ESRF	**Experiment title:** Understanding the chemical transformations enabling Ag liver excretion upon exposure to non-toxic concentrations of silver nanoparticles	**Experiment number**: LS2858
Beamline: ID16B	**Date of experiment**: **from:** 18/10/2018 (8 a.m.) **to:** 22/10/2018 (8 a.m.)	**Date of report**: 21/02/2020
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Report

Over the last years, we deciphered the hepatic fate of silver nanoparticles (AgNPs) in 2D cell culture **[1, 2]** and more recently using 3D cell culture. The latter mimicks liver structure and function including bile canaliculi that are required for the excretion of xenobiotics out of the body. Our latest experiment (LS2710) enabled to visualize for the first time the excretion of Ag species within bile canaliculi. However, the molecular processes involved in this mechanism and the speciation of Ag in the different compartment remained unknown. The aim of the study was to disclose Ag speciation in intracellular Ag hot spots and in bile canaliculi in the different exposure conditions and assess the role of the main canalicular excretion pathways, such as drug transporters or Cu transporter, for Ag excretion. In the proposal, we proposed to combine nanoXRF on cells with various inhibitors to try to block Ag excretion and nanoXANES to analyze Ag speciation in specific cellular and intercellular compartments.

Methods:

The experiment was carried out in 7/8 + 1 filling mode, with a monochromatic beam. The beam was focused to ~60nm(v)x60nm(h) with KB-mirrors, providing a photon flux of ~10^9 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) in order to identify areas of high intensity in silver corresponding to vesicles with NP upon transformation and bile canaliculi loaded with silver species. Regions of interest were scanned with 100x100 nm^2 or 200x200 nm^2 step size and 300 ms/point dwell time. On these regions, high resolution maps (50x50 nm2 step size, 200 ms/pt) were acquired on Ag-rich spots, in order to select the spots for nanoXANES. For fluoXAS experiments, fluorescence maps were acquired in areas of ~ 1x1 μm^2 or less, with 100x100 nm^2 resolution, in 135 energy points over the XANES range 25.45 – 25.70 eV. 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 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 epon resins using a protocol preserving the ionic content of the cell. Cell sections of 400 nm thicknesses were then deposited onto Si_3N_4 support before XRF analysis. A reference material consisting of atomic layers of elements in known concentration sputtered over a Si_3N_4 window (from AXO) was measured, and used to extract the detector parameters for quantitative XRF analysis. Standards corresponding to AgNPs, Ag(I)-GSH, $AgNO_3$, and Ag_2S were also measured.

Results:

NanoXRF maps enabled to perform quantification of silver in the cytosol in different conditions. The blockage of three pathways involved in canalicular excretion showed that Mrp and MDR membrane transporters are not involved in the translocation of Ag species into bile canaliculi. On the contrary, the deletion of the protein ATP7B led to a two- to three-fold increase of cytosolic Ag, meaning that Ag is mainly excreted as Ag(I) ions by this Cu(I)-transporter.

To investigate the canalicular speciation and go beyond in the analysis of Ag fate in hepatocyte spheroids, nanoXANES was used. We performed several tests to define the optimum way to acquire Ag K-edge XANES spectra in the silver-containing regions identified by nano-XRF, in particular in the canaliculi, i.e. where the excretion takes place. Ag was detected in ~ 500 nm areas in a strongly inhomogeneous distribution from one pixel to another. Indeed, single-point XANES provided very distorted spectra, as a consequence of inhomogeneity and of the beam drift in the energy range of the XANES scan. Applying a correction (pb2) to compensate for the beam drift induced a clear improvement in the data quality. The correction was more effective in step-by-step XANES than in zap scans. However, step-by-step XANES required a longer irradiation of a single spot and induced radiation damage. The best signal-to-noise ratio and spectral features, with no damage, were obtained with the fluoXAS mode. FluoXAS maps acquired at different energies were aligned to compensate for the beam drift over the energy range, and the XANES signal was extracted. The resulting signal showed a pronounced edge jump and a relatively featureless post-edge trend, regardless of the exposure condition (Figure 1). The comparison with the spectra of reference compounds suggests that AgNPs are not the main species found in cellulo, and that Ag-S species are predominant instead.

Ag_2S-like spectral features were encountered both in vesicles and canaliculi, suggesting that a transformation of AgNPs occurrs already in the former, well before excretion.

The investigation of 400 nm cell sections allowed us to interrogate NPs (or their degradation products) that had unumbiguously been internalized by cells. This experiment improved our knowledge about the local speciation of Ag released from AgNPs in a liver-like model. However, fluoXAS is a very time consuming technique, and only a few silver spots in hepatocytes could be probed. In order to add statistical significance to our results, we mean to probe the average speciation of Ag transformed from AgNPs in spheroids. XAS on frozen cell pellets can provide this information, as we previously demonstrated **[2, 3]**.

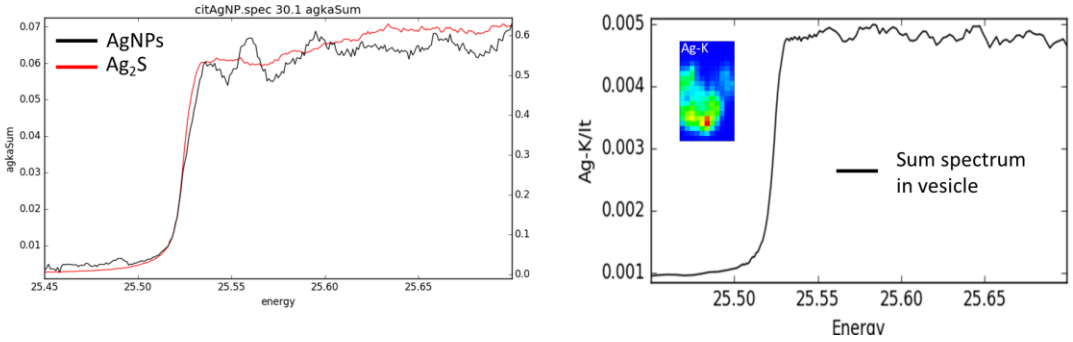

Figure 1. Left: XANES spectra of reference compounds, pristine NPs in colloidal suspension (black) and Ag_2S acanthite powder (red). Right: sum spectrum of fluoXAS acquisition in a silver-containing vesicle of 3D culture of hepatocytes. Inset: Ag distribution in the vesicle extracted from a single map of the fluoXAS stack.

[1] Veronesi, G.*, Deniaud, A.* *et al. Nanoscale*, **2016**, *8*, 17012-21. [2] Tardillo Suárez V. *et al. BioRχiv*, **2019**, 825919. [3] Veronesi G. *et al. Nanoscale*, **2015**, *7*, 7323-30.