Electronic Supplementary Information

Nuclear Translocation of Silver Ions and Hepatocyte Nuclear Receptor Impairment upon Exposure to Silver Nanoparticles

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Supplementary figures



Fig. S1. HepG2 cell viability upon AgNP exposure. Hepatocytes were exposed to cit-AgNPs or PVP-AgNPs at 12.5 and 25 μ M Ag, respectively. Cells were exposed for the indicated time (24 to 96 hours) before being harvested, suspended in PBS and mixed in one volume of trypan blue. Total and viable cells were counted in a TC20 Automated Cell Counter (Bio-Rad).



Fig. S2. mRNA levels of redox and metal stress indicators. (A) HMOX mRNA fold increase in HepG2 cells exposed to AgNPs. (B) MT1 mRNA fold increase in HepG2 cells exposed to AgNPs. Results are expressed as the relative change in expression compared to control (ctl), corresponding to cells unexposed to AgNPs. The results are expressed as means +/- standard error of the mean of at least three independent experiments.



Fig. S3. Ag K-edge XANES of HepG2 cells exposed to AgNPs. Experimental spectra of reference compounds (solid black curves) and of cell pellets (black circles) exposed for 24 h to 12.5 μ M cit-AgNPs or 25 μ M PVP-AgNPs, or for 48h to 12.5 μ M cit-AgNPs. The best-fitting curves obtained by linear combination of reference spectra are drawn in red over the corresponding experimental spectra.

A- cit-AgNP



Fig. S4. Dynamics of AgNPs in HepG2 cells. Confocal fluorescence and reflectance microscopy of HepG2 cells exposed to 12.5 μ M of cit-AgNPs (A) or 25 μ M PVP-AgNPs (B) for 24 hours. AgNPs were observed in reflection mode (red) and Lamp1 by immunofluorescence (green). White arrows pinpoint some of the lysosomes (Lamp1) containing AgNPs. N stands for nuclei.



Fig. S5. Visualization and analysis of cit-AgNPs in the perinuclear region. (A) Large field of view STEM micrograph showing the presence of a lysosome containing NPs in contact with a nucleus in HepG2 cells exposed for 24 hours to $12.5 \,\mu$ M cit-AgNPs. (B) EDX map of the orange area in (A) showing HAADF map in black and white, Ag map in red and S map in green. The different colored boxes correspond to the area selected for spectra extraction presented in (C). (C) EDX spectra of different regions in the energy range 1 - 3.5 keV. The position of Si, S, Cl and Ag peaks is highlighted.



Fig. S6. Visualization and analysis of PVP-AgNPs in the perinuclear region. (A) Large field of view STEM micrograph showing the presence of a lysosome containing NPs in contact with a nucleus in HepG2 cells exposed for 24 hours to 25 μ M PVP-AgNPs. (B) STEM micrograph of orange area in (A) at higher magnification. The different colored boxes correspond to the area scanned by EDX with spectra presented in (C). Lower panels show EDX Ag and S maps of the black area. (C) EDX spectra of different regions in the energy range 1 – 3.5 keV. The position of Si, S, Cl and Ag peaks is highlighted.



Fig. S7. Semi-thin sections of HepG2 cells analyzed by Nano-XRF. Two-color maps (Ag in red and Os in green) for 400 nm sections (left panels) and Ag areal density maps of the same regions (right panels) of cells exposed for, (A) 48h to 12.5 μ M cit-AgNPs, (B) 96h to 12.5 μ M cit-AgNPs, (C) 72 h to 25 μ M PVP-AgNPs. Areal densities are expressed in μ g/cm²; pixel size is 100x100 nm². Scale bars correspond to 10 μ m. c: cytoplasm; n: nuclei; N: nucleoli. The upper areal density limit in color bars is set to values that allow the visualization of the low areal density signal.



Fig. S8. Normalized XRF spectra from different sub-cellular areas. (A) two-color map (Ag in red and Os in green) for a 400 nm section of a HepG2 cell exposed for 72 h to 12.5 μ M cit-AgNPs. Different areas in the map are defined as background (b), hot spots of Ag (h), cytoplasm (c), nucleus (n) and nucleolus (N). (B) normalized XRF spectra extracted from these different areas are presented with AgNP hot spots (red), cytosol (blue), nucleolus (green), nucleus (yellow) and background (black) regions. In the inset, a magnification of the Ag K α emission region is shown.



Fig. S9. False color representation of Os (green) and Ag (red) distribution in HepG2 cells exposed to citrate-coated AgNPs for (A) 48h, (B) 72h, (C) 96h. Starting from these images, we extracted the concentration of silver in the different subcellular compartments. The relative regions of interest, where silver has been quantified, are reported over the maps: yellow = nuclei; blue = nucleoli; white = background. The average values for Ag concentrations in nuclei and nucleoli at each exposure time are reported as an histogram in Fig. 2 of the main text.



Fig. S10. Kinetics of cellular AgNP uptake. The amount of Ag per million cells was determined by ICP-AES after 24, 48, 72 and 96 hours of exposure to 12.5 μ M cit-AgNPs or 25 μ M PVP-AgNPs. For each condition, the experiment was repeated three times independently. * stands for data statistically different between different exposure duration with p<0.05 and ** stands for data statistically different between different exposure duration with p<0.01.



Fig. S11. Variations of nuclear receptor mRNA levels upon ligand addition and/or AgNP exposure. (A) FXR mRNA fold variation in HepG2 cells exposed to AgNPs and CDCA or DMSO. (B) LXR β mRNA fold variation in HepG2 cells exposed to AgNPs and 22R-HC or ethanol. Results are expressed as the relative change in expression compared to the control (ctl), corresponding to cells unexposed to AgNPs. The results are expressed as means +/- standard error of the mean of at least three independent experiments.



Fig. S12. Kinetics of cellular AgNP uptake under very low dose AgNP exposure. The amount of Ag per million cells was determined by ICP-AES after 3, 7 and 10 days of exposure to 1 μ M cit-AgNPs or 2 μ M PVP-AgNPs. For 3 and 10 days of exposure, the experiments were repeated three times independently and for 7 days of exposure, they were repeated 4 times independently.



Fig. S13. Dynamics of cit-AgNPs in HepG2 cells. Confocal fluorescence and reflectance microscopy of HepG2 cells exposed to 12.5 μ M of cit-AgNPs for the indicated times. Nuclei were labeled with Hoechst (blue) and AgNPs were observed in reflection mode (green). At t= 3 hours, a bright field image is also shown. The images correspond to the full field of the images shown in Fig. 4A. White arrows pinpoint particles close to nuclei. The scale bars represent 20 μ m.



Fig. S14. Dynamics of PVP-AgNPs in HepG2 cells. Confocal fluorescence and reflectance microscopy of HepG2 cells exposed to 25 μ M of PVP-AgNPs for the indicated times. Nuclei were labeled with Hoechst (blue) and AgNPs were observed in reflection mode (green). At t = 0 hour, a bright field image is also shown. The images correspond to the full field of the images shown in Fig. 4B and all the points in the kinetic. White arrows pinpoint particles close to nuclei and red arrows particles bound to the cell membrane. The scale bars represent 20 μ m.



Fig. S15. Volume projections from confocal reflectance microscopy kinetics. Nuclei were labeled with Hoechst (blue) and AgNPs were observed in reflection mode (green). Images represent a maximum z-projection of all acquired planes, (A) cit-AgNPs, (B) PVP-AgNPs. The scale bar on all images is $20 \,\mu$ m.



Fig. S16. Impact of AgNP exposure on FXR activity in hepatocyte spheroid. FXR activity followed by CDCA-induced BSEP expression. HepG2/C3a cells grown as spheroids were exposed or not to 12.5 μ M cit-AgNPs or 25 μ M PVP-AgNPs for 48 hours and to CDCA at 80 μ M or DMSO during the last 17 hours. The RNA levels of BSEP were quantified in the different conditions using qPCR. 100% FXR activity was set using control conditions (unexposed to AgNPs) by calculating the ratio: amount of BSEP-RNA in ctl+CDCA / amount of BSEP-RNA in ctl without CDCA. This ratio (+CDCA/-CDCA) was measured for each condition and divided by the control ratio in order to determine the percentage of FXR activity in cells exposed to AgNPs (for details see experimental section). These experiments were performed three times independently. ** stands for data statistically different from the control with p<0.01.



Fig. S17. Visualization and analysis of AgNP-containing vesicles close to nuclei. (A) and (B) STEM micrographs from 240 nm thin sections showing the presence of AgNP-containing vesicles close to nuclei (white and black arrows). Scale bars represent 2 μ m. (C) Zoom in on the vesicle in (B) pointed by the black arrow. Scale bar represent 200 nm. (D) EDX map of the white box shown in (C) with Ag EDX map (pink) superimposed on the STEM map. (E) EDX spectra of two regions of the map shown in (D) noted A and B. The position of specific peaks of Os, S, Cl, Ag and U are highlighted in the spectra and proves the presence of Ag in the region A but not in the region B.

Supplementary tables

	Hydrodynamic diameter (polydispersity index)	Zeta potential
PVP-AgNP	89 nm (13.9%)	-6.8 mV
cit-AgNP	160 nm (16.3 %)	-27.1 mV

Table S1. Characterization of AgNPs. Diameter and polydispersity index (in parenthesis) of the main species observed in the dynamic light scattering distribution (in intensity) of cit-AgNPs and PVP-AgNPs in complete culture medium (Minimum Essential Medium + 10% Foetal Calf Serum). The zeta potential was measured in storage buffer using a Malvern Nano ZS.

Gene	Forward primer sequence	Reverse primer sequence	
HPRT	ATGGACAGGACTGGACGTCTTGCT	TTGAGCACACAGAGGGGCTACAATG	
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA	
MT1X	GCTTCTCCTTGCCTCGAA	TGACGTCCCTTTGCAGATG	
ABCG5	CTCCTACAGCGTCAGCCAC	AGGATGCACATGATCTGCCC	
BSEP	ACATGCTTGCGAGGACCTTTA	GGAGGTTCGTGCACCAGGTA	
FXR	GCATGCAGATCAGACCGTGA	TCAGTTTTCTCCCTGCATGACT	
LXR-β	CAGAGCGCAAGCGAAAGAAG	GCTGAGCACGTTGTAGTGGA	
45S pre- rARN	ACCTGTCGTCGGAGAGGTT	GACGCGCGAGAGAACAGCA	
HMOX1	ATGACACCAAGGACCAGAGC	GTGTAAGGACCCATCGGAGA	

Table S2. Quantitative real time polymerase chain reaction primer sequences.

Exposure conditions	AgNP fraction [%]	Ag-GSH fraction [%]	R_{fit}
PVP-AgNP 24h	24 ± 2	76 ± 2	$1.8 \cdot 10^{-4}$
cit-AgNP 24h	30 ± 2	70 ± 2	$2.3 \cdot 10^{-4}$
cit-AgNP 48h	23 ± 2	$\overline{77 \pm 2}$	$2.3 \cdot 10^{-4}$

Table S3. Results of Linear Combination Fitting of Ag K-edge XANES spectra of HepG2cells exposed to 12.5 μ M cit-AgNPs or 25 μ M PVP-AgNPs.

Caption for Movie S1. This movie shows, first, a complete stack of 2D micrographs and then segmentation of the different elements with nuclei in green, mitochondria in red, AgNPs in black (all AgNPs are inside vesicles as shown in Fig. 5B-C) and intercellular space in grey.