Electronic Supplementary Information

FITC-quencher based, caspase 3-Activatable nanoprobes for effectively sensing caspase 3 in vitro and in cells

Anming Tang,[a] Bin Mei,[a] Weijuan Wang,[a] Wanglai Hu,[b] Fang Li,[a] Jun Zhou,[a] Qing Yang,[a] Hua Cui,[a] Mian Wu,[b] and Gaolin Liang*[a]

[a] CAS Key Laboratory of Soft Matter Chemistry, Department of Chemistry, University of Science and Technology of China, 96 Jinzhai Road, Hefei, Anhui 230026, China

[b] School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China

***Corresponding author:**

Gaolin Liang, Ph. D.,

Department of Chemistry

University of Science and Technology of China

96 Jinzhai Road, Hefei, Anhui 230026

P. R. China

Tel.: +86-551-63607935

Fax: +86-551-63600730

E-mail: gliang@ustc.edu.cn

Homepage: http://lianglab.ustc.edu.cn

Supplementary Methods

General methods. Synthesis and characterization are in the online Supplementary information. All the starting materials were obtained from Adamas or Sangon Biotech. Commercially available reagents were used without further purification, unless noted otherwise. All other chemicals were reagent grade or better. Active recombinant mouse CASP3 was obtained from BioVision as lyophilized powder (~300,000 U/mg, 1 U corresponds to the amount of enzyme which cleaves 1 nmol of the caspase substrate DEVD-pNA per hour at 37 ℃ in a reaction solution containing 50 mM Hepes, pH 7.2, 50 mM NaCl, 0.1% Chaps, 10 mM EDTA, 5% Glycerol, and 10 mM TCEP). All the enzymatic studies were performed in CASP3 reaction solution described above. HPLC analyses were performed on an Agilent 1200 HPLC system equipped with a G1322A pump and an in-line diode array UV detector using an Angilent Zorbax 300SD-C_{18} RP column with CH_3OH (0.1% of TFA) and water (0.1% of TFA) as the eluent. ^1H NMR spectra were obtained on a 300 MHz Bruker AV 300. MALDI-TOF/TOF mass spectra were obtained on a time-of-flight Ultrflex II mass spectrometer (Bruker Daltonics). UV-vis absorbance spectra were recorded on a U-2910 UV-visible spectrophotometer (Hitachi High-Techonologies Corporation, Japan) at room temperature. Time-dependent fluorescence emissions for caspase-3 digestion of the two nanoprobes were recorded on a F-2500 fluorescence spectrophotometer (Hitachi High-Techonologies Corporation, Japan) with excitation wavelengths set to 465 nm. Temperature of the detector cell was fixed at 37 °C. Transmission electron micrographs (TEM) were obtained on a JEOL 2100 high resolution transmission electron microscope, operating at 200 kV. The samples were prepared as following: a copper grid coated with carbon was dipped into the suspension and placed into a culture dish. Water was removed from the frozen specimen by a lyophilizer. Fluorescence microscopic images were taken on a IX71 fluorescence microscope (Olympus, Japan) equipped with a FITC filter. 254 nm UV irradiation used for inducing apoptosis was monitored by a UV radiometer (Everfine Photo-E-Info, Hangzhou, China).

Cell culture. The hepatocellular carcinoma HepG2 cells were obtained from Department of Biochemistry and Molecular Biology, Anhui Medical University. HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, Thermo Fisher Scienfitic, United States) supplemented with 10% fetal bovine serum (FBS) (Sijiqing Biological Engineering Materials, Hangzhou, China) and 5% CO_2 in humid atomosphere at 37 °C.

Enzymatic study. Stock solution of nanoprobe **1** at 4.25 nM in CASP3 buffer (50 mM Hepes, 50 mM NaCl, 0.1% Chaps, 10 mM EDTA, 5% Glycerol, and 10 mM TCEP, pH 7.2) was treated with 0.425 $pmol \cdot U^{-1}$ of CASP3 at 37 °C. The time-dependent fluorescence (FL) enhancement of the reaction mixture was monitored by a F-2500 fluorescence spectrophotometer. The solution was excited at 465 nm. Stock solution of nanoprobe **2** at 0.26 μM in CASP3 buffer (50 mM Hepes, 50 mM NaCl, 0.1% Chaps, 10 mM EDTA, 5% Glycerol, and 10 mM TCEP, pH 7.2) was treated with 5.2 $pmol \cdot U^{-1}$ of CASP3 at 37 °C. The time-dependent fluorescence (FL) enhancement of the reaction mixture was monitored by a F-2500 fluorescence spectrophotometer. The solution was excited at 465 nm.

Quenching efficiency of nanoprobe 1 and nanoprobe 2. The amount of FITC released by CASP3 cleavage in enzymatic study of nanoprobe **1** and nanoprobe **2** were calculated according to calibration curve in **Figure S7B**. Quenching efficiency (E_q (%)) was obtained following equation below:

$$E_q\ (\%) = \{1-F_0*R_c/[F_n-F_0*(1-R_c)]\}*100,$$

where F_0 and F_n are fluorescence intensity at the starting time point and the ending time point of Casp3 activity study respectively, R_c is the ratio of FITC released by nanoprobe **1** or nanoprobe **2** at the ending time point in enzymatic study of Casp3 (**Figure 4**).

Fluorescence enhancement studies of nanoprobes 1 and 2 in cell lysate. HepG2 cells were cultured in DMEM supplemented with 10% FBS at 37 °C. 6×10^6 cells were exposed to a 254 nm UV irradiation at a dosage of 10000 μJ·cm^{-2}, incubated for 60 min at 37 °C. After that, the cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer. 4.25 nM of nanoprobe **1** or 0.26 μM of nanoprobe **2** was incubated with the lysate at 37 °C. Time course of fluorescence emissions were recorded on a F-2500 fluorescence spectrophotometer.

Live cell imaging using nanoprobe 2. For UV-induce group, HepG2 cells were cultured in DMEM without FBS at 37 °C for 4 h. Then the cells were incubated with nanoprobe **2** (25 μM in DMEM) for 30 min at 37 °C and washed three times with phosphate-buffered saline (PBS). After that, the cells were exposed to a 254 nm UV irradiation at a dosage of 10000 μJ·cm^{-2} at room temperature. After that, the cells were cultured in fresh DMEM supplemented with 10% FBS at 37 °C and time-coursely imaged under a fluorescence microscopy. For inhibitor group, after 30 min incubation of the cells with nanoprobe **2**, we added 100 μM of Ac-DEVD-CHO (a commercial caspase-3/-7 inhibitor) into the culture medium for another 30 min before UV irradiation. For negative control group, neither inhibitor nor UV irradiation was involved. The cells were imaged under a fluorescence microscope equipped with a GEFP filter. The exposure time was 100 ms.

Western blot analysis. Western blot was performed as described by Mei et al.[1, 2] HepG2 cells were treated with PBS buffer (0.2 M, 1 μL) (Negative Control, NC), nanoprobe **2** (25 μM) for 30 min, UVC (254 nm) at 10000 μJ·cm^{-2}, nanoprobe **2** (25μM) for 30 min then UVC (254 nm) at 10000 μJ·cm^{-2} or camptothecin (CPT, 10μM) for 6 h. The following antibodies were used from the indicated sources: the primary antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (FL-335, Santa Cruz) is a rabbit polyclonal antibody raised against amino acids 1-335 representing full length GAPDH of human origin, primary antibody against poly ADP-ribose polymerase (PARP) (F-2, Santa Cruz) is a mouse monoclonal antibody raised against amino acids 764-1014 mapping at the C-terminus of PARP of human origin, primary antibody against Caspase-3 (pAb, Stressgen) is a rabbit polyclonal antibody against recombinant human Caspase-3 protein (full length), Alkaline phosphatase (AP)-conjugated secondary antibodies against mouse and rabbit IgG (Promega). Blots were developed by Thermo Western Blot stabilized substrate and images of the blots were obtained using Image Quant LAS-4000 (Fujifilm, Japan).

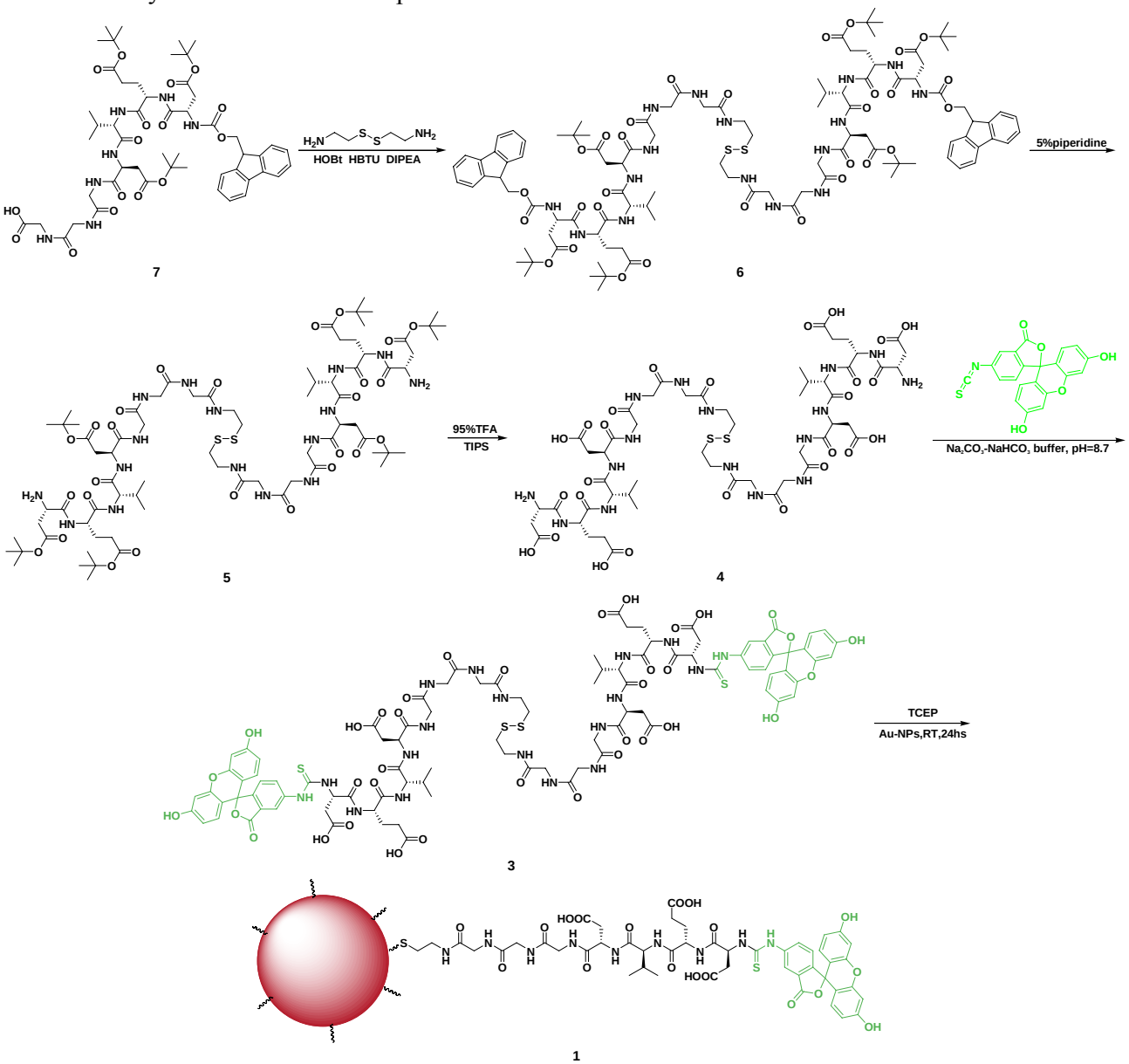
Synthesis and Characterizations

The preparations of nanoprobes **1** and **2** were described as below:
Fmoc-Asp(OtBu)-Glu(OtBu)-Val-Asp(OtBu)-Gly-Gly-Gly-OH **7** was synthesized following the literature method (Bernadett Bacsa & C Oliver Kappe. Rapid solid-phase synthesis of a calmodulin-binding peptide using controlled microwave irradiation. *Nature Protocols*, **2007**, Vol.2 No.9, 2222-2227). Au NP (Φ=16nm) was synthesized following the literature method (G. Frens. Controlled Nucleation for Regulation of Particle-Size in Monodisperse Gold Suspensions. *Nature Physical*

Science, **1973**, Vol.241, 20-22).

*Preparation of FITC-DEVDGGG-AuNP (**1**)*:

Scheme S1. Synthetic route for nanoprobe **1**.



*Synthesis of Cystamine-[Gly-Gly-Gly-Asp(OtBu)-Val-Glu(OtBu)-Asp(OtBu)(Fmoc)]$_2$ (**6**)*: Compound **7** (140.1mg, 0.135 mmol), HBTU (56.5mg, 0.149 mmol), and HOBt (19.1 mg, 0.141 mmol) in DMF (3 mL) were stirred for 0.5 h in presence of DIPEA (92.8 mg, 0.718 mmol).Then cystamine dihydrochloride(13.1mg, 0.058 mmol) was added and stirred for 3 hs at room temperature. The pure product of **6** (89.0 mg, yield: 70%) was obtained after HPLC purification. MS: calc. M^+ = 2191.01, obsvd. ESI-MS: m/z 1096.64, $[(M+2H)^{2+}/2]$.

*Synthesis of Cystamine-[Gly-Gly-Gly-Asp(OtBu)-Val-Glu(OtBu)-Asp(OtBu)]$_2$ (**5**)*: The Fmoc protecting group of **6** was cleaved with 5% piperidine in DMF for 0.5 h at room temperature to yield compound **5** in good yield (59.0 mg, yield: 83%) after HPLC purification. MS: calc. M^+ = 1746.88, obsvd. ESI-MS:

m/z 1747.70, $[(M+H)^+]$.

*Synthesis of Cystamine-(Gly-Gly-Gly-Asp-Val-Glu-Asp)$_2$ (**4**)*: The OtBu protecting group of **5** was cleaved with 95% TFA in DCM for 3 h at room temperature to yield compound **4** (22.8 mg, yield: 47.8%) after HPLC purification. MS: calc. M^+ = 1410.50, obsvd. ESI-MS: m/z 1411.3 $[(M+H)^+]$.

*Synthesis of Cystamine-(Gly-Gly-Gly-Asp-Val-Glu-Asp-FITC)$_2$ (**3**)*: Compound **4** (8.5 mg, 0.006mmol) was solved in Na_2CO_3-$NaHCO_3$ buffer (2mL, pH 9), Fluorescein Isothiocyanate (FITC, isomer I, 9.2mg, 0.023mmol) was solved in DMSO (400 µL), and added into the buffer solution dropwise, then stirred for 3 h at room temperature. Pure compound **3** (8.0 mg, yield: 61%) was obtained after HPLC purification. ^{1}HNMR of compound **3** (300 MHz, DMSO-d_6, δ, ppm) (Figure S1): 10.26-10.44 (s, 2H), 8.38-8.44 (s, 2H), 8.27-8.38 (t, 3H), 8.12-8.23 (t, 2H), 8.01-8.12 (m, 6H), 7.93-8.01 (t, 2H), 7.63-7.83 (t, 4H), 7.26-7.37 (s, 1H), 7.07-7.23 (t, 2H), 6.65-6.77 (t, 4H), 6.48-6.65 (m, 8H), 5.15-5.25 (d, 2H), 4.47-4.62 (d, 4H), 4.36-4.44 (d, 2H), 3.71-3.79 (s, 12H), 3.63-3.72 (d, 8H), 3.27-3.43 (d, 6H), 3.14-3.19 (s, 2H), 2.74-2.85 (m, 8H), 2.23-2.37 (m, 4H), 1.88-2.06 (m, 4H), 1.77-1.86 (m, 2H), 1.26-1.34 (d, 2H), 0.68-0.91 (t, 12H). MS: calc. for $C_{94}H_{105}N_{18}O_{36}S_4$ $[M+H^+]^+$ = 2189.5816. obsvd. HR-MALDI-MS: m/z 2189.5743.

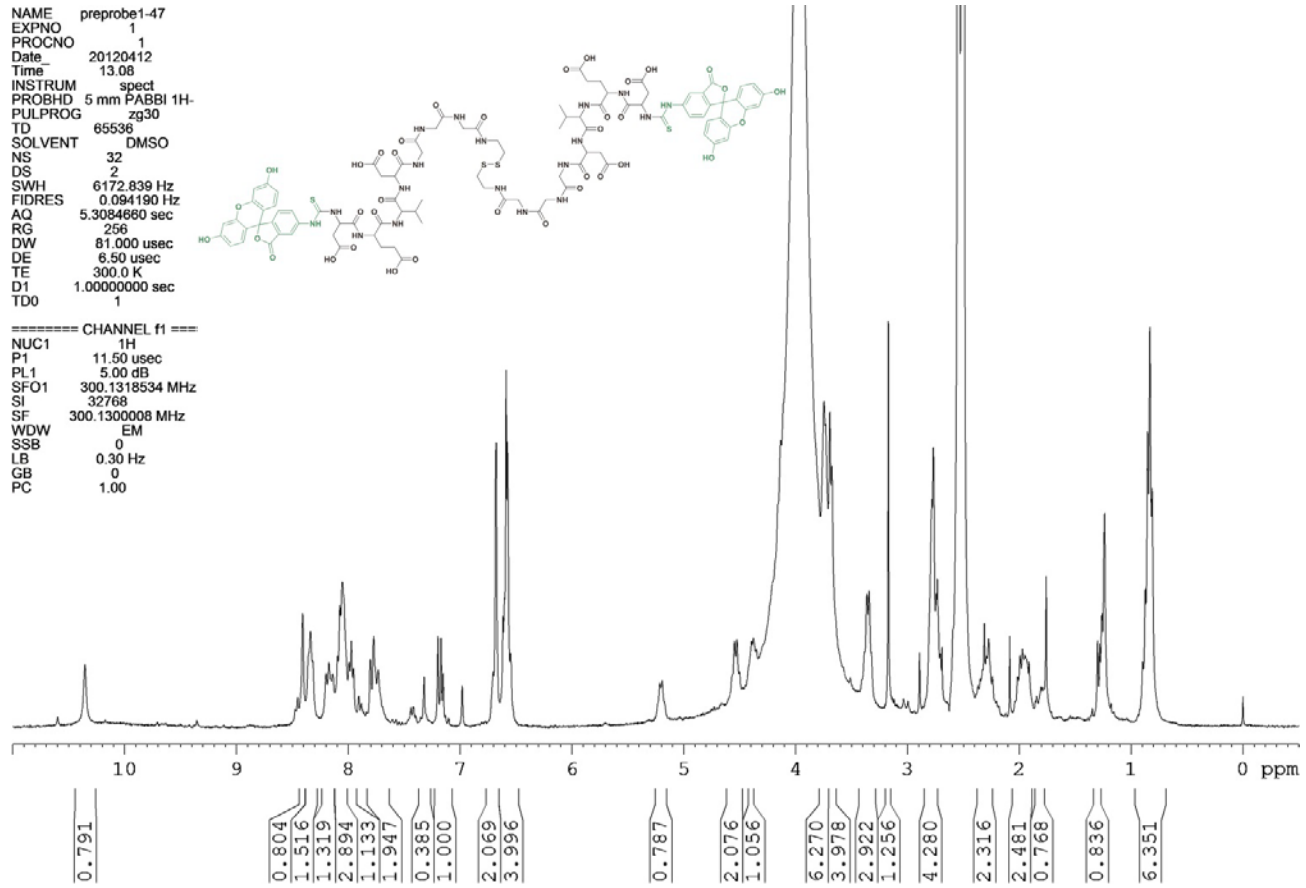


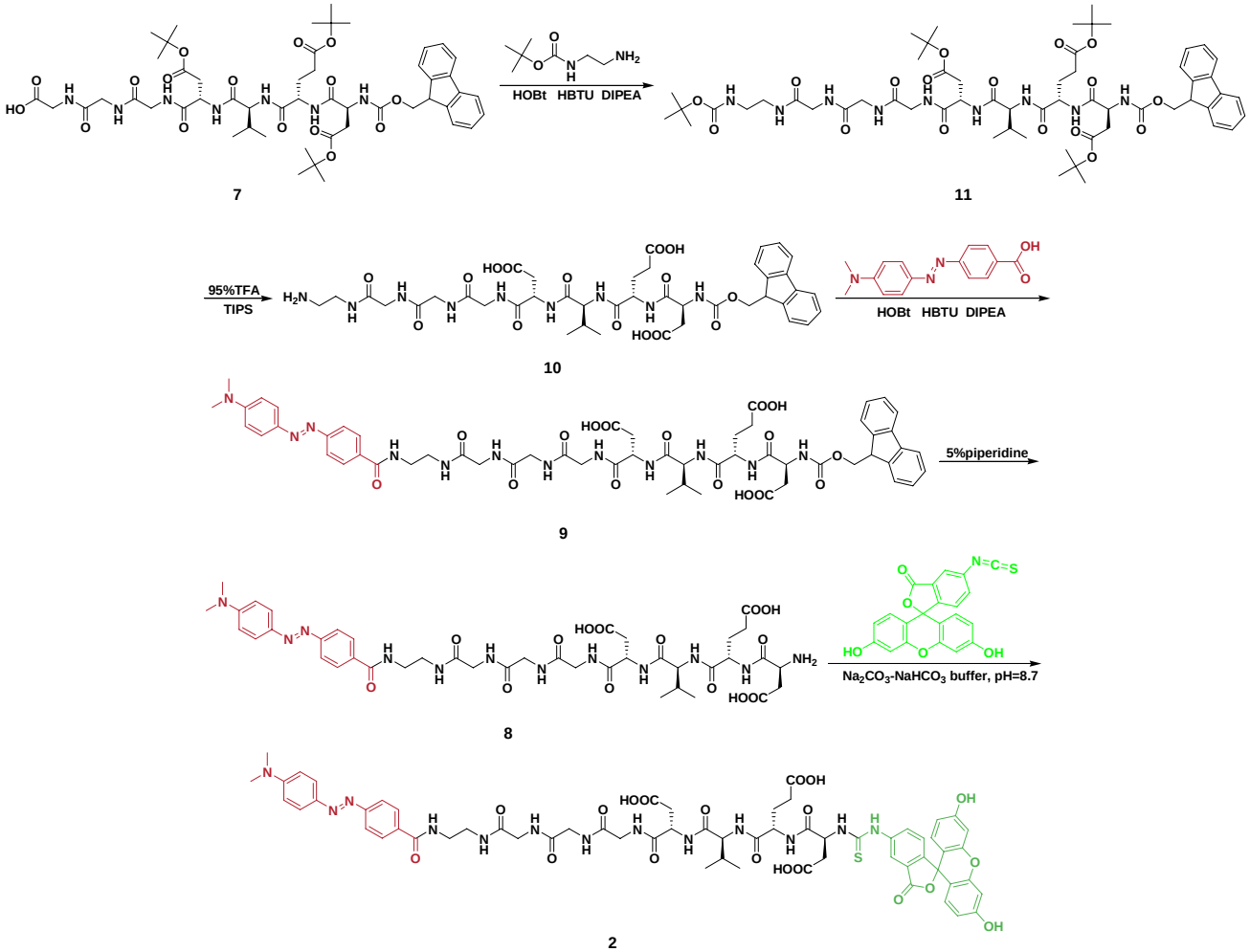
Figure S1. ^{1}H-NMR spectrum of **3**.

*Preparation of FITC-DEVDGGG-AuNP (**1**):* Compound **3** (0.30 mg, 0.14µmol) was disolved in 0.6mL DMSO, then diluted with 1 mL ultrapure water. 5 µL of above solution was diluted to 0.1 mL then 2 µL

of the solution was added with 10 μL TCEP at 6.27 μ M. The mixture was then added into 5 mL AuNPs solution (Φ=16nm, 1.7 nM in sodium citrate). Then the reaction mixture was stirred for 3 h and stored in refrigerator overnight. The above reaction mixture was centrifuged at 12500 r·min^{-1} for 5 min. After that, the precipitates were collected and dispersed in 0.5 mL ultrapure water and centrifuged. This washing step was repeated triply before the nanoprobe **1** was dispersed in Casp3 buffer for use.

Preparation of FITC-DEVDGGG-EDA-DABCYL (**2**):

Scheme S2. Synthetic route for nanoprobe **2**.



Synthesis of Fmoc-Asp(OtBu)-Glu(OtBu)-Val-Asp(OtBu)-Gly-Gly-Gly-EDA-Boc ***(11)***: Compound **7** (62.9 mg, 0.06 mmol), HBTU (23.6 mg, 0.06 mmol), and HOBt (8.2 mg, 0.06 mmol) in DMF (3 mL) were stirred for 0.5 h in presence of DIPEA (8.2mg, 0.06 mmol).Then N-Boc-Ethylenediamine [Boc-EDA] (8.0 mg, 0.05 mmol) was added and stirred for 3 hs at room temperature. The pure product of **11** (49.8 mg, Yield: 84%) was obtained after HPLC purification. MS: calc. M^+ = 1179.6, obsvd. ESI MS: m/z 1179.3 [M^+].

Synthesis of Fmoc-Asp-Glu-Val-Asp-Gly-Gly-Gly-EDA ***(10)***: The Boc and OtBu protecting groups of **11** were cleaved with 95% TFA in DCM for 3 hs. Compound **10** was obtained after HPLC purification (37.5 mg. Yield: 97%). MS: calc. M^+ = 911.4, obsvd. ESI MS: m/z 912.2 [$(M+H^+)^+$].

*Synthesis of Fmoc-Asp-Glu-Val-Asp-Gly-Gly-Gly-EDA-DABCYL (**9**)*: 4-((4-(dimethylamino)phenyl)azo) benzoic acid [DABCYL] (13.6 mg, 0.050 mmol), HBTU (18.6 mg, 0.049 mmol), and HOBt (7.6 mg, 0.056 mmol) in DMF (3 mL) were stirred for 0.5 h in presence of DIPEA (6.38 mg, 0.049 mmol). Then Compound **10** (37.5mg, 0.041 mmol) was added and stirred for 3 h at room temperature. The pure product of **9** (38.8 mg, yield: 81%) was obtained after HPLC purification. MS: calc. M^+ = 1162.5, obsvd. ESI MS: m/z 1163.2 $[(M+H)^+]$.

*Synthesis of Asp-Glu-Val-Asp-Gly-Gly-Gly-EDA-DABCYL (**8**)*: The Fmoc protecting group of **9** was cleaved with 5% piperidine in DMF for 20 mins at room temperature to yield compound **8** (21.8 mg, yield: 69%) after HPLC purification. MS: calc. M^+ = 940.4, obsvd. ESI-MS: m/z 939.3, $[(M-H^+)^-]$.

*Synthesis of FITC-DEVDGGG-EDA-DABCYL (**2**)*: Compound **8** (18.4 mg, 0.020 mmol) was disolved in Na_2CO_3-$NaHCO_3$ buffer (3 mL, pH 8.7), Fluorescein Isothiocyanate (FITC, isomer I, 9.14 mg, 0.023 mmol) was disolved in DMSO (300 µL), then added into the buffer solution dropwise. The reaction mixture was then stirred for 3 h at room temperature. Pure compound **2** (21.2 mg, Yield: 80%) was obtained after HPLC purification. ^{1}HNMR of compound **2** (300 MHz, DMSO-d_6, δ, ppm) (Figure S2): 10.29-10.46 (s, 1H), 9.94-10.27 (s, 1H), 8.56-8.66 (t, 1H), 8.38-8.44 (s, 1H), 8.29-8.38 (t, 2H), 8.16-8.23 (d, 1H), 8.04-8.12 (m, 2H), 7.96-8.02 (t, 2H), 7.92-7.96 (s, 1H), 7.79-7.86 (t, 4H), 7.70-7.78 (d, 1H), 7.12-7.23 (t, 1H), 6.81-6.88 (d, 2H), 6.65-6.72 (s, 2H), 6.58-6.63 (d, 2H), 6.52-6.58 (m, 2H), 5.16-5.26 (m, 1H), 4.50-4.59 (m, 1H), 4.34-4.43 (m, 1H), 4.10-4.17 (t, 1H), 3.74-3.77 (s, 4H), 3.69-3.73 (d, 6H), 3.31-3.39 (t, 2H), 3.22-3.31 (t, 2H), 2.98-3.16 (s, 6H), 2.75-2.84 (m, 3H), 2.69-2.74 (d, 1H), 2.53-2.61 (m, 1H), 2.20-2.37 (m, 2H), 1.88-2.03 (m, 2H), 1.72-1.85 (m, 1H), 1.25-1.31 (m, 1H), 0.73-0.94 (t, 6H). MS: calc. for $C_{62}H_{67}N_{13}O_{19}S$ $[M+H^+]^+$ = 1330.4470. obsvd. HR-MALDI-MS: m/z 1330.4397.

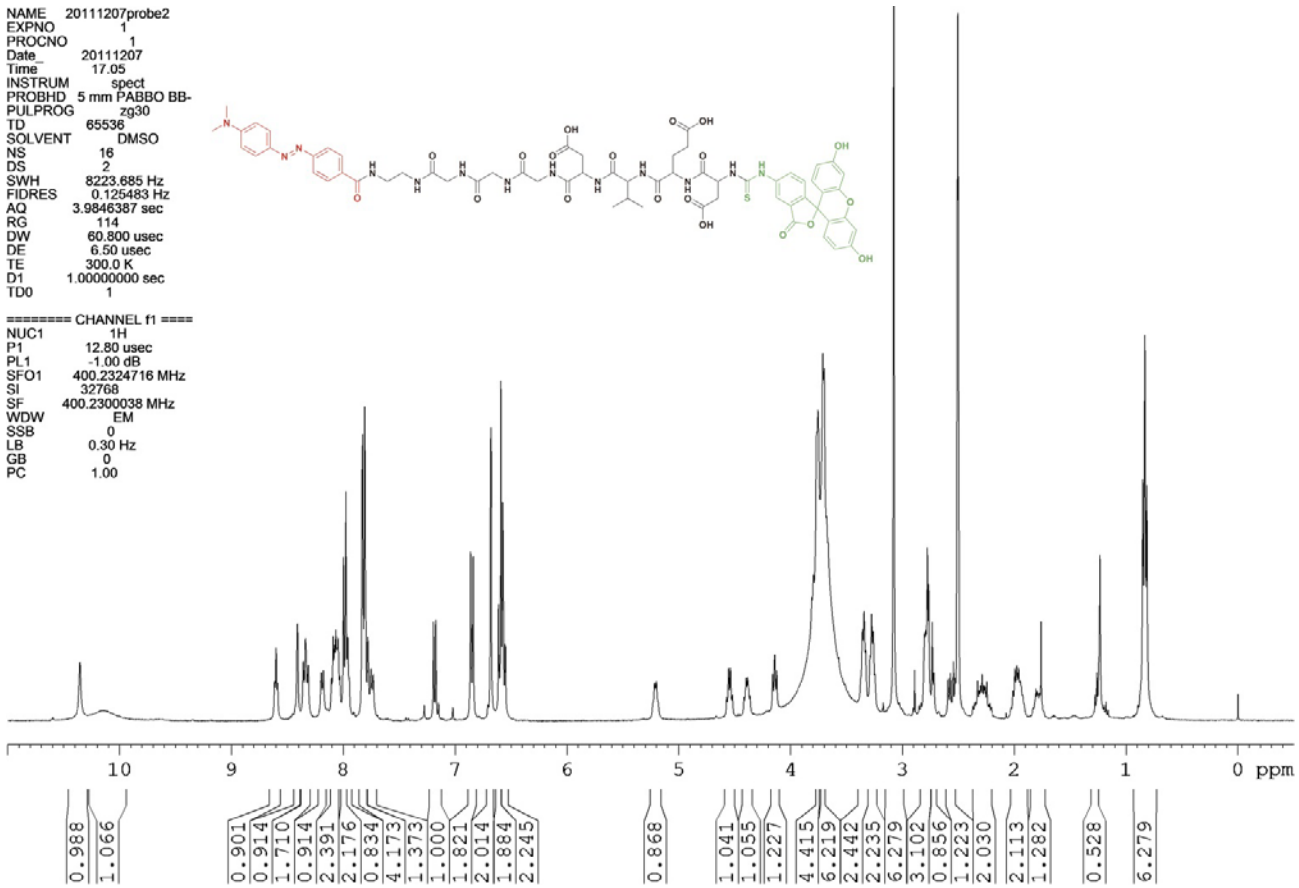


Figure S2. ^{1}H-NMR spectrum of **2**.

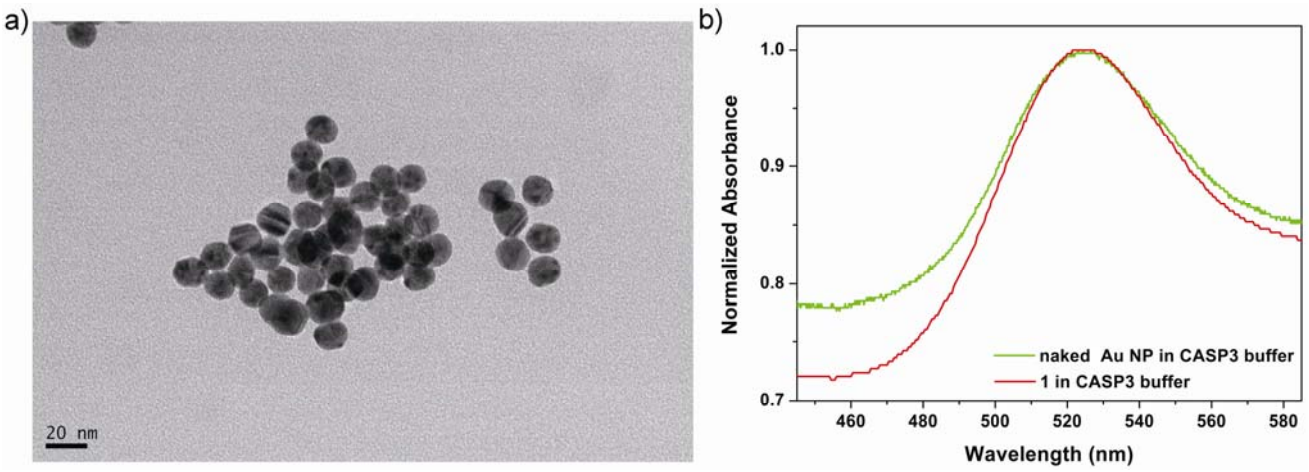


Figure S3. (A) TEM image of naked AuNPs in Casp3 buffer. (B) UV-vis absorbance spectra of naked AuNPs (green) and nanoprobe **1** (red) in Casp3 buffer.

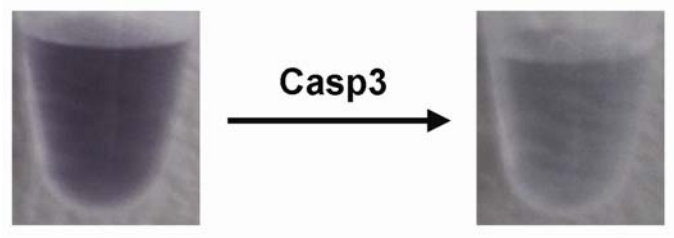


Figure S4. Optical images of nanoprobe **1** before and after Casp3 cleavage.

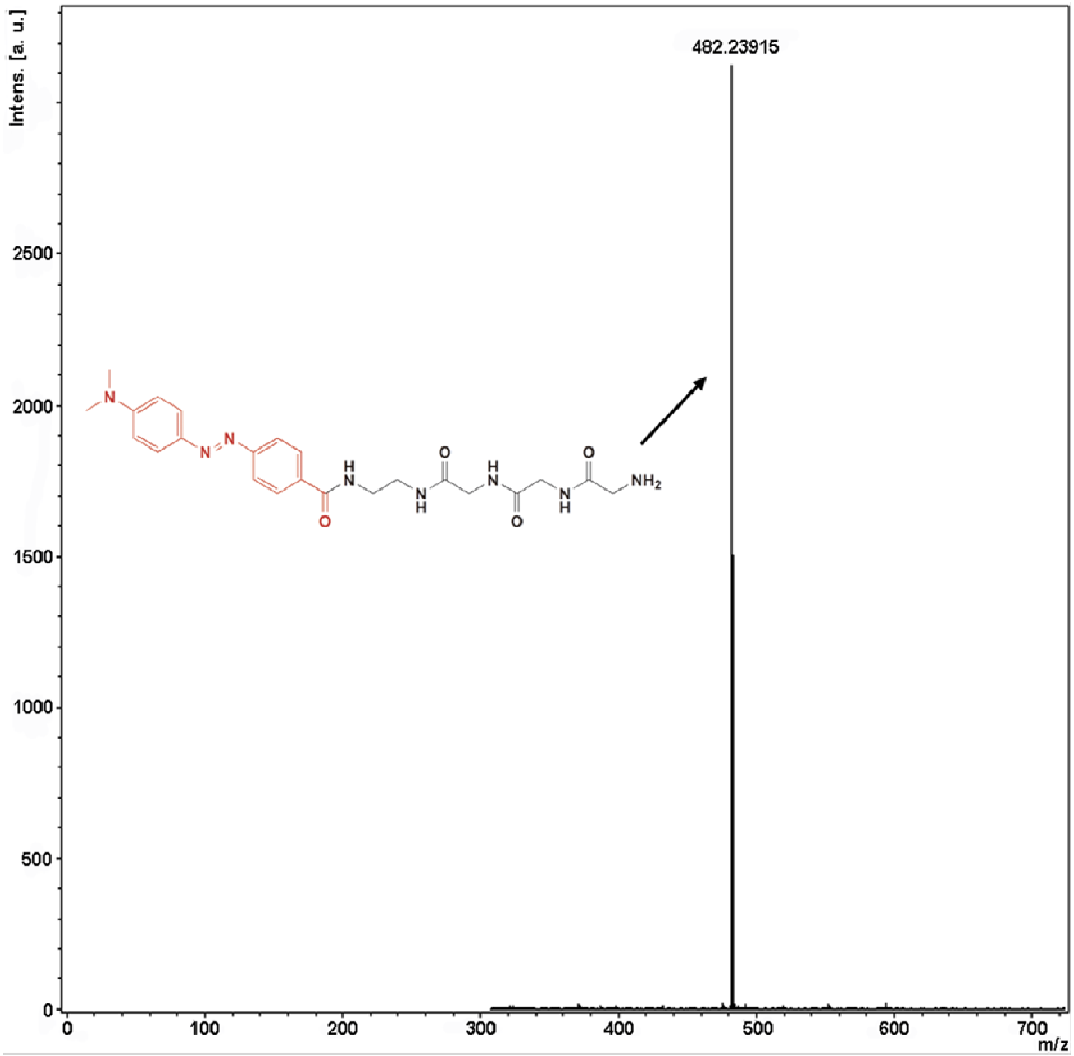


Figure S5. MALDI mass spectrum of HPLC peak **3** in Figure 3B.

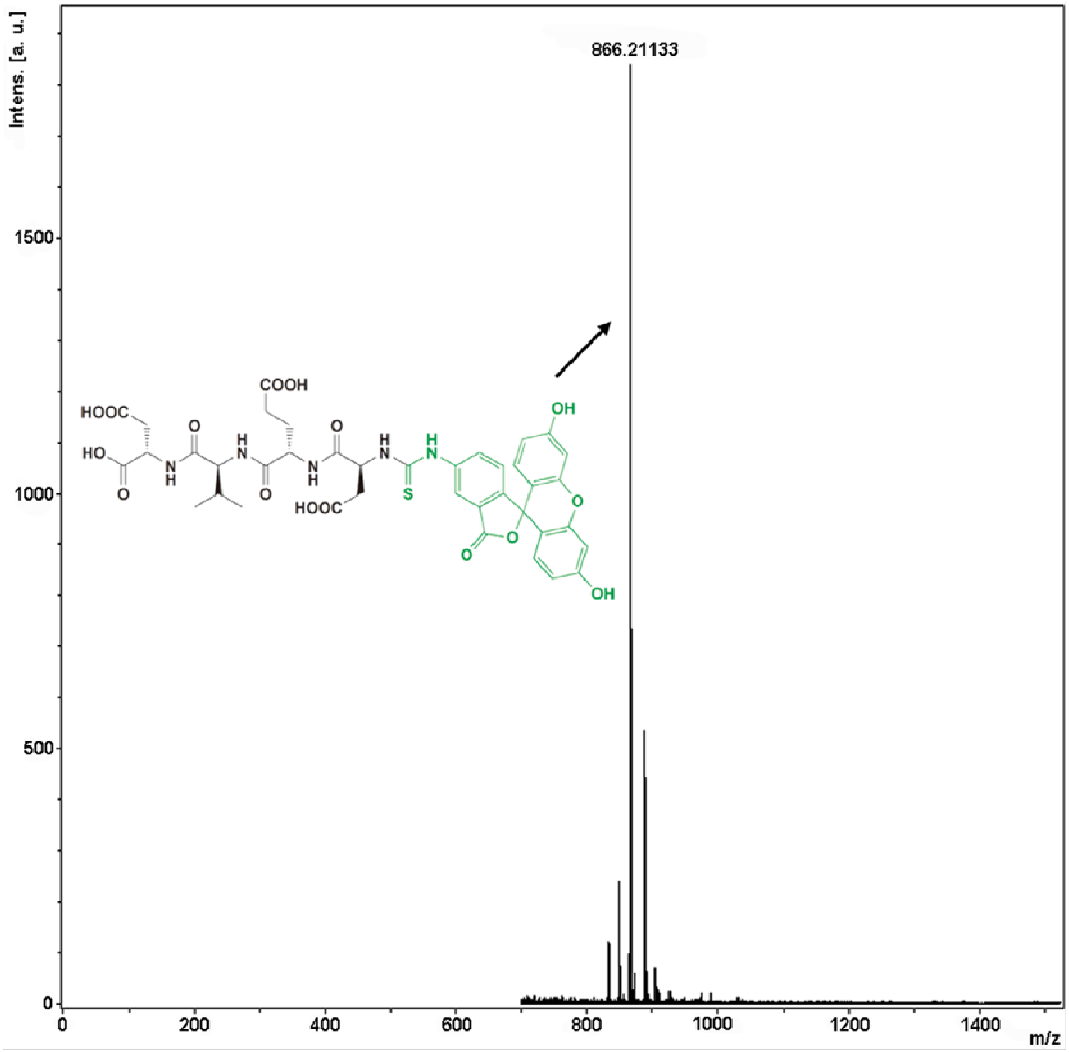


Figure S6. MALDI mass spectrum of HPLC peak **4** in Figure 3B.

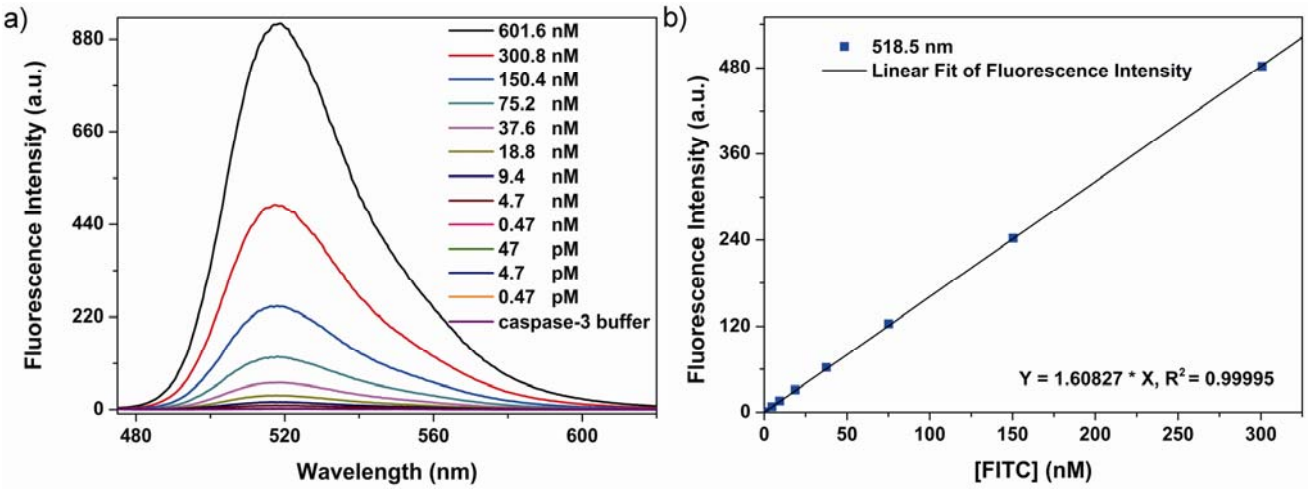


Figure S7. (A) Fluorescence emission spectra of different concentrations of free FITC in Casp3 buffer at 37 °C. Wavelength for excitation: 465 nm. (B) Calibration curve of Fluorescence Intensity at 518.5 nm versus concentration of FITC in A. Linear range of concentration of FITC: 0-300 nM.

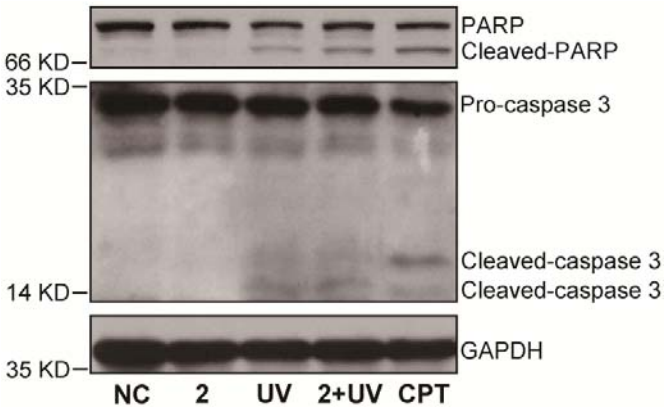


Figure S8. Western blot analyses. Western blot analysis of expression of Casp3 and PARP in HepG2 cells incubated with DMEM containg 1% DMSO (Negative Control, **NC**), nanoprobe **2** (25 μM) for 30 min, UVC (254 nm) at 10000 μJ·cm^{-2}, nanoprobe **2** (25μM) for 30 min then UVC (254 nm) at 10000 μJ·cm^{-2}, or camptothecin (CPT, 10μM) for 6 h at 37 °C.

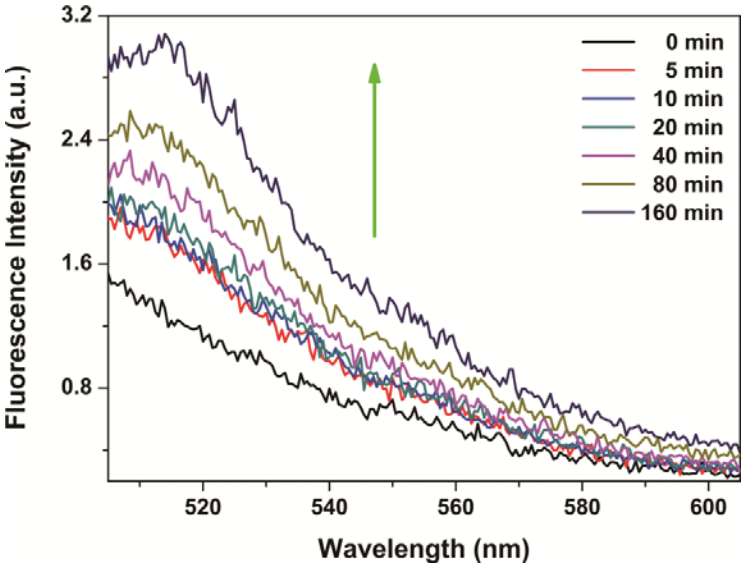


Figure S9. Time course of fluorescence emission spectra of 4.25 nM of nanoprobe **1** incubated with UV-induced apoptotic HepG2 cell lysate at 37 °C. Wavelength for excitation: 465 nm.

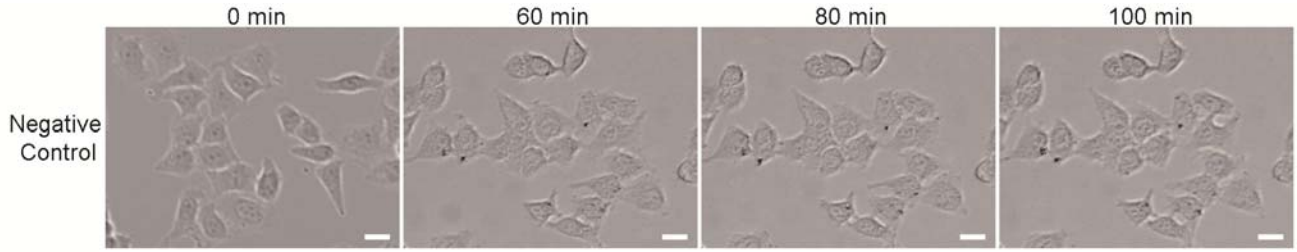


Figure S10. Real-time merged microscopic images (white field and EGFP channel) of HepG2 cells treated after incubation with 25 μM of nanoprobe **2** for 30 min at 37 °C. Scale bar: 20 μm.

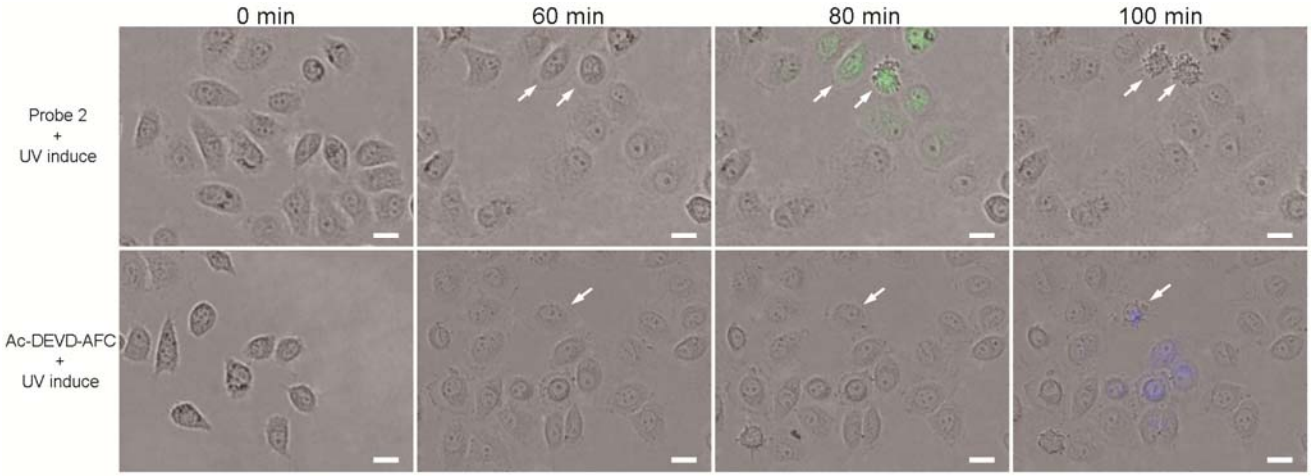


Figure S11. Real-time merged microscopic images of apoptotic HepG2 cells with nanoprobe **2** (white field and EGFP channel) or Ac-DEVD-AFC (white field and DAPI channel). Upper panel: HepG2 cells were incubated with 25 μM of nanoprobe **2** for 30 min at 37 °C, followed by 254 nm UV irradiation at 10000 $\mu J \cdot cm^{-2}$ prior to time course imaging. Lower panel: HepG2 cells were incubated with 25 μM of Ac-DEVD-AFC for 30 min, followed by 254 nm UV irradiation at 10000 $\mu J \cdot cm^{-2}$ prior to time course imaging. White arrows indicate those cells undergoing apoptosis with obvious morphological changes. Scale bar: 20 μm.

Supplementary Table S1. HPLC conditions for the purification of compound **3**.

Time (minute)	Flow (mL/min.)	H_2O %	CH_3CN %
0	3.0	95	5
3	3.0	95	5
39	3.0	40	60
42	3.0	40	60
43	3.0	95	5
45	3.0	95	5

Supplementary Table S2. HPLC condition for the purification of compound **2**.

Time (minute)	Flow (mL/min.)	H_2O %	CH_3CN %
0	7.0	80	20
3	7.0	80	20
35	7.0	20	80
37	7.0	20	80
38	7.0	80	20
40	7.0	80	20

Reference

1 Y. Mei, W. Du, Y. Yang and M. Wu, *Oncogene*, 2005, **24**, 7224-7237.
2 Y. Mei, C. Xie, W. Xie, X. Tian, M. Li and M. Wu, *Neoplasia*, 2007, **9**, 871-881.