A common European approach to the regulatory testing of nanomaterials

Test item preparation, exposure, dose and fate for regulatory purposes and toxicology Deliverable 2.08

Introduction

In ecotoxicology and (submerged) *in vitro* testing, exposure to manufactured nanomaterials (MNM) occurs via dispersion into a liquid medium containing both inorganic and organic compounds. For a meaningful interpretation and comparison of data generated in such tests, it is important to understand the variation in physicochemical exposure-dose characteristics ("fate") during the experiment. Relevant parameters include (i) the dispersion stability / sedimentation rate and (ii) particle agglomeration/size-fractionation in the exposure medium, and (iii) MNM reactivity including their interaction with medium constituents and dissolution as well acid-base and redox activity. This deliverable reports on work carried out within Tasks 2.4 e, f and I, which also contributed to development of the NANoREG Technical Guidance Document (TGD) for (eco-)toxicological testing.

Description of Work

The work carried out in D2.08 encompassed the development and demonstration of procedures for:

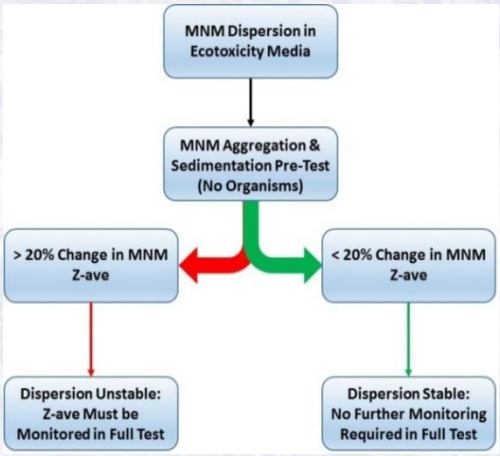
- Quantification of MNM **exposure and fate** in dispersions for **ecotoxicological** studies.
- Quantification of MNM **exposure and fate** in dispersions for ***in vitro*** studies.
- Characterization of MN **hydrochemical reactivity** in **synthetic biological fluids.**

Main results and evaluation

*Procedures for quantification of MNM **exposure and fate** in dispersions for **ecotoxicological** studies*

In this sub-task, the "NANoREG ECOTOX Dispersion Characterisation Technical Guidance Document (TGD)" was developed as a methods-supported framework requesting:

- Characterisation of the MNMs at the start and at the end of the test in order to identify potential changes in particle size and morphology over time.
- The total concentration of the MNM (C_{total} given in specific surface area (SSA) and mass-concentrations) present in the water phase of the exposure at both the start and at end of the experiment.
- The SSA and mass-concentration of both dissolved MNM and particulate MNM in the water phase (actual) at both the start and the end of the experiment.
- Estimation of the amount of test MNM (in both SSA and mass), which has either sedimented out of the water phase or adsorbed to the surfaces of the exposure system during the test.



Pre-test for aggregation and sedimentation

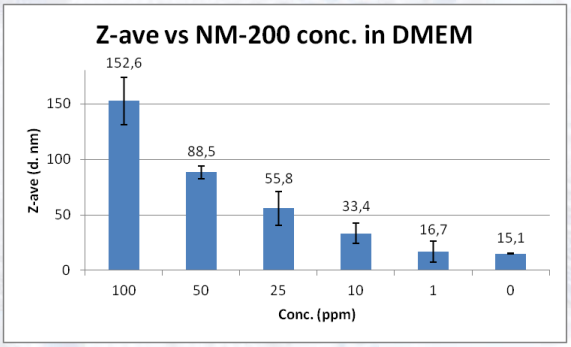
While D2.8 provides example data of using the ECOTOX TGD, its application in ecotoxicity studies in NANoREG WP4 clearly highlights the need for conducting detailed physicochemical characterisation of MNM dispersion exposures throughout the duration of an ecotoxicity test. Application of the ECOTOX TGD enables a clear and more reproducible decision-making process for exposure-fate characterisation in aquatic ecotoxicity tests.

*Procedures for quantification of MNM **exposure and fate** in dispersions for **in vitro** studies*

In this sub-task, procedures for providing the key physicochemical MNM exposure-dose characteristics requested in the NANoREG TGD for *in vitro* studies were developed and/or demonstrated. The procedures include:

- A centrifuge Liquid Sedimentation (CLS) and two Dynamic Light-Scattering (DLS) procedures to assess dispersion stability (plus zeta-potential by DLS) and size-evolutions during testing in cell culture mediums.

- A Proton Induced X-ray Emission (PIXE) procedure for quantification of deposited (elemental) dose to cells.
- An ELISA-procedure for quantifying the MNM-biomolecule interaction (LDH, IL-6 and IL-8) in cell culture mediums.
- A Sensor-Dish Reader (SDR) procedure for real-time measurement of the pH- and O_2-reactivity and offline determination of particle dissolution rate during in vitro testing.
- A method for measuring the dissolved fraction of MNM using Ultrafiltration Inductive Coupled Plasma Atomic Emission Spectrometry (UF/ICP-AES).



Z-ave sizes of dispersions of different concentrations of NM-200 in DMEM measured with DLS

Even-though CLS has a higher size-resolution than DLS, the DLS results obtained following the NANoREG TGD minimum characterization requests (initial and final hydrodynamic size in the test) are still useful. A proper MNM concentration should be selected when the media itself contain particles, because Z-ave diameter is strongly dependent from particles size and their relative amount. Employing a proper MNM concentration, the contribution to the total light scattered by the media particles can be neglected in favour to the one from MNM. DLS size-distributions should also be assessed and verified by qualitative electron microscopy imaging. Continuous DLS monitoring enables assessment of the temporal dispersion behaviour. Using the derived count rate to assess particle sedimentation rates showed that 50% of NM-110 (0.256 mg/mL RPMI with 0.2% glutamax) were deposited after 2.7 hours. For NM-400, only 25% were deposited after 8.4 hours. Quantification of deposited dose by PIXE is a more precise chemical dose determination, but it cannot be used for e.g., carbon. The effective PIXE dose can differ significantly from the nominal dose. A PIXE dose of only ~50% nominal dose was observed in a 72-hour test with NM-110.

Deposition times assessed from relative intensity of the derived count rate measured by DLS

Deposited fraction	Deposition time NM-110 [hour]	Deposition time NM-400 [hour]
25%	1.0	8.4
50%	2.7	16.4[€]
75%	4.5	24.4[€]
99%	6.2[£]	32.2[€]

[£]The suspension near the bottom may start to agglomerate and accumulate at the base of the vial. [€]Apparent deposition time projected from initial slope. The NM appears to form a suspended accumulation layer in the lower volume of test vial.

The SDR-procedure to identify MNM reactivity and dissolution during incubation was demonstrated and showed a serious increase in pH during the initial stages of ZnO (NM-110 and NM-111) dissolution in HAMs F12 cell medium. Incubation of Ag (NM-300K) was associated with important changes in the O_2 concentrations, but only minor changes in pH. The pH and O_2 concentrations were not affected during incubation with silica (NM-200), which however undergoes extensive dissolution. In the biomolecule interaction tests, important LDH and interleukin interactions were observed with different MNM under *in vitro* test conditions, which need attention. In conclusion, exposure-fate characterization is essential to understand MNM toxicological test results.

Procedures for characterization of MN ***hydrochemical reactivity*** *in* ***synthetic biological fluids***

In this task, two different example studies were shown to demonstrate the use of an:

- Atmosphere-Temperature-pH-controlled stirred batch reactor (ATempH-SBR) with online monitoring of redox potential (E_h) to assess the MNM reactivity and dissolution in a simulant phagolysomal fluid (PSF; pH 4.5).
- Acellular *in vitro* test procedure to assess the MNM behaviour in the different fluids encountered along the gastro-intestinal (GI) tract.

Demonstration of results obtained using the ATempH-SBR was made with NM-110 and NM-300K. NM-110 showed rapid dissolution and a short-term destabilization of pH and reduction on E_h. Dissolution of NM-300K was slower and resulted in a continuous reduction in E_h during extensive buffering with NaOH to maintain pH.

A comprehensive demonstration of exposure-fate and reactivity characterization was made using GI-fluids. Zeta-potential measurements clearly showed differences in particle charge with test mediums, which was also reflected in UV-vis and DLS size-measurements. TEM was needed to show morphological changes in the different fluids. Chemical analysis showed that the MNM dissolution in the different mediums varied with MNM type. Overall, it can be concluded that MNM are generally reactive and do not maintain their primary characteristics in liquid mediums. The changes affect the exposure characteristics which should influence on cellular permeability and uptake (for *in vitro* studies) and ADME parameters (in *in vivo* studies).

Zeta-pot of three MNM in synthetic human digestive compartments. CTRL refers to the Zeta-pot of MNMs pre-dispersed in 0.05% (v/v) BSA-water

	Nominal Charge (mV)	CTRL charge (mV)	Saliva (mV)	Stomach (mV)	Intestine (mV)
NM-110	-24.3	-20.6	-36.0	-9.0	-35.0
NM-200	-47.5	-28.0	-30.0	-10.0	-39.0
NM-300K	-11.0	-24.4	-35.0	-3.0	-32.0

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

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