Supplementary Information (SI)

An Activatable Molecular Probe for Early Detection of Obesity-Associated Aminopeptidase N Activity in Vivo and in Serum

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Supplementary Section

Materials and Characterization.

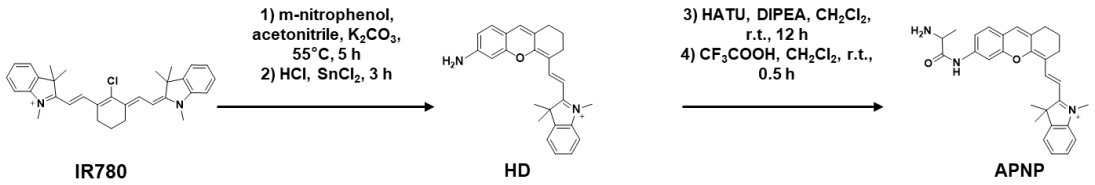
All the chemical materials used in the experiment were purchased from commercial suppliers. Indolenine and iodomethane were purchased from Tianjin Heowns Biochemical Technology Co., Ltd. Cell culture plates were purchased from NEST biotechnology Co. Ltd. (Wu Xi, China). Ultrapure water was obtained from the Milli-Q system. The silica gel used for column chromatography was purchased from Leyan.com.

The absorbance was recorded by absorption spectrometry (UV-2600, Shimadzu). Fluorescence measurement was measured on a HITACHI F4600 fluorescence spectrophotometer at room temperature. H&E images were observed by a digital slice scanning system (Pannoramic MIDI). ^{1}H NMR was performed on a Bruker DRX-400 spectrometer (Bruker) system. A matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (ultrafleXtreme) was used for mass spectrometry. Infrared spectra were measured by IR SHIMADZU Spirit.

Synthesis of APNP. Under argon protection, a mixture of 3-nitrophenol (125 mg, 1.0 mmol) and K2CO3 (138 mg, 1.0 mmol) in anhydrous MeCN (10 mL) was stirred for 10 min at room temperature. IR780 (485 mg, 1.0 mmol) in MeCN (8 mL) was then added dropwise. After solvent removal, the crude product was extracted with CH2Cl2, washed with water, and redispersed in methanol (20 mL). This intermediate solution was treated with SnCl2 (4 g) in concentrated HCl (4 mL) under nitrogen at 70°C for 6 h. Standard workup and column chromatography yielded pure hemicyanine.

N-Boc-Ala (0.5 mmol) was activated with HATU/DMAP (1 mmol each) in CH2Cl2 (10 mL) at 0°C for 1 h. hemicyanine (200 mg, 0.5 mmol) in CH2Cl2 (10 mL) was then added slowly, with overnight stirring at room temperature. After aqueous workup, the Boc-protected intermediate was deprotected using TFA/CH2Cl2 (5:10 mL) for 30 min. Final purification by silica gel chromatography (CH2Cl2/MeOH) afforded APNP as a stable solid. 1H NMR (400 MHz, Methanol-d4) δ 8.79 (d, J = 15.0 Hz, 1H), 7.96 (s, 1H), 7.83 – 7.75 (m, 1H), 7.63 (dd, J = 7.8, 2.7 Hz, 1H), 7.56 (t, J = 3.3 Hz, 2H), 7.50 (d, J = 2.0 Hz, 2H), 7.34 (s, 1H), 6.62 – 6.49 (m, 1H), 4.15 (q, J = 7.1 Hz, 1H), 3.89 (s, 3H), 2.82 (t, J = 6.2 Hz, 2H), 2.74 (t, J = 6.1 Hz, 2H), 1.98 (t, J = 6.1 Hz, 2H), 1.84 (s, 6H), 1.65 (d, J = 7.1 Hz, 3H). 13C NMR (101 MHz, MeOD) δ 171.15, 162.34, 161.36, 155.41, 142.27, 141.85, 141.21, 130.76, 129.43, 122.30, 121.74,

117.62, 115.70, 114.09, 113.36, 112.91, 109.70, 99.21, 50.31, 49.77, 49.72, 49.67, 33.29, 28.40, 28.29, 27.63, 27.56.



Scheme S1. Synthesis route of APNP

MTT assays

The cytotoxicity assays were performed on RAW264.7 cells. Three replicate wells of the same concentration were used, and each measurement was assayed three times. The cells were grown in a culture medium and treated with various levels of **APNP** for 24 h at 37 ℃. Then 10 μL MTT (5 mg/mL) was added to each well and the plate was placed in a 37 ℃ incubator for 4 h. Finally, the cell supernatant was removed and the residue was suspended in 150 μL DMSO. The absorbance was monitored on a Tecan Microplate Reader.

Animal experiments

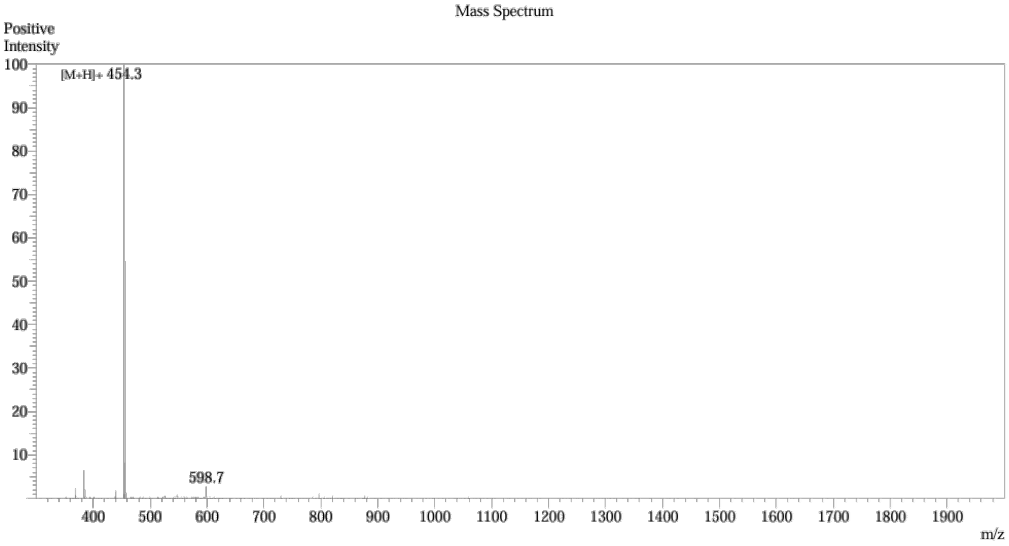
All experiments were performed in compliance with the relevant guidelines, receiving approval from the Institutional Animal Care and Use Committee of Jiangnan University (WXCH2025-05-101). For photoacoustic imaging, a commercial small animal photoacoustic imaging system was used: Model: inVision 256-TF (iThera Medical GmbH, Munich, Germany); Optical parametric oscillator (OPO): Nd: YAG laser; Excitation pulses: 9 ns; Wavelengths range: 680 nm to 980 nm; Repetition rate: 10 Hz; Wavelength tuning speed:10 ms; Peak pulse energy: 100 mJ; Center frequency of ultrasound transducers: 5 MHz (60% bandwidth).

The organ was separated from mice and embedded in optimal cutting temperature (OCT) compound directly, frozen at -80°C for 30 minutes, then sliced at a thickness of 10 μm.

For hematoxylin-eosin (H&E) staining, frozen sections of the organ were stained with 10 μL hematoxylin for 5 min at room temperature, then those sections were rinsed three times with deionized water and stained with 10 μL eosin for 10 s. Next, those sections were dehydrated three times with 10% ethanol and added one drop of neutral balsam. Finally, those sections were covered with a coverslip and scanned by the digital slice scanning system.

Serum test process. Serum samples were collected by centrifugation at 3000 rpm. Following this, the probe was mixed with 50 μL of the serum samples, and PBS, with the solution composition adjusted to serum samples/PBS in a 1:1 ratio (v/v/v).

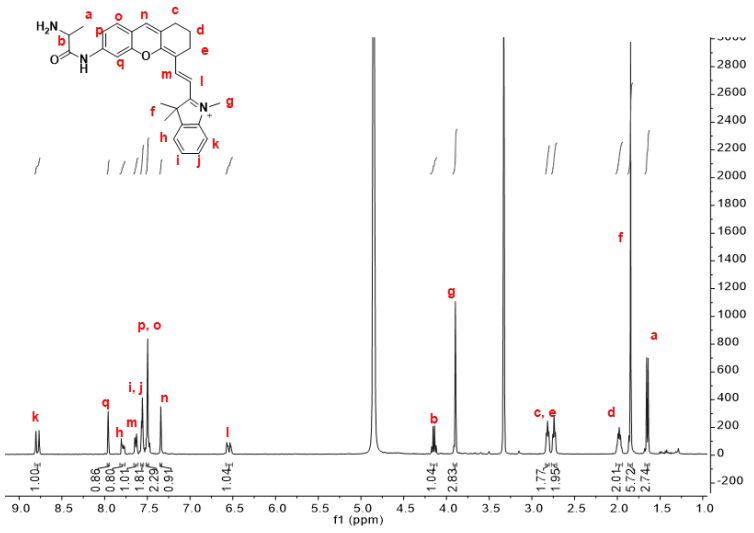
Mass Spectrum



Sample Information
Month-Day Processed : 03/12/25
Time Processed : 8:37:45
Injection Volume : 0.5
Sample Name : 化合物4
Sample ID : C3352207G0-5
Theoretical MW : 454.25
Observed MW : 453.3

Interface :ESI
Nebulizing Gas Flow :1.5L/min
CDL Temp :250
Block Temp :200

Equipment :ZJ22010150
Interface Bias : +4.5 kV
Drying Gas Flow :5 L/min
T.Flow :0.2 ml/min
B,conc :50%H2O/50%MeOH



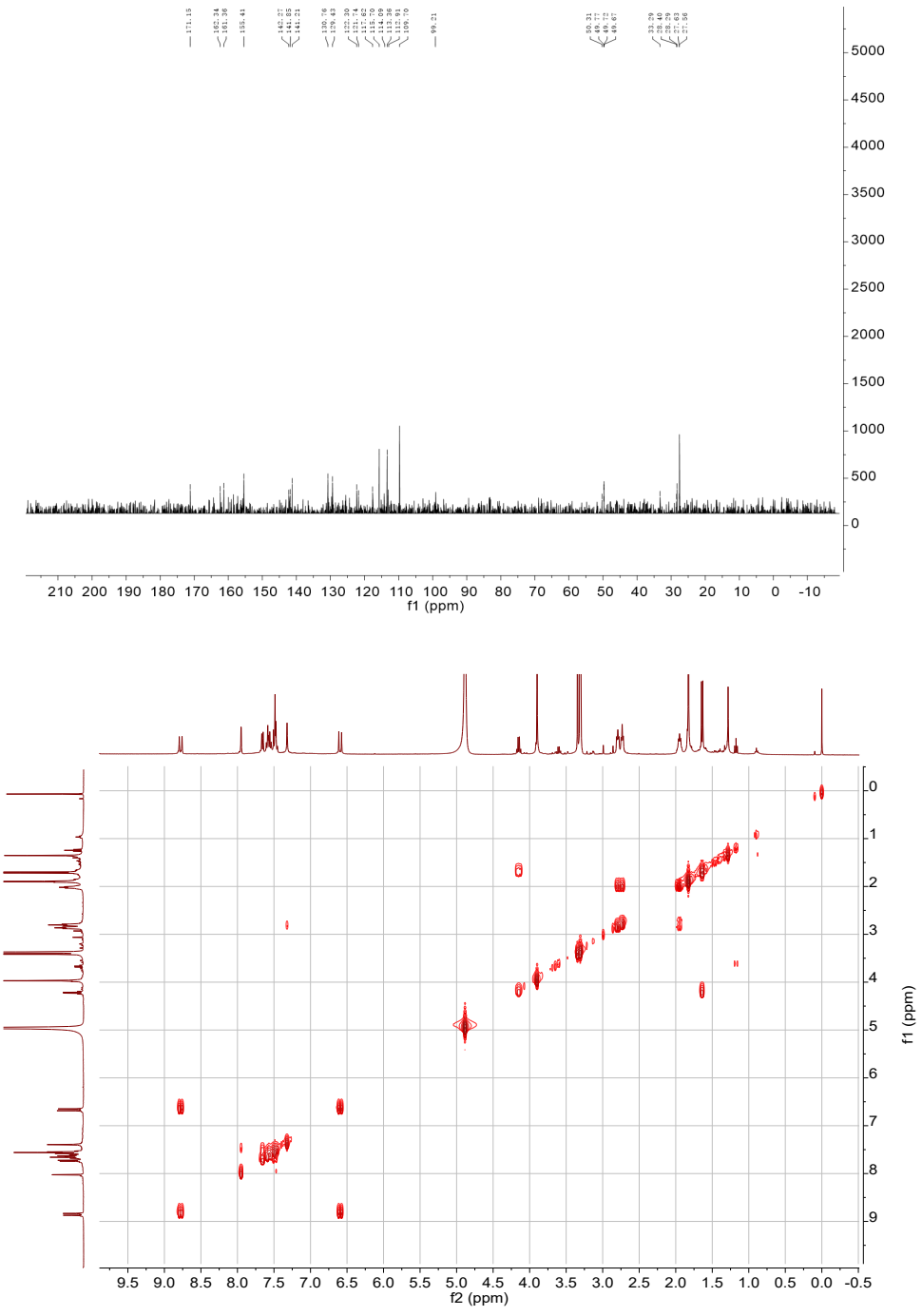


Figure S1. MS and NMR spectra of APNP.

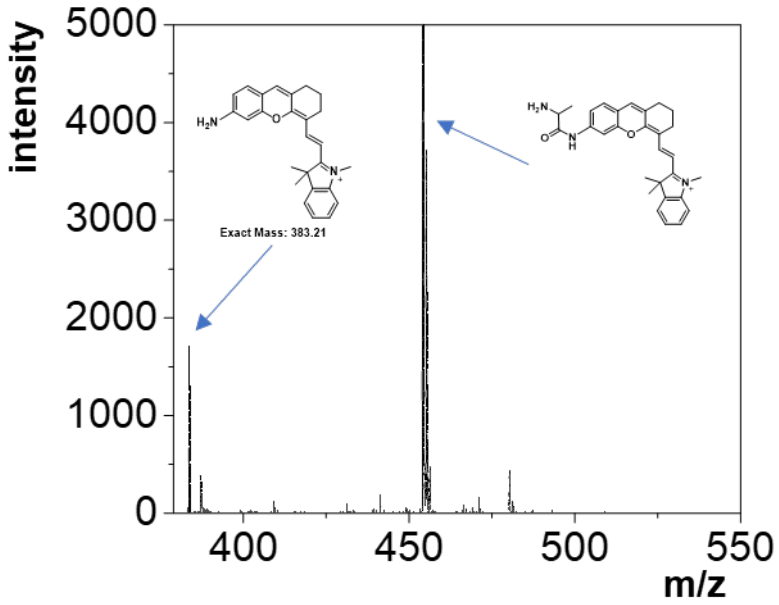


Figure S2. MS spectrum of the mixture of APNP and APN..

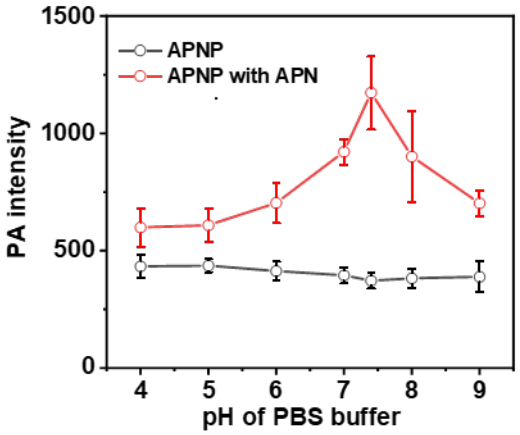


Figure S3. PA signal of APNP with or without APN at different pH buffers.

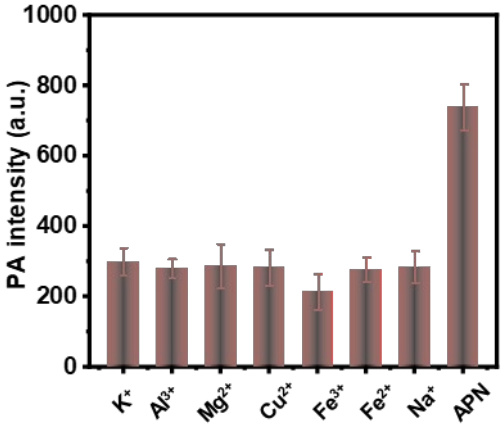


Figure S4. Selective testing: APNP adds different metal ions and changes in the PA signal after incubation.

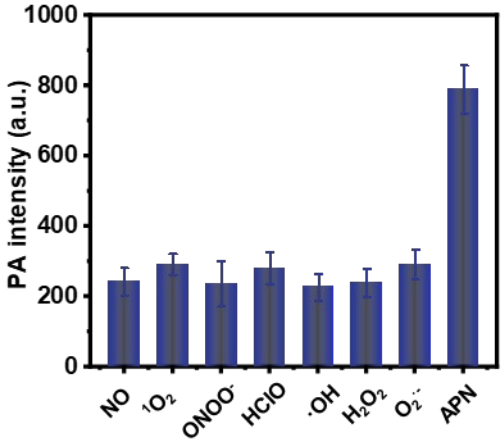


Figure S5. Selective testing: APNP adds different reactive species and changes in the PA signal after incubation.

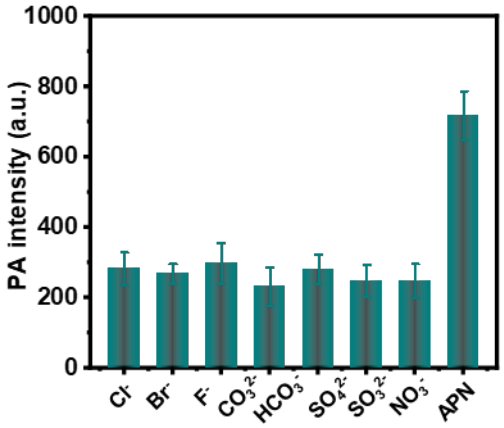


Figure S6. Selective testing: APNP adds different anions and changes in the PA signal after incubation.

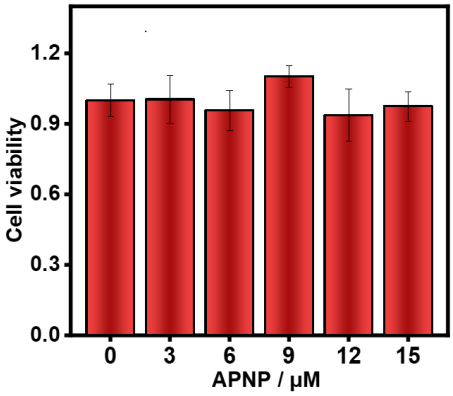


Figure S7. Cell viability of RAW264.7 cell after incubation with different concentration probe.