ESRF	**Experiment title:** Nanoparticle translocation through lung or digestive epithelium, consequences for cell homeostasis and fate of nanoparticles	**Experiment number**: MD464
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Shifts: 18	**Local contact(s)**: Barbara Fayard	*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 ranging from 12 to 140 nm. 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 performed on Ti-rich regions identified inside cells.

Experimental method

Caco-2 cells (ATCC HTB-37), a human colon tumor cell line, were used as a model for gastrointestinal epithelium, since they are known to display several characteristics of differentiated enterocytes in the small intestine (Alvarez-Hernandez *et al.* 1991). They were cultured 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 and 3 µm) of these samples were then cut using a ultramicrotome (collaboration D. Jaillard, Centre Commun de Microscopie Electronique d'Orsay), 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, with a spatial resolution of 0.6 x 0.6 µm², 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

Experimental setting

XRF analyses show that even if mapping of Ti on a 1 µm-thick section is achievable, the quality of the images is much higher when analysing 3 µm-thick sections. Transwell membranes could be identified because the whole sample section, except the transwell region, contains high amounts of Cl (probably contained in epoxy resin). Cells were identified by mapping Os/P distribution (fluorescence peaks of these two elements are partly superimposed). By drawing these two maps, it was thus possible to identify the apical pole (exposed to NP) and the basolateral pole (in the transwell region) of epithelia. Mapping of Ti distribution (Fig. 1) enabled to observe TiO_2-NP internalized and translocated in cells.

TiO_2-NP accumulation and translocation in Caco-2 epithelia

TiO_2-NP were accumulated in cells, although in low amounts. Accumulation was time-dependent: cells exposed for 48h has accumulated more TiO_2-NP than cells exposed for shorter periods. Only one or two cells out of 20 contained TiO_2-NP. When accumulated in cells, TiO_2-NP were most of the time sequestered in the upper regions of the epithelium, and rarely reached the basolateral pole of cells (Figure 1A). In these locations, TiO_2-NP seemed to be grouped into heterogeneous clusters of 1-2 µm. Interestingly, this distribution was observed whatever NP diameter, meaning that even large NP (140 nm) were uptaken by cells. This is of major importance since we previously demonstrated that large NP were less cytotoxic than very fine NP: uptake of NP is thus not always correlated with cytotoxicity. This distribution of TiO_2-NP was observed when Caco-2 epithelia were exposed to either anatase or rutile TiO_2-NP.

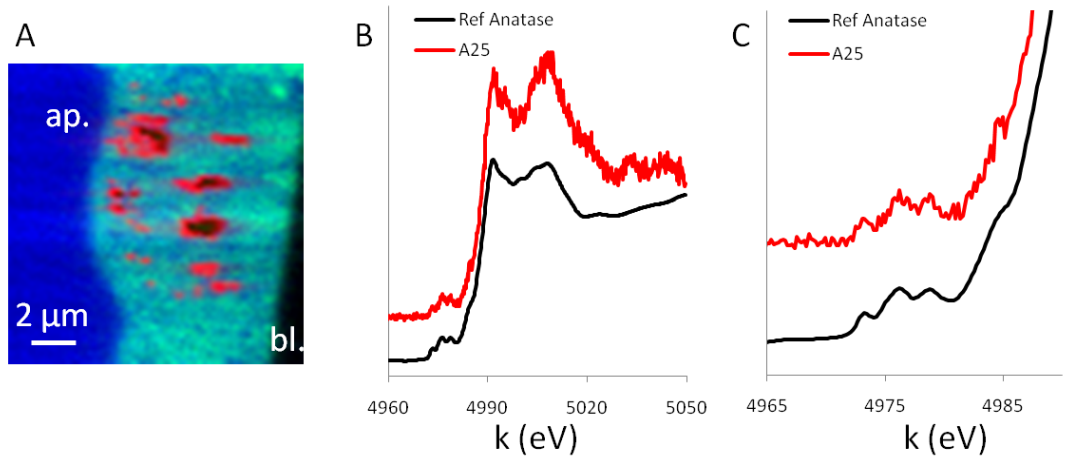

Figure 1. µXRF map of TiO2-NP distribution in a Caco-2 epithelium, and in situ XAS analysis of a Ti-rich region. Caco-2 epithelium is identified by the distribution of Os (A, green), the transwell membrane is visible in black on the right side of the image (A), unabling to identify the apical (ap.) and basolaterla (bl.) poles of the epithelium. TiO2-NP distribution in the epithelium is mapped through the fluorescence signal of Ti (A, red). XANES spectrum (B) and a zoom on the pre-edge feature (C) of a Ti-rich region, inside the cell.

Analysis of Ti speciation after TiO_2-NP internalization in gastrointestinal cells.

XAS spectra were recorded in Ti-rich regions of cell sections (Figure 1B), 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.

Conclusions and perspectives

In conclusion we demonstrated in this experiment that raw, pure TiO_2-NP, either anatase or rutile, were uptaken by cells but did not cross a reconstituted gastrointestinal epithelium during the first 48 h of exposure. TiO_2-NP did not dissolve, their crystalline phase did not change in the intracellular environment. A future experiment will aim at studying the translocation of TiO_2-NP in more relevant exposure conditions: gastrointestinal fluids are acidic and contain high concentrations of proteins. These conditions may alter TiO_2-NP surface chemistry, and consequently may modify their affinity for cell membrane receptors and transporters, and thus change their bioavailability, *e.g.* their cellular uptake, transport and epithelial translocation. These conditions may also cause their dissolution. Moreover a more relevant

epithelium model should now be used, particularly epithelia obtained by co-culture of Caco-2 and HT29-MTX cells, model for a mucus-secreting epithelium (Mahler *et al.*, 2009), or by co-culture of Caco-2 and Raji-B cells, model for follicule-associated epithelium (des Rieux *et al.*, 2007).

Thanks to the allocation of this beamtime, we were able to obtain innovative results which will be completed and will lead to the redaction of a publication. The possibility to perform both XAS and XRF on the same sample is particularly interesting for this type of sudy, and XAS is the only way to demonstrate that nanoparticles did not dissolve in biological environment. For these reasons allocation of this beamtime was an excellent opportunity.

Bibliography

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