Supporting Information

A Single Probe for Imaging and Biosensing of pH, Cu^{2+} ions, and pH/Cu^{2+} in live cells with Ratiometric Fluorescence Signals

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1. Supplementary Experimental Section

Chemicals and Reagents. Tris(2-aminoethyl)-amine, 2-pyridinecarboxaldehyde, hydrogen peroxide (30%), sodium nitrite, sodium hypochlorite, potassium superoxide, 2,2-azobis(2-methylpropionamidine)-dihydrochloride (AAPH) and ouabain were acquired from Aladdin reagent company. Ethylene Sulfide was obtained from TCI America. Gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$, 99%), fluorescein isothiocyanate (FITC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), cysteamine, methyl thiazolyl tetrazolium (MTT), human ceruloplasmin, copper, zinc-superoxide dismutase (SOD) from bovine liver, and lipopolysaccharides (LPS) from E. coli were obtained from Sigma-Aldrich. 7-diethylaminocoumarin-3-carboxylic acid (DEAC) was purchased from AmyJet Scientific Co. Ltd. Amino acids, potassium phosphate dibasic trihydrate, dichloromethane, methanol, and silica gel (100−200 meshes) were purchased from Sinopharm Chemical Regent Co. Ltd. Dialysis tube (MWCO: 3500) was obtained from Ebioeasy Corporation. Cell culture media. Apoptosis assay was executed by an Annexin V-FITC apoptosis detection kit. All other chemicals were commercially available and of analytical grade. Metal ions were obtained from their chloride salts and were acquired from Aladdin Reagent Company. All aqueous solutions were prepared with ultrapure water (18.2 MΩ cm^{-1}) from a Millipore water purification system.

Instruments and Methods. NMR spectra were collected by using a Bruker-400 MHz NMR spectrometer at 25°C. All chemical shifts were recorded in the standard δ notation of parts per million. High-resolution mass spectral analysis was carried out at IonSpec 4.7 Tesla Ultima FTMS (IonSpec, USA). High-resolution transmission electron microscopy (TEM) images were obtained with a JEOL JEM-2100 microscope. UV-Vis absorption spectrum was recorded by an Agilent 8453 UV-Vis spectrometer. Infrared spectroscopic data were collected by a NEXUS470 infrared spectrometer (Nicolet). The XPS images were performed by an X-ray photoelectron Spectrometer (PHI 5000 ESCA, Perkin-Elmer, USA) equipped with an Al Kα source (1486.6 eV photons). X-Ray diffraction (XRD) measurements were obtained with an XRD-6000 powder diffractometer (Shimadzu, Japan). Fluorescence spectra were measured with a Hitachi F-2700 Fluorescence Spectrophotometer with a 450 W xenon lamp in a 1 cm × 1 cm quartz cuvette. Confocal laser scanning images were obtained at a confocal laser scanning biological microscope (Fluoview 1000, Olympus, Japan). All the experiments were performed at room temperature.

Synthesis of TPAA and TPAASH. TPAA was prepared according to the previous procedure. In a 250 mL flask, 9.60 g 2-pyridinecarbaldehyde (90 mmol) was dissolved in 100 mL ethanol under nitrogen. Then, 4.38 g tris(2-aminoethyl)amine (30 mmol) in 80 mL ethanol was dropwise added. The mixture was refluxed for 3 h in dark, and then cooled to room temperature. After added 1.14 g $NaBH_4$ (30 mmol), the reactant was continuously stirred overnight. After solvent evaporation, the crude product was extracted with CH_2Cl_2 (3 × 30 mL) in saturated Na_2CO_3 aqueous solution. The organic layer was combined and dried over Na_2SO_4, filtered and evaporated to get yellow oil. ^{1}H NMR (400 MHz, CDCl3): δ (ppm) = 8.48 (dd, 3H), 7.58 (td, 3H), 7.31 (d, 3H), 7.12 (dd, 3H), 3.90 (s, 6H), 2.75 (t, 6H), 2.70 (br, 3H), 2.65(t, 6H); ^{13}C NMR (100 MHz, CDCl3): δ (ppm) = 159.79, 149.07, 136.24, 121.99, 121.69, 55.00, 54.33, 47.22.

Next, TPAASH was synthesized based on literature procedure with modifications. Briefly, 1.1 mL ethylene sulfide (18 mmol) was added dropwise into a solution of 1.26 g TPAA (3 mmol) in 20 mL $CHCl_3$ under argon. The reactant mixture was stirred at 65 ℃ for 4 days. After filtration, the extra solvent was eliminated by rotary evaporation. The rough product was purified through a silica-gel column chromatography using a mixture of methanol and ethyl acetate (1:9 v/v) as eluent to get yellow oil with odorous smell. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.51 (t, 3H), 7.65 (m, 3H), 7.50 (d, 3H), 7.16 (t, 3H), 3.74 (s, 6H), 2.70-2.78 (m, 10H), 2.56-2.63(m, 14H), 1.71(s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) = 159.67, 148.87, 136.52, 122.50, 121.81, 60.63, 59.19, 53.27, 52.58, 24.79. TOF MS EI$^+$: calculated for $[M+H]^+$ 600.2977, found 600.2971.

Preparation of AuNCs and AuNC@FITC@DEAC. AuNCs were synthesized by a literature procedure with minor modifications. In a typical synthesis, 20 mg $HAuCl_4$•$3H_2O$ (0.1 mmol) and 104 mg TPAASH (0.3 mmol) were dissolved in 17.5 mL mixed solvent (acetic acid:methanol = 1:6 v/v) and stirred for 30 min. After the brown solution became clear, 5 mL cold $NaBH_4$ solution (0.1 M) was added into the reactant solution under rapid stirring and ice bath. The reactant solution was stirred continuously for another 3 h. After solvent evaporation, the raw product was dispersed in 15 mL ultra-high purity water and purified through dialysis tube (MWCO: 3500) in ultra-high purity water for 4 days. The as-prepared AuNCs capped with TPAASH was collected into a glass container with a tight lid and stored at 4 ℃.

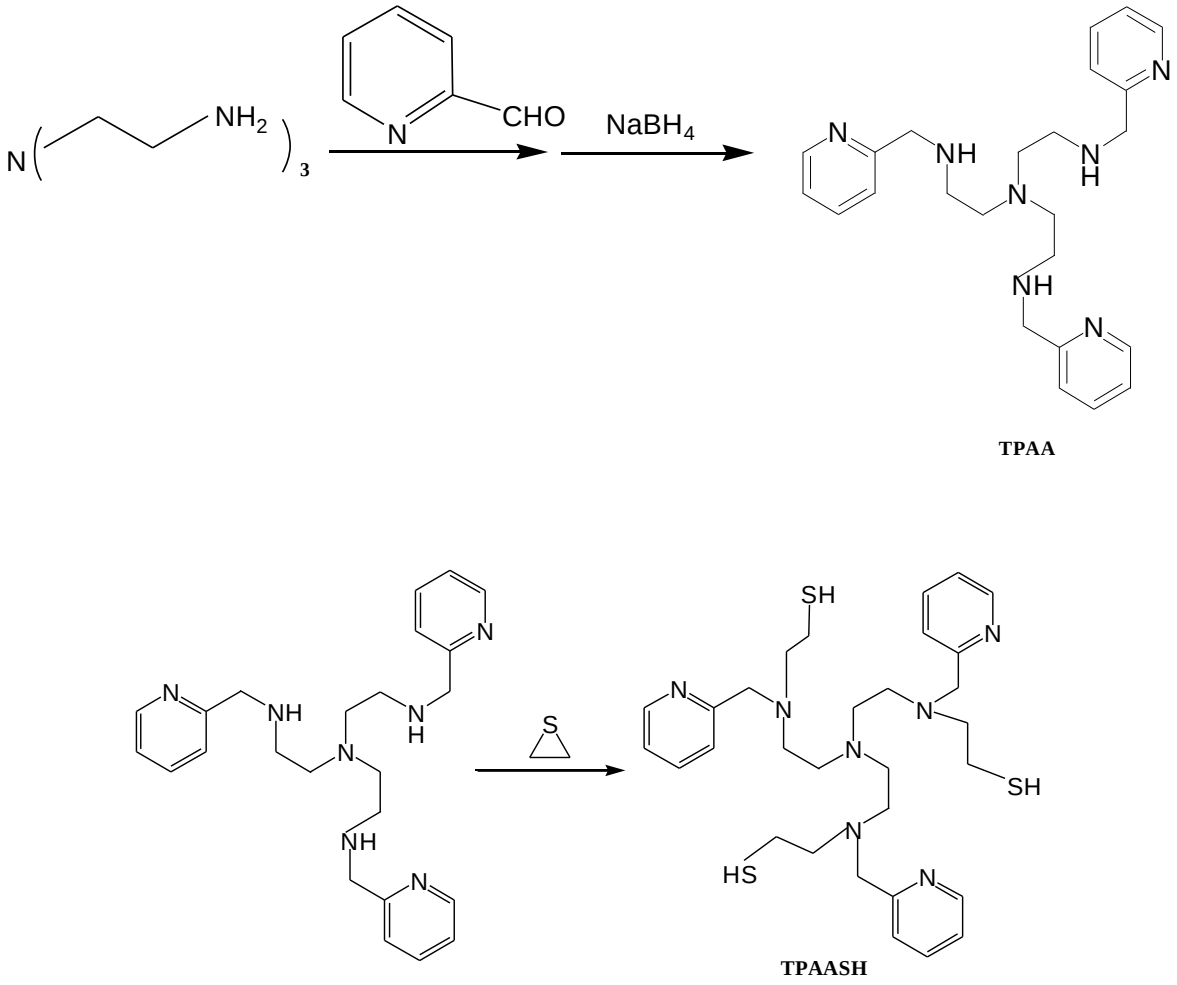
To prepare AuNC@FITC@DEAC probe, FITC was firstly conjugated onto the surface of AuNCs. The AuNCs were added into a solution of FITC and cysteamine and stirred for 5 h to form AuNC@FITC@Cys. After purified through dialysis in ultrapure water, the as-prepared AuNC@FITC@Cys was mixed with DEAC activated by EDC and NHS and stirred overnight. Finally, the nanohybrid probe AuNC@FITC@DEAC was dialyzed in ultrapure water for 8 h by using dialysis tube (MWCO: 3500).

MTT Assay and Apoptosis Assay. Hela cells were used to determine cytotoxicity and biocompatibility of the as-prepared hybrid probe AuNC@FITC@DEAC. In a typical MTT assay, cells were cultured in 96-well plates with 1 × 105 cells per well in the culture media (DMEM with 10% fetal bovine serum, 2 mM L-glutamine, and 100 μg mL^{-1} penicillin/streptomycin). After incubated at 310 K in 5% CO_2 / 95% air for 24 h, the culture media was removed and the cells were incubated in culture medium containing the as-prepared AuNC@FITC@DEAC probes with different concentrations (0.25, 1.0, 2.5, 5.0, and 10.0 μM). Fresh PBS-containing media was used as negative controls. After another 24 h or 48 h incubation, 20 μL MTT (5 mg mL-1) was added into every well and incubated at 310 K for another 4 h in dark. After removing the medium, 150 μL DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 490 nm in ELx800 reader (BioTek Instruments, Inc, Winooski, VT). Cell viability values were determined according to the following formulae: cell viability (%) = the absorbance of experimental group/the absorbance of blank control group × 100%.

The percentage of apoptotic cells was determined by using an Annexin V-FITC apoptosis detection kit (Key-GEN Biotech) according to the standard protocol. In an apoptosis assay, Hela cells were incubated with AuNC@FITC@DEAC probes at the concentrations of 0.25, 1.0, 2.5, 5.0, and 10.0 μM respectively for 48 h. After removing the culture media, the cells were harvested by using EDTA-free trypsin and collected by centrifugation. After washed with PBS, the cell pellets were suspended in 500 μL binding buffer and incubated with 5 μL FITC−Annexin V and 5 μL propidium iodide solution from 30 min in dark. A Becton-Dickinson flow cytometer was used to evaluate cells apoptosis by monitoring the fluorescence of FL-1 (530 nm) and FL-2 (585 nm) at an excitation wavelength of 480 nm.

Confocal Fluorescence Imaging and Biosensing in Hela Cells. One day before fluorescence imaging experiments, Hela cells were plated on a 35-mm Petri dish with 14-mm bottom well in culture media at 37°C. Then, the culture media were exchanged into PBS (pH = 7.4) with 10 μM AuNC@FITC@DEAC probe and incubated for 1 h. After washed with PBS three times to remove the extracellular probe, the cell fluorescence im-age was obtained by using an Olympus FV1000 confocal laser scanning microscope equipped with an oil immersion 60 × objective at an excitation wavelength of 405 nm. The fluorescence emissions were collected simultaneously from the DEAC channel, FITC channel, and AuNCs channel in the range of 430−485 nm, 500−600 nm, and 655–755 nm, respectively. Then, exogenous Cu^{2+} was introduced directly into the Petri dish and the cells were incubated for 30 min to determine the Cu^{2+} changes in cells. The pH sensing was operated by monitoring the Na^+/H^+ exchange in cells. In the experiment set, Hela cells incubated with hybrid probe AuNC@FITC@DEAC were firstly washed into 2 mL of Cl^--free solution with 0.1 mM ouabain and incubated for 45 min. Next, the cells were incubated in 2 mL Na^+-free Ringer's solution for 3 min, followed by adding 100 mM NaCl to incubate for another 4 min. The fluorescence intensities of three fluorescence channels: DEAC channel (430−485 nm), FITC channel (500−600 nm), and AuNCs channel (655–755 nm) were collected at the same excitation of 405 nm to indicate the changes in the concentration of Cu^{2+} and/or pH in live cells.

2. Synthetic route for TPAA and TPAASH (Scheme S1)



Scheme S1. Synthetic route for TPAA and TPAASH.

3. NMR and MS Data of TPAA and TPAASH (Figure S1-S5)

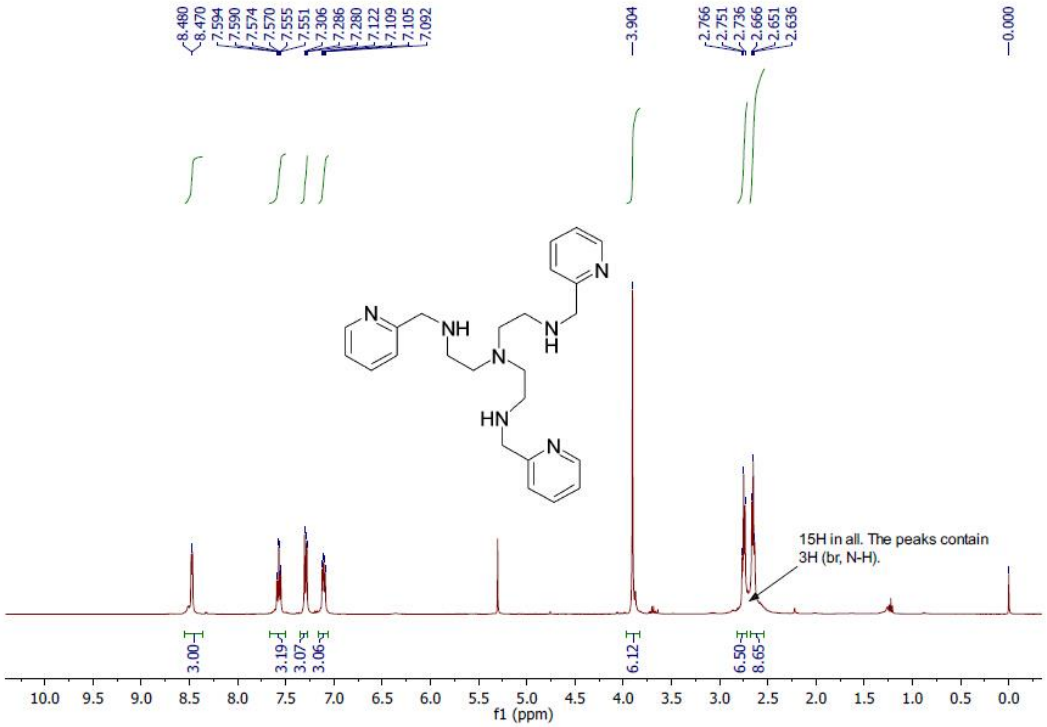


Figure S1. ^{1}H NMR spectrum (400 MHz) of TPAA in $CDCl_3$.

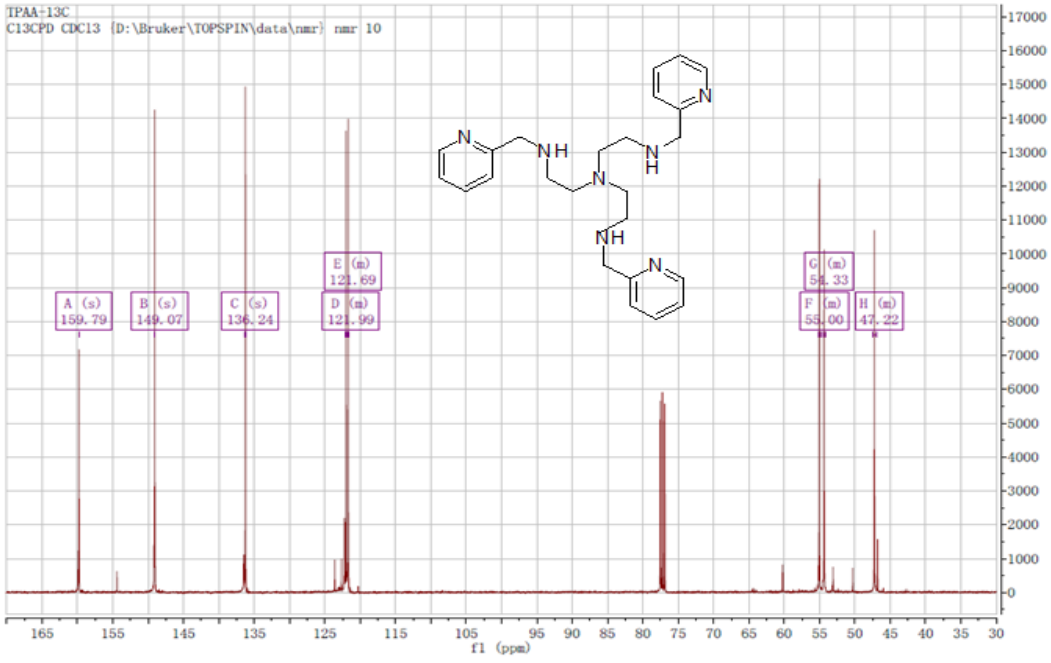


Figure S2. ^{13}C NMR spectrum (100 MHz) of TPAA in $CDCl_3$.

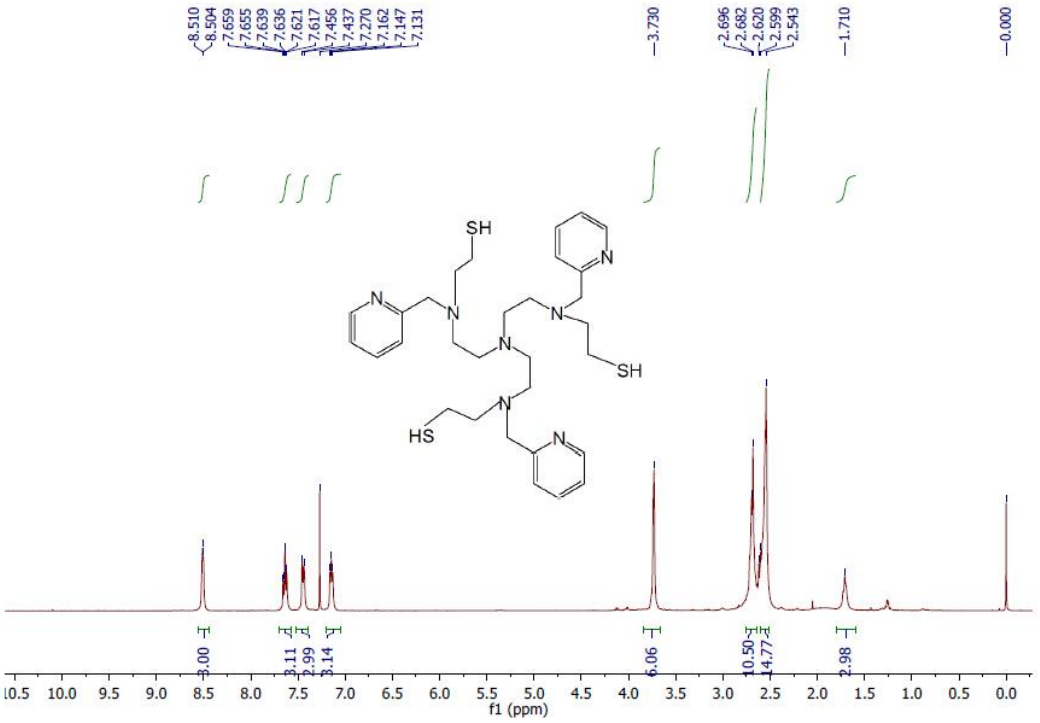


Figure S3. ^{1}H NMR spectrum (400 MHz) of TPAASH in $CDCl_3$.

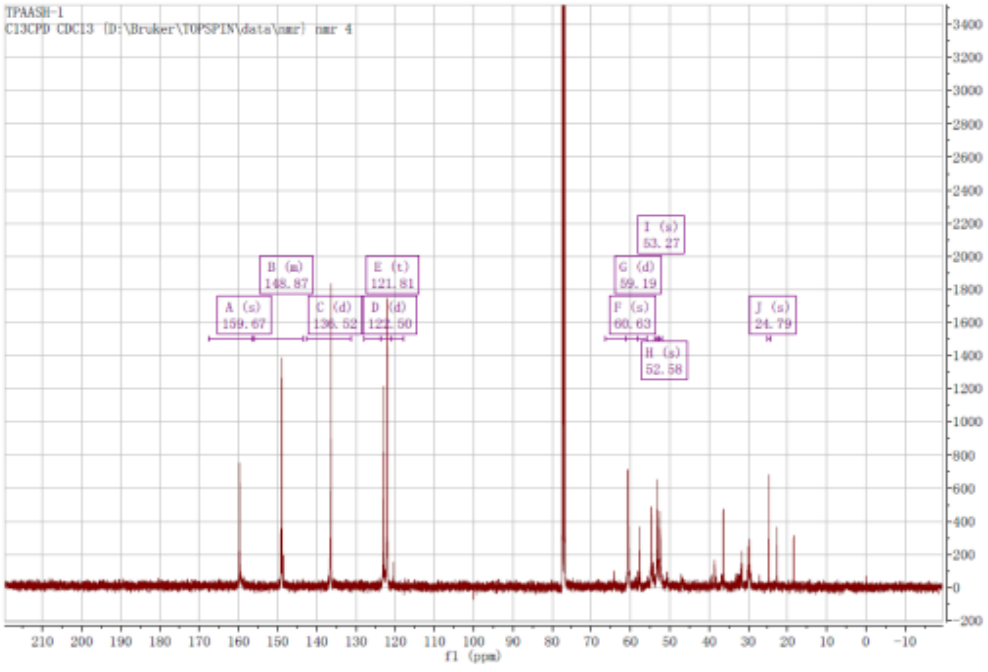


Figure S4. ^{13}C NMR spectrum (100 MHz) of TPAASH in $CDCl_3$.

Shanghai Mass Spectrometry Center
Shanghai Institute of Organic Chemistry
Chinese Academic of Sciences
High Resolution MS DATA REPORT

Instrument: IonSpec 4.7 Tesla FTMS

Card Serial Number : WI13 0730

Sample Serial Number: 2

Operator : HuaQin Date: 2013/03/29

Operation Mode: MALDI/DHB

Elemental Composition Search Report:

Target Mass:
Target m/z = 600.2977 ± 0.002
Charge = +1

Possible Elements:

Element:	Exact Mass:	Min:	Max:
C	12.000000	0	100
H	1.007825	0	100
N	14.003074	0	10
S	31.972071	0	5

Additional Search Restrictions:
DBE Limit Mode = Both Integer and Half-Integer
Minimum DBE = 0

Search Results:
Number of Hits = 3

m/z	Delta m/z	DBE	Formula
600.29801	-0.00031	5.0	$C_{32}H_{56}S_6^{+1}$
600.29713	0.00057	11.5	$C_{30}H_{46}N_7S_3^{+1}$
600.29960	-0.00190	26.0	$C_{40}H_{36}N_6^{+1}$

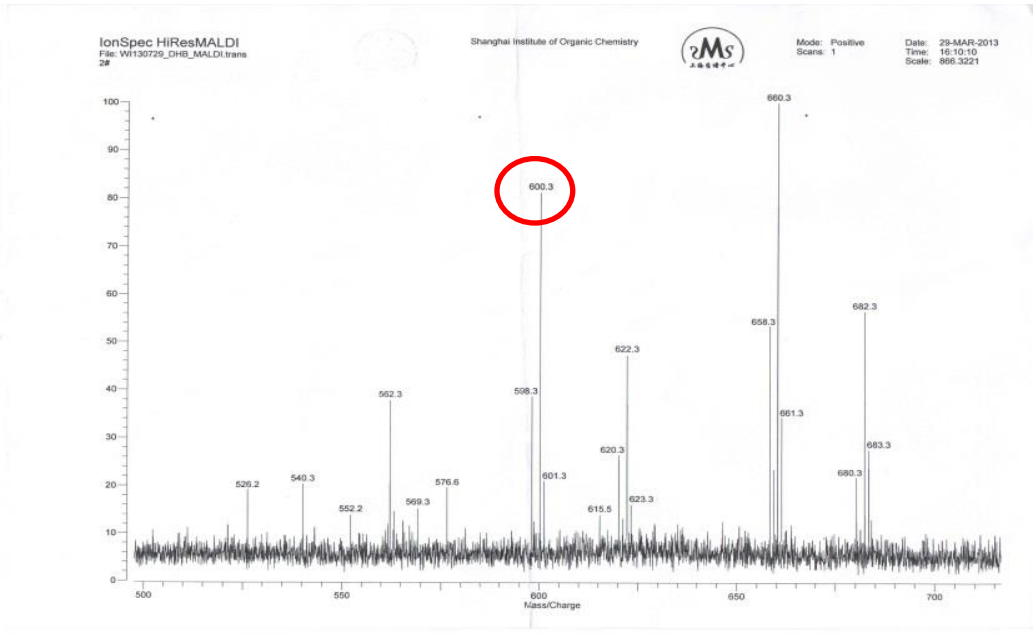


Figure S5. Elemental composition search report by HR-MS for TPAASH.

4. FT-IR spectra (Figure S6)

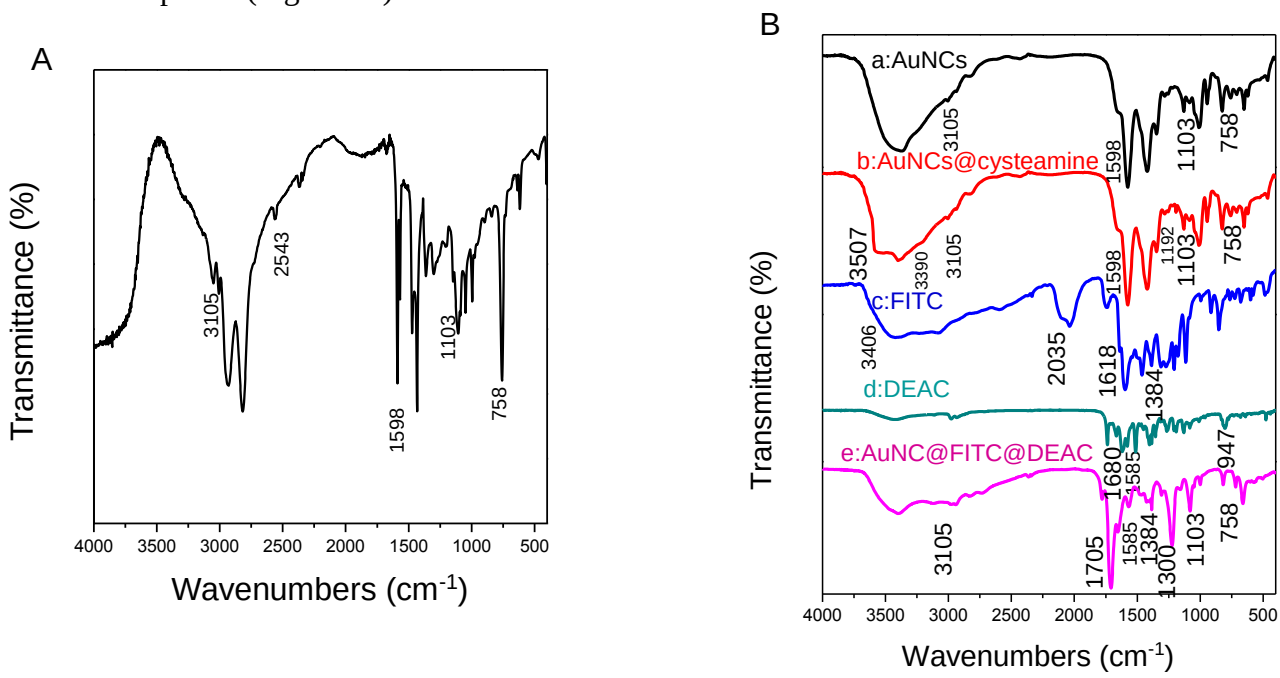


Figure S6. FT-IR spectra of (A) TPAASH; (B) the as-prepared AuNCs (a), AuNCs@cysteamine (b), FITC (c), DEAC (d), and AuNC@FITC@DEAC (e).

The FT-IR spectrum for the TPAASH-ligand AuNCs showed four peaks located at 3105 cm^{-1} (ν_{C-H}), 1598 cm^{-1} ($\nu_{C=N}$), 1103 cm^{-1} (ν_{C-N}), and 758 cm^{-1} (ν_{C-H}), ascribed to polycyclic aromatic hydrocarbons from TPAA. Compared with that of TPAASH (Supporting Information, Figure S6, curve a), S-H band (2543 cm^{-1}) disappeared, possibly due to the binding of thiol to Au surface to form Au-S bond. As given as curve b, three peaks were observed at 3507 cm^{-1} (ν_{NH2}), 3390 cm^{-1} (ν_{NH2}), and 1192 cm^{-1} (ν_{C-N}) for AuNC@Cys, suggesting the successful attachment of cysteamine on the AuNCs surface. On the other hand, FT-IR spectrum for FITC (Figure S6, curve c) demonstrated four characteristic peaks located at 3406 cm^{-1}, 2035 cm^{-1}, 1620 cm^{-1}, and 1384 cm^{-1}, respectively, attributed to the vibration of O−H, N=C=S, C=O, and C−O−C. Meanwhile, three peaks were clearly observed at 1680 cm^{-1} ($\nu_{C=O}$), 1585 cm^{-1} (ν_{C-C}), and 947 cm^{-1} (ν_{O-H}) in the FT-IR spectrum of DEAC (Figure S6, curve d). After FITC and DEAC were conjugated on AuNCs surface, five peaks were clearly obtained at 3105 cm^{-1} (ν_{C-H}), 1705 cm^{-1} (Amide $\nu_{C=O}$), 1300 cm^{-1} (Amide ν_{C-N}), 1585 cm^{-1} (ν_{C-C}), and 1384 cm^{-1} (ν_{C-O-C}) in the FT-IR spectrum of AuNC@FITC@DEAC (Figure S6, curve e), confirming the successful modification of FITC and DEAC on AuNCs.

5. UV-vis absorption spectrum and photoemission spectrum of the luminescent Au NCs (Figure S7)

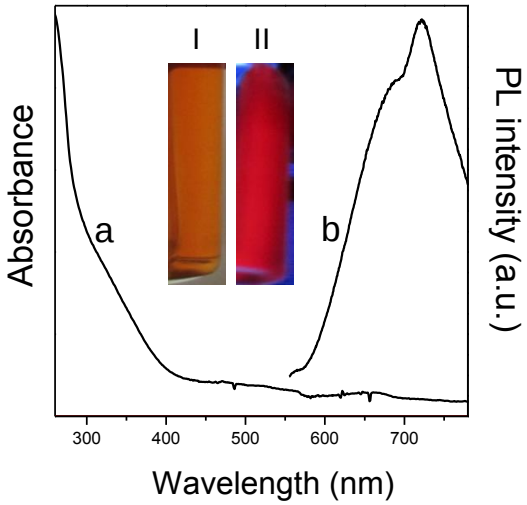


Figure S7. Absorption (line a) and emission spectra (line b, λ_{ex} = 405 nm.) of the luminescent Au NCs dispersed in water.(Insets) Digital photos of luminescent Au NCs in water under (I) visible and (II) UV light.

6. Quantum yield (Figure S8)

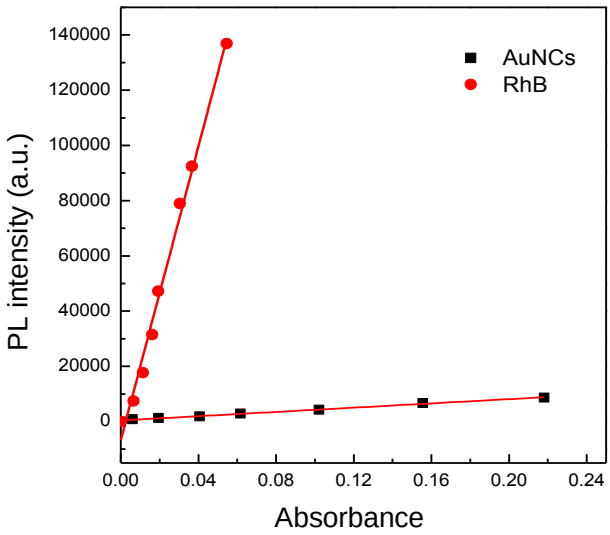


Figure S8. Photoluminescence (excited at 405 nm) and absorbance (at 722 nm) of AuNCs probe and rhodamine B (RhB).

7. Fluorescence responses of Au NCs towards H^+ ions and FITC towards Cu^{2+} ions (Figure S9)

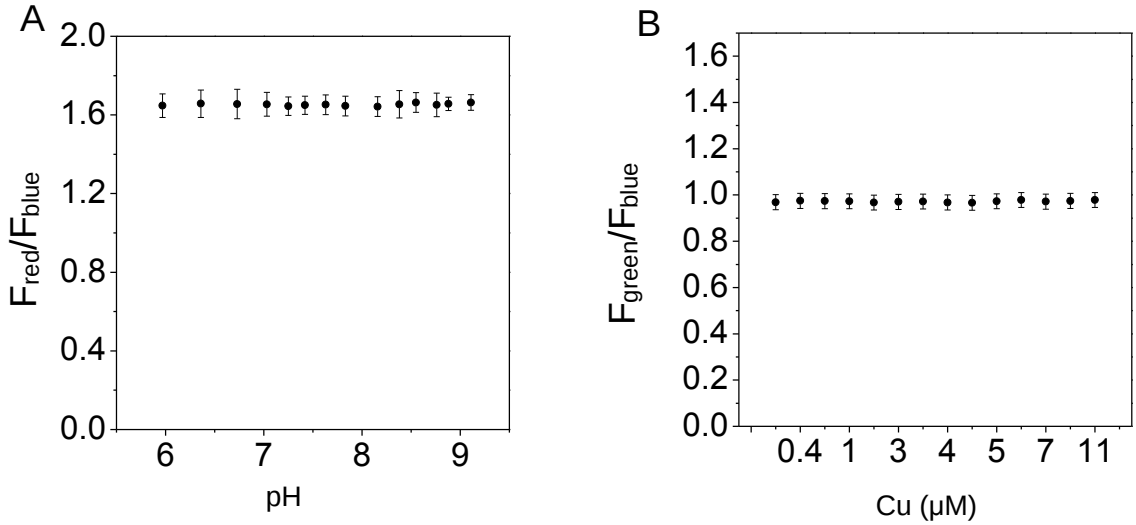


Figure S9. (A) Fluorescence responses of AuNCs towards H^+ ions. (B) Fluorescence responses of FITC towards Cu^{2+} ions.

8. Reaction Time (Figure S10)

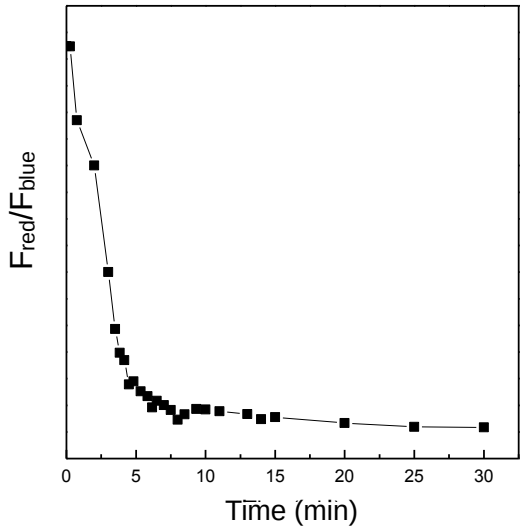


Figure S10. The relationship between the luminescence ratio (F_{red}/F_{blue}) and reaction time.

9. Time-Resolved Fluorescence measurements (Figure S11)

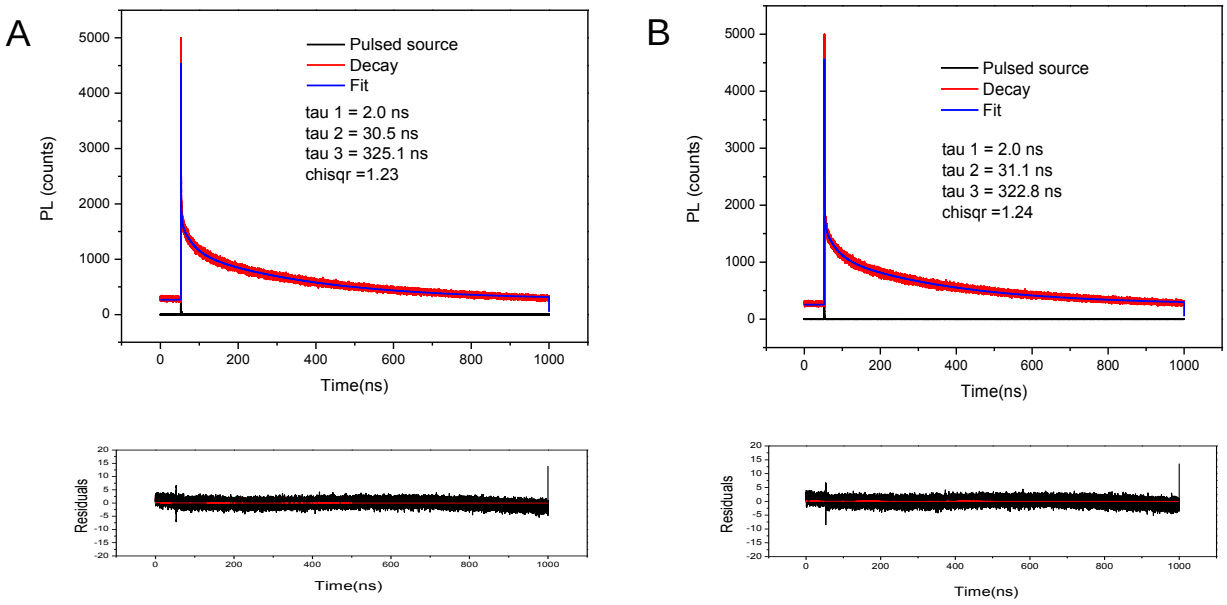
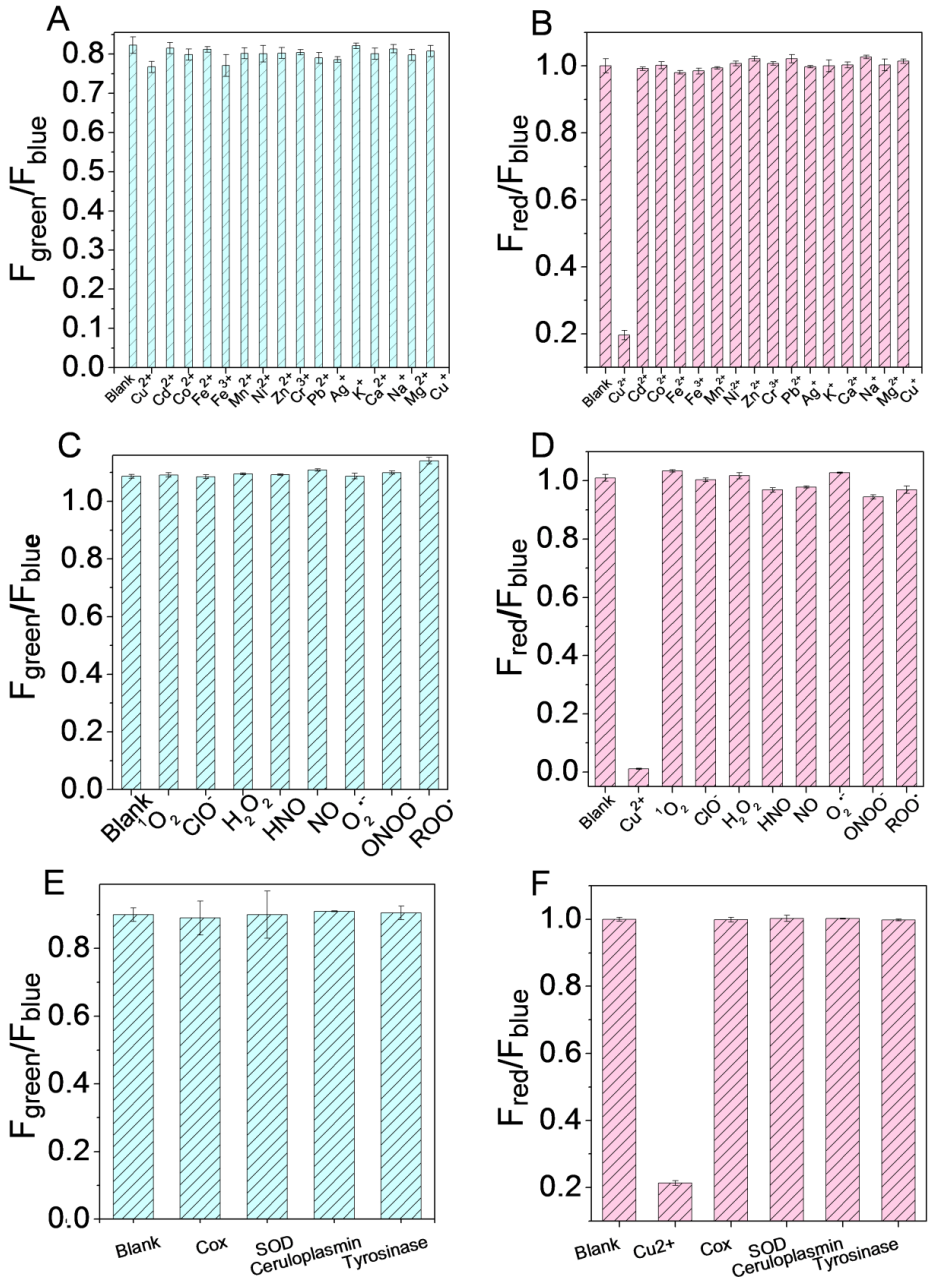


Figure S11. Photoluminescence lifetime measurements for AuNCs@FITC@DEAC probe: (A) before the addition of 2.5 μM $CuCl_2$, (B) after the addition of 2.5 μM $CuCl_2$.

10. Selectivity (Figure S12)



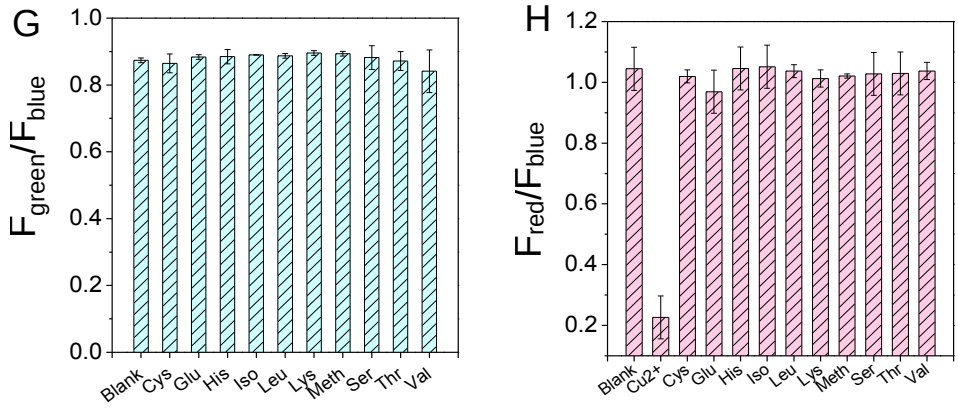


Figure S12. Selectivity of the AuNC@FITC@DEAC probe toward (A, B) metal ions, (C, D) various ROS, (E, F) Cu-containing proteins, and (G, H) amino acids. The cyan bars mean the addition of potential interferences to FITC. The pink bars mean the addition of potential interferences to AuNCs. (1 mM for Na^+, K^+, Ca^{2+}, and Mg^{2+}, 10 μM for other cations; 10 μM for ROS and RNS; 1 equiv of different Cu-containing proteins, including COX, SOD, ceruloplasmin, and tyrosinase; 10 μM for amino acids).

11. Photostability (Figure S13)

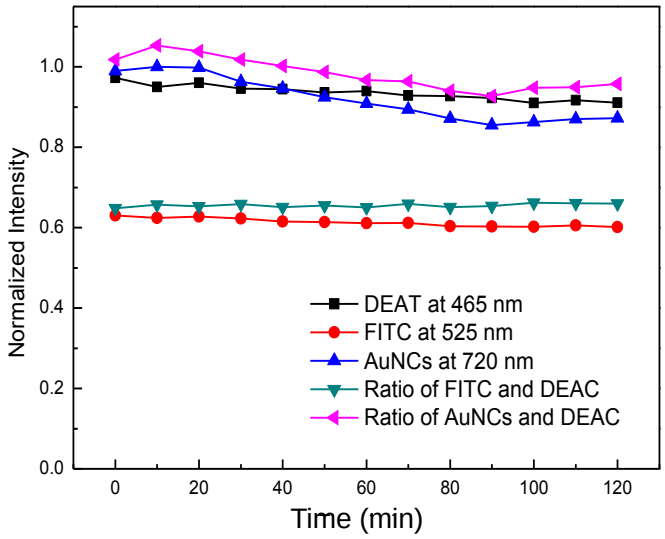


Figure S13. Time course of AuNCs@FITC@DEAC nanohybrid measured by fluorescence spectrophotometer (λ_{ex} = 405 nm, λ_{em} = 465 nm, 525 nm, and 720 nm).

12. MTT Assay (Figure S14)

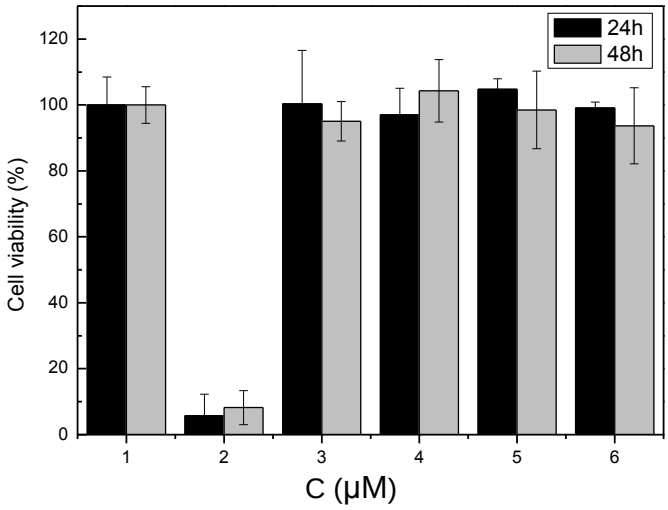


Figure S14. Cells viability values (%) estimated by MTT proliferation tests. Hela cells were incubated with the concentrations of AuNC@FITC@DEAC of (1) 0, (2) positive control, (3) 0.5, (4) 2.5, (5) 5.0, and (6)10.0 μM for 24 (black) and 48 h (gray) at 37 °C, respectively.

13. Apoptosis Assay (Figure S15)

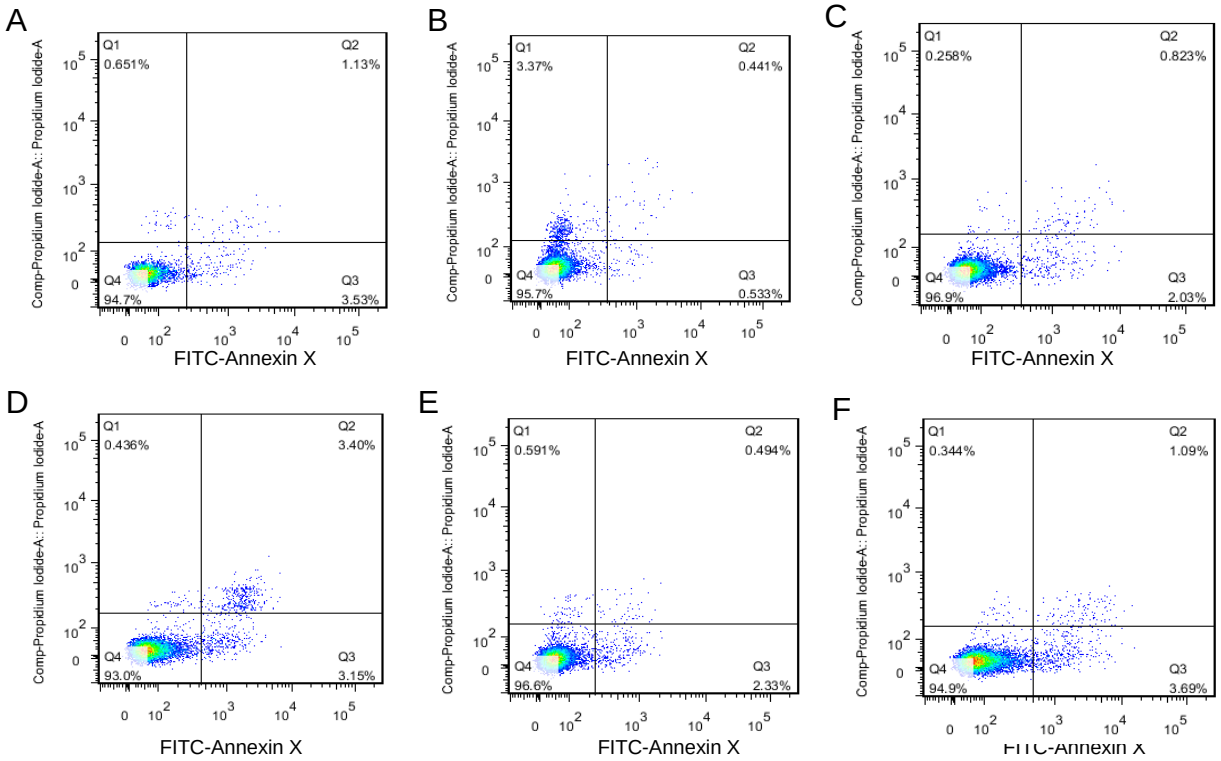


Figure S15. Apoptosis assay of Hela cells incubated with AuNCs@FITC@DEAC probes at concentrations of (A) 0, (B) 0.25, (C) 1.0, (D) 2.5, (E) 5.0, and (F) 10.0 μM for 48 h. Q1, Q2, and Q3 represent the regions of dead cells, late apoptotic cells, and early apoptotic cells, respectively.