

***In vitro* and *in vivo* evaluation of TiO₂ oral absorption**

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Abstract

The Caco-2 monolayer permeation test is a widely used *in vitro* test to predict oral absorption of organic compounds, particularly by the pharmaceutical industry (Artursson et al. 2001). A few reports also exist on the use of this test system for NMs (e.g., Al-Jubory and Handy 2012; Antunes et al. 2013; Jin et al. 2013). But the predictive value of the data obtained in such test is still unclear. The main processes that determine uptake for chemicals are diffusion and active transport through membrane transporters (Grassi 2007), and the Caco-2 monolayer permeation test is a good model for both processes. However, these are not the main processes governing the oral uptake of particles. Three main pathways for absorption of particles across intestinal barriers have been described. First, paracellular transport can take place for small molecules that can pass the tight junctions and the pore-diameter which is reported to be around 0.6–5 nm (Ruenaroengsak et al. 2010). Second, transcytosis may appear across enterocytes, but mostly across M-cells located in the Peyer's patches (Powell et al. 2010). An third, particles (nano and micro sized) can be transported across degrading enterocytes (Hillyer and Albrecht 2001; Volkheimer 1993). The three mechanisms that have been described for NMs oral absorption could theoretically occur in the Caco-2 model, particularly if this could be modified to incorporate M-cells.

In the present report, we have used a single type of TiO₂ NPs (spherical, 18 ± 8 nm diameter, surface area was 89.8 m²/g) in different *in vitro* and *in vivo* systems in order to better understand how results from these studies can be extrapolated to other cell types or levels of organization. In particular, we describe the results of five studies: an *in vitro* cell uptake using A549 cells, an *in vitro* permeation test using differentiated Caco-2 cells, an *in vitro* permeation test using a coculture of differentiated Caco-2 cells and M-cells, and two *in vivo* oral absorption tests in rats (with and without fasting conditions prior to administration). To overcome the analytical challenges associated to the tracking of unlabelled TiO₂ NPs, both inductively coupled plasma mass spectrometry (ICP-MS) and transmission electron microscopy (TEM) were used.

TiO₂ NPs were present in form of agglomerates of different sizes within cytoplasmic vesicles of A549 cells after 72 hours exposure. In none of the cells evaluated, particles were found freely in the cytoplasm or in the nucleus. This efficient cell internalization and the fact that NPs were found as aggregates in cytoplasmic vesicles is consistent with previous reports in a variety of human cell lines.

A very small proportion of the NPs was able to cross the differentiated Caco-2 cell membrane (below or close to the detection limit, i.e., 0.1 ppm or 0.4% of the applied concentration). In an attempt to increase the biological relevance of this permeation model, we introduced Raji cells to induce the differentiation of Caco-2 cells into M-cells. The Caco-2/M-cell coculture model was established and characterized through measuring TEER values during the differentiation process, and performing morphological (histological sections and scanning electron microscopy; Figure 1) and immunostaining (ZO1 and occludin tight junction proteins). All data confirmed that a proportion of Caco-2 cells consistently differentiated into M-cells. In such system, a very low permeation rate for TiO₂ NP was recorded, although qualitatively higher (higher frequency of values above the detection limit) than that for Caco-2 cell monocultures.

The readily internalization in A549 cells and in most cell lines contrasts with an extremely low absorption by Caco-2 cells in our study and the low uptake by these cells in the study by Fisichella et al. (2012). The fact that we used the same TiO₂ NPs for *in vitro* studies with both A549 and Caco-2 cells indicate that these differences are related to the cell line properties and not to the TiO₂ NPs used. The apparent contradiction is probably related to the cell membrane morphology of the latter. Differentiated Caco-2 cells are polarised cells with microvilli in their apical side (where exposure takes place).

To evaluate *in vivo* absorption Sprague-Dawley male rats were administered the vehicle or the TiO₂ NPs by oral gavage. Two independent experiments were performed, one in which administration of the NPs was performed in fasting conditions and one without food restrictions. Administered rats were weighted and observed to assess clinical signs of toxicity on the day of administration and on the following day, before sacrifice. At termination, spleen, liver, small and large intestines, and mesenteric lymph nodes were removed. The intestines were carefully washed with phosphate buffer to remove their

content. Peyer's patches were excised and separated from the rest of the small intestine. The caecum was separated from the rest of the large intestine. One of the Peyer's patches and a piece of smooth small intestine were immediately preserved in buffered glutaraldehyde-paraformaldehyde for later TEM analyses. The remaining samples were kept at -20°C until acid digestion and analysis by ICP-MS.

There was no detectable increase in titanium levels in any of the tissues evaluated 24 hours after the administration of 100 mg/kg TiO₂ NPs, regardless of the food restriction conditions (only some tissues under fasting conditions: Peyer's patches, smooth small intestine, and mesenteric lymph nodes). Smooth sections and Peyer's patch sections of the small intestine of the animals that received TiO₂ NPs without food restrictions were examined by TEM. No TiO₂ NPs were observed in the smooth sections. In contrast, we did observe at least one cell containing considerable amounts of TiO₂ NPs aggregates in a Peyer's patch section (Figure 2). In this cell, the TiO₂ NPs were not surrounded by membranes, but they were freely distributed in the cytoplasm. We did not observe NPs inside mitochondria or the nucleus.

The low bioavailability of TiO₂ NPs in this report contrasts with the relatively high oral bioavailability study reported by Jani et al. (1994). We had hypothesized that these differences could be due to the fact that Jani et al. (1994) administered the particles after several hours of fasting (Janer et al., 2014), but the experiment that we conducted in fasting conditions does not support such hypothesis.

In summary, we showed that A549 cells readily uptake the TiO₂ NPs used in this study. The results were consistent with most literature reports for TiO₂ NPs and other type of NMs, suggesting a limited modulating effect of the physicochemical properties of NMs on cell uptake. However, such rapid uptake contrasted with a very low oral absorption in the *in vitro* and *in vivo* studies that we performed. The results of this study support that M-cells play an important role in the absorption of nanoparticles, and suggest that the Caco-2/M-cell coculture model is a more relevant model for the prediction of oral absorption of nanoparticles than the Caco-2 monoculture model.

References

- Al-Jubory AR, Handy RD, Nanotoxicology, **7** (2013) 1282-301.
Antunes F, Andrade F, Araujo F, Ferreira D, Sarmiento B, Eur J Pharm Biopharm **83** (2013) 427-35.
Artursson P, Palm K, Luthman K, Adv Drug Deliv Rev **46** (2001) 27-43.
Grassi M GG, Lapasin R, Colombo I, A Physical And Mathematical Approach. CRC Press. (2007).
Powell JJ, Faria N, Thomas-McKay E, Pele LC, J Autoimmun **34** (2010) J226-33.
Jin X, Zhang ZH, Li SL, Sun E, Tan XB, Song J, Jia XB, Fitoterapia **84** (2013) 64-71.
Ruenraroengsak P, Cook JM, Florence AT, J Control Release **141** (2010) 265-76.
Hillyer JF, Albrecht RM, J Pharm Sci **90** (2001) 1927-36.
Volkheimer G. Pathologie **14** (1993) 247-52.
Fisichella M, Berenguer F, Steinmetz G, Auffan M, Rose J, Prat O, Part Fibre Toxicol **9** (2012) 18.
Jani PU, McCarthy DE, Florence AT, Int. J. Pharmaceutics **105** (1994) 157-168.
Janer G, Mas del Molino E, Fernández-Rosas E, Fernández A, Vázquez-Campos S. Toxicol Lett. **228** (2014) 103-10.

Figures

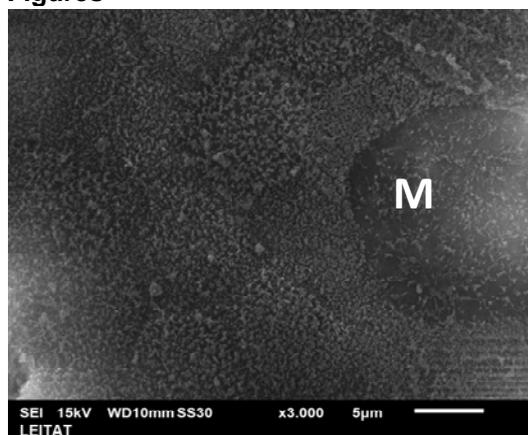


Figure 1. SEM images of Caco-2/Raji cocultures. Caco-2 cells show dense microvilli and contrast with larger M-cells with only rudimentary microvilli (M).

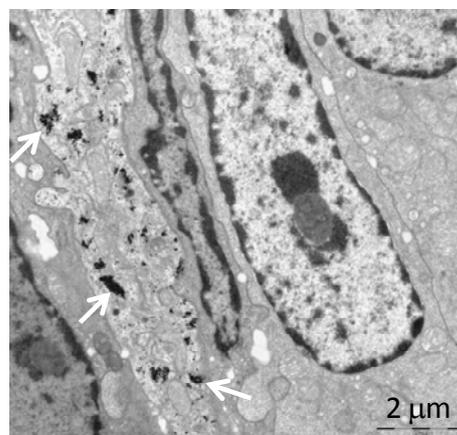


Figure 2. TEM micrograph showing the presence of TiO₂ nanoparticles in a cell from a Peyer's patch section. The arrows point to some of the NP aggregates.