Supporting Information

Aminopeptidase N based color-convertible fluorescent nano-probe for cancer diagnosis

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Experimental section

Materials: ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR) of PEG-PPa and PEG-RB was characterized by 400 MHz spectrometer (Varian), 600 MHz spectrometer (AVANCE NEO 700MHz, Bruker) and 700 MHz spectrometer (AVANCE NEO 700MHz, Bruker), respectively. Fluorescent spectra and quantum yield of the fluorescent molecules were employed using steady state and time-resolved photoluminescence spectroscopy (FLS1000). The size and morphology of nanoparticles were measured by transmission electron microscopy (TEM, Tecnai G2 Spirit Biotwin) and dynamic light scattering (DLS, Malvern Zetasizer Nano S apparatus). Ultraviolet-visible (UV-Vis) absorption spectrum was processed by Thermo Electron-EV300 UV-Vis spectrophotometer. Fluorescent spectra were characterized by steady-state & time-resolved fluorescence spectrofluorometer (USA/CAN). Confocal images were obtained by super-resolution multiphoton confocal microscope (TCS SP8 STED 3X, Leica). Liquid chromatography-mass spectrometry (LC-MS) was performed on a Water ACQUITY UPLC system equipped with a binary solvent delivery manager and a sample manager coupled to a Waters Q-TOF Premier Mass Spectrometer equipped with an electrospray interface (Waters Corporation, Milford, MA).

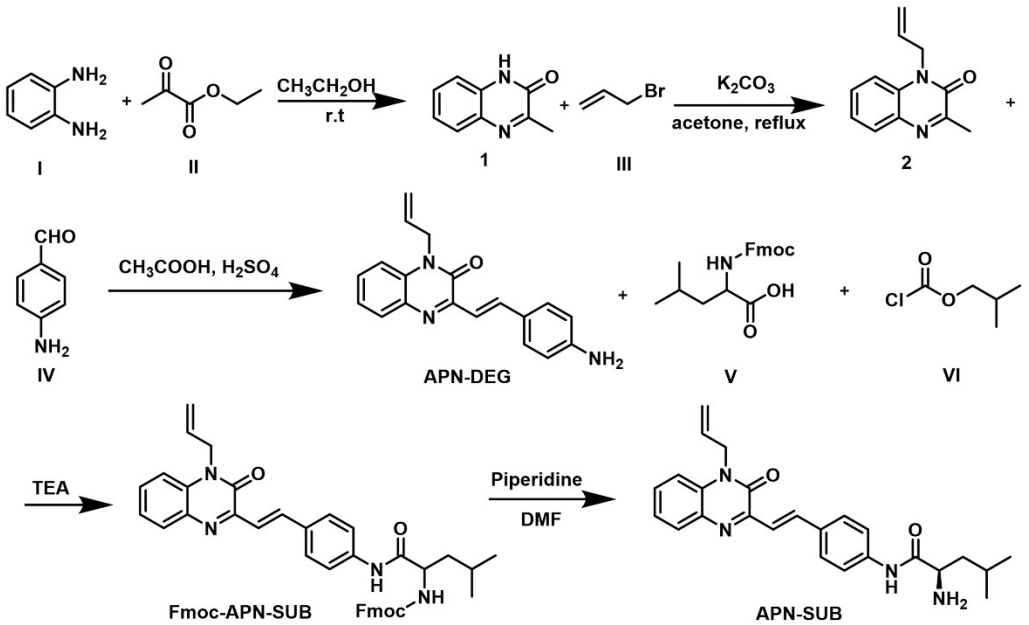
Synthesis of **1**: To a suspended solution (150 mL) of 1,2-phenylenediamine (0.1 mol, 10.81 g) in ethanol, the ethyl pyruvate (0.12 mol, 13.92 g) solution in ethanol (10 mL) was added slowly under stirring at ice bath. The solution was stirred at room temperature for 12 h. The resulting precipitate was filtered, washed with ethanol, and dried under vacuum to obtain the product **1** (14.76 g, yield: 91%) as a white powder.

Synthesis of **2:** To the suspended solution (50 mL) of compound **1** (10 mmol, 1.61 g) and K_2CO_3 (12 mmol, 1.66 g) in acetone, allyl bromide (12 mmol, 1.45 g) was added slowly and stirred in 62 ^{o}C for 12 h. When compound **1** was completely consumed, the solvent was concentrated under vacuum. The residue was dissolved in water and extracted with ethyl acetate (EA). The organic layer was dried with anhydrous $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash column chromatography (hexane:ethyl acetate = 10:1, v:v) to give compound **2** (1.72 g) as a light yellow powder, yield 86%.

Synthesis of **APN-DEG:** A solution of compound **2** (2.5 mmol, 0.51 g), 4-amino-benzaldehyde (3 mmol, 0.36 g) and H_2SO_4 (0.5 mL) in acetic acid (10 mL) was heated to 55 ^{o}C for 48 h. The solvent was removed in vacuo. The crude product was dissolved in water and extracted with EA. The organic layer was collected and the water phase was basified by K_2CO_3 and extracted by EA again. The combined organic phase was dried with anhydrous $MgSO_4$, and purified by flash column chromatography (PE:EA = 6:1) to obtain target compound **APN-DEG** (0**.**49 g) as red powder, yield 65%.

Synthesis of **APN-SUB:** Fmoc-L-Leucnie (0.52g, 1.74 mM) was dissolved in 10 mL anhydrous tetrahydrofuran solution, then the reaction mixture was stirred 2 h under ice-salt bath. Then APN-DEG (0.41 g, 1.32 mM) was added in the reaction mixture and stirred 6 h at room temperature. The crude reaction product was then purified through column chromatography to obtained pure Fmoc-APN-SUB (0.61 g, yield: 72%). To the solution of Fmoc-APN-SUB (0.5 g, 0.7 mM) in 5 mL DMF was added 1 mL piperidine. The mixture was stirred 5 h at room temperature. After the completion of reaction, the purified target product APN-SUB (yellow powder) was obtained by precipitation with water and ice-chilled diethyl ether (0.25 g, yield: 87%).

Synthesis of **PEG-NHNH$_2$:** mPEG5K-OH (5 g, 1mM) was dissolved in anhydrous methylene chloride (20 mL) and 4-nitrophenyl chloromate (0.605 g, 3 mM) was added. Then pyridine (0.4 g) was added slowly at 0 ℃. The reaction was processed for 12 h under nitrogen atmosphere at room temperature. Then hydrazine dissolved in DMF was added in the above reaction mixture and stirred continuously another 12 h at room temperature. The resulting PEG-$NHNH_2$ was purified by precipitation in ice-chilled diethyl ether. After dried under reduced pressure, PEG-$NHNH_2$ was characterized by 400 M nuclear magnetic resonance (NMR) spectroscopy. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.13 (s, 2H), 4.25 (s, 1H), 3.63 (s, 255H), 3.36 (s, 3H).



Scheme S1. Synthesis of fluorescent molecule APN-SUB.

Synthesis of **PEG-Hz1:** To the solution of PEG5K-$NHNH_2$ and 4-(2-hydroxyethyl) benzaldehyde in anhydrous methanol solution, was added anhydrous $MgSO_4$. Then, the mixture was reflux 24 h under nitrogen atmosphere. After the reaction was completed, the reaction solution was filtrated and the solvent was removed by reduced pressure to obtain the crude product. The crude product was purified by precipitation in ice-chilled diethyl ether. ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.90 (s, 1H), 7.85 (d, *J* = 8.7 Hz, 1H), 7.65 (d, *J* = 8.4 Hz, 1H), 7.10 (s, 1H), 7.04 (d, *J* = 8.7 Hz, 1H), 6.92 (d, *J* = 8.8 Hz, 1H), 4.19 – 4.17 (m, 1H), 4.13 – 4.10 (m, 1H), 4.02 – 4.00 (m, 1H), 3.98 – 3.96 (m, 1H), 3.64 (s, 486H), 3.38 (s, 3H).

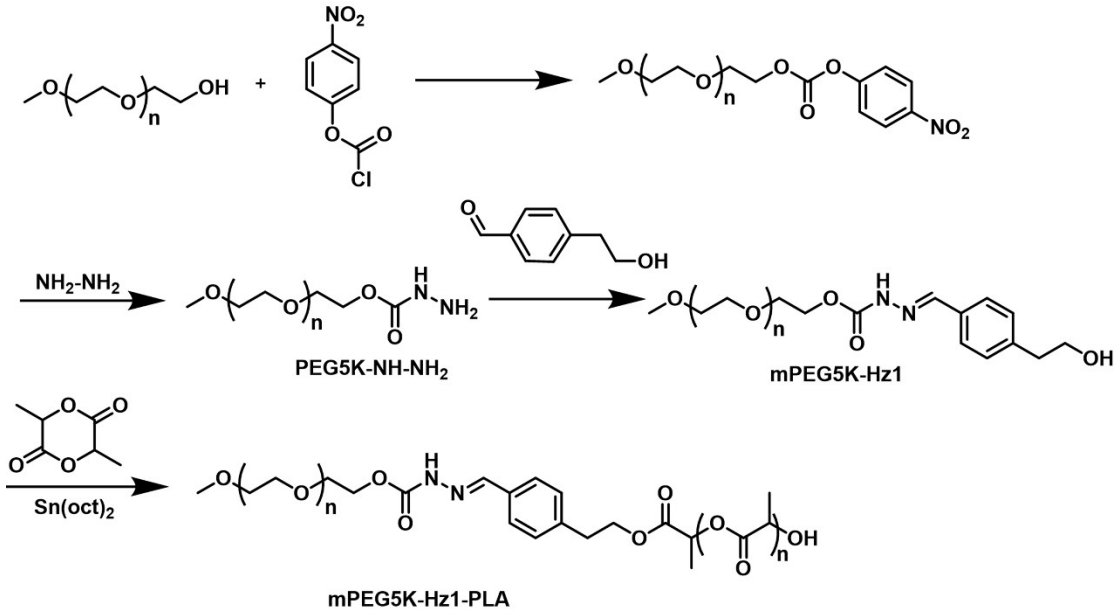
Synthesis of **PEG-Hz1-PLA:** L-lactide (3.21 g, 22 mmol) and mPEG-Hz1 (0.5 g, 0.1 mmol) were heated to 120 °C under nitrogen atmosphere for complete melting. Then stannous octoate in anhydrous toluene was added into the melting mixture with a monomer/catalyst molar ratio of 500:1 to initiate ring opening polymerization (ROP). Polymerization was carried under reflux condition at 120 °C under nitrogen atmosphere for 24 h. After the completion of the reaction, the crude product was cooled to room temperature, stirred with cold water to hydrolyze unreacted L-lactide monomers, and extracted with dichloromethane. The synthesized PEG-Hz1-PLA were purified by precipitation in cold diethyl ether and dried under vacuum.

$ONOO^-$ stock solutions were freshly prepared each time prior to usage. To a solution of 3 M NaOH that was cooled at 0 °C, 0.7 M H_2O_2, 0.6 M $NaNO_2$ and 0.6 M HCl were added simultaneously. The $ONOO^-$ solution was analyzed spectrophotometrically whereby the concentration of $ONOO^-$ was estimated through ε = 1670 ± 50 L/cm·M at 302 nm in 0.1 M NaOH (aq.). Hydrogen peroxide (H_2O_2) is commercially available whereby the concentration of H_2O_2 was determined through spectrophotometrical analysis with ε = 43.6 L/cm·M at 240 nm. Sodium hypochlorite (NaOCl) is commercially available whereby the concentration of ^-OCl was determined through spectrophotometrical analysis with ε = 250 L/cm·M at 292 nm. $^1O_2$ was generated by the reaction of H_2O_2 (1 mM) with NaClO (1 mM). In detail, H_2O_2 was slowly added to aq. NaOCl and stirred for 2 min.

Kinetic constant determination

All assays were performed in 96-well polystyrene flat-bottom microtiter plates. 10 μL of diluted protein solution (or buffer for the blank) was added to 190 μL of PBS buffer (pH 7.4, concentration of APN-SUB is 100 μM) to yield the appropriate protein concentration and incubated at 37 °C for 30 min in a 96-well plate. The fluorescent properties of different probes before and after the addition of APN were recorded.

Kinetic constants (Vmax and Km) were determined for APN-SUB. An enzyme concentration of 0.24 mU/ml and [S] final between 0.2 and 80 µM were used. The assays were performed in quadruplicate. Km and Vmax were calculated by nonlinear fitting of the Michaelis–Menten equation using the enzyme kinetic module of SigmaPlot 9.01 (Systat Software). The results are given as the averages ± standard deviations of at least three separate measurements.



Scheme S2. Synthesis of PEG-Hz1-PLA.

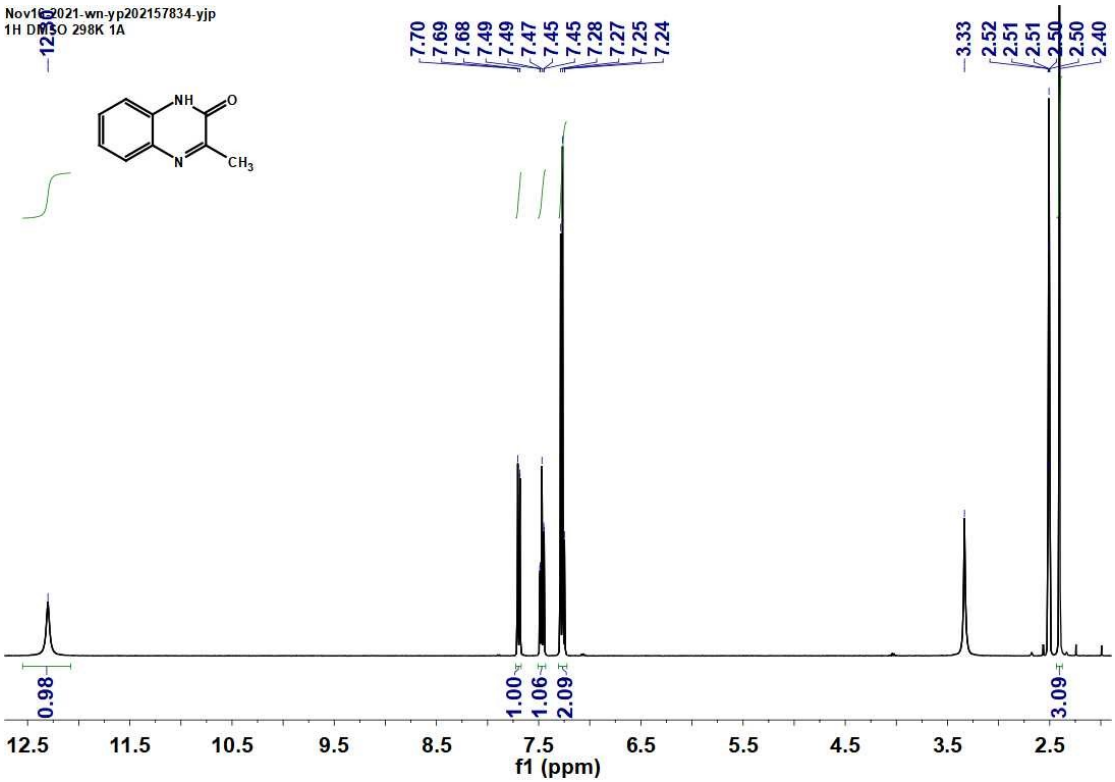


Figure S1. ^{1}H-NMR spectrum of mediate compound 1.

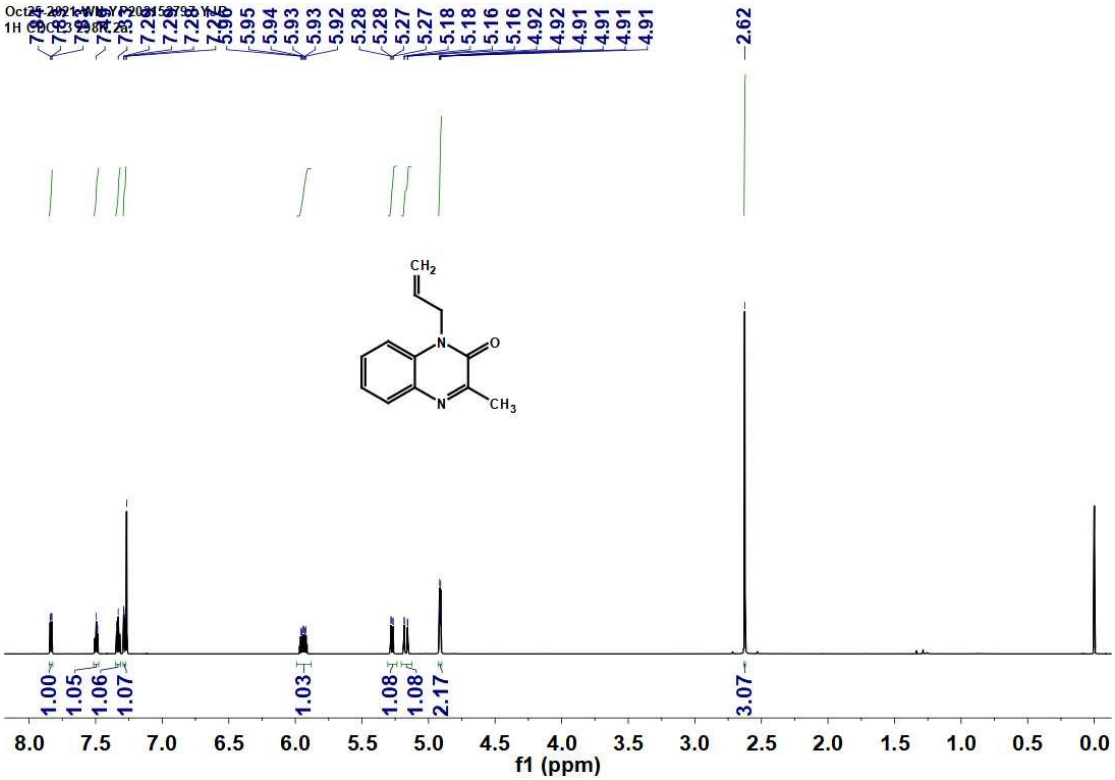


Figure S2. ^{1}H-NMR spectrum of mediate compound 2.

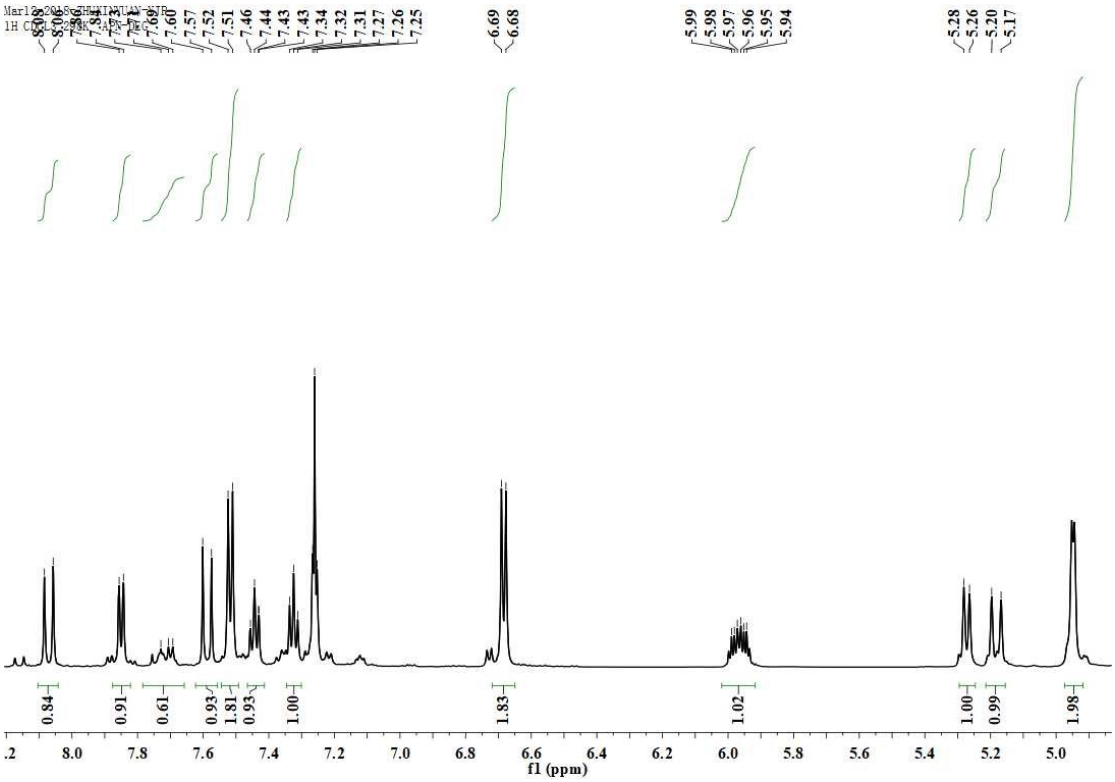


Figure S3. ^{1}H-NMR spectrum of APN-DEG.

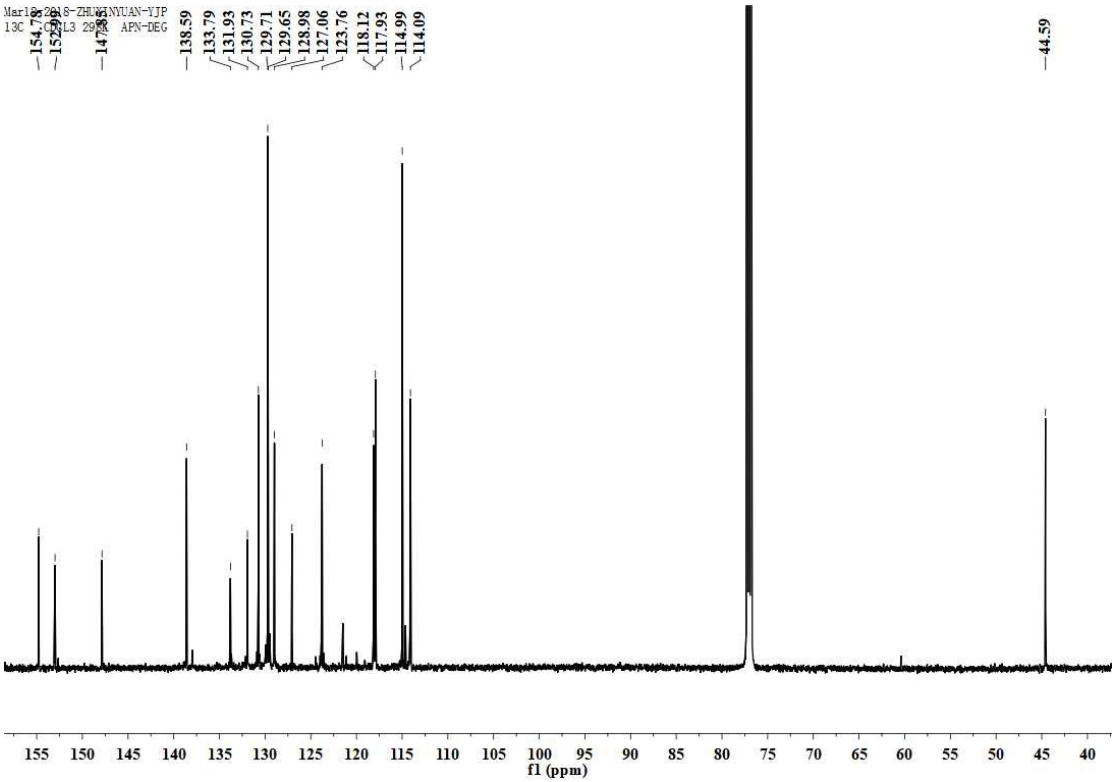


Figure S4. ^{13}C-NMR spectrum of APN-DEG in $CDCl_3$.

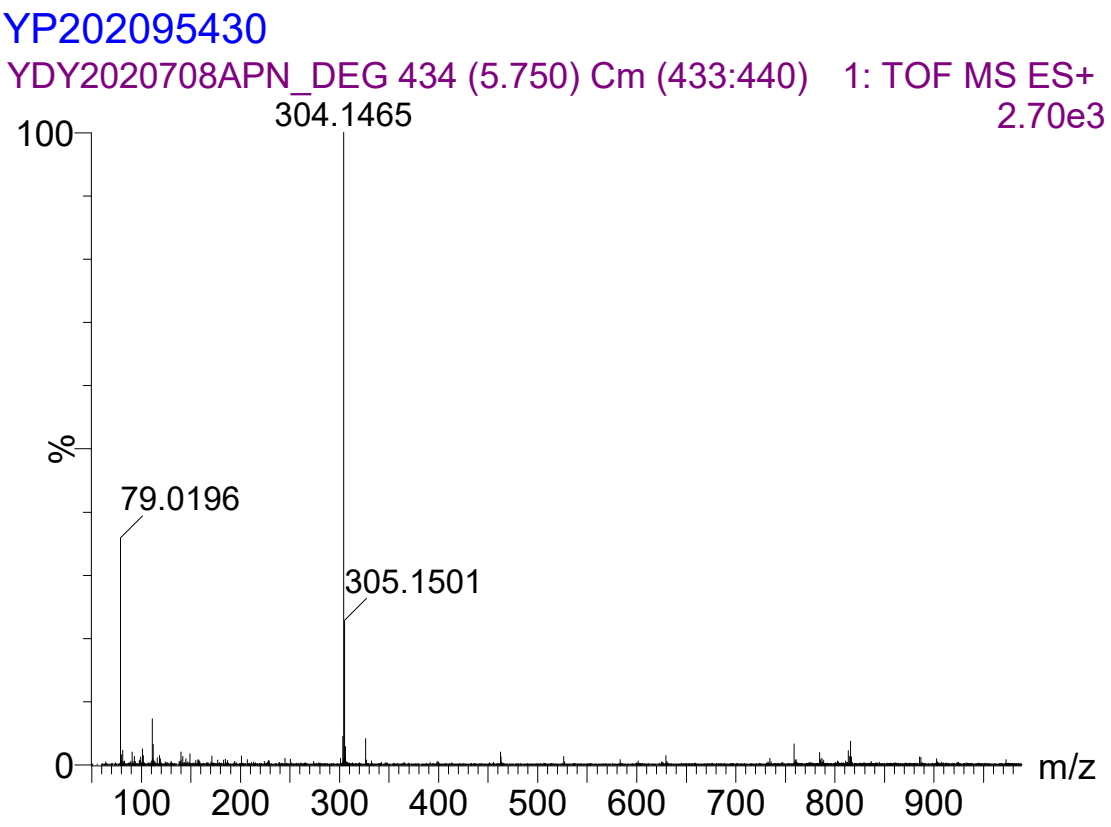


Figure S5. High resolution mass spectrum of APN-DEG.

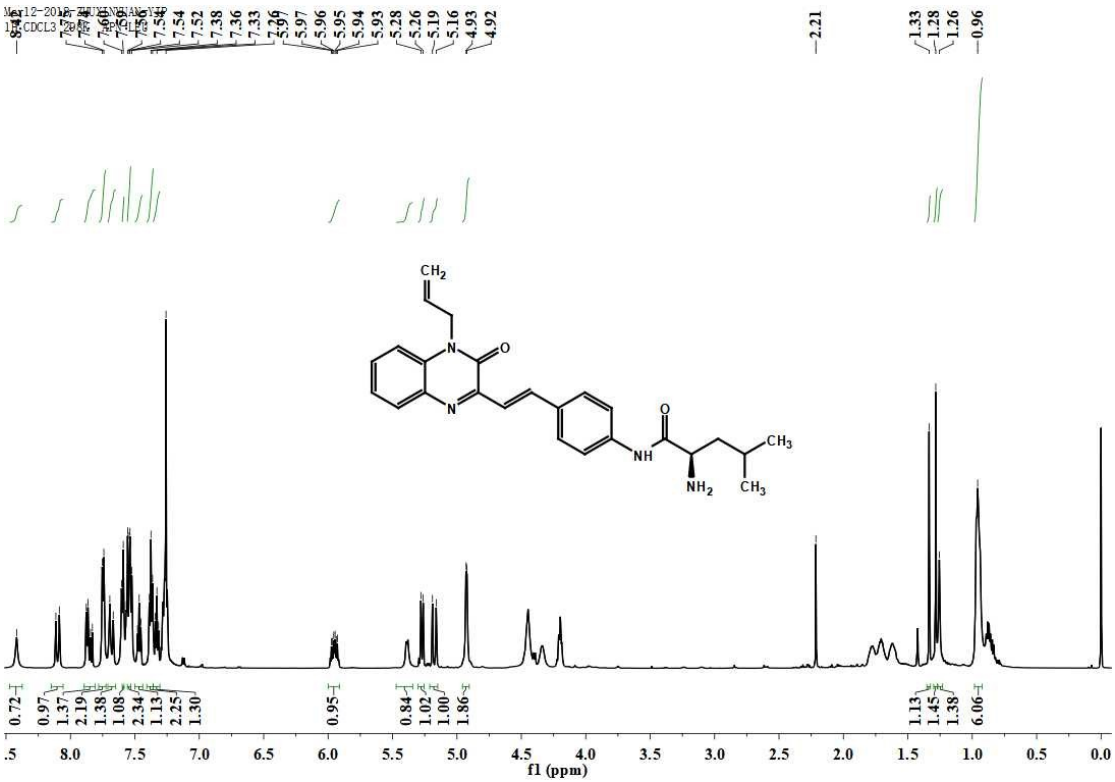


Figure S6. ^{1}H-NMR spectrum of APN-SUB.

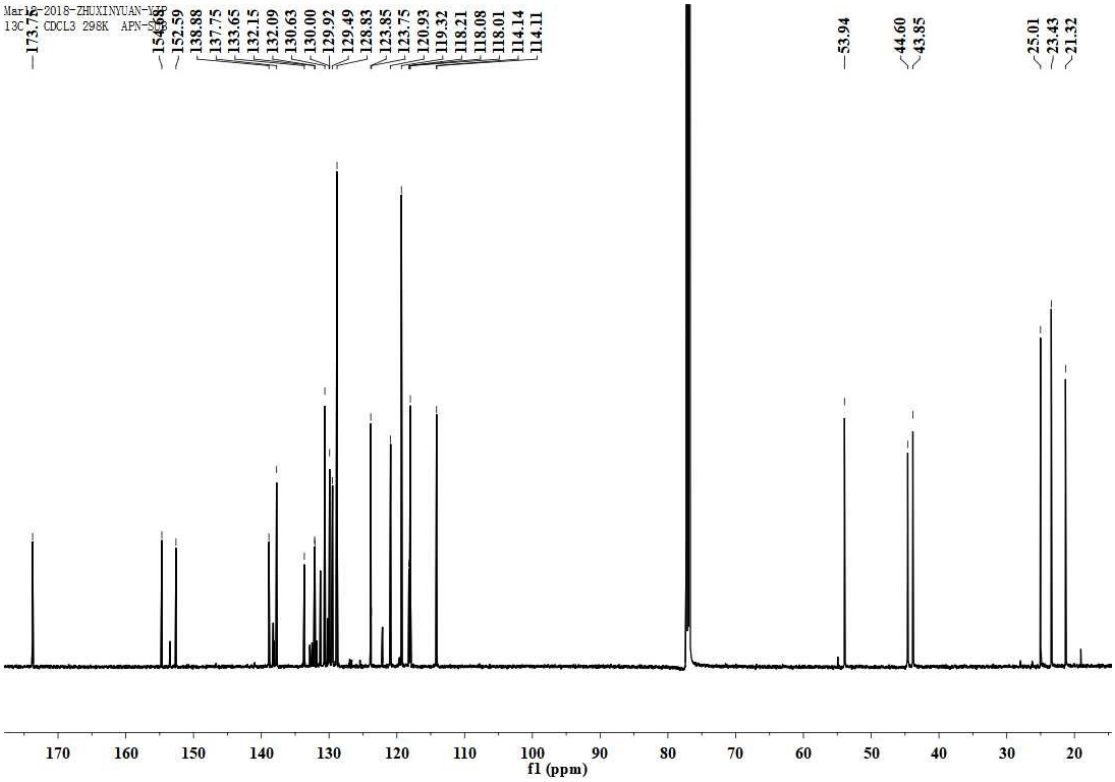


Figure S7. ^{13}C-NMR spectrum of APN-DEG in $CDCl_3$.

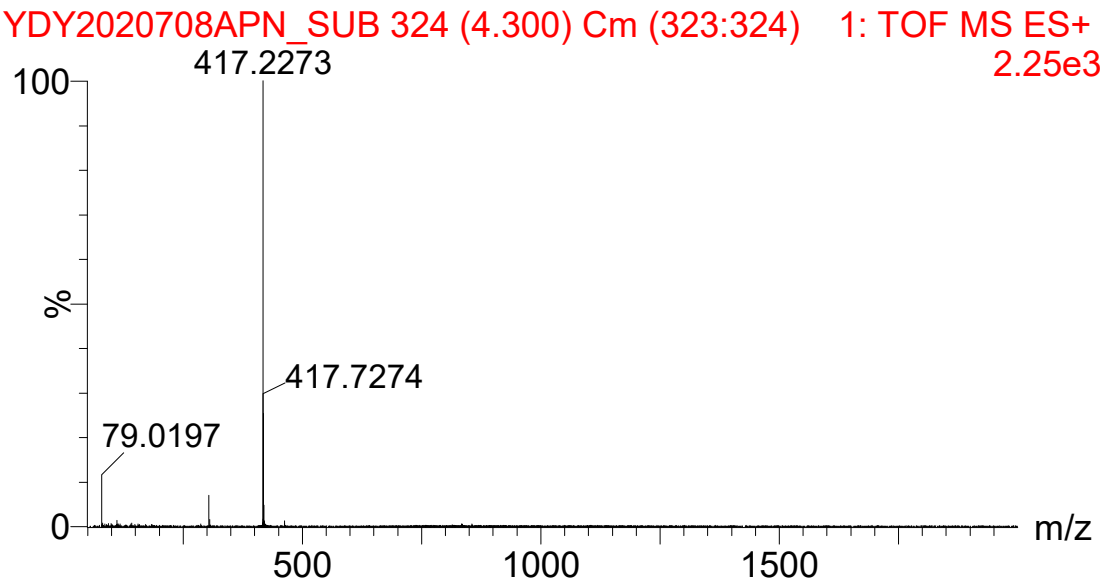


Figure S8. High resolution mass spectrum of APN-SUB.

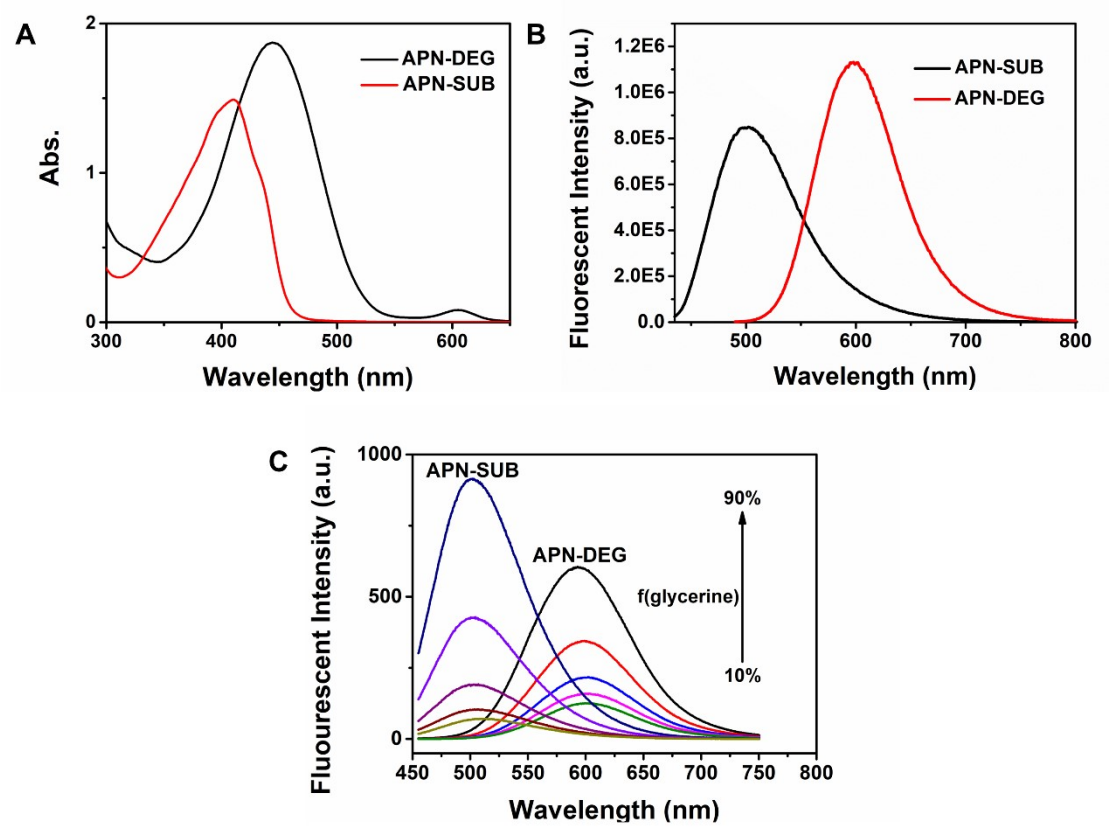


Figure S9. The absorption spectra and the fluorescent emission spectra of APN-SUB and APN-DEG. A) The absorption spectra of APN-DEG and APN-SUB in DMSO. B) The fluorescent spectra of APN-DEG and APN-SUB in DMSO. C) The fluorescent spectra of APN-DEG and APN-SUB in glycerine/methanol mixed solutions with different ratios.

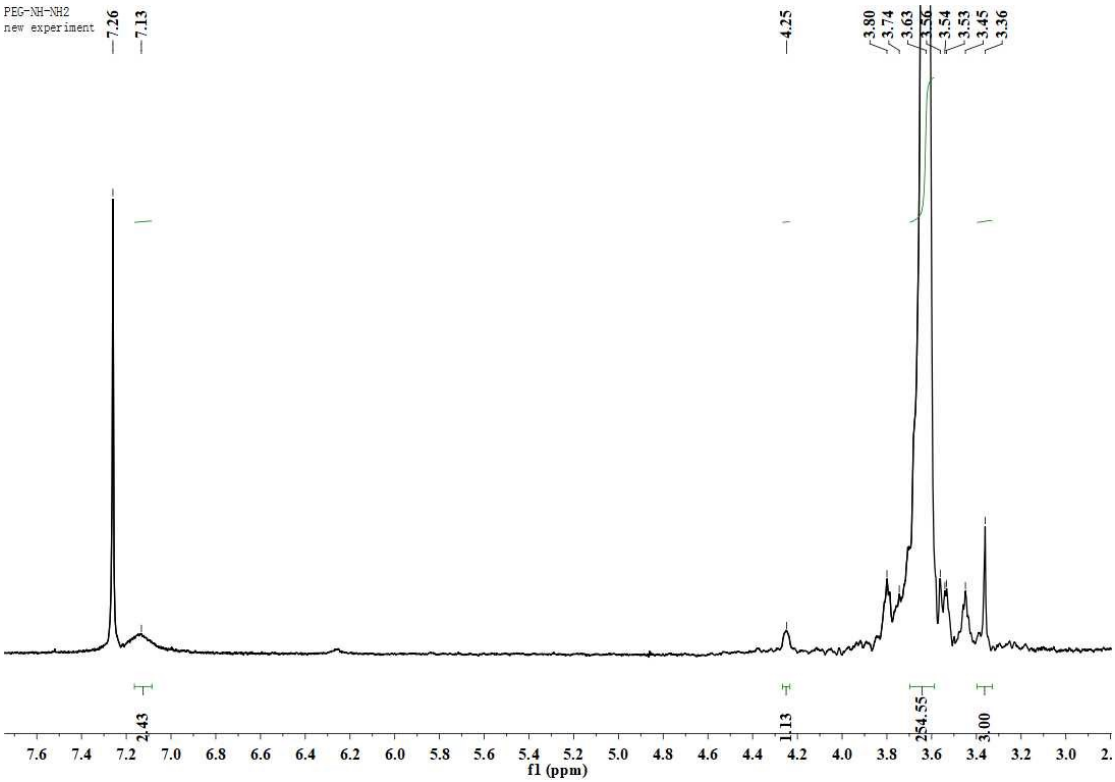


Figure S10. ^{1}H-NMR spectrum of **PEG-NHNH$_2$** (400 MHz, $CDCl_3$).

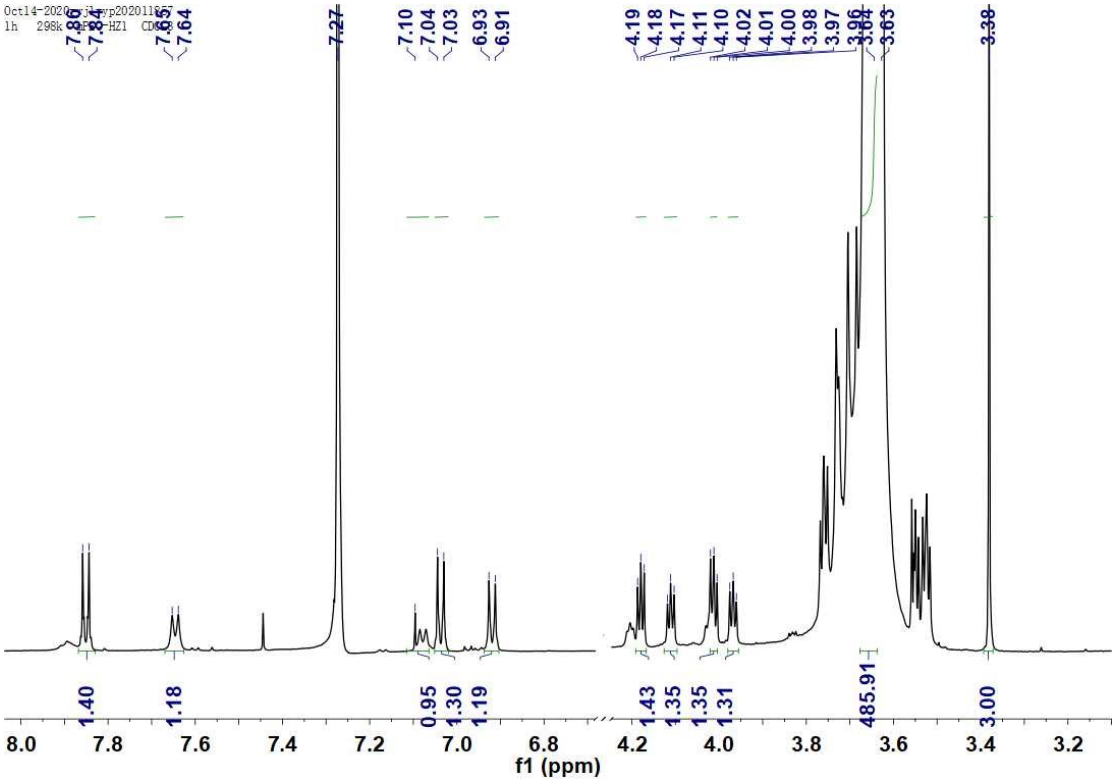


Figure S11. ^{1}H-NMR spectrum of PEG-Hz1.

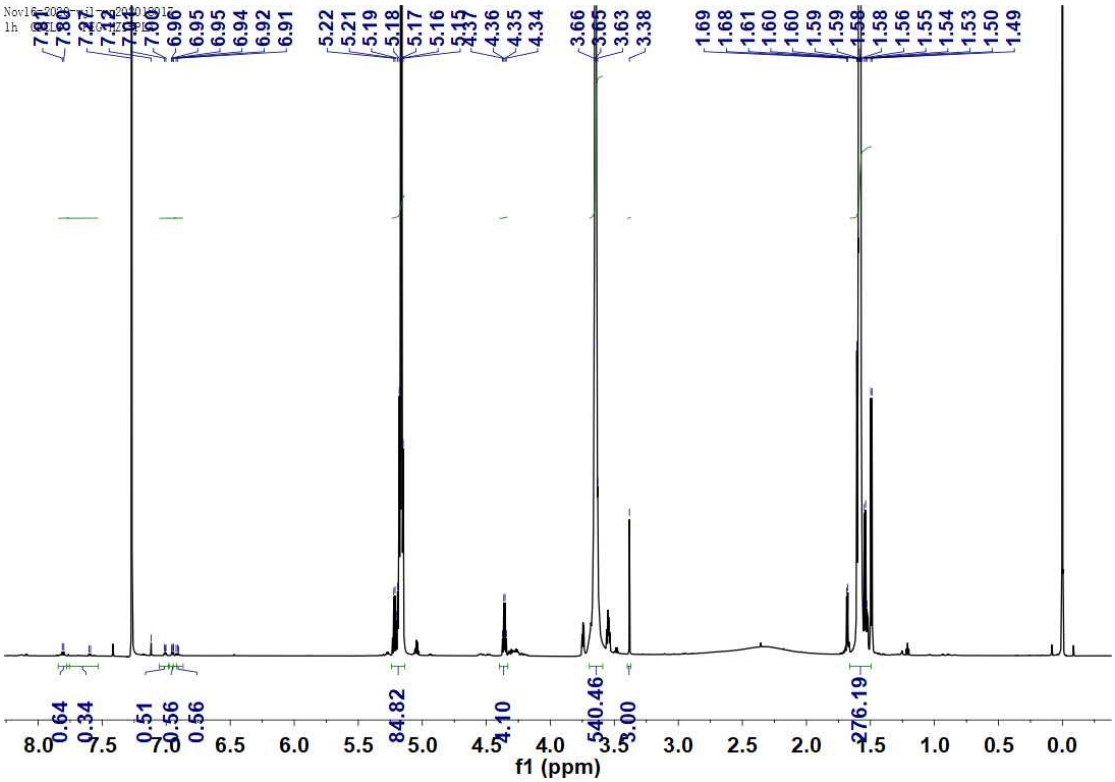


Figure S12. ^{1}H-NMR spectrum of PEG-Hz1-PLA.

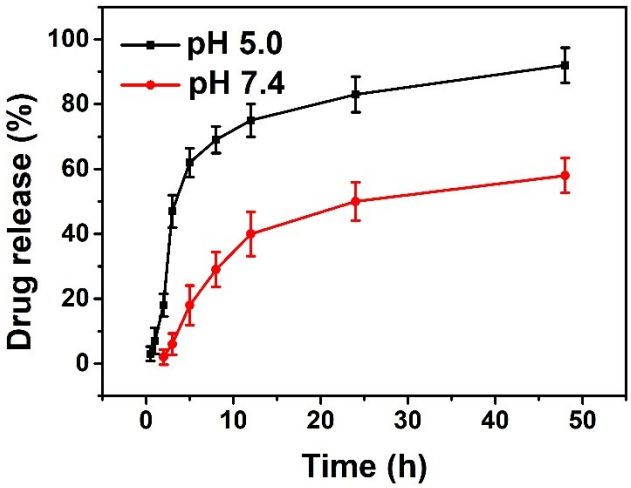


Figure S13. APN-SUB release profile from APN-SUB nanoprobe at pH=5.0 and pH=7.4 PBS buffer under 37 ^{o}C.

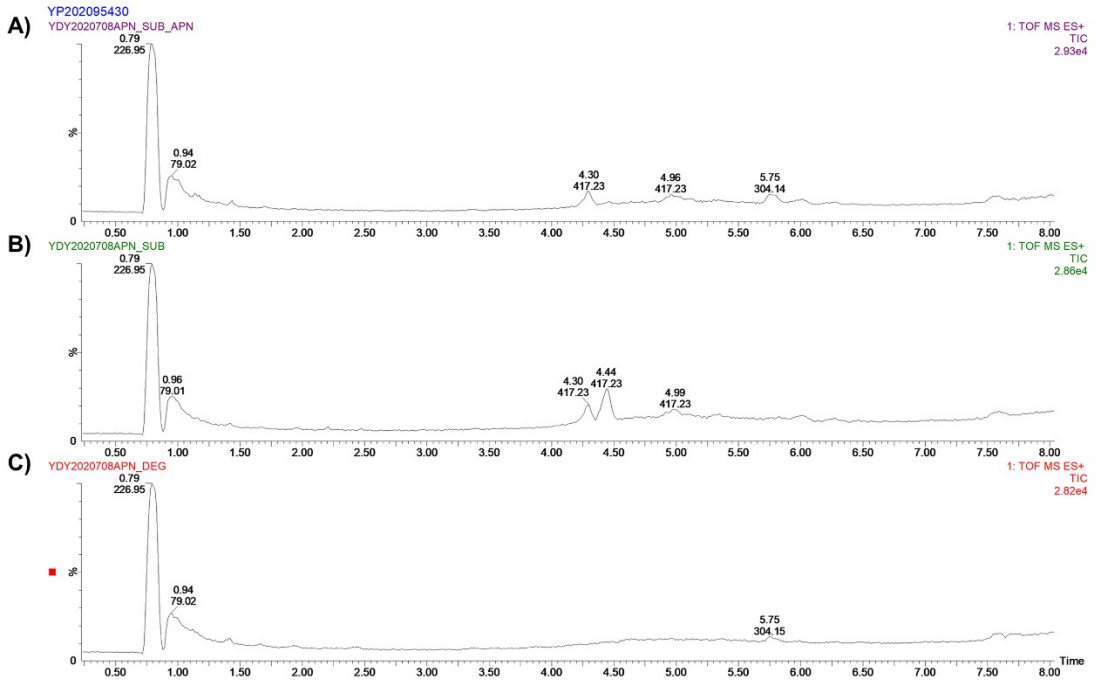


Figure S14. Chromatogram spectra of A) APN-SUB nanoprobe treated with APN, B) APN-SUB nanoprobe and C) APN-DEG nanoprobe.

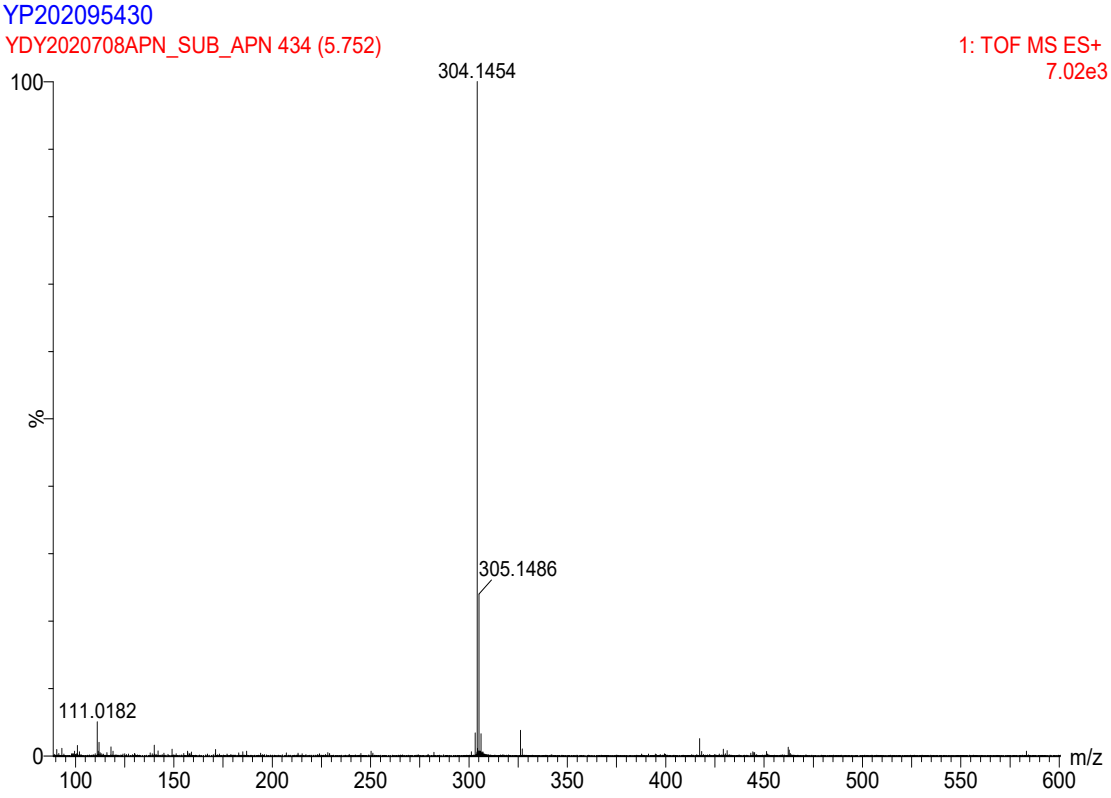


Figure S15. Mass spectrum extracted by chromatogram spectrum of APN-SUB nanoprobe treated with APN.

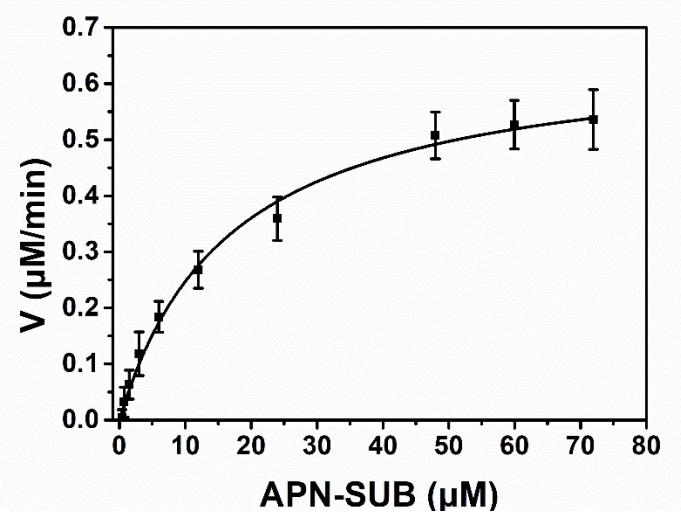


Figure S16. Michaelis-Menten curve for APN-SUB catalyzed by APN (2.4 mU).

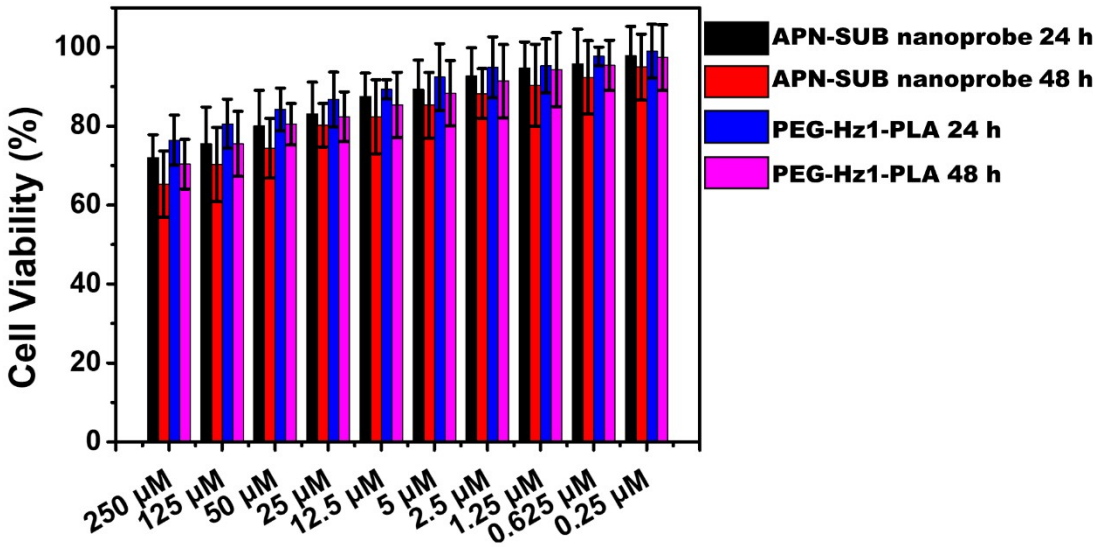


Figure S17. Cell viabilities of HeLa cells incubated with different concentrations of APN-SUB nanoprobe and PEG-Hz1-PLA for 24 h or 48 h.

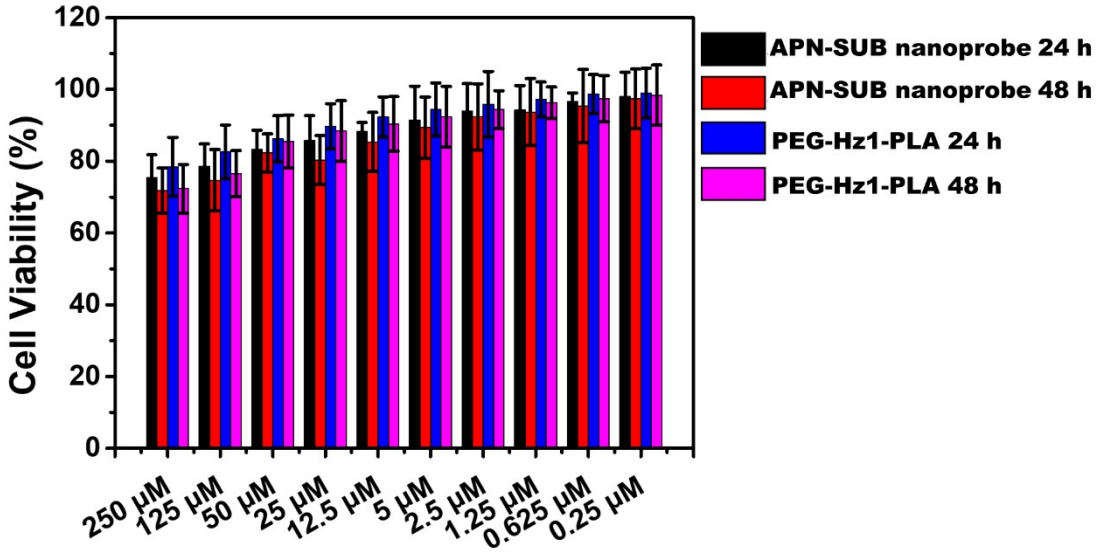


Figure S18. Cell viabilities of BT-549 cells incubated with different concentrations of APN-SUB nanoprobe and PEG-Hz1-PLA for 24 h or 48 h.

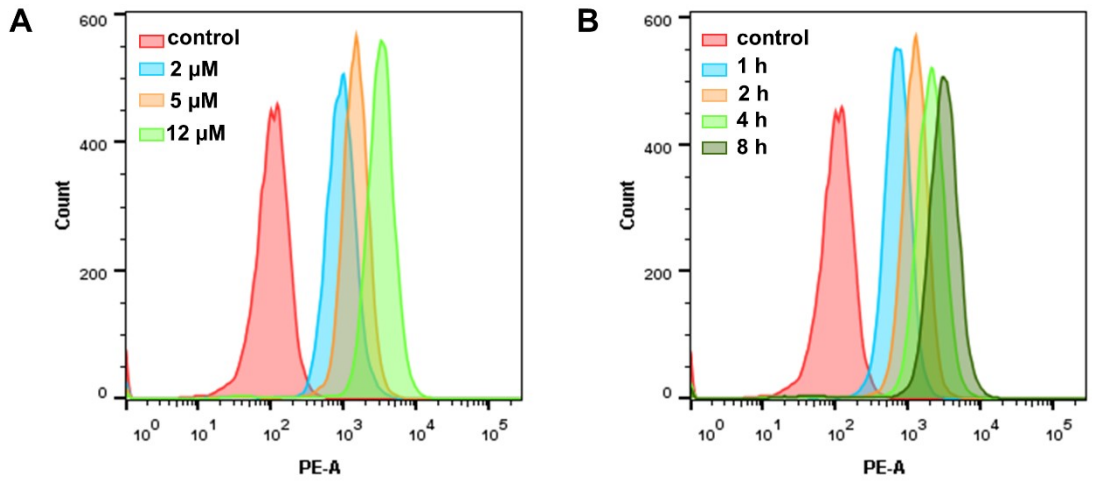


Figure S19. Cellular uptake of APN-SUB nanoprobe. A) Flow cytometric analysis of HeLa cells exposed to APN-SUB nanoprobe at different concentration (2, 5, 12 μM) for 6 h. B) Flow cytometric analysis of HeLa cells treated with APN-SUB nanoprobe (12 μM) for different incubation time (1 h, 2 h, 4 h, 8 h).

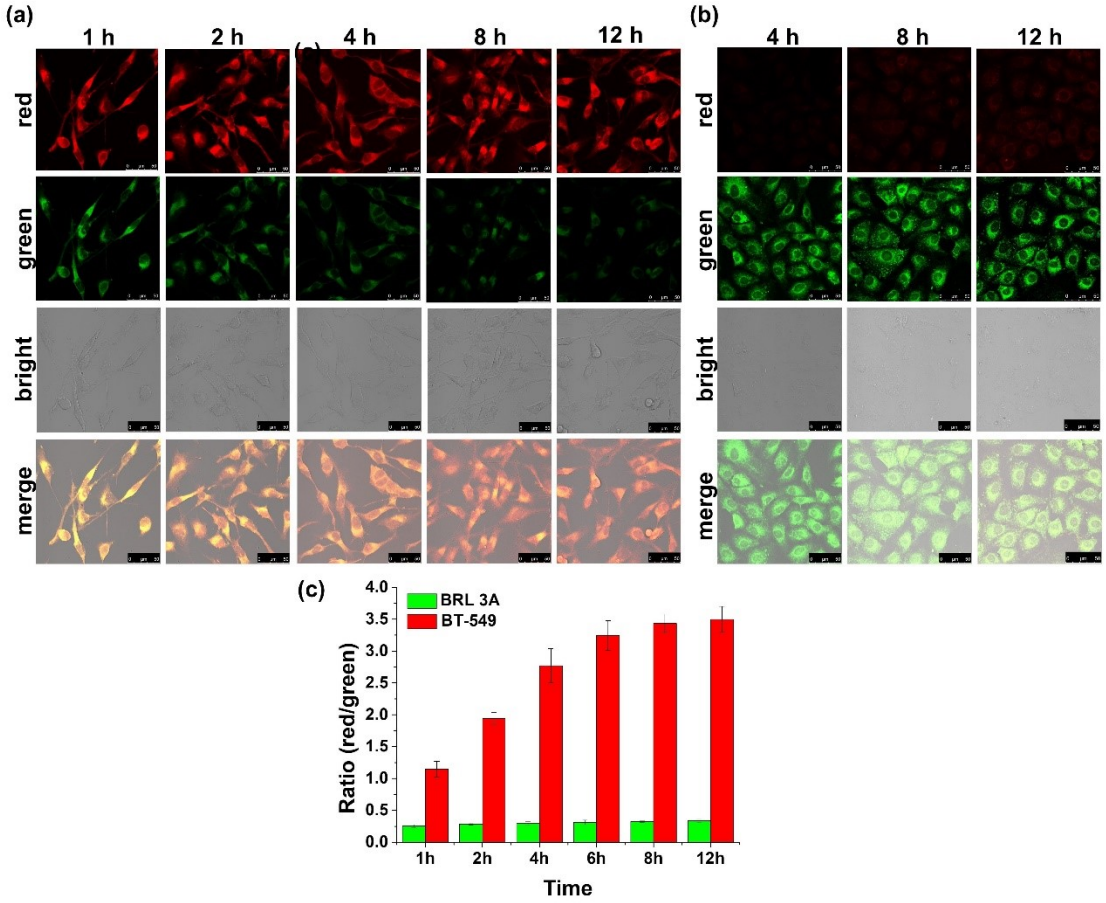


Figure S20. Confocal fluorescent images of (a) BT-549 cells and (b) BRL 3A cells incubated with APN-SUB nanoprobe (2 μM) for 1h, 2h, 4h, 8h and 12h. Green channel: λex=405 nm, λem=470-530 nm; red channel: λex=405 nm, λem=580-640nm; (c) Average intensity ratios from red channel to green channel in confocal fluorescent images. Data represent mean standard error (n=3). The scale bar is 50 μm.

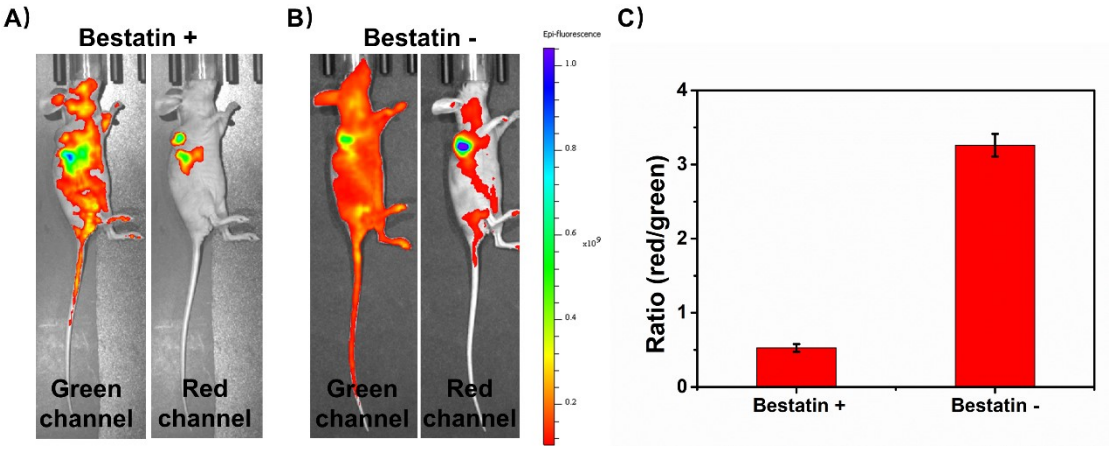


Figure S21. Imaging of endogenous APN in nude BABL/c mice bearing HeLa xenograft tumor. Whole-body imaging of female mice treated with A) or without B) bestatin (100 μM, 50 μL), followed by injection of APN-SUB nanoprobe (8 mg/kg) for 8 h. The signals were collected from the green channel (480−520 nm, left panel) and red channel (580−620 nm, right panel) simultaneously with excitation at 450 nm. C) The ratio of fluorescence intensity (red/green) in images captured by small animal imaging system.