	Experiment title: Interference between Ag nanoparticles and metal homeostasis in hepatic cells: insight into the role of dissolved Ag in the mechanism of toxicity	**Experiment number**: MD-867
Beamline:	**Date of experiment**: from: 27/11/2014 to: 3/12/2014	**Date of report**:
Shifts:	**Local contact(s)**: Julie Villanova, Sylvain Bohic	*Received at ESRF:*

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Report:

The aims of the experiment were to visualize silver nanoparticle (AgNP) distribution in hepatic cells upon exposure, evaluate the concentration of Ag(I) ion released from AgNPs in cells and its dependence on the nanoparticle coating, and highlight eventual variations of intracellular Cu concentration induced by the NPs.
In the original proposal, we planned to use µXAS in order to retrieve the chemical environment of Ag(I) released from AgNPs and recombined with biomolecules; however, since the µXAS setup was not fully operational at the time of the experiment, this part of the project was not tackled; nevertheless, a careful and exhaustive µXRF study was carried out.

Experimental details:
µXRF was performed with 29.6 keV pink beam. The beam was focused to 60x70 nm (VxH) with the KB mirrors; the flux on the sample was ~$2 \cdot 10^{11}$ ph/s. Two SDD detectors were contemporarely used to detect the emitted fluorescence, a 3-elements and a Vortex single-element. The general experiment strategy was the following: first full cells (~30 µm lateral size) were scanned with 250x250 nm^2 step size and 1 s/point dwell time in order to acquire

images of single cells and allow the identification of cellular compartments (nuclei vs cytoplasm); the dwell time was chosen in order to optimize the detection of basal metals (Zn and Cu), whose concentration in cells is low (~1 µM). Then, on selected areas of interest high-resolution maps (70x70 nm^2 step size) were acquired with an integration time of 300 ms/pt, in order to visualize the size of nanoparticles in cellulo.
Finally, the incoming beam energy was tuned to 17.5 keV (pink beam) and samples were scanned in order to obtain a better detection of basal Zn and Cu; in this configuration Ag was not directly detected because its L-emission lines overlap to the K-emission of the Ar present in the air; however, Ag distribution will be retrieved through data analysis thanks to the deconvolution of fluorescence emission lines performed with PyMca.
Samples were HepG2 hepatocytes grown on Si_3N_4 windows and exposed to 100 µM AgNPs for 6 or 24 hours, or to highly-toxic silver salt ($AgNO_3$) for 30 minutes; after exposure cells were rinced and fixed with methanol. AgNP were either 100 nm size PVP-coated NPs, or 20 nm size citrate-coated NPs. Three cells per exposure condition were analyzed, in order to ensure the reproducibility of the results.
A reference AXO thin film and a bovine liver NIST standard were also measured for quantification of XRF maps.

Results:
High-quality µXRF maps could be acquired, and data treatment aimed at elemental quantification based on comparison with standard compounds is currently under development.
Figure 1 shows the general experimental strategy used: select isolated cells (Fig.1a), acquire low spatial resolution images on a whole cell (Fig.1b), zoom on areas of interest and acquire high-resolution maps (Fig.1c). The most remarkable result is that we were able to visualize the distribution of Ag(I) released from AgNPs *in cellulo*: maps in Figure 1b,c show that silver is present in exposed cells both as highly concentrated isolated spots (i.e. NPs), and as a low-concentration background (ionic Ag(I)).
This effect is clearly visible when cells are exposed for 24 hours to citrate-coated AgNPs (Figure 1), but it is much weaker when they are exposed for 24 h to the same dose of PVP-coated NPs. Figure 2a shows a single cell exposed for 24 h to PVP-coated AgNPs and Fig.2b the colocalisation of Ag (green) with basal Zn (red, that gives the cell's shape): Ag is found mainly in form of highly concentrated aggregates (NPs) in the perinuclear region; however, the logarithmic scale visualisation of Ag distribution (Fig.2c) highlights that a low-concentration Ag background is present throughout the cell, suggesting that Ag(I) release occurs as well for PVP-coated NPs, but to a lower extent with respect to citrate-coated NPs. These effects are even weaker, but still existing, when the exposure time is 6 h.
Non-exposed control cells were also measured and no Ag was detected; the evaluation of the background signal in the energy region of Ag fluorescence emission in the maps of non-exposed cells allowed the estimation of the detection limit for Ag: the estimated value is 0.01 $µg/cm^2$ at the excitation energy of 29.6 keV.

These results have been correlated to the cytotoxicity of AgNPs measured in our home laboratory, and to the dissolution of AgNP assessed *in vitro*; it all is the subject of a publication in preparation.

Figure 1

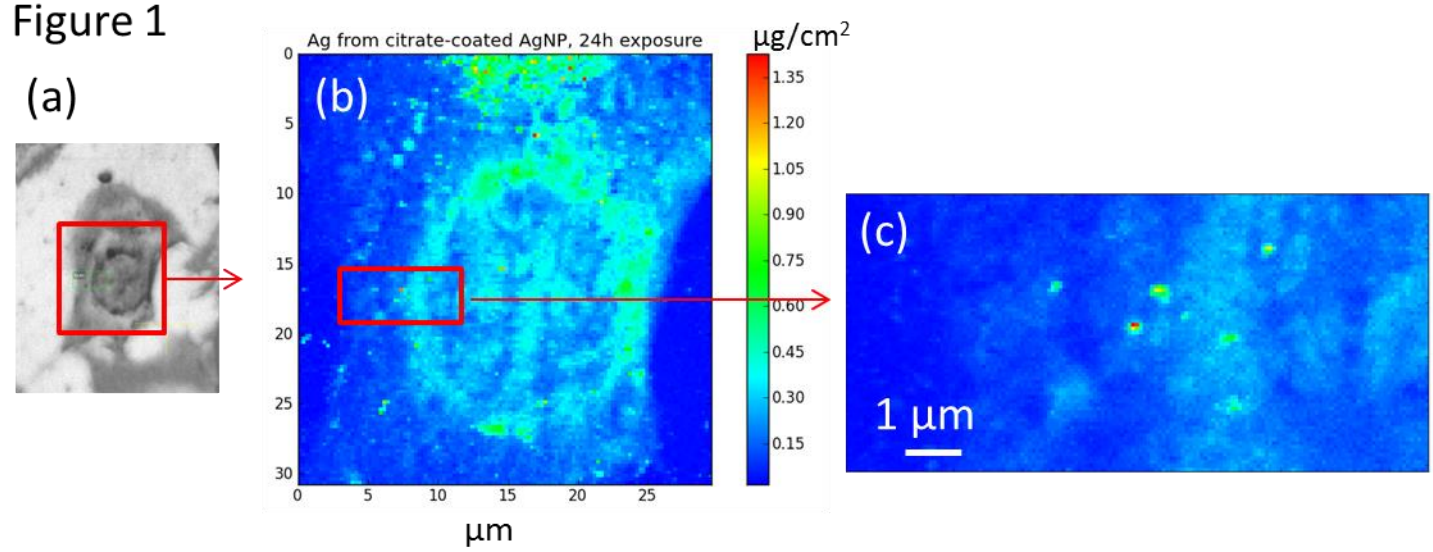

Figure 2

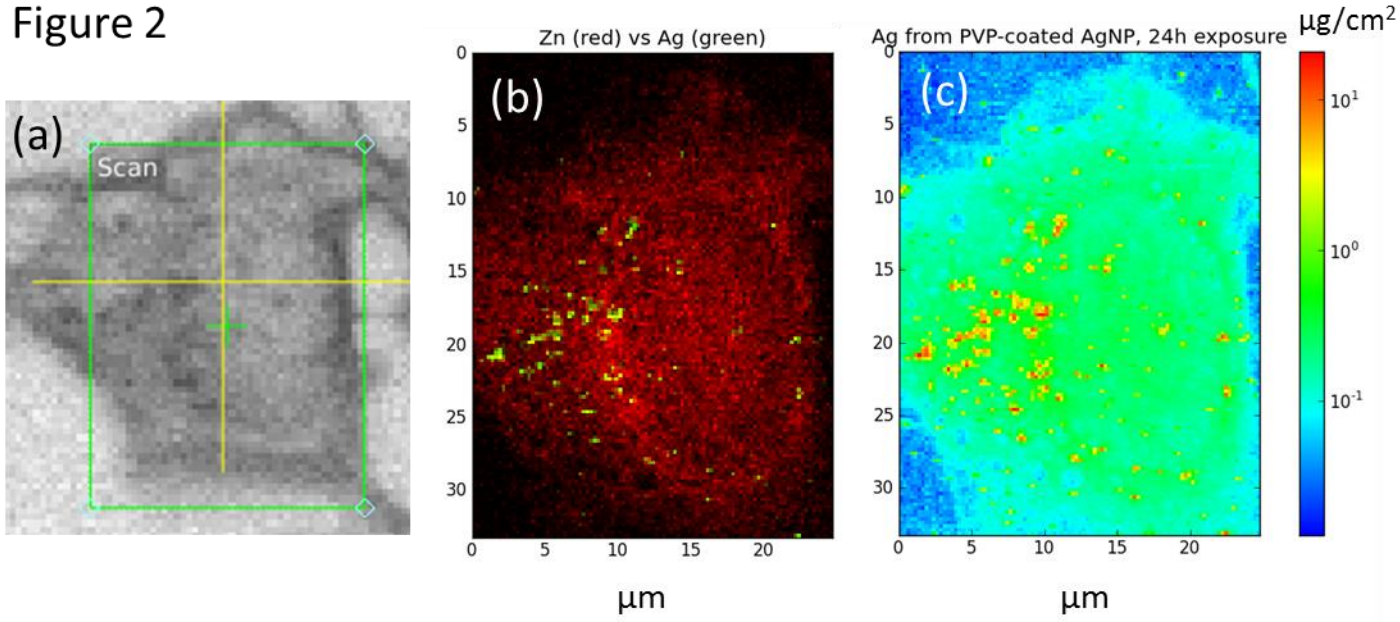

Figure 1. Optical image of a HepG2 cell (a) exposed for 24h to 100 µM citrate-coated AgNPs; quantitative distribution of Ag in the cell area (b), extracted from a µXRF map acquired with 250x250 nm^2 step size; Ag distribution (c) in a selected region of the cell, extracted from a high resolution (70x70 nm^2) µXRF map.
Figure 2. Optical image of a HepG2 cell (a) exposed for 24h to 100 µM PVP-coated AgNPs; co-localization of Ag (green) and Zn (red) in the cell area (b); quantitative distribution of Ag (log scale) in the whole cell extracted from a µXRF map acquired with 250x250 nm^2 step size.

Morover, we recently investigated by XAFS spectroscopy the dissolution of AgNPs in macrophages and hepatocytes, and developed an analysis protocol to determine the complexation of the released Ag(I) ions in cellulo[1]; these experiments were performed on cellular pellets (experiment LS-2331 on FAME-BM30B), without spatial resolution. Further studies on the local speciation of Ag(I) in a single cell with a µXAFS approach would be highly beneficial.

References:

1. Veronesi et al., *Exposure-dependent Ag^+ release from silver nanoparticles and its complexation in AgS_2 sites in primary murine macrophages*. Nanoscale (2015), DOI: 10.1039/C5NR00353A