ESRF	**Experiment title:** Silver nanoparticle fate in 3D-hepatocyte culture mimicking liver architecture and impact on metal homeostasis	**Experiment number**: LS2710
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Report

The aim of the study was to analyze silver nanoparticles (AgNPs) fate in a model mimicking liver structure and function: hepatocyte spheroids. This system possesses intercellular contacts and communications including bile canaliculi that are required for the excretion of xenobiotics out of the body. Our objectives were thus to use this realistic model to observe the putative excretion of AgNP and/or Ag(I) species through canaliculi. In the initial proposal, we also planned to analyze Cu, Fe and Zn distribution in these spheroids following long-term exposure to AgNPs. However, since this would have required a change in the setup (move from an excitation energy of 29.6 keV optimized for Ag to 17 keV for Fe, Cu, Zn), we limited our analysis to Ag subcellular distribution.

Methods:

Experiment was carried out in 7/8 + 1 filling mode, using an excitation energy of 29.6KeV in pink-beam mode. The beam was focused to 66nm(v)x58nm(h) with KB-mirrors, providing a photon flux of ~1011 photons/sec. The XRF signal from the sample was recorded with two 3-element SDD detectors.

The acquisition strategy was the following: spheroids were first rapidly scanned at low resolution (1x1 µm2 steps, 50 ms dwell time) and then regions of interest were scanned with 100x100 nm2 step size and 500 ms/point dwell time in order to acquire images including different compartment of the cells with intercellular spaces (bile canaliculi for instance).

Samples were spheroids made of HepG2-C3A hepatocytes repeatedly exposed to citrate- or PVP-coated AgNPs or Ag(I) ions at non-toxic concentrations for 2 to 7 days or exposed for 4 days and then unexposed for 3 days. After the chosen exposure time, spheroids were frozen under high pressure, stained with Os and included into plastic resins using a protocol preserving the ionic content of the cell. Cell sections of 200 nm thicknesses were then deposited onto Si3N4 support before XRF analysis. At least three areas from 2 or 3 different spheroids per condition were mapped in order to strengthen our conclusions. A reference material consisting of atomic layers of elements in known concentration sputtered over a Si3N4 window (from AXO) was measured, and used to extract the detector parameters for quantitative XRF analysis.

Results:

High-quality XRF maps have been acquired. The combination of Os staining and small thickness of the sections provide a very accurate visualization of the cells and of the subcellular and intercellular compartments (**Figure 1A**). As previously observed on 2D cell cultures, AgNPs were only observed in

vesicles. They are present throughout the whole volume of the spheroid (~ 200 µm diameter), including inner and outer cell layers. This observation opens the question of the diffusion and/or transfer process of AgNPs towards inner areas of the spheroid.

The data are still under process and the accurate determination of Ag areal densities in the different maps is not yet completed. However, it is already possible to visualize a low intensity Ag signal, previously assigned to Ag(I) complexes [1], everywhere in the cells. This signal increases with exposure time and is very low following 3 days of excretion. These results are similar with both PVP- and citrate-coated AgNPs. Interestingly, the transformation processes, at least in terms of kinetics, are very different between PVP and citrate AgNPs. Indeed, we did not catch an event of Ag excretion in bile canaliculi with citrate AgNPs in the different conditions we tested, while we did observe Ag hot spots in canaliculi with PVP AgNPs (**Figure 1B**). The intensity of the signal is lower compared to individual AgNPs but higher than the diffuse signal usually observed for Ag(I) species released in the cytosol. Altogether, this is **the first visualization of the excretion of Ag species out of cells**. As a control, we also assessed the fate of Ag ions following the exposure of spheroids to AgNO3. From these conditions, we observed two very interesting phenomena: first, within cells Ag is not only observed as diffuse signal throughout the cell corresponding to soluble ion bound to biomolecules, but also as intense signal. We hypothesize that this corresponds to Ag precipitates formed inside cells, as previously observed in organs from rodents orally exposed to Ag salt. Second, intense Ag signal is also localized in bile canaliculi proving Ag excretion though them.

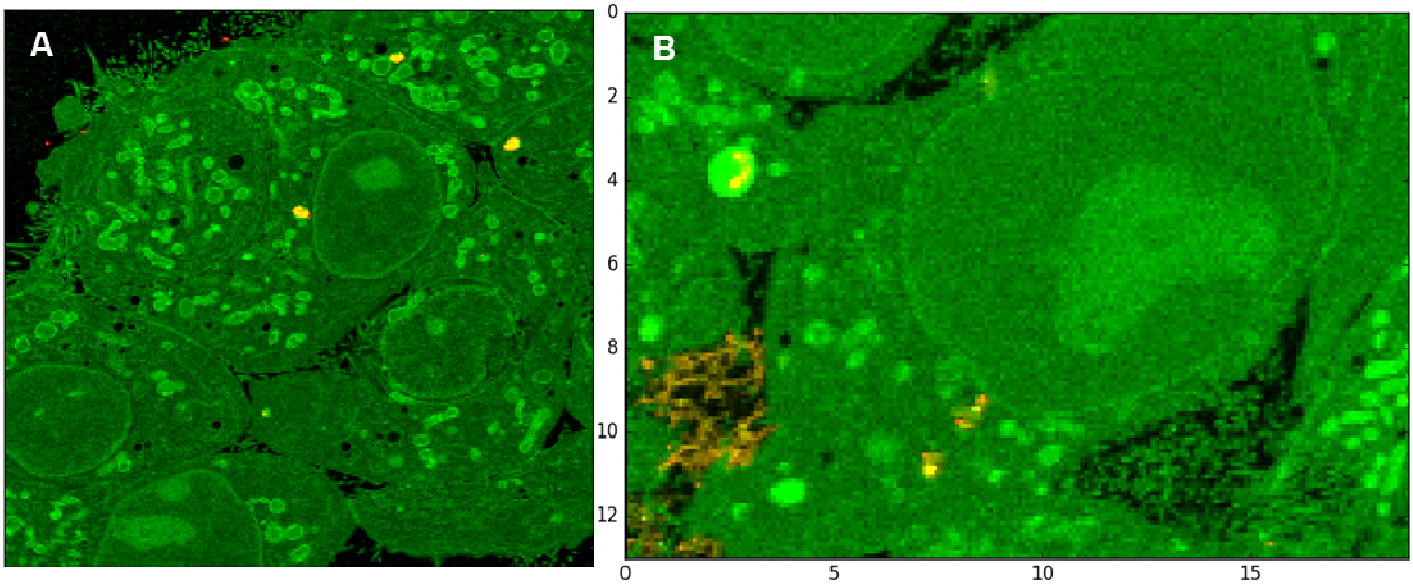

Figure 1: A) *Spheroid section exposed to 25 µM citrate-coated AgNPs for 2 days.* ***B)*** *Spheroid section exposed to 50 µM PVP-coated AgNPs for 7 days. Bile canaliculi with intense Ag signal (red) is observed of the bottom left side of the image. Os is in green and Ag is in red.*

These observations are very interesting and pave the way towards the understanding of the excretion mechanisms set up by hepatocytes to excrete Ag out of the body. Several questions emerge from this first study. How can we explain the differences between citrate- and PVP-coated AgNPs? How are Ag(I) ions from Ag salt exposure transformed into particulate, and what is the speciation of Ag in these particles? What is the speciation of Ag in bile canaliculi and what is the exact molecular process enabling this excretion? These question need to be further explored by a combination of cellular and molecular biology experiments and speciation analysis, i.e. using nanoXANES.

[1] Veronesi G., Deniaud A. Nanoscale (2016)