	Experiment title: Antioxidative activity of Cerium Oxide nanoparticle in biological environments studied by XANES	**Experiment number**: CH-4218
Beamline: BM23	**Date of experiment**: from: 21/11/2014 to: 25/11/2014	**Date of report**: 06/03/2015
Shifts: 12	**Local contact(s)**: Giovanni Agostini	*Received at ESRF:*

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Report:

Background CeO_2 nanoparticles are at the moment actively investigated as promising agents in the therapy of different pathogenesis involving free radicals or oxidative stress, like Alzheimer and Parkinson. Although the antioxidant property of Ceria nanoparticles (CNP) has been largely demonstrated, the exact mechanism that makes CNP such a powerful tool is not completely elucidated yet. It is not clear, for example, which is the evolution of the oxidative state of the CNP during the process of cell internalization; how it is influenced by the initial Ce(III)/Ce(IV) ratio; how the presence of a protein corona and surface functionalization can modify the response of CNP as free radical scavenger.
Aim To address some of these questions and further elucidate the possible mechanism involved, we planned to investigate the Ce(III)/Ce(IV) ratio, on the surface of cell internalized CNP using XANES spectroscopy at the Ce-L_{III} edge.

Experimental description We used human epithelial cultured cells (HeLa) incubated with CNP with a diameter of 5-10 nm, which allowed a reasonable ratio of surface sites/bulk sites and therefore a better contrast between (surface) Ce(III) and (bulk) Ce(IV). Prior to cell incubation, the CNP were exposed to both highly oxidative and highly reducing environment in order to modify significantly the Ce(III)/Ce(IV) ratio on their surface. After 24 h of incubation with the CNPs, cells were recovered and the Ce oxidation state of the internalized CNPs analyzed. In order to investigate whether a specific oxidation state correlates with specific intracellular localization, we fractionated the cells previously incubated with CNP into endosomes/lysosomes and nuclei. All the biological samples, either the intact cells or the cell fractions, were immediately quenched

to liquid nitrogen temperature to prevent modification in the oxidation state of CNP during storage and transport to the ESRF. The XANES samples analyses were performed at 10 K. Due to the low amount of Ce present into the cells, the spectra have been acquired in fluorescence mode.

Results The XANES analysis of the CNP exposed only to the cell culture medium showed that the fully oxidized CeO_2 (X6, Figure 1) remained fully oxidized, while the partially reduced CNP (X10, Figure 1) showed the presence of a quite considerable amount of Ce(III), *in addition to Ce(IV)*. By fitting the edge structure of sample X10 with a linear combination of the spectra of CeO_2 and $Ce(NO_3)_3$ an amount of Ce(III) equal to ca. 20 % (atomic percentage) was estimated. Once established the Ce oxidation state of the CNP just before entering the cells, we then investigated whether the process of internalization modified the initial Ce(III)/Ce(IV) ratio. By analyzing the internalized CNP for the two types of NP incubated with the cells (4Cbis, cell incubated with fully oxidized CNP and 13C, cell incubated with partially reduced CNP), we observed in both cases a significant amount of Ce(III), regardless the initial Ce oxidation state. This result suggested that cells can "process" CNP favouring the formation of Ce(III). To further investigate whether lysosomes/endosomes, the subcellular structure where in general NP accumulate within the cell, we analysed the subcellular fraction (endo-lysosome and nuclear). For the endo-lysosome samples obtained from cells previously incubated with fully oxidised (X6) and the partially reduced (X10) CNP, we observed a significant amount of Ce(III) in the case of cells incubated with X10 (4OR) and a less pronounced amount for cells incubated with X6 (13OR). Figure 2 shows the normalised spectra of 4Cbis, 4OR, 13 C, and 13OR, along with the spectra of CeO_2 and $Ce(NO_3)_3$. This suggests that the surface of the CNP accumulated inside these compartments are actively modified producing an increase of Ce(III) compared to the CNP originally incubated with the cells. For the nuclear fraction, we could not detect any signal due to either the extremely low level of CNP inside the nuclei and the high background coming from apparatus. In fact, the Ce background coming from beamline and cryostat, excited by scattered photons, was not negligible (Figure 3, sample (4CT_1) vs background), causing the presence of a spurious signal that prevents drawing sensible conclusions for the most diluted samples. The combination of low signals and the presence of a not-negligible background, prevented us from performing analysis on samples of cells incubated with CNP for shorter times than 24 h (e.i.15 min, 1 h, and 6 h) either in the case of whole cells-measurements or subcellular fractions, as proposed in the relative proposal CH-4218.

Conclusion By "feeding" cells with NP characterized by a known Ce(III)/Ce(IV) ratio, we could detect an intracellular transformation showing an increased level of Ce(III).This provided the first direct evidence the Ce oxidative states can be altered intra-cellularly by an active cell-mediated process. However, the presence of spurious Ce signal coming from beamline and sample environment prevented to exploit a full systematic investigation.

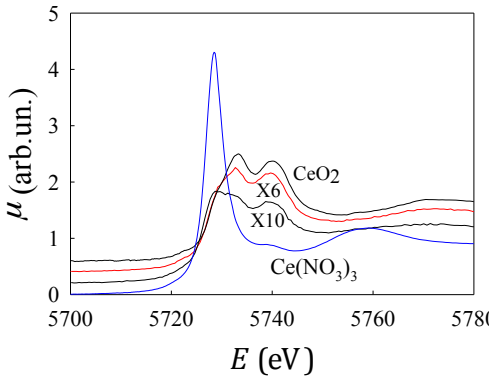

Fig.1

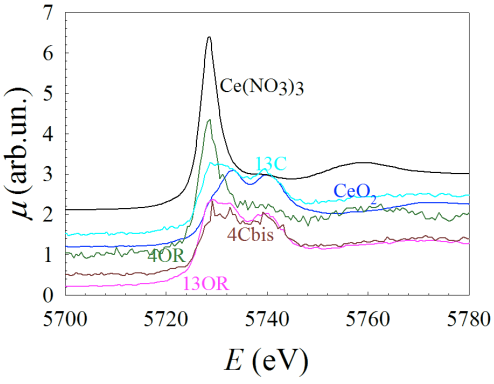

Fig. 2

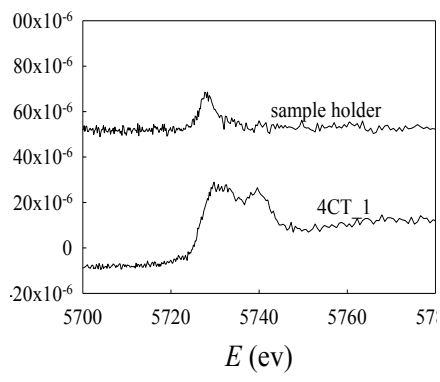

Fig. 3