Digestion Simulation for Nanoparticle Toxicity Testing: Particle Characterization and *In-vivo In-vitro* Comparison

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Background:

Oral exposure to engineered nanomaterials is increasingly likely due to their frequent use in consumer products. We investigate the influence of simulated gastro-intestinal (GI) pH conditions on particle agglomeration, cytotoxicity and DNA damage induction in intestinal epithelial cell lines and validate the results against *in vivo* DNA damage of the colon.

PVP-capped silver (Ag-PVP) and titanium dioxide (TiO_2) nanoparticles (NP) were tested on Caco-2 and HT29-MTX-E12 (E12) cells as well as in 28-day feeding studies. The pesticide chlorpyrifos (CPF) served as a non-particulate control both *in vitro* and *in vivo*.

The results are part of a pilot study to facilitate studying the effects of ENM in advanced *in vitro* models of the intestine.

Conclusions & Outlook

- Simulation of GI pH conditions induced agglomeration of Ag-PVP and TiO_2 NP, but did not influence the average toxicity of the NP
- The simulated digestion reduced the cytotoxic potential of CPF in Caco-2 cells
- TiO_2 NPs caused moderate DNA damage in Caco-2 and E12 cells in absence of cytotoxicity, and no effects in murine colon cells
- Ag-PVP NP caused pronounced DNA damage with & without concurrent cytotoxicity in Caco-2 and E12 cells, respectively, but no effects in murine colon cells
- Digestion simulation can enhance the applicability of *in vitro* methods and will be adapted for use in an advanced intestinal co-culture model (Fig. 1) within the PATROLS project*

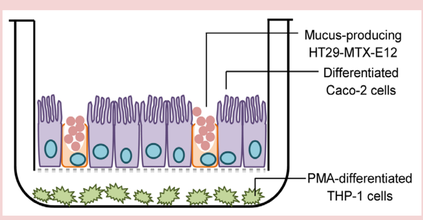

Fig. 1 Advanced intestinal co-culture

Simulation of gastro-intestinal pH conditions

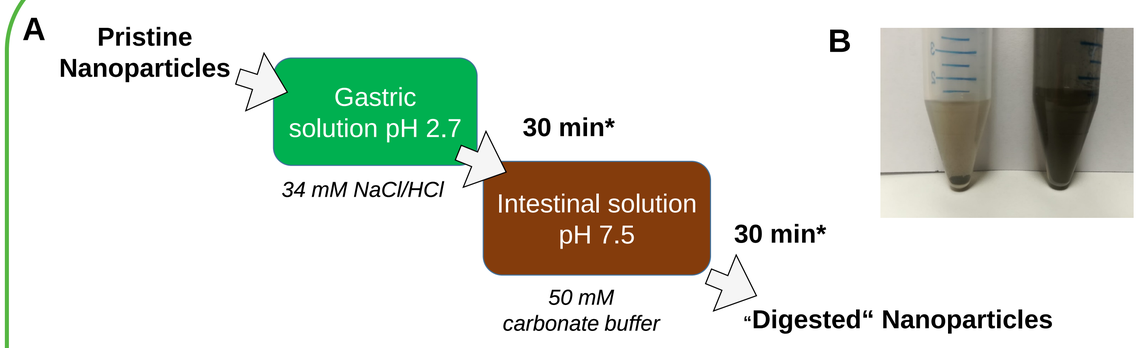

Fig. 2 (A) Schematic description of the artificial digestion process mimicking the gastro-intestinal pH conditions (Method based on: Gerloff et al. 2013 Nanotoxicology); **(B) Example of Ag-PVP NP** before (right) and after (left) digestion [*Shaking with 120 rpm at 37 °C]

Nanoparticle characterisation

Tab. 1 Charakterisation of Ag-PVP and TiO_2 NP

Nanomaterial	SEM Ø [nm]	Z-average (DLS) [nm]	D50 (DLS) [nm]
Ag-PVP (UD)	888 ± 1181	773	472
Ag-PVP (D)	200 ± 137	29219	5040
TiO_2 (UD)	438 ± 586	627	567
TiO_2 (D)	1539 ± 1601	2196	1823

*UD: undigested, D: digested

- SEM Ø based on analysis of 300 agglomerates
- Simulation of GI-pH conditions caused a strong increase in hydrodynamic diameter in Ag-PVP NP, but only a moderate increase in TiO_2 NP
- SEM analysis confirms size increase for TiO_2 NP, but not Ag-PVP NP

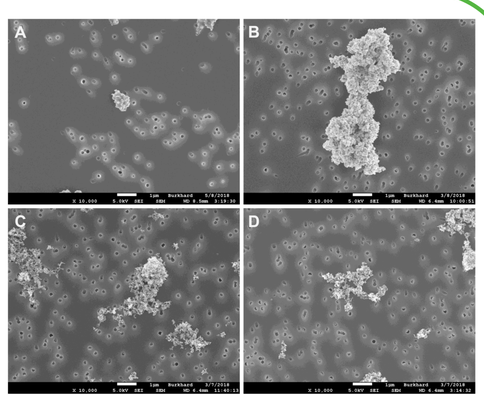

Fig. 3 SEM images of Ag-PVP NP before (A) and after (B) digestion and TiO_2 NP before (C) and after (D) digestion

In vitro: Cytotoxicity

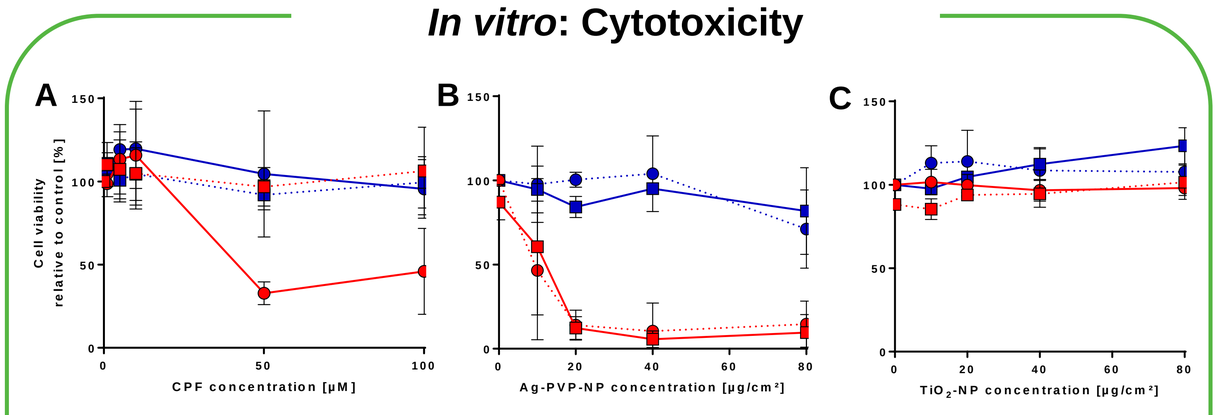

Fig. 4 Metabolic activity in Caco-2 (red) and E12 (blue) cells after 24h exposure to (A) CPF, (B) Ag-PVP NPs, and (C) TiO2 NPs in their pristine form (circles) or after incubation in simulated gastro-intestinal pH conditions (squares) (Average ± SD of N=3)

In vitro: DNA damage

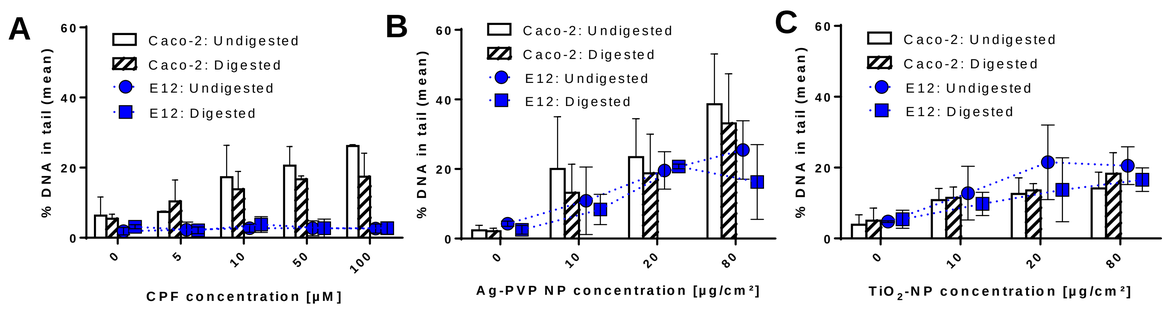

Fig. 5 DNA damage in Caco-2 and E12 cells after 4h exposure to (A) undigested (Caco-2: white bars; E12: blue circles) or digested (Caco-2: striped bars; E12: blue squares) CPF, (B) PVP-capped Ag NPs or (B) TiO_2 NPs. DNA damage was assessed using the alkaline comet assay. (Caco-2: CPF: Average±SD N=2, Ag-PVP & TiO_2: N=3; E12: Average±SD N=2)

In vivo: DNA damage in colon epithelial cells

- 2 cm of proximal colon was cut open, fixated with a microscopic glass slide and epithelial cells were scraped off using as second slide
- The cell suspension was homogenised and pressed through a 70 µM nylon filter
- After centrifugation the supernatant was discarded and the cell pellet re-suspended in 1 ml buffer.* *Method based on: Risom et al. 2007 Toxicology Letters

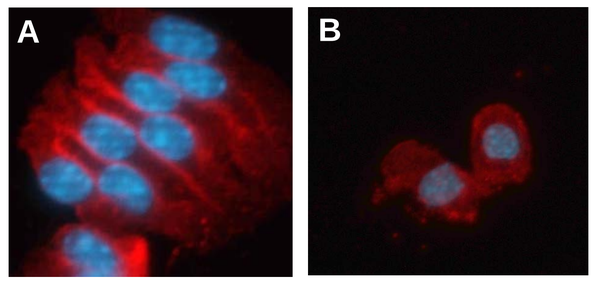

Fig. 6 CD326 Immunostaining of isolated murine colon epithelial cells (40x magnification). (A) intact connective epithelial tissue; (B) single cells as used for Comet Assay (Red (AlexaFluor 594): Epithelial cell adhesion molecule (EpCAM), Blue (Hoechst): nuclei)

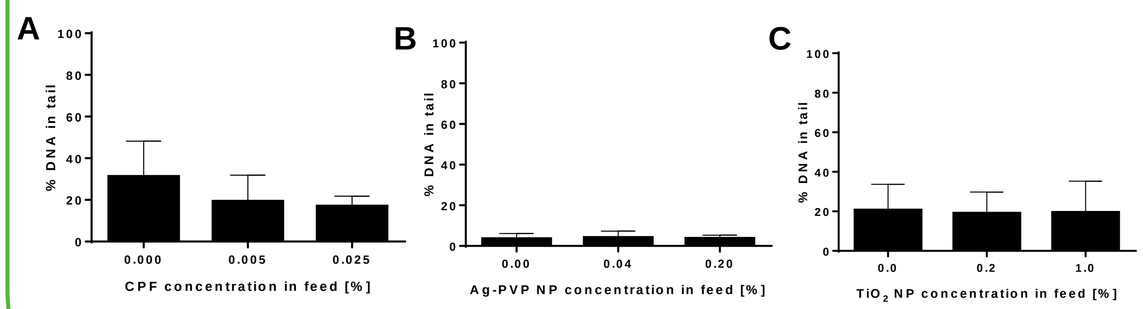

Fig. 7 DNA damage in isolated murine colon epithelial cells after 28-day feeding studies exposing mice to (A) CPF (0,005 or 0,025 % in feed), (B) Ag-PVP NPs (0,04 or 0,2 % in feed) and (C) TiO_2 NPs (0,2 or 1 % in feed) (Average±SD of 5 animals per group; control animals were included in each feeding study)

Materials & Methods

Ag-PVP (Sigma, <100 nm) and TiO_2 NP (P25) were suspended in H_2O (4 mg/mL) and sonicated according to the NanOxiMet suspension protocol (www.nanoximet.eu).

The particle characterisation using Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM) was performed by the Institute *of Energy and Environmental Technology e. V. (IUTA, Duisburg, Germany).*

Caco-2 and HT29-MTX-E12 cells were grown for 48 h before treatment. 16 – 20 h before treatment, the cell culture medium was changed to MEM (Caco-2) or DMEM (HT29-MTX-E12) containing 1 % FCS. Cytotoxicity was assessed via **WST-1 assay**, using the Roche Cell Proliferation the Ready-To-Use Reagent WST-1 after 24 h of treatment.

DNA damage was analyzed via **alkaline comet assay** after 4 h of treatment.

For the **feeding studies**, five week old C57BL/6J mice were used. Five animals per concentration were fed for 28 days before euthanization and dissection.

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*** Physiologically Anchored Tools for Realistic nanOmaterial hazard aSsessment**

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