Supplemental Information

Human serum albumin stabilizes aqueous silver nanoparticle suspensions and inhibits particle uptake by cells

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METHODS

Materials. PVP- and citrate- stabilized 20 nm silver particles were purchased form Nanocomposix as aqueous suspensions. Human serum albumin (HSA) (Sigma-Aldrich) was used to protect silver citrate nanoparticles to form the HSA protected samples. For these samples 5 µG of HSA was added to a 5mL solution of 20 µg/mL silver citrate particles. This resulted in a 50x by weight suspension of HSA-protected particles. Particles were then aliquoted and lyophilized overnight. The dry yellow powders were then stored at 4 degrees Celsius in the dark.

UV-Vis Spectroscopy. Samples were run on a Varian Cary 5000 UV-Vis spectrometer at ambient temperature under a nitrogen atmosphere using both plastic (Brand) and quartz cuvettes. Samples for time courses were prepared as 2:1 mixtures of DMEM (no phenol red) with 10% FBS and silver particles suspended in water at 20 µg/mL. This results in a final concentration of 6.7% FBS and 13.3 µg/mL silver nanoparticle in a total 3 mL volume.

Dynamic Light Scattering. Samples were run on a Malvern Zetasizer Nano-ZS. Samples were run in plastic cuvettes (BRAND) with a 400 µL sample volume. Each sample was measured 5 times and the reported values are the average of the z-average value and calculated standard errors are from repeating the measurement 5 times.

Transmission Electron Micorscopy.

Sample preparation: The Ag NP powders were suspended in DI water to the concentration of 0.02 mg/ml. Carbon film supported TEM grids were glow discharged. About 8µl droplet of Ag NP suspension was placed on TEM grid. After one minute, excess liquid on grid was blotted away with filter paper and TEM grids with Ag NPs on were dried at ambient air before being analyzed in TEM.

Image acquisition: Bright field (BF) TEM images of Ag NPs were carried out on JEOL 2200FS TEM, which uses a 200kV Schottky - emission gun with an in-column energy filter. A magnification/image resolution combination is selected to provide the pixel resolution of 0.49 nm. Images with particles untouched were selected and the irregular shaped particles (not sphere) were not excluded. At least 400 particles from multiple images were used for size analysis.

Particle and data Analysis: Open source software ImageJ was used for particle analysis. The brightness and contrast of all images were checked having the histograms centered and wide enough to cover at least 80% of the possible grey levels. The threshold for particles in images was adjusted and applied. The area and shape descriptor were set as measurands. The analysis results were saved as spreadsheet after performing the analyzing particles operation. OriginPro was used for data analysis. The diameters (d) of particles were calculated from the result of area (*A*) obtained from ImageJ. The formula $A = \pi r^2$ and $d = 2r$ were used. Histogram of particle size was plotted and the Gaussian curve fitting model was used for the mean and standard deviation.

Cell Culture. SH-SY5Y, Neuro-2A, U87-MG and HepG2 cells (American Tissue Culture Center) were all grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (50 µg/ml, Gibco) under standard culture conditions (37 °C, 5% CO_2). Cells were grown in T75 flasks (Falcon) and trypsin-edta solution

(Gibco) was used for passaging cells (3mL per T75 flask for HepG2 and Neuro-2A cells and 2 mL for SH-SY5Y and U87 cells). For passaging, SH-SY5Y cells were treated with trypsin-edta at room temperature for 5 minutes, while the other three cell lines were incubated for 10 minutes at 37 degrees.

MTT Assay. Cells were seeded into wells in a 96-well plate (Falcon) (1 x 10^5 cells/ml, 100 µl per well) to cover a 9x6 grid, filling 54 wells. Remaining wells were filled with 200 µl of PBS. After 24 hours, 100 µl volumes of dilutions of particles in either water or PBS spanning from 20 µg/mL to 0.01 µg/mL were added to the seeded wells (final concentrations spanning 10 µg/mL to 0.05 µg/mL). For each nanoparticle eight dilutions were prepared and for each dilution six replicates were performed. In the remaining 6 wells, 100 of µL PBS was added as a control. Cells were then incubated with nanoparticles for 72 h. After 72 h, 50 µL of a PBS solution of MTT (2.5 mg/ml) was added to each well and then incubated for 3 h. After 3 h, media was aspirated from all wells, leaving purple formazan crystals in those wells with viable cells. To each well, 150 µl of DMSO was added. Plates were then agitated for 10 s and analyzed using a plate reader (Fluorstar Omega, BMG Labtech.) to determine the absorbance of each well at 570 nm. This reading divided by the average from the reading of the six control wells was plotted to determine the IC_{50} value of each complex for each cell line. Six replicates were performed for each sample on each cell line for each experiment, and each experiment was repeated three times. The values and errors reported are calculated from 18 unique measurements.

t-tests were used to ascertain if real differences in IC_{50} values were arising. For both cell lines that exhibited cytotoxicity (SH-SY5Y and HepG2), statistically significant differences in IC_{50} values arose between the 20 nm HSA-AgNPs compared to the PVP- and citrate-AgNPs. The differences between citrate and PVP particles was not significant.

Metal Analysis. To determine the Ag nanoparticle uptake into each cell line, 5 mL cell suspensions of 10^5 cells/mL cells were plated into 3 cm Petri dishes. After 24 h, 250 µL of nanoparticles (stock suspensions of 20 µg/mL) were added to the cells. These samples were incubated for 24 h, at which times the media was removed and the cells rinsed twice with PBS. Trypsin-EDTA (2 mL of 0.25 %) was then added to detach the cells from the plate surface, and an additional 3 mL of PBS added to resuspend the cells. These suspensions were transferred to 15

mL conical Falcon tubes and centrifuged for 5 min at 800 rpm. The supernatant was discarded and the cells resuspended and rinsed twice with PBS in this manner. Cell pellets were then resuspended in 2 mL of PBS and counted using a LUNA Automated Cell Counter (Logos Biosystems). Cell suspension ranged between 2 to 4 x 10^6 cells per sample for HepG2 cells and between 0.5 and 1.5 x 10^6 cells per sample for SHSY5Y cells. After counting the cells in each sample, the cells were centrifuged again for 5 min at 2000 rpm and the supernatant discarded. The cell pellet was dried overnight. To each dried pellet, 100 µL of concentrated nitric acid was added and the sample left for 24 h to be digested. Samples were then diluted with H_2O and submitted for ICP-MS analysis to determine the silver content. The results were then normalized to the number of cells in each sample. Each experiment was repeated 3 times and the values and errors reported are the average of these 3 measurements.

t-tests were used to ascertain if real differences in silver uptake were arising. For 20 nm HSA- and citrate- AgNPs the uptake of silver between each of the four cell lines was different. For the PVP-AgNPs, there is no difference in metal uptake between HepG2 and U87-MG cells but there is when compared with either the SH-SY5Y cells or Neuro-2A cells. Within a given cell line, there is not difference in uptake for Neuro-2A cells. For SH-SY5Y cells there is no difference in uptake between PVP- or citrate-AgNPs, but uptake of HSA-AgNPs is lower. For both HepG2 and U87-MG cell lines, citrate-AgNPs have the highest uptake. Uptake of PVP-AgNPs is lower, and uptake of HSA-AgNPs is significantly lower that either PVP- or citrate-stabilized particles.

UV-Vis spectra of resuspended particles.

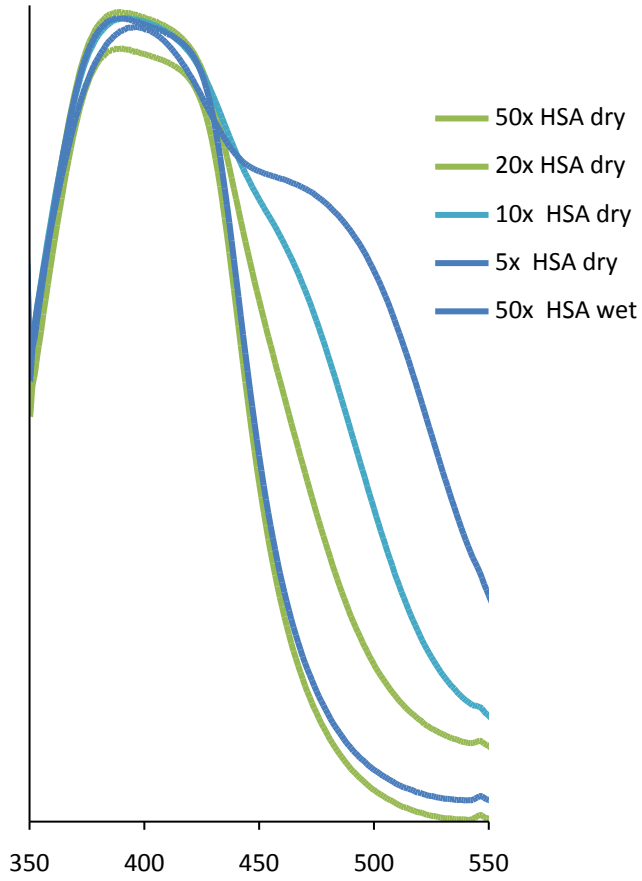

Figure S1. HSA treated particles were measured by UV-Vis. The spectra of all ratios of protein to silver were the same before lyophilization. Only one is shown for clarity (50x HSA wet). Addition of only 5-fold excess of HSA resulted in a large shoulder upon resuspending the dried solid. As the ratio of protein to silver was increased, the resulting shoulder decreased. At 50:1 HSA:silver the shoulder no longer appears.

DLS Data.

	z-average hydrodynamic diameter and PDI of particles in water (nm)	z-average hydrodynamic diameter and PDI of particles in DMEM with 6.7% FBS (initial) (nm)	z-average hydrodynamic diameter and PDI of particles in DMEM with 6.7% FBS (72 h) (nm)
20 nm PVP stabilized AgNPs	**39.0(0.5) / 0.12(0.01)**	**40(1) / 0.45(0.05)**	**136(1) / 0.22 (0.01)**
20 nm resuspended HSA stabilized AgNPs	**35.4(0.3) / 0.27 (0.01)**	**42(1) / 0.35(0.04)**	**104(2) / 0.27 (0.01)**
20 nm citrate stabilized AgNPs	**35.5(0.3) / 0.22(0.01)**	**35(1) / 0.30 (0.01)**	**91(1) / 0.25 (0.01)**

Table S1. DLS measurements are the average of 3 subsequent readings reported with standard errors reported in parentheses. PDI values are also reported as an average of 3 measurements for each sample with standard error in parentheses. Particles in media were measured as a 2:1 mixture of DMEM with 10% FBS mixed with particles in water. Measured hydrodynamic radii for 20 nm PVP and 20 nm citrate samples are in agreement with those provided by Nanocomposix for these materials.

Representative plates for MTT data highlighting scatter of data for viable cells.

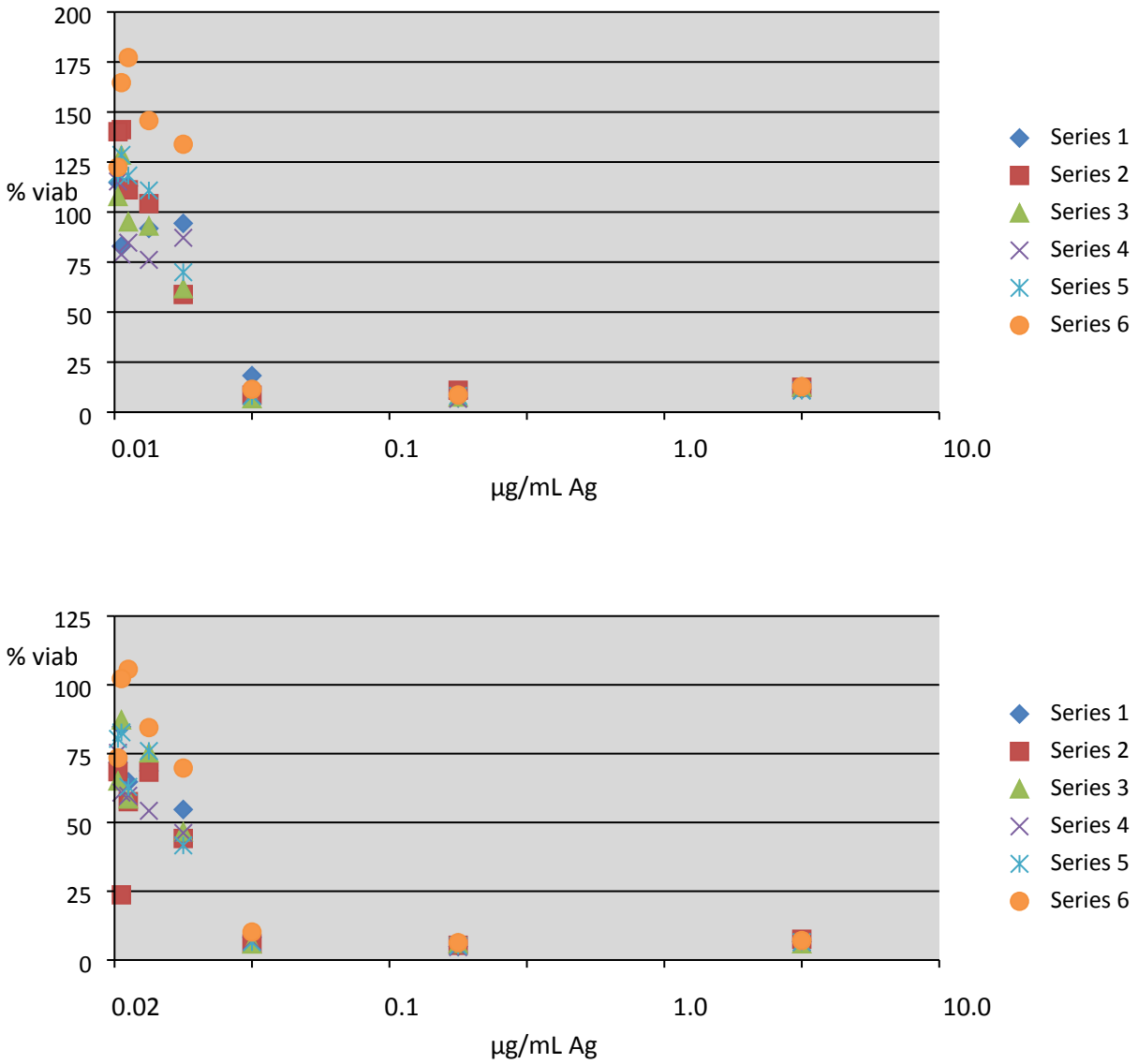

Figure S2. Two data sets of 6 replicates, each from a single plate for MTT data for SH-SY5Y cells treated with HSA –protected 20 nm AgNPs. This data is representative of typical plates and highlights the need for several replicates in order to obtain reproducible IC_{50} values.