Supplementary information for:

Cellular repair mechanisms triggered by exposure to silver nanoparticles and ionic silver in embryonic zebrafish cells

Ana C. Quevedo, Iseult Lynch*, Eugenia Valsami-Jones

School of Geography, Earth and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

* Corresponding author: Iseult Lynch, i.lynch@bham.ac.uk

Section 1. Methodology:

1. Comet assay: buffer preparation

Lysis stock solution (1 L)

Chemical	Concentration	Amount
NaCl	2.5 M	146.4 g
$Na_2EDTA - 2H_20$	0.1 M	37.2 g
Tris HCl	8 mM	1.21 g

Preparation: Dissolve the salts in 800 mL of dH_2O and adjust pH to 10 using approximately 8 g of solid NaOH pellets, bring up to 1 L, autoclave and store at 4 °C.

NaOH 10M solution (500 mL)

Chemical	Amount
NaOH pellets	195 g

Preparation: Dissolve the NaOH pellets and bring solution up to 500 mL. Keep the solution at 4 °C. Note: This produces a dangerous exothermic reaction so use the appropriate measures during its preparation.

Na_2EDTA 200mM solution (500 mL)

Chemical	Amount
Na_2EDTA	37.2 g

Preparation: Dissolve the EDTA pellets to water and bring up 500 mL. Adjust the pH to 8.

Electrophoretic Buffer solution (2.5 L)

Solution	Concentration	Amount
NaOH 10M	300 mM	75 mL
Na_2EDTA 0.2M	1 mM	12.5 mL

Preparation: The individual solutions can be prepared in advance and kept at 4ºC for 1 month. However, on the day of the assay, the electrophoretic buffer needs to be prepared freshly with cold water (4 °C) by mixing the specified volume of each solution in final volume of 2.5 L.

Neutralization solution (500 mL)

Chemical	Concentration	Amount
Tris HCl	0.4 M	31.5 g

Preparation: Dissolve in 500 mL H_2O and adjust to pH 7.5 with Hydrochloric acid (HCl). Store the solution at 4 °C.

Preparation of the precoated slides (1 %)

Glass slides were boiled in water for 5 minutes and then set to dry overnight. Then, Normal Melting point Agarose (NMA) (1g) was dissolved in 99 mL of PBS for a total volume of 100 mL by microwave. Then, 50 mL of the diluted NMA suspension was kept warm by placing 45 mL into a falcon tube and set at 50 °C in a heat block. The rest of the NMA solution was stored at 4 °C.

Slides were dipped into the NMA for 10 seconds, the back was wiped, and the slides were set to dry onto a flat surface overnight.

Preparation of the low melting point agarose (0.7 %)

Low melting point agarose was diluted in PBS for a final concentration of 0.7 % in total volume of 50 mL.

Section 2: Results

1. Characterisation of the NPs

Table S1. Characterisation by dynamic light scattering (DLS) of PVP-capped silver nanoparticles (AgNPs) in serum free medium (SFM) and complete culture medium (CCM) containing 10 % Foetal bovine serum) at 0 and 24 hours. The Table shows the characterisation of the AgNPs in different media and at different time points. The results are the average of three individual replicates and their standard deviation.

AgNPs size	Hydrodynamic size (nm)			
	SFM		CCM	
Sample	Time (hours)			
	0	24	0	24
Medium	**1.20** ± 0.11	**1.36** ± 0.20	**12.95** ± 0.64	**12.85** ± 0.69
10 nm	47.86 ± 0.01	617.21 ± 0	94.49 ± 0.03	105.66 ± 0.3
30 nm	94.82 ± 0.01	690 ± 0.1	95.10 ± 2.85	102.65 ± 0.02
100 nm	135.9 ± 0.05	540.93 ± 0.03	160.60 ± 0.52	175.06 ± 0.5
	PDI			
	SFM		CCM	
Medium	0.10 ± 0.01	**0.09** ± 0.00	0.50 ± 0.05	0.51 ± 0.00
10 nm	0.23 ± 0.01	1 ± 0	0.53 ± 0.03	0.52 ± 0.07
30 nm	0.24 ± 0.01	1 ± 0	0.45 ± 0.03	0.43 ± 0.02
100 nm	0.03 ± 0.01	0.52 ± 0.03	0.06 ± 0.07	0.12 ± 0.08
	Zeta potential (mV)			
	SFM		CCM	
10 nm	-7.44 ± 0.74	-7.31 ± 0.58	-10.55 ± 1.22	-10.47 ± 0.83
30 nm	-6.98 ± 0.55	-5.80 ± 0.28	-12.13 ± 1.20	-11-73 ± 1.09
100 nm	-6.69 ± 1.10	-6.25 ± 0.90	-11.0 ± 0.45	-12.33 ± 1.43

Abbreviations: Dynamic Light Scattering (DLS), Serum Free Media (SFM), Complete Culture Media (CCM), nanometer (nm), and Polydispersity index (PDI).

2. Lactate dehydrogenase (LDH) assay

Table S2. Viability of ZF4 cells after treatment with AgNPs or $AgNO_3$ in SFM. Cell viability was measured using the LDH assay at different times post exposure. The table shows the mean of three individual replicates, and results are presented in percentages (%).

10 nm AgNPs							
	Concentrations in µg/mL						
Time (hours)	Naive	0.25	0.5	1	1.5	2	2.5
3	99.33 ± 0.57	92.11 ± 0.85	89.55 ± 2.25	86.99 ± 7.58	84.15 ± 4.92	83.29 ± 4.97	80.73 ± 3.55
6	99 ± 1.03	90.12 ± 2.46	85.71 ± 3.01	79.74 ± 1.80	63.11 ± 3.71	55.72 ± 3.44	51.17 ± 6.43
12	99 ± 1.01	84.79 ± 2.68	76.74 ± 4.25	67.08 ± 5.97	59.09 ± 3.82	53.83 ± 5.43	41.40 ± 7.45
24	98.33 ± 1.15	73.76 ± 6.28	65.81 ± 8.86	49.23 ± 1.52	31.68 ± 2.11	31.07 ± 1.55	31.68 ± 0.42
30 nm AgNPs							
3	99.33 ± 0.57	92.39 ± 0.98	90.12 ± 4.20	88.13 ± 3.55	86.14 ± 2.25	84.43 ± 0.85	73.91 ± 0.98
6	99 ± 1.03	90.12 ± 2.99	92.39 ± 1.30	86.14 ± 3.07	82.16 ± 4.20	79.88 ± 4.85	62.54 ± 5.48
12	99 ± 1.01	88.89 ± 1.72	85.71 ± 4.09	77.01 ± 1.65	82.85 ± 2.12	70.11 ± 6.74	58.79 ± 5.03
24	98.33 ±	91.57 ±	84.33 ±	70.53 ±	51.18 ±	43.14 ±	33.39 ±

	1.15	6.09	7.88	7.15	5.80	1.46	0.84
100 nm AgNPs							
3	99.33 ± 0.57	90.68 ± 2.60	87.56 ± 3.44	86.70 ± 1.30	81.59 ± 4.20	72.77 ± 2.74	67.94 ± 2.60
6	99 ± 1.03	85.85 ± 0.98	84.71 ± 0.98	82.44 ± 7.15	70.22 ± 5.67	62.26 ± 5.59	59.41 ± 9.51
12	99 ± 1.01	87.27 ± 3.31	83.26 ± 1.88	80.60 ± 5.32	70.05 ± 2.66	60.05 ± 4.15	58.12 ± 4.16
24	98.33 ± 1.15	87.81 ± 2.77	83.78 ± 4.74	69.95 ± 8.15	53.87 ± 0.42	47.53 ± 1.26	41.92 ± 4.97
$AgNO_3$ (ionic control)							
	Concentrations in µg/mL						
Time (hours)	**Naive**	**0.5 $AgNO_3$**	**1 $AgNO_3$**	**2 $AgNO_3$**	**3 $AgNO_3$**	**5 $AgNO_3$**	**8 $AgNO_3$**
3	98.81 ± 2.06	99.10 ± 1.54	99.10 ± 0.89	98.21 ± 3.09	74.88 ± 2.23	55.35 ± 7.62	54.16 ± 4.40
6	100 ± 0.49	95.45 ± 7.45	82.96 ± 3.55	50.37 ± 2.36	20.76 ± 1.92	15.71 ± 1.03	13.06 ± 1.27
12	99.26 ± 1.27	38.07 ± 1.72	36.93 ± 4.55	28.41 ± 5.50	20.59 ± 3.49	16.71 ± 0.47	13.14 ± 0.34
24	93.36 ± 5.98	31.51 ± 6.38	22.71 ± 2.25	19.27 ± 0.02	14.96 ± 0.61	12.14 ± 1.60	10.65 ± 1.91

3. AgNP internalisation by ZF4 cells

The analysis was performed by manually drawing a region of interest (ROI) in the acquired image by FIJI. First, the image was separated into the various channels (green, red and blue) (Fig. S1 A), then FIJI was set to record the mean grey value for the NPs (Fig. S1 B), the ROI was carefully selected based on the outline of the cells provided by the cellular membrane staining with Alexa 488 (green) and added to the ROI toolbar (Fig. S1 C). Then the intracellular background fluorescence, from an adjacent area of equal size without cells, was subtracted. The corrected intensity values were normalised to a total number of 1000 cells. A similar method was implemented to calculate the total intensity of fluorescence per NP (visible intracellular accumulations of NPs): first an area with visible NPs was selected, then the intensity values for the selected area were subtracted to obtain a corrected intensity, as described in the literature [1-3]. Three individual samples per AgNP treatment were prepared; then, four cells per replicate were analysed, for a total of twelve cells per treatment (n=12). The intensities were plotted using GraphPad software.

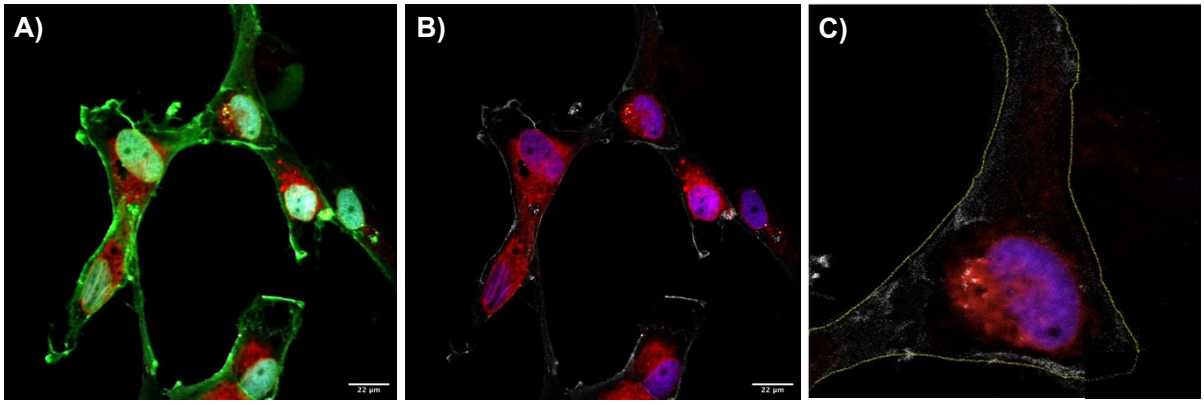

Figure S1. Example of the manual analysis of the AgNP reflectance intensity following exposure of the ZF4 cells to 10 nm AgNPs for 2 hours at 2.5 µg/mL. A) Composite image of a group of cells. Green shows the cell membrane, blue/light green the nucleus, red are the lysosomes and white are the NPs. Due to interference between the reflectance channel and the dye for cell membrane (A), the green channel was removed to visualize better the presence of the NPs for images B and C. **B)** Reflected intensity of the AgNPs – comparison with A indicates that the NPs are broadly associated with the cellular membranes. **C)** Zoomed image of a cell in figure B to show the manual drawing of the region of interest (ROI) by FIJI. The scale bar for all the images is 22 µm. Images were taken with a NIKON A1R 808 series microscope at 60X objective.

Table S3. Mean of the AgNP cellular intensities from 12 cells over three independent replicates. The table shows the mean of the reflected intensities analysed by FIJI after their incubation for either 2 or 24 hours.

Size	2.5 AgNPs (µg/mL)	5 AgNPs (µg/mL)	10 AgNPs (µg/mL)
2 hours			
10 nm	196.47 ± 18.35	176.95 ± 49.34	171.10 ± 42.70
30 nm	172.40 ± 56.72	130.59 ± 24.75	111.15 ± 24.79
100 nm	129.45 ± 49.90	125.29 ± 23.64	114.46 ± 24.54
24 hours			
10 nm	49.45 ± 2.24	59.94 ± 20.53	61.04 ± 17.18
30 nm	77.92 ± 15.84	80.81 ± 18.78	89.26± 16.14
100 nm	88.54 ± 17.28	87.62 ± 18.302	97.58 ± 14.64

Table S4. Calculation of mass concentration and NPs/mL. The mass concentration (10 µg/mL) was calculated to obtain the number of particles per millilitre (NPs/mL).

AgNPs size	NPs/mL
10 nm	1.76E+12
30 nm	5.85E+10
100 nm	1.67E+09

Table S5. Number of NP spots per cell area after 2 or 24 hours of exposure to different concentrations of the AgNPs. Results represent the average of three individual samples per AgNP concentration and size.

Size	2.5 AgNPs (µg/mL)	5 AgNPs (µg/mL)	10 AgNPs (µg/mL)
2 hours			
10 nm	0.006 ± 0.001	0.003 ± 0.002	0.003 ± 0.001
30 nm	0.002 ± 0.004	0.002 ± 0.001	0.003 ± 0.001
100 nm	0.001 ± 0.001	0.003 ± 0.001	0.004 ± 0.001
24 hours			
10 nm	0.007 ± 0.006	0.014 ± 0.007	0.020 ± 0.007
30 nm	0.005 ± 0.001	0.005 ± 0.001	0.012 ± 0.003
100 nm	0.012 ± 0.007	0.008 ± 0.004	0.07 ± 0.007

3.1 Confocal images

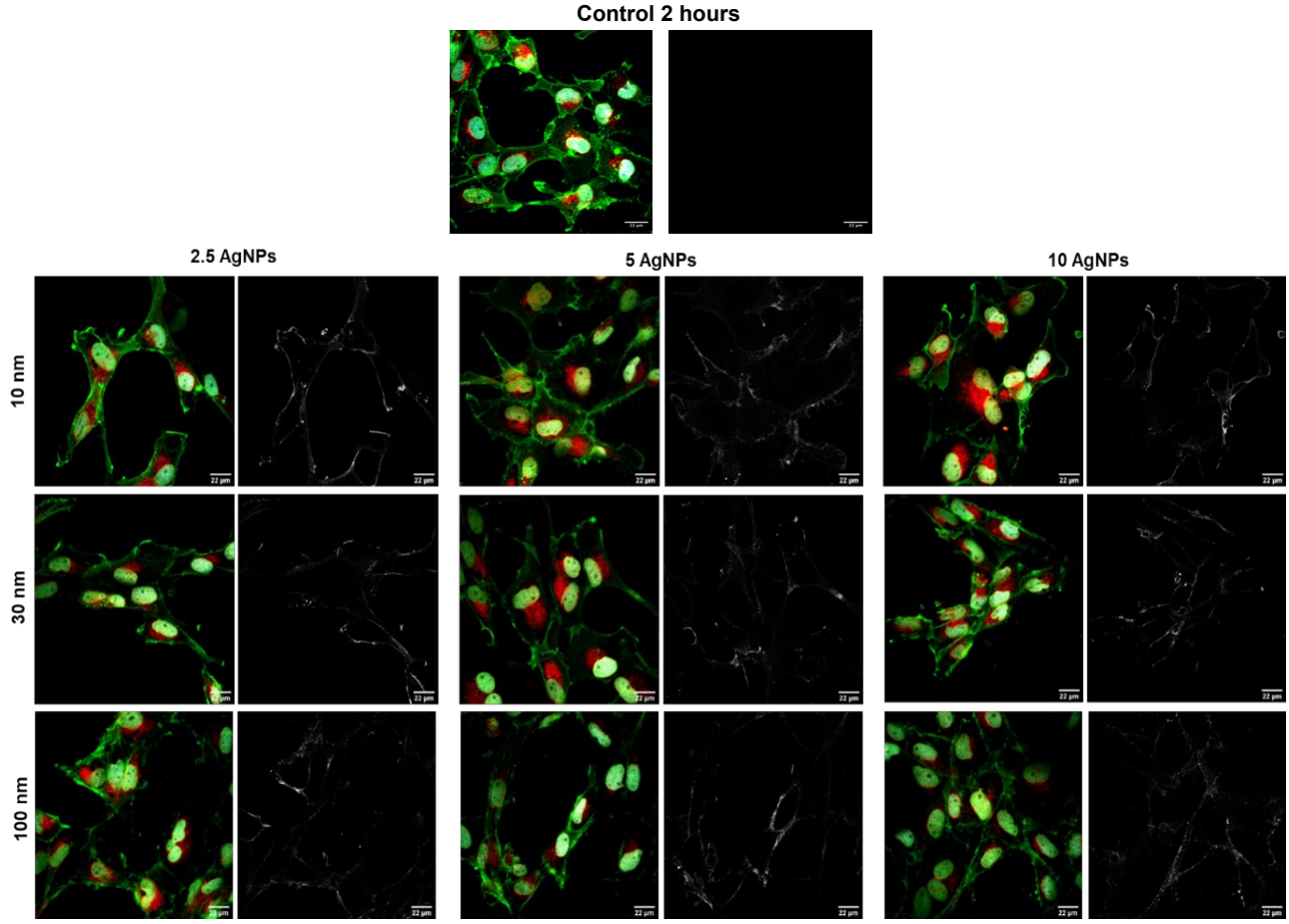

Figure S2. Confocal images of ZF4 cells treated with AgNPs for 2 hours. All the images show the composite image which includes the cellular dyes used for organelle identification. Green dye stains the cell membrane. Blue/light green represents the nucleus and red the lysosomes. The images next to the composite (right panels) shows the reflectance channel (NPs) acquired from the same images. The AgNP concentration in µg/mL used for the treatments is shown above the images, while the AgNP size is shown on the left. The control represents untreated (naïve) cells. Images were taken using a 60x objective. The scale bar is 22 µM.

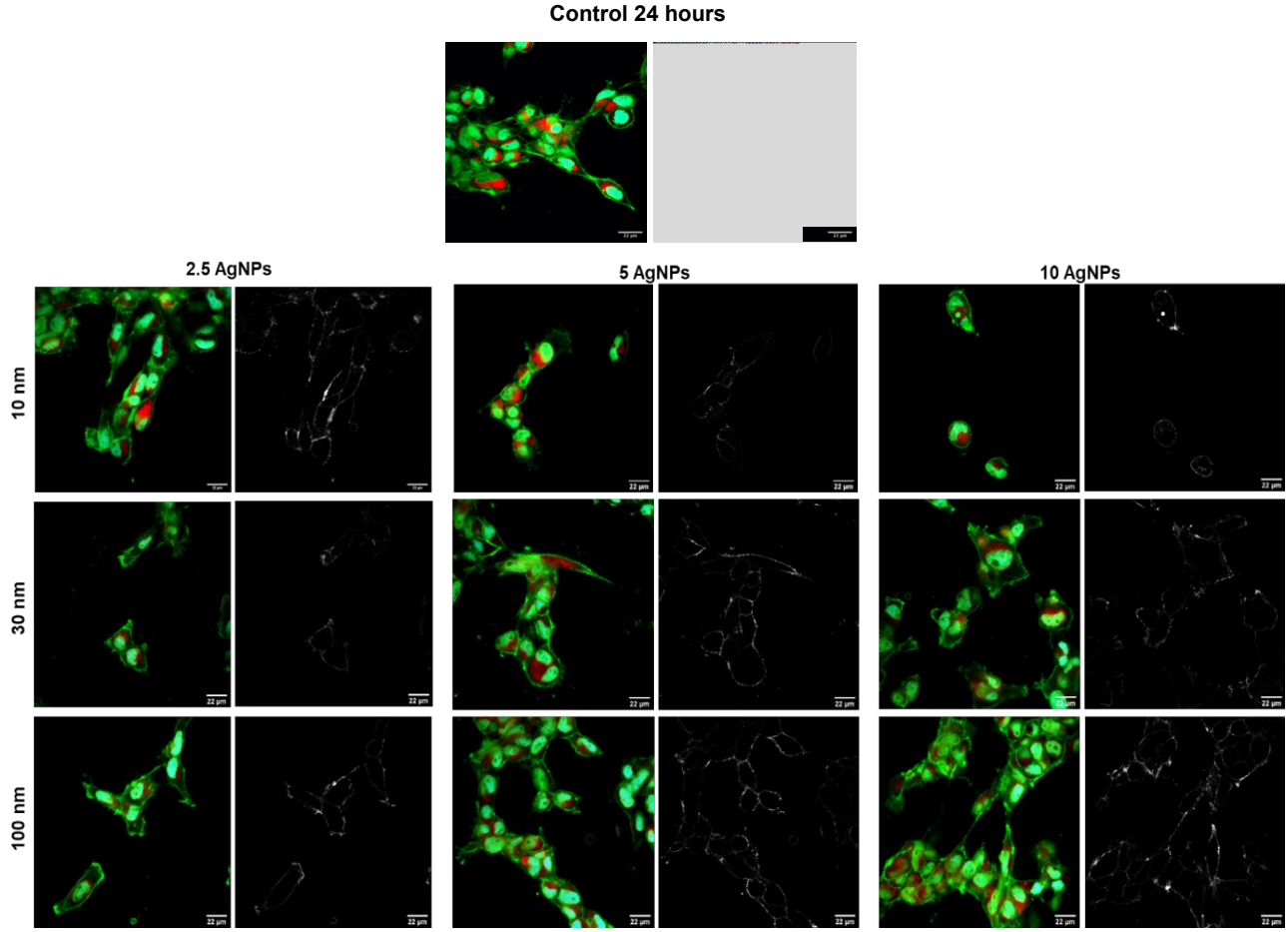

Figure S3. Confocal images of ZF4 cells treated with AgNPs for 24 hours. All images show the composite image which includes the cellular dyes used for organelle identification. Green dye stains the cell membrane. Blue/light green indicates the nucleus and red represents the lysosomes. The images next to the composite (right panel) show the reflectance channel (NPs) acquired for the same images. The AgNP concentration in µg/mL used for the treatments is shown above the images, while the the AgNP size is indicated on the left. The control represents untreated cells. Images were taken using a 60x objective. The scale bar is 22 µM.

4. Calcium homeostasis

Table S6. Intracellular calcium concentrations (expressed as %) following exposure to AgNPs or $AgNO_3$. Intensities are normalised relative to the naïve control. First, intensity results were corrected by subtracting the naïve values, then the corrected values were divided per number of viable cells and then normalised to percentage (%) against the naïve cells. Results represent the mean and standard deviation of three individual replicates.

AgNPs size	**Naive**	**2.5 AgNPs (µg/mL)**	**5 AgNPs (µg/mL)**	**10 AgNPs (µg/mL)**
10 nm	100 ± 0.0	219.99 ± 80.12	178.53 ± 43.17	164.19 ± 18.75
30 nm	100 ± 0.0	98.34 ± 29.67	116.40 ± 53.92	255.68 ± 26.63
100 nm	100 ± 0.0	101.03 ± 3.01	109.04 ± 6.37	164.34 ± 23.21
$AgNO_3$	**Naive**	**1 $AgNO_3$**	**1.5 $AgNO_3$**	**2 $AgNO_3$**

		(µg/mL)	(µg/mL)	(µg/mL)
	100 ± 0.0	77.81 ± 4.31	14.16 ± 3.14	14.74 ± 0.88

5. Oxidative stress

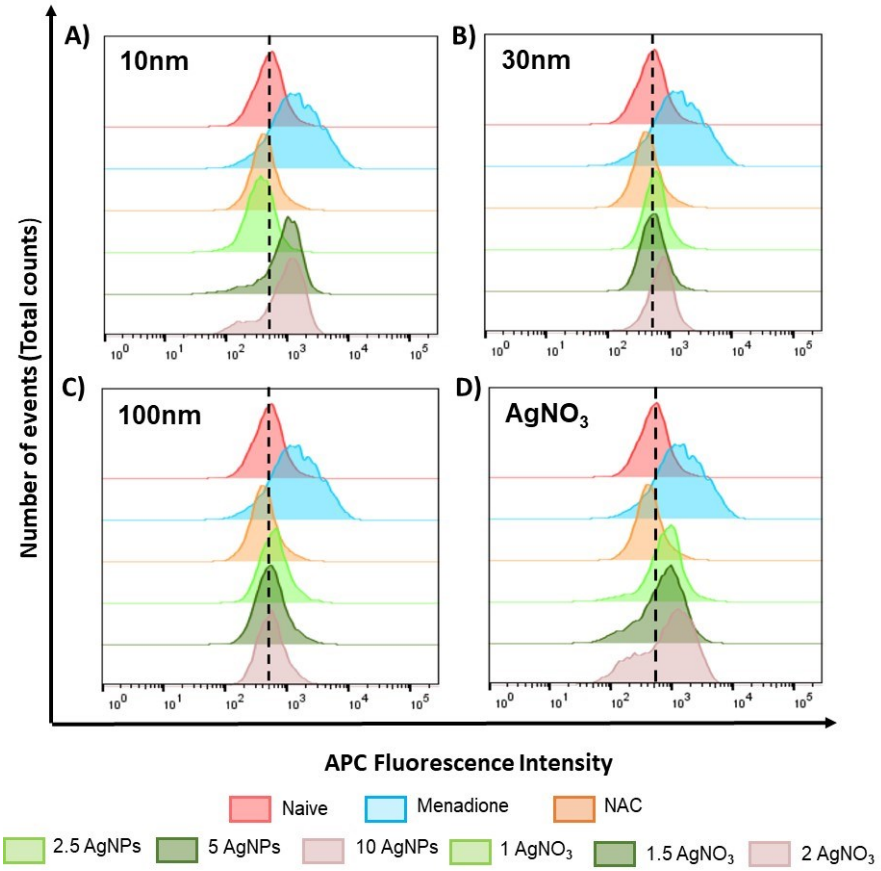

Figure S4. Oxidative stress histogram plots for ZF4 cells. Histograms representing the number of cell counts versus the Allophycocyanin (APC) dye fluorescence intensities obtained from the analysis by Flowjo software. **A), B)** and **C)** show the results for 2.5, 5 and 10 µg/mL of 10, 30 and 100nm AgNPs respectively, while **D)** shows the histograms for the $AgNO_3$ treatments (1, 1.5 and 2 µg/mL). The dashed lines in the middle of the pictures represent the mean intensity of the naïve cells and can be used a reference for the shifting of peaks induced by the various treatments.

Table S7. Normalised intensities for the oxidative stress results. The mean APC intensities were normalised to percentages (%) relative to the naïve.

AgNPs size	**Naive**	**2.5 AgNPs (µg/mL)**	**5 AgNPs (µg/mL)**	**10 AgNPs (µg/mL)**
10 nm	100 ± 18.95	106.2 ± 32.11	211.8 ± 22.96	217.2 ± 16.85
30 nm	100 ± 18.95	136.1 ± 4.77	121.9 ± 7.9	173.7 ± 8.0
100 nm	100 ± 18.95	141.1 ± 9.80	123.3 ± 6.33	135.6 ± 11.78
$AgNO_3$	**Naive**	**1 $AgNO_3$**	**1.5 $AgNO_3$**	**2 $AgNO_3$**

		(µg/mL)	(µg/mL)	(µg/mL)
	100 ± 13.02	162.01 ± 27.89	166.5 ± 5.07	208.2 ± 9.92

6. Cell cycle

Table S8. Cell cycle percentages for each phase.

10 nm				
Cell cycle phase	**Naive**	**2.5 AgNPs (µg/mL)**	**5 AgNPs (µg/mL)**	**10 AgNPs (µg/mL)**
G1	65.46 ± 2.2	62.26 ± 3.65	58.43± 4.36	61.26 ± 1.38
S	17.50 ± 1.25	16.60.1 ± 0.65	13.56 ± 3.97	13.86± 3.81
G2	10.03 ± 1.6	11.30 ± 0.3	5.83 ± 2.29	8.66 ± 1.08
30 nm				
G1	65.46 ± 2.2	64.8 ± 1.44	67.70 ± 2.78	65.06 ± 4.6
S	17.50 ± 1.25	14.0 ± 0.7	14.03 ± 1.65	13.33 ± 2.13
G2	10.03 ± 1.6	12.36 ± 3.15	13.18 ± 1.37	12.53 ± 0.37
100 nm				
G1	65.46 ± 2.2	63.0 ± 3.88	64.86 ± 1.44	65.73 ± 2.45
S	17.50 ± 1.25	14.57 ± 5.68	16.96 ± 1.41	16.01 ± 0.87
G2	10.03 ± 1.6	11.02 ± 4.18	12.46 ± 2.15	10.95 ± 1.97
$AgNO_3$				
Cell cycle phase	**Naive**	**1 $AgNO_3$ (µg/mL)**	**1.5 $AgNO_3$ (µg/mL)**	**2 $AgNO_3$ (µg/mL)**
G1	65.46 ± 2.2	62.13 ± 2.81	68.35 ± 1.06	71.0 ± 2.40
S	17.50 ± 1.25	17.8 ± 1.22	13.05 ± 4.06	12.09 ± 2.61
G2	10.03 ± 1.6	11.76 ± 0.94	7.87 ± 0.92	6.75 ± 0.78

6.1 Cell cycle histograms

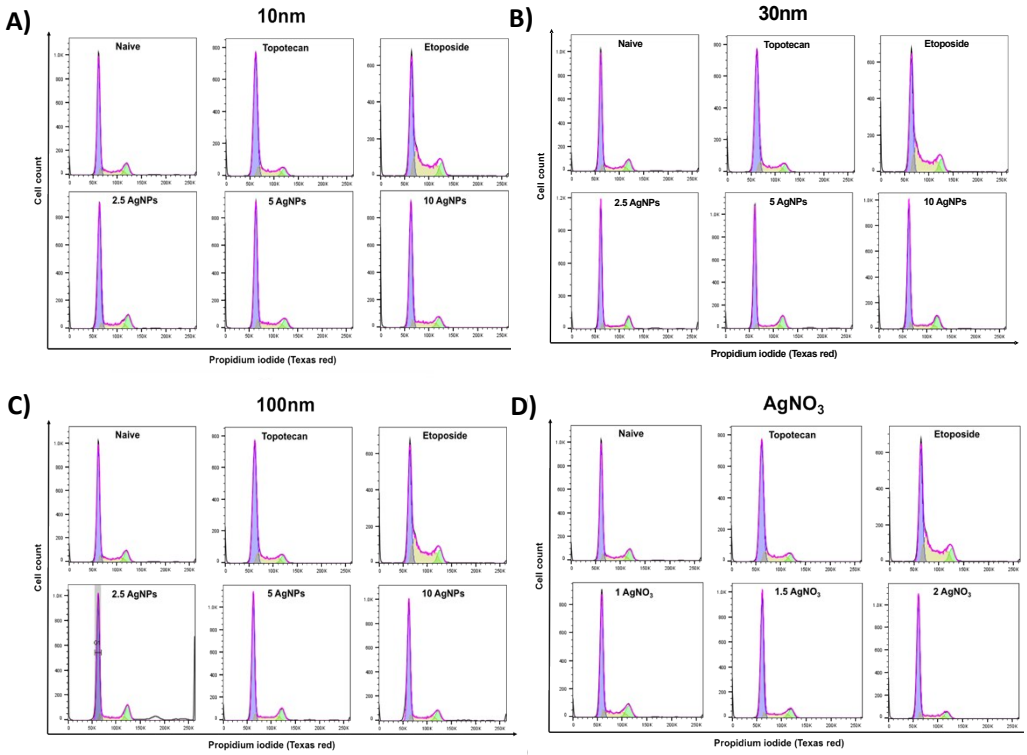

Figure S5. Propidium iodide intensities versus the total cell counts (10,000) were analysed by FlowJo V10 software using the cell cycle tool. **A)** shows data for 10 nm, **B)** for 30 nm, **C)** for 100nm AgNPs, and **D)** for the ionic control ($AgNO_{3)}$. Cell cycle images represent one of three individual replicates used for the analysis.

7. DNA Damage determined by Comet assay

7.1 **Images of the acquired comets.**

Three individual replicates were used to calculate the mean intensity of the scored tail percentage (%) using comet IV assay macro software by Instem solutions, https://www.instem.com/solutions/genetic-toxicology/comet-assay.php.

Table S9. Summary of the DNA percentage strand breaks (%). Results represent the mean DNA tail percentage of three individual replicates, obtained by scoring 50 comets

per replicate, for a total of 150 comets per treatment. Comets were analysed and scored using IV comet macro software. H_2O_2 was used as the positive control.

AgNPs					
AgNPs Size (nm)	**Naive**	**H_2O_2 200µM**	**2.5 AgNPs**	**5 AgNPs**	**10 AgNPs**
10nm	1.88 ± 1.45	46.81 ± 6.02	7.55 ± 2.61	8.70 ± 4.52	11.45 ± 3.53
30nm	1.88 ± 1.45	46.81 ± 6.02	8.32 ± 6.03	8.88 ± 9.69	10.46 ± 5.02
100nm	1.88 ± 1.45	46.81 ± 6.02	6.36 ± 0.86	6.62 ± 2.63	10.13 ± 4.56
$AgNO_3$					
$AgNO_3$	**Naive**	**H_2O_2 200µM**	**1 $AgNO_3$ (µg/mL)**	**1.5 $AgNO_3$ (µg/mL)**	**2 $AgNO_3$ (µg/mL)**
	1.88 ± 1.45	46.81 ± 6.02	79.48 ± 2.67	82.67 ± 1.46	82.90 ± 0.91

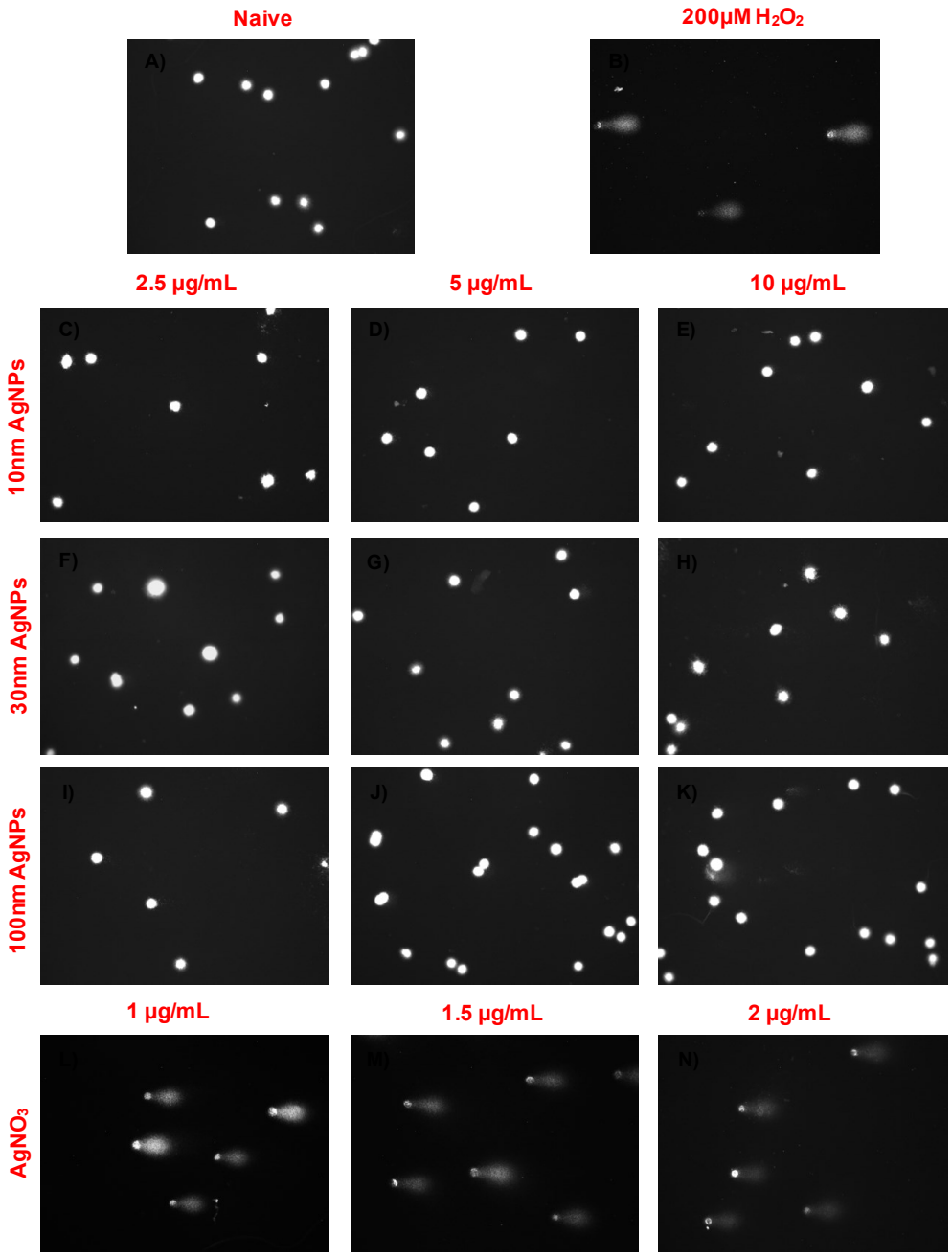

Figure S*6*. Comet assay images. The mass concentrations used are shown above the images while the details of the treatment (AgNP size or ionic control) are shown on the left. The images represent one of the three replicates for each treatment.

Estimation of the uptake mechanism via membrane bound or fluid phase.

A calculation was used to quantitatively estimate the likelihood of NPs entering cells through either direct membrane bound interactions or through fluid encapsulation, based upon the properties of the vesicles and the NPs (e.g., NP size) as described in [2] and [1]. First, the number of NPs/mL was calculated (10 μg/mL) (Figure 8) for the three sizes and then substituted into the equations. Three possible diameters of the vesicles (150, 300 and 500 nm) were used to further understand the likelihood of the NPs entering the cells via the

different routes. In each case, the calculations are likely to be an over-estimation, partly due to neglecting the membrane surface curvature in the NP attachment at the surface. The calculations are based on the hypothesis that the membrane surface has the ability to be coated in NPs as described in Guggenheim *et al.*, (2020) and Smith *et al.*, (2012).

Table 10. Summary of the calculations of the likelihood of NPs entry into the XF4 cells via fluid phase of membrane bound mechanisms. The table shows the results obtained using the calculations previously described for three vesicle diameters and the three different AgNP sizes (10, 30 and 100 nm).

AgNPs size (10 nm)				
Vesicle diameter	Fluid phase (clathrin-coated)		Membrane bound / receptor mediated (non clathrin coated)	
	NPs on surface	NPs in lumen	NPs on surface	NPs in lumen
150 nm	52.56	8.15E+17	131.04	2.53E+18
300 nm	464.79	1.37E+19	663.39	2.25E+19
500 nm	1651.43	8.18E+19	2010.19	1.08E+20
AgNPs size (30 nm)				
Vesicle diameter	Fluid phase (clathrin assumption)		Membrane bound (receptor mediated) no Clathrin coat	
	NPs on surface	NPs in lumen	NPs on surface	NPs in lumen
150 nm	1.31	2.71E+16	6.47	8.41E+16
300 nm	34.98	4.56E+17	53.49	7.47E+17
500 nm	150.65	2.72E+18	186.95	3.60E+18
AgNPs size (100 nm)				
Vesicle diameter	Fluid phase (Clathrin assumption)		Membrane bound (receptor mediated) no Clathrin coat	
	NPs on surface	NPs in lumen	NPs on surface	NPs in lumen
150 nm	0.98	7.74E+14	0.33	2.40E+15
300 nm	0.19	1.30E+16	0.74	2.13E+16
500 nm	5.51	7.76E+16	7.65	1.03E+17

References

1. P. J. Smith, M. Giroud, H. L. Wiggins, F. Gower, J. A. Thorley, B. Stolpe, J. Mazzolini, R. J. Dyson and J. Z. Rappoport, *International journal of nanomedicine*, 2012, **7**, 2045-2055.
2. E. J. Guggenheim, J. Z. Rappoport and I. Lynch, *Nanotoxicology*, 2020, **14**, 504-532.
3. Christine Labno, Basic Intensity Quantification with ImageJ, https://www.unige.ch/medecine/bioimaging/files/1914/1208/6000/Quantification.pdf).