Electronic Supplementary Information

Simultaneous fluorescence imaging of selenol and hydrogen peroxide under normoxia and hypoxia in HepG2 cells and in vivo

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Contents

1. Materials and instruments

Chloroauric acid ($HAuCl_4 \cdot 3H_2O$, 99.99 %), sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), sodium dodecylsulfate (SDS), cesium carbonate (Cs_2CO_3), potassium iodide (KI), sodium acetate (NaOAc), acetic anhydride (Ac_2O), acetonitrile and mercaptoethanol (ME) were all of analytical grade and purchased from China National Pharmaceutical Group Corp. (Shanghai, China). 5-FAM-labled peptide chains 5-FAM-Gly-Cys was synthesized and characterized by Kangbei Biological Chemical Company (Ningbo, China). Cysteine (Cys), selenocystine ($(CysSe)_2$), glutathione (GSH), sodium selenite (Na_2SeO_3), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and selenoprotein TrxR were obtained from Sigma-Aldrich. 4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl bromide was purchased from Tianjin Heowns Biochemical Technology Co., Ltd. (Tianjin, China). All solutions were prepared using ultrapure water (18.2 MΩ·cm, Millipore). Anhydrous dimethyl sulfoxide (DMSO) was obtained by molecular sieve dehydration. Other solvents and regents were of analytical grade and used without further purification. The pH buffer solutions (PBS, pH 3−9) were prepared using 0.1 M citric acid and 0.2 M disodium hydrogen phosphate adjusted with 2 M NaOH or HCl solutions. The human hepatocellular liver carcinoma cell line, HepG2, was obtained from the Committee on Type Culture Collection of the Chinese Academy of Sciences.

Transmission electron microscopic (TEM) images were taken on a JEOL transmission electron microscope (JEM-100CX II) operated at 200 kV. UV-vis absorption spectra were measured on a UV-visible spectrophotometer (UV-1700 Pharmaspec, Shimadzu, Japan). X-ray photoelectron spectroscopy (XPS, ESCALAB250Xi, ThermoFisher Scientific) was used to determine the Au 4f, S 2p and Se 3d binding energies (BEs) of surface species using Al Kα X-ray sources (150 W). ^{1}H-NMR and ^{13}C-NMR spectra were taken on a Varian Advance 600-MHz or Bruker Advance 300-MHz spectrometer, δ values are in ppm relative to TMS. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai LeiCi Device Works, Shanghai, China) with a combined glass-calomel electrode. High-resolution mass spectral analyses (HRMS) were carried out on Bruker maxis UHR-TOF Ultra High Resolution Quadrupole-time of flight mass spectrometer (Bruker Co., Ltd., Germany). Fluorescence spectra were obtained on the FLS-920 Edinburgh Fluorescence Spectrometer with a Xenon lamp. Confocal fluorescence imaging was performed with a TCS SP5 confocal laser scanning microscopy (CLSM, Leica Co., Ltd. Germany) with an objective lens (×40). In vivo fluorescence imaging was

conducted on the in vivo imaging system (IVIS, PerkinElmer). Absorbance was measured with the microplate reader (Synergy 2, Biotek, USA) in the MTT assay.

2. Experimental section

2.1 Preparation of Gold Nanoparticles (Au NPs).

Au NPs were prepared according to the classic method proposed by M. J. Natan with minor modification. [1] All glassware was cleaned in aqua regia (HCl/HNO_3, 3:1), rinsed with ultrapure water, and oven-dried before the experiments. After that, 100 mL $HAuCl_4$ (0.01 wt%) was heated to boiling with vigorous stirring, then 3.6 mL trisodium citrate (1 wt%) was added under stirring. The color of the solution turned from bright yellow to burgundy. Boiling was continued for additional 20 min. After stopping heating, the colloid was stirred until it reached room temperature. Then the colloid was filtered through a 0.45 μm Millipore membrane filter. The prepared Au NPs were stored at 4 °C. The particle size and morphology of the AuNPs were characterized with TEM and images indicated that the particle sizes are 13 ± 2 nm (100 particles sampled, Fig. S1).

2.2 Assembly of the 5-FAM-peptide-AuNPs nanoprobe. The peptide 5-FAM-Gly-Cys was synthesized and characterized by Kangbei Biological Chemical Company (Ningbo, China). SDS solution (10 %) was added dropwise to the as-synthesized Au NPs colloid (13 nM) under slow stirring, and the final concentration of SDS was 0.1 %. After 30 min, the peptide 5-FAM-Gly-Cys solution was added to the mixture, and the final mole ratio of Au NPs to the peptide was maintained at 1:300. After being continuously stirred away from light for 48 h, the resulted nanoprobe was centrifuged (14000 rpm, 20 min) and resuspended in PBS three times. Then, the nanoprobe was sterilized using a 0.22 μm acetate syringe filter and resuspended in PBS as a stock solution stored at 4 ^oC away from light.

2.3 Synthesis route of QCy7-H_2O_2

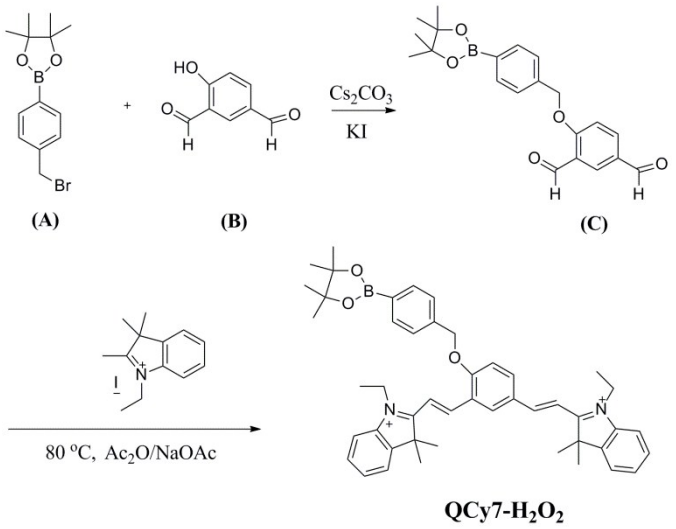
According to previous report [2], QCy7-H_2O_2 was synthesized (Scheme S1) and obtained as a brown solid, the synthesis procedure was as follows. All reactions requiring anhydrous conditions were performed under an Argon atmosphere.

Compound C:

A mixture of commercially available **Compound A** (0.89 g, 3 mmol) and Cs_2CO_3 (0.98 g, 3 mmol) was dissolved in dry acetonitrile and the solution was stirred at 60 ^oC for 10 min. Then, commercially available **Compound B** (0.23 g, 1.5 mmol) and KI (0.25 g, 1.5 mmol) were added. The reaction mixture was stirred for 6 h at 60 ^oC and monitored by thin layer chromatography (TLC). After completion, the reaction mixture was washed with dry acetonitrile three times, and the filtrate was concentrated to give **Compound C** as white solid.

QCy7-H_2O_2:

A mixture of **Compound C** (0.37 g, 1 mmol), NaOAc (0.16 g, 2 mmol) and indolium-iodide (0.63 g, 2 mmol, homemade) was dissolved in Ac_2O. The reaction mixture was stirred for 30 min at 80 °C. After completion, the reaction mixture was washed with cold diethyl ether to yield brown solid. The crude product was purified by silica gel chromatography on silica gel ($CH_3OH/CHCl_2$ =1:10, vol. ratio) give QCy7-H_2O_2 as a brown solid. HRMS (Fig. S9): *m/z* calc. for $C_{47}H_{55}BN_2O_3^{2+}$: 353.2151; found: 353.2124 $[M]^{2+}$.



Scheme S1 Synthesis route of QCy7-H_2O_2

2.4 XPS characterization of 5-FAM-peptide-AuNPs and the product of 5-FAM-peptide-AuNPs with Sec.

The two samples were precipitated through centrifugation (14000 rpm, 20 min, 4 °C) and washed with ultrapure water three times. Before characterization, the samples were dried in the drying cabinet at 80 °C for 12 h. The survey scans were acquired with analyzer pass energy of 100 eV and the step size was 1.0 eV. The narrow scans with high resolution were acquired with analyzer pass energy of 30 eV and the step size was 0.1 eV.

2.5 Fluorescence analysis. The fluorescence analysis was carried out on FLS-920 Edinburgh with a 1.0 cm quartz cells at the slits of 10/10 nm. For the experiment on the spectroscopic property of QCy7-H_2O_2, the probe stock solution was diluted to 1 μM with 0.01 M PBS and incubated with 200 μM H_2O_2 for 30 min at 37 °C, and then the excitation and emission spectra were measured. For the fluorescence response to H_2O_2, the concentration of QCy7-H_2O_2 was 10 μM. For the fluorescence response to selenol, Sec was generated *in situ* by means of the reaction of Cys and $(CysSe)_2$ at an equivalence ratio of 1:2 at 37 °C 10 min before use. After the probe stock solution was diluted to 1 nM with 0.01 M PBS buffer solution (pH=7.4), *in situ* generated Sec was added into the quartz cells. For the simultaneous fluorescence

responses of the two probes, with an excitation at 488 nm, fluorescence intensities at 520 and 710 nm were measured.

2.6 Cell culture. HepG2 cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin). Cells were maintained in a humidified incubator at 37 ^{o}C, in 5 % CO_2 / 20 % O_2 and 5 % CO_2 /1 % O_2 for normoxic and hypoxic conditions, respectively.

2.7 Confocal fluorescence imaging. Under both normoxic and hypoxic conditions, the HepG2 cells were pre-incubated with Na_2SeO_3 (10 μM) for 0, 1, 6 and 12 h. After the excess Na_2SeO_3 was removed, the nanoprobe 5-FAM-peptide-AuNPs (1 nM) was added and incubated for 2 h. Subsequently, QCy7-H_2O_2 was added and incubated for 30 min. Prior to imaging, the medium was removed. The responses of the two probes in living cells were investigated using CLSM through two channels. After excitation at 488 nm, emissions were collected using a META detector between 490 to 590 nm and 600 to 700 nm.

2.8 MTT assay. HepG2 cells (1 × 106 cells/well) were dispersed within 96-well microtiter plates with 200 μL per well. Plates were maintained at 37 °C in a 5% CO_2/ 20% O_2 air incubator for 24 h. After the supernate was discarded, the HepG2 cells were incubated with nanoprobe 5-FAM-peptide-AuNPs (1 nM) and AuNPs (1 nM) for 3, 6, 12 and 24 h. The HepG2 cells incubated with the culture medium served as the blank control. With the supernate removed, 100 μL MTT solutions (0.5 mg·mL^{-1} in PBS) were added to each well away from light. After 4 h, the remaining MTT solution was removed, and 150 μL DMSO was added to each well to dissolve the formed formazan crystals. The absorbance was measured at 488 nm with the microplate reader. The experiment was repeated three times, and the data are shown as the mean ± SD.

2.9 Animal models. All the experiments in vivo were performed in compliance with the Guidelines for the Care and Use of Research Animals (1996), US National Research Council, and approved by the local Animal Care and Use Committee. All animal experiments involved 6-8-week-old Kunmin male mice (Shanghai SLAC Laboratory Animal Co., Ltd.). During procedures, the mice were injected 1 × 10^6 H22 cells into the enterocoelia of each Kunmin mouse, ascites were formed after 5 to 7 days, which were further used after three passages. 6-8-week-old mice received a subcutaneous injection of 1 × 10^6 H22 ascites tumor cells into the axillary lateral subcutaneous of right forelimbs. Tumors were allowed to grow over a period of 15 to 20 days until reaching 0.5-1.5 cm in diameter and then used in the in vivo experiments.

2.10 Imaging in vivo. Kumin mice bearing subcutaneously implanted H22 tumors were used in the in vivo imaging. Kumin mice bearing tumors were subcutaneously injected with Na_2SeO_3 (10 μM); after a specific time, they were then injected with the buffer solutions containing fluorescence probe (1 nM 5-

FAM-peptide-AuNPs for imaging selenol and 10 μM QCy7-H_2O_2 for imaging H_2O_2). After fifteen minutes, mice were imaged immediately on the IVIS spectrum imaging system using autoexposure with excitation at 480 ± 10 nm, emission spectral were collected at 520 and 710 ± 10 nm for 5-FAM-peptide-AuNPs and QCy7-H_2O_2, respectively.

3. TEM characterization of Au NPs and 5-FAM-peptide-AuNPs

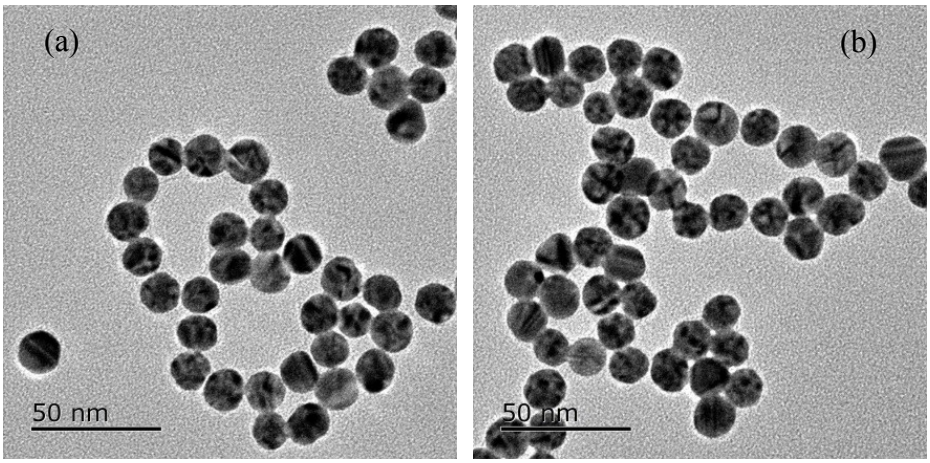


Fig. S1 TEM images of Au NPs (a) and 5-FAM-peptide-AuNPs (b).

4. UV-vis absorption spectra of Au NPs and 5-FAM-peptide-AuNPs

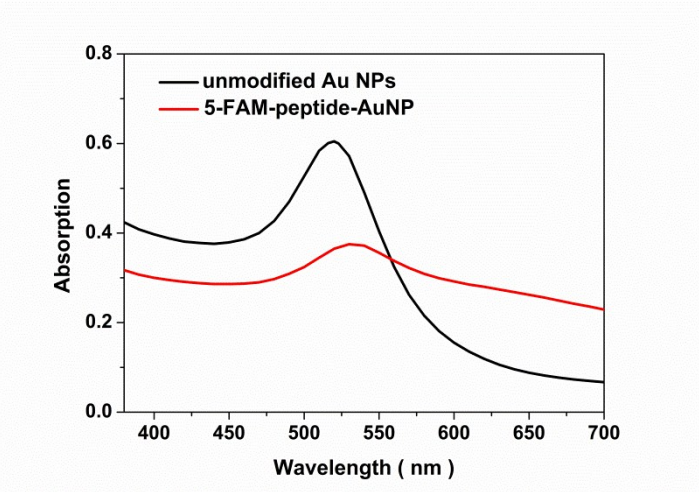


Fig. S2 UV-vis absorption spectra of Au NPs and 5-FAM-peptide-AuNPs.

5. Quantification of 5-FAM-Gly-Cys loading on 5-FAM-peptide-AuNPs

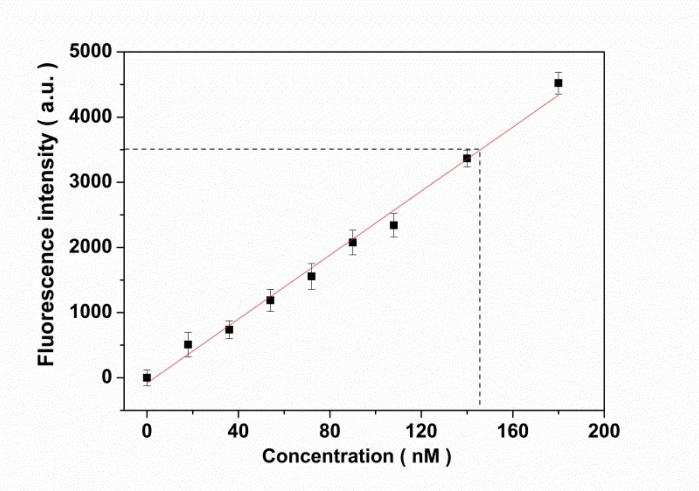


Fig. S3 Standard linear calibration curves of the peptides 5-FAM-Gly-Cys. Error bars were estimated from three replicate measurements. Each Au NP carried approximately 142 ± 1 5-FAM -labeled peptides.

6. XPS characterization of 5-FAM-peptide-AuNPs

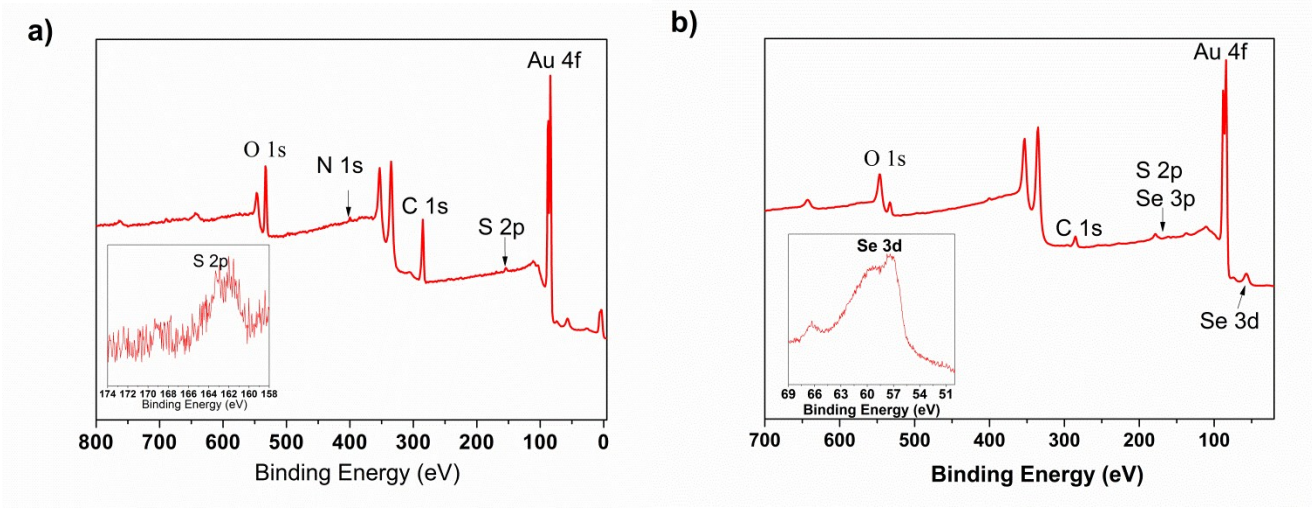


Fig. S4 XPS investigations on 5-FAM-peptide-AuNPs (sample 1, a) and the product of 5-FAM-peptide-AuNPs with Sec (sample 2, b). The inset in (a) is the S 2p peak of sample 1. The inset in (b) is the Se 3d peak of sample 2.

7. The spectroscopic property of 5-FAM-peptide-AuNPs

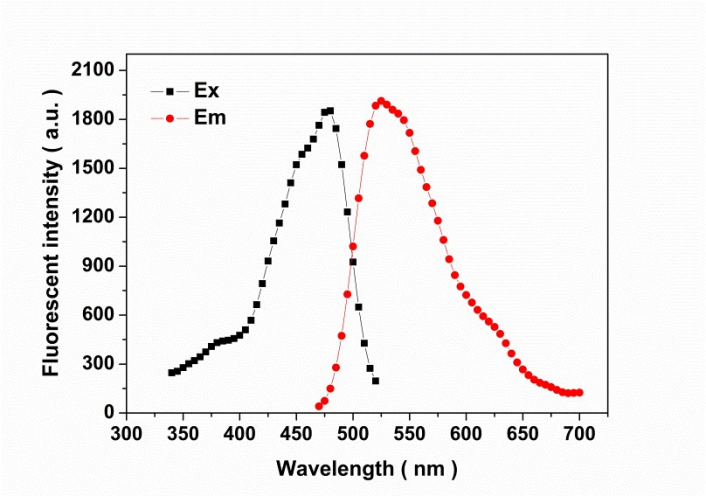


Fig. S5 The spectroscopic property of 5-FAM-peptide-AuNPs.

8. Kinetics study of 5-FAM-peptide-AuNPs with Sec

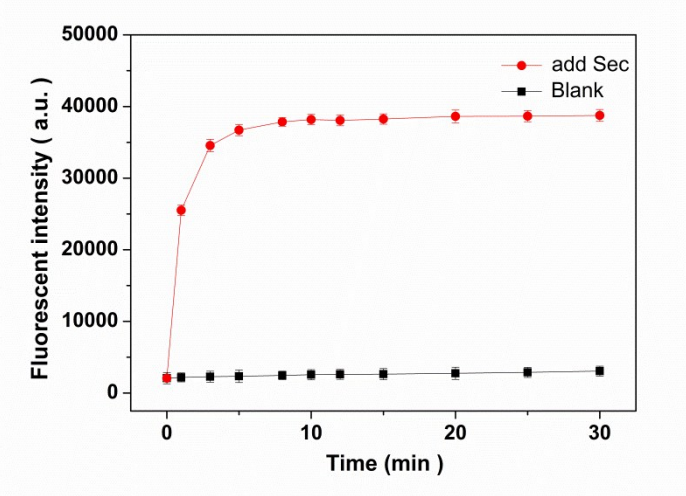


Fig. S6 Fluorescence intensities of 5-FAM-peptide-AuNPs (1 nM) in the presence (red) and absence (black) of Sec (100 μM) in PBS 7.4 (0.01 M) at 37 °C (λ_{ex} / λ_{em}= 488 / 520 nm).

9. The pH effect on the response of 5-FAM-peptide-AuNPs to Sec

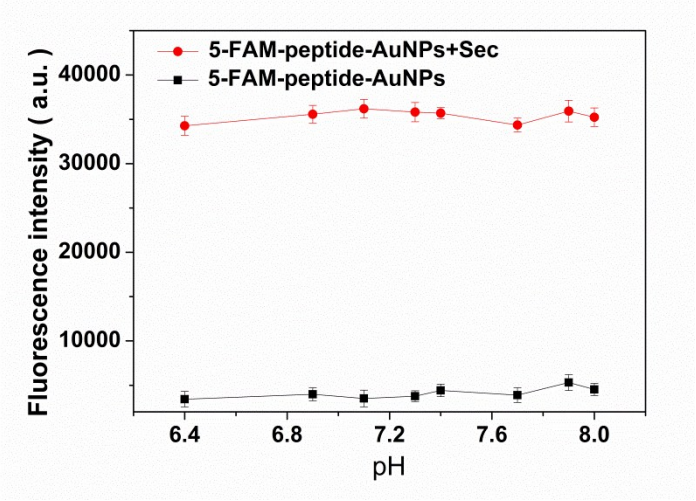


Fig. S7 pH Effect on fluorescence intensities of 5-FAM-peptide-AuNPs (1 nM) toward Sec (100 μM) in PBS buffer (0.01 M) at 37 °C for 10 min (λ_{ex} / λ_{em} = 488 / 520 nm).

10. Selectivity of 5-FAM-peptide-AuNPs toward Sec

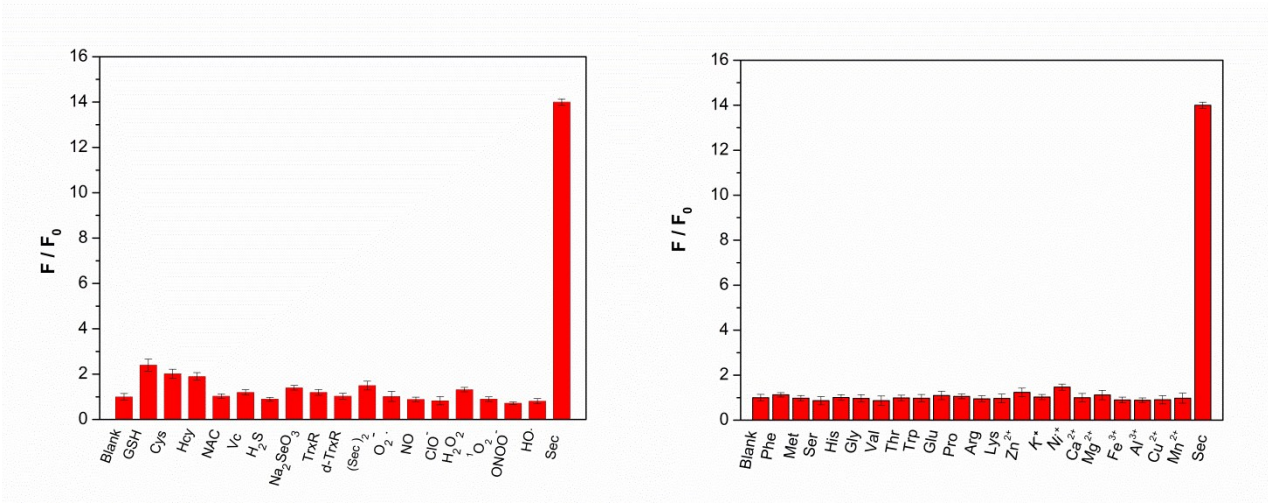


Fig. S8 Fluorescence responses of 5-FAM-peptide-AuNPs (1 nM) to Sec (100 μM) and other common compounds (100 μM for H_2S, 10 mM for GSH, 0.25 mg/mL for TrxR and denatured TrxR (d-TrxR), 1 mM for other interfering substances present in cell. (λ_{ex} / λ_{em} = 488 / 520 nm).

11. The MTT assay of 5-FAM-peptide-AuNPs

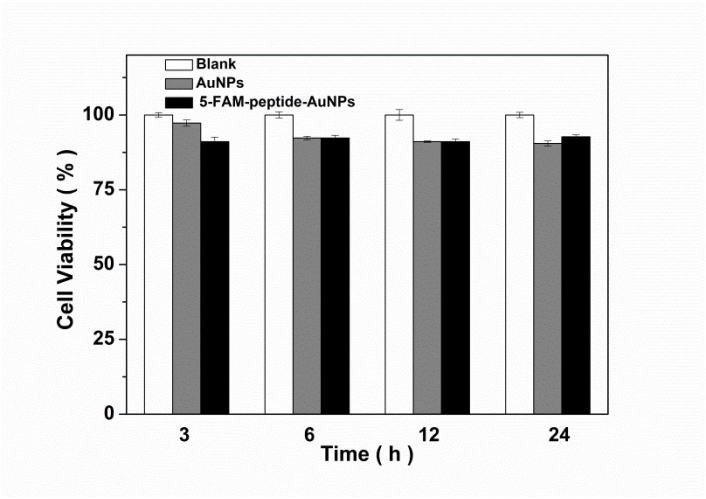


Fig. S9 MTT assay. HepG2 cells incubated with 1 nM Au NPs (gray) and 1 nM 5-FAM-peptide-AuNPs (black) for 3, 6, 12 and 24 h (the blank control is white).

12. Photobleaching test of 5-FAM-peptide-AuNPs

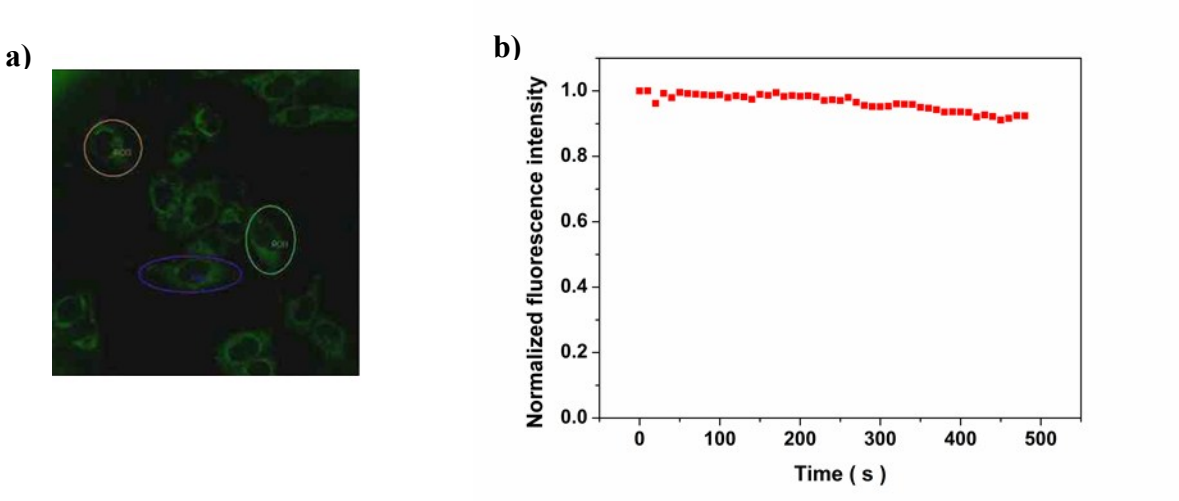


Fig. S10 Photostability Test of 5-FAM-peptide-AuNPs. Fluorescence images were achieved by means of time-sequential scanning of the HepG2 cells incubated with 5-FAM-peptide-AuNPs (1 nM) for 2 h. Normalized fluorescence intensity was recorded in three regions from 0 to 500 s.

13. Comparison of fluorescent probes for selenol

Table S1. Comparison of fluorescent probes for selenol

Probe	**Detection Wavelength (λ_{ex}/ λ_{em}, nm)**	**Detection medium**	**Limit of Detection ($\times 10^{-9}$ mol·L^{-1}) [a]**	**ref**
HB	460/580	PBS buffer, pH 7.4, 1% DMSO	7.0	3
19 (Sel-green)	370/502	PBS buffer, pH 7.4, 1% DMSO	62	4
HD-Sec	650/712	PBS buffer, pH 7.4, 5% DMSO	—	5
PMPC-Dns	490/595	PBS buffer, pH 7.4	50	6
5-FAM-peptide-AuNPs	488/520	PBS buffer, pH 7.4	0.18	this paper

[a] Limit of Detection = 3σ/K. σ is the standard deviation by performing 11 parallel measurements of blank sample; K is the slope of linear curve.

14. HRMS spectrum of QCy7-H_2O_2

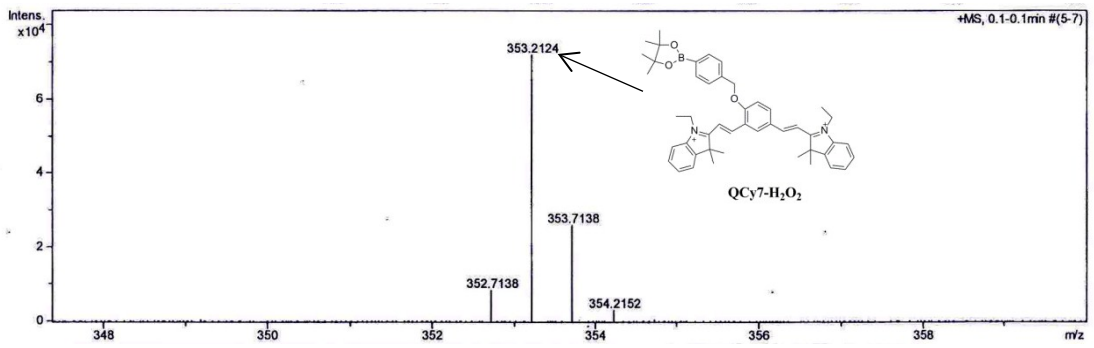


Fig. S11 HRMS Spectrum of QCy7-H_2O_2.

15. The spectroscopic property of QCy7-H_2O_2

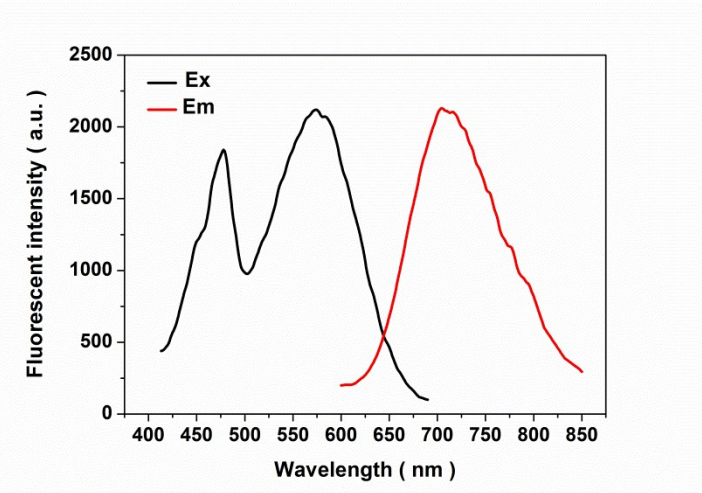


Fig. S12 The spectroscopic property of QCy7-H_2O_2.

16. Fluorescence response of QCy7-H_2O_2 to H_2O_2

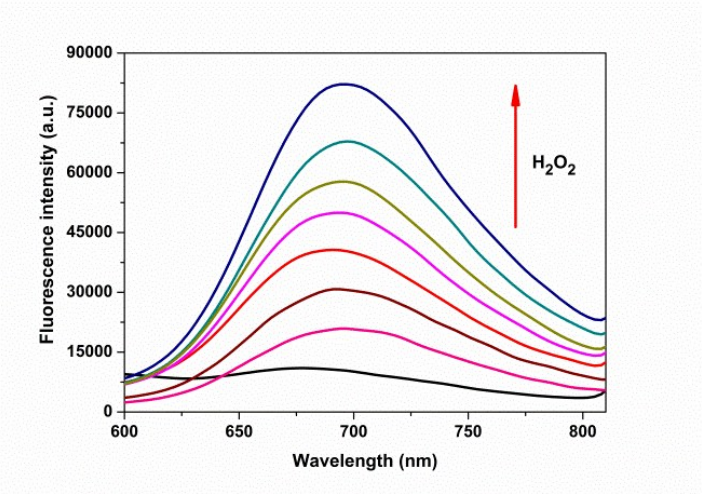


Fig. S13 Fluorescence response of 10 μM QCy7-H_2O_2 toward H_2O_2 in PBS 7.4 (0.01M) at 37 °C (λ_{ex} / λ_{em} = 488 / 710 nm).

17. Selectivity of QCy7- H_2O_2 toward H_2O_2 over Sec

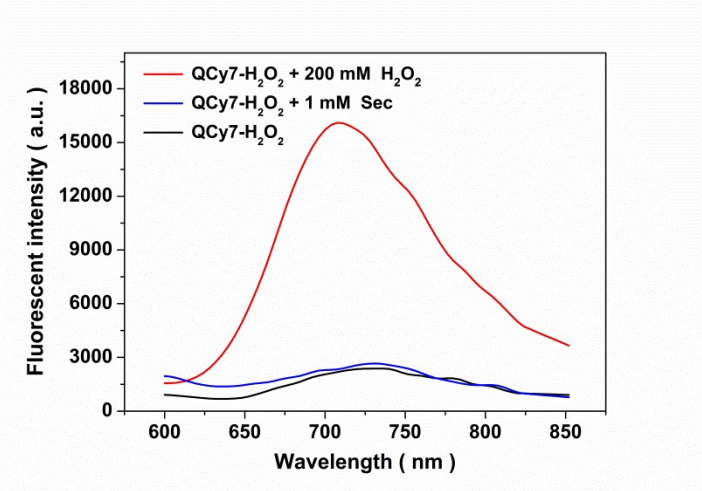


Fig. S14 Selectivity of QCy7- H_2O_2 toward H_2O_2 over Sec. The fluorescence spectra were determined upon incubation of QCy7- H_2O_2 (10 μM) in the absence and presence of Sec (1 mM) and H_2O_2 (200 mM) in PBS 7.4 (0.01M) at 37 °C for 30 min (λ_{ex} / λ_{em}= 488 / 710 nm).

18. The overlapping fluorescence spectra of 5-FAM-peptide-AuNPs and QCy7-H_2O_2

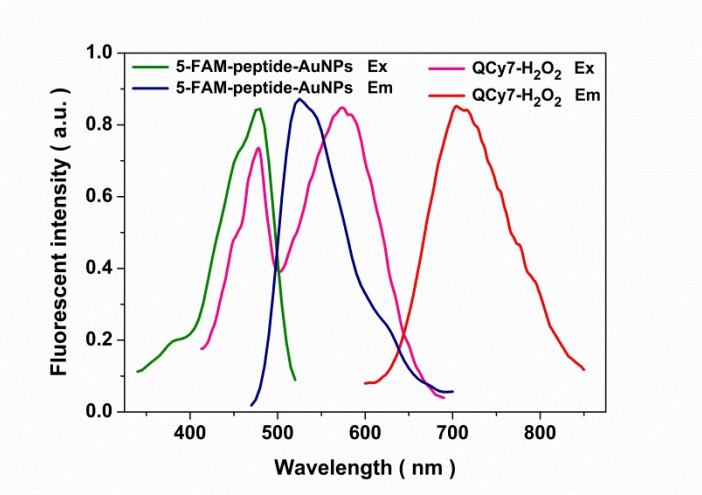


Fig. S15 The overlapping fluorescence spectra of 5-FAM-peptide-AuNPs and QCy7-H_2O_2.

19. Co-staining experiment of HepG2 cells treated with 5-FAM-peptide-AuNPs and QCy7-H_2O_2

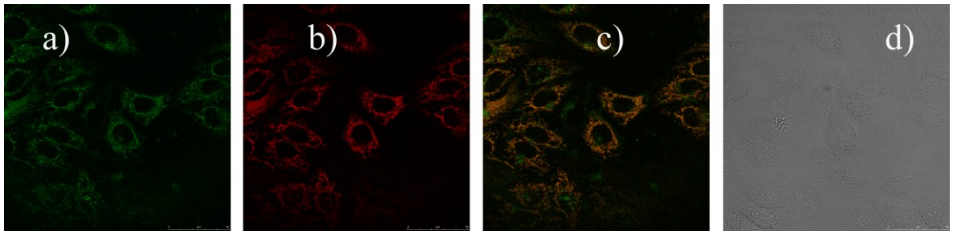
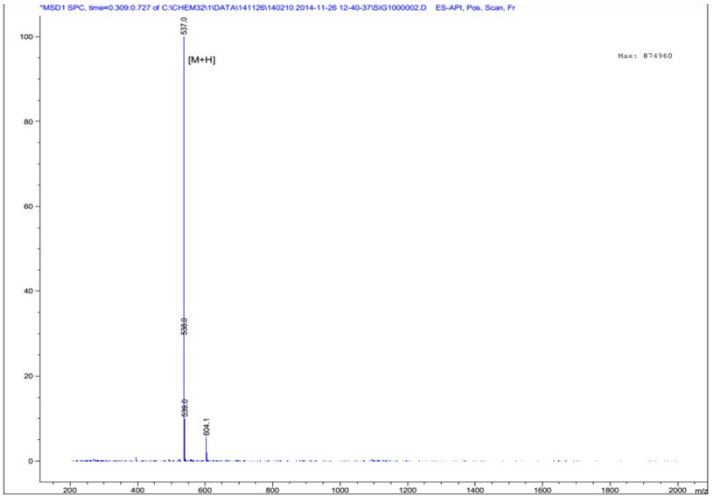
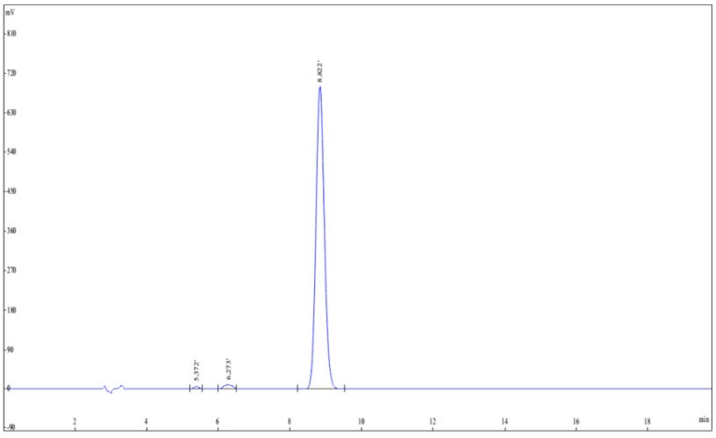


Fig. S16 Co-staining images of HepG2 cells treated with 5-FAM-peptide-AuNPs and QCy7-H_2O_2. (a) green channel, (b) red channel, (c) overlay of (a) and (b), (d) bright-field image. λ_{ex} = 488 nm.

20. MS and HPLC spectra of 5-FAM-Gly-Cys





Signal	Peak#	RT(min)	Area%	Area(mAU*s)	Height(mAU)
	1	5.372	0.5535	63728	5879
	2	6.273	1.546	177940	9786
	3	8.822	97.9	11271628	690966
Total			100	11513296	706631

Fig. S17 MS and HPLC spectra of 5-FAM-Gly-Cys.

21. Reference

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