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Investigation of TiO$_2$ nanoparticles translocation through a Caco-2 monolayer

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Abstract. Nanoparticles (NPs) are introduced in a growing number of commercial products, including food and beverage but their effects on gastrointestinal tract are poorly investigated. Here we focused on the translocation of TiO$_2$ NPs through Caco-2 monolayers exposed to anatase and rutile NPs up to 24 h. Internalization was followed by transmission electronic microscopy and µ-XRF elemental mapping, coupled to XAS analysis of Ti atoms environment. This innovative technique is among the best techniques to get insights on NP fate after internalization. The originality of this project relies on the panel of microscopy techniques implemented to investigate digestive barrier translocation, bringing together biologists, chemists and physicists in a pluridisciplinary research program.

1. Introduction

Due to their remarkable properties, nanoparticles (NPs) are presently introduced in a fast growing number of commercial products as these have known a 379% increase since March 2006 [1]. As its counterparts, the food industry is starting to introduce various NPs as food additives, food packaging components or as dietary supplements. Among them, titanium dioxide (named E171 in Europe) is used for whitening and brightening foods [2] but can also be found in daily use hygiene products such as toothpaste or sunscreens, or orally-administered drugs. Little is known however about the effects of NPs within the gastrointestinal tract and there is still a controversial debate whether they are able to cross this physiological barrier or not. Three main routes can be thought of, for describing NPs...
crossing the gut. First, NPs could be toxic enough to cross the epithelium via holes resulting from dead cells. Second, they could damage the barrier integrity and squeeze through live cells. Last, they could be taken in charge by cells and transported from the apical to the basolateral poles in a process called transcytosis. Among the *in vitro* models of gastrointestinal barrier, Caco-2 cell monolayers are a cornerstone to probe and predict intestinal drug permeability and absorption as many of the *in vivo* brush border enzymes and transport proteins are present and functional [3].

In the present study, an original approach, combining several microscopies, has been developed to assess TiO$_2$ NPs transport across Caco-2 epithelium. In particular, not only microbeam X-ray fluorescence proves to be a valuable tool for elemental imaging at a single cell level but also allows *in situ* speciation analyses of NP identified inside the cells by X-rays absorption spectroscopy (XAS).

2. Materials and Methods

2.1. Nanoparticles

TiO$_2$ NPs synthesis and characterization have already been described in [4,5]. Briefly, 95% anatase and 100% rutile NPs have mean diameters of 12 nm and 21 nm, and specific surface areas of 82 m$^2$/g and 73 m$^2$/g, respectively. To make the notations clearer, they will be referred to as A12 and R20 throughout this manuscript. NPs suspensions were prepared in ultrapure sterile water by pulsed sonication at a concentration of 10 mg/mL. Agglomeration state of NPs suspensions was determined by photon correlation spectroscopy (Malvern ZetaSizer 3000HS, Worcestershire, UK).

2.2. Cells

Caco-2 cells (ATCC HTB-37, passages from 39 to 43) were cultured in a 37°C incubator with a 5% CO$_2$ humidified atmosphere in Dulbecco Modified Medium supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine, 1% (v/v) non essential amino acids, 50 U/mL penicillin and 50 µg/mL streptomycin. Cells were seeded at a density of 50,000 cells/cm$^2$ in 6-well porous transwell®-clear polyester inserts (0.4 µm pores, Corning, Costar) and were cultured to confluence over 21 days, medium being changed every other day. They were exposed on their apical pole to A12 and R20 diluted in culture medium at a concentration of 50 µg/mL for 6, 12 or 24 h.

2.3. Cytotoxicity assays

Cells were seeded in 96-well plates at a density of 30,000 cells/cm$^2$. After 24 h, they were exposed to 100 µL per well of NPs suspensions from 0 to 500 µg/mL up to 24 h. Cell metabolic activity, reflecting NPs cytotoxicity, was assessed by using 3-(4,5-dimethylthiazol-z-yl)-2,5-diphenyl-tetrazotium bromide (MTT) [6]. Mitochondrial dehydrogenases of viable cells reduce MTT to water-insoluble blue formazan crystals. At considered endpoints, exposure medium was replaced by 100 µL of 0.5 mg/mL MTT solution in culture medium. After 2 h at 37°C, medium was replaced by 100 µL DMSO and mixed to dissolve formazan crystals. Absorbance was measured at 550 nm and metabolic activity was determined as a percentage of the negative controls. To probe the interference of NPs with this test, absorbance of exposed cells, treated as described below but without MTT, were recorded.

2.4. TEER measurements

Millicell-ERS (Millipore) and chopstick electrodes were used to measure transepithelial electrical resistance (TEER). A blank insert without cells but immerged in medium was used to obtain the transwell resistance. TEER was calculated as follows: TEER = (R$_{\text{cell}}$ – R$_{\text{blank}}$) x A, where TEER is expressed in Ω cm$^2$, cell and blank resistances in Ω and A, the surface area of the insert, in cm$^2$. As electrodes height positioning had a great influence on the displayed resistance value, at least five measurements were carried out for each insert.
2.5. Electron microscopy
After exposure to NPs, cells were rinsed with phosphate saline buffer, fixed in 2% glutaraldehyde in cacodylate buffer and in 1% osmium tetroxide solution, dehydrated through a graded series of ethanol and embedded in Epon resin, taking care of preserving their polarity. Ultra-thin sections were cut, then stained with 1% uranyl acetate for observation in transmission electronic microscopy (TEM) with a Philips CM120 electron microscope operating at 80 kV.

2.6. µ-SR-XRF and XAS analysis
Embedded samples used for TEM were also analyzed by synchrotron-based micro X-ray fluorescence (µ-SR-XRF) and micro X-ray absorption spectroscopy (µ-XAS). Transversal sections (thickness: 1 µm and 3 µm) of these samples were cut using an ultramicrotome, deposited on kapton polymer and covered by a thin layer of kapton. These sections were directly analyzed.

Cartographies presented here were obtained on LUCIA beamline (SOLEIL synchrotron). Scanning parameters were fixed as follows: cartography 100 µm x 100 µm, step: 2 µm x 2 µm, counting time: 1 s on each point. Ti, K, Ca, P/Os and Cl maps were drawn. Transwell membranes could be identified because they do not contain Cl, cells were identified by mapping K or P/Os distribution. Epoxy resin did not cause any interference in P/Os and Ti fluorescence signals. XAS analyses were performed on cell regions containing high amounts of Ti. XAS spectra were registered at the Ti K-edge (4966 keV).

3. Results and discussion

3.1. TiO2 NPs cytotoxicity
Caco-2 cells were chosen as a first model of gastrointestinal barrier as they exhibit properties of the small intestine in vitro when fully differentiated [3]. As a prerequisite for translocation experiments, we had to determine the sub-toxic concentrations of A12 and R20 NPs. Note that NPs suspensions in culture medium led to their aggregation, as their hydrodynamic diameters, evaluated by photon correlation spectroscopy were higher than 1 µm (data not shown). Their cytotoxicities were assessed in a concentration- and time-dependent manner by evaluating the cell metabolic activity through MTT reduction. As mentioned recently by Jones and Grainger [7], spectral properties of NPs should be carefully quantified in order not to bias dye-based assays. As shown in Figure 1A, interference of R20 NPs with MTT assay was to be taken into account as the residual absorbance of TiO2 cannot be neglected for high concentrations. This may be due to firmly adsorbed NPs on cells, remaining after washings. A12 NPs exposure led to the same observations and absorbance corrections. For both NPs, no modification of cellular metabolic activity, as measured by MTT reduction, was detected as the concentration of TiO2 increased (from 1 to 100 µg/mL) and after the longer exposure time of 24 h. The sub-toxic concentration of 50 µg/mL was then chosen for further experiments.
Figure 1. Caco-2 cells viability when exposed to A12 and R20 NPs in culture medium. Two time exposures were tested: 6 and 24h. Experiments were performed in n=5. Values are expressed as means and standard deviations. A: Residual absorbance of cells treated during 6 or 24 h to R20 NPs. The coefficients of determination of the linear regressions are 0.981 and 0.997 for 6 and 24 h respectively. B: Results from the MTT assay after absorbance correction for A12 and R20 after a 24h exposure.

3.2. Translocation of TiO$_2$ NPs across the epithelium
When cultivated on membranes over confluence, Caco-2 cells form a monolayer in which cells are maintained together by tight and adherens junctions. Its resistance testifies for its integrity. After 3 culture weeks and subsequent exposure to NPs, transepithelial electrical resistances were measured. According to Figure 2, a 50 µg/mL treatment had no significant effect on TEER for both NPs, suggesting that the epithelium remains intact, as observed previously for mixed anatase-rutile NPs [8].

Figure 2. Transepithelial electrical resistance measurements after exposure to A12 and R20 for 6, 12 and 24 h. Three inserts were measured five times for each condition. Values are expressed as means and standard deviations.

We then analysed transversal sections of Caco-2 cells monolayers with TEM and µ-SR-XRF in parallel. The advantages of synchrotron radiation over others X-rays sources are an improvement of sensitivity and spatial resolution of fluorescence analyses, allowing a single cell level imaging [9]. Regions of Interest (ROI) were chosen in order to draw images of P/Os (Figure 3A), Ti (Figure 3B) from the X-ray fluorescence spectrum (Figure 3C). These µ-SR-XRF analyses showed that even if mapping of Ti on a 1 µm-thick section is achievable, the quality of the images is much higher when analyzing 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 whereas cells were identified by mapping Os/P distribution as 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 NPs and the basolateral pole near the transwell region. Mapping of Ti
distribution enabled to observe NPs internalized in cells. After 12h of exposure, R20 NPs were observed on cell surfaces but also deeper inside the cells (Figure 3B). However they were not detected on the basolateral pole. This distribution was also observed when cells were exposed to A12 NPs and for both NPs after a 24 h exposure.

TEM observations consolidated this statement, along with giving a sub-cellular localization of NPs. As can be seen in Figure 3C, NPs were most of the time sequestered in the upper regions of the epithelium and they rarely reached the basolateral pole of cells. This is in good agreement with µ-SR-XRF mapping. Moreover, they were mainly grouped together inside the cells into heterogeneous clusters of 1-2 μm, in intracytoplasmic vacuoles or compartments resembling late endosomes. Far few NPs were observed isolated, as already reported for A549 human type II alveolar epithelium cell line [5]. Due to their size in medium culture, agglomerates of NPs would rather be internalized through macropinocytosis, the main pathway for particles around 1 μm, even if we did not observe any characteristic membrane ruffling. As a conclusion, such TiO₂ NPs accumulated in cells in low amounts and did not cross Caco-2 monolayers during the first 24 h of exposure.

![Figure 3. Ti distribution in Caco-2 cells. Cells were exposed for 12 h to R20 NPs on their apical pole (ap.). P/Os signals (A) allow cells identification. Translocation of NPs to the basolateral pole (bl.) is evaluated through mapping of Ti distribution (B) inside the cells. Elemental signals are extracted from total X-ray fluorescence spectrum (C). NPs distribution in cells is also observed by TEM (D). Note the presence of microvilli (m), one main feature of enterocytes on the apical side.](image)

3.3. Fate of internalized NPs
Elemental mapping at the cellular level is not the only strong point of synchrotron radiation based analysis as it also provides a unique way to gain insights into internalized NPs by recording XAS
spectra in Ti-rich regions of cell sections. Compared to reference compounds spectra, they inform on Ti speciation, on TiO2 crystalline phase and on the size of TiO2 NPs.

Ti pre-edge features are characteristic of the crystalline phase and size of TiO$_2$ NPs. 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) [10]. The intensity of A2 peak is related to the distortion of the octahedral TiO$_6$ unit, particularly the distortion observed on the surface of NP. The intensity of this peak is thus related to the size of the particle: the smaller the diameter, the higher this A2 peak [11]. Meanwhile, the intensity of the A1 peak also decreases as the particle size decreases [12]. As can be seen in Figure 4, 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 local changes of the crystalline phase, on NPs surface after cell internalization, and on their dissolution. In the present experiment, no modification of these pre-edge features was observed after cell internalization of R20 or A12, 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.

![Figure 4](image_url)

**Figure 4.** XAS spectra of Ti in cells exposed to TiO$_2$ NPs. Cells were exposed to R20 NPs. Spectra were recorded at the Ti K-edge (4.966 keV) at room temperature on locally Ti-rich regions determined on µ-SR-XRF maps (Cell), and compared to the spectrum of a reference nanopowder (Ref.). A: whole XAS spectrum, B: pre-edge feature.

4. Conclusions

In brief, the present results show that our forward-looking approach, associating TEM with µ-SR-XRF and XAS was effective and powerful to address the issue of potential translocation of TiO$_2$ NPs across a gastrointestinal model and to obtain in situ information on internalized NPs. Caco-2 epithelia were exposed to bare, pure, anatase and rutile TiO$_2$ NPs. NPs accumulated in cells, although in low amounts as shown in very good agreement by µ-SR-XRF mapping and TEM. Moreover Ti XAS spectra recorded on Ti-rich intracellular regions indicate that after internalization, Ti is still in the TiO$_2$ nanoparticulate state, that crystalline phase and speciation remain identical and that no partial dissolution occurs.

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**References**

[1] www.nanotechproject.org