Institute of Toxicology and Genetics

Karlsruhe Institute of Technology

Developing in vitro and in vivo high throughput screening platforms for hazard prediction of manufactured nanomaterials

Katrin Volkmann[1], Iris Hansjosten[1], Juliane Rapp[1], Iseult Lynch[2], Eugenia Valsami Jones[2], Silvia Diabaté[1], Carsten Weiss[1]

[1]Institute of Toxicology and Genetics, Karlsruhe Institute of Technology, Campus North, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany

[2]School of Geography, Earth and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom

The growing number of manufactured nanomaterials (MNMs) used in consumer products and industrial applications will have an unpredictable impact on human health and on the ecosystem. Thus there is an urgent need to further develop high throughput testing for hazard prediction in nanotoxicology. *in vitro* tests in cultured cells are widely used in nanotoxicology and provide quantitative information about MNM induced toxicity e.g. cell death. Here automated microscopy and software aided image analysis can be developed into high throughput assays with multiple testing conditions and endpoints. The zebrafish (*Danio rerio*) embryo emerges as a vertebrate model organism for nanotoxicity tests, as it provides superior characteristics for microscopy-based screening and accounts for the complex interactions of MNMs with a living organism. A new OECD guideline for toxicity tests of chemicals (OECD (2013),Test No. 236: Fish Embryo Acute Toxicity (FET) Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing.) defines testing conditions and endpoints for comparable results. This guideline might be adapted for tests with MNMs. Recently, automated microscopy in conjunction with automated image analysis has facilitated quantification of various endpoints (e.g. lethality, hatching, malformations) upon MNM exposure. As a partner of the EU funded NanoMILE consortium we will develop a screening platform and carry out systematic toxicity screening for up to 100 MNMs by combining high throughput in vitro testing in cultured cells and in vivo whole organism exposure of zebrafish embryos with help of high content imaging and image analysis. The NanoMILE consortium will provide well characterized MNMs with various specific physico-chemical properties (chemical composition, size, charge, hydrophobicity, shape and morphology). For the first test phase nine metal oxide MNMs with a mean particle size of 10-140 nm were selected. Among these are e.g. four 10nm TiO2 nanoparticles with different coatings, allowing systematic tests of selected physical-chemical properties on toxicity. The large volume of data generated by this work will be instrumental to establish quantitative structure (property)-activity relationships (QS(P)ARs) and to connect observed impacts with the fate of the MNMs *in vitro* / *in vivo*. The results shall help to predict the toxicity of new MNMs to humans and the environment.

1 Manufactured Nanomaterials (MNM)

Metal Oxide	MNM	Size (nm)	Variable Phys-Chem Property
TiO_2	Surface-modified TiO_2 - surface 1 (Uncoated)	10	1st in series of 5 coatings
TiO_2	Surface-modified TiO_2 - surface 2 - PVP coated	10	Surface coating
TiO_2	Surface-modified TiO_2 - surface 3 - Pluronic F127 Coated	10	Surface coating
TiO_2	Surface-modified TiO_2 - surface 4 - Dispex AA4040 Coated	10	Surface coating
TiO_2	TiO_2 (rutile, hydrophilic) NM-104	20	Surface coating
CeO_2	Cerium(IV) oxide (Undoped)	20	Redox (doped versions in preparation)
CeO_2	Cerium(IV) oxide (precipitated, uncoated) NM-212	33	Size & Surface area
ZnO	ZnO Uncoated Hydrophilic (NM-110)	150	Coated and Uncoated Industrial ZnO
ZnO	ZnO TECS Coated Hydrophobic (triethoxycaprylyl silane) (NM-111)	140	Coated and Uncoated Industrial ZnO
Control			
Polystyrene	NH_2 modified polystyrene	52	Amino modified

First set of 9 metal oxide particles from NanoMILE.

Aims: Establish high throughput assays for *in vitro* and *in vivo* MNM toxicity screening.
Correlate nanomaterial toxicity with physico-chemical properties.
Help to predict toxicity for humans and the environment.

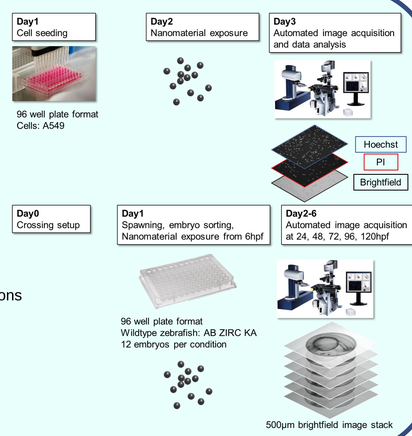
2 *in vitro* and *in vivo* models for MNM toxicity screening

A549 cells
- Human alveolar epithelial cell line
- First contact upon exposure by inhalation
- Established *in vitro* model for nanotoxicity

Zebrafish embryos (AB ZIRC KA wildtype)
- Rapid, extracorporal development with high degree of developmental homology to mammals
- Embryos are excellent specimen for microscopy (small, transparent)
- Transgenic reporter lines and mutants available
- Cost effective maintenance, high numbers of specimen
- Suitable for microscopy based high throughput *in vivo* screening
- Already used for drug screens, toxicity screens
- OECD guidelines available

3 Experimental Design

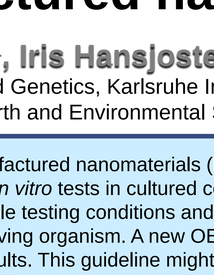
***in vitro* screening**
A549 cells, 24h exposure, 1-125µg/ml.
Staining with Hoechst 33342, Propidium iodide.
High throughput/content fluorescent imaging.
Automated image analysis with
ScanR software (Olympus) for cell death.

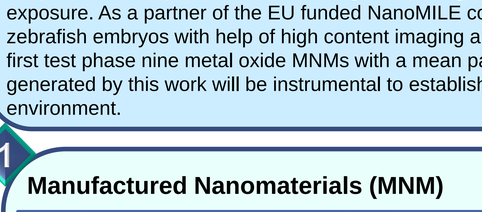


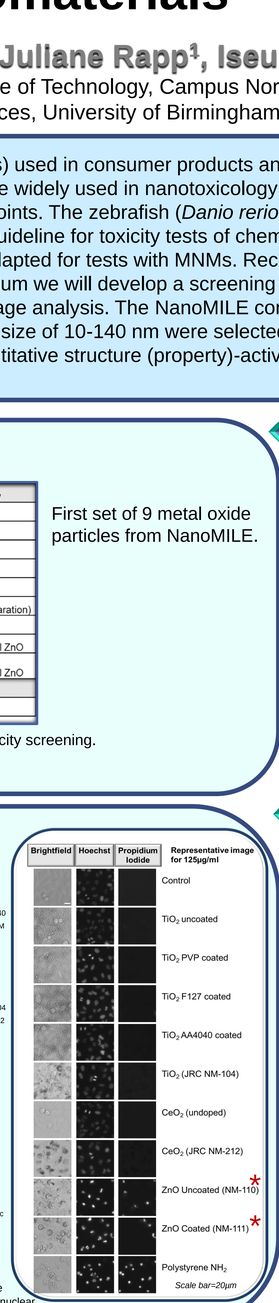
***in vivo* screening**
Wildtype zebrafish embryos and larvae,
24-120h exposure, 1 and 125µg/ml.
High throughput/content brightfield imaging
Manual image analysis for mortality, malformations and hatching.

4 Results *in vitro*

Only ZnO particles and the positive control (polystyrene NH_2) show cytotoxic effects, along with markedly reduced cell numbers at the highest concentrations.
Particle agglomerates are visible in brightfield images, except for CeO_2 (undoped) and polystyrene, indicating particle deposition on the cells.



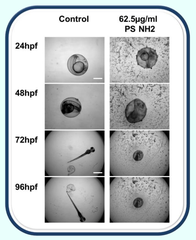




* Apoptotic hallmarks like membrane blebbing and nuclear fragmentation were reduced at 24h.

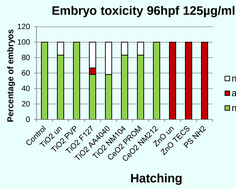
5 Results *in vivo*

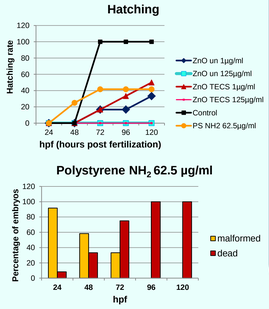
Agglomerates are visible for all particles at the higher concentration. Both ZnO particles block hatching completely at 125µg/ml and partially already at 1µg/ml, with no increase in mortality and malformations up to 96hpf.
Polystyrene NH_2 leads to a 90% malformation rate at 24hpf and 100% mortality at 96hpf. No obvious adverse effects could be observed for the other particles.

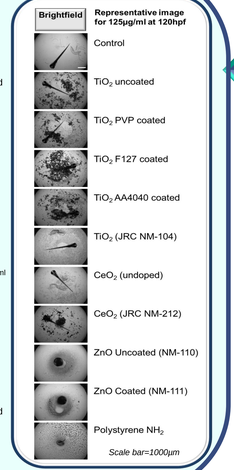


Comparison of a representative untreated and a polystyrene NH_2 treated (62.5µg/ml) embryo over time.

Scale bar at 24, 48hpf = 500µm; 72, 96hpf = 1000µm







6 Conclusions and Perspectives

- We have developed a high throughput screening assay for *in vitro* testing of MNM cytotoxicity.
- Development of a high throughput *in vivo* assay is on it's way.
- Of the 10 tested particles only the uncoated and TECS coated ZnO and amino-modified polystyrene particles showed adverse effects.
- First results show high similarities between toxicity of the MNMs *in vitro* and *in vivo*.
- Particles dispersed in cell and embryo medium will be characterized with DLS and TEM.
- Additional particles will be screened as soon as available.
- For those particles that show no effect on cells at 24h, extended exposure periods for the highest concentration are planned.
- Additional cell lines will be used for *in vitro* screening (HCT116).
- Zebrafish embryo sorting will be automated.
- For those particles that show an effect on embryos, dose response curves will be generated.

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