptides were provided by ChinaPeptides Co., Ltd. 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 carboxylated tetraphenylene (TPE-COOH)

In order to obtain TPE conjugated prodrug, TPE-COOH was first prepared according to our previous articles with slight modification [S1,S2]. In brief, benzophenone (36.4 g, 0.2 mol), 4-hydrobenzophenone (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 (1M) 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 (Bruker DMX500) and MS (Bruker Esquire 3000 plus) of TPE-COOH were shown in Figure S1 and Figure S2.

***In vitro* release of GEM from prodrug 1**

The release profile of GEM from prodrug **1** was investigated in 10 mM phosphate buffer saline (PBS, pH 5.0) with various concentration of cathepsin B (0, 0.1, 0.3, 0.5 UN mL^{-1}) at 37 °C. In a typical experiment, 1 mL of prodrug solution (10 mM) was added to each dialysis bag with cathepsin B and then immersed in 4 mL PBS mL^{-1} in

a prepared tube. The tubes were kept at 37 °C in a thermostatic incubator with constant shaking (100 rpm). At regular time points, 1 mL of media were taken out and replaced with fresh medium. The samples were subjected to HPLC. Prodrug **2** incubated with 0.5 UN mL^{-1} cathepsin B was used as control.

Caspase-3-responsive fluorescence light up

10 μM prodrug **1** in PBS (pH 7.4, 10 mM) was incubated with caspase-3 at room temperature, and the change of fluorescence intensity was measured. The PL spectra were collected from 420 to 650 nm under excitation at 320 nm.

Cell Culture

Human pancreatic cancer cells BxPC-3 cells were cultured in RPMI 1640 medium containing penicillin (100 units mL^{-1}), streptomycin (100 units mL^{-1}), and fetal bovine serum (10%) in 5% CO_2 at 37 °C.

***In vitro* cell cytotoxicity assay**

In order to prove the intracellular cathepsin B-responsive release of GEM in pancreatic cells, the cell viabilities of free GEM, dual-responsive prodrug **1**, no cathepsin B-responsive prodrug **2**, and cathepsin B-responsive prodrug **3** but without caspase-3-responsiveness were investigated by MTT assay. 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, 180μL of free GEM, prodrug **1**, prodrug **2**, and prodrug **3** (final concentration from 1 to 30 μM equivalent to GEM) in fresh medium 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 490 nm using Thermo Scientific Multiskan® FC. Data were expressed as average ± SD (n = 3).

Caspase-3 Activity Assay

The expression of caspase-3 in BxPC-3 cells with different treatment was investigated in accordance with previous literatures with slight modification [S3, S4]. 3.0×10^6 BxPC-3 cells were seeded in 10 cm dishes for 12 h and then incubated with prodrug **1**, prodrug **2**, and prodrug **3** with the same final concentration of 20 μM equivalent to GEM for 72 h. BxPC-3 cells without any treatment were used as control. After washing cells with cold PBS twice, 2 mL of cold PBS was added to each dish, and the cells were scraped and collected in centrifuge tube. The cells were collected by centrifugation at 800 rpm for 5 min at 4 °C and then resuspended in 200 μL of cell lysis buffer on ice for 30 min. The microcentrifuge tubes were adopted for the centrifugation of lysates at 10000 rpm for 2 min at 4 °C. BCA protein assay was provided to test the content of protein in the supernatant and then adjusted the protein content of every sample to the same concentration. 70 μL of the protein solution was distributed to each well of a 96-well plate. Then, 50 μL of 2 × reaction buffer (containing 10 mM DTT) and 5 μL of the Ac-DEVD-pNA caspase-3 substrate were added into each sample to ensure the final concentration (200 μM). Each sample was carried three parallel tests. After incubating for 2 h at 37 °C, the protein activity was analyzed by monitoring the absorbance at 405 nm with Thermo Scientific Multiskan® FC. The caspase-3 protein activity was calculated as fold of the measured optical density (OD) values obtained from untreated control cells, and all the values of samples should subtract the OD value of the blank.

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 prodrug **1**, prodrug **2**, and prodrug **3** in fresh culture medium. After incubation for 3 h, the medium was removed and the cells were washed with PBS for three times. The fluorescence images of cells were observed by fluorescence microscope.

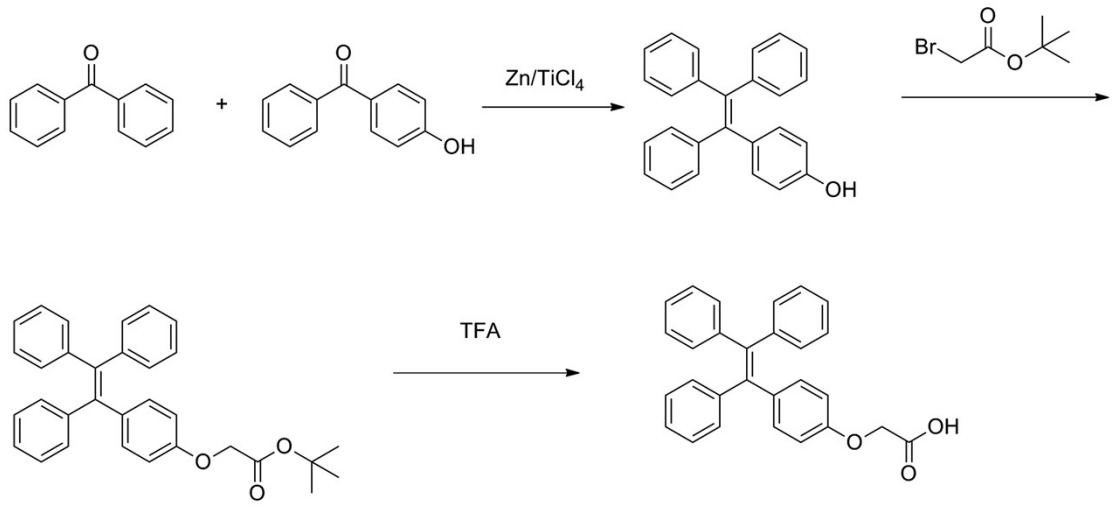
References

[S1] H. Han, Q. Jin, H. Wang, W. Teng, J. Wu, H. Tong, T. Chen and J. Ji, *Small*, 2016, **12**, 3870–3878

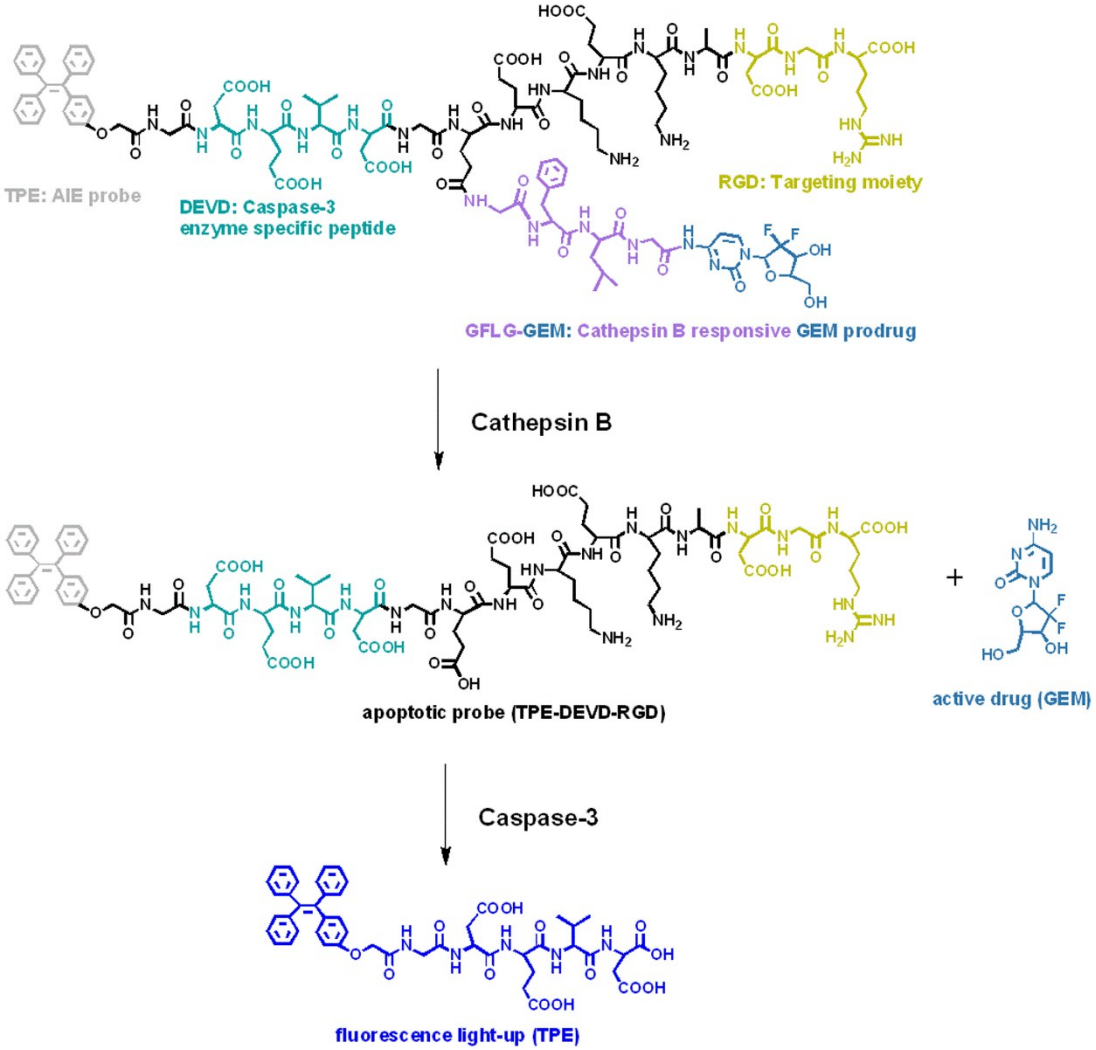
[S2] Y. Chen, H. Han, H. Tong, T. Chen, H. Wang, J. Ji and Q. Jin, *ACS Appl. Mater. Interfaces*, 2016, **8**, 21185–21192

[S3] T. Zhang, P. Huang, L. Shi, Y. Su, L. Zhou, X. Zhu and D. Yan, *Mol. Pharmaceutics*, 2015, **12**, 2328−2336

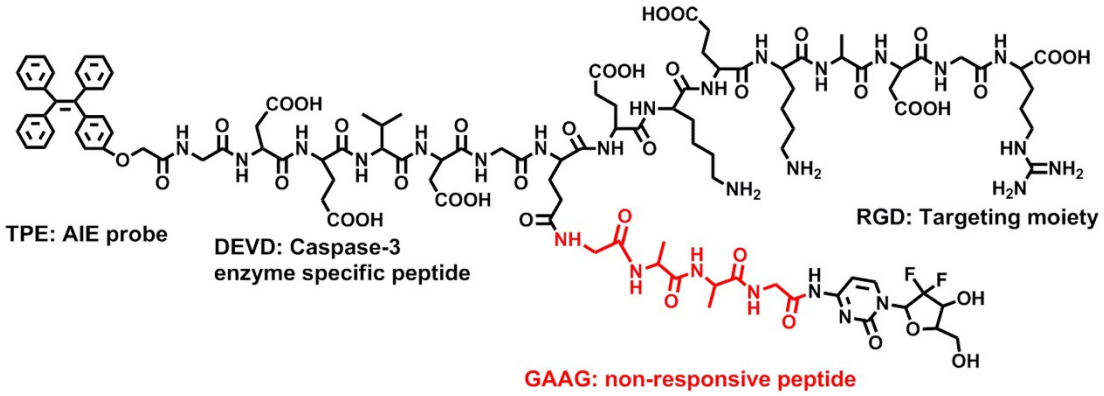
[S4] P. Huang, M. Hu, L. Zhou, Y. Wang, Y. Pang, G. Tong, W. Huang, Y. Su and X. Zhu, *RSC Adv.*, 2015, **5**, 86254–86264



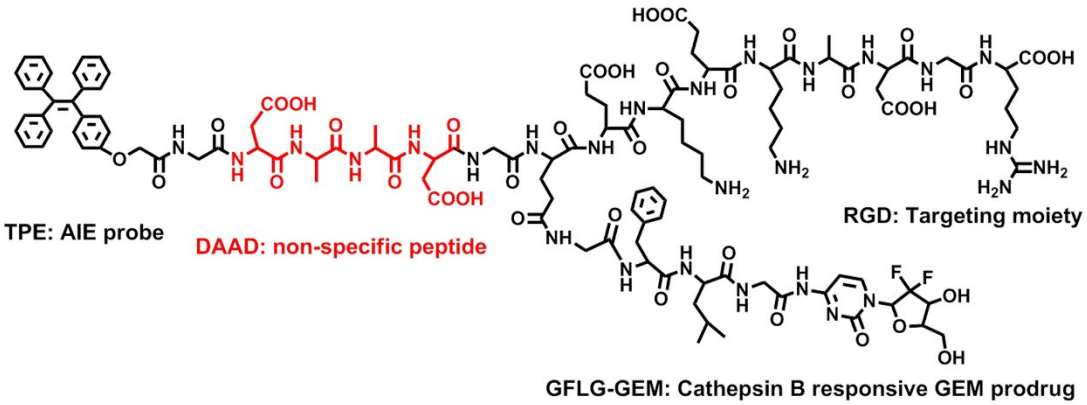
Scheme S1. Schematic illustration of the synthesis of TPE-COOH.



Scheme S2. Schematic illustration of prodrug **1** for cathepsin B-responsive active drug GEM release and caspase-3 activated fluorescence light-up of the hydrophobic TPE residues with AIE characteristics.



Scheme S3. The chemical structure of caspase-3 responsive GEM prodrug **2** but without cathepsin B- responsiveness.



Scheme S4. The chemical structure of cathepsin-B responsive GEM prodrug **3** but without caspase-3-responsiveness.

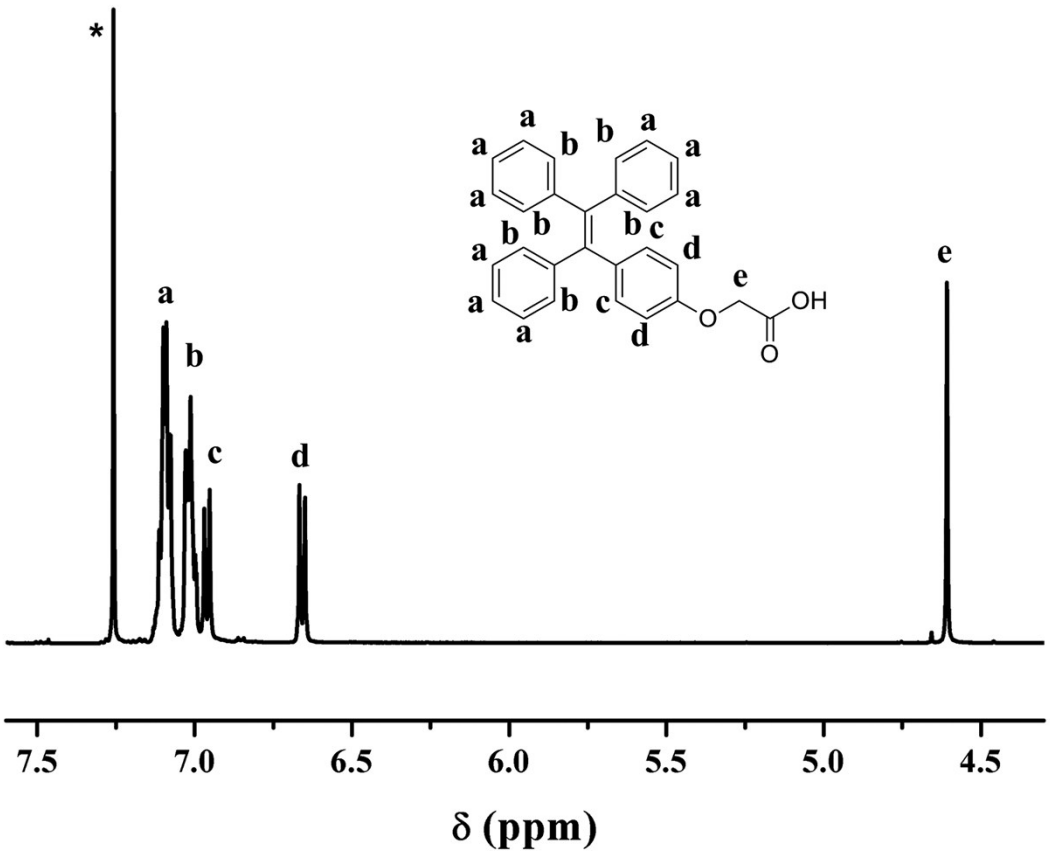


Figure S1. ^{1}H NMR spectrum of TPE-COOH. * Indicates residual NMR solvent.

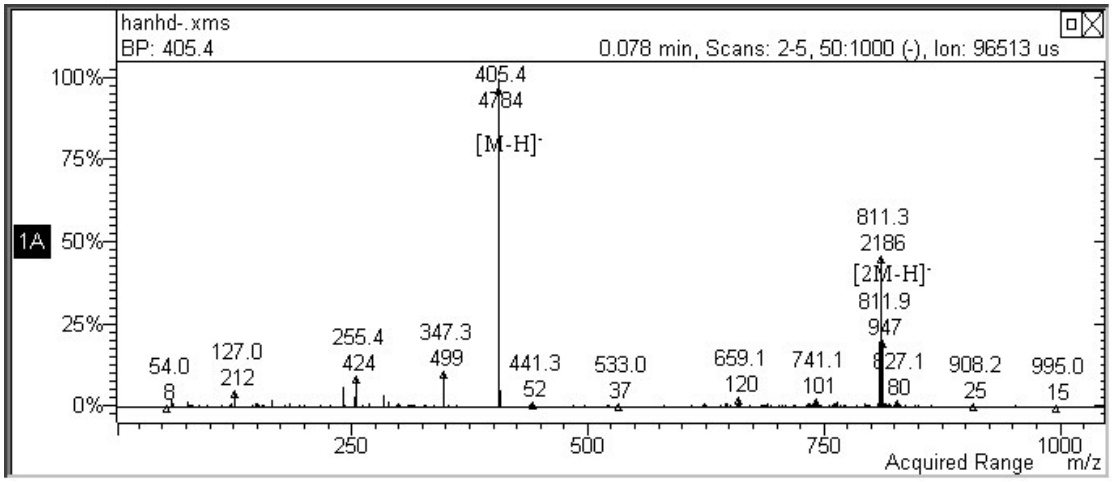
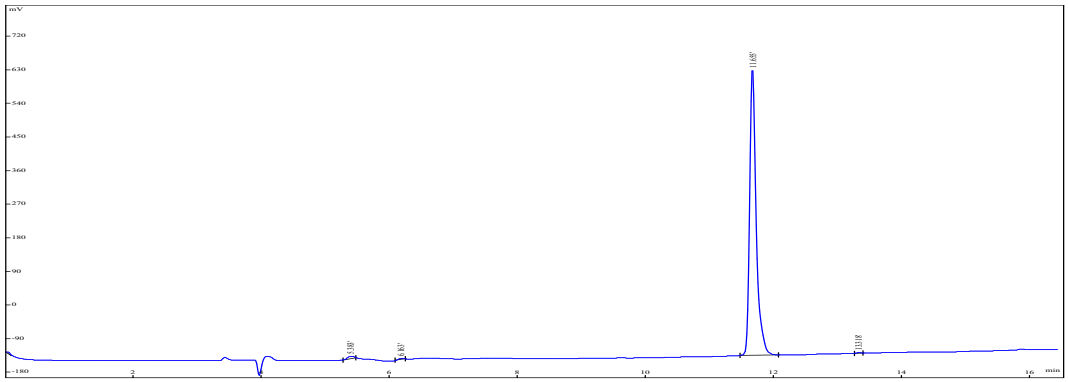


Figure S2. MS spectrum of TPE-COOH.



Rank	Time	Conc.	Area
1	5.383	0.8938	49717
2	6.163	0.2927	16280
3	11.655	98.7	5489853
4	13.318	0.1187	6602
Total		100	5562452

Figure S3. HPLC Spectrum of prodrug **1**.

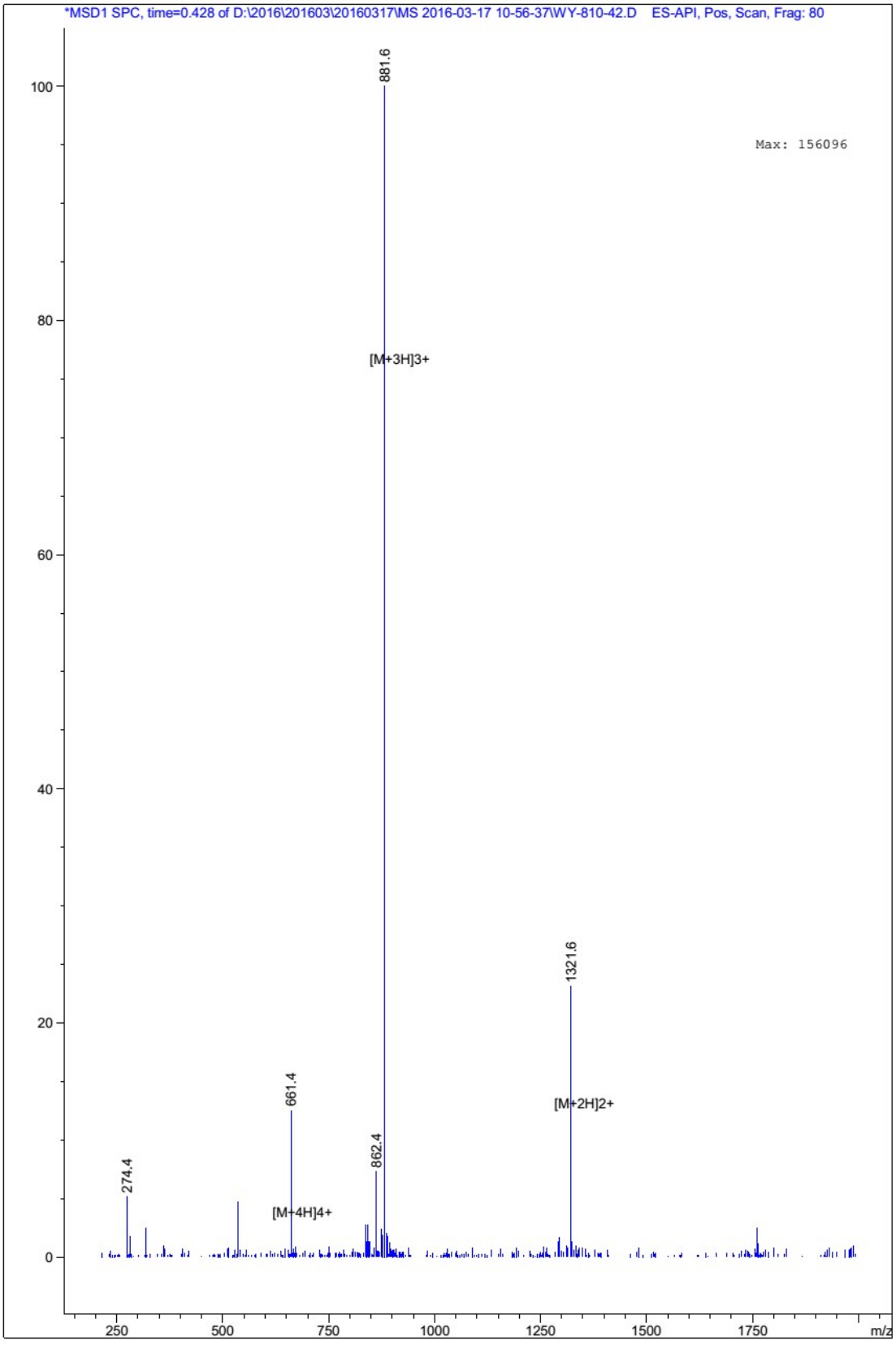
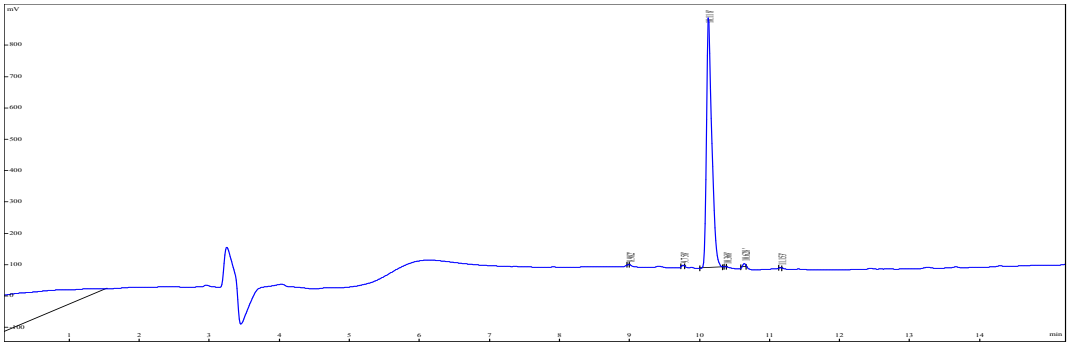


Figure S4. MS Spectrum of prodrug **1**.



Rank	Time	Conc.	Area
1	8.982	0.03291	1403
2	9.758	0.1627	6934
3	10.118	98.99	4218393
4	10.386	0.01473	628
5	10.628	0.7569	32253
6	11.153	0.03707	1580
Total		100	4261191

Figure S5. HPLC Spectrum of prodrug **2**.

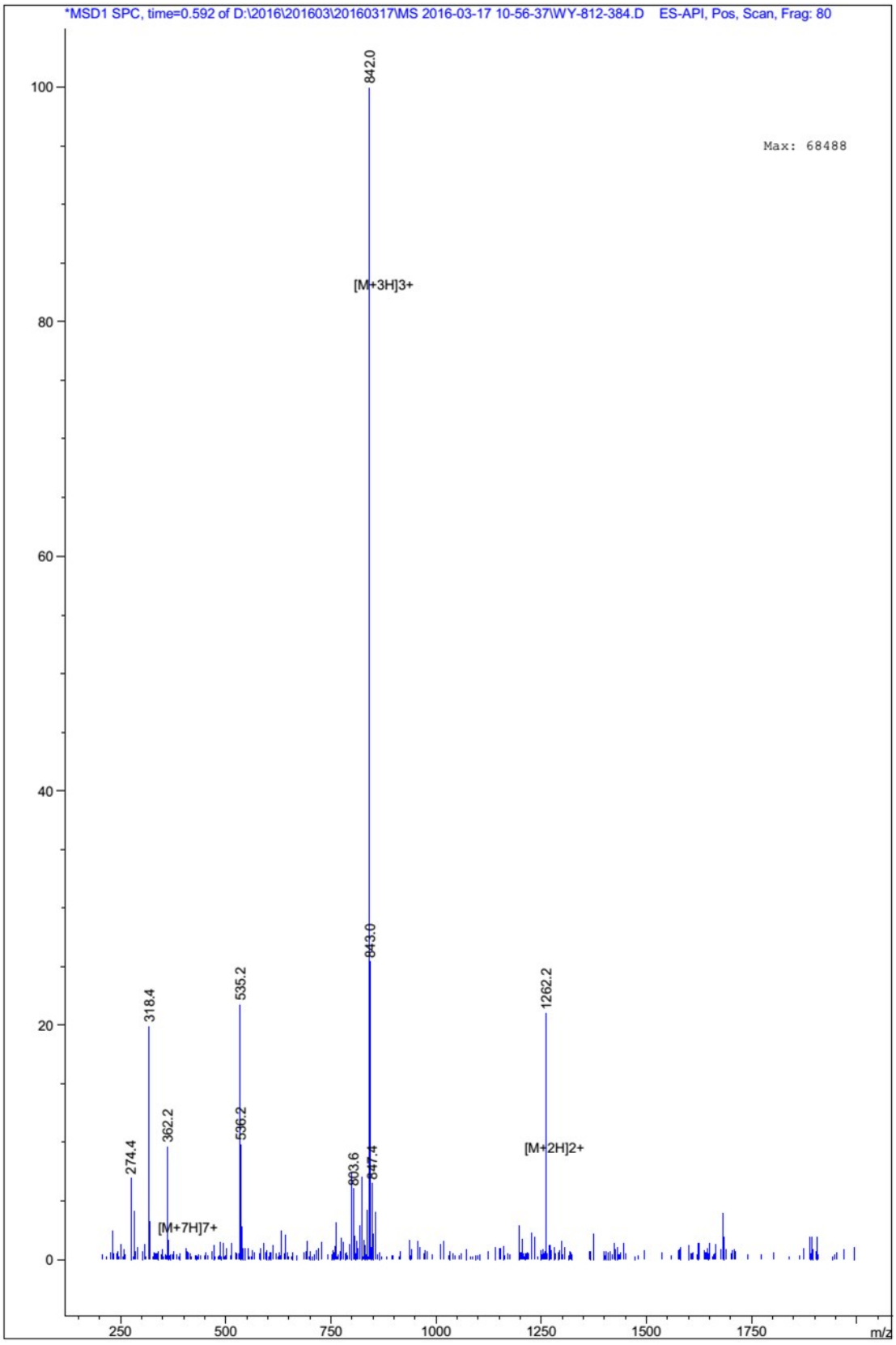
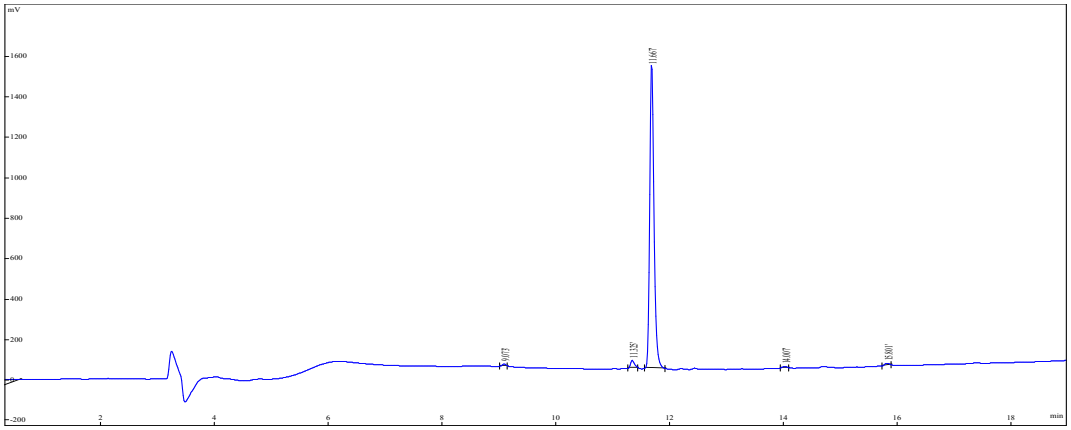


Figure S6. MS Spectrum of prodrug **2**.



Rank	Time	Conc.	Area
1	9.073	0.601	48984
2	11.325	2.143	174693
3	11.667	96.24	7843622
4	14.007	0.3317	27038
5	15.801	0.6906	56284
Total		100	8150621

Figure S7. HPLC Spectrum of prodrug **3**.

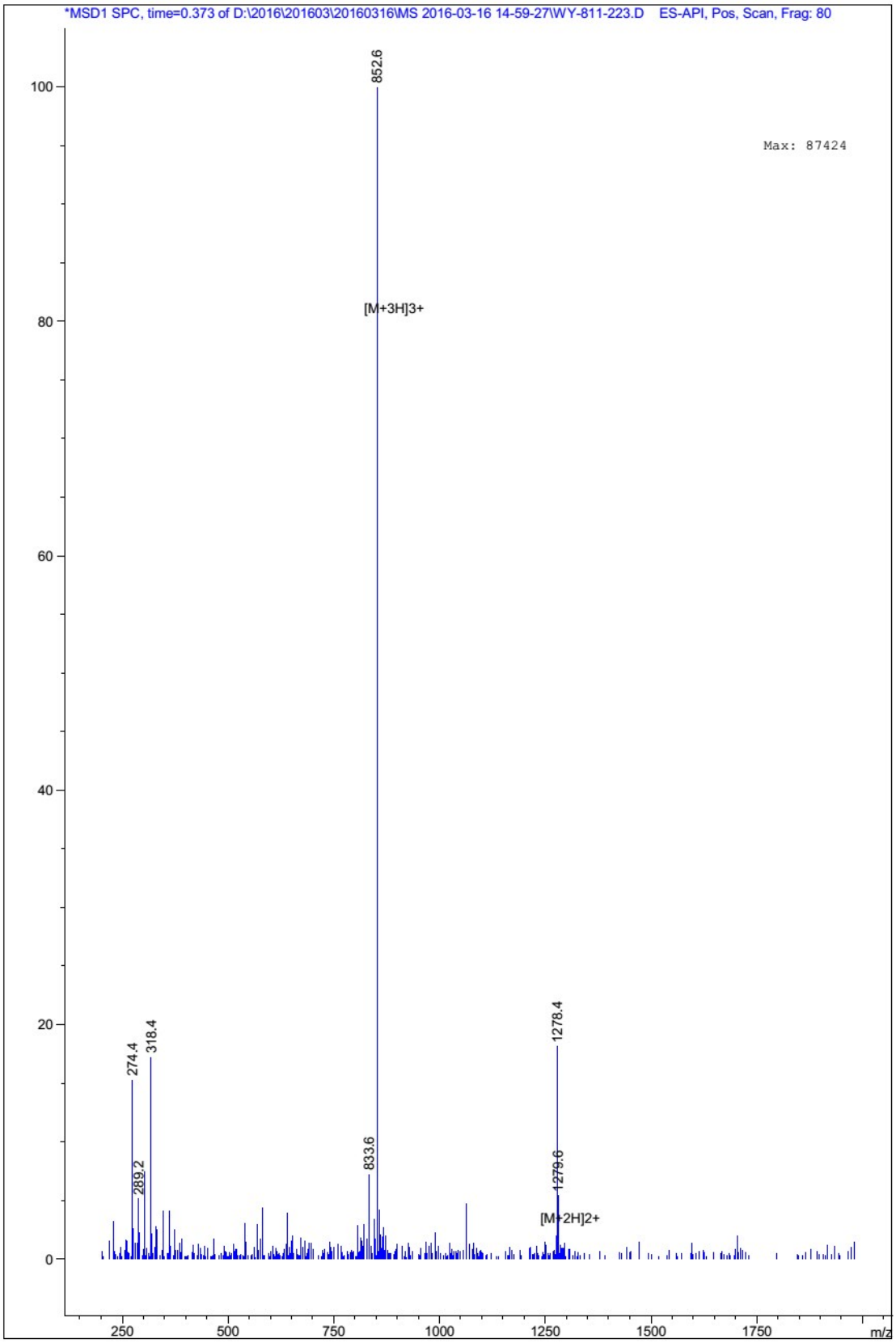


Figure S8. MS Spectrum of prodrug **3**.

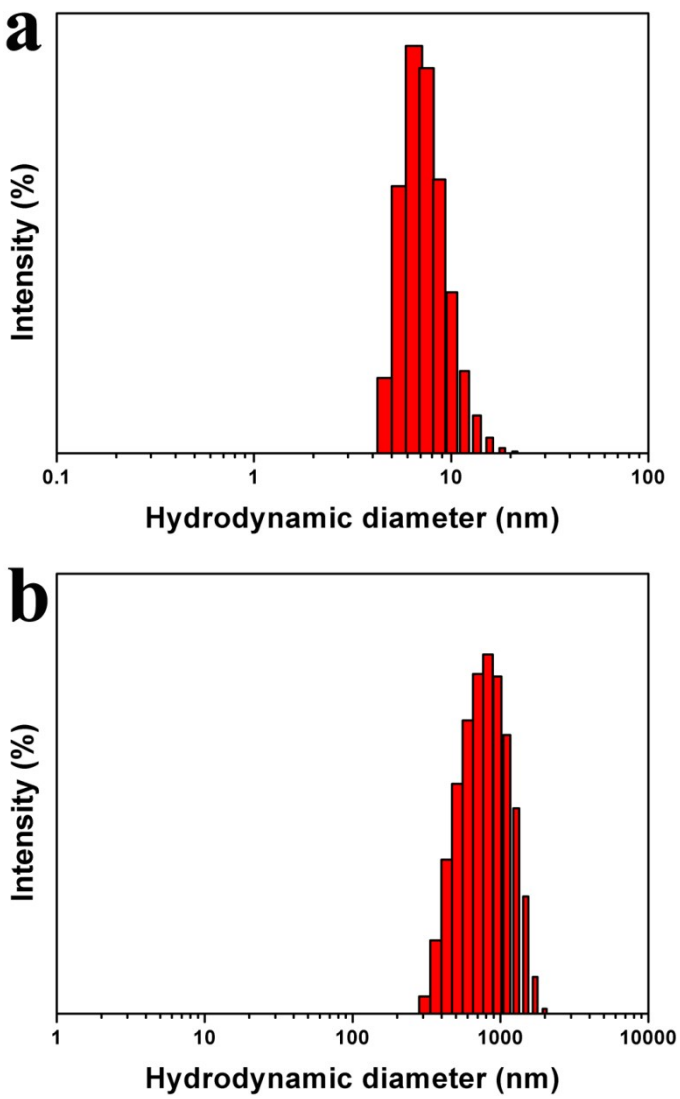


Figure S9. Intensity-average hydrodynamic diameter of (a) prodrug **1** and (b) TPE-COOH in DMSO/PBS mixture (1/199, v/v).