ESRF	**Experiment title:** INTERACTION OF BIOLOGICAL SULPHUR LIGANDS WITH SILVER NANOPARTICLES AND RELATION WITH THEIR TOXICITY	**Experiment number**: LS-2331
Beamline:	**Date of experiment**: from: 09/07/2014 to: 15/07/2014	**Date of report**: 30/08/2014
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Report:

In order to bring insight into the mechanisms that rule the toxicity of silver nanoparticles (Ag-NP) to humans, we undertook this Ag K-edge XAS study. Our strategy consisted in the investigation of:
1) The **complexes that Ag^+ forms with biological ligands in solution**, especially in relation to its competition with Cu^+ binding.
2) The **release of Ag^+ from Ag-NP in exposed cells and its recombination *in cellulo***.

Experimental details:
Samples were prepared in a glove box in our home laboratory and measured at ~16 K in the He-cryostat of FAME-BM30B. Ag concentration in the solutions was low (down to 1 mM, i.e. ~100 ppm), however, thanks to the high photon flux provided in the 200 mA current mode and to the sensitivity of the 30-elements Ge detector, high quality EXAFS spectra could be acquired in fluorescence mode. All spectra were acquired in the 25.20-26.25 keV energy range (up to k=14 $Å^{-1}$), the total integration time for each sample was chosen in order to provide 10^6 fluorescence counts above the absorption edge. A 3µm Ru filter was used to reduce the elastic scattering reaching the detector.

Results:

Ag^+ complexation with biological ligands was first studied by incubating the metal with biomimetic compounds synthesised in our home institute: **P3**, a peptide providing two metal-binding Cys side chains, and **L1**, a tripode presenting three converging metal-binding Cys. These ligands were designed by mimicking the Cu-binding motifs of proteins involved in Cu homeostasis, such as metallothioneins (MT) and metallochaperones [1, 2]; they were proven to efficiently bind also Ag^+, as expected considering the similar electronic configurations of Cu^+ and Ag^+. Their affinity for Ag^+ binding might therefore suggest a mechanism of Ag-NP toxicity based on the disruption of Cu homeostasis.

Our UV/VIS and circular dichroism experiments proved that the two chelators form compounds with Ag^+ up to stoichiometries of [Ag]:[**P3**]=1.5:1 and [Ag]:[**L1**]=2:1. The EXAFS data were collected on four samples characterized by different Ag^+/ligand ratios: Ag^+/**P3**=1, 1.5 and Ag^+/**L1**=1, 2. Ag^+ concentration in sub-stoichiometric samples was below the detection limit. The data reveal that, for Ag^+/ligand=1, the Ag^+ binding sites in the Ag**P3** and Ag**L1** complexes present only S ligands, in number of 2.2±0.2 and 3.0±0.3 respectively. The AgS_2 site in Ag**P3** is compatible with a digonal planar geometry with an Ag-S average distance of 2.477 (7) Å; the presence of an Ag atom at 2.99 (3) Å from the absorber was revealed (Fig.1 (A) and (B)). The complex Ag-glutathion (GSH) showed a similar XAS spectrum and the same ligation pattern, confirming the pertinence of **P3** as a model for the complexation of Ag with biomolecules. We are currently comparing our 1st shell results with a survey of Ag^+-thiolate complexes encountered in the CSD [3], in order to identify the putative models to fit the full EXAFS spectrum.

The AgS_3 site in the Ag/**L1**=1 sample presents a trigonal planar geometry, with an average Ag-S distance of 2.489 (7) Å and no Ag-Ag interaction in the radius probed by EXAFS (Fig.2 (A) and (B)); this is compatible with the formation of a S-bridged network of AgS_3 sites. Unexpectedly, in the Ag/**L1**=2 sample the Ag coordination sphere is unchanged and no Ag-Ag interaction is detected, contrary to what we found in Cu**L1** complexes [4].

Two Ag-MT complexes were as well studied by Ag K-edge XAS, revealing a number of S ligands of 2.5±0.2; this value suggests that the MT metal-binding site is populated with a cluster where Ag^+ shows both digonal AgS_2 and trigonal AgS_3 coordination.

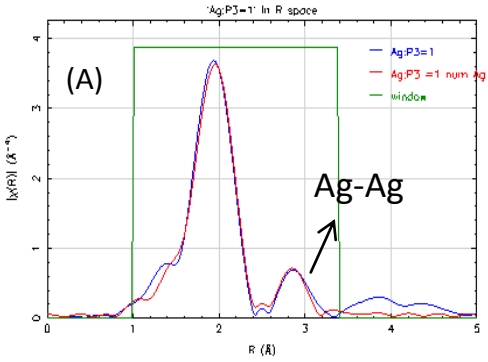
The **release of Ag^+ from Ag-NP and its complexation *in cellulo*** was investigated in two cellular models: primary murine macrophages and HepG2 hepatocytes cell line.

Cells were exposed to sublethal doses of Ag-NP for 6- to 24-hours, washed and then frozen in liquid N_2 and analyzed by Ag K-edge XAS at 16 K. In both cell lines, the features of the XANES spectra are progressively smoothed with increasing the exposure time to NP (Figure 3 (A)-macrophages and (B)-hepatocytes); the XANES and EXAFS spectra were fitted as linear combinations of model compounds, in order to discriminate the fraction of intracellular dissolved and recombined Ag^+ ions. As reference compounds we used the Ag-thiols complexes described in the previous section, as well as AgCl and $AgNO_3$ (prepared in pellet from commercial powders). Our data suggest the preferential recombination of Ag with SH groups *in cellulo*.

A more advanced analysis of the EXAFS region of the spectra is currently in progress, aimed at discriminating the average coordination geometry of Ag^+ in the AgS_x complexes formed.

Interestingly, the same effect was encountered in the two cellular models, with a slightly faster kinetics in macrophages than in hepatocytes; the effect of the NP coating was also

investigated (only in hepatocytes): Ag^+ release and recombination were more prominent in citrate- than in PVP-coated Ag-NP.



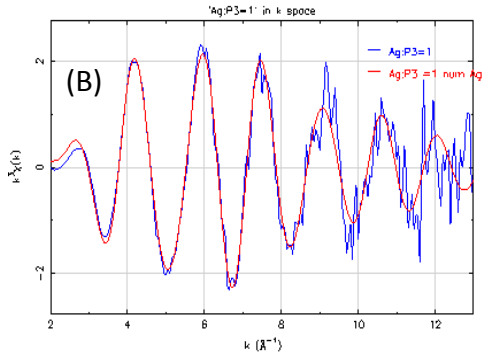
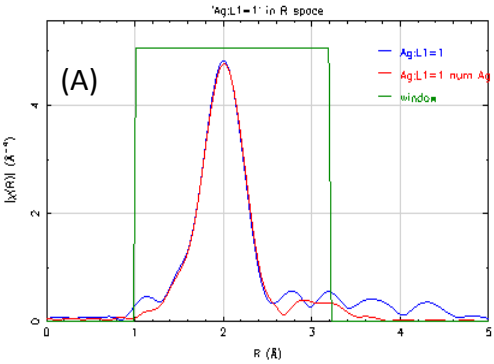


Figure 1: (A) Fourier-Transformed Ag K-edge EXAFS signal of the Ag**P3** complex (blue) and its R-space fit based on the AgS_2 digonal planar model. (B) EXAFS signal (blue) and its best-fitting curve (red) in the k-space.



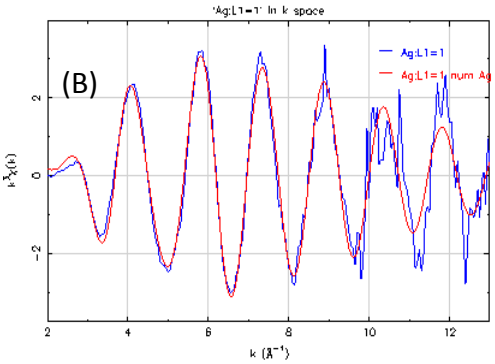
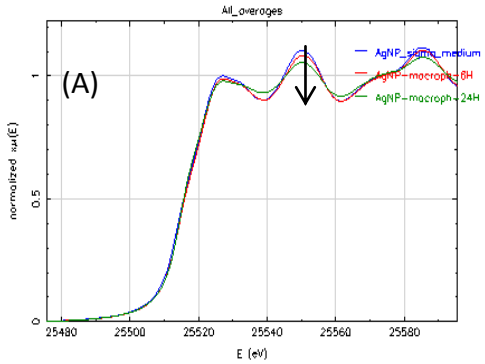


Figure 2: (A) Fourier-Transformed Ag K-edge EXAFS signal of the Ag**L1** complex (blue) and its R-space fit based on the AgS_3 trigonal planar model. (B) EXAFS signal (blue) and its best-fitting curve (red) in the k-space.



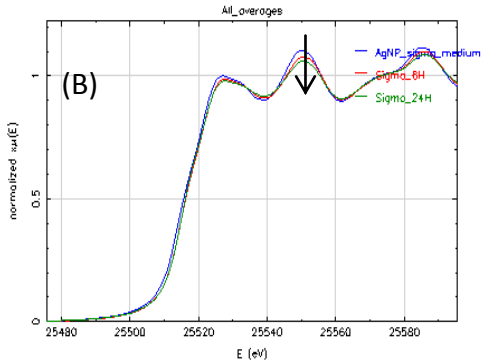


Figure 3: Ag K-edge XANES spectra of raw Ag-NP (commercial product from Sigma, blue line) and cellular models exposed to Ag-NP for 6 hours (red) and 24 hours (green). Cell lines were either macrophages (A) or hepatocytes (B).

<u>Perspectives:</u>

The allocated beamtime was fully exploited, and the experiment provided satisfactory results that will be the subject of two publications (one in preparation). The experimental protocol was validated, and the results open the possibility to new investigations, like:

1) Interaction between **Ag-NP and biological ligands** (biomimetic compounds and other Cu homeostasis proteins like the chaperon Atox1).

2) Ag^+ release and recombination in cells (macrophages and hepatocytes) upon **chronic exposure** to Ag-NP.

3) **Dissolution and recombination kinetics** *in cellulo* (several exposure conditions between 6- and 24-hours) and **influence of the NP coating** on the kinetics.

<u>References:</u>

[1] Pujol, A. M. et al. *Journal of the American Chemical Society*, **2011**, *133*, 286-296.
[2] Pujol, A. M. et al. *Chemistry - A European Journal*, **2011**, *17*, 4418-4428.
[3] Leung, B. O. et al. *Inorganic Chemistry*, **2013**, *52*, 4593-4602.
[4] Jullien, A.-S. et al. *Inorganic Chemistry*, **2013**, *52*, 9954-9961.