The Au Clusters Induce Tumor Cell Apoptosis via Specifically Targeting Thioredoxin Reductase 1 (TrxR1) and Suppressing its Activity

Ru Liu, ‡ Yaling Wang, ‡ Qing Yuan, Deyi An, Jingyuan Li, and Xueyun Gao*

Key Lab for Biomedical Effects of Nanomaterials and Nanosafet, Institute of High Energy Physics, Chinese Academy of Science, Beijing 100049, P. R. China.

SI1: Synthesis of $Au_{25}petide_9$

All chemicals were purchased from Sigma-Aldrich, unless mentioned. Ultrapure water (18MΩ) was used throughout the experiments. The peptide (Cys-Cys-Tyr-Gly-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-Gly) was synthesized by a solid phase method (China Peptides Co. Ltd, Purity: 95%). All glassware was washed with aqua regia ($HCl:HNO_3$, volume ratio = 3:1), and further rinsed with ultrapure water and ethanol. In a typical experiment, an aqueous solution of $HAuCl_4$ (25 mM, 16 μL) was slowly added to peptide solution (1.06 mM, 376 μL) in a 5 mL vial under vigorous stirring, then NaOH (0.5 M, 8 μL) was added within 30 seconds to give a final pH of ~10. The sample was sealed and stored in the dark for 15 hours without any disturbance to produce the $Au_{25}peptide_5$ products. The as-synthesized products were dialyzed for 12h (Dialysis Tube MWCO=500) to remove free $HAuCl_4$ and NaOH, and the sample was further concentrated by ultrafiltration tube (Millipore, MWCO: 3000) to remove free peptide. The $Au_{25}peptide_9$ products was sealed and stored in dark for later studies. The picture of the cluster products was as follows, see Figure S1.

After synthesis polyacrylamide gel electrophoresis experiment was carried out to verify the product is pure. A typical procedure of the separation with single gel is described as follows: The separating and stacking gels were prepared by acrylamide monomers with the total contents of 30 and 3 wt % (acrylamide/bis-(acrylamide) =94:6), respectively. The eluting buffer consisted of 192 mM glycine and 25 mM tris(hydroxymethylamine). The sample solution (10μl) was loaded onto the stacking gel without lanes and eluted for 9 h at a constant voltage mode (150 V) to achieve sufficient separation. The PAGE result of the cluster was shown in Figure S2. It could be seen that there are only one fraction (black arrow), suggesting the cluster should be pure.

Figure S1. The picture of the $Au_{25}peptide_9$ cluster.

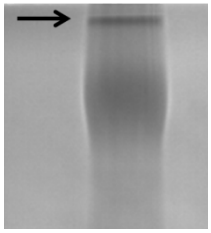

Figure S2. PAGE result of the $Au_{25}peptide_9$ clusters.

SI2: TEM and Optical studies of $Au_{25}petide_9$

The size of Au cluster was characterized by HRTEM. Sample was prepared by casting and evaporating on a 300-mesh holey carbon-coated copper grid (Electron Microscopy Sciences, Washington, USA). High resolution images were acquired with a TENCAI F20 high resolution transmission electron microscopy at 200KV accelerating voltage.

Excitation/Emission spectra of $Au_{25}peptide_9$ were carried out by a PerkinElmer (LS-55) fluorescence spectrometer. Xe lamp, scanning speed: 400 nm/min, repeated 3 times. Excitation and emission slits were both 10 nm.

SI3: MALDI TOF MASS studies of $Au_{25}petide_9$

The molecular weights of $Au_{25}peptide_9$ were analyzed by MALDI-TOF MS on an ABI MALDI-TOF system in positive ion linear mode. The α-Cyano-4-hydroxycinnamic acid was used as the matrix.

SI4: Molecular dynamics (MD) simulation of interaction between TrxR1 and $Au_{25}peptide_9$

The crystal structure of thioredoxin reductase 1 (TrxR1, PDB ID code 1H6V) was used in this work.[1] There are at least 10 negatively charged residues around the active sites of TrxR1 (within the 4 Å separation from active site), resulting in the surface region with net negative charge.

As indicated in previous literature,[2] the Au_{25} cluster is composed of a centered icosahedral Au13 core and an exterior shell formed by the remaining twelve Au atoms. The Au_{25} cluster is modified by eighteen thiolate (-SR) ligands. Each of six Au-Au pairs in the exterior shell is bridged by an -SR ligand and there are two other -SR ligands bridge between the exterior Au atoms and the icosahedral core. Hence, there are two gold and three sulfur atoms arranged in a -S-Au-S-Au-S- pattern.

In our case, the Au_{25} cluster is coated by nine peptides with the sequence of Cys-Cys-Tyr-Gly-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-Gly. For each peptide, there are two successive Cys in C-terminus. Hence, the Au_{25} cluster is similarly modified by 18 cysteine residues. It should be note that the peptide is composed of five consecutively positive residues of Lys-Lys-Lys-Arg-Lys. The gold cluster is highly positively charged, which can induce considerable electrostatic attraction to the region around the active site of TrxR1.

The structure of gold cluster in solution was equilibrated by 2 ns molecular dynamics (MD) simulation. Several representative structures of gold cluster were chosen for the following molecular docking. The rigid-body protein-protein docking program ZDOCK (version 3.0.2) was used to predict possible binding site of gold cluster to TrxR1.[3] The binding site around the active site (Cys497 and Sec 498) was successfully identified.

The structure of the complex of TrxR1 and gold cluster was further refined by 3 ns MD equilibration simulations, during which atomic constraints were employed as follows: (i) firstly, both the TrxR1 and gold cluster were restrained to their positions (1ns); (ii) next, restraint were applied to most of TrxR1, with the exception of the region in the C-terminus, i.e. Ile492, Leu493, Gln494, Ser495, Gly496, Cys497, Sec498 and Gly499. Meanwhile, distance restraint was applied to the pairs between the selected gold atoms and the sulfur atom of Cys497 andor the selenium atom of Sec498, to keep gold cluster in direct contact with TrxR1. Finally, 50 ns MD simulation was performed without any restraint to study the binding stability of gold cluster and TrxR1.

The dimension of whole simulation system was $14\times14\times14$ nm^3, with ~280000 atoms. The TIP3P water model was used, and 31 chlorine ions were added to neutralize system. The OPLS-AA force field was used in this workour system. The bonds involved by hydrogen atoms were constrained by LINCS algorithm. A cutoff distance of 10 Å was used for the calculations of short-range electrostatic and van der Waals interactions. For long-range electrostatic interactions, the Particle Mesh Ewald (PME) method was used. Periodic boundary conditions were applied for the simulations with a time step of 1 fs. The MD simulations were performed with NPT ensemble at 1bar and 300K, the pressure and temperature of system were maintained by using the Parrinello-Rahman method and the velocity rescaling method, respectively. All the simulations were performed by GROMACS 4.5.5 package.

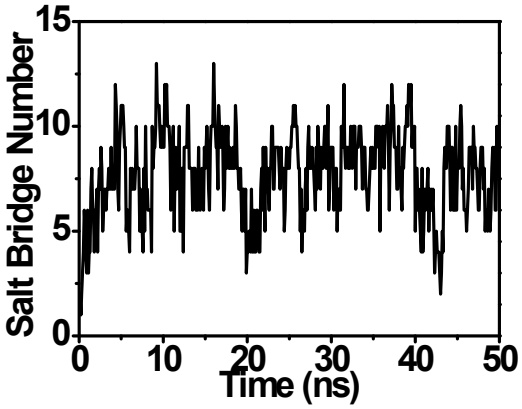

Figure S3. Number of salt bridges between gold cluster and TrxR1. The average number of salt bridges is 8.

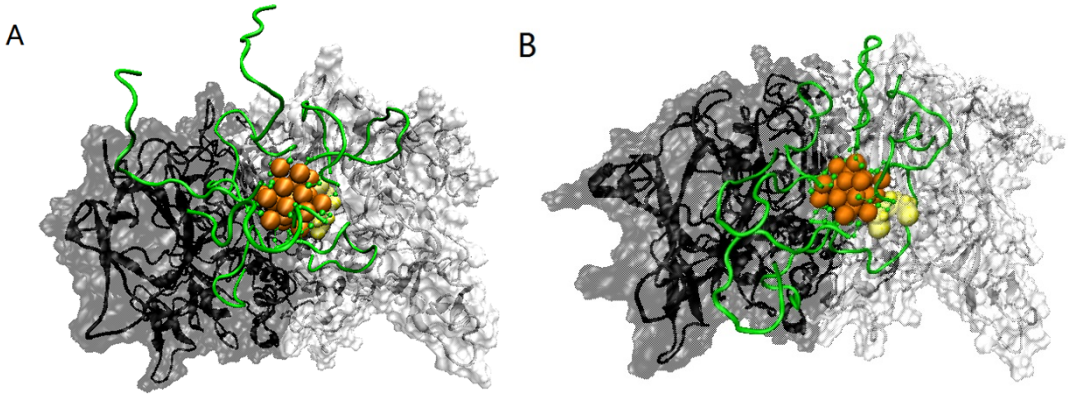

Figure S4 Representative configurations about the complex of gold cluster and TrxR1 in MD simulation (A, B). Gold cluster remains bound to the active site at the inter-domain region of TrxR1 throughout the simulation. The gold atoms, peptide chain, Cys/Sec residues in active site are in orange, green and yellow separately.

SI5: Tube experiment of TrxR1 activity suppressed by $Au_{25}peptide_9$

Pure TrxR1 (Thioredoxin Reductase from rat liver buffered aqueous glycerol solution, ≥100 units/mg protein) were diluted by assay buffer and incubated with different concentration of Au cluster at 0, 4, 16, 64 μM for 30min, respectively. And then the TrxR activity was measured by the Thioredoxin Reductase Assay Kit following the manufacturer's instructions. The relative TrxR1 activity of each sample is expressed by X/Y, where the X is the enzyme activity of gold treated samples and Y is the enzyme activity of none gold treated ones. And the experiments were mesured in triplicate.

For excluding the effect of the serum protein, TrxR1 was introduced into the $Au_{25}Peptide_9$ system after pre-incubated with the culture medium with 10% FBS,. Then the activity of TrxR1 was tested and the results were shown in Figure S5. It is clear that TrxR1 activity is suppressed via dose dependent manners in tube experiment in present of serum proteins.

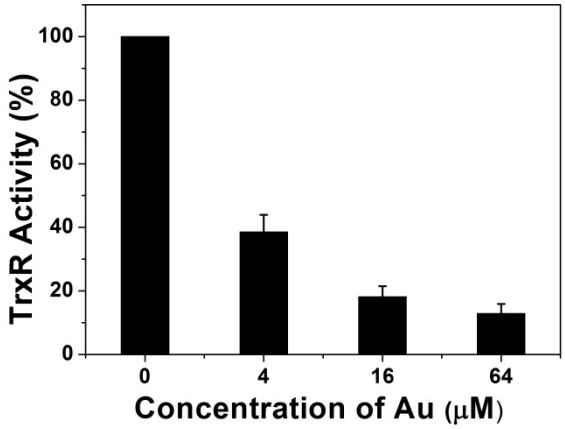

Figure S5. TrxR1 activity suppressed by serial dose of $Au_{25}peptide_9$ in tube study.

SI6: Confocal microscope observation the Hela cell uptake $Au_{25}petide_9$

Hela (human cervical cancer cell line) cells were grown exponentially as monolayers in a culture flask (25 cm^2) in DMEM/high glucose (1×) medium supplemented with 10% (v/v) fetal bovine serum and 4 mM L-glutamine. Cells were cultured in a humid incubator at 37 °C, under

an atmosphere containing 5% CO_2. The $Au_{25}peptide_9$ was introduced and the final gold concentration in culture media is 16μM, the Hela cell exposed to $Au_{25}peptide_9$ for 36hr. Before confocal observation, the culture media is discarded and the cell was washed twice by with fresh PBS buffer, then new culture media was applied to Hela cell. Cells were imaged using an UltraVIEW Vox (PerkinElmer) confocal system attachment and a Nikon Ti-e microscope with 60×1.4NA plan apochromat oil immersion lens. Excitation wavelengths were set at 561 nm and emission wavelengths were set at 615 nm (red).

In order to identity the accurate location of the $Au_{25}peptide_9$ cluster in Hela cells, lysosome probes were used. After incubated with cluster for 36hr, lysotracker green were added in the system and observed by confocal microscope, see Figure S6. It could be found that most of $Au_{25}peptide_9$ cluster were located in cytoplasm evidenced by the strong red emission and a small quantity of cluster localized in the lysosome as shown by the weaker yellow emission overlaid by the red emission of the cluster and the green emission of lysotracker Green co-localized. Hence, there are only a few clusters distribute in the endosome or lysosome and the clusters are very likely to interact with TrxR1 after cellular uptake.

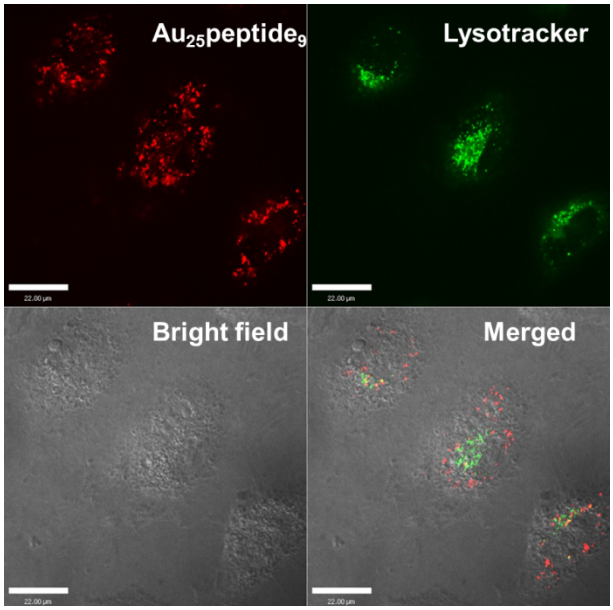

Figure S6. Confocal microscopy images of the intracellular distribution of $Au_{25}peptide_9$ cluster.

SI7: Cell viability studies of free peptide and $Au_{25}peptide_9$ in Hela cell

Cell viability was measured by Cell Counting Kit-8 system (CCK-8) according to manufacturer's instructions (DOJINDO Lab., Japan). Hela cells were seeded at 1×10^4 cells per well in 96-well plates and allowed to attach for 24h. After incubation with different concentrations of Au cluster or free peptide at 0, 0.25, 1, 4, 16μM for 36h, cells were immediately washed with PBS and then incubated with fresh medium containing 10μl of CCK-8 reagent for 2h at 37°C. Then the absorbance at 450 nm was measured by using a microplate reader (SpectraMAX M2, Sunnyvale, California) with a reference at 600 nm. All data were presented as mean percentages ± standard deviation in triplicate compared to the optical density (OD) values of controlled cells.

SI8: The studies of TrxR1 activity suppressed by $Au_{25}peptide_9$ in Hela cell

Hela cells were seeded into a 6-well plate and grown to 80% confluence prior to treatment with Au cluster. After 12h incubation, the media was replaced by fresh culture media containing $Au_{25}peptide_9$ of 0, 0.25, 1, 4, 16μM for another 36h incubation. The cells were then lysed by Mammalian Cell Lysis/Extraction Reagent (CelLytic™ M from sigma) and centrifuged at 18,000 g/min to get total protein of cells. Bradford methods are used to measure the protein concentration of each sample. Thioredoxin Reductase Assay Kit was used to measure the TrxR activity extracted from of different dose gold treated Hela cells. The relative TrxR activity of each sample is expressed by X/Y, where the X is the enzyme activity of gold treated cell and Y is the enzyme activity of none gold treated cell.

For the control experiments, after $Au_{25}Peptide_9$ was added into the cell lysate containing TrxR1, the activity of TrxR1 was tested and the results were shown in Figure S7. It is clear that TrxR1 activity is suppressed via dose dependent manners in cell lysate experiment.

In order to evaluate the interaction between the Au and other selenium-containing enzymes or proteins, such as glutathione reductase, similar activity experiments were carried out. $Au_{25}Peptide_9$ clusters were first added into the cell lysate containing GPx, Cellular Glutathione Peroxidase Assay Kit was used to test the activity of GPx. The results were shown in Figure S8. Within whole concentration scale the activity of GPx is almost unchanged. It suggests that the decrease of ROS level does not result from the impact to GPx. As indicated by our results, the suppression of TrxR1 activity is largely attributed to the coordination interaction of Au with the –SeH and –SH groups of active site in TrxR1. Even though the structure of glutathione reductase is similar toTrxR1, there is not C-terminal redox active site, Cys-Se-Cys, leading to speculations that the selenol group could be the only target. This means inhibiting effect of Au on TrxR1 is much sensitive and obvious than on the GPx.

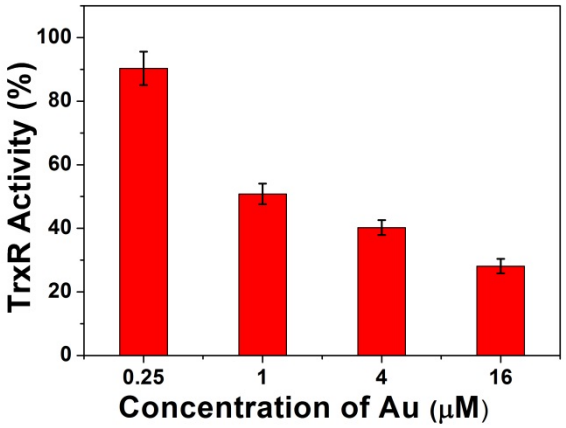

Figure S7. TrxR1 activity suppressed by serial dose of $Au_{25}peptide_9$ in cell lysate experiment.

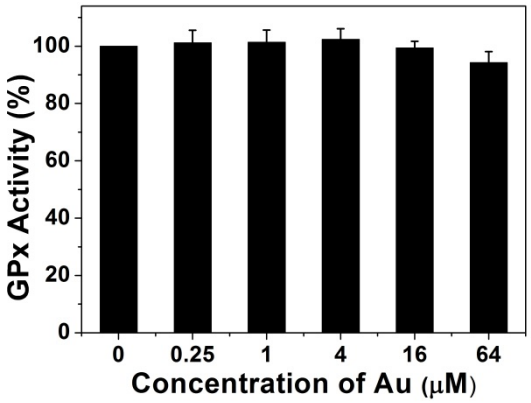

Figure S8. Glutathione reductase activity test by serial dose of $Au_{25}peptide_9$ in cell lysate.

SI9: Confocal microscope observation the radical oxygen species (ROS) induced by $Au_{25}petide_9$ in live Hela cell

Hela (human cervical cancer cell line) cells were grown exponentially as monolayers in a culture flask (25 cm^2) in DMEM/high glucose (1×) medium supplemented with 10% (v/v) fetal bovine serum and 4 mM L-glutamine. Cells were cultured in a humid incubator at 37 °C, under an atmosphere containing 5% CO_2. The $Au_{25}peptide_9$ was introduced and their final gold concentration in culture media is 16μM, the Hela cell exposed to $Au_{25}peptide_9$ for 36hr. After gold compound treatment, Hela cell were exposed to CM-H_2DCFDA e.g. General Oxidative Stress Indicator, in cell cultured media for 30 minutes. Before confocal observation, the culture media is discarded and the cell was washed twice by with fresh PBS buffer, then new culture media was applied to Hela cell. In Hela cell, ROS were highlighted by CM-H_2DCFDA and imaged using an UltraVIEW Vox (PerkinElmer) confocal system attachment and a Nikon Ti-e microscope with 60×1.4NA plan apochromat oil immersion lens. Excitation wavelengths were set at 488nm nm and emission wavelengths were set at 527nm (green).

SI10: The immunoblotting of PARP, TrxR1, and actived PARP from $Au_{25}petide_9$ treated Hela cell

Hela cells were seeded into a 6-well plate and grown to 80% confluence prior to treatment with Au cluster. After 12 h incubation, the media was replaced by new culture media containing Au cluster of 0, 0.25, 1, 4, 16 μM for further 36 h incubation. The cells were then harvested and lyzed with RIPA buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 50 mM NaF and 1mM ethylenediaminetetraacetic acid), protease inhibitor cocktail tablet (Roche Molecular Biochemicals) for 15min at 4℃. Loading buffer was added and samples were denatured for 5 min at 95℃, samples with equal amount were subjected to 12% SDS-PAGE gel and transferred to PVDF membranes. Primary antibodies of rabbit anti- PARP and Thioredoxin 1 (Trx 1) were purchased from Cell Signaling Technology (MA, USA), rabbit anti-β-actin was purchased from Beijing Bioss Biological Technology (Beijing, China), rabbit anti-TrxR1 was purchased from Santa Cruz Biotechnology (Texas, USA). The primary antibodies were diluted as 1:1,000. Samples in PVDF membrane were washed three times with Tris-buffered saline with 0.1% Tween-20, and then horseradish peroxidase-conjugated goat anti-rabbit secondary antibody diluted as 1:5,000 (Jackson, PA, USA) was added. Antibody-antigen reactions were visualized by Amersham ECL™ Prime Western Blotting Detection Reagent (GE healthcare, UK).

SI11: The studies of $Au_{25}petide_9$ induced Hela cell apoptosis

Apoptosis was detected by flow cytometry using an Annexin V-FITC Apoptosis Detection Kit following the manufacturer's instructions (Oncogene Research Products). Hela cells were seeded into a 12-well plate at 1×10^5 cells per well. After growth for 24 h under normal cell culture conditions, cells were treated with Au cluster at 0.25, 1, 4, 16 μM for 36 h, respectively. After 36 h incubation, the culture media were replaced and cells were stained with annexin V-FITC and propidium iodide (PI). The percentage of apoptotic and necrosis cells was

quantified using an Accuri C6 flow cytometer and the data were analysed by Cflow software. The original apoptosis data were displayed as Figure S9.

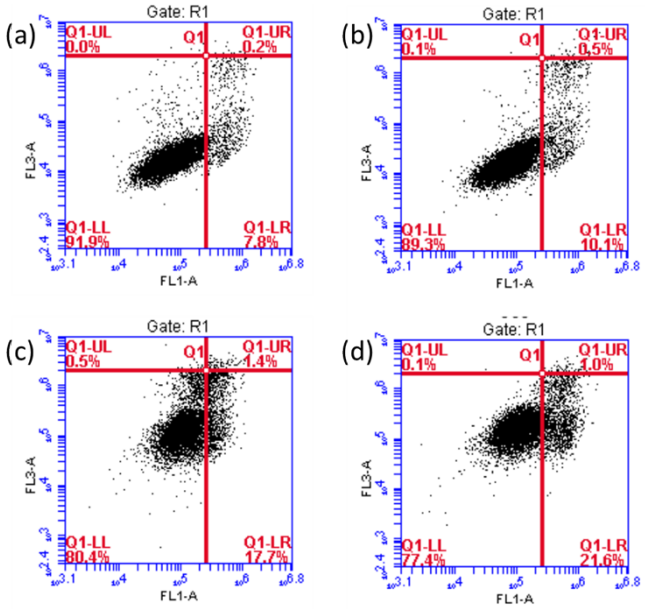

Figure S9. Hela cell apoptosis induced by serial dose of $Au_{25}peptide_9$, 0.25μM (a), 1μM (b) , 4μM (c) , 16μM (d).

SI12: The control studies of $Au_{25}peptide_9$ incubted with normal cell lines

The other two cell lines include human-embryo lung fibroblasts (MRC5) as the normal control and human non-small cell lung carcinoma (H1299) as positive control, see Figure S10. Clearly, under the same concentration, the change of the cell viability of MRC and H1299 cell lines is almost negligible as compared with the Hela cell. Meanwhile, the suppression of TrxR1 activity is much less in the MRC-5 and H1299 cells. These results further suggest that the Au cluster could be developed in tumor therapy.

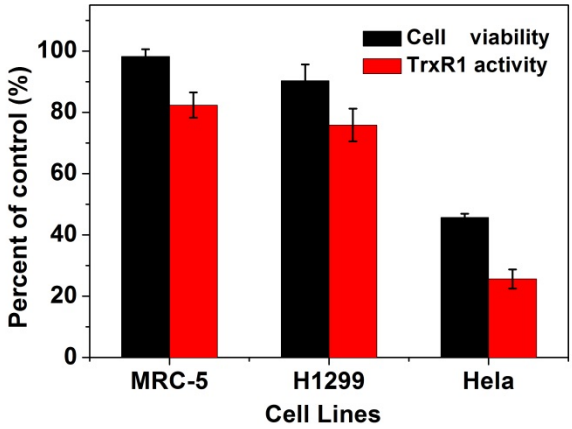

Figure S10. The cell viability and TrxR1 activity of cell lines by incubated with $Au_{25}peptide_9$ clusters at 16 μM concentration.

SI13: Statistical analysis of data

The data were presented as the mean standard error from at least three independent experiments. Statistical significance was tested using a Student's t-test.

Reference

(1) Sandalova, T.; Zhong, L.; Lindqvist, Y.; Holmgren, A.; Schneider, G. *Proc. Natl. Acad. Sci. USA* **2001,** *98,* 9533.
(2) Zhu, M.; Aikens, C. M.; Hollander, F. J.; Schatz, G. C.; Jin, R. *J. Am. Chem. Soc.* **2008,** *130,* 5883.
(3) Chen, R.; Li, L.; Weng, Z. *Proteins* **2003,** *52,* 80.