ESRF	**Experiment title:** *Nanoparticle translocation through digestive epithelium, consequences for cells, fate of nanoparticles*	**Experiment number**: MD588
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Shifts: 18	**Local contact(s)**: Giulia Veronesi	*Received at ESRF:*

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Introduction

Nanoparticles, particularly TiO_2-NP, are included in a growing number of commercial products, and among them it is used as additive in drugs and food. The first objective of this study was to determine if TiO_2-NP are able to be internalized in gastrointestinal cells and to cross a reconstituted gastrointestinal epithelium, which would be a proof of potential contamination of consumers. This was studied by µ-X-ray fluorescence (µ-XRF) mapping of Ti distribution in cells, cultivated on semi-permeable transwell membranes, and exposed for 6-48h to anatase or rutile TiO_2-NP, with diameters 12 and 20 nm, respectively. The second objective was to prove that TiO_2-NP did not dissolve and that their cristalline phase was not modified after cell uptake and transepithelial translocation. Local X-ray absorption spectroscopy analysis (µ-XAS) at the Ti K-edge was carried out on Ti-rich regions identified inside cells. This experiment was the following of experiment MD464), where this analytical strategy had been applied to Caco-2 enterocytes exposed to TiO2-NPs. In the present experiment, we addressed these questions on more representative cell models, i.e. the Caco-2/HT29-MTX coculture which is a good model of mucus-secreting epithelium that lines the intestine, and the Caco-2/RajiB coculture which is a model of follicle-associated epithelium (FAE), present in the most distal part of the ileum.

Experimental method

The Caco-2/HT29-MTX (Mahler *et al.*, 2009) and Caco-2/RajiB (Des Rieux et al., 2007) co-cultures were grown on porous transwell®-clear polyester membranes (Costar) until they reached late confluence, ensuring that epithelia were fully differenciated. They were then exposed for 6h, 12h, 24h or 48h, on their apical side, to 12 nm anatase TiO_2-NP (A12) or 20 nm rutile TiO_2-NP (R20). Cells were then fixed using a two step procedure (paraformaldehyde and osmium), dehydrated and embedded in epoxy resin. Transversal sections (thickness: 1 µm) of these samples were then cut using a ultramicrotome, deposited on ultralen polymer and covered by a thin layer of ultralen. These sections were directly analyzed (µXRF and µXAS). µXRF maps of Ti, K, Ca, Os/P and Cl were drawn, at 5.05 keV. XAS analyses were performed on cell regions containing high amounts of Ti; spectra were registered between 4900 and 5050 eV. Epoxy resin did not cause any interference in fluorescence signals.

Results

TiO_2-NP accumulated in both cell models, in much higher amounts in these two co-cultures than in the Caco-2 monoculture that we used for experiment MD464. Accumulation was time-dependent: cells exposed for 48h accumulated more TiO_2-NP than cells exposed for shorter periods. On the Caco-2/HT29-MTX model, we did not observe any translocation of TiO2-NPs through the epithelium, contrary to the Caco-2/RajiB epithelium, where some NPs were observed in the basolateral compartment. This proves that TiO_2-NP are able to cross a reconstituted FAE, whereas they are unable to cross a reconstituted regular ileum epithelium. Mucus secretion increases TiO_2-NP accumulation in gut cells, since NP accumulation

was much higher in the Caco-2/HT29-MTX model than in the Caco-2 monoculture model. This was confirmed by PIXE analysis, that enabled quantification of TiO_2-NP intracellular accumulation. Our results were also confirmed by transmission electron microscopy. This distribution of TiO_2-NP was observed independently of their crystal phase.
During experiment MD588, we also analyzed the translocation of TiO_2-NP through ex vivo-exposed mice gut epithelium. This experiment confirmed the results obtained in vitro, i.e. TiO_2-NP can cross the gut epithelium. These results are currently being published (article in revision in Particle and Fibre Toxicology).

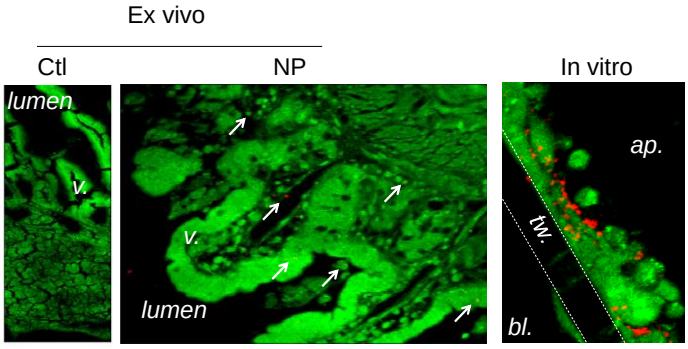

Figure 1. µXRF map of TiO2-NP distribution in a mice gut exposed ex vivo and in the Caco2/RajiB model of FAE. Biological matrix is identified by the distribution of Os (green), where intestinal villi (v.) are easy to recognize, enabling the identification of the lumen side of the epithelium. In vitro, the transwell membrane can be distinguished, unabling the identification of apical (ap.) and basolateral (bl.) poles of the epithelium. TiO2-NP distribution in the epithelium is mapped through the fluorescence signal of Ti (red, arrows).

XAS spectra were recorded in Ti-rich regions of cell sections, and compared to spectra of reference compounds, *e.g.* anatase and rutile TiO_2-NP which have not been exposed to cellular environment. As described in several publications, Ti pre-edge features are characteristic of the crystalline phase and size of TiO_2-NP. Anatase displays a typical triplet feature (A1, A3 and B peaks), with a weak shoulder on the low-energy side of the central A3 peak (A2) (Manzini *et al.*, 1995). The intensity of A2 peak is related to the distorsion of the octahedral TiO_6 unit, particularly the distorsion observed on the surface of nanoparticle. The intensity of this peak is thus related to the size of the particle: the smaller the nanoparticle, the higher this A2 peak (Luca *et al.*, 1998). Meanwhile, the intensity of the A1 peak also decreases as the particle size decreases (Chen *et al.*, 1997). In rutile XAS pre-edge feature, no A2 peak is observed, A3 peak is more intense while B peak is shifted to +1.3 eV as compared to B peak in anatase pre-edge. Analysis of this pre-edge region may then inform on changes in the crystalline phase of nanoparticles after cell internalization, and on their dissolution. In the present experiment, no modification of these pre-edge features was observed after cell internalization of anatase or rutile TiO_2-NP, meaning that Ti is still in the nanoparticulate TiO_2 chemical form inside cells, and that their crystalline phase did not change. Moreover the size of NP remained constant after cell internalization meaning that no partial dissolution of NP occurred. G. Veronesi further analyzed the EXAFS spectra of rutile TiO_2-NPs accumulated in the Caco-2/RajiB model, and showed that the local environement around Ti atoms was exactly the same in intracellularly stored TiO2-NPs than in control nanopowders. These results were published (Veronesi *et al.*, 2012).

Conclusions and perspectives

In conclusion we demonstrated in this experiment that raw, pure TiO_2-NP, either anatase or rutile, were translocated through an in vitro reconstituted FAE, but not through an in vitro model of regular ileum. TiO_2-NP did not dissolve, their crystal phase did not change in the intracellular environment upon sequestration for up to 48 h.
TiO2-NPs are not the unique NPs introduced in food. Now that we optimized the experimental procedure to prepare cells for µXRF/µXAS analysis, it would be interesting to use this acquired expertise to analyze the fate of other types of NPs that are also presnet in food products, such as silver NPs.

Bibliography

Chen *et al.* (1997) J Phys Chem 101, 10688 ; Luca *et al.* (1998) J Phys Chem 102, 10650 ; Manzini *et al.* (1995) Physica B 208&209, 607 ; Mahler *et al.* (2009) J Nutr Biochem 20: 494-502 ; Des Rieux *et al.* (2007) Eur J Pharma Sci 30: 380-391 ; Veronesi et al. (2012) Appl Phys Lett 100(21).