Electronic supplementary information

For

Size dependent anti-invasiveness of silver nanoparticles in lung adenocarcinoma cells

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Experimental Details

Materials

$AgNO_3$ (99.8%, Tongbai Xinhong Silver Products Co. Ltd, Henan, China), sodium borohydride (NaBH4) and trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) were commercially available and used as received. Dihydroethidium was acquired from Apexbio Technology (USA). Other commercial reagents were analytical reagent grade without further purification. The solutions were prepared using ultrapure water, which was obtained through a Milli-Q Integral-5 water purification system (Millipore, U.S.A.) and had an electric resistance of 18.2MΩ. The glass vials used in this work were cleaned with aqua regia (HCl/HNO_3 in volume = 3:1) and rinsed with copious amounts of Milli-Q purified water before use.

Apparatus

The plasmon resonance absorption (PRA) of AgNPs was measured with a Hitachi U-3010 spectrophotometer (Tokyo Japan). Scanning electron microscopy (SEM)

observations were carried out on a Hitachi S-4800 scanning electronmicroscopy (Tokyo, Japan). Bright-field and fluorescent optical imaging of the cells was performed with an Eclipse Ti-S inverted fluorescent microscope (Nikon, Japan), and the dark-field imaging of Ag NPs and cells was acquired with the Eclipse Ti-S inverted fluorescent microscope equipped with a highly numerical dark field condenser.

Preparation of Silver Nanoparticles

The preparation of silver nanoparticles (AgNPs) was performed according to the reference with small modification. 13 nm AgNPs[1]: 1 mL of 50 mM $AgNO_3$ and 1 mL of 5% (w/w) trisodium citrate were added into 48 mL of purified water under vigorous stirring, followed by the supplementary of a small amount of $NaBH_4$ solid. Under continuous stirring for about 10 min, color of the solution is yellow, indicating the formation of AgNPs. The colloidal solution of AgNPs were centrifuged and the supernatant solution was collected for further use.

45 nm AgNPs[2]: 1.5 mL of 2% (w/w) trisodium citrate was added into a boiling 50.0 mL solution containing 1.0 mM $AgNO_3$ in a conical flask. Under continuous stirring and boiling for about 30min, the color of the aqueous mixture changes to brown-yellow via yellow, and the colloidal solution of AgNPs was continuous stirred until it cooled down to room temperature.

92 nm AgNPs[3]: A 50 mL glycerol/water mixture (40 vol% glycerol) was vigorously stirred and heated up to 95°C. Then, 9 mg silver nitrate, and 1 min later, 1 mL sodium citrate (3%) were added to the solvent. The reaction solution was stirred for 1 h at 95°C until the reaction was completed, and the colloid of AgNPs was continuous stirred and stored in a plastic tube at 4 °C.

The size AgNPs as prepared were then measured with SEM, and the concentration was calculated according to the absorbance of the colloidal solution[4], during which molar decadic extinction coefficient ε of the 15, 45 and 92 nm AgNPS are used as 1.57×10^{9}, 1.63×10^{10} and 1.62×10^{11} $cm^{-1}\cdot M^{-1}$, respectively.

Cell culture

Human lung adenocarcinoma A549 (Cat#CBP60084) and mouse subcutaneous connective tissue and fat fibroblasts L929 (Cat#CBP60878) are both commercially from Shanghai Cell Bank (Chinese Academy of Sciences) and routinely cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture (DMEM) supplemented with 10% FBS, penicillin and streptomycin.

MTT assay for evaluation of cytotoxicity

A549 and mice fibroblast L929 cells were seeded at a density of 5, 000 cells in 100 µl of media/well in a 96-well plate, and incubated with various amount of 15, 45 or 92 nm AgNPs for 24h, 48 h or 72h, and then the cells were treated with EnoGeneCell Counting Kit-8 (CCK-8) at 37 °C. After 2-3 hrs incubation, the absorbance was measured using a micro plate reader (Thermo Scientific Multikcan Go, America) at 450 nm. Based on the cell viability, the percentage of cytotoxicity was calculated.

Internalisation of AgNPs

A549 cells were treated with AgNPs overnight, after removal of the unbound particles through washing, the cells then fixed with 4% paraformaldehyde for 30 min. For nucleolus dyeing, Hoechst 33258 (Sigma) was dissolved in 1 mL of water to give a stock solution of 1.2 mg mL^{-1} (2 mM). Cells were stained with PBS mixed with Hoechst 33258 for 10 min, washed with PBS and mounted with glycerin for final imaging.

Reactive Oxygen Species (ROS)

The level of intracellular ROS in A549 cells treated with AgNPs was measured using ROS Fluorescent Probe-DHE (Dihydroethidium). An autoclaved cover-slip was placed at the bottom of a sterile 24-well plate and 1×10^6 cells in 450 µl of media/well were seeded and cultured overnight. Then, 50 µl of AgNPs/well (10.0, 30.0 and 200 µg/ml) of different sizes were supplemented and incubated for 12 h. The media was then removed and the cells were washed twice with PBS and 1 ml of DHE solution (10 □m/L) was added and incubated for 30 min in CO_2 incubator. At the end of the treatment, the cover slip with stained cells was taken out and placed on a microscopic slide and the fluorescence micrographs were taken using fluorescent microscopy.

Evaluation of cell migration

3 groups of cells with the concentration of 1×10^6 cells/well were seeded in a 12-well plate and cultured overnight. A trace on the surface of cells in culture was draw with a 200 μL pipette tip, and the scratches "healing" case was observed under phase contrast microscope after culturing with different size of nanoparticles in serum-free medium for 24 hours and 48 hours. Scratch marks width were then measured using Image J. The experiment was performed at least three independent times.

Transwell assay

6×10^4 A549 cells/well were seeded in the inserts of 24-well Boyden chamber (Corning, USA) that was pre-coated with 60-μl diluted Matrigel (Matrigel: serum-free medium, 1:12). DMEM high glucose medium (Hyclone) containing 10% Australia fetal bovine serum (GIBCO) and AgNPs of different sizes were placed in the lower chamber. After 12 hours incubation, non-migrating cells on the upper surface of the membrane were erased by a cotton swab with serum-free medium. Membrane was then immersed in 95% ethanol for 10 min and stained by crystal violet solution. The cells that penetrate the membrane of the lower chamber were observed with an inverted fluorescence microscope, and number of the invaded cells in five randomly chosen fields per insert were counted and analyzed using Image J software.

Protein expression studies

Total protein was extracted from cultured cells with lysis buffer solution (EnoGene™, Nanjing, China), and the protein concentration was quantified using a BCA Protein Assay Kit (EnoGene™, Nanjing, China). Then, equal amounts of protein were subjected to electrophoresis on a 12% SDS–PAGE and transferred to a PVDF membrane (Millipore, Boston, MA, USA). Membranes were incubated with 5% non-fat dry milk in TBST and probed with anti-Bcl-2, Bax and nuclear factor (NF)-κB antibody in TBST (0.1% Tween 20 in TBS). Horseradish peroxidase-conjugated anti-rabbit IgG (Sigma) was used for detection of immunoreactive proteins by chemiluminescence (Western Blot Chemiluminescence Reagent Plus). Densitometric signals obtained in each independent membrane were quantified using Image J.

Samples of the cells that incubated with 13, 45 and 92 nm AgNPs were measured at least three independent times to assess reproducibility. Results were averaged and plotted using the Prism GraphPad software (GraphPad, La Jolla, CA). To additionally control total protein load, all samples were independently probed against the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and GAPDH signals were assessed in separated blots loaded with the same volume of materials.

Figures

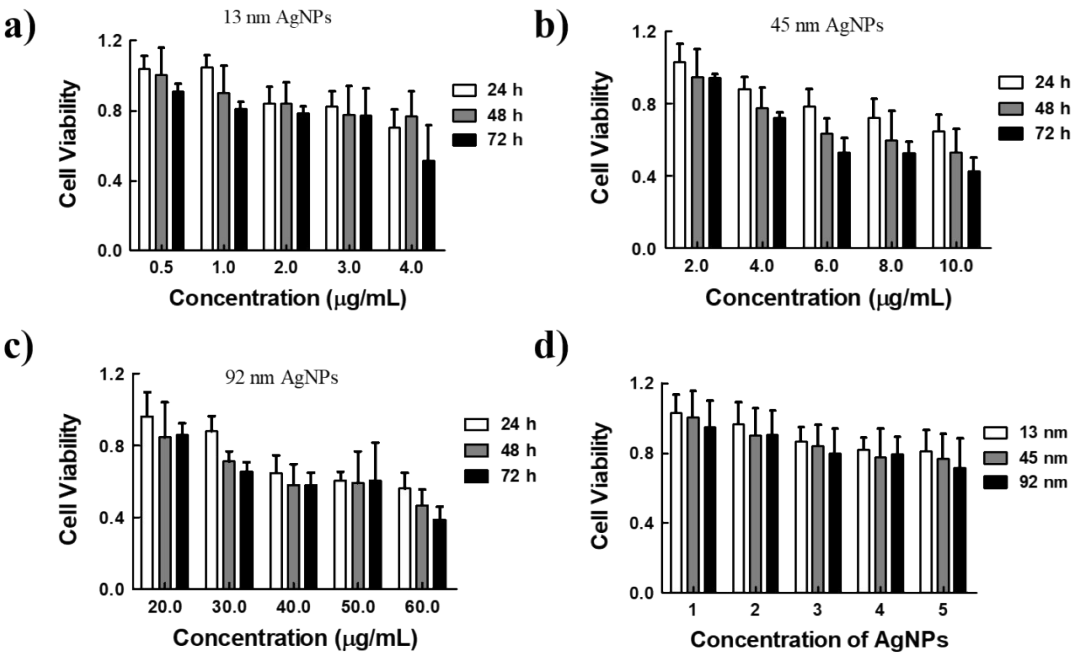

Fig.S1 Concentration-dependent cytotoxicity against A549 cells of 13 nm (a), 45nm (b) and 92 nm (c) AgNPs after 24, 48 and 72 h of incubation. Cell viability assays of the L929 cells treated with of AgNPs at 48 h. The concentrations for 13 nm, 45 nm and 92 nm AgNPs are 0.5, 1.0, 2.0, 3.0, 4.0; 2.0, 4.0, 6.0, 8.0, 10.0 and 20.0, 30.0, 40.0, 50.0, 60.0 μg mL^{-1}, respectively.)

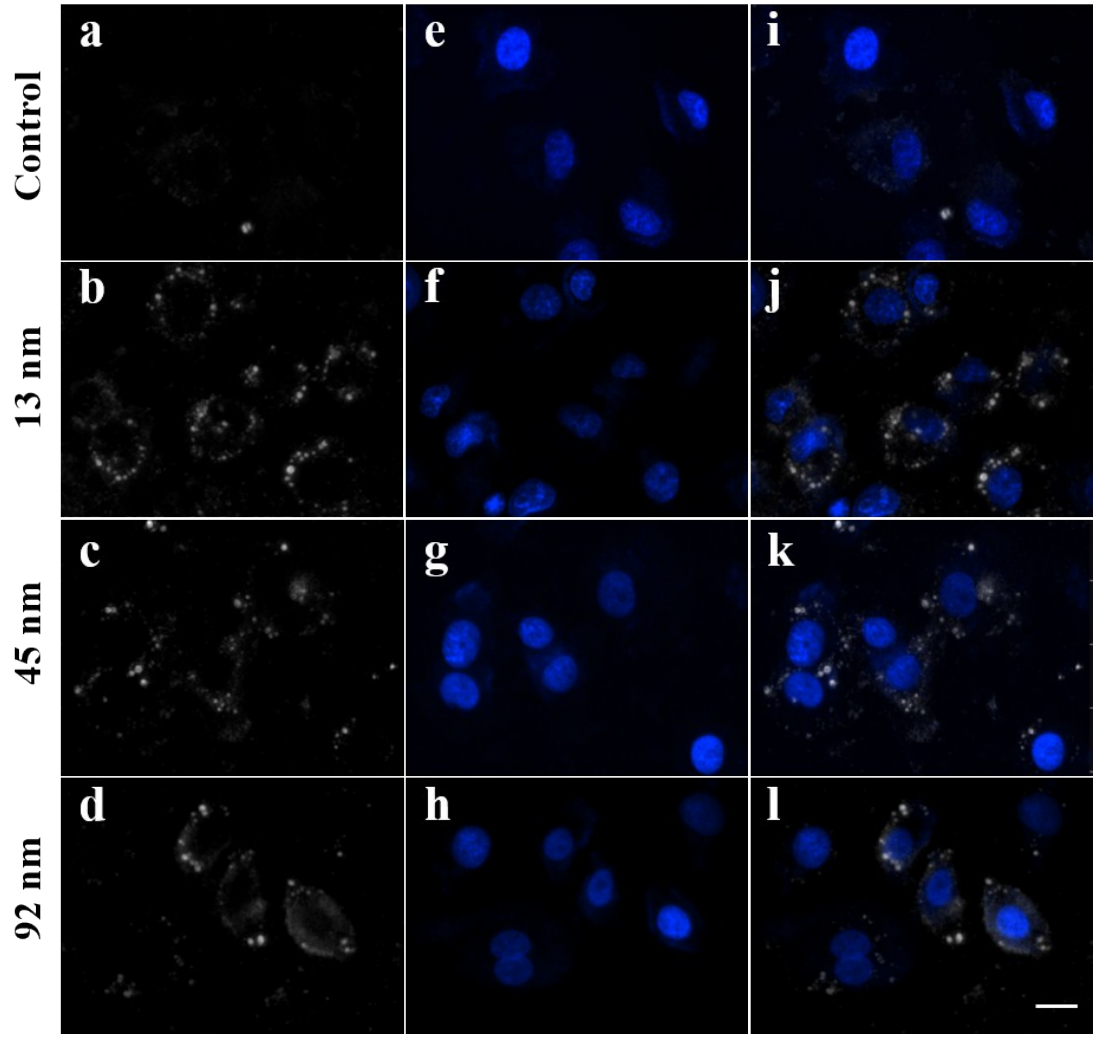

Fig.S2 Delivery of AgNPs into living A549 cells showed with the dark field (a-d), fluorescence (e-g), and the overlay of dark field and fluorescence mode (i-l). The images (b, f, g), (c, g, k) and (d, h, l) demonstrate that all the AgNPs may penetrate through the cell membrane and the dark-field light scattering signal of AgNPs can be observed clearly, while nearly no AgNPs could be found in control cells (a, c and i). Scale bar 30 μm.

Notes and references

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