	Experiment title: Investigation of cerium oxide bioactivity by high-energy-resolution X-ray spectroscopy	**Experiment number:** MD 1213
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Shifts: 18	**Local contact(s): Kristina Kvashnina, BM20, ESRF**	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

*Kristina Kvashnina (ESRF, Grenoble)

*Tatiana Plakhova, Anna Romanchuk, Stepan Kalmykov (Lomonosov Moscow State University)

*Vladimir Ivanov (Kurnakov Institute of General and Inorganic Chemistry)

Report:

Nanoceria is receiving much attention as a potential antioxidant agent in vivo. Initial biological studies have highlighted cell protective, neuro-protective cardio-protective effects, improvement of stem cell adhesion suggesting potential use in tissue engineering, and anti-inflammatory properties of nanoceria. Despite the described beneficial effects of CeO_2 NPs in many medical conditions, the in vivo mechanisms are not yet totally elucidated and they are difficult to clarify via biological experiments. There is a hypothesis that the main factor affecting the biological activity of CeO_2 is the presence of Ce(III) in the structure. However, with the development of modern non-vacuum techniques of solids investigation, this hypothesis is subject to discussion. HERFD is currently the most informative method for studying cerium oxidation state. In our previous study, we investigated CeO_2 NPs after the synthesis procedure by the HERFD method [Plakhova et al., Nanoscale 2019]. Ce(III) in the structure was not indicated regardless of NPs size and thermal treatment conditions. Herein, we performed HERFD experiments on nanoceria samples (2, 5 and 8 nm) obtained by the same synthesis route, but after interaction with solutions of biological meaning: phosphate buffer (PBS), adenosine triphosphate (ATP), simulated lung fluid, cell culture media (CCM) and fetal bovine serum (FBS). We also investigated ceria stable colloid before and after interaction with fibroblasts cells.

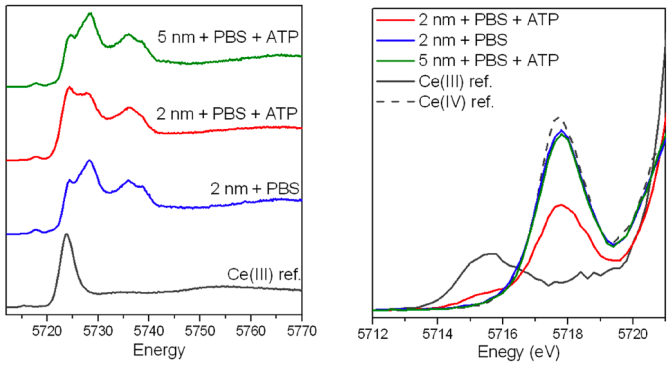

Fig.1. (a) HERFD data near Ce L_3 edge for 2 and 5 nm samples after interaction with PBS or PBS+ATP media; (b) enlarged pre-edge region of the HERFD spectra.

1. CeO_2 interaction with PBS and ATP

Phosphorylated molecules are ubiquitous in a biological system, participate in protein regulation and energy transfer, and are an essential part of DNA structure. After storing 2 nm CeO_2 NPs in PBS for two weeks (Fig.1), there were no changes in the spectrum compared to CeO_2 before interaction [Plakhova et al., Nanoscale 2019]. However, when an ATP is added to PBS, Ce(III) is generated the sample at the same interaction time. The signal attributed to Ce(III) is observed in the enlarged pre-edge area and the main spectra edge. At the same time, no trivalent cerium in the CeO_2 5 nm + PBS + ATP system. This indicates that no redox reaction occurs in this system. It could be because 5 nm NPs are less reactive towards ATP molecules due to less surface area.

2. CeO_2 interaction with simulated lung fluids

CeO_2 NPs of 2 and 5 nm were stored in multicomponent media, which are models of lung fluids: simulated lung fluid (SLF) and Gamble's solution. Analysis of the HERFD spectra from CeO_2 2 nm sample reveals the partial reduction of the initial Ce(IV) to Ce(III) in case of SLF and Gamble media (Fig. 2). In Gamble's solution, the Ce(III) generation process is faster, probably, due to the presence of glycine - the reduction agent - in the Gamble solution. In a sample with an initial particle size of 5 nm, the maximum from Ce(III) in the absorption pre-edge region is hardly distinguished. This indicates slower kinetics of interaction of 5 nm NPs in comparison to 2 nm with components of simulated lung solution.

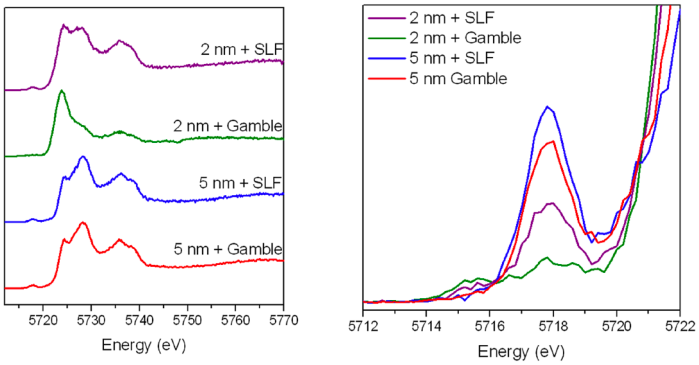

Fig.2. (a) HERFD data near Ce L_3 edge for 2 and 5 nm CeO_2 samples after storing in Gamble's solution and SLF; (c) enlarged pre-edge region of the HERFD spectra.

3. Interaction with fetal bovine serum in cell culture media

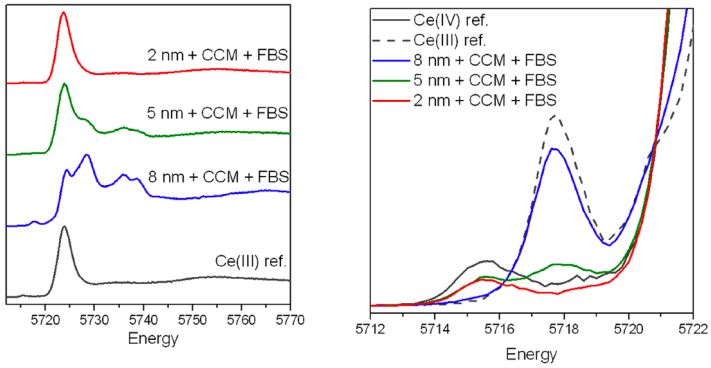

Fig.3. (a) HERFD data near Ce L_3 edge for CeO_2 of different sizes after interaction with CCM+ FBS media; (b) enlarged pre-edge region of the HERFD spectra.

According to HERFD, after storage of a 2 nm CeO_2 sample in CCM+PBS medium, all Ce(IV) in sample is reduced to Ce(III) oxidation state. In a sample with an initial particle size of 5 nm, cerium is partially reduced to Ce(III) state. CeO_2 NPs with initial size 8 nm nanoparticles did not change after interaction with serum. Thus, the changes observed in systems depend on the particle size.

All samples after HERFD measurements were also analyzed by XRD, XFR and microscopy methods to understand the Ce(III) peak's origins in spectra. CeO_2 to cerium phosphate phase transformations after interaction with solutions of biological meaning was revealed. The structure, morphology and size of generated cerium phosphates depend on initial NPs size (surface-to-volume ratio) and the presence of reducing agents in the systems.

Ce L_3 edge spectra were also collected during in-situ reactions of CeO_2 NPs interaction with a solution of biological meaning in specially constructed cells. There were no significant changes in the cerium spectra during in-situ measurements conducted for 12 - 48 hours. Therefore, the process of redox-mediated phase transformation occurs in more than 48 hours.

4. Interaction with cells (mouse fibroblasts)

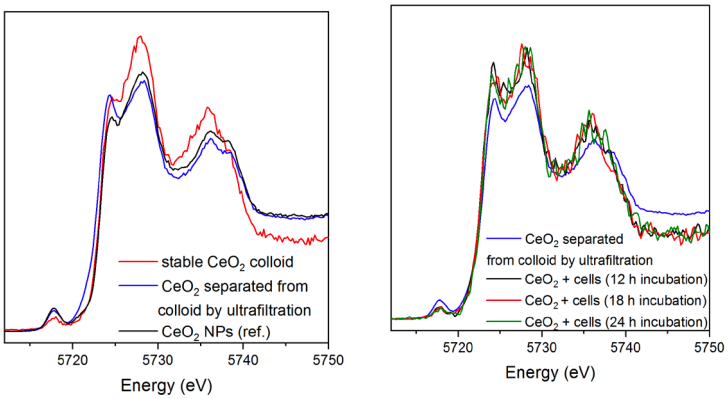

Fig.4. HERFD data near Ce L_3 edge for CeO_2 stable colloids (a) before and (b) after interaction with fibroblasts cells.

To study the antioxidant properties of CeO_2 in living cells, generally, the NPs additionally stabilize in solution by adding citrate-contained compound. However, at the moment, there is no data on citrate effect on the structure of CeO_2 particles and cerium oxidation state in such stable colloids. Within the framework of this project, a citrate-stabilized CeO_2 colloid was analyzed by HERFD. We measured CeO_2 colloidal solution and additionally separate solid from that. The solid phase was separated from the solution by ultrafiltration with 3kD filters. It is shown that the spectra of the colloid and the separated solid phase are different (Fig. 4a). However, The pre-edge structure for separated CeO_2 solid and colloid reveals solely the presence of the Ce(IV) oxidation state. After interaction with fibroblasts cells during the day, the formation of Ce(III) in the system is not observed (Fig. 4b).

Based on the presented data, at least three articles are being prepared for publication.