Electronic Supplementary Information

of

Cancer Targeted Functional Gold Nanoparticles for Apoptosis Inducing and Real-Time Imaging Based on FRET

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1. Materials

N-Fluorenyl-9-methoxycarbonyl (Fmoc) protected D-amino acids (Fmoc-D-Lys(Boc)-OH, Fmoc-D-Ala-OH, Fmoc-D-Leu-OH and Fmoc-Lys(Dde)-OH) and *N*-fluorenyl-9-methoxycarbonyl (Fmoc) protected L-amino acids (Fmoc-Gly-OH, Fmoc-Asp(otBu)-OH, Fmoc-Val-OH, Fmoc-Glu(otBu)-OH and Fmoc-Cys(Trt)-OH), 2-chlorotrityl chloride resin (100-200 mesh, loading: 0.4 mmol/g, 1%DVB), o-benzotriazole-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), triisopropylsilane (TIS) and piperidine were purchased from GL Biochem. Ltd. (Shanghai, China) and used as received. Diisopropylethylamine (DIEA) was acquired from GL Biochem. Ltd. (Shanghai, China) and used after distillation. Trifluoroacetic acid (TFA), hydrazine hydrate, anhydrous ether, Rhodamine B, gold(III) chloride trihydrate and trisodium citrate dehydrate were obtained from Shanghai Chemical Co. (China) and used directly. Folic acid and (4-carboxybutyl) triphenylphosphonium bromide were purchased from Adamas Reagent Co. Ltd. N,N-dimethylformamide (DMF), dichloromethane (DCM) and methanol were provided by Shanghai Chemical Co. (China) and distilled prior to use. All other reagents and solvents were of analytical grade and used directly.

Recombinant human caspase-3 and the inhibitor Ac-DEVD-CHO were purchased from R&D Systems. RPMI-1640 Medium was purchased from Sigma-Aldrich. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zoliumbromide (MTT), trypsin, penicillin-streptomycin, Dulbecco's phosphate buffered saline (PBS) and the mitochondria fluorescence probe (Mito Tracker Green FM) were purchased from Invitrogen.

2. Synthesis of RB-DEVD and DTP

RB-DEVD and DTP (Figure S1) were synthesized manually employing a standard Fmoc chemistry through the solid phase peptide synthesis. Briefly, peptide chains were grown on 2-chlorotriyl chloride resin. The coupling of the first residue used 4 eq. (relative to the substitution degree of resin) Fmoc-protected amino acid and 6 eq. of DIEA in a DMF solution for 2 h. Other amino acid couplings were carried out with 4 eq. of Fmoc-protecting amino acid, 4 eq. of HBTU and 6 eq. of DIEA in a DMF solution for 4 h. During the synthesis, Fmoc protected groups were deprotected with 20% piperidine/DMF (v/v) for twice and every time for 15 min. For the synthesis of RB-DEVD, after obtaining the DEVDGG-C sequence, Rhodamine B was conjugated by using the standard method on solid phase (4 eq. Rhodamine B, 4 eq. of HBTU, 4 eq. of HOBt and 6 eq. of DIEA). For the synthesis of DTP, after obtaining the $_{D}$(KLAKLAK)$_2$-C sequence, the specific lysine (Fmoc-Lys(Dde)-OH) was used as a linker. Following by Fmoc protected groups were deprotected with 20% piperidine/DMF (v/v), (4-carboxybutyl) triphenylphosphonium bromide was coupled using the standard method on solid phase (4 eq. (4-carboxybutyl) triphenylphosphonium bromide, 4 eq. of HBTU, 4 eq. of HOBt and 6 eq. of DIEA). Then the pendant group (Dde) was removed with 2% hydrazine hydrate in DMF, and the resin was stirred overnight with a folic acid solution containing 4 eq. folic acid, 4 eq. of HBTU, 4 eq. of HOBt and 6 eq. of DIEA. After the completion of the synthesis, the resin was finally washed with DMF (4 times) and DCM (4 times) and dried under vacuum for 24 h. Cleavage of the expected peptide and the removal of side chain protected groups from the dried resin were performed by suspending the resin in a cleavage cocktail containing TFA (95%), TIS (2.5%) and H_2O (2.5%) for 2 h. The filtration was concentrated to a viscous solution by rotary evaporation. After the precipitation in cold ether, the crude product was collected and vacuum dried, then

dissolved in distilled water and freeze-dried. The purity of the product was examined by HPLC with a C18 column and using a linear gradient of acetonitrile and DI water containing 0.1% TFA. The molecular weight of peptide analogs was analyzed by ESI-MS. RB-DEVD: purity, 93.4%; ESI-MS, calculated 1118.4, found $[M-H]^-$: 1117.5; DTP: purity, 90.2%; ESI-MS, calculated 2522.4, found $[M+2H]^{2+}/2$: 1262.0 and $[M+3H]^{3+}/3$: 842.1.

3. Synthesis of AuNPs

The gold nanoparticles (AuNPs) were synthesized via the classical Turkevich-Frens method. Briefly, 100 mL of 1 mM the $HAuCl_4 \cdot 3H_2O$ was added in 250 mL round-bottomed flask and refluxed. Then 10 mL of 38.8 mM sodium citrate was rapidly added to the boiling solution and an apparent color changing from light blue to crimson was observed. After the color changed, the solution was refluxed for an addition 15 min, cooled to room temperature naturally, subsequently filtered through 0.45 μm aqueous phase membrane filter and the gold nanoparticles were prepared prior to analysis and use.

4. Preparation of RB-DEVD-AuNP and RB-DEVD-AuNP-DTP

The RB-DEVD and DTP were conjugated on the surface of gold nanoparticle via the covalent bonding interactions between thiol and AuNPs. As a caspase-3 imaging probe, we assumed that the fluorescence of RB-DEVD was completely quenched by AuNPs. To select optimal feed ratio, we fixed the concentration of the caspase-specific peptide RB-DEVD at 1 μM and then increased the concentration of AuNPs from 0 to 5 nM in the complex solution. After stiring 24 h at the room temperature, we detected the fluorescence intensity changes of RB-DEVD and observed that the fluorescence of RB-DEVD was completely quenched when the concentration of AuNPs fixed at 2 nM. Therefore, in the progress of prepraing RB-DEVD-AuNP-DTP,

for ensuring the fluorescence of RB-DEVD completely quenched by AuNPs, before adding excess DTP (20 μM) to modify the AuNPs, we preferentially conjugated the RB-DEVD (1 μM) with AuNPs (10 nM). All the reactions were under the nitrogen atmosphere and in the dark. After the complete of reactions, the complex solution dialysized (MWCO:14000 Da) against DI water for 3 days and lyophilized, then the functional AuNPs was prepared. The concentration of RB-DEVD conjugated on AuNPs was analyzed by RF-530/PC spectrofluorophotometer after RB-DEVD-AuNP-DTP digesting by aqua regia. The concentration of DTP conjugated on AuNPs was analyzed by UV-Vis spectrophotometer. Through the standard fluorescence curve of RB-DEVD and the standard UV-visible spectroscopy of DTP (Fig. S4), the conjugated amount of RB-DEVD and DTP was determined respectively.

5. Characterization of AuNPs, RB-DEVD-AuNP and RB-DEVD-AuNP-DTP

UV-Vis spectrophotometer (Lambda Bio40, Perkin-Elmer), RF-530/PC spectrofluorophotometer (Shimadzu), dynamic light scattering (DLS) techniques with a Zetasizer Nano ZS (Malvern Instruments) and X-ray photo-electron spectroscopy (XPS) (KRATOS XSAM800) were employed to monitor the reaction process of preparing RB-DEVD-AuNP-DTP. Morphologies of AuNPs, RB-DEVD-AuNP and RB-DEVD-AuNP-DTP were observed by transmission electronic microscopy (TEM, JEOL-2100).

6. Study of the fluorescence of RB-DEVD-AuNP-DTP recovered by caspase-3 or GSH

For studing the fluorescence recovery of RB-DEVD-AuNP-DTP *in vitro*, the general procedure of caspase-3 assay was conducted. 2 mL of RB-DEVD-AuNP-DTP solution were added in the PBS buffer solution containing recombinant human caspase-3 (1 Unit) or PBS buffer solution containing recombinant human caspase-3

(1 Unit) and the caspase inhibitor Ac-DVED-CHO (20 μM), and incubated at 37 °C. As the control, glutathione (GSH) was co-cultured with RB-DEVD-AuNP-DTP and we detected the fluorescence intensity of Rhodamine B at given time intervals. Then the fluorescence emission spectra of Rhodamine B was detected by RF-530/PC spectrofluorophotometer (Shimadzu) at given time intervals. The emission and excitation slit widths were set at 5 nm with λ_{ex} = 532 nm.

7. Cell culture

Human mouth epidermal carcinoma cells (KB) were cultured in folic acid-depleted RPMI-1640 media supplemented with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10000 U/mL) at 37 °C in a humidified atmosphere containing 5% CO_2. Human cervix carcinoma cells (HeLa) and African green monkey kidney fibroblast cells (COS7) were incubated in DMEM medium with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10000 U/mL) at 37 °C in a humidified atmosphere containing 5% CO_2.

8. Co-incubation of RB-DEVD-AuNP with HeLa cells and live-cell imaging of caspase-3 activity

HeLa cells were seeded in a glass bottom dish at a density of 1×10^5 cells/well for 24 h. Thereafter, 1 mL RB-DEVD-AuNP (containing 0.5 μM of RB-DEVD) dispersed in DMEM containing 10% FBS and 1% antibiotics was added in each dish. After incubation at 37 °C for 12 h, the medium was wiped off and washed with PBS three times; then the cells were treated with fresh medium containing staurosporine (STS) of a given concentration and incubated for additional 4 h and 12 h, respectively. Finally, after removing the medium and washing with PBS buffer, the cells were observed by using Confocal Laser Scanning Microscopy (CLSM, Nikon C1-si, BD Laser).

9. *In vitro* cytotoxicity of RB-DEVD and RB-DEVD-AuNP-DTP evaluated by MTT assay

In vitro cytotoxicity was performed with KB cells and COS7 cells by MTT assay. Briefly, KB cells and COS7 cells were seeded in 96-well plates at a density of 6000 cells/well, and then cells were incubated in 100 μL folic acid-depleted RPMI 1640 media supplemented with 10% FBS and 1% antibiotics for KB cells, or DMEM containing 10% FBS and 1% antibiotics for COS7 cells 1 day prior to adding RB-DEVD and RB-DEVD-AuNP-DTP. After co-incubation for 2 days, the medium was replaced with 200 μL of fresh medium. Then 20 μL MTT solutions (5 mg/mL) was added to each well and further incubated for 4 h. After that, the medium was removed and 200 μL DMSO was added. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Model 550, USA). The relative cell viability was calculated as: cell viability = $(OD_{570\ (samples)}/OD_{570\ (control))} \times 100\%$, where $OD_{570\ (control)}$ was obtained in the absence of RB-DEVD and RB-DEVD-AuNP-DTP, and $OD_{570\ (samples)}$ was obtained in the presence of RB-DEVD and RB-DEVD-AuNP-DTP. Each value was averaged from four independent experiments. As shown in Fig. S7, The RB-DEVD showed no cytotoxicity for both KB cells and COS7 cells. The selective hunting KB cells property of RB-DEVD-AuNP-DTP was caused by the specific damage ability of DTP.

10. Western blotting analysis of the caspase-3 expression in KB cells and COS7 cells

KB and COS7 cells were respectively seeded in 24-well plates at a density of 5×10^5 cells per well and then incubated for 1 day prior to experiments. Then, RB-DEVD-AuNP-DTP (containing 10 μM of DTP) was added and the cells were allowed to culture for additional 12 h and 24 h, respectively. After washing with PBS for 3 times,

the cells were lysed using 50 μL RIPA buffer (PBS, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 10 μg/mL PMSF, 2 μg/mL aprotinin, and 100 mM Na-orthovanadate) and resuspended in 50 μL 2 × SDS sample buffer containing 1% β-mercaptoethanol. Subsequently, the samples were boiled for 5 min and separated on a 10% SDS-PAGE (15 μL per lane). After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore) by semi-dry transfer cell (Bio-rad). After blocking in PBS solution (containing 5% skim milk) for 1 h, the caspase-3 was detected by respectively incubating the membranes with mouse monoclonal anti-caspase-3 antibody (1:3000 dilution, Cell Signaling Technology) overnight at 4 °C and subsequently treated with the secondary antibody HRP-labeled goat anti-rabbit IgG (1:3000 dilution, Santa Cruz Biotechnology) for 1 h. Specific protein was detected by enhanced chem-iluminescence (ECL; Pierce). Mouse monoclonal anti-GAPDH antibody (Santa Cruz Biotechnology) was used as protein loading control.

11. Confocal laser scanning microscopy observation and flow cytometry analysis

KB and COS7 cells were respectively seeded in a glass bottom dish at a density of 1×10^5 cells per well and then incubated for 1 day prior to experiments. Then, RB-DEVD-AuNP-DTP (containing 10 μM of DTP) was added and the cells were allowed to culture for another 12 h and 24 h, respectively. Subsequently, 100 nM Mito Tracker Green FM in DMEM was added to stain the mitochondria for 30 min. After washing with PBS for 3 times, the cells were observed under a laser scanning confocal microscopy (CLSM, Nikon C1-si TE2000, BD Laser). For the flow cytometry analysis, the cells were digested by trypsin (no EDTA), collected in centrifuge tube, wished with PBS for 3 times and resuspended in 0.5 mL PBS buffer, finally analyzed by flow cytometry (BD FACSAria TM III).

Supplementary Figures

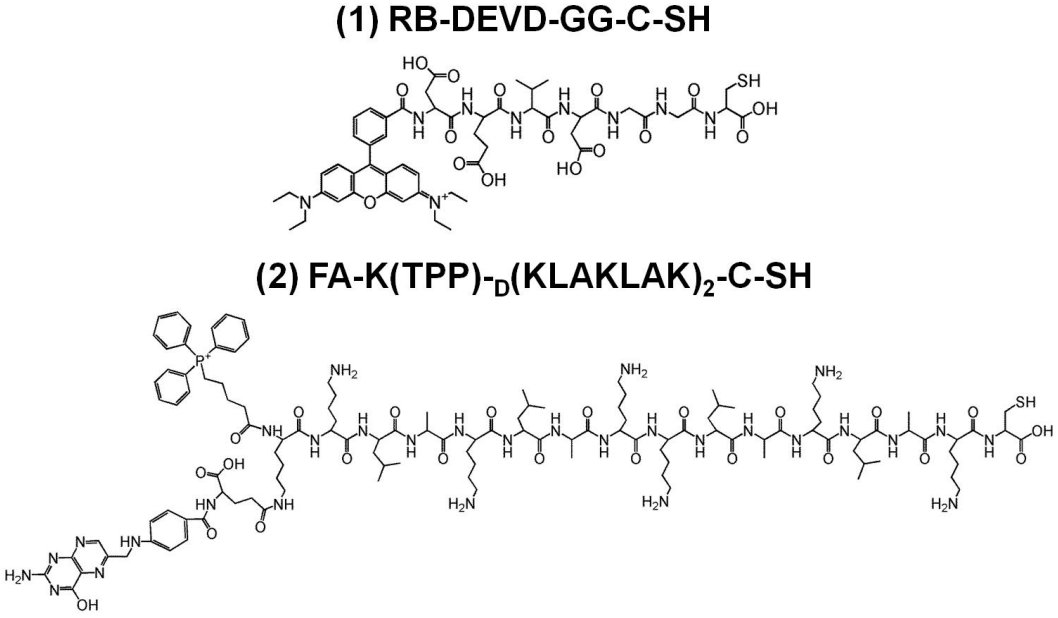

Fig. S1 Chemical structures of (1) The Rhodamine B conjugated caspase-specific peptide sequence: RB-DEVD-GG-C-SH (RB-DEVD) and (2) The dual-targeting pro-apoptotic peptide sequence: FA-K(TPP)-$_D$(KLAKLAK)$_2$-C-SH (DTP).

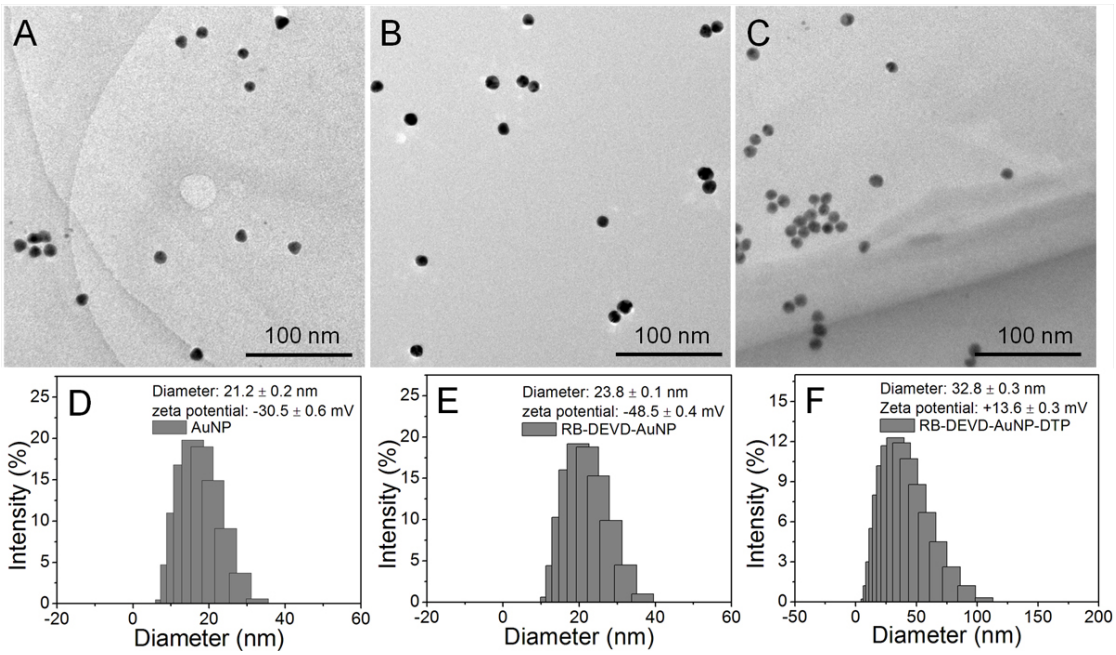

Fig. S2 Characterizations of AuNPs, RB-DEVD-AuNP and RB-DEVD-AuNP-DTP by TEM and DLS. (A) TEM image of AuNPs. (B) TEM image of RB-DEVD-AuNP. (C) TEM image of RB-DEVD-AuNP-DTP. (D) The hydrodynamic size and zeta potential of AuNPs determined by DLS. (E) The hydrodynamic size and zeta potential of RB-DEVD-AuNP determined by DLS. (F) The hydrodynamic size and zeta potential of RB-DEVD-AuNP-DTP determined by DLS.

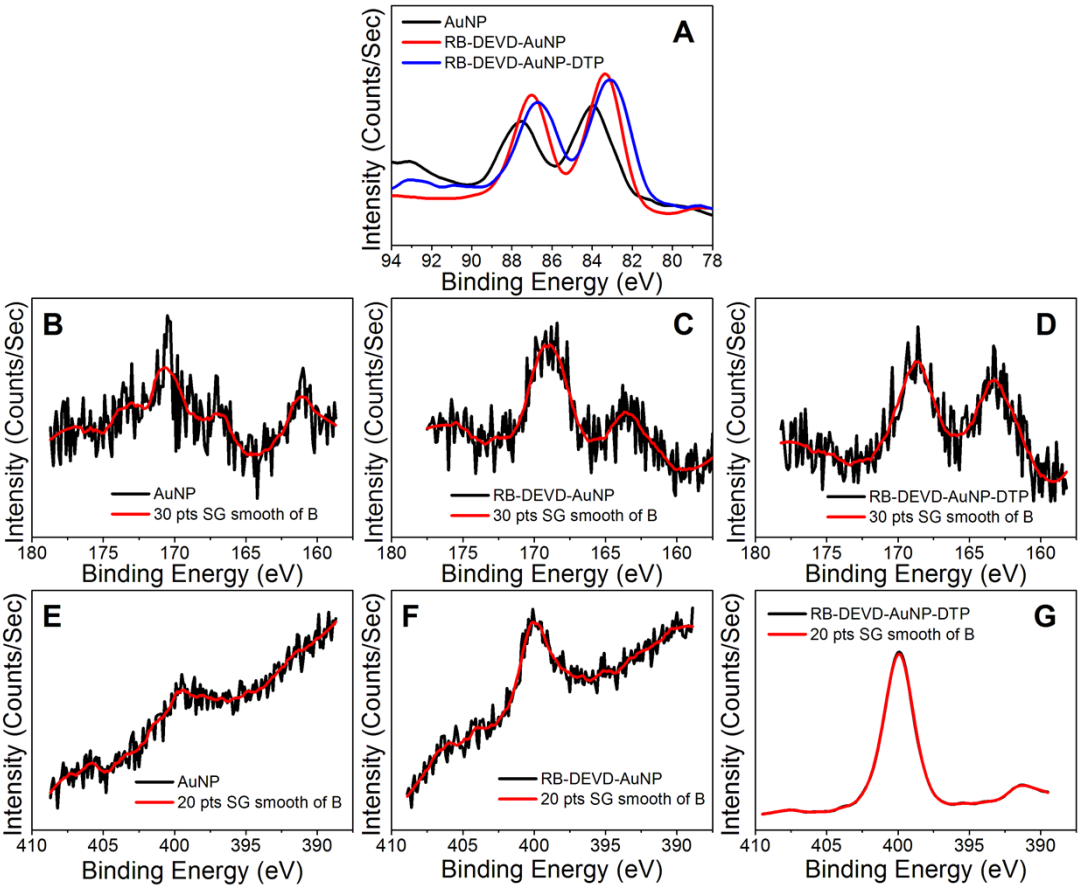

Fig. S3 XPS was used to monitor the progress of surface functionalization of AuNPs. (A) XPS spectra of AuNPs, RB-DEVD-AuNP and RB-DEVD-AuNP-DTP, the signal of $Au4f_{7/2}$ region. (B) XPS spectra of AuNPs, the signal of S2s and S2p region. (C) XPS spectra of RB-DEVD-AuNP, the signal of S2s and S2p region. (D) XPS spectra of RB-DEVD-AuNP-DTP, the signal of S2s and S2p region. (E) XPS spectra of AuNPs, the signal of N1s region. (F) XPS spectra of RB-DEVD-AuNP, the signal of N1s region. (G) XPS spectra of RB-DEVD-AuNP-DTP, the signal of N1s region.

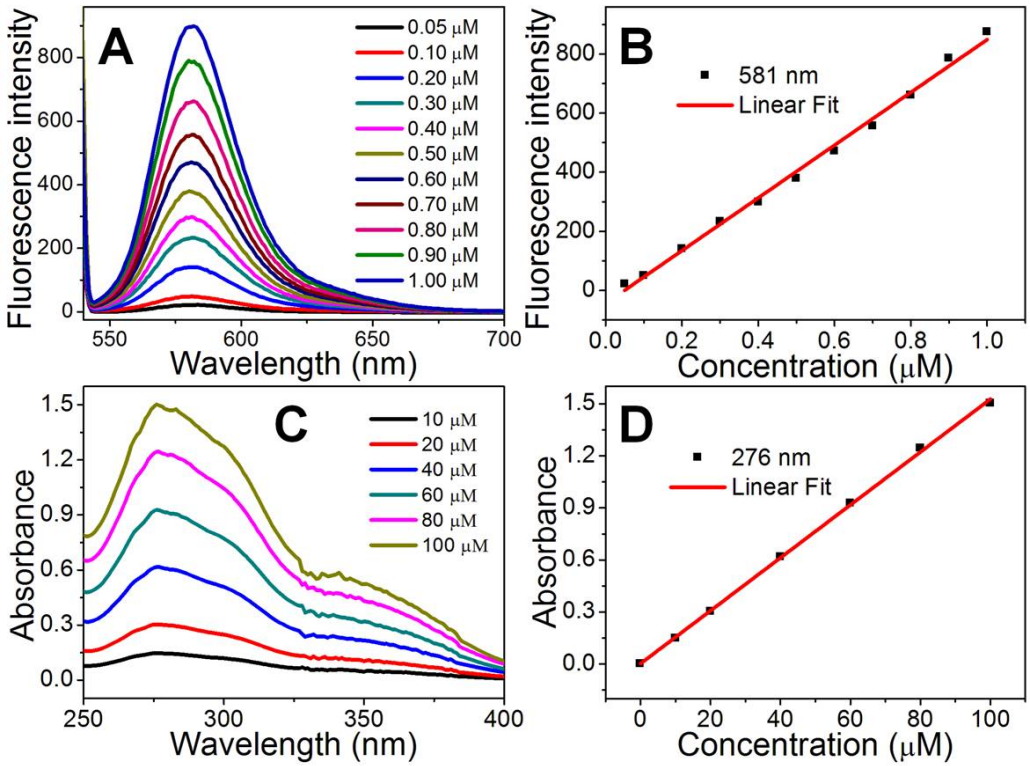

Fig. S4 The fluorescence spectra of RB-DEVD and the UV-visible spectroscopy of DTP. (A) The fluorescence spectra of RB-DEVD, and the excitation wavelength was 532 nm. (B) RB-DEVD standard fluorescence curve (Y = a + bX, a = -32.9, b = 877.1, $R^2 = 0.994$). (C) The UV-visible spectroscopy of DTP. (D) DTP standard UV-visible spectroscopy curve (Y = a + bX, a = 0.002, b = 0.01525, $R^2 = 0.999$).

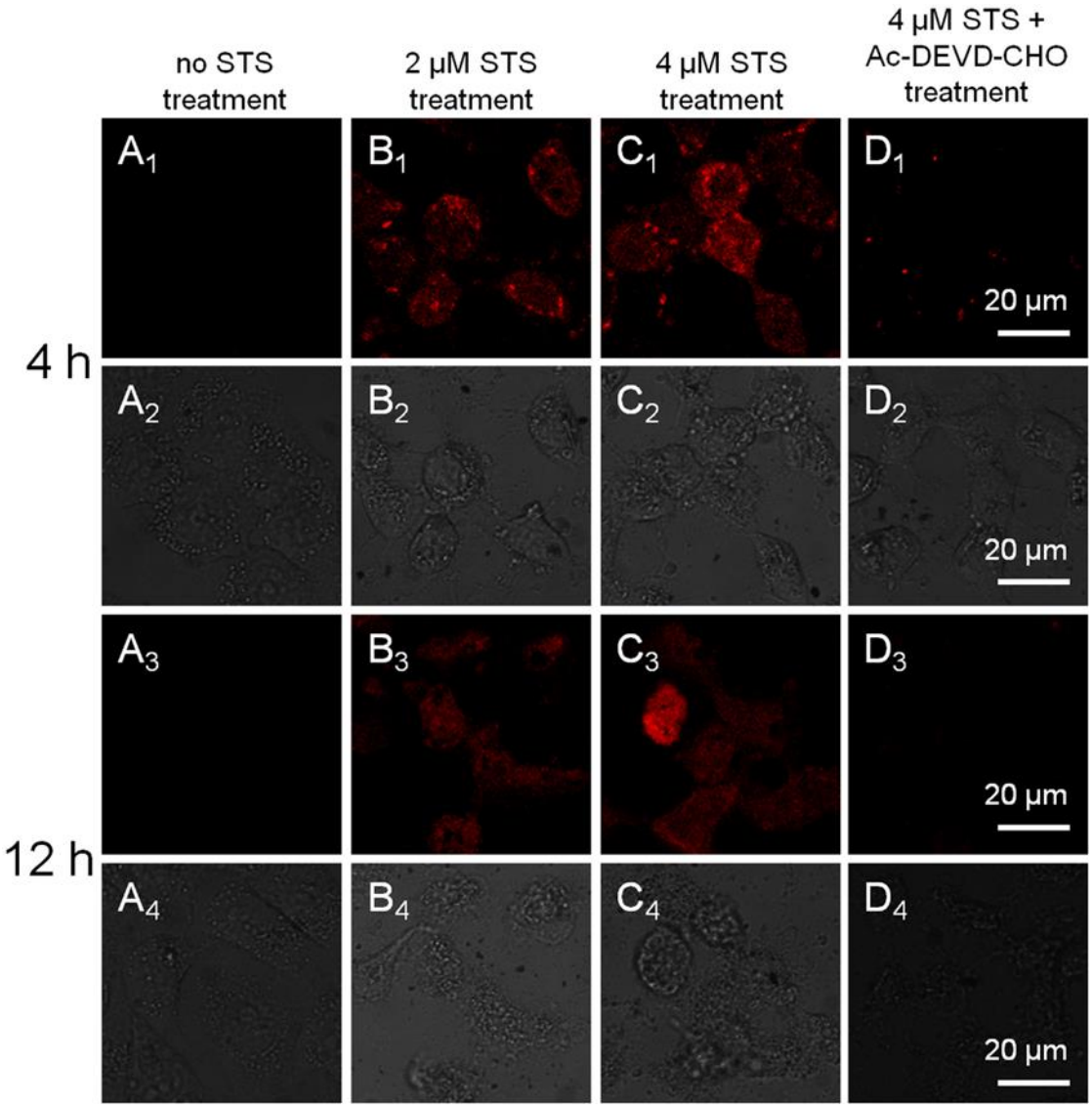

Fig. S5 CLSM images of HeLa cells treated with different concentrations of STS after incubating with RB-DEVD-AuNP 12 h. (A_1, A_2) no STS 4 h; (B_1, B_2) 2 μM STS 4 h; (C_1, C_2) 4 μM STS 4 h; (D_1, D_2) 4 μM STS and 20 μM Ac-DEVD-CHO 4 h. (A_3, A_4) no STS 12 h; (B_3, B_4) 2 μM STS 12 h; (C_3, C_4) 4 μM STS 12 h; (D_3, D_4) 4 μM STS and 20 μM Ac-DEVD-CHO 12 h. (A_1-D_1 and A_3-D_3) Fluorescence images; (A_2-D_2 and A_4-D_4) Bright-field images.

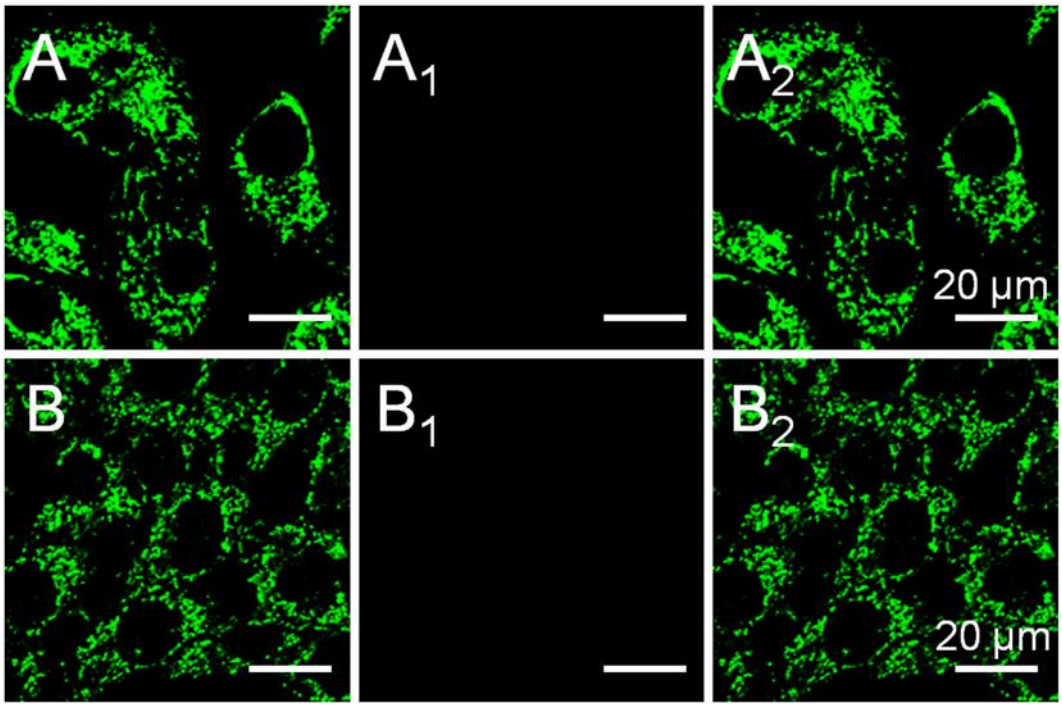

Fig. S6 CLSM images of KB cancer cells and COS7 normal cells incubated without RB-DEVD-AuNP-DTP (A) KB cancer cells; (B) COS7 normal cells.

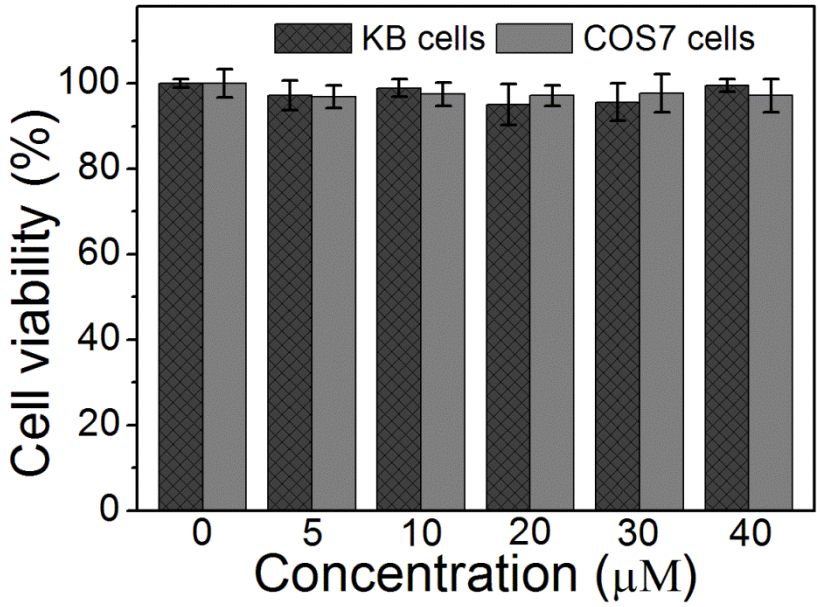

Fig. S7 The cytotoxicity of RB-DEVD in KB cells and COS7 cells tested by MTT assay. The RB-DEVD showed no cytotoxicity for both KB cells and COS7 cells. The selective hunting KB cells property of RB-DEVD-AuNP-DTP was caused by the specific damage ability of DTP.

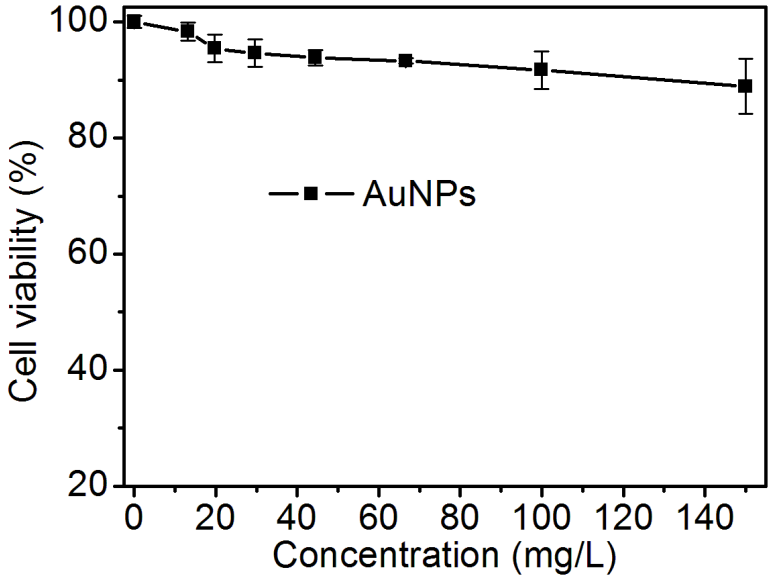

Fig. S8 The cytotoxicity of pure AuNPs in COS7 cells tested by MTT assay.

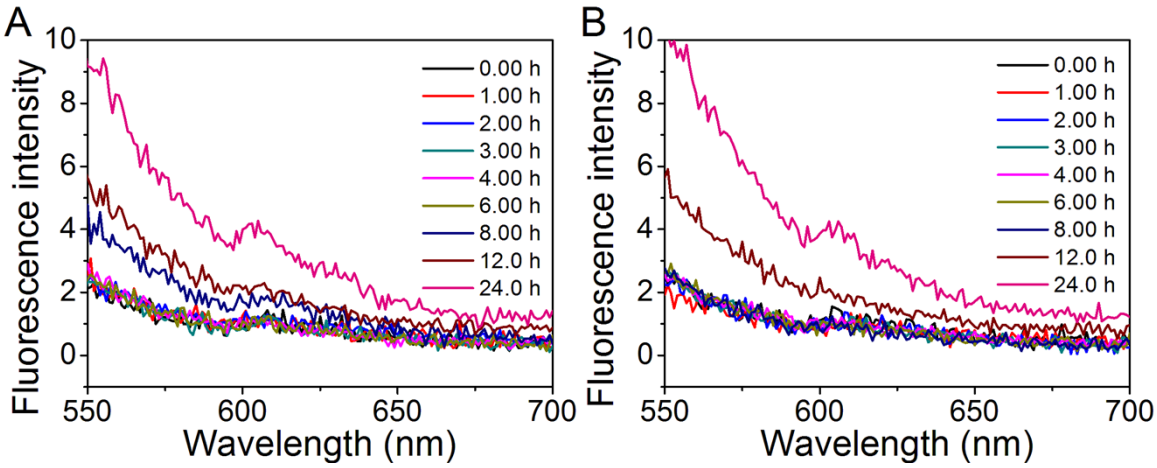

Fig. S9 Fluorescence emission spectra of RB-DEVD-AuNP-DTP upon incubating with (A) GSH (5 mM) and (B) GSH (10 mM).