Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Supplementary material for:

Toxicity and chemical transformation of silver nanoparticles in A549 lung cells: dose-rate-dependent genotoxic impact

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Materials and methods

1. Physico-chemical characterization

Materials and methods for measurement of primary diameter of nanoparticles (NPs) are described elsewhere (for Ag-PVP, see[1] and for NM300 K, see http://publications.jrc.ec.europa.eu/repository/handle/JRC60709). Primary particle diameters were analyzed from TEM images (Figure 1), where diameter was deduced from the analysis of the area of ~200 particles using Image J. Hydrodynamic diameters, polydispersity indexes (PdI) and zeta potentials were measured using dynamic light scattering (DLS), on a Malvern Nano-ZS zetasizer (Malvern Instrument). For these measurements, NPs were diluted in either water or medium (DLS and PdI measurements only) to 10 µg/mL. DLS measurements were repeated 3 times independently, and consisted of 12 runs of 10 s. Zeta potential measurements were repeated 3 times independently and consisted of 20 runs for Ag-PVP and 50 runs for NM300K.

2. 53BP1 immunostaining and counting using High Content Analysis (HCA)

The amount of p53-binding protein 1 (53BP1) foci per cell nucleus was evaluated by immunostaining of cells fixed for 15 min in 4% paraformaldehyde, using anti-53BP1 antibody (Acris, 1/1500, vol./vol., exposure 1 h at room temperature) and anti-rabbit secondary antibody coupled to Atto488 (1/2000,vol./vol., exposure 1 h at room temperature). Cell nuclei were stained for 5 min with DAPI (1 µg/mL). As positive control, A549 cells were exposed to 10 µM of etoposide for 24 h. The total number of 53BP1 foci was automatically counted, as well as the total number of nuclei, on a Cell Insight CX5 (Thermofisher).

3. Cytokinesis-block micronucleus assay, HCA

After exposure to NPs, cells were cultured for another 24 h in complete cell culture medium containing 4 µg/mL cytochalasin B in order to block cytokinesis. They were then fixed for 15 min in 4% paraformaldehyde and stained with DAPI for 5 min (1 µg/mL). As positive control A549 cells were exposed to 25 or 50 ng/ml of mitomycin c for 24 h. 5 replicates per condition were used. Micronuclei were automatically counted (% micronuclei), as well as % binuclear cells, proliferation index and % targeted cells, on a Cell Insight CX5 (Thermofisher).

Figure S1. Exposure scenarios. Cells were seeded at day 0, then exposed at day 1 (acute exposure) or at days 2, 3 and 4 (repeated, cumulative exposure). Assays were performed 24 h after the last exposure, i.e., at day 2 (acute exposure) or at day 5 (repeated exposure). In the repeated exposure procedure, the exposure medium was not discarded from one day to another. NPs were added in the exposure medium from the past day.

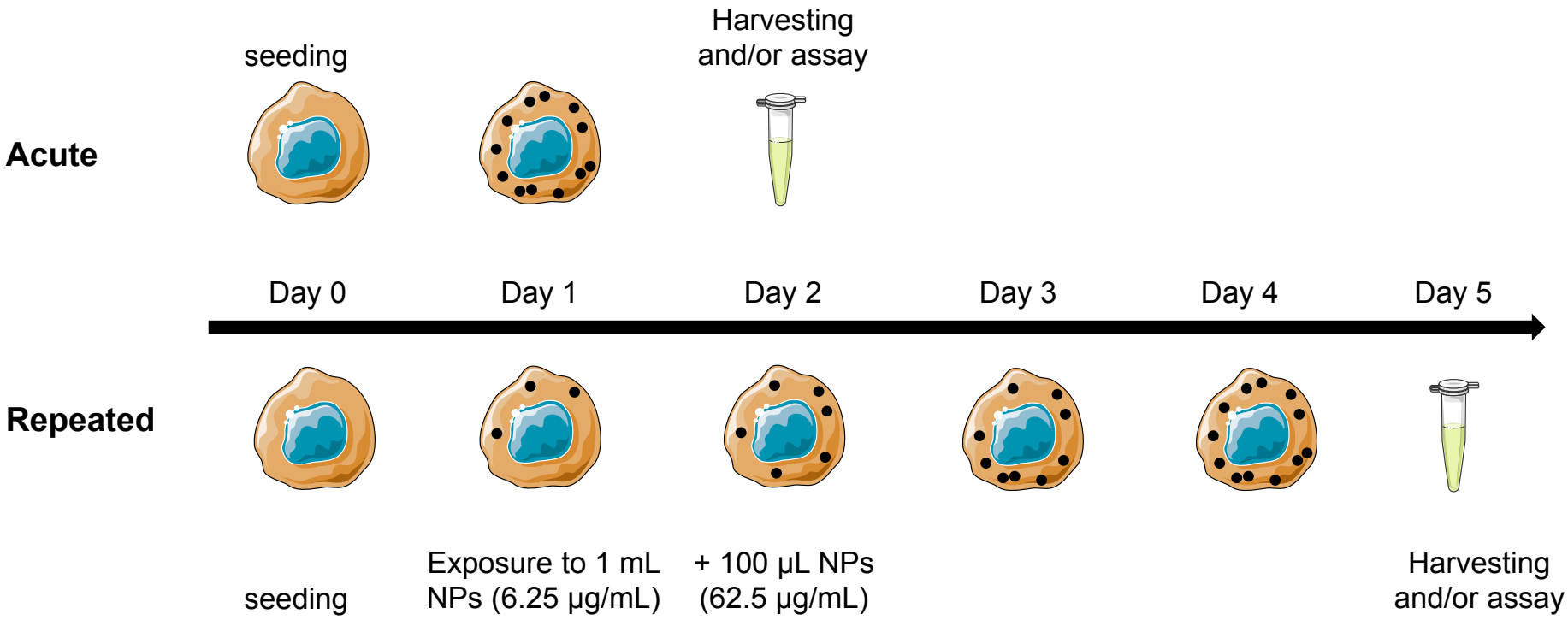


Table S1. Comparison of exposure concentrations in µg/mL and µg/cm²

Assay	Type of plate	volume	Concentration (µg/mL)	Concentration (µg/cm²)
Cytotoxicity	96 wells	100 µL	10, 25, 50, 100, 200	3.1, 7.8, 15.6, 31.2, 62.5
H2-DCF-DA	96 wells	100 µL	3.25, 12.5, 25	1.9, 3.9, 7.8
ICP-MS	24 wells	500 µL	25	6.25
XAS	Petri dishes, 56 cm²	10 mL	25	4.5
TEM	24 wells	1 mL	25	6.25
Comet	24 wells	500 µL	25	6.25
8-oxo-dGuo (HPLC-MS/MS)	Petri dishes, 56 cm²	10 mL	25	4.5
53BP1	96 wells	100 µL	25	7.8
Micronucleus assay	96 wells	100 µL	25	7.8
RT-qPCR	6 wells	2 mL	25	10
DNA repair activities	6 wells	2 mL	25	10
Cell cycle (flow cytometry)	12 wells	1 mL	25	12.5

Table S2. Interference with WST-1, H2DCFDA and DHR123 assays[a]

	WST-1				H2-DCF-DA assay	
	no PS-NH_2		+ PS-NH_2			
	NM300K	Ag-PVP	NM300K	Ag-PVP	NM300K	Ag-PVP
CTL	3.93±0.07	3.99±0.01	0.25±0.01	0.28±0.01	896553±11524	886382±21374
3.125	3.83±0.10	3.80±0.05	0.30±0.01	0.36±0.01	nd	nd
6.25	3.74±0.12	3.84±0.07	0.32±0.01	0.41±0.03	855950±51178	788834±14265
12.5	3.76±0.14	3.84±0.08	0.36±0.01	0.53±0.02	770255±29136	763321±11932
25	3.90±0.06	3.96±0.04	0.46±0.02	0.67±0.02	714160±15804	718955±16316

[a] In the WST-1 assay, interference was evaluated on control cells (no PS-NH_2) and cells exposed to 200 µg/mL PS-NH_2 (+ PS-NH_2), leading to 100% cells death. These cells were then exposed for 24 h to 3.125, 6.25, 12.5 or 25 µg/mL Ag-NPs, either NM300K or Ag-PVP, then incubated with WST-1. Absorbance was measured at 450 nm; data presented in this table are mean ± standard deviation of absorbance measured over 5 replicates. In H2-CF-DA assay, the fluorescence emitted by 3.125, 6.25, 12.5, 25 µg/mL of Ag-NPs diluted in cell culture medium was measured at λexc/λem 480/530 nm.

Table S3. Primer sequences for qPCR

Gene	Forward (5'->3')	Reverse (5'->3')
MT1	GCTTCTCCTTGCCTCGAAA	TGACGTCCCTTTGCAGATG
MT2	TCTTCAGCTCGCCATGGAT	TTGTGGAAGTCGCGTTCTTTA
SOD1	AGGGCATCATCAATTTCGAG	ACATTGCCCAAGTCTCCAAC
SOD2	TCCACTGCAAGGAACAACAG	TCTTGCTGGGATCATTAGGG
CAT	AGCTTAGCGTTCATCCGTGT	TCCAATCATCCGTCAAAACA
GSR	GATCCCAAGCCCACAATAGA	CTTAGAACCCAGGGCTGACA
GCLM	CCTCCTGCTGTGTGATGCCAC	CGTGCGCTTGAATGTCAGGAATGC
NRF2	CAGTCAGCGACGGAAAGAGT	ACCTGGGAGTAGTTGGCAGA
PARP1	GCTCCTGAACAATGCAGACA	CATTGTGTGTGGTTGCATGA
OGG1	TGGAAGAACAGGGCGGGCTA	ATGGACATCCACGGGCACAG
APE1	GCTGCCTGGACTCTCTCATC	GCTGTTACCAGCACAAACGA
PCNA	GGCTCTAGCCTGACAAATGC	GCCTCCAACACCTTCTTGAG
XRCC1	CAGCCCTACAGCAAGGACTC	GCTGTGACTGGGGATGTCTT
POLβ	GAGAAGAACGTGAGCCAAGC	CGTATCATCCTGCCGAATCT

Table S4. Physico-chemical characteristics of NM300K and Ag-PVP NPs

	NM300K	Ag-PVP
Diam. TEM (nm)	5.42±1.72 and 15.03±1.13[a]	59±18
Diam. Range TEM (nm)	8-47	25-100
Zeta water (mV)	-4 mV	-23 mV
Z-average in water (nm)	33.6	103.3
PDI in water	0.226	0.153
Z-average in growth medium[b] (nm)	40.3	109.8
PDI in medium	0.460	0.113

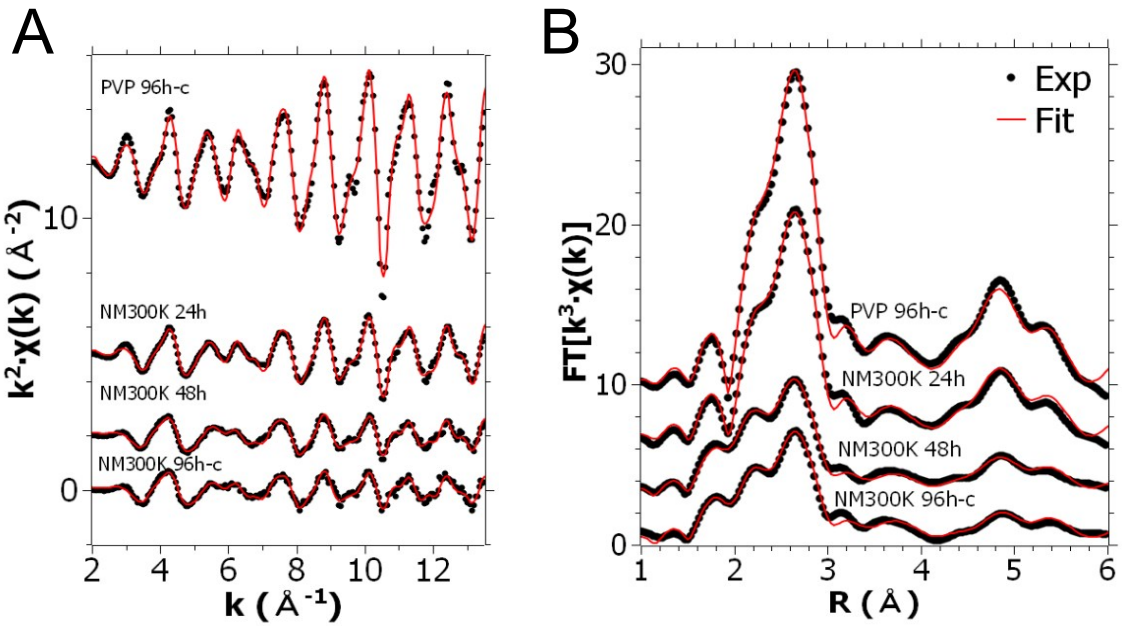
[a] As reported in the JRC report. [b] Growth medium was DMEM containing 10% FBS and antibiotics.

Table S5. Fit results of the EXAFS region of Ag k-edge absorption spectra as a linear combination of reference compounds.[a]

	AgNP (%)	AgGSH (%)	Ag**L**^{1} (%)	R_{factor} (10^{-2})
NM300K-24h	40.0±0.4	22±3	38±3	1.9
NM300K-48h	20.3±0.4	15±2	65±2	3.9
NM300K-96h-c	18.9±0.5	30±3	51±3	7.7

[a] The three components used to fit the data represent Ag in undissolved nanoparticles (AgNPs) or released from NPs and forming Ag-thiol complexes in AgS_2 (AgGSH) or AgS_3 (AgL1) coordination.

Figure S2: Ag K-edge experimental EXAFS spectra (black dots) of A549 cells after 24 h (NM300K 24 h), 48 h (NM300K 48 h) of acute exposure or 4 days of repeated exposure (NM300K 96 h-c) to NM300K silver NPs. Fourier-Transformed EXAFS spectra obtained after 24 h or 48 h of acute exposure to 25 µg/mL of NM300K, or after 96 h of repeated exposure (6.25 µg/mL per day for four successive days) to NM300K or PVP-coated Ag-NPs. Relative best-fitting curves (red) based on an *ab initio* model given by a combination of Ag^0 in the crystalline fcc phase (i.e. Ag in NPs) and ionic Ag^+ recombined with thiolate groups (A). The extracted and k^2-weighted EXAFS spectra are presented in panel B (black dots: experimental data; red solid lines: best-fitting curves shown in panel A, back-transformed into the k space and overlapped to the experimental data for a cross-check).



Discussion on linear Combination Fitting of EXAFS spectra

The XANES Linear Combination Fitting (LCF) analysis suggests that the main species formed by dissolved Ag ions *in situ* in cells are Ag-S species. Ag can bind in digonal or trigonal coordination in thiolate sites, and we previously demonstrated that the spectral region is most sensitive to the coordination geometry is the EXAFS region[2]. We performed therefore a LCF analysis of the EXAFS regions using two different Ag-SR reference compounds: Ag-GSH for AgS_2 coordination, and Ag**L**^{1} that we previously identified as a model of AgS_3 coordination[2]. EXAFS data were fitted in the range k=[3, 12] $Å^{-1}$. This analysis could only be performed when the fraction of dissolved Ag atoms became predominant, i.e. in samples exposed to NM300K AgNP. The results are reported in Table S5 and the best-fitting curves are shown in Figure S3. The majority of ionic Ag is found to form AgS_3 complexes as per the ones formed when Ag replaces Cu/Zn in metallothionein[2].

Figure S3. TEM image of NM300K after 24 h of incubation in exposure medium, at 37°C and 5% CO_2. Scale bar 200 nm (A) and 100 nm (B).

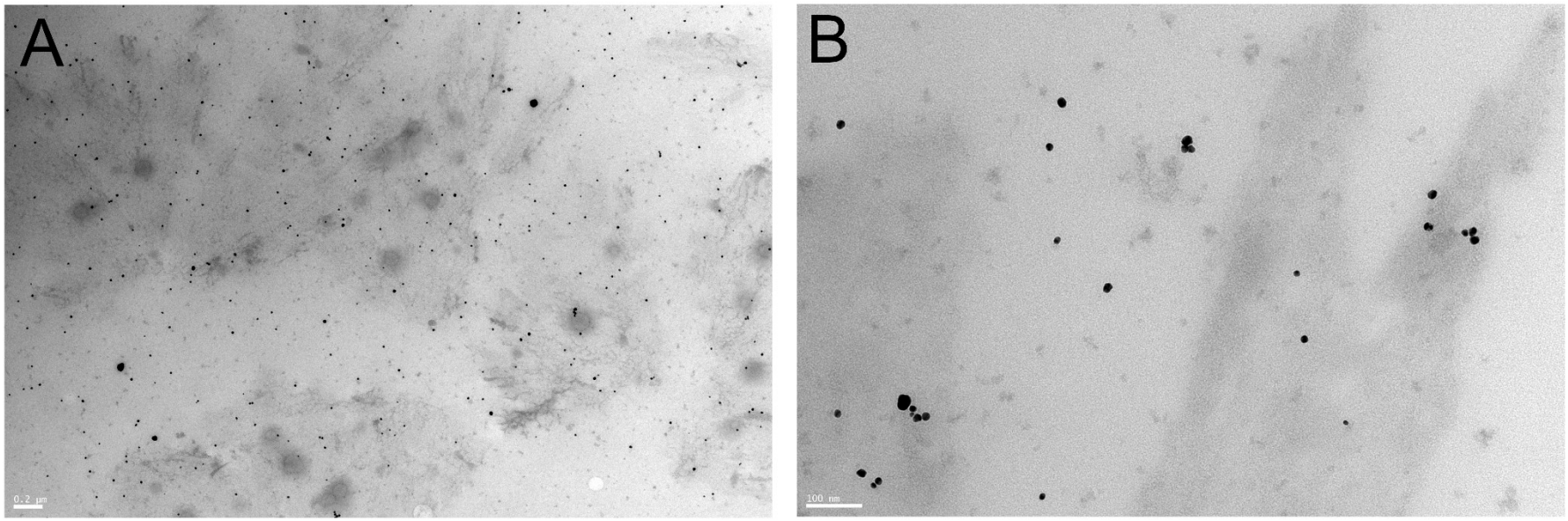


Figure S4. 8-oxo-dGuo level in A549 cells exposed acutely to Ag-NPs

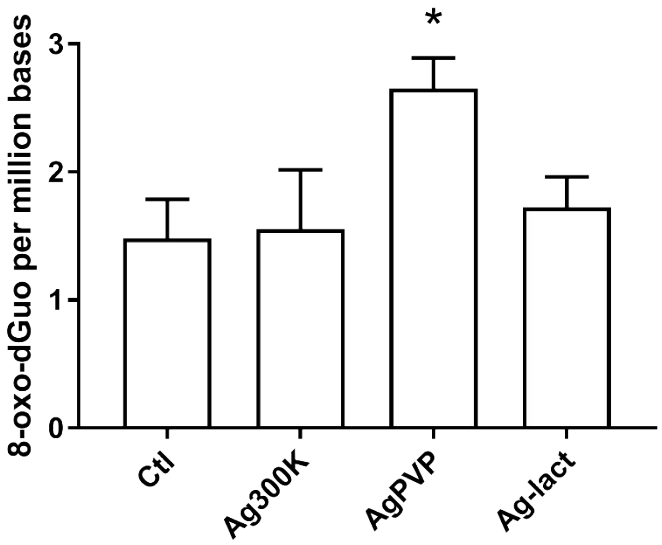


Table S6. Results from 53BP1 immunostaining assay and micronucleus assay[a]

	Nr. 53BP1 foci	Nr. micronucleus
CTL	0.24±0.10	2.35±0.61
Ag NM300K	0.26±0.11	2.25±0.11
Ag-PVP	0.19±0.07	2.41±0.25
Ag-lactate	0.49±0.48	2.48±0.17
Etoposide	1.72±0.82*	n/a
Mitomycin c	n/a	4.67±0.87*

[a]Cells were exposed for 24 h to 25 µg/mL Ag-NP (NM300K or Ag-PVP) or 10 µg/mL Ag-lactate. CTL: unexposed cells. Positive controls: for 53BP1, 10 µM etoposide for 24 h, for micronucleus assay 25 ng/mL mitomycin c for 24 h. Statistical significance, *P<0.05, exposed *vs*. CTL.

References

1. C. Aude-Garcia, F. Villiers, V. Collin-Faure, K. Pernet-Gallay, P. H. Jouneau, S. Sorieul, G. Mure, A. Gerdil, N. Herlin-Boime, M. Carriere and T. Rabilloud, *Nanotoxicology*, 2016, **10**, 586-596.
2. G. Veronesi, T. Gallon, A. Deniaud, B. Boff, C. Gateau, C. Lebrun, C. Vidaud, F. Rollin-Genetet, M. Carriere, I. Kieffer, E. Mintz, P. Delangle and I. Michaud-Soret, *Inorganic chemistry*, 2015, **54**, 11688-11696.