Supporting Information

A multifunctional nanoprobe based on Au-Fe_3O_4 nanoparticles for multimodal and ultrasensitive detection of cancer cells

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Chemicals. $HAuCl_4 \cdot 3H_2O$, tert-butylamine-borane (TBAB), oleylamine, oleic acid, 1,2,3,4-tetrahydronaphthalene (tetralin), 1-octadecene, $Fe(CO)_5$, poly(ethylene glycol) bis(amine) (MW=3400), polyethylene glycol (MW=4000), and fluorescein isothiocyanate (FITC) were purchased from Sigma Aldrich. Folic acid, thioglycollic acid, dicyclohexylcarbodiimide (DCC), N-Hydroxysuccinimide (NHS), 3,3',5,5'-tetramethylbenzidine (TMB) and 3,4-dihydroxybenzaldehyde were from Aladdin in China. H_2O_2 was obtained from Beijing Chemicals Inc (Beijing, China). All chemicals were used without further purification, except DMF, DMSO, $CHCl_3$, triethylamine were used as anhydrous. Aqueous solutions were prepared with double-distilled water (ddH_2O) from a Millipore system (>18 MΩcm). 1,ω-Diaminopolyoxyethylene (MW=4000), PEG-3,4-dihydroxy benzyl amine (DIB-PEG-NH_2) and diethylenetriamine pentaacetic anhydride (DTPAA) were synthesized according to the published method.[1-3] All the dialysis bags (MWCO 8000-14000) were obtained from Shanghai Med.

Instrumentation. ^{1}H-NMR spectra were acquired with Varian 400 MHz NMR. The TEM measurements were carried out with Philips EM 420 (120kV) under ambient conditions deposition of the hexane or H_2O dispersions of the particles on amorphous carbon coated copper grids. The hysteresis loop was obtained at 300K with a LakeShore 7400 VSM system. The fluorescence spectra were recorded on a Hitachi RF-4500 spectrofluorophotometer. FT-IR characterization was carried out on a Therrno Mattson FT-IR spectrometer. UV-vis absorbance measurements experiments were carried out on a Lambda 950 spectrophotometer (Perkin Elmer, USA). Fluorescent images were taken on Zeiss Leica inverted epifluorescence/reflectance laser scanning confocal microscope.

Synthesis of Au NPs. 5 nm Au NPs are prepared by reduction of hydrogen tetrachloroaurate ($HAuCl_4 \cdot 3H_2O$) according to literature. Briefly, 0.1 g $HAuCl_4 \cdot 3H_2O$ was added to 10 mL tetralin, followed by 10 mL oleylamine to

form a yellow solution. The solution was then stirred for 10 min at room temperature. TBAB (1 mmol), tetralin (1 mL), and oleylamine (1 mL) were mixed by sonication and quickly injected into the above solution. The reaction mixture was further stirred at room temperature for 1 h. 40 mL ethanol was added to the solution, and gold particles were separated by centrifugation, washed by ethanol, and then redispersed in hexane.

Synthesis of Au-Fe_3O_4 nanoparticles. 5 nm Au NP seeds (20 mg) in oleylamine (2 mL) were added to a solution of 20 mL of 1-octadecene with oleic acid (1 mL). The mixture was stirred under a gental flow of nitrogen at 120^{0}C for 1 h. Then under a blanket of nitrogen, 0.10 mL $Fe(CO)_5$ was injected into the solution. The solution was heated to reflux (300 ^{0}C) and kept at that temperature for 25 min. After cooled down to room temperature, the particles were separated by adding 40 mL isopropanol, centrifuged and redispersed into hexane.

Synthesis of DIB-PEG-DTPA. 35.7 mg (0.1mmol) DTPAA were suspended in 5 mL of DMF, and then stirred at room temperature. After the mixture solution became clearly, a solution of **1a** (400 mg, 0.1 mmol) in 5 mL anhydrous $CHCl_3$ was added dropwise within 10 min, and continued to stir 12 hours. The product was precipitated by adding diethyl ethe, and collected by centrifugation at 4000 rpm. After washing with $CHCl_3$, DMF and diethyl ethe (1/1/5, v/v/v) 3 times, the product was then stored at -20 ^{0}C.

Synthesis of Au-Fe_3O_4-DIB-PEG-NH_2-DTPA (1b). DIB-PEG-DTPA (200 mg) was dissolved in $CHCl_3$ (10 mL), and then Au-Fe_3O_4 (30 mg) was added. The mixture was stirred overnight at room temperature. The modified Au-Fe_3O_4 nanoparticles were precipitated by adding hexane, and collected by centrifugation at 3000 rpm. The product was washed with $CHCl_3$ and hexane (1/5, v/v) 3 times. Finally, the product was redispersed in dry $CHCl_3$.

Synthesis of Eu: DTPA-PEG-Fe_3O_4-Au. **1b** (90 mg) was dispersed in 20 mL $CHCl_3$, and then a solution of the $EuCl_3.6H_2O$ (9 mg) in 5 mL ethanol was added. The mixture was stirred at 25 ^{0}C for 12 hours. The product was precipitated by adding n-hexane, and collected by centrifugation at 3000 rpm. After washing with $CHCl_3$, ethanol and hexane (1/1/5, v/v/v) 3 times, the product was redispersed in $CHCl_3$.

Synthesis of HS-PEG-NH_2 (1c). A solution of 0.092 g thioglycollic acid (1 mmol) in 20 mL of dry dimethylformamide (DMF) was added 0.40 g (1.5 mmol) of dicyclohexylcarbodiimide (DCC) and 0.14 g (1.2 mmol) of hydroxysuccinimide (NHS). After stirring for 14 hours at room temperature in the dark, the white precipitate was removed by centrifugation. The filtrate was mixed with 3.4 g (1.0 mmol) poly(ethylene glycol) bis(amine) 3400 and allowed to react at 25 ^{0}C for 14 hours. The solvent was evaporated to half under reduced pressure and precipitated out with diethyl ether (100 mL) and dried in vacuo. The product was then stored at -20 ^{0}C. ^{1}HNMR (CDCl3, 400 MHz): δ= 4.2 (s, 2H, SH2CH2), 3.84 (t, 2H, NH2CH2CH2), 3.70 (bs, ~187H, PEG3400), 3.41 (t, 2H, NH2CH2).

Synthesis of HS-PEH-NH-FA (1d). Folic acid (0.044 g, 0.1 mmol) was dissolved in 3 mL of dry dimethyl sulphoxide (DMSO) into which 0.040 g (0.15 mmol) of dicyclohexylcarbodiimide (DCC) and 0.014 g (0.12 mmol) of hydroxysuccinimide (NHS) were added. The reaction mixture was stirred for 14 hours at room temperature in the dark. The byproduct, dicyclohexylurea, was filtered off. The filtrate was mixed with 0.23 g (0.11 mmol) **1c** and allowed to react at 25 ^{0}C for 14 hours. The product was precipitated by adding diethyl ether (20 mL) and dried in vacuo. The product was then stored at -20 ^{0}C. ^{1}HNMR (DMSO, 400 MHz): δ=8.62 (1H, s, C8), 7.61 (2H, d, C13 and C15), 6.61 (2H, d, C12 and C16), 4.47 (2H, s, C9), 4.33 (2H, dd, C19), 3.80 (s, 2H, SH2CH2), 3.64 (t, 2H, NH2CH2CH2), 3.50 (bs, ~187H, PEG3400), 2.81 (t, 2H, NH2CH2), 2.65 (2H, m, C25), 2.55 (2H, m, C26), 2.20

(2H, m, C20), 1.80 ppm (2H, m, C19).

Synthesis of HS-PEH-NH-FITC (1e). FITC (3.9 mg, 10.0 μmol) and **1c** (23.0 mg, 11.0 μmol) was dissolved in 2 mL ethanol and 2 mL $CHCl_3$ at room temperature and stirred in the dark. After 4 h, the reaction was precipitated in diethyl ether (20 mL) and dried in vacuo. The product was then stored at -20 ^{0}C. ^{1}HNMR (CDCl3, 400 MHz): δ=7.15 (m, 6H, 12,14,15), 6.45~6.78 (m, 6H, 1,3,4,8,9,11) 4.2 (s, 2H, SH2CH2), 3.84 (t, 2H, NH2CH2CH2), 3.70 (bs, ~187H, PEG3400), 3.46 (t, 2H, NH2CH2).

Synthesis of Eu:DTPA-PEG-Fe_3O_4-Au-HS-PEG-FITC, FA (1f). 0.04 g of Eu: DTPA-PEG-Fe_3O_4-Au in 5 mL of $CHCl_3$ was added to 0.05 g of **1d** and 0.02 g **1e** in $CHCl_3$. The mixture was stirred 2h in the dark at room temperature. The particles were then collected by centrifugation, washed with $CHCl_3$ and hexane (1:5 V/V), and then dispersed in water and dialysised with H_2O for 24 h to remove unreacted organic molecules.

1f Characterization. Magnetic measurements show minimal changes in the magnetic properties observed in the as-synthesized Au-Fe_3O_4 Nps and **1f** (Figure S2). **1f** had a 53 nm hydrodynamic diameter, as measured by Dynamic light scattering (Figure S3). The zeta potential value (ζ) of **1f** was -3.1± 0.1 mV, which was in agreement with the measured values of Bae et al.[4] In addition, the hydrodynamic diameters of the **1f** dispersed in PBS for 72 h were unchanged (Figure S4), suggesting the robust stabilization of **1f**. Furthermore, the 1f also has high photostability (Figure S5).

The covalently grafted Eu complex onto Fe_3O_4 side was confirmed by FT-IR and fluorescence measurements. The FT-IR spectrum (Figure S6) of oleic acid and oleyl amine-coated Au-Fe_3O_4 Nps showed strong absorption bands at 2850 cm^{-1} and 2915 cm^{-1}, arising from symmetric and asymmetric C-H stretch in the oleyl chains, respectively. After the attachment of DTPA ligands, the characteristic bands of oleic acid remarkably decreased, and a strong characteristic peak at 1625 cm^{-1} for C=O stretch vibrations was observed. Conjugation of FA to Au side was confirmed by FT-IR measurements. A new peak at 1694 cm^{-1} in the FT-IR spectrum indicates the presence of CO-NH groups on the Au side, which is further supported by a characteristic absorption peak of FA at 283 nm and 350 nm in the UV spectra (Figure S8). **1f** shows fluorescent emission at 513 nm, blue-shifted from the free FITC emission at 525 nm (Figure S7). However, Eu:DTPA-PEG-Fe_3O_4-Au-HS-PEG-FITC, without FITC, exhibits no fluorescent emission, which indicates that FITC is anchored on the Au side. The absorbance at 493 nm in the UV (Figure S8) spectra also proved that FITC was anchored on the Au side.The final conjugate (**1f**) can be dispersed in PBS, and there are no NP morphology change and no evidence of aggregation after surface modification (Figure S1B).

Bioassay. Kinetic measurements of different NPs for oxidation of TMB in the presence of H_2O_2 were performed using UV-vis spectrophotometer in time course mode at 653 nm. Experiments were carried in a reaction volume of 2 mL buffer solution (0.2 M HAc-NaAc, pH=4.0), with 0.5 mL H_2O_2 (different concentrations), using 30 μL TMB(20 mM) as substrates, and 20 μL NPs (Fe, 12.4 mM) as catalyst. The kinetic parameters were calculated based on the function $v = v_{max} C /(K_m + C)$, where v is the initial reaction rate, v_{max} is the maximal reaction rate, C is the substrate concentration and K_m is the Michaelis-Menten constant. The v_{max} was converted into molar change from UV absorbance according to the formula A = εlc (A is the absorbance, ε is the absorbance coefficient, l is the path length and c is the molar concentration) with ε =3.9×10^4 $M^{-1}cm^{-1}$ and l =10 mm.

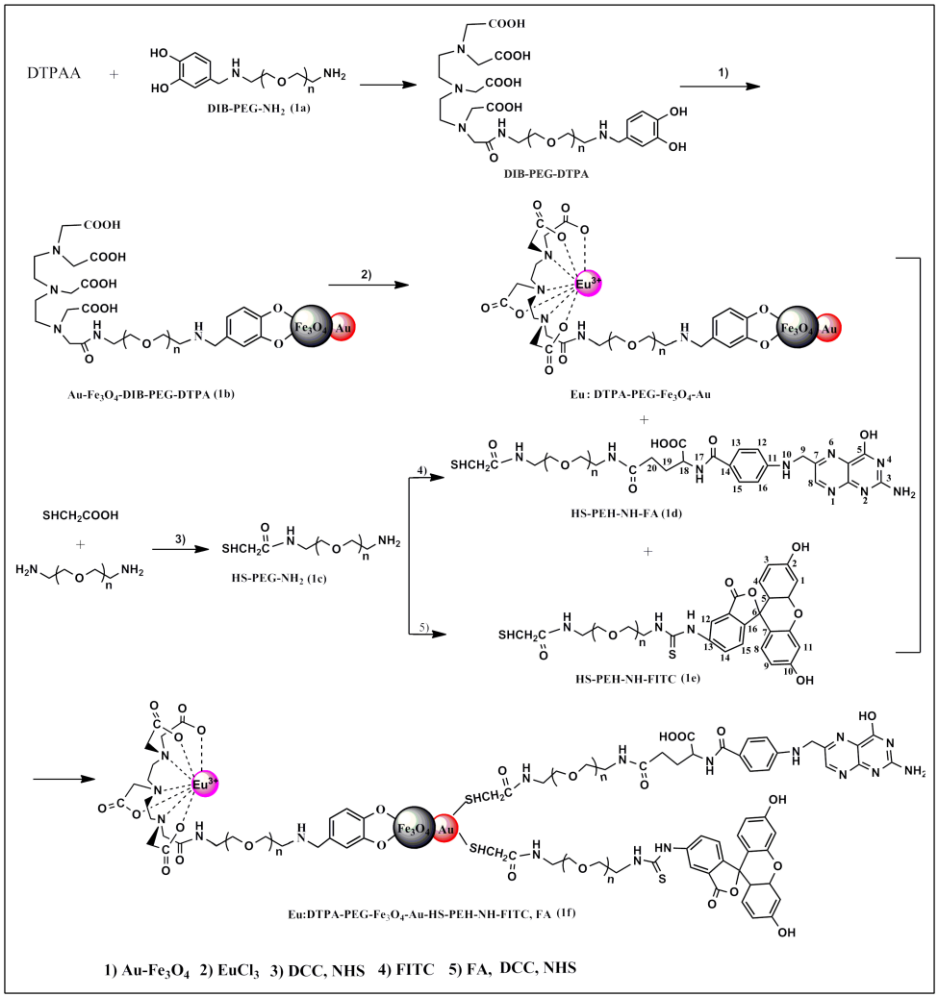
Cytotoxicity assay. In vitro cytotoxicity of the **1f** was evaluated by performing methyl thiazolyl tetrazolium (MTT) assay of the HeLa cells incubated with the particles. Cells were seeded into a 96-well cell culture plate with a density of 5×10^4 cells/well in DMEM with 10% FBS at 37 ^{0}C under 5% CO_2 for 24 h. Then, the cells were incubated with the **1f** with different concentrations (1, 5, 50, 100 μg/mL in DMEM) for 24 h，48h and 72h respectively at 37^{0}C under 5% CO_2. Thereafter, MTT (20 mL, 5 mg/mL) was added to each well and the plate was incubated for 4 h at 37 ^{0}C. After the addition of dimethyl sulfoxide(DMSO, 100 μL/well), the cell plate was allowed to stand at 37^{0}C for 15 min. The optical density was measured at 492 nm using a microplate reader (Shanghai Sanco Instrument Co., Ltd. 318C-microplate reader).

Cell imaging. HeLa cells and NIH-3T3 cells were plated in 6-well plate with a coverslip in it and cultured 1 day in DMEM medium without FBS. The medium was removed and the cells were washed twice with DMEM medium. Then DMEM medium (1mL) solution containing 0.5 μM **1f** was added to the cells and incubated for 1 h at 37 ^{0}C with 5 % CO_2. Then the cells were washed twice with PBS (1 mL) and illuminated under a Zeiss Leica inverted epifluorescence/reflectance laser scanning confocal microscope (Ex 482 nm, Em 515 nm).

Cell MRI. Hela cells (5×10^6) were incubated with **1f** with different concentrations for 6 h at 37 ^{0}C. For comparison, NIH-3T3 cells, which do not overexpress the folate receptor (5×10^6), were incubated with **1f** at different concentrations under similar conditions. After incubation, the cells were washed with PBS buffer three times and resuspended in PBS buffer with a cell density of 1×10^6 cells/mL before MR imaging. All MR imaging measurements were performed with a 3.0 T systems (3 T Siemens Magnetom Trio). T2-weighted images were acquired using a multi-slice spin echo sequence. The parameters were set as follows: field of view (FOV) 120 mm, base resolution, 192×160, slice thickness 1.5mm, multiple echo times (TE) 20, 40, 60, 80, 100, 120, 140 ms, repetition time (TR) 2000 ms.

Study peroxidase-like activities of 1f. Previous studies have suggested that the catalytic activity of NPs based peroxidase mimetics and horseradish peroxidase (HRP) depends on pH, temperature, and H_2O_2 concentration. With this mind, we measured the peroxidase-like activity of **1f** while varying the pH from 2.5 to 9.0, the temperature from 0 ^{0}C to 60 ^{0}C, and the H_2O_2 concentration from 0 to 20 mM. The experimental results in Figure S11 indicated that the catalytic activity of **1f** increased along with the increase in pH from 2.5 to 4.0, above which it sharply decreased with the increasing pH value. Thus, the optimized pH was 4.0. Likewise, the optimal temperature and H_2O_2 concentration were 35 ^{0}C and 5 mM, respectively (Figure S11).

To better understand and compare the peroxidase-like activities of the different kinds of NPs reported in this paper, we determined the steady-state kinetic for the reaction by changing the concentration of H_2O_2 in this oxidation reaction. From Figure S10, the typical Michaelis-Menten curves were observed for theses Nps. The Michaelis-Menten constant (K_m) and the maximum initial velocity (v_{max}) were obtained using Lineweaver-Burk plot[5] (Figure S12) and listed in Table S1. The apparent K_m value for the dumbbell NPs is only half the value of that from the physical mixture of Fe_3O_4 and Au NPs, indicating that the dumbbell NPs have a much higher affinity for TMB than the physical mixture of Fe_3O_4 and Au NPs.



Scheme S1. Synthetic route of Eu:DTPA-PEG-Fe_3O_4-Au-HS-PEG-FITC,FA (**1f**).

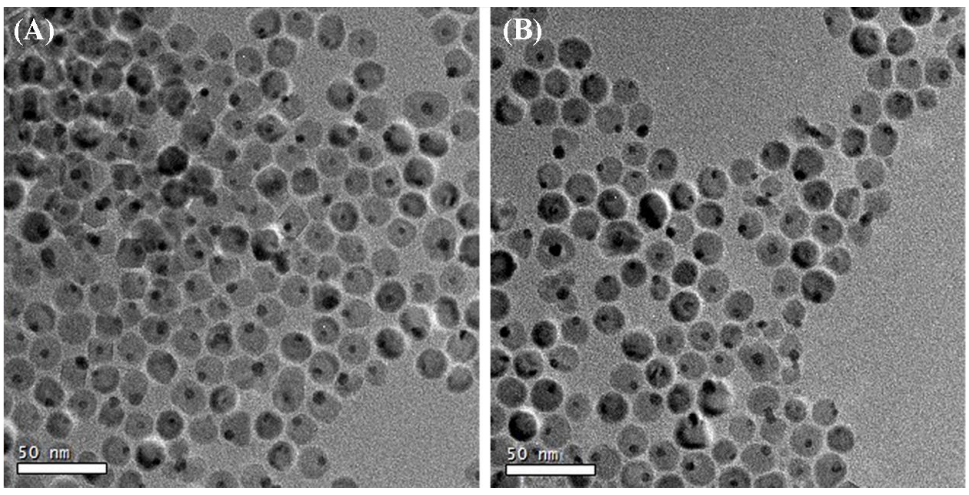


Fig S1. TEM images of (A) as-synthesized Au-Fe_3O_4 nanoparticles dispersed in hexane; (B) **1f** dispersed in H_2O.

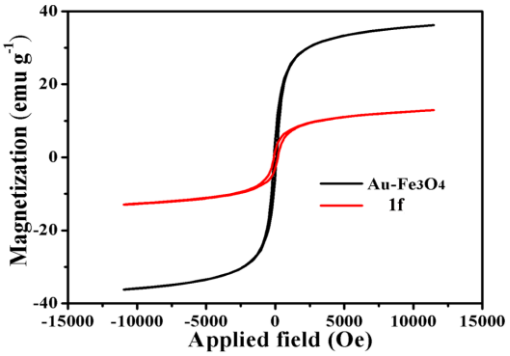


Fig S2. Magnetization measured at 300 K. The saturated magnetization (*Ms*) of as-synthesized Au–Fe_3O_4 Nps and **1f** are 36.2 and 17.6 emu/g.

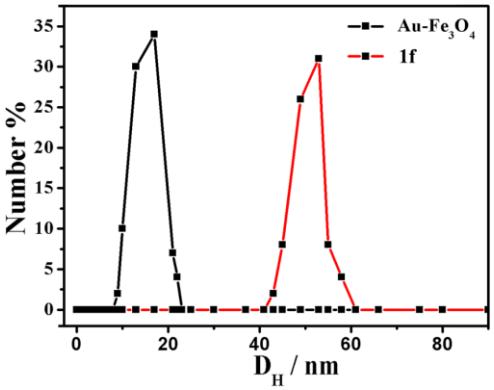


Fig S3. Hydrodynamic diameters measured by DLS for the as-synthesized Au-Fe_3O_4 NPs dispersed in hexane and the **1f** dispersed in PBS.

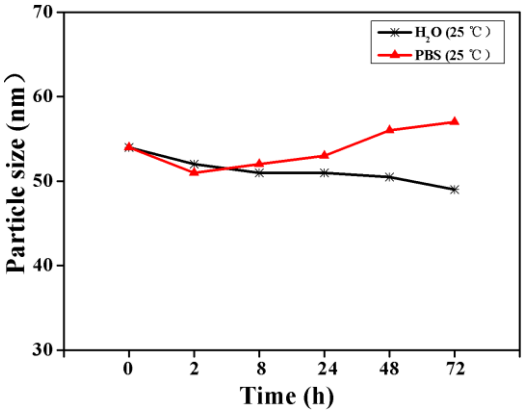


Fig S4.The hydrodynamic diameters of the **1f** dispersed in PBS and H_2O for 72 h were changed slightly, suggesting the robust stabilization of **1f**.

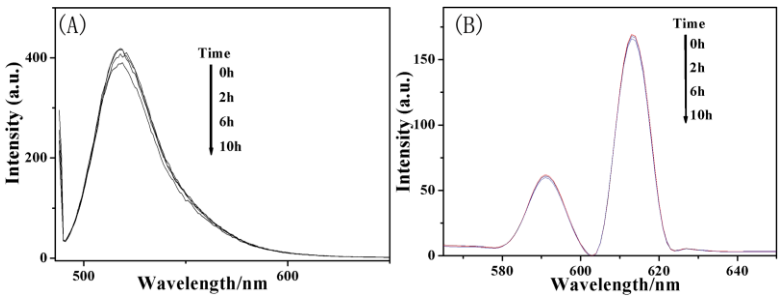


Fig S5. The fluorescence intensity of **1f** in H2O at different time (A: FITC, B: Eu complex).

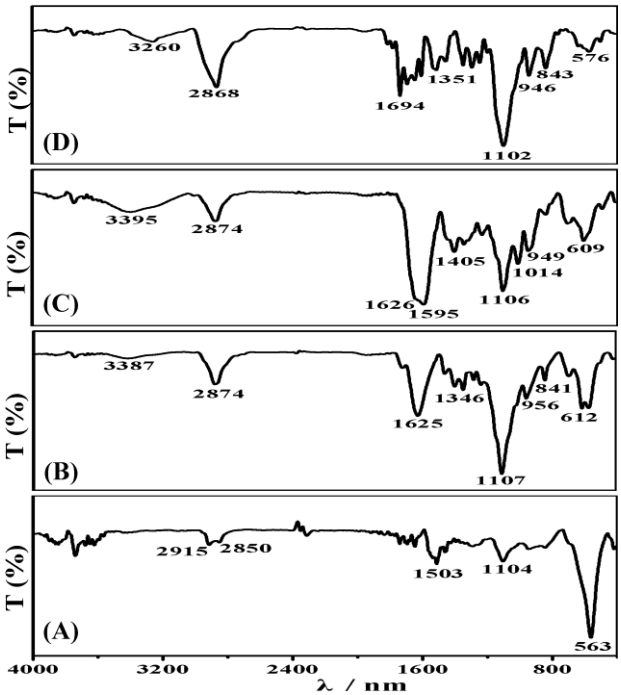


Fig S6. The IR spectra of (A) as-synthesized Au-Fe_3O_4 nanoparticles; (B) Au-Fe_3O_4-PEG-DTPA; (C) Eu:DTPA-PEG-Fe_3O_4-Au; (D) FA-PEG-SH-Au-Fe_3O_4.

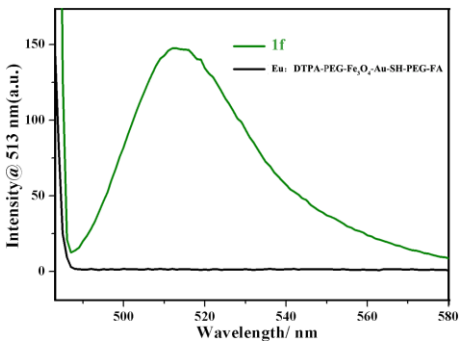


Fig S7. Fluorescence spectra of Eu:DTPA-PEG-Fe_3O_4-Au-HS-PEG-FA and **1f** in aqueous solution.

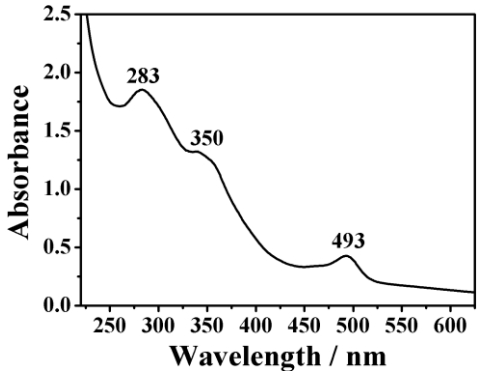


Fig S8. UV-vis detection of **1f**. The absorbance peaks at 283 nm and 350 nm belong to folic acid (FA), and 493 nm to fluorescein isothiocyanate (FITC).

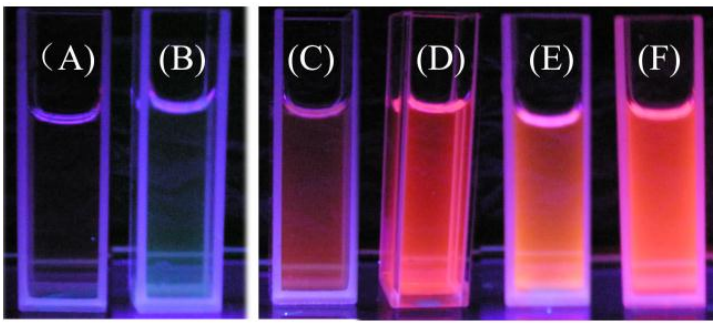


Fig S9. Different fluorescence intensity after adding different concentration of CaDPA (under a UV-lamp). From left to right: (A) H_2O without **1f**, (B) H_2O without CaDPA, (C)~(H) increasing concentrations of CaDPA.

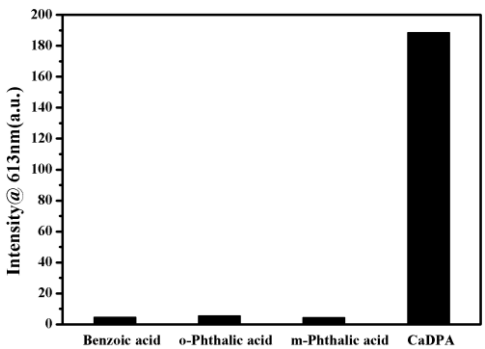


Fig S10. Plot of fluorescence intensity of sensor **1f** with CaDPA, benzoic acid, m-phthalic acid and o-phthalic acid (from left to right, concentration: 1 mM; monitored at the maximum fluorescence intensity).

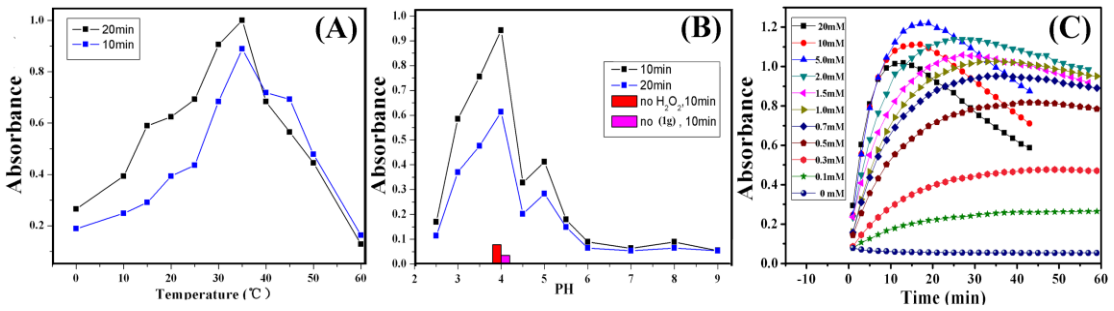


Fig S11. The peroxidase-like activity of the **1f** is pH, temperature, H_2O_2 concentration dependent. Experiments were carried out using 20 μL **1f** (12.4 mM) in a reaction volume of 2.0 mL HAc-NaAc buffer (0.2 M), with 30 μL TMB (15.0 mM) as substrate. The H_2O_2 concentration was 2.0 mM, PH=4.0, and the temperature=35℃, unless otherwise stated. (A) **1f** showed an optimal temperature around 35 ℃; (B) optimal PH=4.0; (C) optimal concentration of H_2O_2 was 2.0 mM (All the concentration was defined with the original condition).

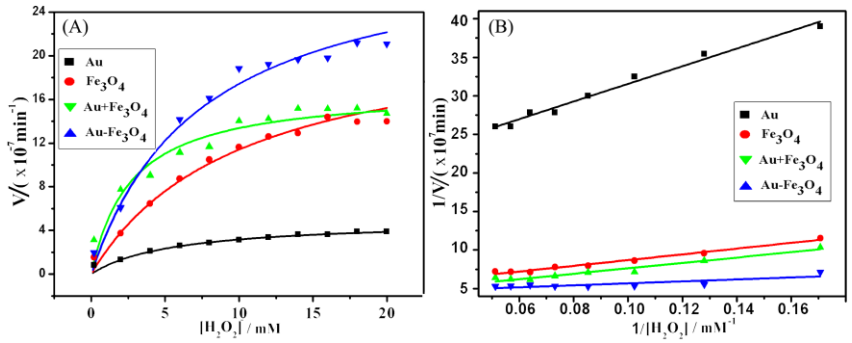


Fig S12. A) The reaction rate of different NPs in 1.5 mL PBS (pH = 4.5) in the presence of 0.3 mM TMB and H_2O_2 with different concentrations at room temperature. B) Double-reciprocal plots of activities of different NPs at a fixed concentration of TMB (0.3 mM) and varying concentrations of H_2O.

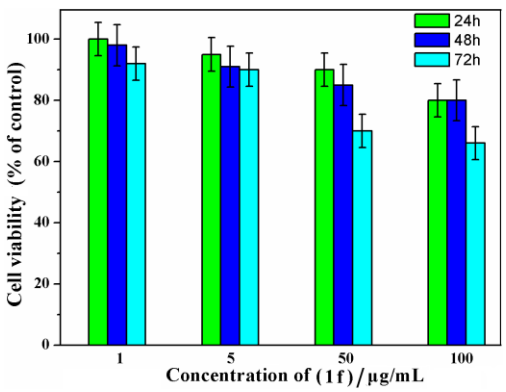


Fig S13. In vitro cell viability of Hela cells incubated with **1f** with different concentrations (1, 5, 50, 100 μg/mL) incubated for 24, 48 and 72 h at 37 ℃.

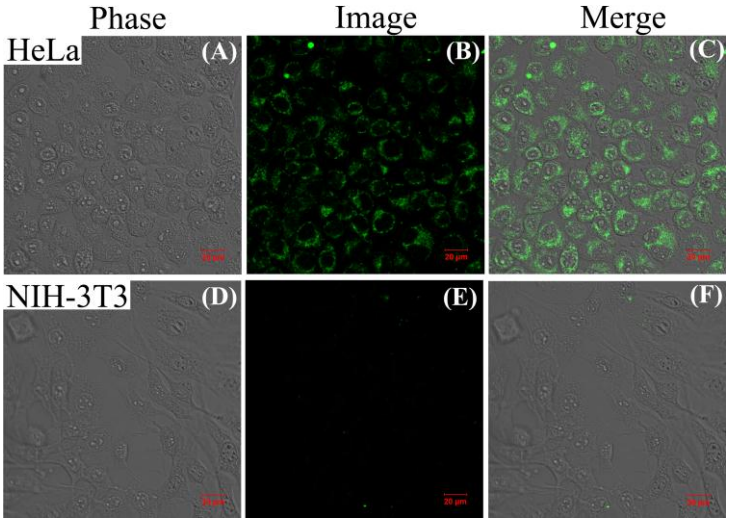


Fig S14. Tests of specificity. Bright field (A), confocal fluorescence (B) and merge images (C) of **1f** in Hela cells. Bright field (D), confocal fluorescence (E) and merge images (F) of **1f** in NIH-3T3 cells.

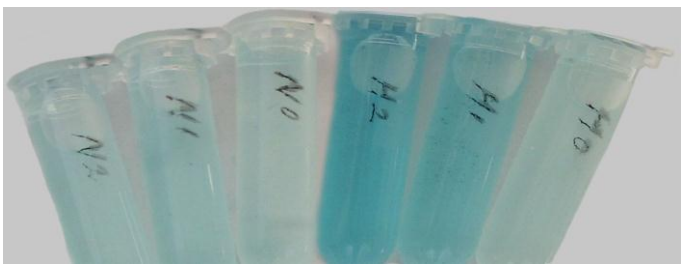


Fig S15. **1f** for cancer cell detection. Different kinds of cells (N, H refer to NIH-3T3 cell, HeLa cell, respectively) and different cell number (0, 1, 2 refer to 0 cells, 10^4 cells, 10^8 cells, respectively) can be distinguished by naked eyes. (Condition: different number of these two kinds cells was incubated with 20 μL **1f** in PBS+10% FBS at 37 ℃ incubator for 2 hours. The cells were then washed with PBS three times and centrifuged in eppendorf tube at 10000 rpm for 30 s. Sbusquently, incubated with TMB (10.0 mM) for 30 minutes.)

Table S1. Comparison of the Kinetic Parameters of Different NPs (TMB as a substrate). K_m is the Michaelis constant, v_{max} is the maximal reaction rate.

	K_m [mM]	v_{max} [M min^{-1}]
Au-Fe_3O_4 dumbell	0.021	2.11×10^{-6}
Au and Fe_3O_4 mixture	0.059	1.51×10^{-6}
Fe_3O_4	0.062	1.43×10^{-6}
Au	0.190	3.86×10^{-7}

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