Electronic Supplementary Information

of

A New Anti-Cancer Strategy of Damaging Mitochondria by Pro-apoptotic Peptide Functionalized Gold Nanoparticles

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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-Gly-OH) 2-chlorotrityl chloride resin (100-200 mesh, loading: 0.4 mmol/g, 1%DVB), o-benzotriazole-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU), 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), 12-aminododecanoic acid, anhydrous ether, ninhydrin, thioglycolic acid, 1,4-dithiothreitol (DTT), gold(Ⅲ) chloride trihydrate and trisodium citrate dehydrate were obtained from Shanghai Chemical Co. (China) and used directly. N,N-dimethylformamide (DMF), methanol and dichloromethane (DCM) were provided by Shanghai Chemical Co. (China) and distilled prior to use.

JC-1 (5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolycarbocyanine iodide) fluorescent dye was purchased from Sigma-Aldrich (USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin streptomycin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zoliumbromide (MTT), trypsin and Dulbecco's phosphate buffered saline (PBS) were purchased from Invitrogen (CA, USA). Molecular probe (Hoechst 33258) and the mitochondria fluorescence probe (Mito Tracker Red CM-H_2XRos) were purchased from Invitrogen (CA, USA). All other reagents and solvents were of analytical grade and used directly.

2. Synthesis and characterization of Fmoc-ADDA-OH

The Fmoc-ADDA-OH was synthesized according to a previous report.[S1] 4.31 g (20 mM) of 12-aminododecanoic acid (ADDA) was suspended in 100 mL 10% Na_2CO_3 solution in 250 mL round-bottomed flask and cooled to 0 °C. 40 mL of 5.18 g (20

mM) fluorenylmethoxy carbonyl chloride (Fmoc-Cl) in dioxane was dropped slowly, stirred for 4 hours at 0 °C and allowed to warm up to room temperature and stirred overnight. The dioxane was removed by rotary evaporation and 200 mL DI water was added. Then the solution was acidified to pH=3 by the addition of concentrated HCl. The product was extracted into ethyl acetate and dried with magnesium sulphate. After removed ethyl acetate, we could get the crude product. The crude product was recrystallised from acetonitrile twice and formed the pure product, the productivity was 78.3%. ^{1}HNMR recorded on a Mercury VX-300 spectrometer at 300 MHz (Varian, USA) by using DMSO-d_6 as a solvent and TMS as an internal standard: δ (ppm): 1.15-1.45 (e, 18H, -CH_2-), 2.1-2.5 (d, 4H, -CH_2-), 4.10-4.40 (c, 2H, -CH_2-), 7.25-7.85 (b, 8H), 12 (a, 1H, -COOH).

3. Synthesis of the pro-apoptotic peptide

The pro-apoptotic peptide HS-ADDA-ADDA-GG-$_D$(KLAKLAK)$_2$ (PAP) was 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 equiv (relative to the substitution degree of resin) Fmoc-protected amino acid and 6 equiv of DIEA in a DMF solution for 2 hours. Other amino acid couplings were carried out with 4 equiv of Fmoc-protecting amino acid, 4 equiv of HBTU, and 6 equiv of DIEA in a DMF solution for 4 hours. During the synthesis, Fmoc protected groups were deprotected with 20% piperidine/DMF (v/v) for twice and every time for 15 minutes. At the end of the synthesis, thioglycolic acid was conjugated to the peptide segments. After the completion of the synthesis, the resin was finally washed with DMF (four times) and DCM (four times) and dried under vacuum for 24 hours. 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 (90%), TIS (3%), H_2O (3%) and DTT (4%) for 2 hours. 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 molecular weight of pro-apoptotic peptide was analyzed by MALDI-TOF-MS and found in 2089.3. The actual molecular weight was 2106.8 and lose a molecular H_2O was 2089.3 (Fig S1b).

Purity (Fig S1c): 90.2% determined by high-pressure liquid chromatography (HPLC) with a C18 column and using a linear gradient of acetonitrile and DI water containing 0.1% TFA.

4. Preparation of gold nanoparticles

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. 10 mL of 38.8 mM sodium citrate was then 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 minutes, 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.

5. Preparation of pro-apoptotic-gold nanoparticles hybrid materials

The fabrication of pro-apoptotic peptide-gold nanoparticles (PAP-AuNPs) nanocomposites were mainly used the covalent bonding interactions between thiol and AuNPs. The filtered AuNPs and thiolated pro-apoptotic peptide conjugated with weight ratios PAP: AuNPs = 2:1. In the process of preparing, 39.4 mg of PAP was dissolved in 5 mL deionization water and then dropped in the 100 mL filtered AuNPs solution. The mixture of pro-apoptotic peptide and AuNPs were stirred 24 h at room

temperature in a nitrogen round-bottom flask, dialysized (MWCO:14000 Da) against DI water for 3 days and liophilized, then the PAP-AuNPs hybrid nanomaterial was prepared.

6. Characterization of AuNPs and PAP-AuNPs

UV-Vis spectrophotometer (Lambda Bio40, Perkin-Elmer, USA), zeta potential instrument (Nano-ZS ZEM3600, Malvern Instruments Co. Ltd, UK), Circular dichroism (CD, Jasco J-810 spectropolarimeter, Jasco, Japan) and Fourier transform infrared spectroscopy (Perkin–Elmer Spectrum One, USA) were employed to monitor the reaction processes of PAP-AuNPs. Morphologies of AuNPs and PAP-AuNPs were observed by transmission electronic microscopy (TEM, JEOL-2100, Japan). Thermal gravitational analysis (TGA) was performed with a thermal analyzer (Thermo Gravimetric Analyzer，TGS-II, Perkin-Elmer) at 5 °C/min under nitrogen flow 40 mL/min.

7. Cell culture

Human cervix carcinoma cells (HeLa) 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. In vitro cytotoxicity assay

The cytotoxicity assay was performed with HeLa cells by MTT assay. Briefly, the HeLa cells were seeded in 96-well plates at a density of 6000 cells/well, and then cells were incubated in 100 μL DMEM containing 10% FBS for 1 day prior to adding PAP or PAP-AuNPs. After PAP or PAP-AuNPs was added for 2 days, the medium was replaced with 200 μL of fresh medium. Then 20 μL MTT (5 mg/mL in PBS buffer) solutions were 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 (%) = ($OD_{570\ (samples)}/OD_{570\ (control)}$) ×100, where $OD_{570\ (control)}$ was obtained in the absence of PAP or PAP-AuNPs and $OD_{570\ (samples)}$ was obtained in the presence of PAP or PAP-AuNPs. Each value was averaged from four independent experiments.

9. Characterization of cell uptake and distribution and specifically damage mitochondria by TEM

To investigate nanoparticles distribution within cells and study the PAP-AuNPs specifically damage mitochondria, HeLa cells were cultured with 150 mg/L PAP-AuNPs for 4 hours, 12 hours and 24 hours. After treatments, cell monolayer on the 6-well plate was washed with PBS three times to remove excess PAP-AuNPs. Then the cells in each well were fixed with 1 mL general fixative (containing 2.5% glutaraldehyde in 0.1 M PBS) at 4 °C and overnight. After washed with PBS for 3 times, the cells were further stained with 4% osmium tetroxide for 0.5 hours at room temperature. After rinsed with distilled water, then dehydrated at room temperature in a graded ethanol series of 30%, 50%, 70%, 90% and followed by three rinses of 100% ethanol for 10 min each. After dehydration, cells were embedded in epoxy resin and the resin was stored at 55 °C for 48 hours to allow resin polymerization. The embedded samples were then sliced with a thickness of 50-70 nm. Finally, the cell section was stained with 5% uranyl acetate for 15 min and 2% lead citrate for 15min before TEM imaging.

10. Observation of mitochondria damage

HeLa cells were cultured with PAP-AuNPs and equivalent PAP in cell culture dish. The concentration of PAP-AuNPs was 150 mg/L and the equivalent PAP was 32 mg/L (through Figure S3b 150×0.21 mg/L). After incubation at 37 °C for another 24 hours,

48 hours and 72 hours, respectively, cells nuclei were stained with 10 μg/mL Hoechst 33258 in 1 mL DMEM containing 10% FBS for 15 min and washed with PBS 3 times and then the cells mitochondria were stained with 100 nM Mito Tracker Red CM-H_2XRos in DMEM (no containing 10% FBS, because Mito Tracker Red CM-H_2XRos easily oxidized by FBS) for 30 min and washed with PBS 3 times. Cells nuclei and mitochondria were observed with a laser scanning confocal microscopy (CLSM, Nikon C1-si TE2000, Japan).

11. Study on the mitochondrial-regulated apoptosis mechanism in cancer cells by JC-1 assay

HeLa cells were cultured with 100 mg/L PAP-AuNPs in cell culture dish. After incubation at 37 °C for another 24 hours, 48 hours and 72 hours, respectively, cells nuclei were stained with 10 μg/mL Hoechst 33258 in 1 mL DMEM containing 10% FBS for 15 min and washed with PBS 3 times and then the cells mitochondria were stained with JC-1 10 μg/mL in DMEM for 30 min and washed with PBS 3 times. Cells nuclei and mitochondria were observed with a laser scanning confocal microscopy, studied the PAP-AuNPs specifically ability of damaging mitochondria by investigating the JC-1 existing forms (the red J-aggregates or the green J-monomer).

12. The quantitative detection of cells with low ΔΨm by flow cytometry

HeLa cells were seeded in 24-well plates at a density of 5×10^4 cells/well and cultured with 1 mL DMEM containing 10% FBS for 1 day. Then cells were cultured with 100 mg/L PAP-AuNPs and equivalent 100×0.21 mg/L PAP for 48 hours and 72 hours respectively. After treatments, cell monolayer on the 24-well plate was washed with PBS 3 times to remove excess PAP-AuNPs and PAP. Then the control cells and the test cells were stained with JC-1 10 μg/mL in DMEM for 30 min and washed with PBS 3 times. All the cells digested by trypsin and collected in centrifuge tube, wished

with PBS 3 times, and then the JC-1 dyeing cells were detected for J-aggregates red fluorescence (PE-A) and J-monomer green fluorescence (FITC-A) by flow cytometry (BD FACSAria TM III, USA) detect.[S2]

13. The apoptosis analysis by flow cytometry

HeLa cells were seeded in 24-well plates at a density of 5×104 cells/well and cultured with 1 mL DMEM containing 10% FBS for 1 day. Then cells were cultured with 100 mg/L PAP-AuNPs and equivalent 100×0.21 mg/L PAP for 24 h, 48 h, and 72 h, respectively. After treatments, cell monolayer on the 24-well plate was washed with PBS 3 times to remove excess PAP-AuNPs and PAP. All the cells digested by trypsin (no EDTA) and collected in centrifuge tube, wished with PBS 3 times and then resuspended cells in 0.5 mL 1× annexin-binding buffer. Hereafter added 5 μL of Annexin V-FITC and 10 μL of propidium iodide (PI) and incubated at room temperature for 5 min in the dark. Then wished with 1× annexin-binding buffer 2 times and signals from Annexin V-FITC and PI were individually analyzed in Channel FL-1 and FL-2 (BD FACSAria TM III, USA).

14. Western blotting analysis the release of cytochrome c and caspase-3

HeLa cells were seeded in 24-well plates at a density of 5×104 cells/well and cultured with 1 mL DMEM containing 10% FBS for 1 day. Then cells were cultured with 100 mg/L PAP-AuNPs and equivalent 100×0.21 mg/L PAP for 24 hours and 48 hours respectively. After treatments, cell monolayer on the 24-well plate was washed with PBS 3 times to remove excess PAP-AuNPs and PAP. The cells were lysed in 50 μL RIPA buffer (1×PBS,1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 10 μg/mL PMSF, 2 μg/mL aprotinin, 100 mM Na-orthovanadate) and resuspended in 50 μL 2 × SDS sample buffer containing 1% β-mercaptoethanol. 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). The membranes were blocked for 1 h in PBS containing 5% skim milk and cytochrome c as well as caspase 3 were detected by incubating the membranes with mouse monoclonal anti-cytochrome c antibody (1:3000 dilution, EPI) and mouse monoclonal anti-caspase-3 antibody (1:3000 dilution, Cell Signaling Technology) overnight at 4 °C and then with the secondary antibody HRP-labeled goat anti-rabbit IgG (1:3000 dilution, Santa Cruz Biotechnology) for 1 h. Specific proteins were detected by enhanced chem-iluminescence (ECL; Pierce). Mouse monoclonal anti-β-actin antibody (Santa Cruz Biotechnology) was used as protein loading control.

Supplementary References

[S1] L. A. Carpino, G. Y. Han, J. Org. Chem. 1972, 37, 3404-3409.

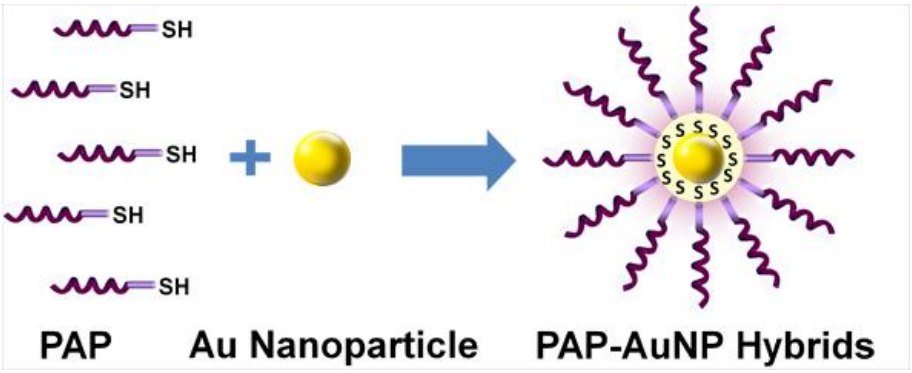
[S2] H. Lecoeur, A. Langonne, L. Baux, D. Rebouillat, P. Rustin, M. C. Prévost, C. Brenner, L. Edelman, E. Jacotot, *Exp Cell Res.* **2004**, *294*, 106-117.

Supplementary Table S1

Table S1. Physical and chemical properties of AuNPs and PAP-AuNPs.

	Diameter (nm)			
Sample	TEM	DLS	Zeta potential (mV)	Max SPR peak (nm)
AuNPs	13.4	22.8	-35.8	525
PAP-AuNPs	14.6	36.2	23.8	528

Supplementary Schemes and Figures



Scheme S1. Schematic illustration of forming PAP functionalized AuNPs hybrid nanomaterial.

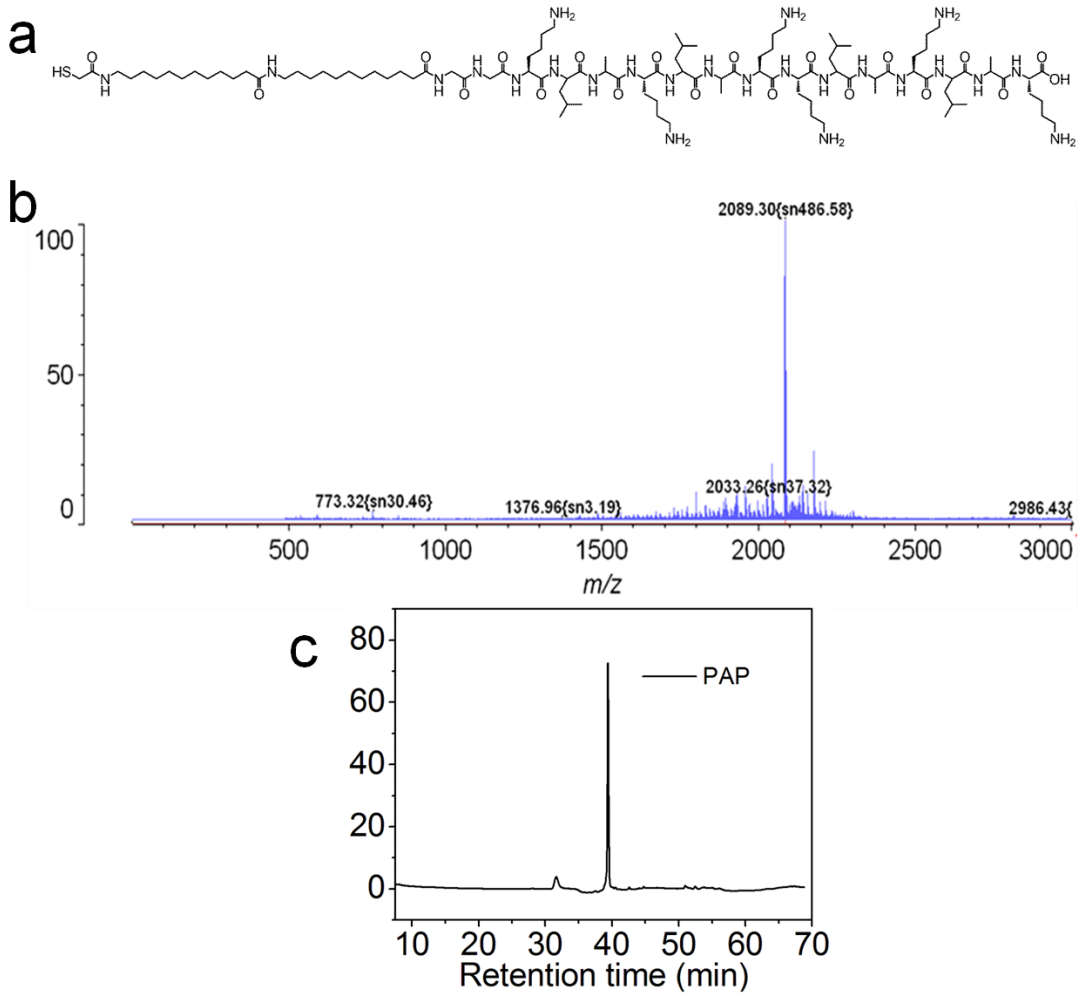


Fig S1. a) The chemical structure of pro-apoptotic peptide HS-ADDA-ADDA-GG-$_D$(KLAKLAK)$_2$. b) MALDI-TOF-MS analysis of pro-apoptotic peptide HS-ADDA-ADDA-GG-$_D$(KLAKLAK)$_2$. c) HPLC analysis of PAP.

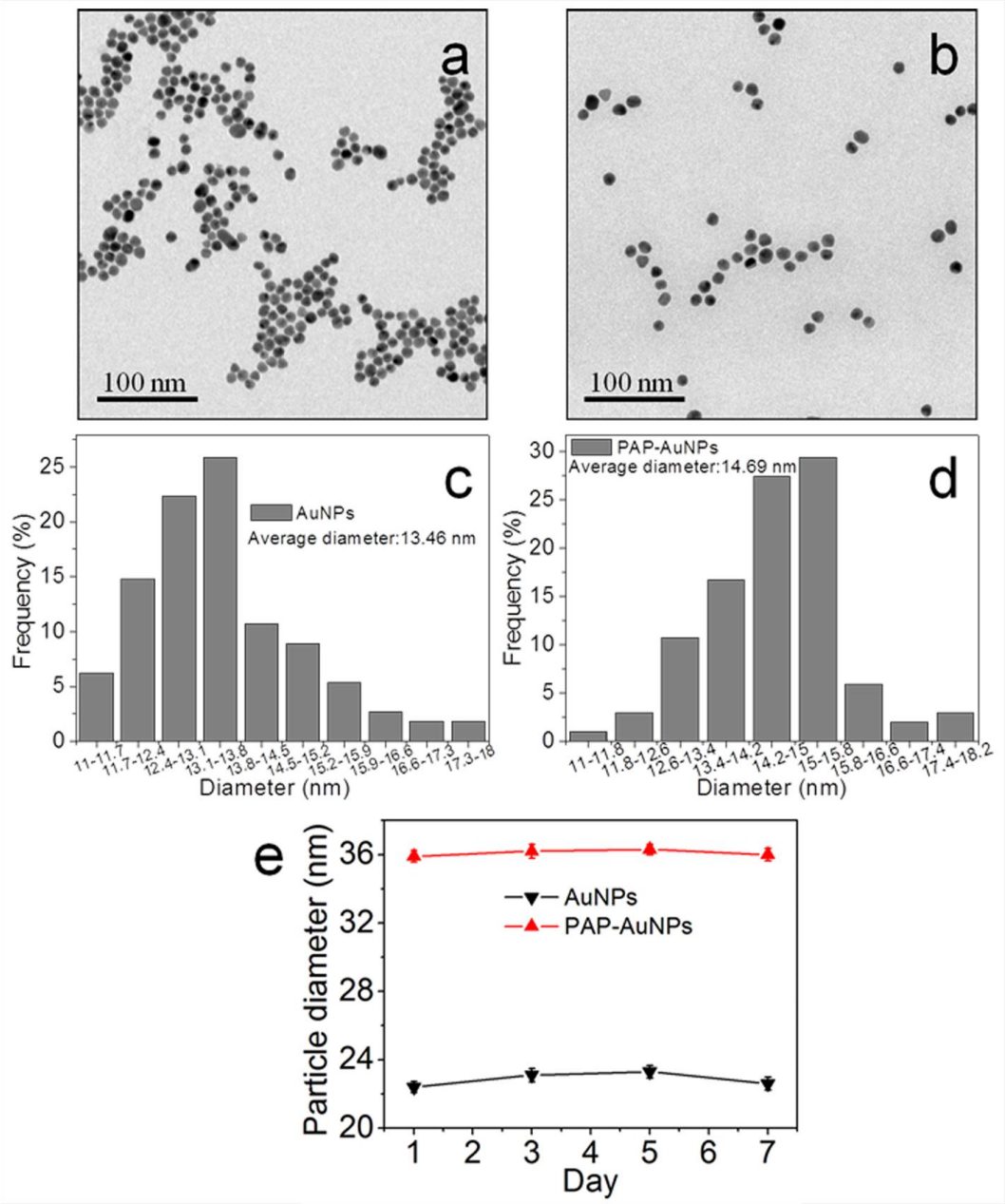


Fig S2. Characterization of AuNPs and PAP-AuNPs by TEM. a) TEM image of AuNPs. b) TEM image of PAP-AuNPs. c) Size distribution of AuNPs. d) Size distribution of PAP-AuNPs. Briefly, more than 200 AuNPs and PAP-AuNPs were statically analyzed. e) Monitoring the particle size changes of AuNPs and PAP-AuNPs for 7 days by DLS.

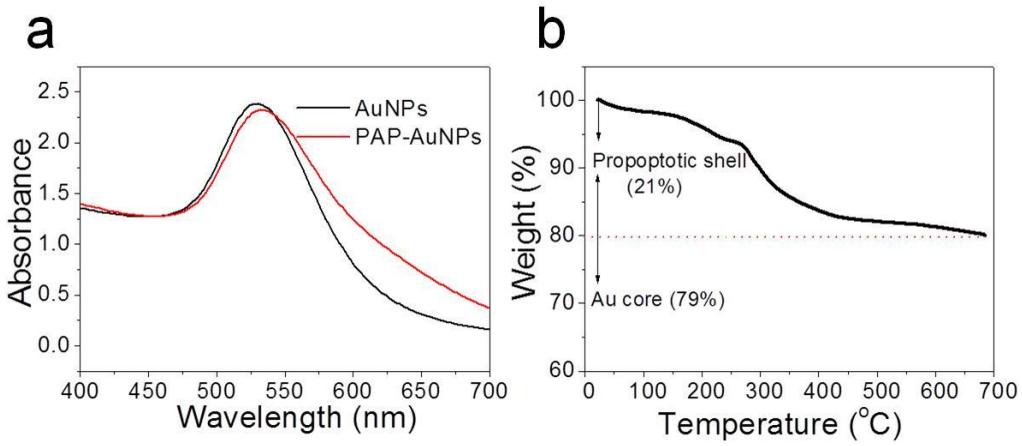


Fig S3. a) Monitoring the conjugate process of AuNPs and PAP by UV-vis spectroscopy, the maximum SPR redshift 3 nm. b) The TGA analysis of PAP-AuNPs.

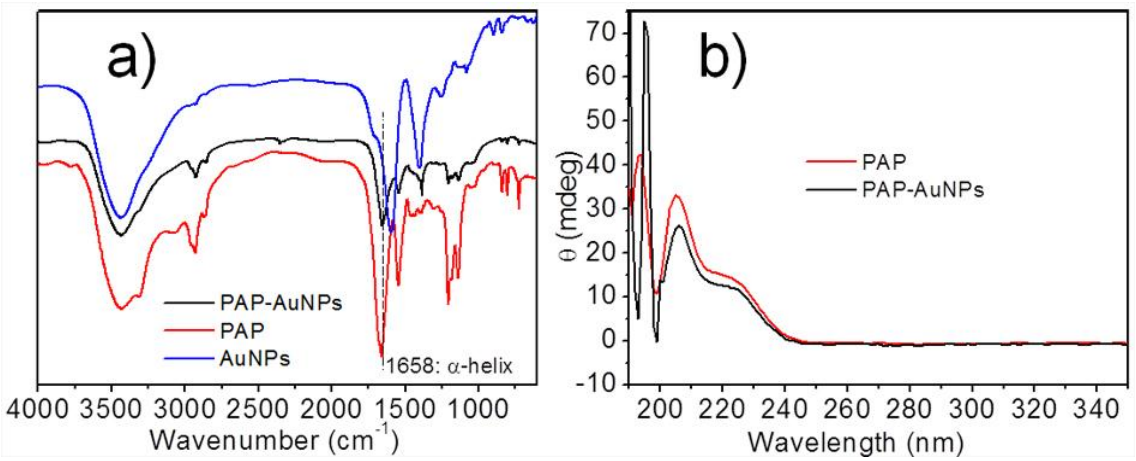


Fig S4. a) The FT-IR spectra of pro-apoptotic peptide, AuNPs and pro-apoptotic peptide functionalized AuNPs. The characteristic absorption band at 1658 cm^{-1} indicated that PAP and PAP-AuNPs mainly adopted α-helical conformation. b) The CD spectra of PAP and PAP-AuNPs. The positive bands near 208 nm and 220 nm were indicative of α-helical conformation of PAP and PAP-AuNPs.

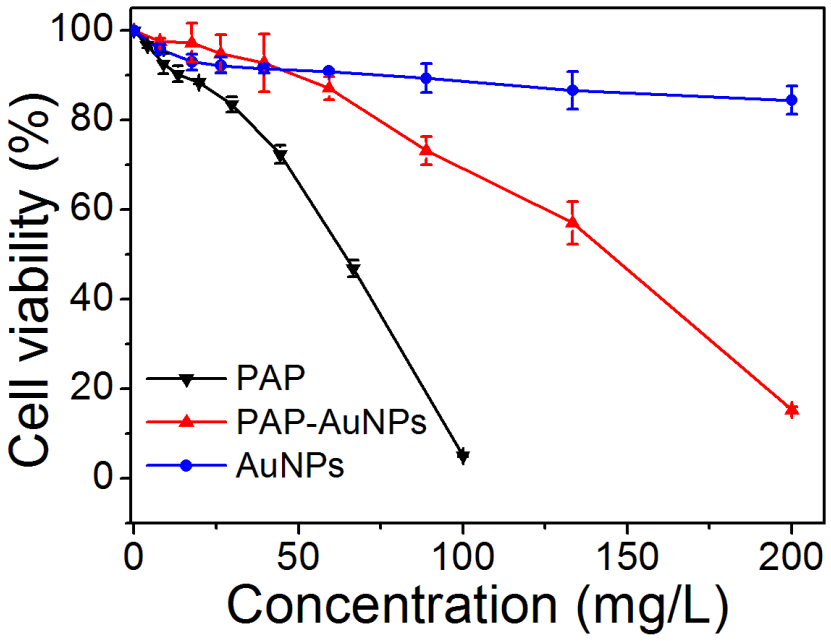


Fig S5. The cytotoxicity of PAP, PAP-AuNPs and pure AuNPs in HeLa cells tested by MTT assay

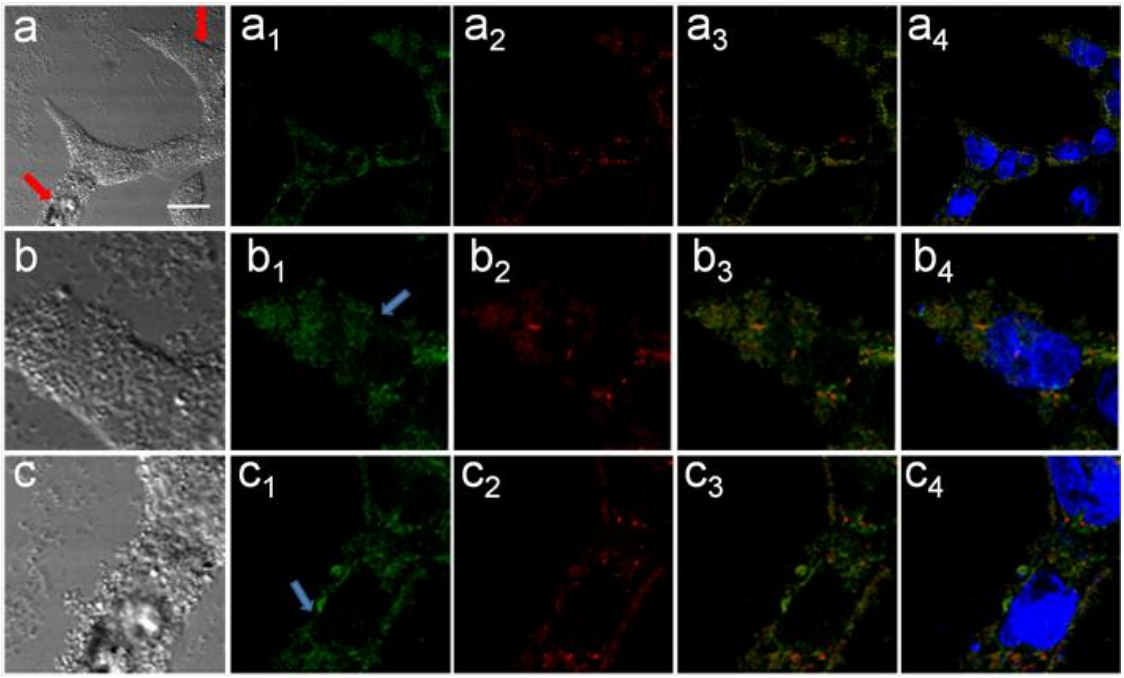


Fig S6. CLSM images of the permeable state of cell membrane. The nuclei are stained by Hoechst 33258 and mitochondria are stained by probe JC-1 fluorescence probe. Green fluorescence represents J-monomer and red fluorescence represents J-aggregates. Fig S6b and c were magnified from Figure S6a (red arrows). The scale bar was 20 μm.

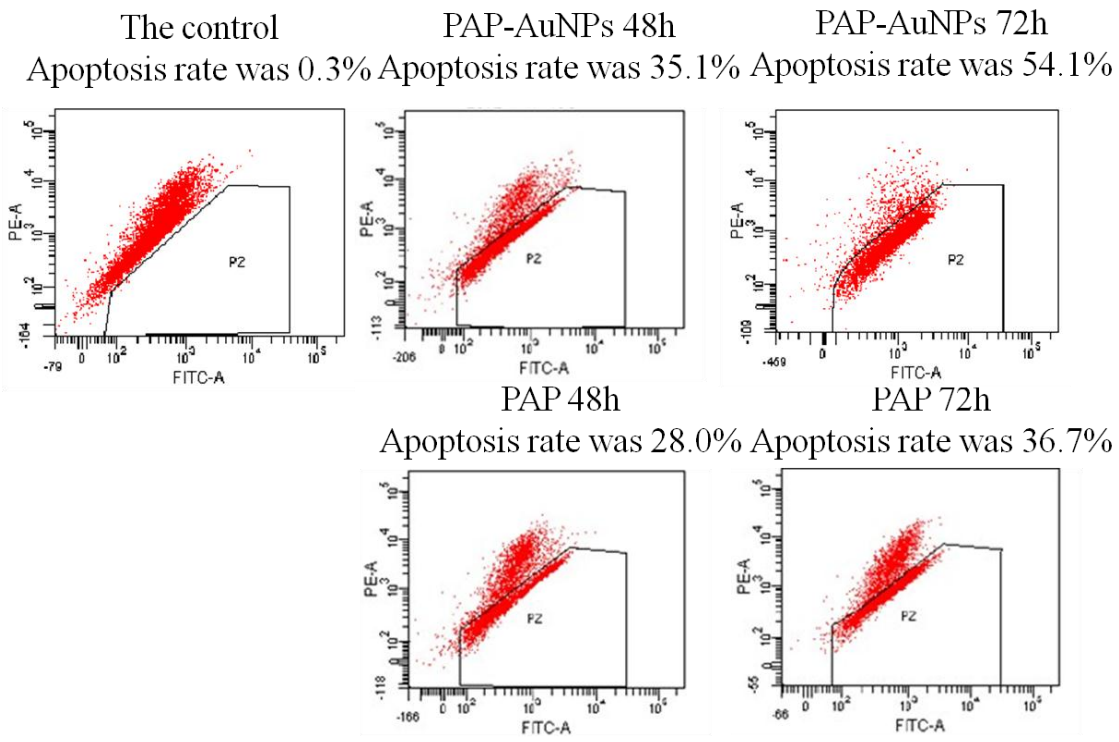


Fig S7. Flow cytometry analysis of apoptotic ability for HeLa cells treated with PAP-AuNPs and PAP respectively. P2 was represented the cell populations with the low ΔΨm, meaning those cells were in the apoptotic state.

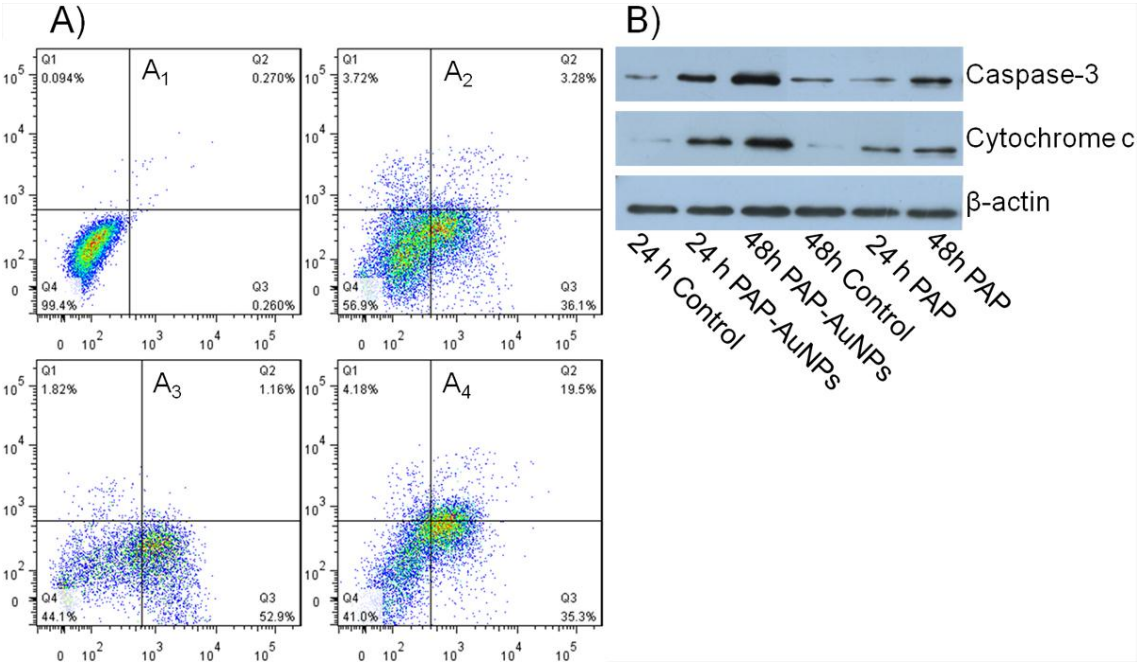


Fig S8. A) Apoptosis and necrosis of HeLa cells analyzed by flow cytometry, A_1 is control, $A_{2\text{-}4}$ are cells incubated with PAP-AuNPs for 24, 48 and 72 h, respectively. B) Western blot analysis for the determination of the release of cytochrome c and expression of caspase-3 by the HeLa cells incubated with PAP or PAP-AuNPs.

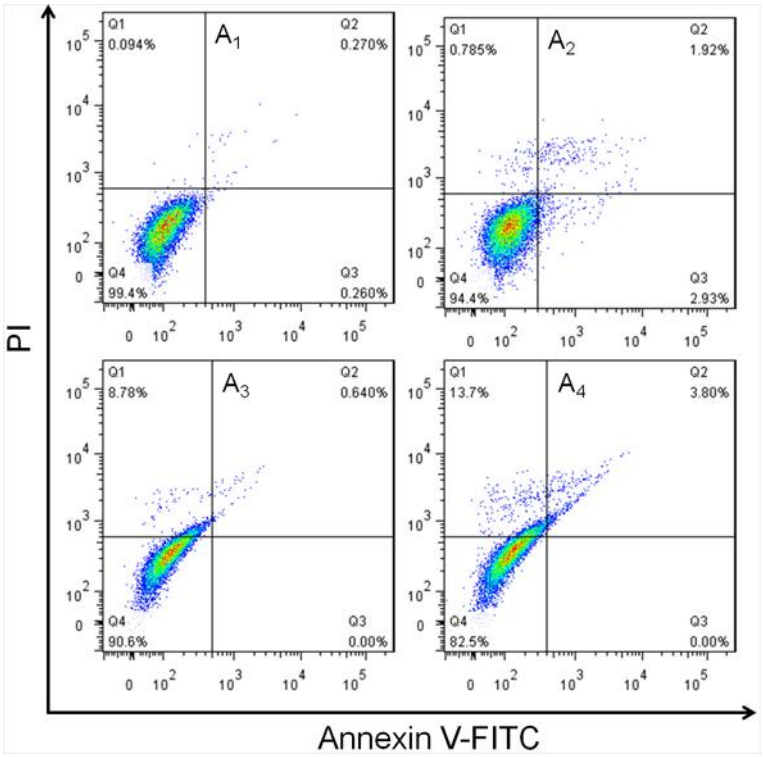


Fig S9. Apoptosis and necrosis of HeLa cells analyzed by flow cytometry, A_1 was control, A_2, A_3, A_4 was cells treated with PAP 24 h, 48 h, 72h, respectively, and cells were stained with Annexin V-FITC and PI.