The cytotoxicity of gold nanoparticles is dispersity-dependent

Dengtong Huang, Hualu Zhou, Hanyu Liu, and Jinhao Gao*

Electronic Supplementary Information

Experimental section

Synthesis, modification and characterization of gold nanoparticles and nanorods

12 nm citrate-capped gold nanoparticles were synthesized according to the methods of Frens et al.[1] with an optimization by Zakaria et al.[2] First, 8 mL of 50 mM $HAuCl_4$ was added to 400 mL boiling ultra-pure water, while kept heating and stirring. Then 7.2 mL of 250 mM fresh sodium citrate solution was rapidly added. The resulted solution was boiled for about 10 min and then stopped heating and cooled to room temperature. We would get the 12 nm gold colloid.

4 nm gold nanoparticles were synthesized using sodium borohydride.[3] In brief, 1 mL of 50 mM $HAuCl_4$ and 0.2 mL 250 mM sodium citrate were add to 194 mL water under magnetic stir. Then 5 mL of 0.1 M $NaBH_4$ was added. The solution turned pink immediately. The gold nanoparticles were modified within 5 h.

23 nm gold nanoparticles were synthesized using seeding growth methods. 16 nm gold nanoparticles synthesized from reduction by sodium citrate was used as seed. 1.7 mL 250 mM sodium citrate and 1.7 mL 50 mM $HAuCl_4$ were added to 200 mL seed solution (0.1 mmol Au) while stirring. 20 mL 0.4 mg/mL hydroxylamine hydrochloride was added at a rate of 0.5 mL/min. The reaction was kept for 6 h.

Gold nanorods were synthesized according to the methods of El-Sayed et al.[4] with an optimization by Zubarev et al.[5] CTAB on the surface of nanorods was replaced by sodium citrate according to the methods provided by Mehtala et al.[6] Then the resulted nanorods were coated with meso-2,3-dimercaptosuccinic acid (DMSA) as follows.

50 mL of as-synthesized gold nanoparticles was diluted to 200 mL with water. 50 mg DMSA dissolved in 20 mL mixture of acetone and water was added into gold colloid solution at a rate of 200 μL/min while kept stirring. The reaction was kept for 12 h. Finally, the resulted Au@DMSA nanoparticles were purified by ultrafiltration (Amicon® Ultra-15, 100 kD, Millipore). Unless otherwise specified, the experimental subject was 12 nm Au@DMSA nanoparticles.

The size and morphology of gold nanoparticles were detected by transmission electron microscopy (TEM). The hydrodynamic diameter of nanoparticles was monitored by dynamic light scattering (DLS) (Nano-ZS90, Malvern). The concentration of gold nanoparticles was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

Regulation of the dispersity of gold nanoparticles

To prepare well dispersed Au nanoparticles, we added culture medium to Au nanoparticles, and then did pipetting immediately for 30 times. To prepare aggregated Au nanoparticles, we added Au nanoparticles to culture medium and kept undisturbed for 1 min or longer. In detail, we set well-dispersed groups (**10m** and **30m**) and aggregated groups (**10** and **30**) as follows: (a) **10m**, 990 μL culture medium was added to 10 μL Au@DMSA nanoparticles and then mixed by pipetting immediately. The final concentration of Au was 32 μg/mL. (b) **10**, 10 μL nanoparticles was added to 990 μL culture medium and kept undisturbed for 1 min or longer. After the nanoparticles were aggregated, gently shook to make them uniform. The final concentration of Au was the same to **10m**. (c) **30m**, 970 μL culture medium was added to 30 μL nanoparticles and then mixed by pipetting immediately. The final concentration of Au was 96 μg/mL. (d) **30**, 30 μL nanoparticles was added to 970 μL culture medium and kept undisturbed for 1 min or longer. After the nanoparticles were aggregated, gently shook to make them uniform. The final concentration of Au was the same as **30m**.

Methyl thiazolyl tetrazolium (MTT) assay

HeLa cells were seeded on a 96-well plate at a density of 5000 cells/well and incubated at 37 °C. We set six wells in each group. Five wells were parallel and the other well was blank control. 12 h later, the wells were replaced by fresh medium containing gold nanoparticles. After incubating for 24 h or 48 h, the parallel wells were replaced by fresh medium containing 0.5 mg/mL MTT while the blank control wells were just replaced by fresh medium. 4 h later, removed the medium and added 100 μL dimethylsulfoxide (DMSO). Shook for 5 min and measured the absorbance of each well at 490 nm using a microplate reader (Multiskan FC, Thermo Scientific).

Reactive oxygen species (ROS) detection

HeLa cells were seeded on a 6-well plate at a density of 3×10^4/well and incubated for 24 h. After cells were treated with gold nanoparticles for 48 h, they were collected by trypsin and stained with 50 μM 2',7'-dichlorofluorenscin diacetate (DCFHDA) for 30 min in the dark. Then the cells were washed three times and resuspended in PBS. The fluorescence intensity was measured using flow cytometer (BD FACSAria II). At least 10,000 events were recorded and analyzed.

Annexin V-Fluos/PI staining

HeLa cells were seeded on a 6-well plate at a density of 3×10^4/well (for 24 h) or 5×10^4/well (for 48 h) and incubated for 24 h. After cells were treated with gold nanoparticles for 24 h or 48 h, they were collected by trypsin and stained with Annexin V-Fluos/PI staining kit (Roche Life Science) according to the guide of manufacturer. The fluorescence intensity was measured using flow cytometer. At least 10,000 events were recorded and analyzed.

Scanning electron microscopy (SEM) imaging of cells

After the cells were treated with gold nanoparticles for 24 h, they were fixed with 2% glutaraldehyde (EM grade, SPI Supplies) for 30 min at room temperature and then washed with PBS for 5 min × 4. Dehydrate cells by ethanol with increasing concentration (30%, 50%, 70%, 90%, 100%, respectively) for 5-10 min each time. Finally, dry the cells under dryer. We coated cell with about 13 nm Pt using auto fine coater (JFC-1600) before acquiring images using Zeiss Sigma SEM.

Transmission electron microscopy (TEM) imaging of cells

After the cells were treated with gold nanoparticles for another 24 h, they were collected by trypsin and centrifuged (400 × g, 4 min) to a pallet. Fixed with 2% glutaraldehyde at 4 °C overnight, the cells were washed with PBS for 5 min × 5. Dehydrate cells in ethanol with increasing concentration (30%, 50%, 70%, 90%, 100%, respectively) for 5-10 min each time. Repeated once in 100% ethanol and then replaced it with acetone for 5 min × 2. Cell pallet was embedded in SPI-Pon 812 resin (SPI Supplies) and polymerized at 35 °C for 12 h, and 60 °C for 24-48 h. Sections (60-80 nm, ultramicrotome Leica Ultracut) were stained with 3% uranyl acetate (EM grade, SPI Supplies) and lead citrate (EM grade, SPI Supplies). TEM images were acquired in JEM-1400, 100 kV.

Quantification by ICP-MS (Inductively coupled plasma mass spectrometry)

Cells were harvested by trypsin and centrifuged to a pellet. 2 mL aqua regia was added. We then heated it at 95 °C to completely digest cells and gold nanoparticles. After that, we dilute it to a certain volume and filtered it using a 0.45 μm filter. The gold concentrations were determined by Agilent 7700X ICP-MS.

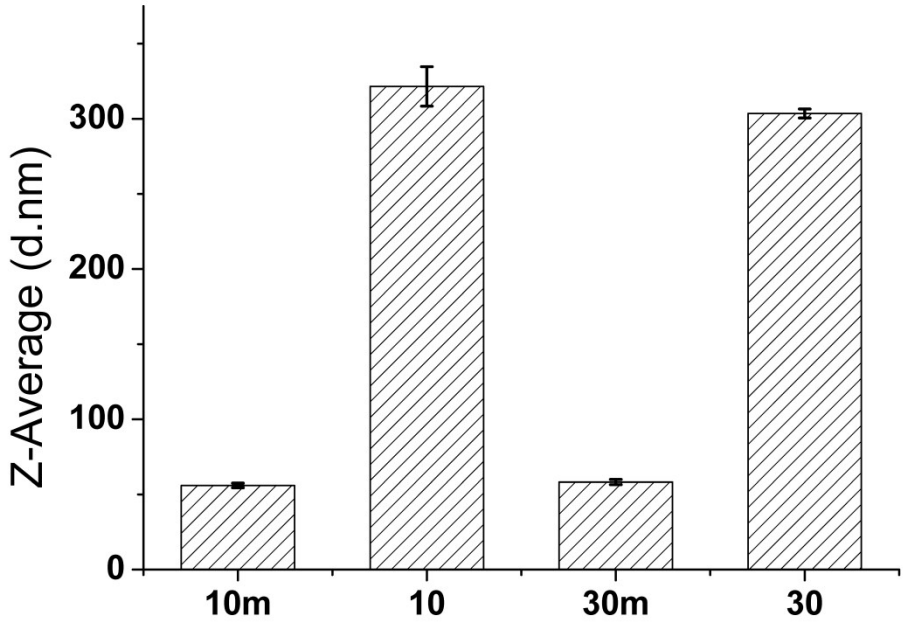

Figure S1. Monitoring aggregation of gold nanoparticles in culture medium by DLS. The aggregation size was about 300 nm in **10** and **30** groups. Pipetting (**10m** and **30m**) reduced the aggregation. Data was represented as the average of three replicate measurements with standard deviation.

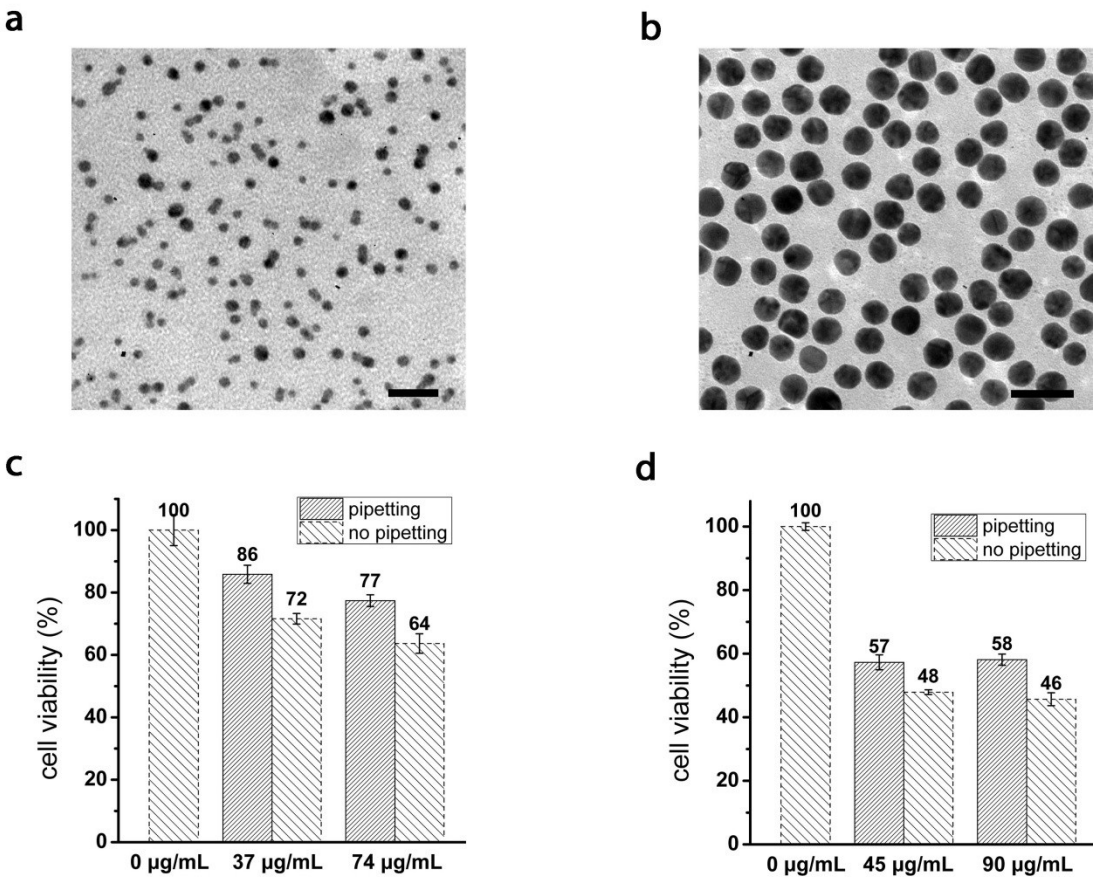

Figure S2. Cytotoxicity of gold nanoparticles with different sizes. (a) TEM image of 4.3 nm gold nanoparticles. Scale bar was 20 nm. (b) TEM image of 23 nm gold nanoparticles. Scale bar was 50 nm. Cell viability after treated with (c) 4.3 nm and (d) 23 nm gold nanoparticles for 24 h. Data was represented as the average of five replicate wells with standard deviation.

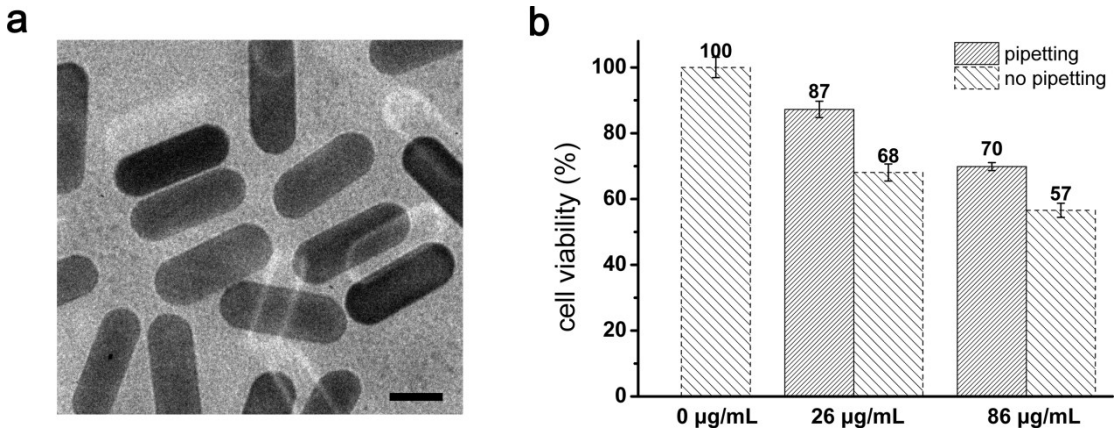

Figure S3. Cytotoxicity of gold nanorods. (a) TEM image of 45×18 nm gold nanorods. Scale bar was 20 nm. (b) Cell viability after treated with gold nanorods for 24 h. Data was represented as the average of five replicate wells with standard deviation.

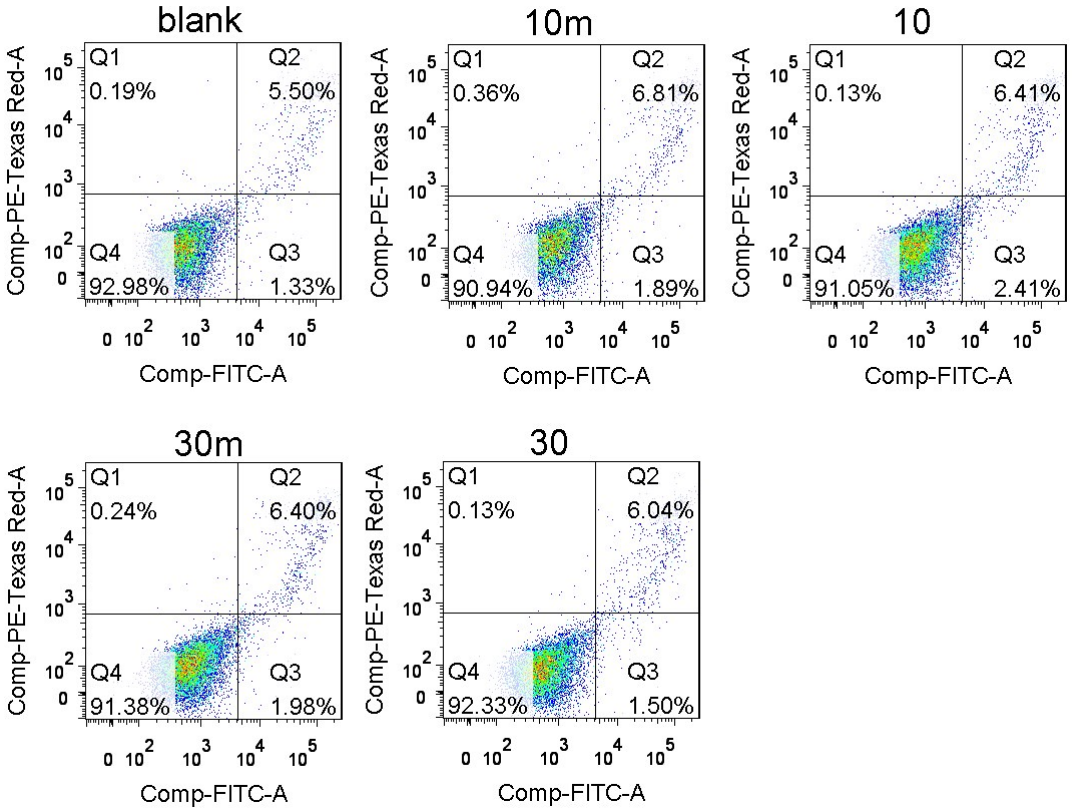

Figure S4. Annexin V-Fluos/PI staining of cells treated Au@DMSA nanoparticles for 24 h. Horizontal axis shows the fluorescence intensity of annexin V-fluos while vertical axis represents PI. Q3 zones with high intensity of annexin while low intensity of PI represent cell populations at early stage of apoptosis.

Table S1. The ratio of apoptotic cells after treated with gold nanoparticles

24 h	The ratio of apoptosis (%)	48 h	The ratio of apoptosis (%)
Blank	1.33	**Blank**	0.83
10m	1.89	**10m**	1.23
10	2.41	**10**	1.06
30m	1.98	**30m**	0.82
30	1.50	**30**	0.39

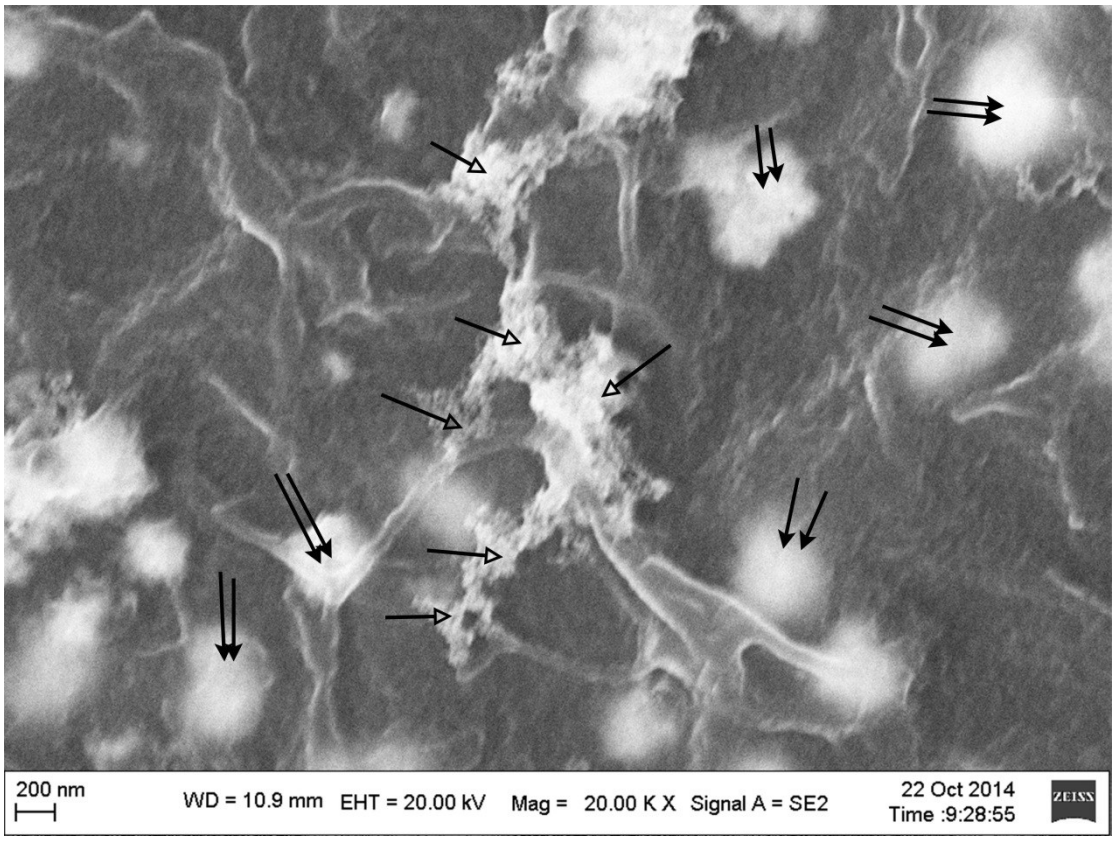

Figure S5. Gold nanoparticles outside and inside cells. Single open arrows pointed to gold nanoparticles outside cells, while double solid arrows pointed to gold nanoparticles inside cells. The data of element analysis was shown in Fig. S4 and Table S2.

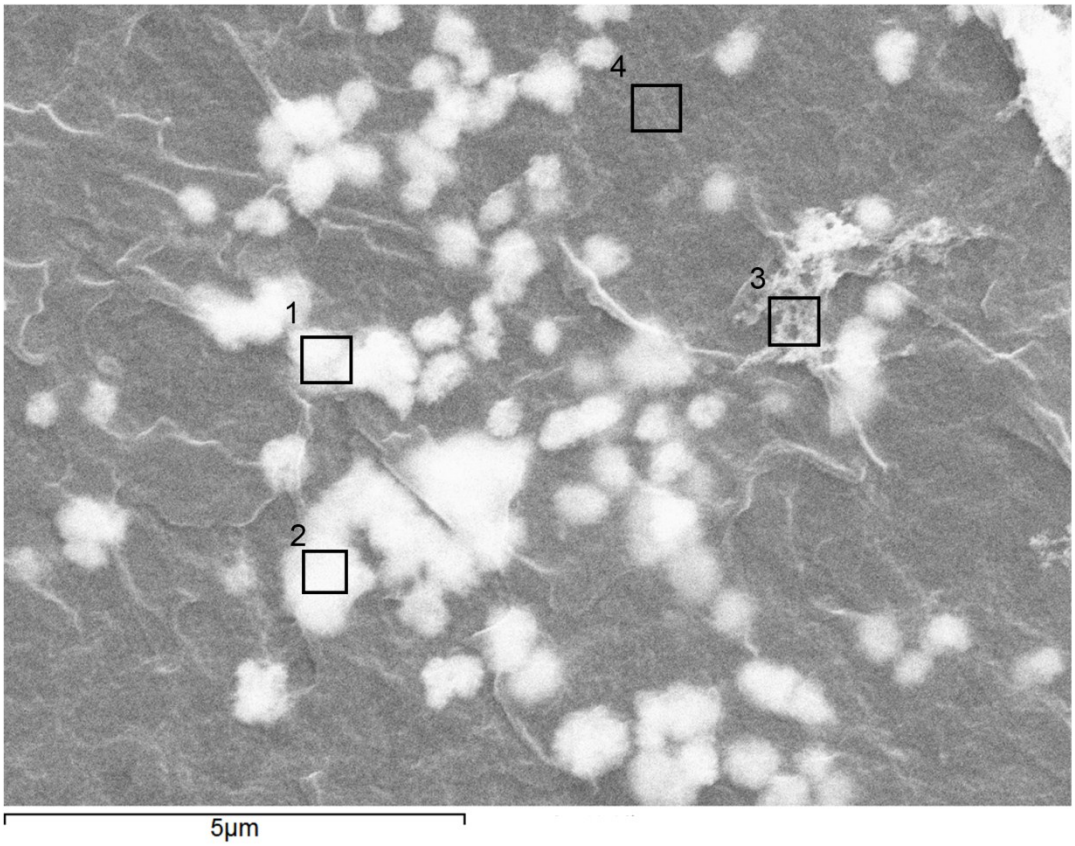

Figure S6. Element analysis of gold nanoparticles outside and inside cells. The element content in each pane was shown in Table S2.

Table S2. Element content of each spectrogram in Fig. S4.

spectrogram	C	N	O	Au	total
1	68.54	14.56	7.03	9.87	100.00
2	60.87	16.37	8.09	14.67	100.00
3	88.28	0.00	8.86	2.86	100.00
4	74.73	16.10	9.17	0.00	100.00

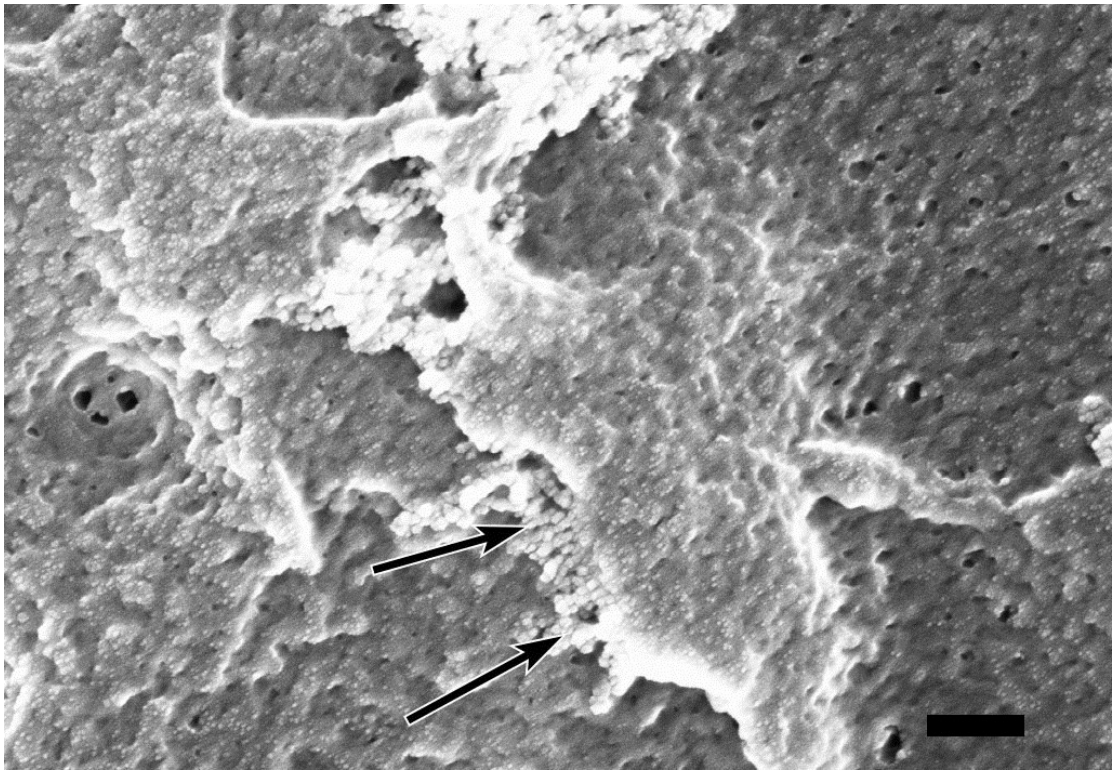

Figure S7. Visualizing macropinocytosis of aggregated gold nanoparticles by SEM. The arrows pointed to the gold nanoparticles internalized by macropinocytosis. Scale bar was 200 nm.

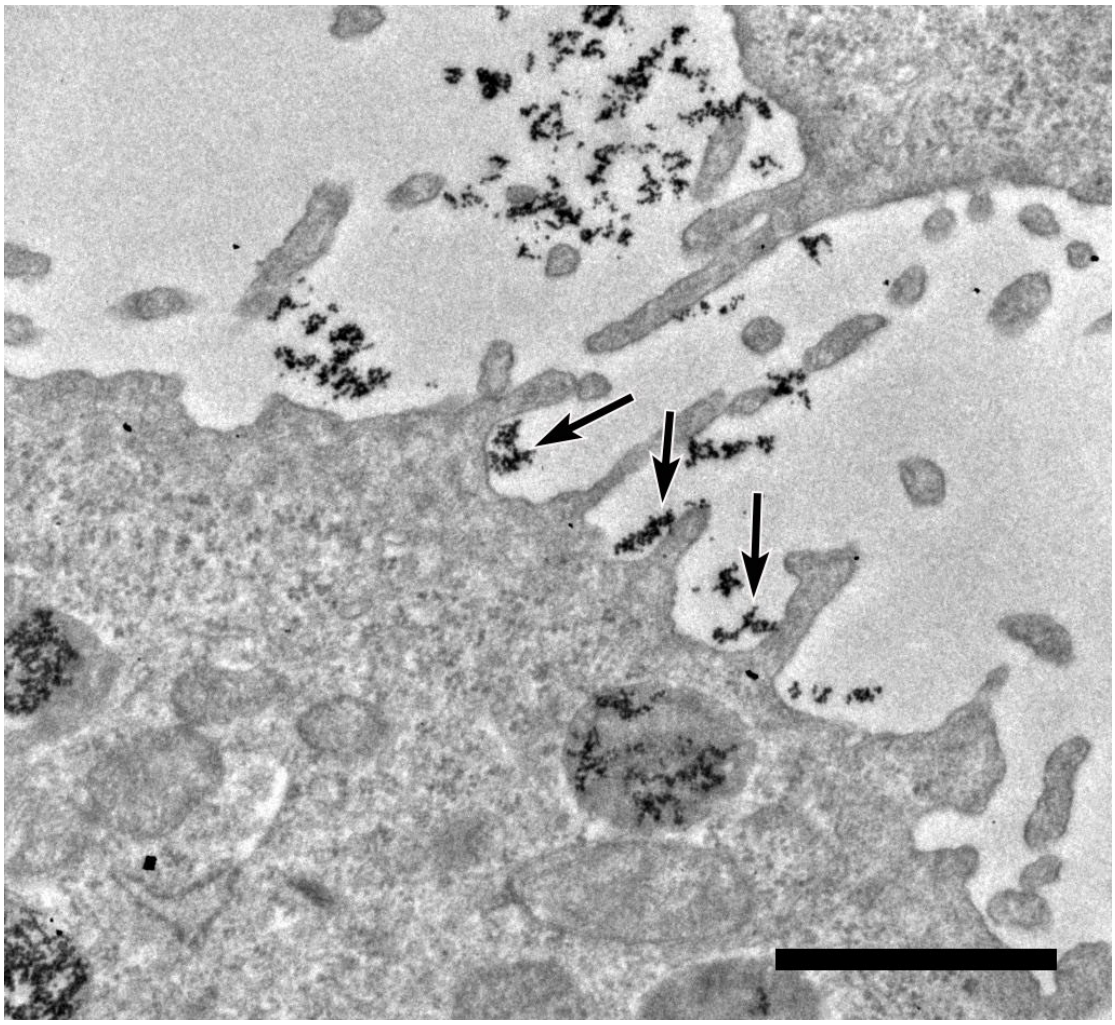

Figure S8. Visualizing macropinocytosis of aggregated gold nanoparticles by TEM. The arrows indicated that the gold nanoparticles being internalized by macropinocytosis. Scale bar was 1 μm.

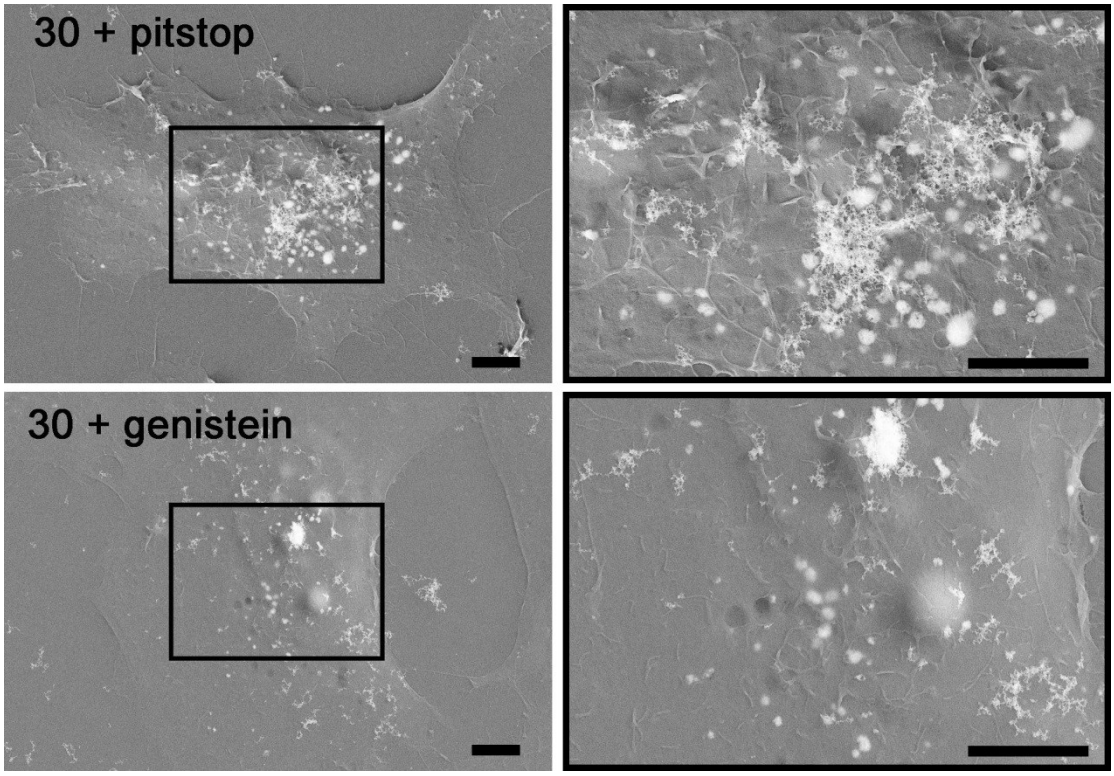

Figure S9. Inhibition of endocytosis. Cells were treated with gold nanoparticles together with 25 μM pitstop or 100 μM genistein for 6 h. The right views were the magnification of the region with a black box in the left views. Scale bars were 5 μm. Note: since macropinocytosis is tyrosine kinase dependent process and genistein is an inhibitor of tyrosine kinase, macropinocytosis can be partially inhibited by genistein.

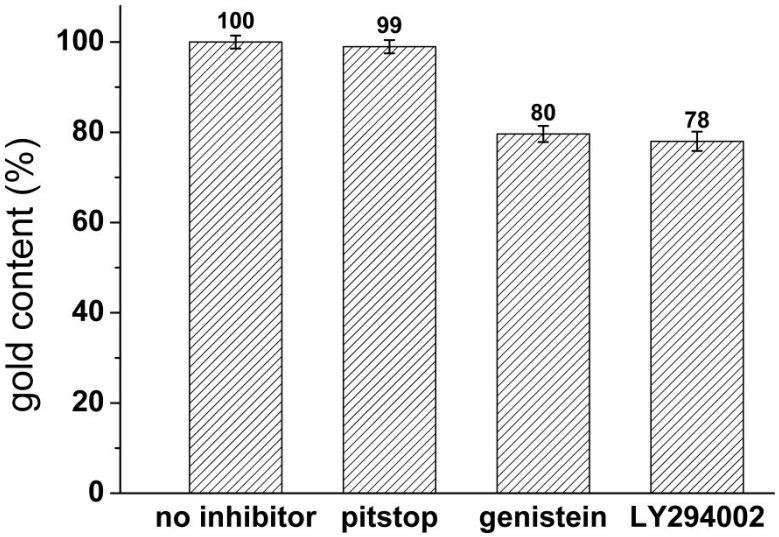

Figure S10. Quantification of endocytosis of gold aggregation in the presence or absence of inhibitors by ICP-MS. Data was represented as the average of three replicate measurements with standard deviation. Note that we could not completely remove the particles adhered to cell surfaces. The ICP-MS data include the gold nanoparticles both inside cells and on cell surfaces. Since macropinocytosis is tyrosine kinase dependent process and genistein is an inhibitor of tyrosine kinase, macropinocytosis can be partially inhibited by genistein.

References:

1 G. Frens, *Nature*, 1973, **241**, 20.
2 H. M. Zakaria, A. Shah, M. Konieczny, J. A. Hoffmann, A. J. Nijdam and M. E. Reeves, *Langmuir*, 2013, **29**, 7661.
3 N. R. Jana, L. Gearheart and C. J. Murphy, *Langmuir*, 2001, **17**, 6782.
4 B. Nikoobakht and M. A. El-Sayed, *Chem. Mater.*, 2003, **15**, 1957.
5 B. P. Khanal and E. R. Zubarev, *Angew. Chem. Int. Ed.*, 2007, **46**, 2195.
6 J. G. Mehtala, D. Y. Zemlyanov, J. P. Max, N. Kadasala, S. Zhao and A. Wei, *Langmuir*, 2014, **30**, 13727.