Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Real-time Monitoring of Caspase-3/8 Activity by Self-assembling Nanofiber Probes in Living Cells

Li-Song Zhang, Hong-Lei Xu, Ying Xia, Jian-Peng Bi, Chuan-Zeng Zhang, Zhen Xi, Lu-Yuan Li, and Zhi-Song Zhang*

*Email: zzs@nankai.edu.cn

S1. Experiment Materials and Instruments

Dulbecco's Modified Essential Medium (DMEM), Trypsin-EDTA (0.25%) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Coumarin 151, 2-Cl-trityl chloride resin (1.0-1.2 mmol/g), Fmoc-OSu and other Fmoc-amino acids were obtained from Gil Biochemical (Shanghai, China).N, N-diisopropylethylamine (DIPEA), DMSO-d6, 2-(1H-benzotriazole-1-yl)-1, 1, 3, 3-tetramethylur onium hexafluoro phosphate (HBTU) and other chemical reagents and solvents were obtained from Bide Pharmaceutical (Shanghai, China); Trifluoroacetic acid (TFA) and other elution reagents were all of analytical grade and used without further purification. Alkaline phosphatase was purchased from Biomatik (Cat. No. A1130, [ALP] >1300U/mg, in 50% glycerol).^{1}H and ^{13}C NMR spectra were recorded at 25 °C on a Bruker AV400 NMR spectrometer, operating at 400 and 100 MHz, respectively. Morphological analyses were conducted on a Thermo Scientific™ Talos™ F200C transmission electron microscopy. Cellular uptake and drug tracking images were taken by a confocal laser scanning microscopy (Leica TSC SP8, Germany).

S2. Synthesis and Characterizations

Fmoc-L-Asp(OH)-AFC: To a solution of AFC (10 mmol) and Fmoc-L-Asp(OtBu)-OH (10 mmol) in dry pyridine, phosphoryl chloride (10 mmol) was added at -15 °C, and the solution was stirred at -15 °C for 60 min. The mixture was poured into 150 mL water and extracted with ethyl acetate. The ethyl acetate layer was separated; washed with 1mol/L hydrochloric acid and brine; and then dried over Na_2SO_4. Filtration, concentration in vacuo, and purification by silica gel flash column chromatography, afforded Fmoc-L-Asp(OtBu)-AFC (yield 85%).Fmoc-L-Asp(OtBu)-AFC was dissolved in 95% TFA, then the mixture was stirred at 0 °C for 2 h. The solvent was removed in vacuo to give Fmoc-L-Asp-AFC (yield 73%).

Two probes were prepared by solid-phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin. The Fmoc-L-Asp (OH)-AFC was loaded onto the resin at the C-terminal, followed by removal of the Fmoc protecting group by treatment with 20% piperidine. The next Fmoc-protected amino acid was coupled to the free amino group using N,N-Diisopropylethylamine/O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (DIPEA/HBTU) as the coupling reagent. As the final step, the resin-bound peptide was cleaved using a cocktail of TFA/triisopropylsilane/water (95: 2.5: 2.5) for 2 hours under a nitrogen atmosphere, and then the cleavage solution was collected. Crude product was obtained after the addition of cold diethyl ether into concentrated filtrate and purified by reverse phase high performance liquid chromatography (HPLC) using a semi-prepare C18 column. The peptide solution was freeze-dried to obtain the pure product (yield 38%/42%).

S3. General Procedures for Hydrogel Preparation and TEM Sample Preparation.

Enzymatic gelation: to evaluate the self-assembling ability of the two probes under the action of ALP. we dissolved the two probes in a PBS (1 mL) solution at the concentration of 5 mmol/L. The pH value of the solution was carefully monitored through a pH paper by adding a 1 mol/L sodium carbonate solution. After the pH of the solution reached 7.4, we added recombinant ALP. Place in a 37 °C incubator for 120 minutes.

In this paper, two gelled sample solution (5 μL) were added to a glow-discharged thin carbon-coated copper grid (400 mesh, Pacific Grid-Tech), Respectively. After 30 seconds, we carefully removed excess liquid from the edges of the grid with filter paper and rinsed the grid twice or three times with deionized water. The grid was dried in air for 48 hours, and a TEM image was obtained by a transmission electron microscope.

S4. Cell Culture and Cell Viability Assay

Hela and MCF-7 cells were purchased from the American Type Culture Collection (ATCC, USA) and cultured in a basic essential medium (MEM) supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The incubation conditions for the cells are at 37 °C in a humidified atmosphere of 5% CO_2.

For cell viability assay, Hela or MCF-7 cells in the logarithmic growth phase were seeded into 96-well plates with 1×10^4 cells per well and cultured for 6 hours. Then cells were incubated with Nap-GFFpYIETD-AFC and Nap-GFFpYDEVD-AFC at a gradient concentration (0 μmol/L as a control) ranging from 0 to 200 μmol/L. After 48 h treatment, the medium was replaced with 200 μL 0.5 mg/mL MTT. And after 4 hours the MTT solution was replaced with 200 μL DMSO solution. Measure the absorbance of each well at 570 nm with a microplate reader. The results are expressed as a percentage of cell survival relative to control group.

S5. Flow Cytometry

Apoptosis was evaluated by Annexin V-FITC apoptosis detection kit (Beyotime, China) following the manufacturer's instructions. Hela cells and MCF-7 cells were seeded into a 6-well plate at a density of 1×10^5 cells/well. After incubation for 24 h, treat the cells with 200 μmol/L Nap-GFFpYIETD-AFC and Nap-GFFpYDEVD-AFC, respectively, using untreated cells as the negative control. After 12 hours, the cells were trypsinized and centrifuged at $1000\times g$ for 5 min. Remove the supernatant, wash the cells with ice-cold PBS and resuspend the cells in 195 μL of binding buffer. Thereafter, 5 μL of Annexin V-FITC and 10 μL of PI were added and the cells were incubated for 20 min in the dark at room temperature. The fluorescence of 10,000 events per sample was analyzed using flow cytometry (BD, USA).

S6. Sample Preparation for Confocal Microscopy

Hela cells and MCF-7 cells in log phase growth were seeded at the density of 1×10^5 cells/well in a dedicated confocal dish. Incubate cells in a 37ºC incubator for 6 hours. Then, cell binding test was achieved by incubating the cells with Nap-GFFpYIETD-AFC (200 μmol/L) and Nap-GFFpYDEVD-AFC (200 μmol/L) at 37 °C. Cells were stained with RedDot 2 (1x) at 37 °C for 2 minutes. The cells were washed for three times with cold PBS. Confocal fluorescence imaging was performed on Leica TSC SP8 confocal laser scanning microscope (CLSM).

S7. Time and Concentration Gradient Release Curves of AFC

Concentration gradient release assay: Prepare Nap-GFFpYDEVD-AFC and Nap-GFFpYIETD-AFC solutions with different concentrations (5, 10, 20, 40, 80 μmol/L) in PBS. Add 2 μg corresponding caspase protein. After 120 min incubation at 37°C, the fluorescence intensity of AFC was measured with a fluorescence spectrophotometer. The curves in Figure 2A exhibit positive correlation between probe concentration and free AFC fluorescence intensity.

Time gradient release assay: Prepare Nap-GFFpYDEVD-AFC and Nap-GFFpYIETD-AFC solutions at 40 μmol/L in PBS. Add 3 μg of the corresponding Caspase-3 or Caspase-8 proteins. After sampling at different time points (0, 10, 20, 30, 40, 60, 80 min), the fluorescence intensity of AFC was detected with a fluorescence spectrophotometer. As shown in Figure 2B, the fluorescence intensity of AFC gradually increased with the extension of the incubation time.

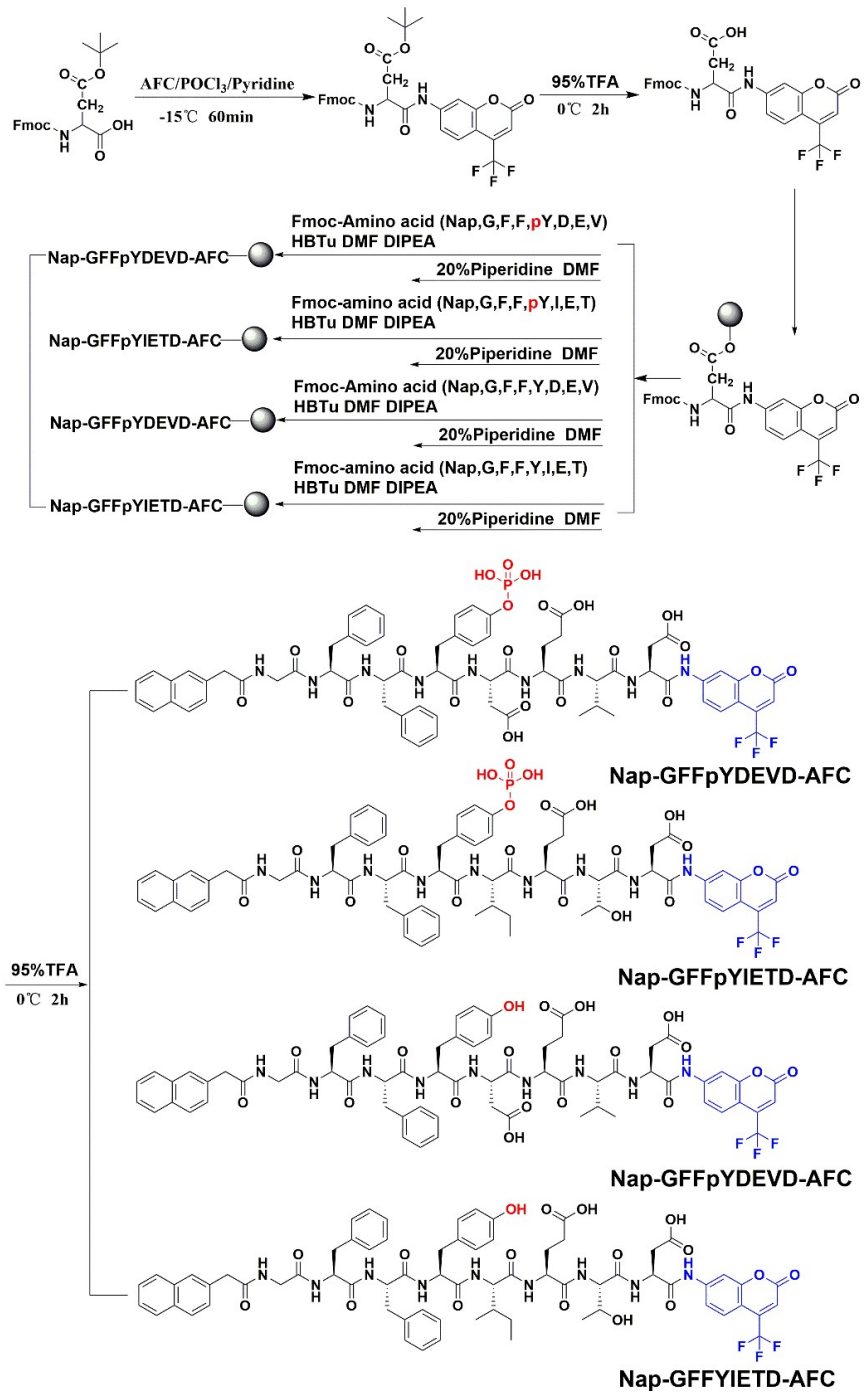

Figure S1. Synthesis route of Nap-GFFpYDEVD-AFC, Nap-GFFpYIETD-AFC, Nap-GFFYDEVD-AFC and Nap-GFFYIETD-AFC.

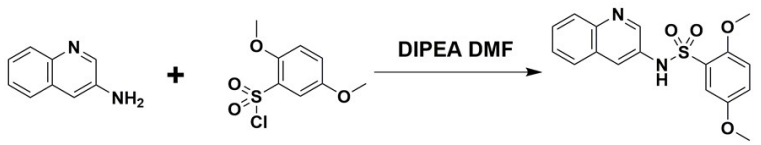

Figure S2. Synthesis routes of ALP inhibitor (DQB = 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide).

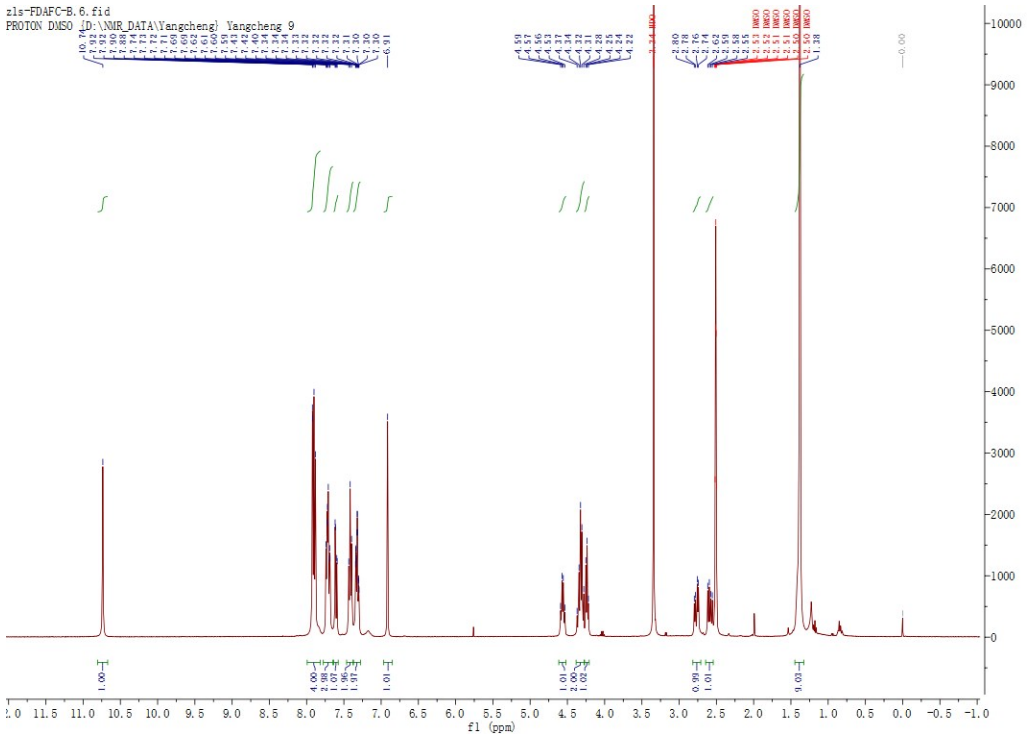

Figure S3A. ^{1}H NMR spectrum (400 MHz) of Fmoc-Asp(OtBu)-AFC in DMSO-d6 at 25 ºC.

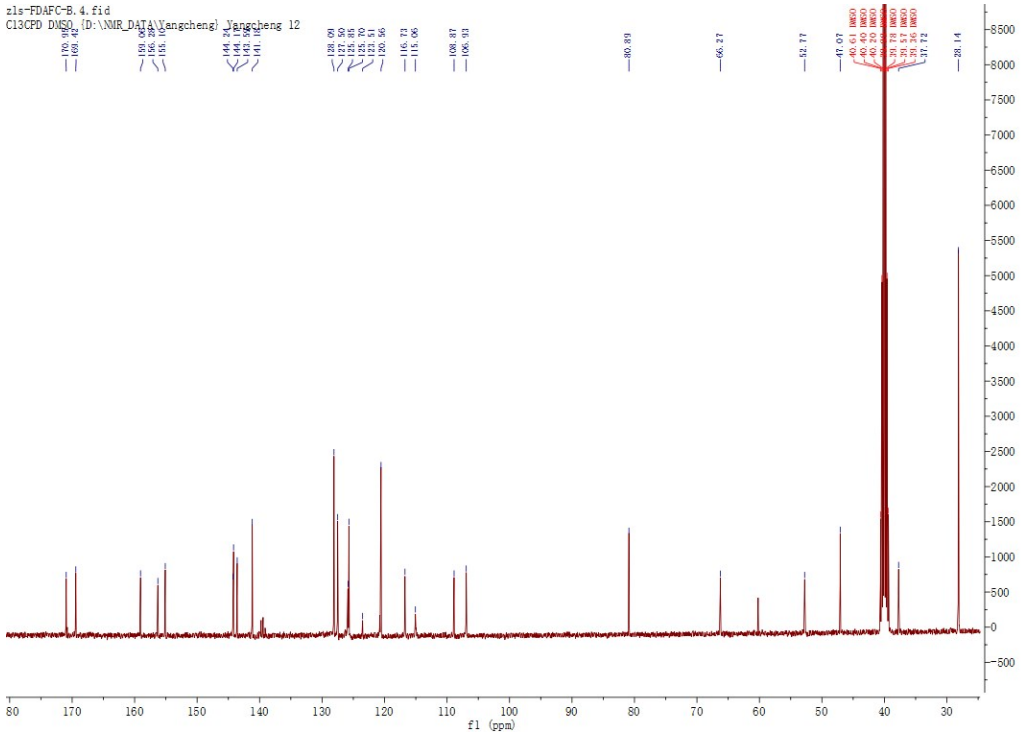

Figure S3B. ^{13}C NMR spectrum (100 MHz) of Fmoc- Asp(OtBu)-AFC in DMSO-d6 at 25 ºC.

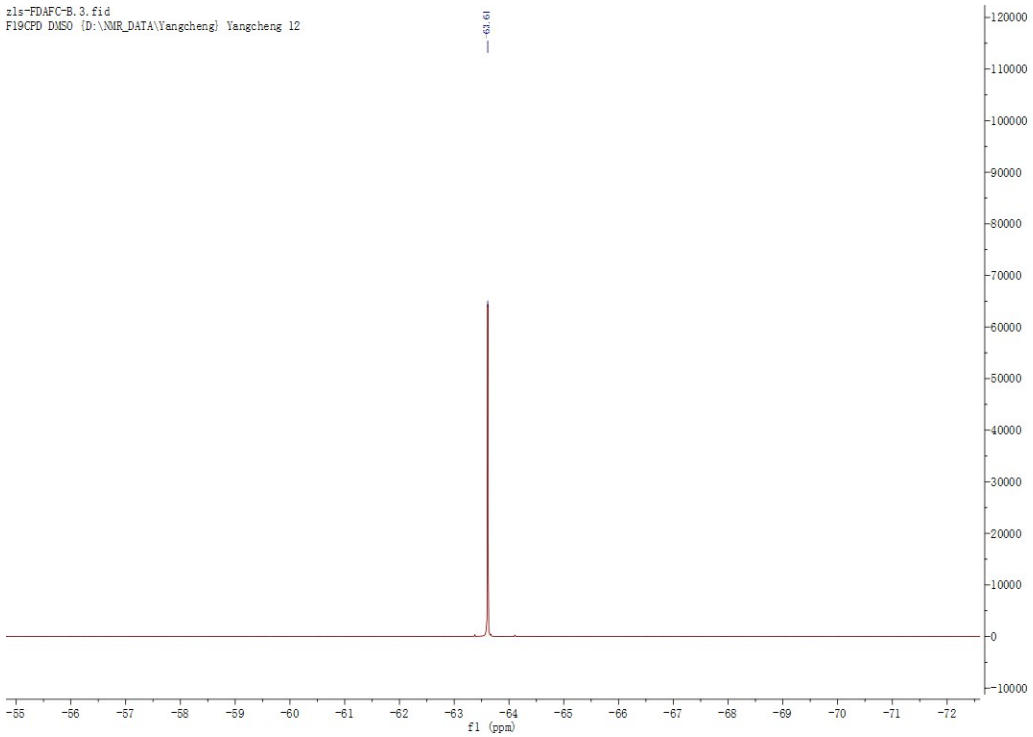

Figure S3C. ^{19}F NMR spectrum of Fmoc- Asp(OtBu)-AFC in DMSO-d6 at 25 ºC.

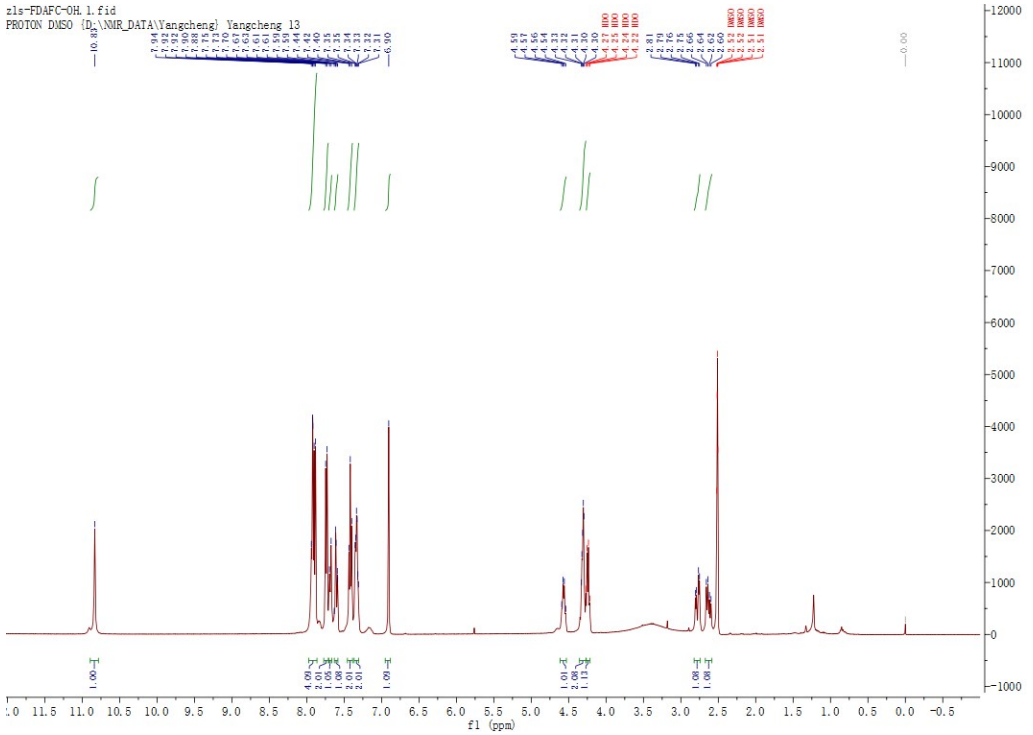

Figure S4A. ^{1}H NMR spectrum (400 MHz) of Fmoc- Asp(OH)-AFC in DMSO-d6 at 25 ºC.

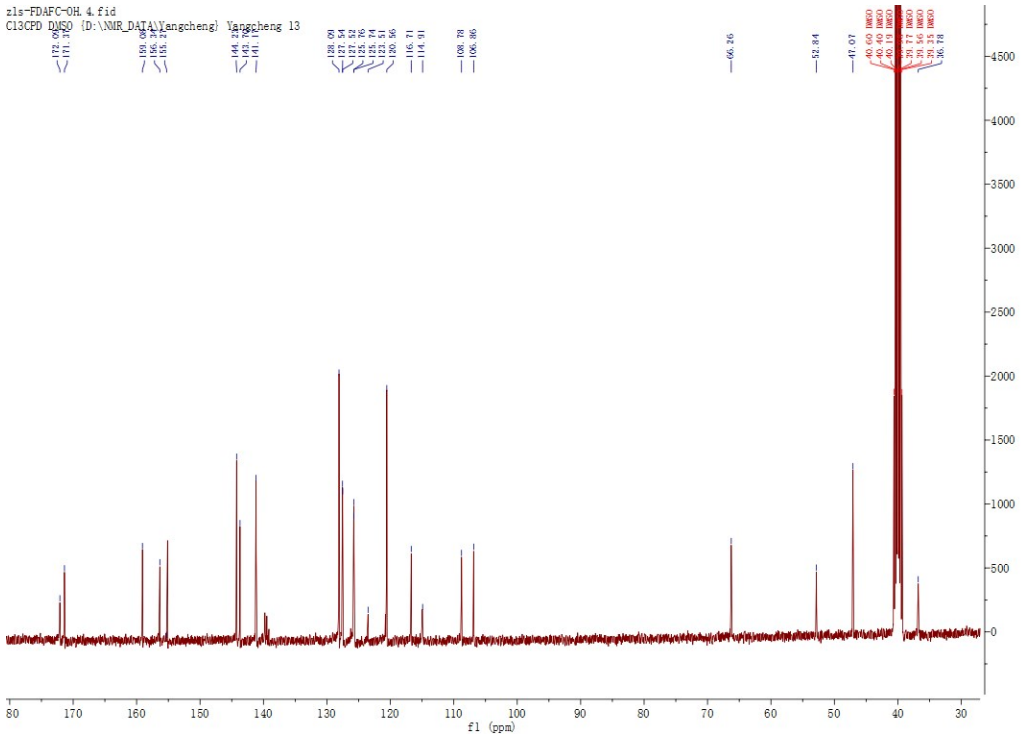

Figure S4B. ^{13}C NMR spectrum (100 MHz) of Fmoc- Asp(OH)-AFC in DMSO-d6 at 25 ºC.

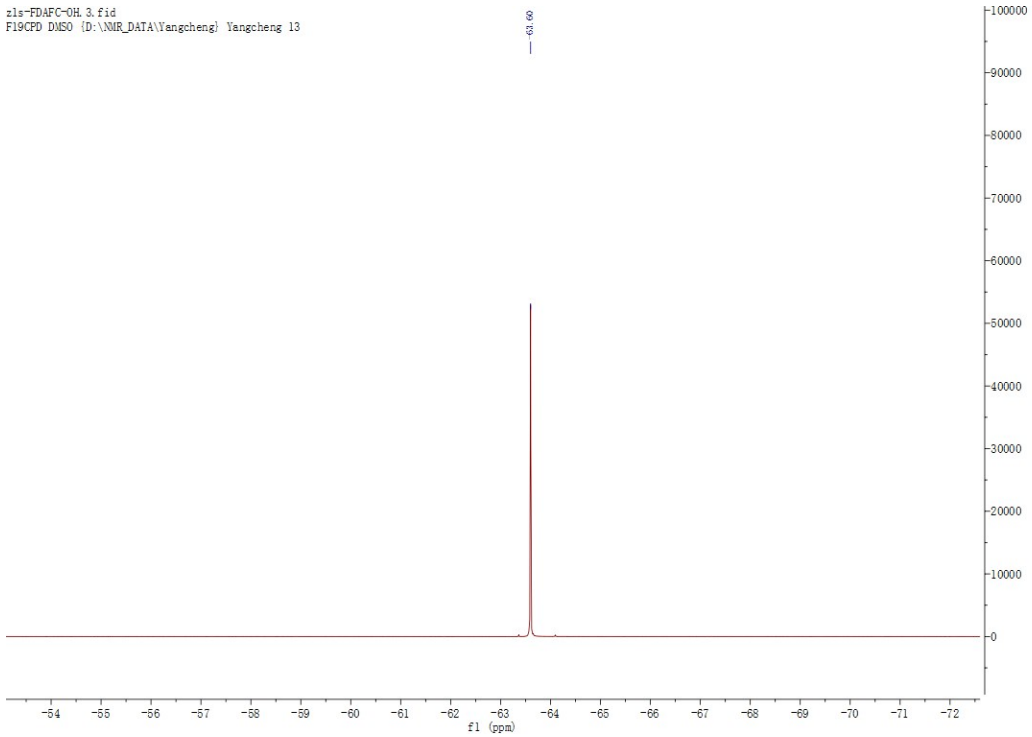

Figure S4C. ^{19}F NMR spectrum of Fmoc- Asp(OH)-AFC in DMSO-d6 at 25 ºC.

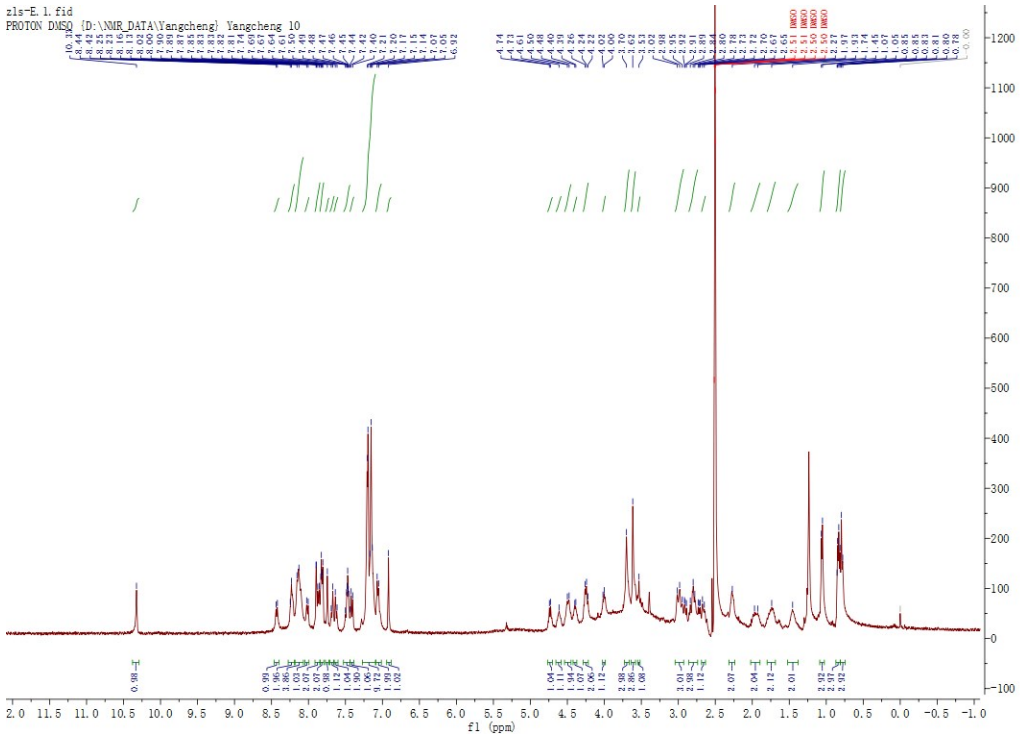

Figure S5A. ^{1}H NMR spectrum (400 MHz) of Nap-GFFpYIETD-AFC in DMSO-d6 at 25 ºC.

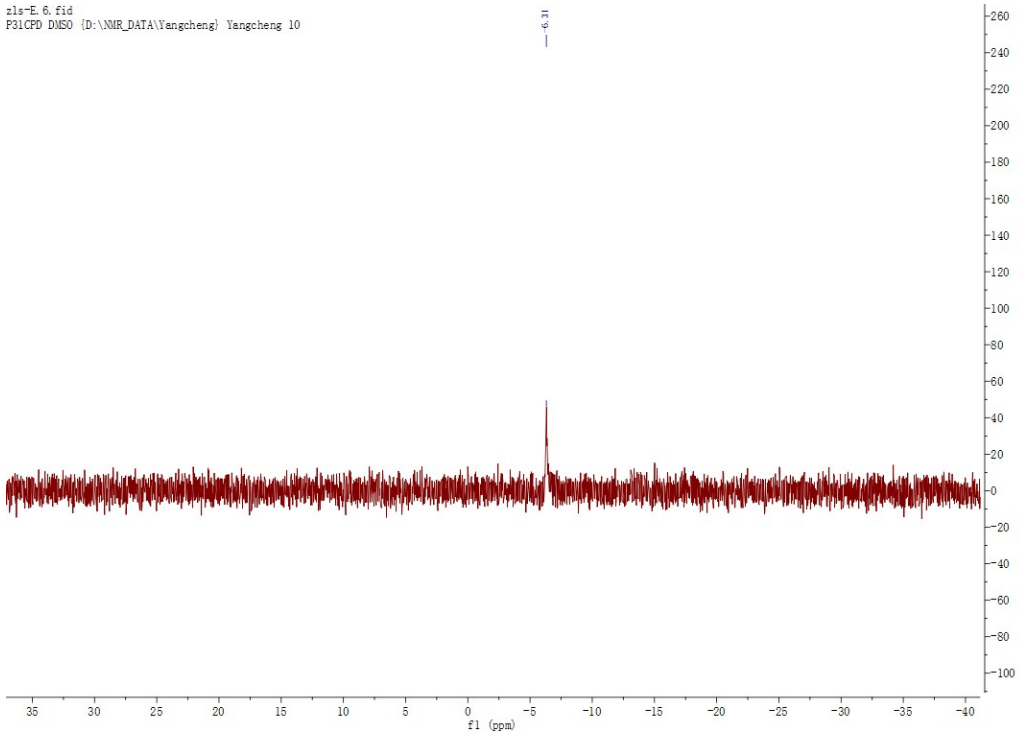

Figure S5B. ^{31}P NMR spectrum of Nap-GFFpYIETD-AFC in DMSO-d6 at 25 ºC.

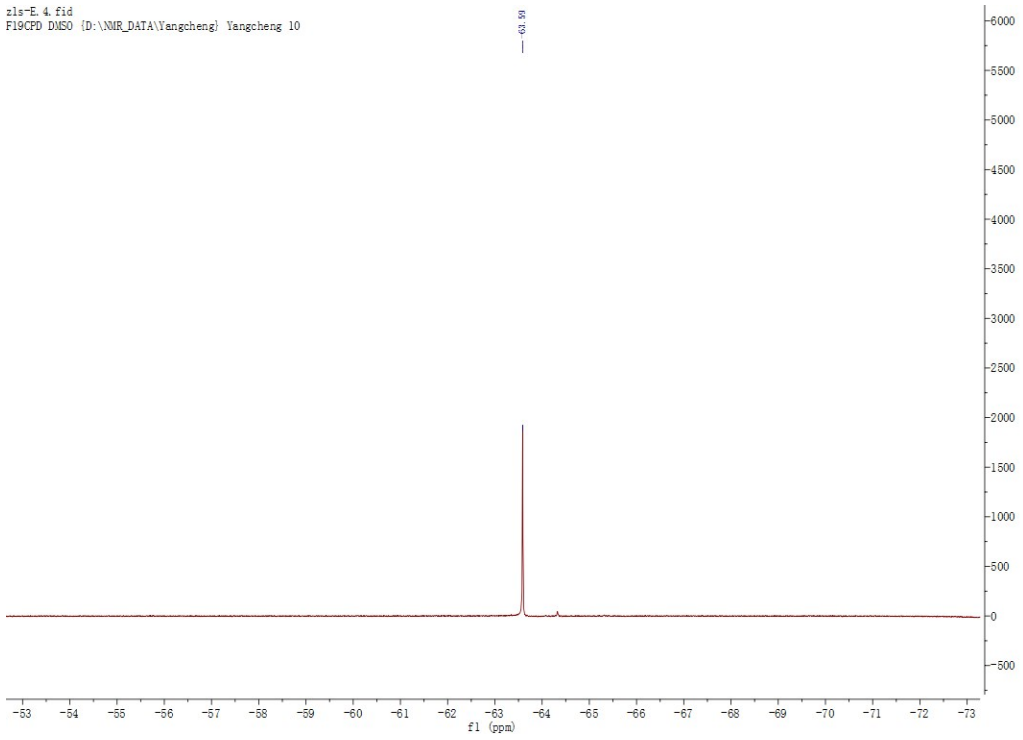

Figure S5C. ^{19}F NMR spectrum of Nap-GFFpYIETD-AFC in DMSO-d6 at 25 ºC.

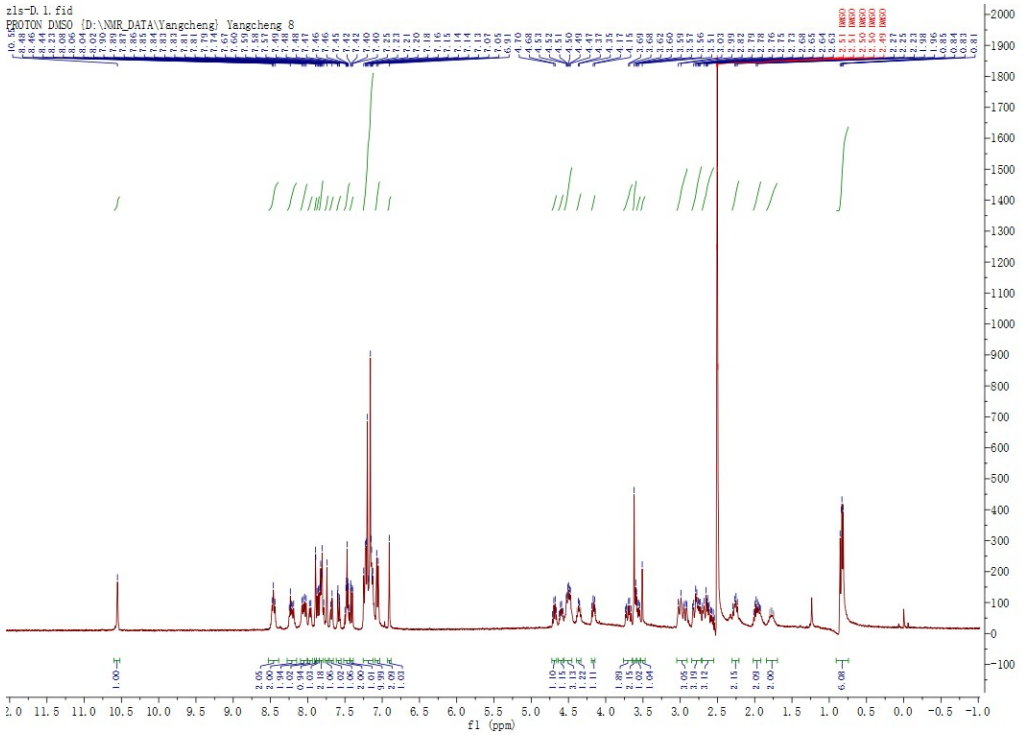

Figure S6A. ^{1}H NMR spectrum (400 MHz) of Nap-GFFpYDEVD-AFC in DMSO-d6 at 25 ºC.

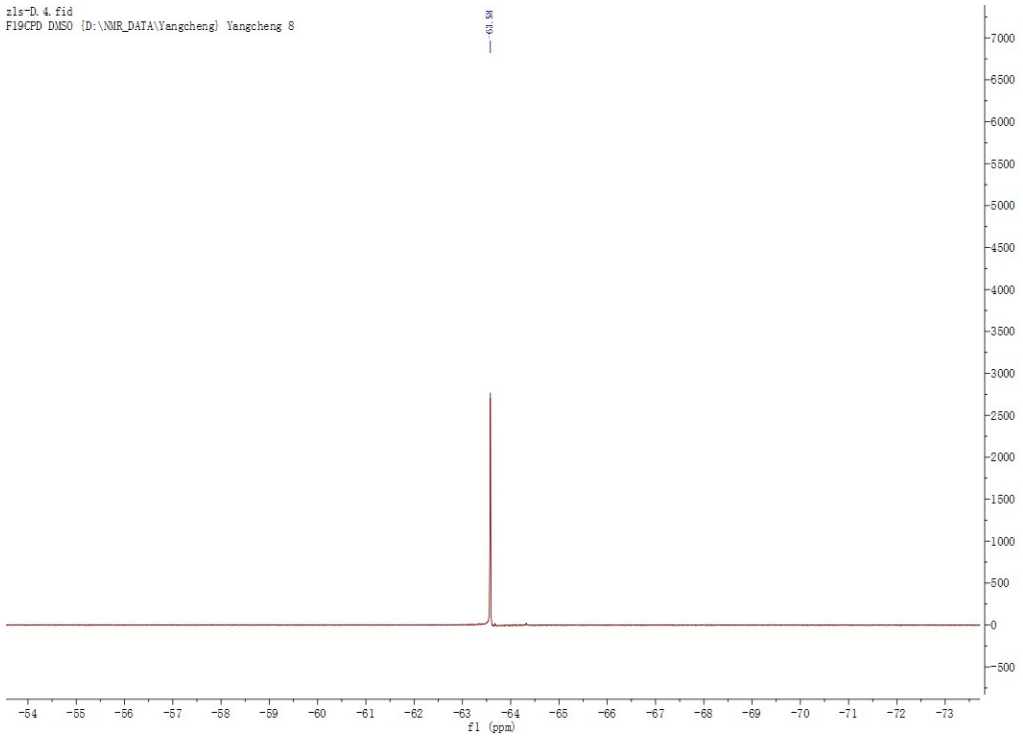

Figure S6B. ^{19}F NMR spectrum of Nap-GFFpYDEVD-AFC in DMSO-d6 at 25 ºC.

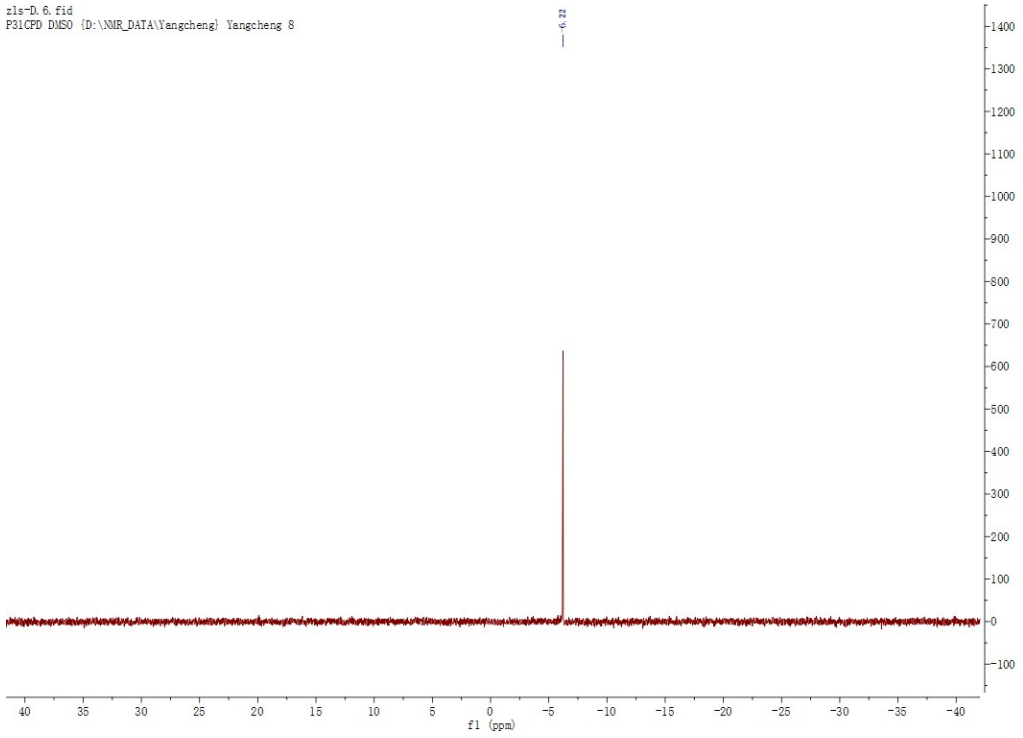

Figure S6C. ^{31}P NMR spectrum of Nap-GFFpYDEVD-AFC in DMSO-d6 at 25 ºC.

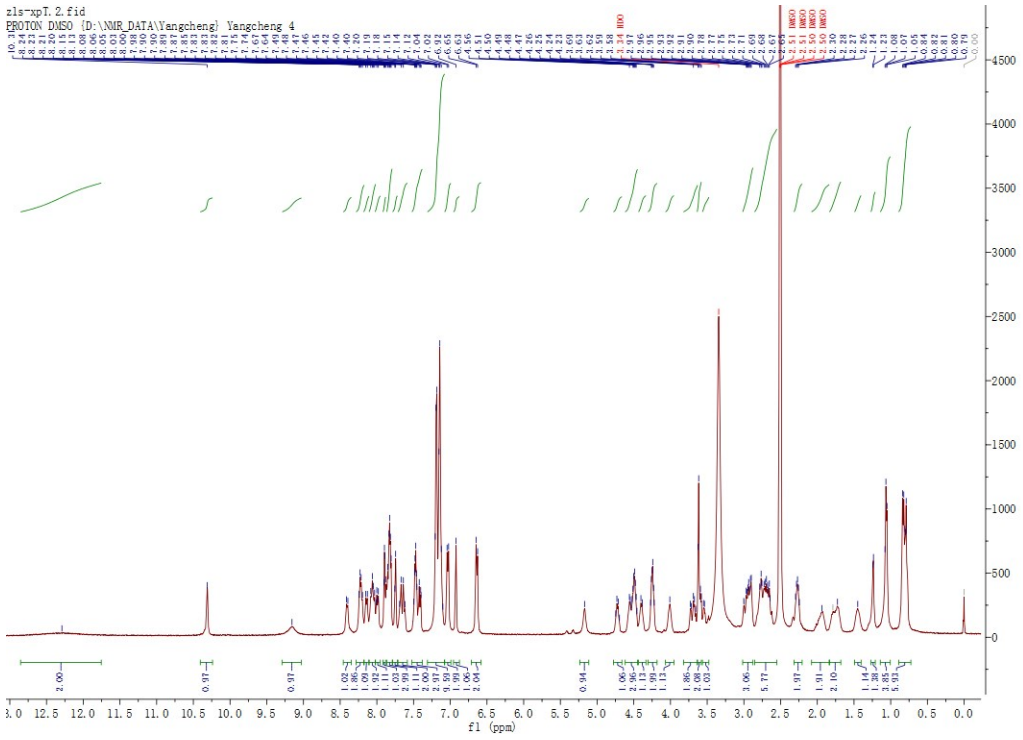

Figure S7A. ^{1}H NMR spectrum of Nap-GFFYIETD-AFC in DMSO-d6 at 25 ºC.

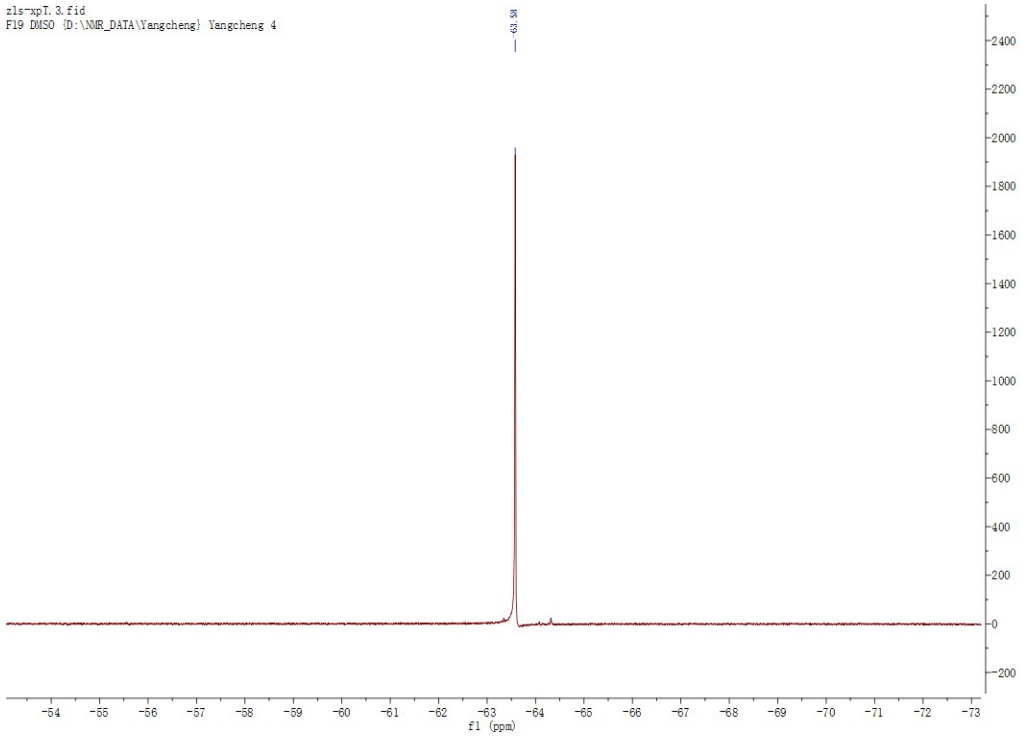

Figure S7B. ^{19}F NMR spectrum of Nap-GFFpYIETD-AFC in DMSO-d6 at 25 ºC.

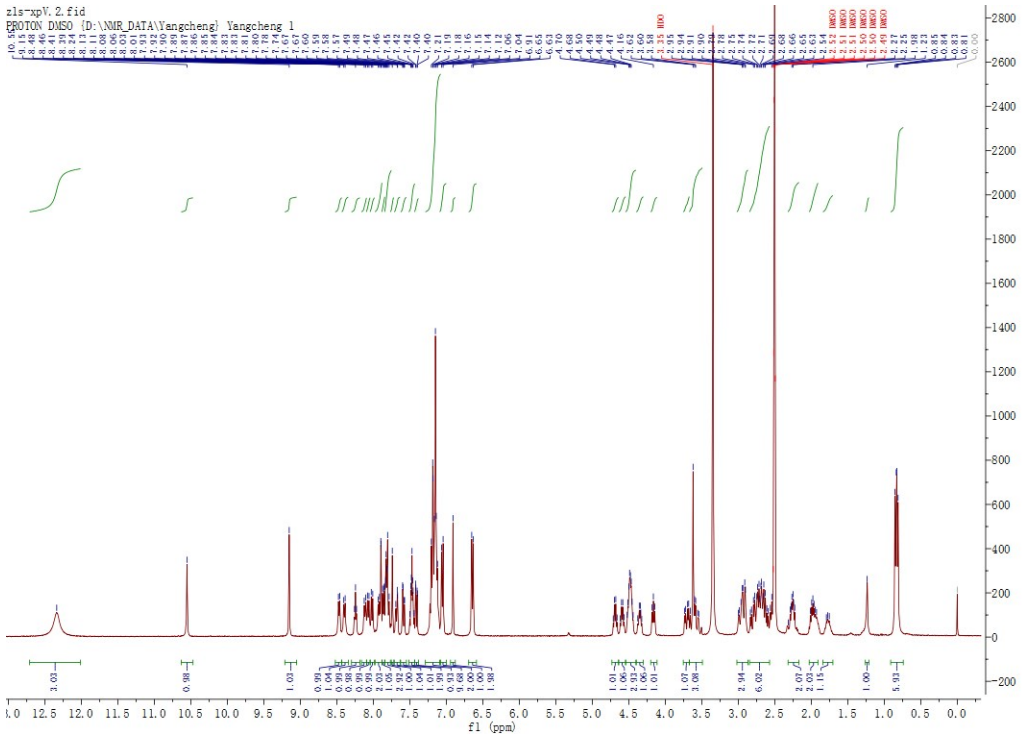

Figure S8A. ^{1}H NMR spectrum of Nap-GFFYDEVD-AFC in DMSO-d6 at 25 ºC.

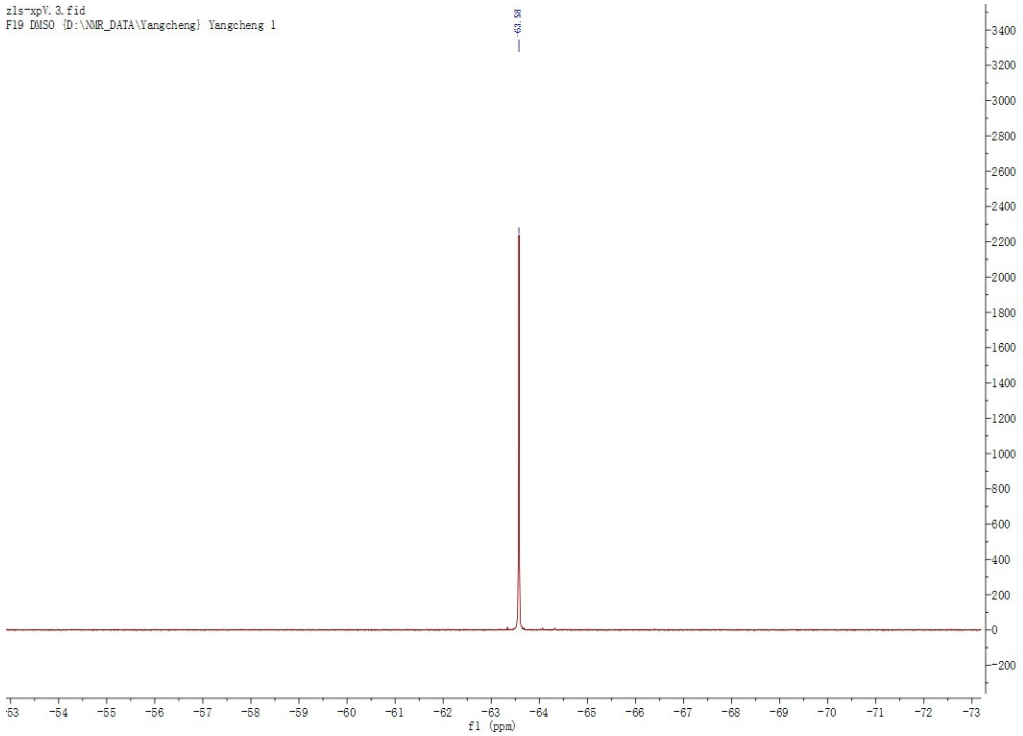

Figure S8B. ^{19}F NMR spectrum of Nap-GFFYDEVD-AFC in DMSO-d6 at 25 ºC.

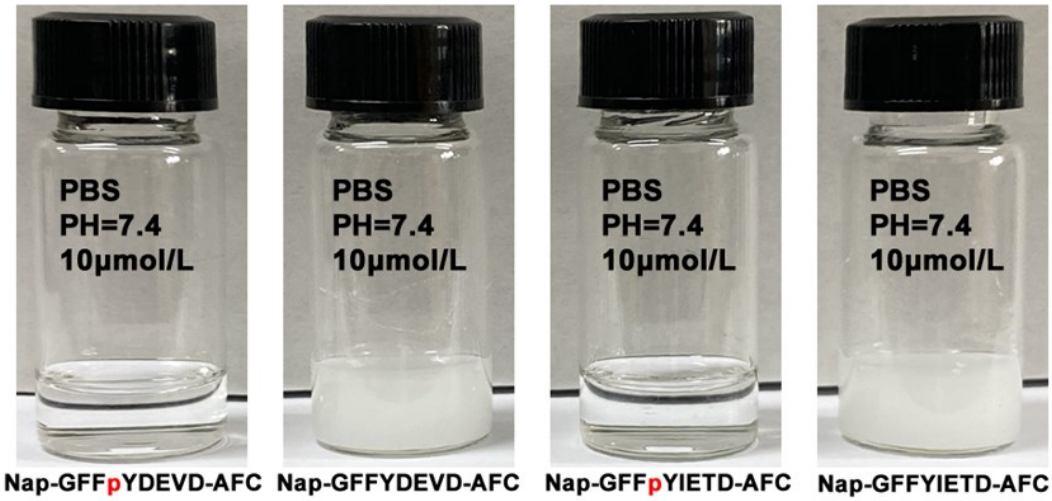

Figure S9. Solubility of self-assembled probes with and without phosphate groups in PBS (pH=7.4). Nap-GFFpYDEVD-AFC and Nap-GFFpYIETD-AFC form a clear and transparent solution; Nap-GFFYDEVD-AFC and Nap-GFFYIETD-AFC form a turbid solution.

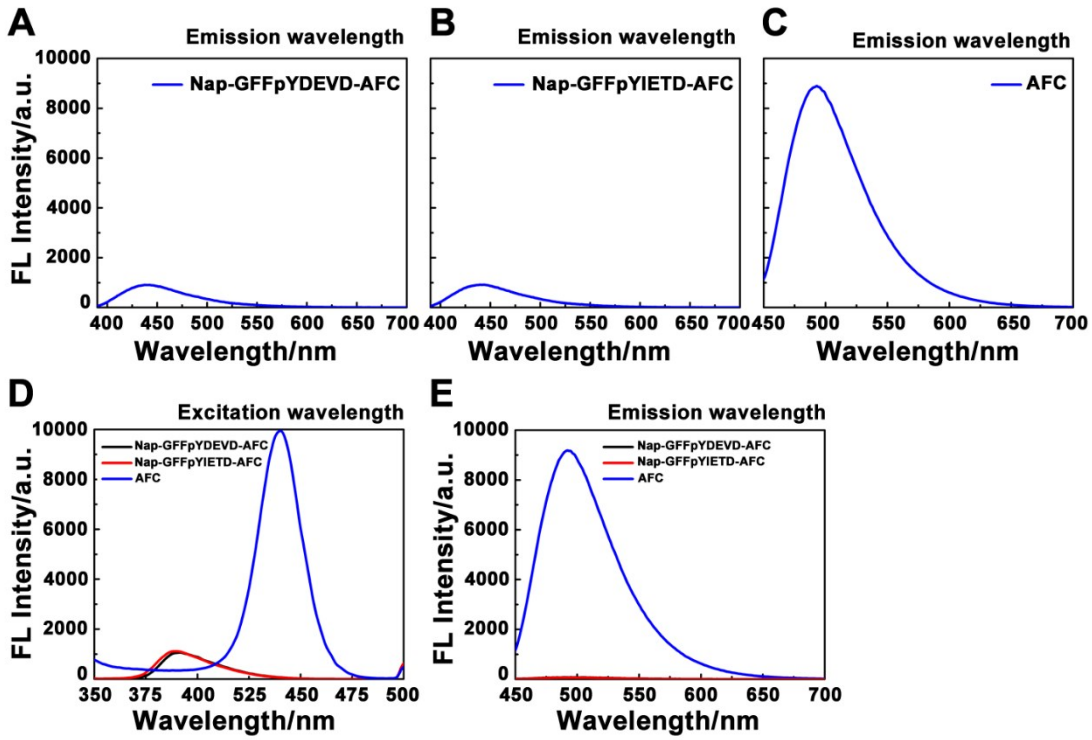

Figure S10. The fluorescence spectrum characteristics of AFC and its derivatives. The maximum emission wavelength and fluorescence intensity of Nap-GFFpYDEVD-AFC (A) and Nap-GFFpYIETD-AFC (B) at an excitation wavelength of 392 nm; C) The maximum emission wavelength and fluorescence intensity of free AFC at 440 nm excitation wavelength; D) The maximum excitation wavelength and fluorescence intensity of Nap-GFFpYDEVD-AFC, Nap-GFFpYIETD-AFC and AFC; E) Fluorescence intensity of Nap-GFFpYDEVD-AFC, Nap-GFFpYIETD-AFC and AFC at 440 nm excitation wavelength.

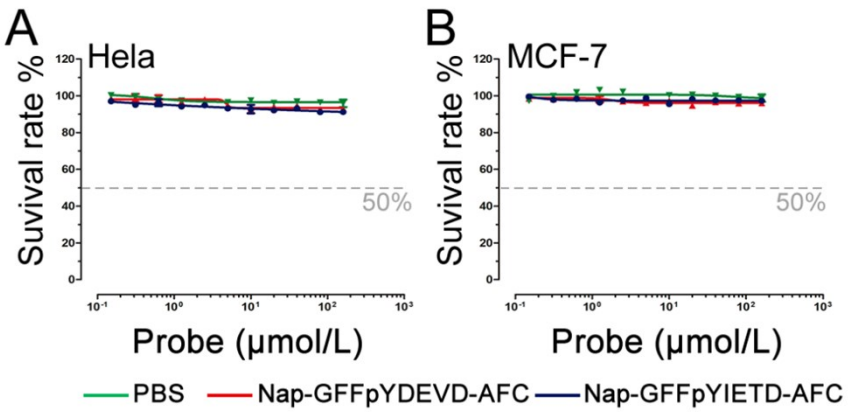

Figure S11. IC_{50} values of Nap-GFFpYDEVD-AFC (red) and Nap-GFFpYIETD-AFC (blue). PBS group as a control. Nap-GFFpYDEVD-AFC and Nap-GFFpYIETD-AFC were evaluated with a cell viability assay (MTT; OD 570 nm)) on cell lines Hela and MCF-7. Probe concentrations range from 0.30625 to 200 μmmol/L, 2000 cells/well, incubation time 48 hrs.

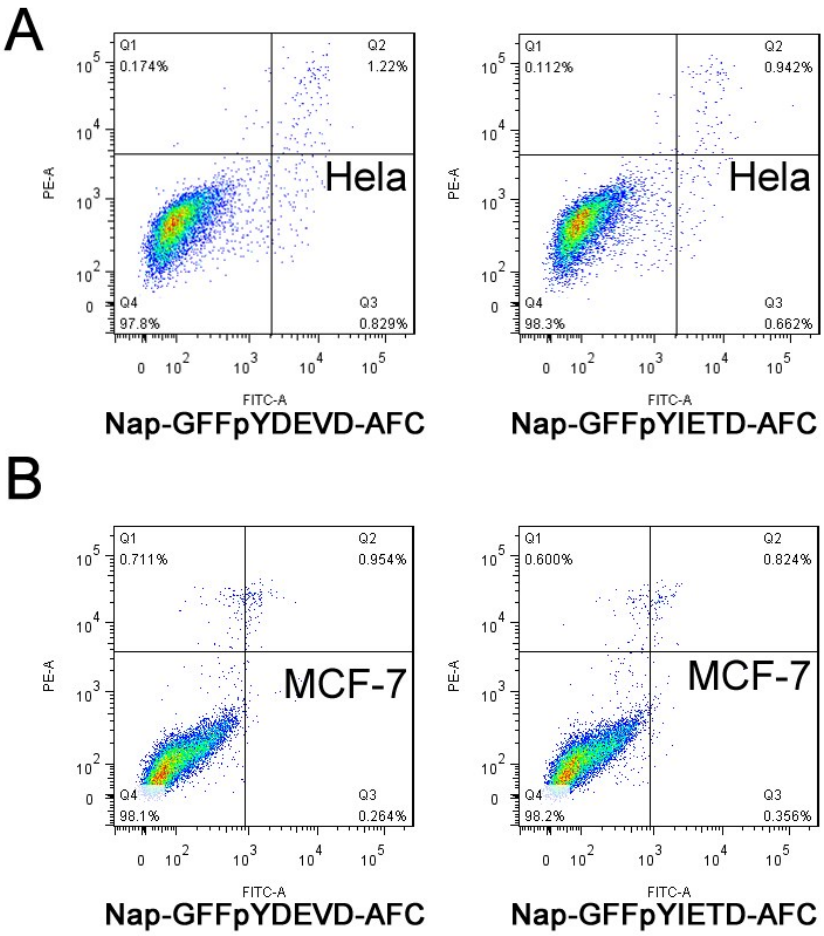

Figure S12. Apoptosis of Hela and MCF-7 cells. Proportion of apoptosis induced by Nap-GFFpYDEVD-AFC and Nap-GFFpYIETD-AFC in two different cell lines. Probe concentration: 200 μmol/L, time to induce cell apoptosis: 48 hours. Number of cells seeded on 6-well plate: 25,000 cells/well.

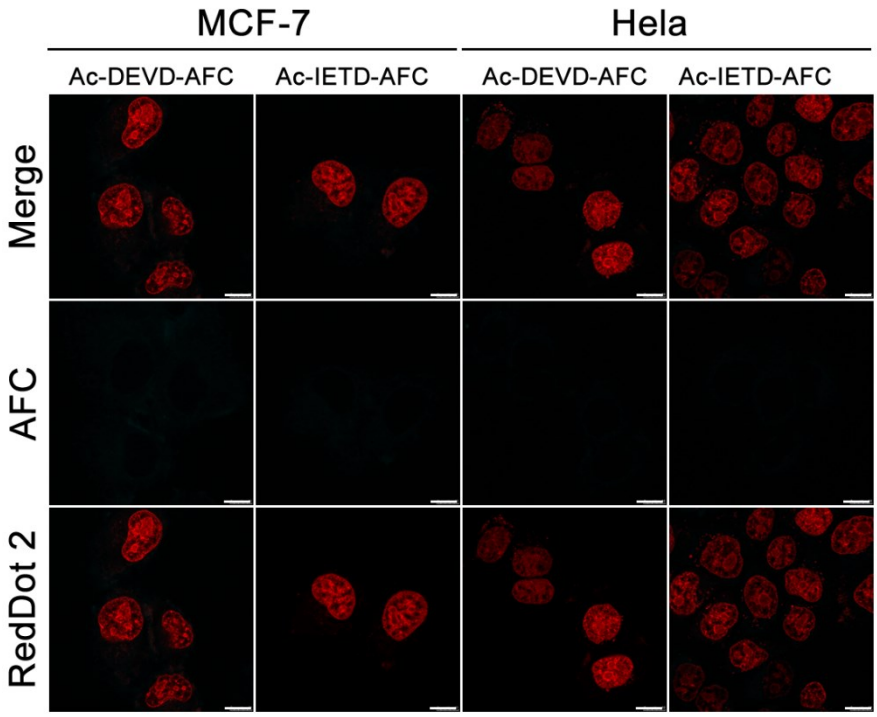

Figure S13. Confocal images of MCF-7 and HeLa cells incubated with 50 μmol/L Ac-DEVD-AFC or Ac-IETD-AFC for 4 h and further treated with H_2O_2 (1.0 mmol/L). AFC: λ_{ex} = 458nm, λ_{em} = 492nm, RedDot 2: λ_{ex} =561nm, λ_{em} = 700nm. Scale bar: 10 μm.

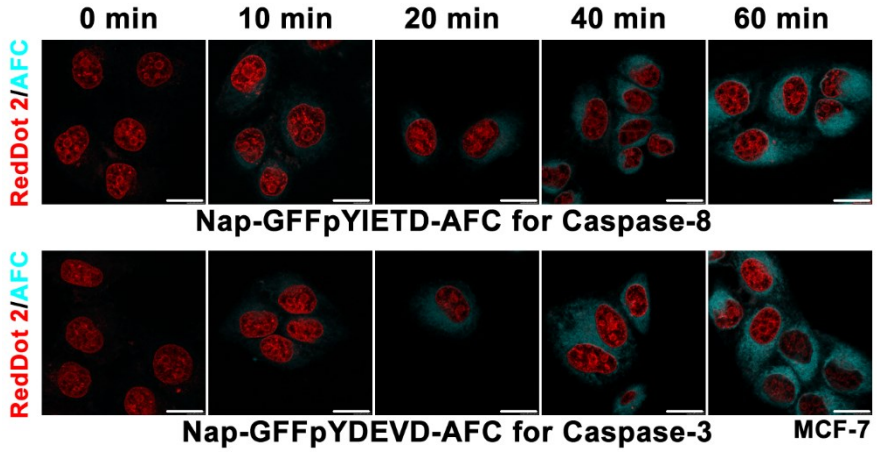

Figure S14. Confocal images of MCF-7 cells incubated with Probe Nap-GFFpYIETD-AFC (50 μmol/L) and Nap-GFFpYDEVD-AFC (50 μmol/L) for 4 h and further treated with H_2O_2 (1 mmol/L) at different times. AFC: λ_{ex} =458nm, λ_{em} = 492nm, RedDot 2: λ_{ex} =561nm, λ_{em} = 700nm. Scale bar: 20 μm.

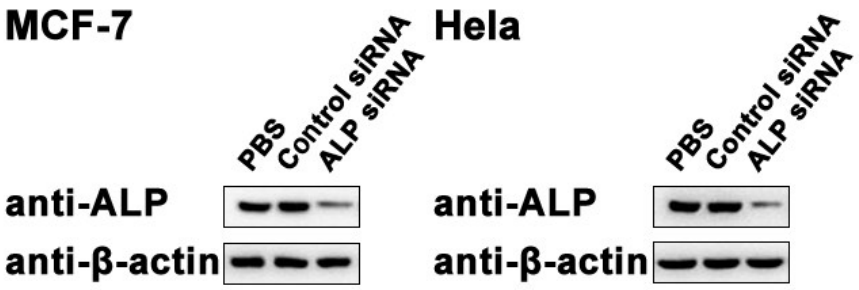

Figure S15. Western Blotting analyses of the expression of the ALP protein in MCF-7 and Hela cells. Lane: PBS, Control siRNA, ALP siRNA.

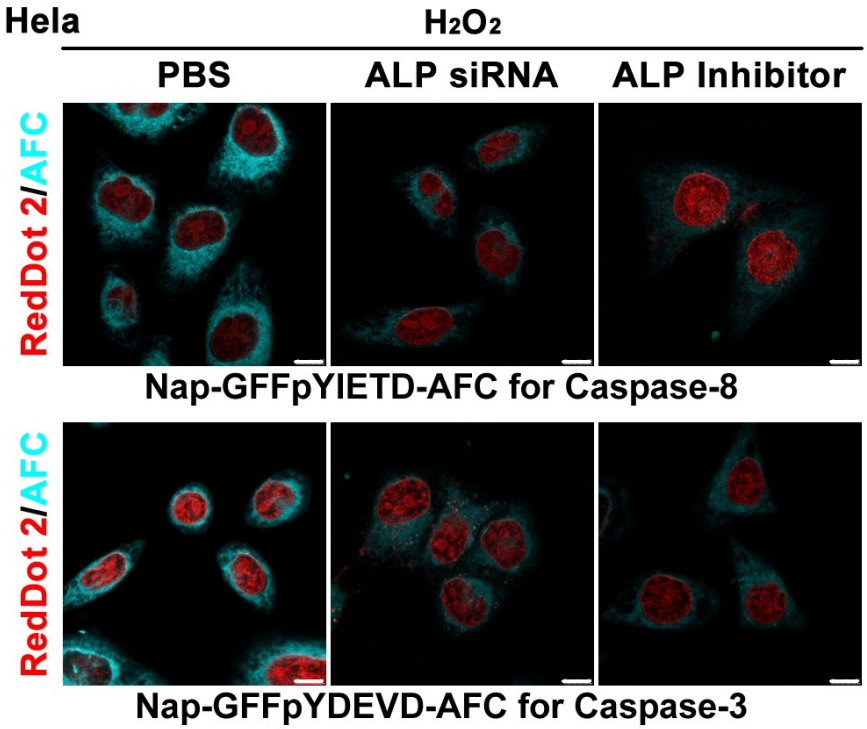

Figure S16. Confocal image of Hela cells incubated with ALP siRNA or inhibitor. Hela cells transfected with ALP siRNA or pre-treated with phosphatase inhibitor (50 μmol/L) were incubated with self-assembled probes for 4 h, and then apoptosis was induced with H_2O_2 (1 mmol/L). AFC: λ_{ex} = 458 nm, λ_{em} = 492 nm, RedDot 2: λ_{ex} = 561 nm, λ_{em} = 700 nm. Scale bar: 20 μm.

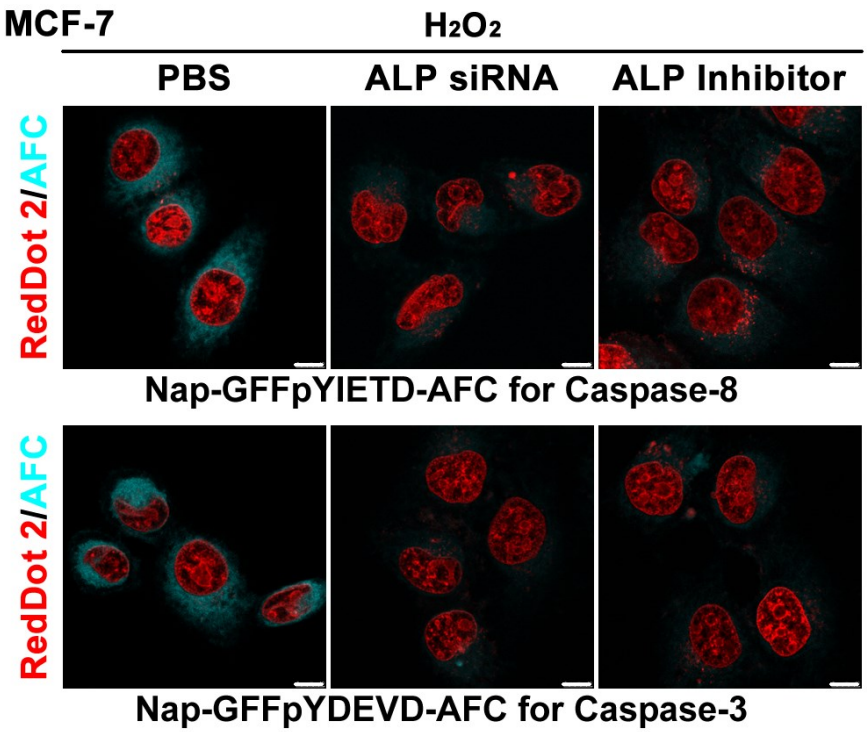

Figure S17. Confocal image of MCF-7 cells incubated with ALP siRNA or inhibitor. MCF-7 cells transfected with ALP siRNA or pre-treated with phosphatase inhibitor (50 μmol/L) were incubated with self-assembled probes for 4 h, and then apoptosis was induced with H_2O_2 (1 mmol/L). AFC: λ_{ex} = 458 nm, λ_{em} = 492 nm, RedDot 2: λ_{ex} = 561 nm, λ_{em} = 700 nm. Scale bar: 20 μm.

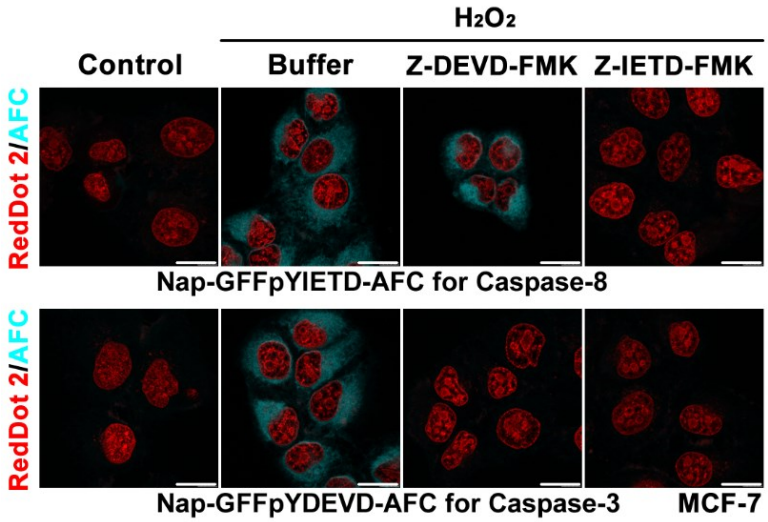

Figure S18. Confocal images of MCF-7 cells incubated with Nap-GFFpYIETD-AFC (50 μmol/L) or Nap-GFFpYDEVD-AFC (50 μmol/L) and Caspase-3/Caspase-8 inhibitors for 2 h with or without further treatment by H_2O_2 (1.0 mmol/L). AFC: λ_{ex} = 458nm, λ_{em} = 492nm, RedDot 2: λ_{ex} =561nm, λ_{em} = 700nm. Scale bar: 20 μm.

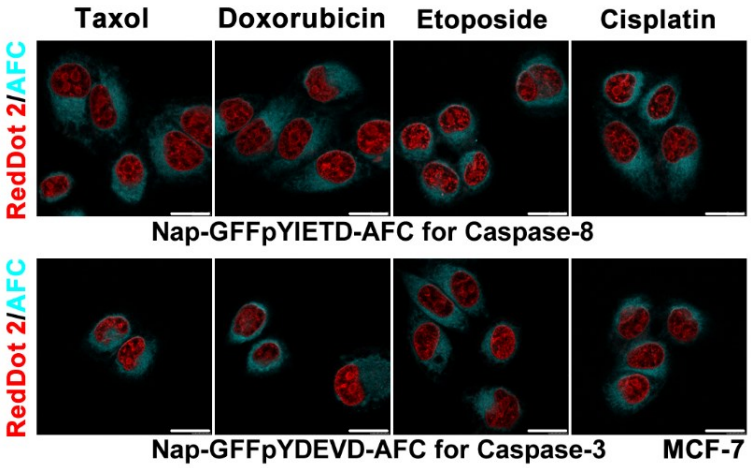

Figure S19. MCF-7 cells were co-incubated with Nap-GFFpYIETD-AFC (50 μmol/L) or Nap-GFFpYDEVD-AFC (50 μmol/L) and four commonly used anticancer drugs, Taxol (5 μmol/L), Doxorubicin (5 μmol/L), Etoposide (20 μmol/L) and Cisplatin (10 μmol/L), the fluorescence of AFC was observed. AFC: λ_{ex} = 458nm, λ_{em} = 492nm, RedDot 2: λ_{ex} =561nm, λ_{em} = 700nm. Scale bar: 20 μm.