A common European approach to the regulatory testing of nanomaterials

In vitro screening methodology for absorption or crossing of other barriers
Deliverable 5.3

Introduction

To understand and predict effects of NMs in organisms, it is essential to determine how these NMs will distribute in an organism. The crossing of biological barriers such as intestinal or alveolar epithelia is a crucial aspect in that context. To date, limited and controversial data are available on NMs uptake by cells of biological barriers and their transport across, both *in vivo* and *in vitro*.

For this reason and due to ethical and economical constrains, it is needed to develop relevant and alternative *in vitro* barrier models and protocols for evaluation of barrier crossing in order to predict the behaviour of nanomaterials in organisms and to assess potential health implications. Deliverable 5.03 describes the result of Task 5.3 of the NANoREG project aimed at providing and validating such models.

Description of Work

The potential internalization and crossing through biological barriers was evaluated for 12 NANoREG core materials and two fluorescent nanomaterials using four different barrier models (*i.e.* intestinal epithelium, alveolar epithelium, oral mucosa, blood-brain barrier; Figure 1). Before starting the crossing assays, the sub-toxic concentrations of the applied NMs have been determined using the MTS viability assay SOP developed in the NanoValid project (http://www.nanovalid.eu/). In order to maintain the same dose units (µg of NMs/cm²) between crossing experiments (using inserts) and cytotoxicity experiments (using 96 well plates), apical assay volumes added to the inserts were adapted.

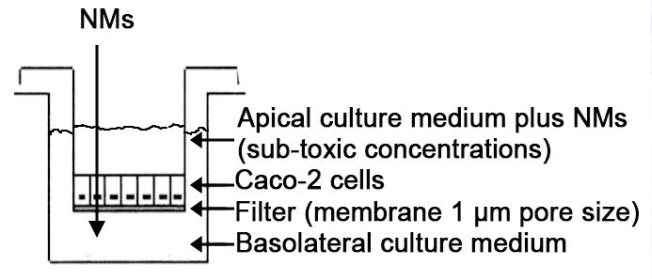

Figure 1: Schematic representation of NMs incubation with intestinal model (adapted from Gullberg et al., 2000; doi:10.1006/bbrc.2000.4038). Caco-2 cells were differentiated on the membrane of transwell to form a tight intestinal monolayer. Intestinal epithelium was incubated for 24h with sub-toxic NMs concentration added in apical medium (350 µl). Crossing of NMs was determined by analysing their presence into basolateral medium. A similar experimental procedure was used with BBB and Alveolar models. ALI (Air-Liquid Interface) Alveolar models were exposed to a limited volume of cell culture media (30 µl) in the apical side. For oral mucosa model, a small droplet (10 – 20 µl, according to the model surface area) of NMs suspension was applied onto the surface of the model (approx. 0.2 cm²) for 24h. After fixation, dehydration and paraffin embedding, exposed oral models were sectioned for imaging.

The paracellular marker Lucifer yellow (LY) assay was used to evaluate the impact of NMs on the integrity of *in vitro* barrier models. This assay was shown to be suitable to evaluate the effect of NMs on intestinal epithelium, alveolar and BBB models integrity. No interference of NMs with this assay was observed. However, LY assay and multiple dye assay were found not suitable for the 3D oral mucosa model and thus the impact of NMs on the barrier integrity of this model could not be determined.

Intracellular NMs localization was performed with Confocal Microscopy (CM), and TEM. Crossing of NMs was evaluated with both qualitative (TEM/SEM coupled or not to EDX detector, flow cytometry, fluorescence microscopy and by ultrahigh resolution microscopy) and quantitative methods (PIXE, ICP-OES and ICP-MS).

Main results and conclusions

The experiments carried out by task 5.3 reveal a number of serious limitations of the *in vitro* barrier models.

The first and main limitation is the interference of the insert membrane playing a physical barrier role in the NMs crossing. It limits the suitability of *in vitro* barrier models (intestinal epithelium, alveolar epithelium and BBB model) to reliably evaluate the crossing of NMs. In case of oral mucosa model, this interference does not arise as the NMs were considered to successfully cross the oral mucosa barrier if they penetrated through the epithelium (*Figure 3*). So, oral mucosa model appears to be suitable for evaluation of NMs crossing. For screening purposes (medium or high throughput) however this model is not suitable since it's a time- and resource (money and manpower) consuming model.

The second limitation is related to the insufficient sensibility of some quantitative methods to evaluate crossing of NMs. (*e.g.* PIXE, ICP-MS for some NMs such as SiO_2 NMs).

Intracellular penetration of NMs was mainly investigated using TEM and CM. CM appears to be suitable to evaluate internalization of metallic and fluorescent NMs. Using labelling of specific cellular components *(e.g.* nuclei and membrane), this technic allowed to investigate whether NMs were localized at surface of epithelia or were internalized (*see Figure 2*). However, no information about NMs morphology and chemical composition is given by this technique render difficult the firmly identification of NMs. So, results obtained by CM should be confirmed by complementary techniques such as TEM-EDX which will allow identification of NMs-like structures with morphology and size similar to that of raw NMs. However, TEM has also shown some limitations in the study of NMs internalization as it is costly and time-consuming. In addition, caution must be taken before drawing final conclusions about the internalization of NMs if no NMs are observed in cells. The absence of NMs inside cells in the selected areas might simply result from a very low frequency of NMs and therefore a low probability of observation. This conclusion is also valid for confocal microscopy. On the other hand, data obtained in task 5.3 indicated that some NM-like structures were in fact not included in the cellular "background" but rather overlaid on the cells. They could be driven during the cutting of tissue

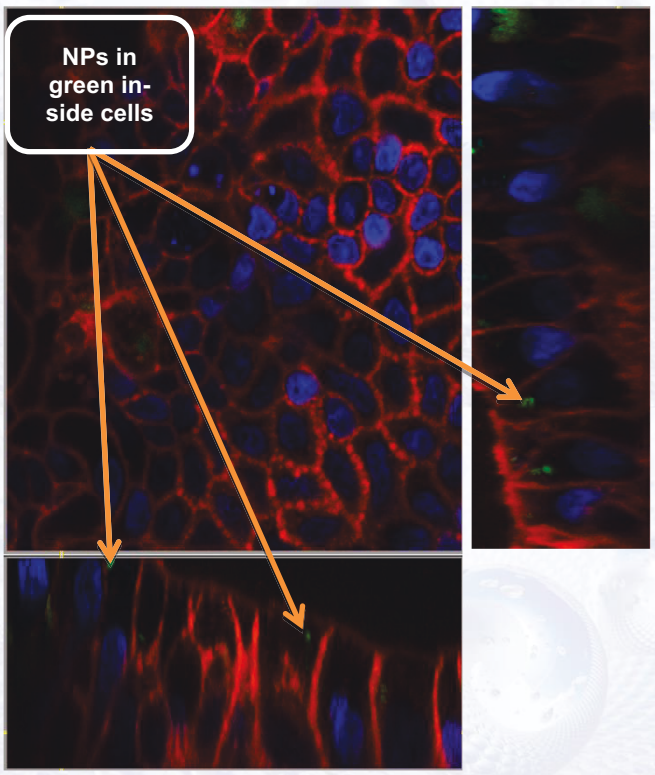

Figure 2: Observation of fluorescent negatively charged SiO_2 NMs within Caco-2 cell monolayers by confocal analysis. Intestinal epithelia were incubated for 24h with 100 µg/ml of fluorescent negatively charged SiO_2 NMs. Cytoplasmic membrane was stained with Cell Mask (red) and nuclei with Hoescht (blue). Negatively charged SiO_2 (green) were mainly localized inside the upper part of Caco-2 cells (arrows).

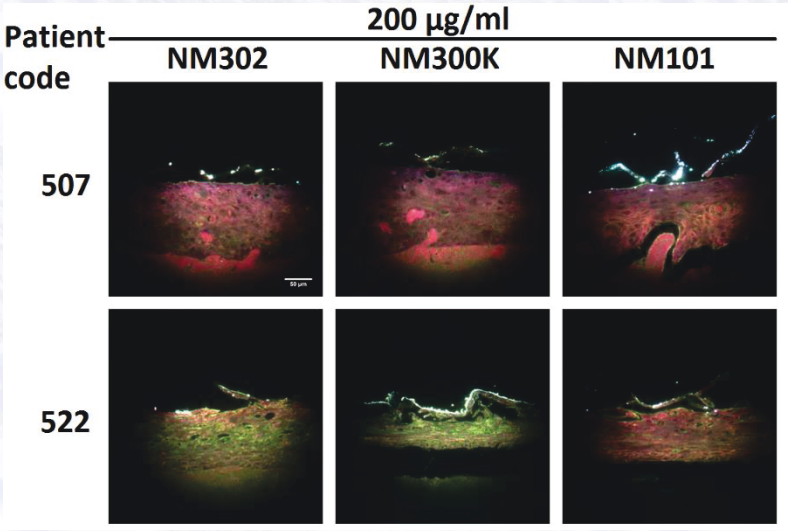

Figure 3: Representative images of 3D oral mucosa models exposed to NM-302, NM-300K and NM-101 at 200 µg/ml for 24h. Images show epithelium in darker purple/green, connective tissue pink/green, and the NMs as bright white spots. NM-302, NM-300K, and NM-101 can all be seen located at the surface or being removed by desquamation from the epithelium. All images were captured with URI system (CytoViva) at 40x magnification. Scale bar = 50 µm.

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slides. This also should be taken into account when interpreting the results of TEM analysis.

A third limitation is the relatively large volume (1,5 ml) of basolateral medium in relation to the quantity of nanomaterials that could cross the barrier and the presence of organic matter in culture media generating an opaque background rendering difficult the reliable detection by SEM/TEM of crossed NMs.

Final conclusion of Task 5.3 is that even if i*n vitro* barrier models developed in task 5.3 such as intestinal epithelium model have proven to be quite reproducible in NMs crossing/internalization evaluation, the use of these *in vitro* barrier models are currently subject to too much analytical technique limitations and interference to be recommended for a reliable evaluation of the potential crossing of NMs through biological barriers.

Given the relevance that absorption data through epithelial barriers have in risk assessment of NMs, particularly for which concern systemic effects evaluation, a further improving step in the set-up of *in vitro* barrier models is necessary. In particular, a combination of complementary analytical techniques (both qualitative and quantitative) is required to avoid under- or overestimation of crossing events.

For more details about NANoREG please visit the official website **www.nanoreg.eu.**

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