Electronic Supplementary Material (ESI) for Nanoscale.

Supplementary information

Silver nanoparticle induced toxicity and cell death mechanisms in embryonic zebrafish cells

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Experimental Methods

Characterisation of AgNPs: detailed methodology

Samples for Transmission Electron Microscopy (TEM) were prepared by diluting the stock suspension (1000 µg/mL) with ultrapure water to a final concentration of 100 µg/mL. The diluted suspension (15 µL) was immediately loaded onto a 200 mesh Formvar coated copper grid (Agar Scientific, AGS138). After 2 hours the sample was gently washed with ultrapure water and left to dry for 24 hours inside a plastic tray to avoid dust.

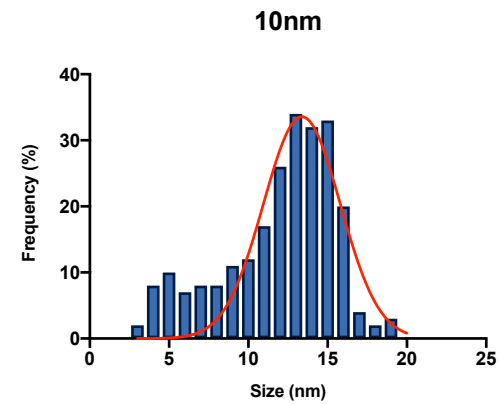
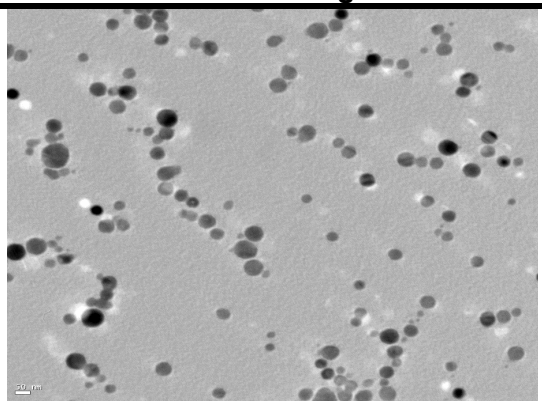
Samples for Nanoparticle Tracking Analysis (NTA) (NS300, Malvern) were prepared by diluting 5 µL of the AgNP stock suspension (1000 µg/mL) in 20 mL of ultrapure water for a final concentration of 0.25 µg/mL. Then 1 mL of the diluted suspension was added to 19 mL of ultrapure water, to achieve a final working solution concentration of 0.012 µg/mL. Afterwards, 1 mL of the diluted NP suspension was loaded into the syringe pump and flushed into the NTA chamber at a 1000 seconds injection time. As soon as the NPs were visualized the flow interval was changed to 50 seconds (circa 5.2 µL/min (1)). The video was recorded and then analysed using the automatic settings of the NTA. The auto setup sets the camera level for standard measurement runs, including the autofocus and optimum camera level as follows: screen gain 1.0 and camera level 7. It is important to mention that these setting may vary depending on the brightness and concentration of the analysed sample, it is recommended to use

a minimum concentration of 10^9 particles per mL to ensure the detection of the sample as suggested in the NTA user manual. The equipment's temperature was set to room temperature (22 ºC). For further information the reader is directed to the manual for the NanoSight NS300 instrument.

Samples for Dynamic Light Scattering (DLS) (Zetasizer Nano ZS, Malvern) were prepared at a final concentration of 10 µg/mL in 1 mL of either ultrapure water (UPW) or complete cell medium (CCM) containing 10% foetal bovine serum (FBS). The hydrodynamic size was analysed as soon as the samples were prepared (7 runs per sample), afterwards the samples in the DLS cuvettes were incubated at 28°C for 24 hours, then the hydrodynamic size was recorded again. Results are representative of the average of three independent experiments, each measurement includes the average of 7 DLS runs. The instrument software was v3.30 and the measurements were recorded by selecting parameters according to the type of measurement (size or zeta potential) and the physical properties of the AgNPs, i.e., refractive index 0.135 and absorption index (k) 3.99. The equipment's temperature was set to 22 ºC.

Results

Characterisation of the AgNPs

Size distribution	TEM image
	

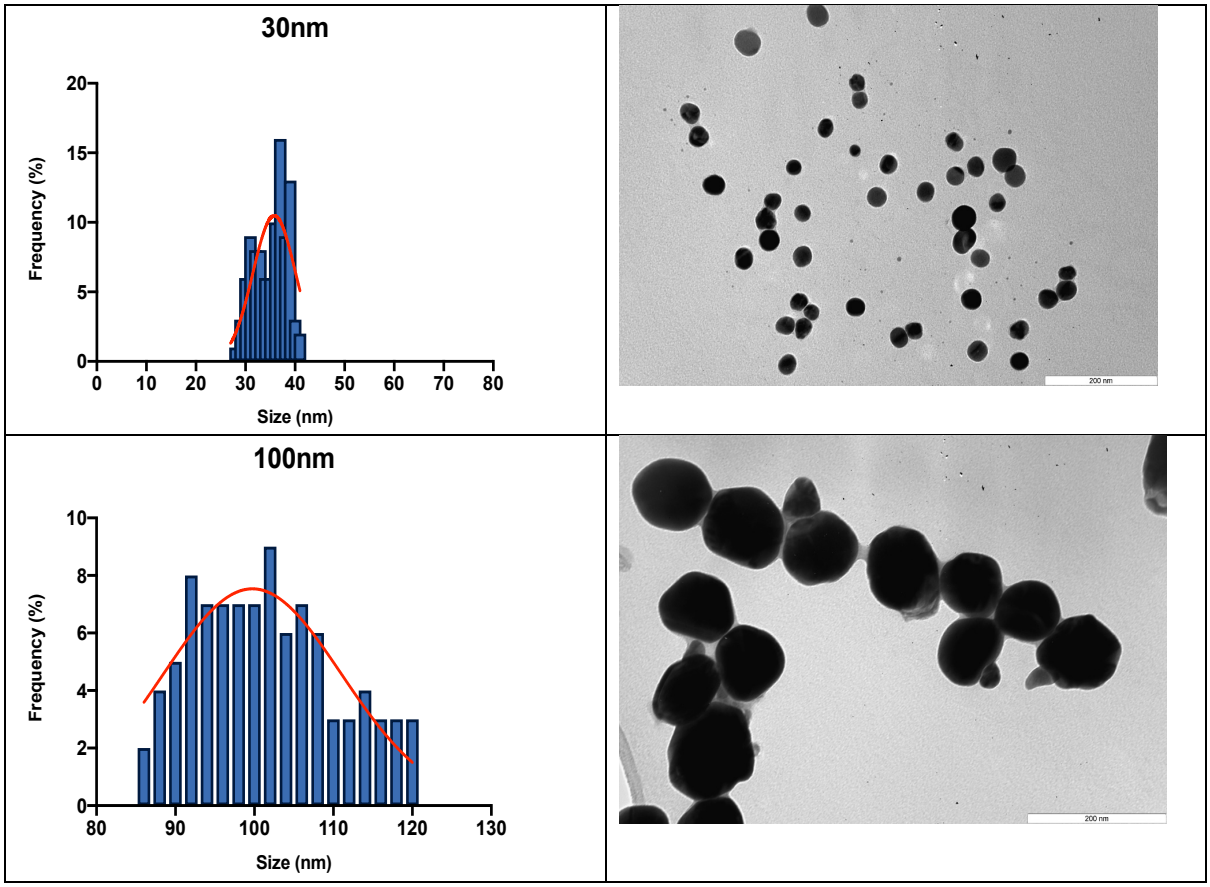


Figure S1. TEM characterisation of the 3 different AgNPs using a JEOL JEM-1400 microscope. Approximately 100 nanoparticles were used to calculate a size distribution graph for each of the three particle sizes using graphpad software.

Table S1. Characterisation of AgNPs by TEM and NTA. The table shows the summary of the results obtained in the assessment of the core size by TEM and hydrodynamic size by NTA. The AgNPs were only prepared in ultrapure water. See detailed methodology for further details about the sample preparation and working concentrations for each sample.

	Water			**Cell medium (+10% FBS)**		
AgNPs size	10nm	30nm	100nm	10nm	30nm	100nm
TEM	13 ± 2.4	34 ± 2.8	101.6 ± 9.2	N/A	N/A	N/A
Hydrodynamic size (NTA)	35.9 ± 11.7	37.8 ± 7.4	107 ± 10.4	N/A	N/A	N/A

Abbreviations: Not applicable (N/A), Nanoparticle tracking Analysis (NTA), Transmission electron microscopy (TEM), Foetal Bovine Serum (FBS).

Table S2. Characterisation of the cell medium alone by DLS. Cell medium was supplemented with 10% FBS and measured after 0 and 24 hours of incubation.

Time	Hydrodynamic size (nm)		Zeta potential (mV)		Polydispersity index (PDI)	
	Result	STDV	Result	STDV	Result	STDV
0 hours	13.61	2.29	14.72	0.52	0.51	0.05
24 hours	13.85	1.49	14.13	0.33	0.41	0.00

Abbreviation: Standard deviation (STDV)

Table S3. Characterisation of the AgNPs in cell medium by DLS. The table shows the characterisation of the size, zeta potential and PDI at 0 and 24 hours using three different AgNPs concentrations (2.5, 5 and 10 µg/mL) in cell medium supplemented with 10% Foetal Bovine Serum (FBS).

AgNPs size	Time	AgNPs concentration µg/mL					
		2.5		5		10	
Hydrodynamic size (nm)							
		Result	STDV	Result	STDV	Result	STDV
10 nm	0 hours	87.94	11.26	88.98	15.26	113.70	3.40
	24 hours	107.4	3.22	105.67	2.12	99.20	1.02
30 nm	0 hours	92.87	3.71	98.68	3.64	106.06	4.85
	24 hours	90.29	3.65	92.66	7.33	92.21	0.13
100 nm	0 hours	143.97	2.51	144.43	1.07	140.93	0.92
	24 hours	152.63	4.97	155.07	0.12	156.66	3.97
Zeta potential (mV)							
10 nm	0 hours	-10.11	1.44	-7.31	0.59	-8.13	1.19
	24 hours	-10.55	1.23	-10.14	0.84	-10.20	0.56
30 nm	0 hours	-6.99	0.55	-6.47	1.44	-8.03	1.05
	24 hours	-12.13	1.20	-11.73	1.10	-12.30	1.57
100 nm	0 hours	-6.70	1.10	-6.25	0.90	-7.01	1.36
	24 hours	-11.00	0.46	-12.33	1.44	-11.77	1.05
Polydispersity index							
10 nm	0 hours	0.57	0.02	0.54	0.03	0.43	0.02
	24 hours	0.57	0.01	0.52	0.01	0.40	0.01
30 nm	0 hours	0.5	0.01	0.46	0.04	0.32	0.02
	24 hours	0.54	0.03	0.43	0.03	0.37	0.01
100 nm	0 hours	0.07	0.02	0.07	0.01	0.04	0.01
	24 hours	0.14	0.02	0.12	0.01	0.08	0.00

Abbreviation: Standard deviation (STDV)

Table S4. Characterisation of the AgNPs in ultrapure water by DLS. The table shows the characterisation of the size, zeta potential and PDI at 0 and 24 hours using three different AgNPs concentrations (2.5, 5 and 10 µg/mL) in ultra-pure water.

AgNPs size	Time	AgNPs concentration µg/mL					
		2.5		5		10	
Hydrodynamic size (nm)							
		Result	STDV	Result	STDV	Result	STDV
10 nm	0 hours	64.05	9.36	64.18	1.71	69.58	8.37
	24 hours	76.3	5.95	75.06	3.6	67.58	8.71
30 nm	0 hours	56.18	3.13	71.9	0.14	72.02	0.50
	24 hours	60.06	7.00	70.22	6.07	59.73	2.03
100 nm	0 hours	102.27	0.75	105.38	0.11	139.47	3.89
	24 hours	107.26	0.04	105.08	1.78	137.63	1.07
Zeta potential (mV)							
10 nm	0 hours	-8.16	0.79	-6.99	0.25	-8.70	0.95
	24 hours	-7.48	2.52	-9.40	0.42	-10.15	0.78
30 nm	0 hours	-6.77	0.56	-6.86	1.80	-7.42	2.55
	24 hours	-7.75	0.78	-8.30	1.13	-9.95	0.78
100 nm	0 hours	-5.64	0.04	-6.34	1.26	-7.75	0.69
	24 hours	-9.95	0.78	-8.30	2.26	-10.80	1.41
Polydispersity index							
10 nm	0 hours	0.13	0.02	0.14	0	0.12	0.03
	24 hours	0.32	0	0.15	0.01	0.29	0.04
30 nm	0 hours	0.15	0.03	0.25	0.03	0.2	0.00
	24 hours	0.26	0.01	0.21	0.01	0.3	0.05
100 nm	0 hours	0.2	0.00	0.25	0.04	0.4	0.01
	24 hours	0.32	0.02	0.35	0.05	0.36	0.01

Dissolution of the AgNPs in water and CCM.

The possible interactions of the filters with ionic Ag in water and cell culture media was evaluated by preparing a stock suspension of 2 µg/mL of $AgNO_3$ in both testing medium. The samples were centrifugated at 20817 x g for 5 and 30 minutes respectively. It is important to mention that the sample in CCM required 2 additional washed to fully pass through the filters. The total Ag concentrations after centrifugation were diluted 20-fold and then analysed by ICP-MS. Results after dilution factor demonstrated minimal differences compared to the initial concentration; therefore, efficiency calculations were not implemented to the dissolution results.

Sample	Total Ag µg/mL
CCM	1.90 ± 0.03
Water	1.93 ± 0.01

Table S5. Dissolution of the AgNPs. Dissolution of the AgNPs was determined in UPW and CCM (DMEM-F12 supplemented with 10% FBS) at different time points. Results show the mean of three individual replicates and their standard deviation. Data is expressed in µg/mL and percentage, which was calculated against their initial concentration (10 µg/mL).

Dissolution in ultrapure water (UPW)						
Time (hours)	AgNP size and results					
	10 nm		**30 nm**		**100 nm**	
	µg/mL	**%**	µg/mL	**%**	µg/mL	**%**
0.25	0.52 ± 0.01	5.20 ± 0.12	0.16 ± 0.18	1.63 ± 1.8	0.002 ± 0.002	0.025 ± 0.020
0.5	0.92 ± 0.06	9.27 ± 0.69	0.59 ± 0.05	5.91 ± 0.56	0.012 ± 0.002	0.126 ± 0.028
1	1.25 ± 0.05	12.59 ± 0.55	0.69 ± 0.01	6.94 ± 0.12	0.020± 0.004	0.207 ± 0.040
1.5	1.432± 0.006	14.30 ± 0.06	0.82 ± 0.03	8.28 ± 0.37	0.023 ± 0.004	0.233 ± 0.040
2	1.52 ± 0.02	15.59 ± 0.20	0.86 ± 0.02	8.70 ± 0.03	0.023 ± 0.001	0.238± 0.010
4	2.03 ± 0.01	20.34 ± 0.10	1.06 ± 0.24	10.68 ± 2.49	0.025 ± 0.001	0.253 ± 0.010
8	2.12 ± 0.20	21.24 ± 2.01	1.07 ± 0.02	10.74 ± 0.23	0.025 ± 0.001	0.250± 0.011
Dissolution in Complete Culture Media (CCM)						
	10 nm		**30 nm**		**100 nm**	
Time (hours)	µg/mL	**%**	µg/mL	**%**	µg/mL	**%**
0.25	0.010 ± 0.009	1.04 ± 0.09	0.095 ± 0.041	0.95 ± 0.41	0.030 ± 0.011	0.30 ± 0.11
0.5	0.017 ± 0.018	1.73 ± 0.18	0.135 ± 0. 015	1.35 ± 0.152	0.039 ± 0.004	0.39 ± 0.04
1	0.206 ± 0.061	2.07 ± 0.06	0.114 ± 0.024	1.14 ± 0.24	0.044 ± 0.030	0.44 ± 0.30
1.5	0.194 ± 0.042	1.95 ± 0.42	0.131 ± 0.099	1.31 ± 0.99	0.042 ± 0.034	0.42 ± 0.34
2	0.208 ± 0.016	2.09 ± 0.16	0.133 ± 0.010	1.33 ± 0.10	0.062 ± 0.011	0.62 ± 0.11
4	0.224 ± 0.009	2.24± 0.09	0.099 ± 0.021	0.99 ± 0.22	0.043 ± 0.001	0.43 ± 1.10
8	0.202 ± 0.012	2.02 ± 0.12	0.109 ± 0.012	1.09 ± 0.12	0.051 ± 0.029	0.51 ± 0.29

Cell viability: LDH assay

Table S6. Viability of ZF4 cells after treatment with AgNPs or $AgNO_3$. Cell viability was measured using Lactate dehydrogenase (LDH) assay at different time points and concentrations. The table shows the mean of three individual replicates, results are presented as percentage cell viability (%) and their standard deviation.

10 nm							
Time (hours)	**Naive**	**5 AgNPs**	**10 AgNPs**	**20 AgNPs**	**30 AgNPs**	**40 AgNPs**	**60 AgNPs**
3	100 ± 0	100 ± 0	100± 0	100 ± 0	100 ± 0	99.01 ± 4.97	99.57 ± 0.83
24	97.18 ± 4.88	69.67 ± 1.53	50.52 ± 4.59	34.98 ± 2.12	32.81 ± 2.68	25.17 ± 28.54	18.84 ± 1.54
48	98 ± 20	39.50± 3.11	33.47 ± 2.28	31.70 ± 3.77	30.37± 0.49	28.54 ± 0.14	20.74 ± 0.38
72	94.98 ± 8.64	19.22 ± 2.89	18.38 ± 2.01	16.80 ± 0.42	18.38 ± 4.45	16.34 ± 0.42	15.59 ± 0.27
30 nm							
3	97.88 ± 2.93	96.28 ± 2.96	93.07 ± 6.22	84.02 ± 2.86	80.96 ± 5.09	72.21± 1.73	63.02 ± 7.73
24	98.33 ± 1.79	80.14 ± 4.35	56.47 ± 2.31	48.85 ± 1.86	47.71 ± 4.50	46.57 ± 2.11	43.42 ± 5.29
48	95.77 ± 6.48	41.04 ± 3.02	41.04 ± 6.69	30.43 ± 1.17	26.36 ± 3.48	25.53 ± 3.15	21.14 ± 1.51
72	96.17± 6.61	20.45 ± 3.02	25.32 ± 2.63	21.72 ± 0.97	22.07 ± 2.12	22.41 ± 3.98	16.51 ± 3.04
100 nm							
3	99.66 ± 0.57	98.75 ± 1.76	97.96 ± 1.84	98.58 ± 1.27	82.91 ± 1.91	75.84 ± 2.69	69.61 ± 2.05
24	98.01 ± 1.76	88.31 ± 6.86	56.34 ± 2.57	51.72 ± 8.87	37.51 ± 1.47	29.01 ± 3.05	24.30 ± 3.28
48	99.72 ± 0.48	56.89 ± 6.18	30.91 ± 4.08	23.18 ± 2.18	19.46 ± 1.63	19.18 ± 0.32	19.08 ± 2.02
72	95.96 ± 6.98	55.52 ± 4.51	26.55 ± 0.95	14.19 ± 0.64	13.07 ± 0.52	13.24 ± 0.70	12.30 ± 0.43
$AgNO_3$							
Time (hours)	**Naive**	**0.5 $AgNO_3$**	**1 $AgNO_3$**	**2 $AgNO_3$**	**3 $AgNO_3$**	**5 $AgNO_3$**	**8 $AgNO_3$**
3	99.66 ± 0.57	99.10 ± 1.54	99.10 ± 0.89	98.21 ± 3.09	74.88 ± 2.23	55.35 ± 7.62	54.16 ± 4.40
24	98.01 ± 1.76	95.45 ± 7.45	82.96 ± 3.55	50.37 ± 2.36	20.76 ± 1.92	15.71 ± 1.03	13.06 ± 1.27
48	99.72 ± 0.48	38.07 ± 1.72	36.93 ± 4.55	28.41 ± 5.50	20.59 ± 3.49	16.71 ± 0.47	13.14 ± 0.34
72	95.96 ± 6.98	31.51 ± 6.38	22.71 ± 2.25	19.27 ± 0.02	14.96 ± 0.61	12.14 ± 1.60	10.65 ± 1.91

Autophagy results

Table S7. Autophagy percentage (%) in ZF4 cells exposed to AgNPs and $AgNO_3$. The table shows the mean and standard deviation of three individual replicates for FITC intensities normalised against naïve.

Size AgNPs	Naive	2.5 AgNPs µg/mL	5 AgNPs µg/mL	10 AgNPs µg/mL
10 nm	0 ± 0	3.12 ± 1.47	4.88 ± 3.17	1.97 ± 1.53
30 nm	0 ± 0	0.36 ± 0.22	3.63 ± 0.69	5.01 ± 0.73
100nm	0 ± 0	2.71 ± 3.03	2.18 ± 1.44	1.77 ± 1.92

	Naive	1 $AgNO_3$ µg/mL	1.5 $AgNO_3$ µg/mL	2 $AgNO_3$ µg/mL
Mean Intensity	0 ± 0	-20.23 ± 5.23	-9.71± -5.97	-2.16 ± 4.41

Figure autophagy

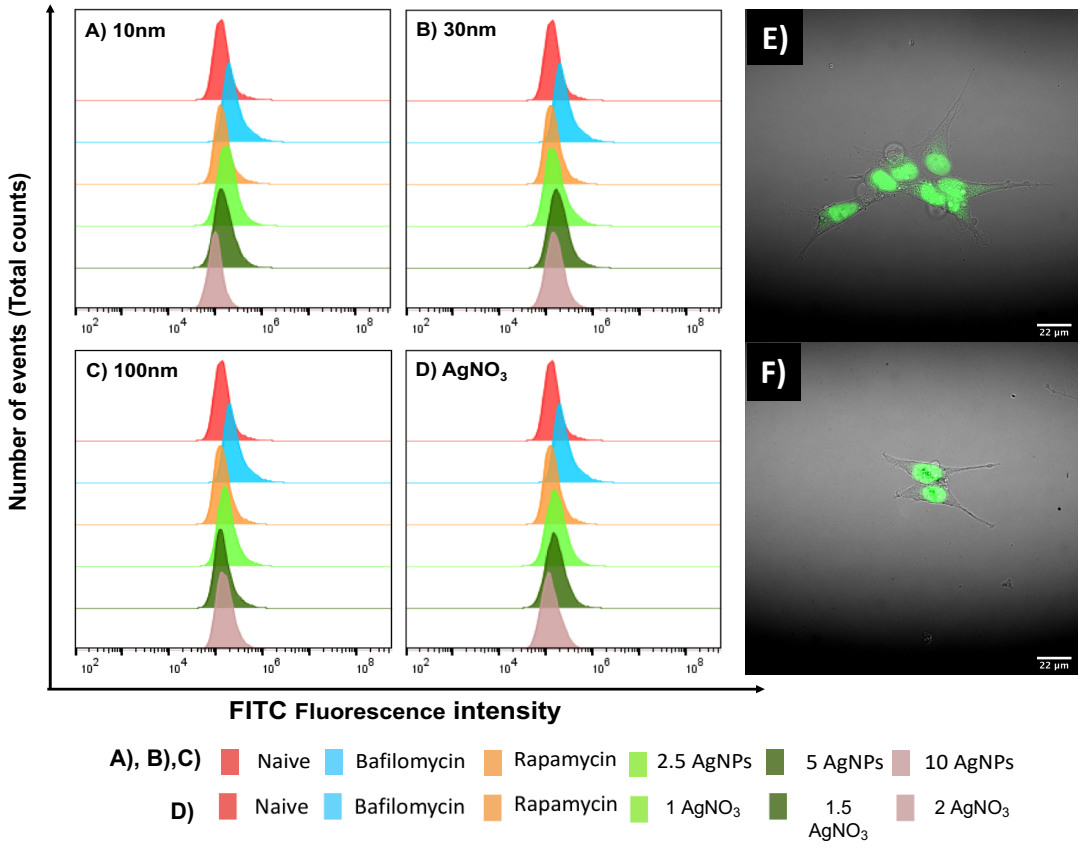


Figure S2. Autophagy results. The A-D shows representative FlowJo histograms for the treatments. Cells were treated with 2.5, 5 and 10 µg/mL of 10, 30n and 100 nm AgNP sizes and $AgNO_3$ 1, 1.5, 2 µg/mL for 24 hours. Controls are shown as naïve (untreated cells), 100 µM Bafilomacin (reduce response) and 10 µM Rapamycin (induce autophagy). E-F images

show cells stained with the autophagosome marker (green) to confirm the viability of the assay. Images were recorded with NIKON air microscopes at 60X objective and using the Fluorescein isothiocyanate (FITC) filter, which has a fluorescence Excitation/Emission of 499/521 nm. Figure E shows naïve and F cells treated with 10 µg/mL of 10 nm AgNPs for 24 hours.

Data analysis for the apoptosis versus necrosis assay

Flow cytometry results were analysed using FlowJo software. The percentage of cells in each of the different populations were sorted as follows:

Quadrant 4 (Q4) represents healthy cells, Q3 apoptotic cells, and Q1 and Q2 were considered as necrotic cells, as shown in Figure S2.

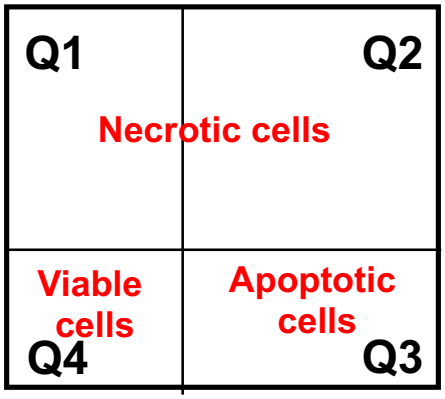


Figure S3. Division of the flow cytometry quadrants by cell death mechanism.

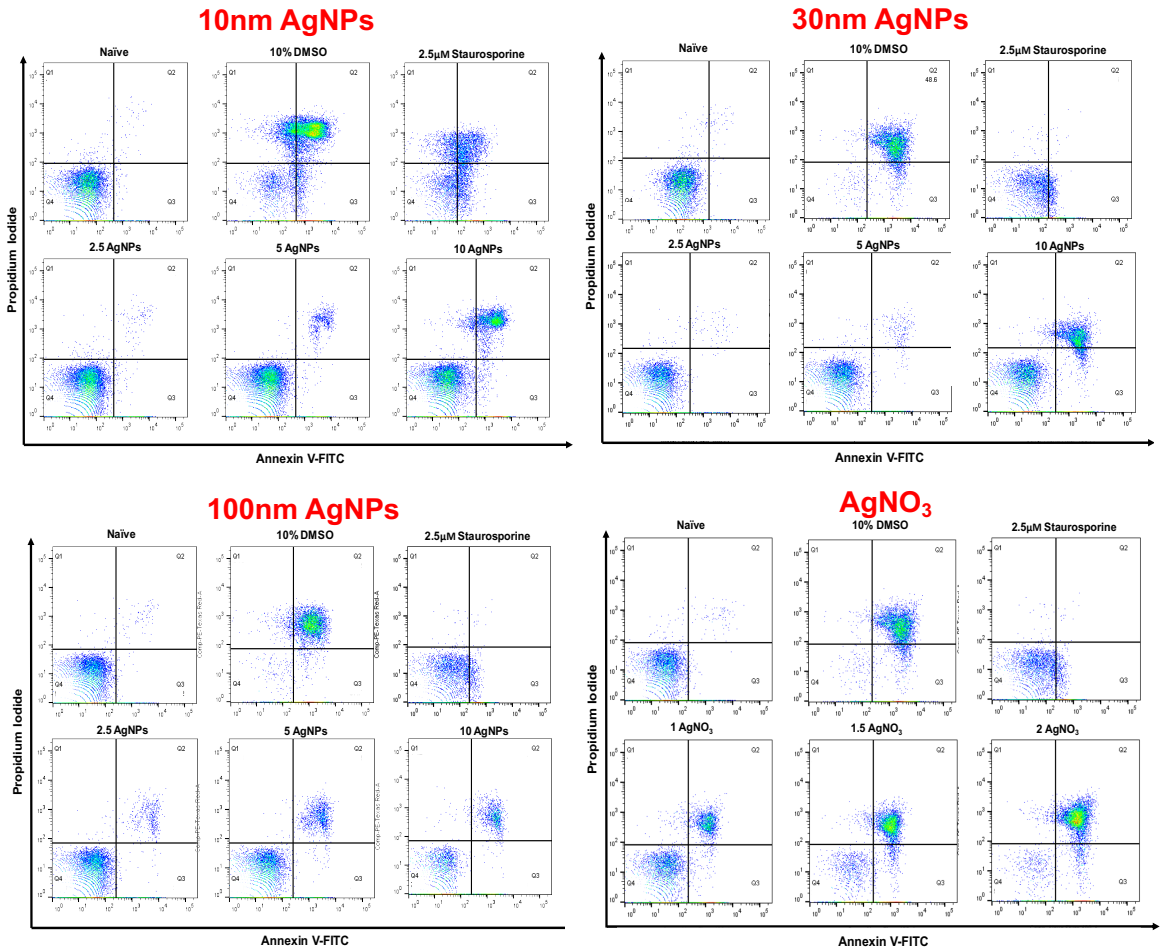


Figure S4. Representative scatter plots of the AgNPs treatments and controls. The figures represent one of the three replicates obtained after analysis with FlowJo software. The AgNPs represent treatments with 2.5, 5 and 10 µg/mL of 10, 30n and 100 nm AgNP sizes and $AgNO_3$ 1, 1.5, 2 µg/mL for 24 hours. Controls are shown as naïve (untreated cells), 10% DMSO (necrosis) and 2.5 µM Staurosporine (apoptosis).

Table S8. Analysis of the mechanisms of cell death - % of the cell population undergoing apoptosis and necrosis. Cells were treated with 2.5, 5 and 10 µ/mL of three different AgNPs sizes and 1, 1.5 and 2 µg/mL of $AgNO_3$ for 24 hours. Data are presented as the mean fluorescence intensity in percentage (%) for apoptosis and necrosis staining of the cells at the different exposure concentrations. Values represent the average of three individual replicates and their standard deviation.

% Cell population	Naive	2.5 AgNPs µg/mL	5 AgNPs µg/mL	10 AgNPs µg/mL
		10 nm		
Live cells	95.43 ± 2.28	72.30 ± 17.5	65.53 ± 21.53	53.27 ± 7.12
Apoptotic cells	3.220 ± 1.85	11.92 ± 4.03	17.95 ± 9.58	21.30 ± 1.65
Necrotic cells	1.357 ± 0.44	15.77 13.11	16.57± 12.31	25.46 ± 5.52
		30 nm		
Live cells	96 ± 1.40	94.80 ± 3.34	88.20 ± 7.97	60.97 ± 1.68
Apoptotic cells	2.137 ± 2.38	1.750 ± 0.62	4.457 ± 0.28	12.16 ± 8.95
Necrotic cells	1.543 ± 0.17	2.123 ± 1.37	4.483 ± 2.92	25.91 5.59

		100 nm		
Live cells	95 ± 1.60	95.13 ± 2.10	91.40 ± 5.58	87.57 ± 14.61
Apoptotic cells	2.68 ± 2.16	2.913 ± 0.89	3.847 ± 0.69	4.833 ± 3.60
Necrotic cells	1.23 ± 0.49	1.95± 1.25	4.47 ± 5.01	7.57 ± 10.99
		$AgNO_3$		
	Naive	**1 $AgNO_3$ µg/mL**	**1.5 $AgNO_3$ µg/mL**	**2 $AgNO_3$ µg/mL**
Live cells	94.20 ± 1.13	55.35 ± 0.35	20.40 ± 1.13	9.385 ± 0.46
Apoptotic cells	4.22 ± 0.93	24.85 ± 0.21	24.40 ± 0.70	18.60 ± 0.56
Necrotic cells	1.595 ± 0.21	19.81 ± 0.07	55.15 ± 0.45	70.54 ± 2.28

Mitochondrial permeability

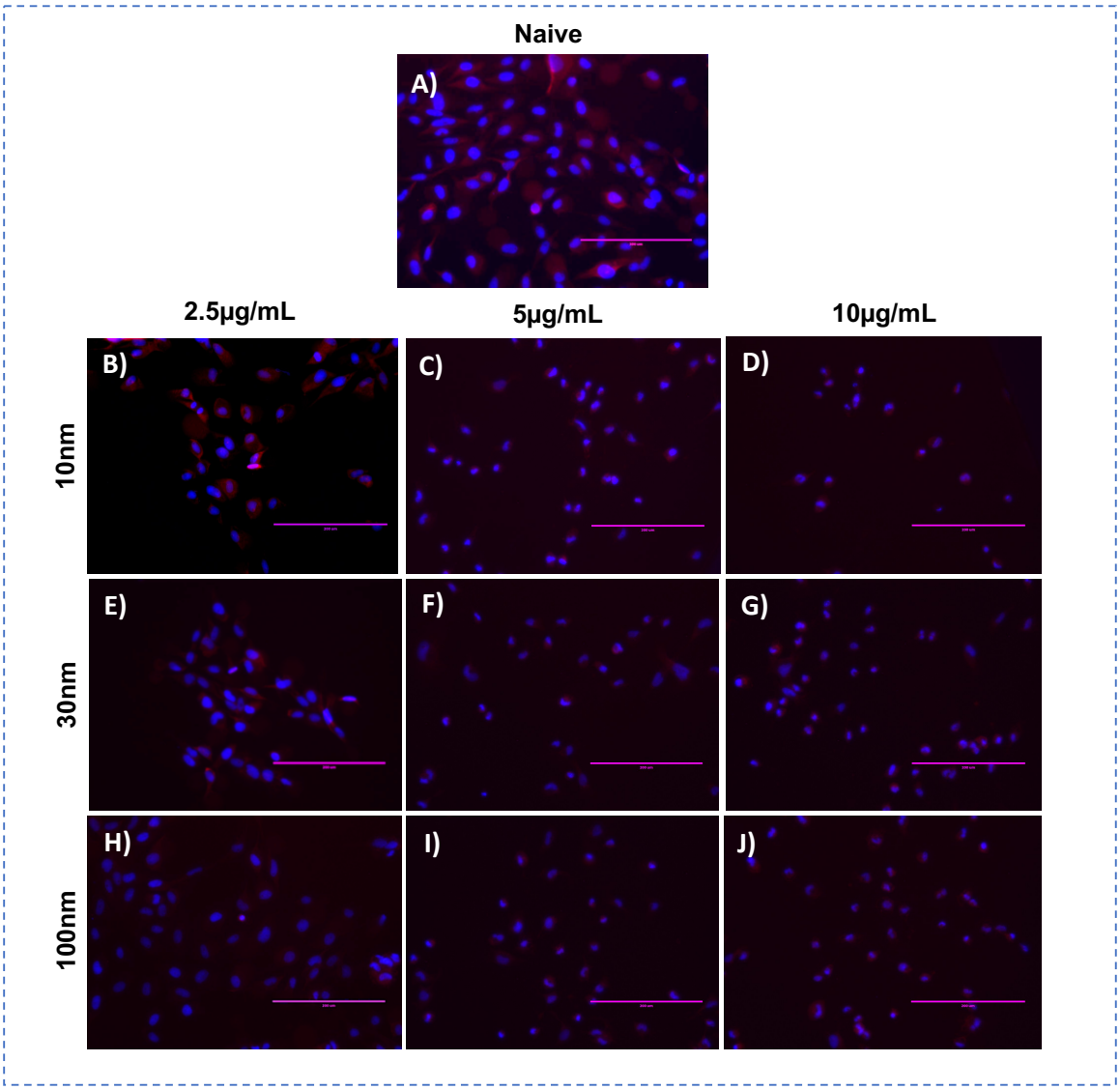


Figure S5. ZF4 cells stained with MitoHealth to assess mitochondrial permeability. Cells were treated with 2.5, 5 and 10 µ/mL of three different AgNPs sizes and 1, 1.5 and 2 µg/mL of $AgNO_3$ for 24 hours. Blue staining shows the cell nuclei, bright red staining shows the MitoHealth dye in healthy mitochondria, permeabilization of the mitochondrial membrane shows a reduction in the red dye intensity. Images were taken with a fluorescence microscope using a 20X objective.

Table S9. Normalized % values for mitochondrial permeability. The table shows the normalized mean intensities for FITC against the untreated control of three individual replicates. Naive cells were used as 100% to represent healthy mitochondrial membranes. Compromised membranes resulted in lower fluorescence intensities values. Cells were treated with 5, 5 and 10 µ/mL of three different AgNPs sizes and 1, 1.5 and 2 µg/mL of $AgNO_3$ for 24 hours. Results are presented in µg/mL unless otherwise stated.

AgNPs Size	***Naive***	***H_2O_2 100µM***	***2.5 AgNPs***	***5 AgNPs***	***10 AgNPs***
10nm	100 ± 4.03	77.46 ± 0.54	87.35 ±5.95	88.98 ± 4.55	90.54 ± 6.97
30nm	100 ± 4.03	77.46 ± 0.54	85.61 ± 3.51	90.74 ± 4.65	87.24 ± 6.65
100nm	100 ± 4.03	77.46 ± 0.54	94.72 ± 4.51	90.25 ±2.72	87.58 ± 0.37

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	Naive	**1.0 $AgNO_3$**	**1.5 $AgNO_3$**	**2.0 $AgNO_3$**
Mean Intensity	100 ± 4.03	95.22 ± 3.66	86.94 ± 7.91	86.89 ± 3.75

Lipid peroxidation results

Table S10. Lipid peroxidation of ZF4 cells after AgNPs and $AgNO_3$ treatments. Results are presented as the ratio of the fluorescence of Texas red to FITC. Treatments are presented in µg/mL unless otherwise stated. The ratios represent the mean and standard deviation of three individual replicates.

AgNPs Size	***Naive***	***100 µM Cumene hydroperoxide***	***2.5 AgNPs***	***5 AgNPs***	***10 AgNPs***
10nm	0.78 ± 0.21	0.58 ± 0.11	0.62 ± 0.15	0.43 ± 0.12	0.33 ± 0.05
30nm	0.78 ± 0.21	0.58 ± 0.11	0.62 ± 0.13	0.61 ± 0.17	0.53 ± 0.14
100nm	0.78 ± 0.21	0.58 ± 0.11	0.56 ± 0.09	0.71 ± 0.10	0.72 ± 0.11

	Naive	***100 µM Cumene hydroperoxide***	***1 $AgNO_3$***	***1.5 $AgNO_3$***	***2 $AgNO_3$***
Mean	0.78 ± 0.21	0.58 ± 0.11	0.38 ± 0.05	0.36 ± 0.09	0.28 ± 0.09

Particle number concentration

Calculation of number of particles (NPs/mL) for each mass concentration used (2.5, 5 and 10 µg/mL).

Calculations were performed as described in the paper by Huk et al. (2). The number of particles for each size was provided by manufacturer in the technical specifications.

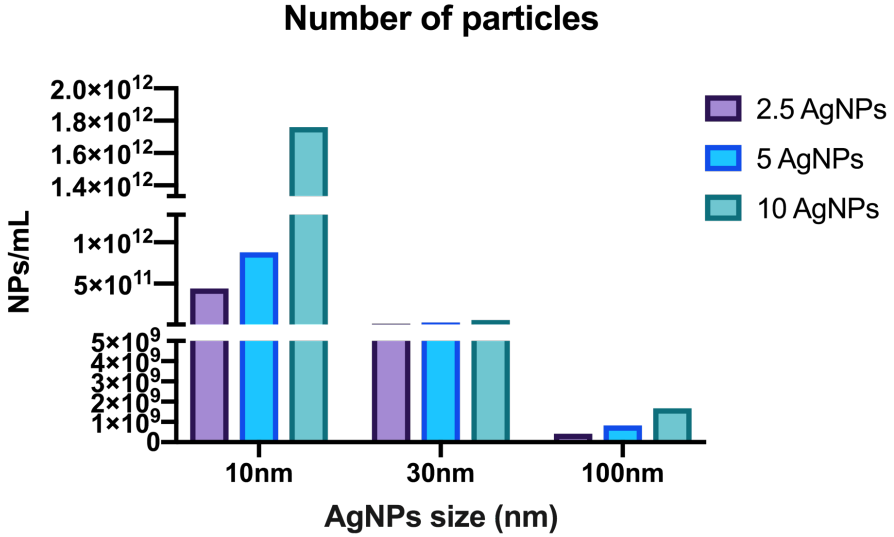

Figure S6. The number of particles in the different AgNPs mass concentrations. The mass concentrations used for the study were re-calculated to show the number of particles per mL. The graph also illustrates the NPs of particles needed to reduce cell viability after 24 hours.

Table S11. Values for the replotting mass to NPs/mL. The table shows the particle numbers per volume (NPs/mL) for the mass concentrations used in the study (2.5, 5 and 10 µg/mL) as well as the % of cell viability that remained after 24 hours treatment with AgNPs using different sizes, estimated number of particles (NPs/mL), and mass concentration.

AgNPs size	Mass concentrations (µg/mL)					
	2.5		5		10	
	NPs/mL	% viability	NPs/mL	% viability	NPs/mL	% viability
10 nm	4.40E+11	80	8.80E+11	69.67	1.76E+12	50.52767
30 nm	1.46E+10	90	2.92E+10	80.14	5.85E+10	53.1428571
100 nm	4.17E+08	92	8.33E+08	82.31	1.67E+09	56.34595

Surface area (SA) calculations

Calculations of total surface area was performed using the density of silver and 10.49 g/cm^{3,} the radius in the test medium as determined by DLS measurements and spherical shape of the NPs as described in by Teeguarden et al. and Book et al., (3, 4).

Table S12. Total surface area for AgNPs at different sizes and mass concertation. The total surface area was calculated for the three different sizes and mass concentration used in the study. The calculated values are in m^2/g.

AgNPs Size	AgNPs concentration		
	2.5 µg/mL	5 µg/mL	10 µg/mL
10 nm	1.34E-05	2.72E-05	5.78E-05
30 nm	1.59E-05	3.11E-05	6.22E-05
100 nm	9.41E-06	1.85E-05	3.67E-05

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