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# Effects of engineered nanomaterials in an inflamed-like intestinal triple culture – acute vs repeated exposure

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**Background:**

Although oral exposure to engineered nanomaterials (ENM) steadily increases, uncertainty remains regarding possible adverse effects on the intestine. Damage to or functional impairment of the intestinal barrier by inflammatory conditions might affect the risk associated with ENM exposure. To facilitate an appropriate risk assessment of ENM in the context of inflammatory processes an intestinal triple culture model of human cell line-derived enterocytes (Caco-2), goblet-like cells (HT29-MTX-E12) and macrophage-like cells (PMA-differentiated THP-1) was developed (Figure 1). An inflammation-like state was induced and the model exposed to silver (Ag-PVP, <100 nm) and titanium dioxide ($TiO_2$, P25, ~21 nm) ENM for an acute (1x for 24h) and a long-term repeated exposure (1x/day for 5 consecutive days).

**Conclusions:**

- The inflamed-like culture was characterised by a disruption of barrier integrity, high levels of pro-inflammatory cytokines, a change in mucin profile, and increased constitutive DNA damage.
- The inflamed-like state of the triple culture showed similar characteristics to inflamed intestinal tissue *in vivo* reported in the literature, making it a suitable *in vitro* model to study ENM-induced toxicity.
- No significant effects were detected in most observed endpoints after ENM treatment. Following repeated exposure to $TiO_2$, a significant reduction in MUC2 expression and a reduction in DNA damage levels was observed.

## Experimental set-up

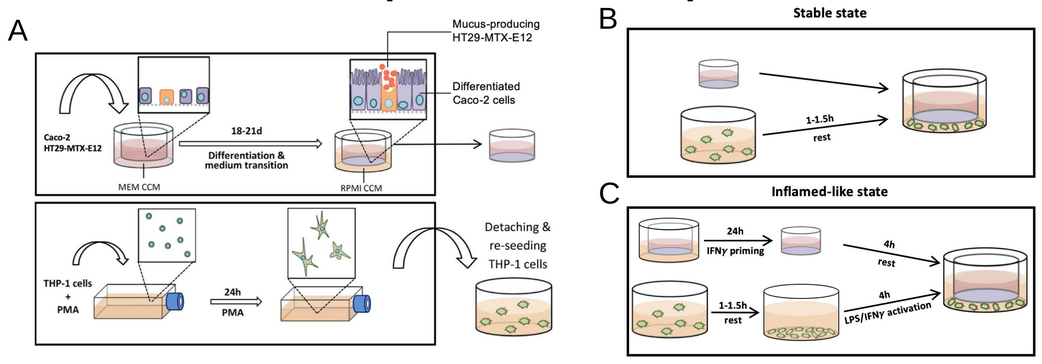


**Fig. 1 Schematic description of the triple culture model** using (A) Caco-2, HT29-MTX-E12, and THP-1 cells in a (B) stable and (C) inflamed-like state (based on [1])

## Characterisation of the triple culture

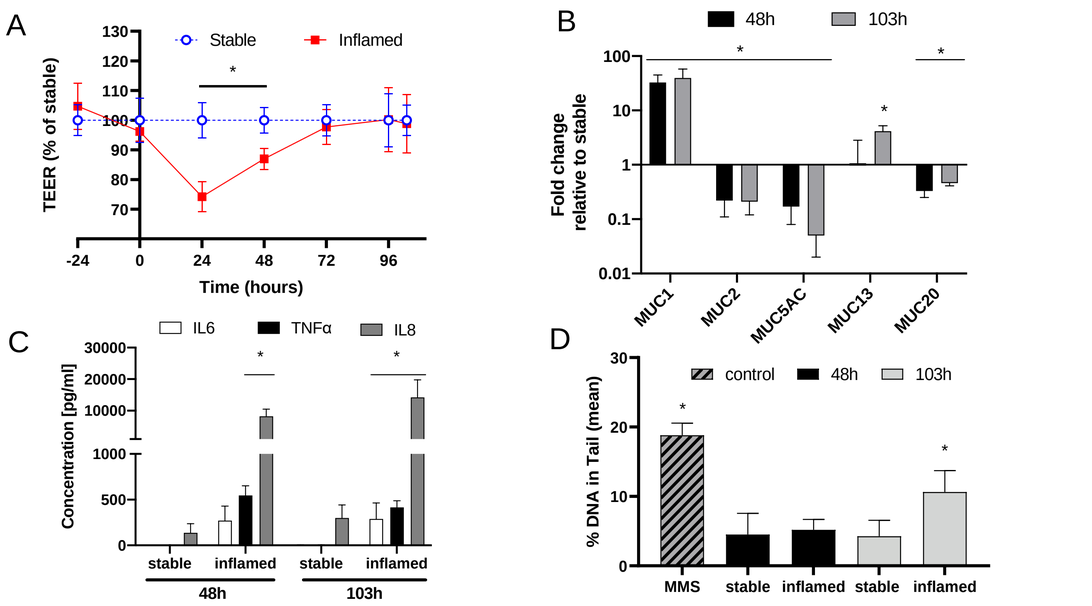


**Fig. 2 Characterisation of the stable and inflamed-like triple culture**
(A) TEER compared to stable state, (B) mucin expression compared to stable (normalized to $\beta$-actin), (C) cytokine release and (D) DNA damage. *p≤0.05 compared to the corresponding stable state values.

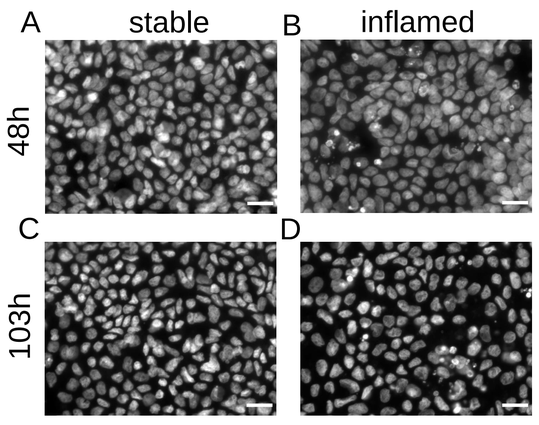


**Fig. 3 DAPI immune staining** after (A,B) 48h and (C,D) 103h in stable (left) and inflamed-like state (right). (Scale bar = 25 µm)

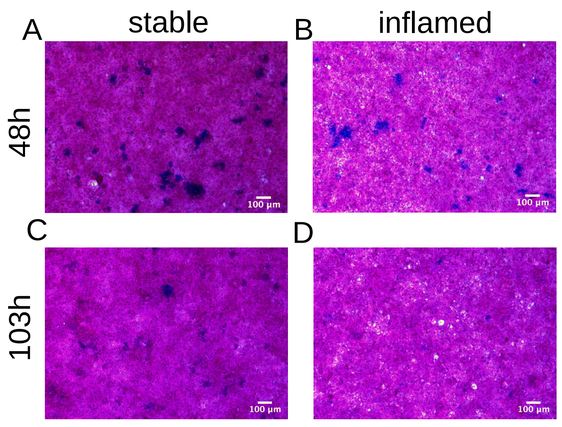


**Fig. 4 Alcian Blue staining and PAS reaction** after (A,B) 48h and (C,D) 103h in stable (left) and inflamed-like state (right).

## No effects: TEER, Cytokines

**Inflamed-like cells exposed to Ag-PVP and $TiO_2$** in
- an acute exposure with 24h equilibration phase and 24h treatment (10, 80 µg/cm²)
- a repeated 5-day exposure 1x/day (3, 16.5, 30 µg/cm²)

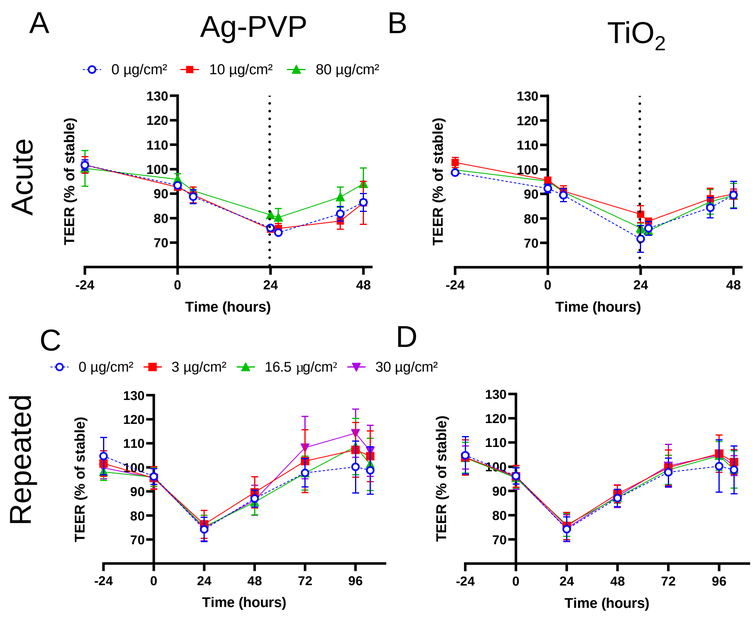


**Fig. 5 Barrier integrity** during (A, B) acute treatment and (C, D) repeated 5-day exposure with Ag-PVP (left) and $TiO_2$ (right) ENM compared to stable triple culture.

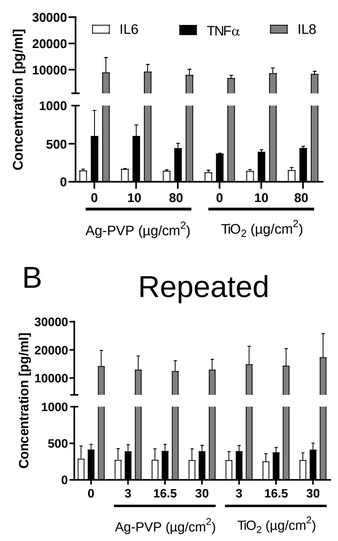


**Fig. 6 Cytokine release** after (A) acute treatment and (B) repeated 5-day exposure with Ag-PVP and $TiO_2$ ENM.

## Altered mucin expression and DNA damage

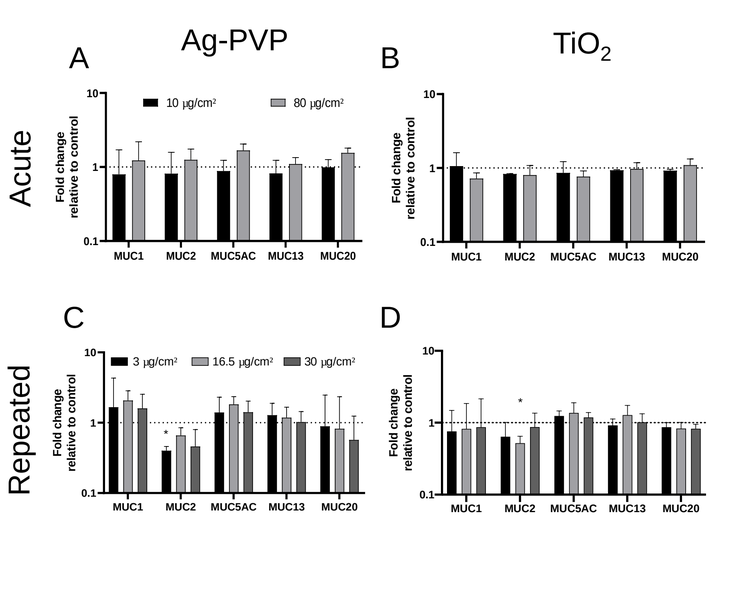


**Fig. 7 Gene expression analysis of mucins** $\Delta\Delta C_T$ – based fold-change after (A,B) acute exposure and (C,D) repeated 5-day exposure with Ag-PVP (left) and $TiO_2$ (right) ENM compared to inflamed-like triple culture (normalized to $\beta$-actin). *p≤0.05 compared to untreated control.

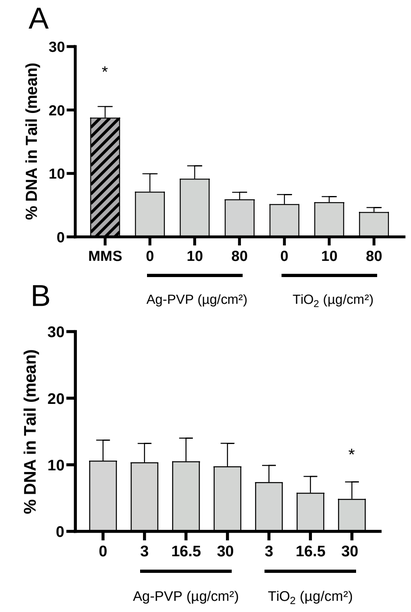


**Fig. 8 Comet tail intensity** after (A) acute and (B) repeated 5-day exposure with Ag-PVP and $TiO_2$ ENM. Methylmethanesulfonate (MMS) was used as a positive control. *p≤0.05 compared to untreated control.

## *Materials & Methods*

Ag-PVP (Sigma) and $TiO_2$ (P25) were suspended in $H_2O$ and sonicated according to the NanOxiMet suspension SOP (www.nanoximet.eu). For epithelial co-culture, Caco-2 and HT29-MTX-E12 cells were seeded (ratio 9:1) on transwell filters (Falcon, PET, 1 µm size), maintained for 18-21 days, and primed (IFN$\gamma$, 24h) 24h before triple culture.
THP-1 cells were PMA-differentiated (100 nM), detached with Accutase and seeded ($1.8x10^5$ cells/well). After re-attachment(1-1.5h) and activation (LPS/IFN$\gamma$, 4h), the transwell inserts were placed onto THP-1 cells. For the acute exposure, cells were treated after 24h triple culture for additional 24h. The repeated long-term exposure started immediately. Particles were prepared in cell culture medium (1% FBS) in 500 µL per well. The **transepithelial electrical resistance** (TEER) was measured repeatedly throughout the experiment. Supernatants were collected for cytokine quantification by ELISA. Cells were washed and fixed with 4% paraformaldehyde for staining of mucus (**Alcian blue staining and PAS reaction;** detection of acidic and neutral mucus) or **DAPI immune staining.** Unfixed cells were detached from the filter using trypsin and used for the **alkaline comet assay** and gene expressions analysis by **RT-qPCR**.

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[1] Kämpfer, AMM; Urbán, P; Gioria, S; et al.: Toxicology in Vitro 2017, 45(Pt 1) 31-43 (https://doi.org/10.1016/j.tiv.2017.08.011)

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