A common European approach to the regulatory testing of nanomaterials

NANoREG

Grant Agreement Number 310584

Deliverable D 4.18

Overall evaluation of data, publication, and transfer to OECD sponsorship programme

Due date of deliverable: *2016/09/30*

Actual submission date: *2016/10/05*

Author(s) and company:	BAuA, Tom Gebel
Work package/task:	WP4 all
Document status:	draft / final
Confidentiality:	confidential / restricted / public
Key words:	

DOCUMENT HISTORY

Version	*Date*	*Reason of change*
1	2016/10/05	late comments from some partners and MC requests
2	2017/03/07	Project Office harmonized lay-out
3		
4		

This project has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 310584

Lead beneficiary for this deliverable:
BUNDESANSTALT FUER ARBEITSSCHUTZ UND ARBEITSMEDIZIN; BAuA, 3

Owner(s) of this document	
Owner of the content	NANoREG work package 4 (total)
Co-Owner 1	
Co-Owner 2	
Co-Owner 3	
…..	

Table of Content

1 Description of task

This deliverable summarizes the final results of all tasks included into NANoREG work package 4. It intends to provide a comprehensive overview on the in vivo experiments carried out in the NANoREG project to assess its regulatory significance.

2 Description of work & main achievements

2.1 Summary

2.1.1 Summary in brief

Conclusion and impact

The in vivo studies performed in NANoREG work package 4 so far have not provided evidence that the nanomaterials studied possess different hazardous properties compared to their analogous bulk materials. The established regulatory standard tests proved to be adequate also for nanomaterials. However, the used test protocols mostly needed some adaptation. There is some but no general evidence that the nanostructure may lead to a certain modulation of the hazard potency. There is no evidence that the established methodology for chemical hazard assessment is not applicable to nanomaterials.

NANoREG WP 4: Main Results	*Impact*
Hitherto no new 'nanomaterial toxicology' evident in in vivo testing	*Contributions to regulatory assessment of granular and fibrous nanomaterials*
Some indications for a refined evaluation for some materials	*Contributions to refined regulatory assessment and material developments safer by design*
Refinements for regulatory ecotoxicity testing methods established	*Basis for more valid result outcome in future regulatory testing*

Relevant commercial forms of nanosized material like carbon black, titanium dioxide or cerium dioxide may be subsumed under the category nanoscaled granular biodurable particles without known specific toxicity. There is evidence that such particles induce lung tumours in rats, possibly also at low exposure doses. Concerns were expressed on a putative long-term accumulation of these particles in the body also leading to toxic effects in other organs. A long-term inhalation study was carried out to study these issues. The currently available first set of results showed that cerium dioxide particles accumulate at a rather low level in the body. After one year of exposure, no resulting damage in organs aside from the lung was found. In the lung, inflammation was detected but no macroscopically visible tumours were found after 24 and 30 months. Further histological evaluation of the available study material will show whether lung tumours were induced also at the lower dust exposures used in the study.

Studies with fibrous nanomaterials found evidence that physical and chemical properties (surface area, surface oxidation, length and diameter) influenced toxicity. Nano- and bulk cellulose materials showed a relatively high toxicity and biopersistence.

A 90 day oral study with amorphous silicon dioxide emphasized low dose toxicity testing. Numerous endpoints were covered but no signs of overt toxicity were found.

Some first PBPK models for nanomaterials were developed which need further development to be usable in regulatory practice.

The performed ecotoxicity studies addressed methodological limitations in the standardisation of manufactured nanomaterials (MNMs) ecotoxicity testing and performed round robin-style testing. Within NANoREG, the existing OECD and ISO standard methods for ecotoxicity assessment using three priority species representing different trophic levels have been adapted specifically for MNM testing and developed into defined Standard Operational Procedures (SOPs). Silver nanoparticles showed a high ecotoxic potency. A lower ecotoxicity was generally observed in the test systems for MWCNTs and titanium, cerium, silicon and zinc oxide MNMs.

2.1.2 Extended Summary

NANoREG WP 4: Main Results	*Impact*
So far no evidence for nano-specific toxicity/ new toxicity endpoints in in vivo testing	*Contributions to adequate regulatory assessment of granular and fibrous nanomaterials*
Systemic low level accumulation of granular CeO_2 nanomaterial did not lead to systemic toxicity so far	*Contributions to adequate regulatory assessment of granular nanomaterials*
No macroscopically visible lung tumours after 24 and 30 months with nano-$BaSO_4$ and including low dose exposures of nano-CeO_2	*Contributions to adequate regulatory assessment of granular nanomaterials*
Physical and chemical properties (surface area, surface oxidation, length and diameter) influenced toxicity of fibrous nanomaterials	*Contributions to safer by design approaches for fibrous nanomaterials*
Biopersistent nano- and bulk nanofibrillated cellulose materials showed a relatively high toxicity and biopersistence.	*Contributions to adequate regulatory assessment of biopersistent nano- and bulk nanofibrillated celluloses*
Fibrillated celluloses: the outcome of the in vivo toxicity tests was not consistently predicted by in vitro toxicity studies	*Contributions to adequateness of in vivo/in vitro test comparison*
No marked and clearly dose-dependent effects after low oral doses of amorphous silicon dioxide	*Contributions to adequate regulatory assessment of amorphous silicon dioxide*
No prenatal toxicity in studies with cerium dioxide, amorphous silicon dioxide and multi-walled carbon nanotubes	*Contributions to adequate regulatory assessment of these materials*
Methodological limitations in the standardisation of manufactured nanomaterials (MNMs) ecotoxicity testing addressed	*Basis for more valid result outcome in future regulatory testing*
Silver nanoparticles showed high ecotoxic potency. A lower ecotoxicity was generally observed for MWCNTs and titanium, cerium, silicon and zinc oxide MNMs.	*Contributions to adequate regulatory ecotoxicity assessment of these materials*

A combined chronic/carcinogenicity whole body inhalation study was performed according to OECD TG 453 with several protocol extensions. Female rats (n=100/group) were exposed to cerium dioxide (NM-212, 0.1; 0.3; 1; 3 mg/m³) and barium sulfate (NM-220; 50 mg/m³) for 24 months. 50/100 animals were kept a further 6 months exposure-free. The aim was to investigate lung carcinogenicity and putative systemic effects of low-dose exposures to biopersistent nanoparticles. The histological examinations are ongoing, results are available from the 12-months interim section. No macroscopically visible tumours were detected at the 24 months and 30 months sacrifices.

After 12 months, CeO_2 exposure-related histopathological findings were exclusively observed in the respiratory tract but not systemically. Adverse effects in the lung included alveolar/interstitial inflammatory cell infiltration, granulomatous inflammation and interstitial fibrosis. Alveolar lipoproteinosis was observed in the 3 mg/m³ high-dose CeO_2 exposure group

only. After 12 months of inhalation exposure neither neoplastic nor pre-neoplastic treatment-related findings were seen in the lungs of CeO_2-exposed animals.

Although statistically not significant, some adverse effects such as alveolar/interstitial inflammatory cell infiltration, granulomatous inflammation, and interstitial fibrosis have already been observed in the 0.1 mg/m³ low-dose CeO_2 exposure group.

The CeO_2 nanoparticles (NPs) were found mainly in macrophages and alveolar septum. NPs were also detected in endothelium of blood vessels and in close vicinity of nucleus in pneumocytes. NPs were able crossing epithelium and endothelium barriers of alveoli and being transferred into other organs.

Independent on the exposure concentration the CeO_2 the lung burden increased from 3 to 24 months in a linear manner with a factor of ~5-7 including the concentrations below overload. The CeO_2 burden of liver, kidney, spleen, brain, heart, lymph nodes, bone and olfactory bulb was generally very low. In brain, maximum CeO_2 levels were 0.005 µg/g tissue which is a factor of 700000 below the lung burden. There was no evidence for systemic toxicity in the interim section after 12 months including the lung-associated lymph nodes although the cerium levels were relatively high in this tissue.

10 commercial different non-rigid short (average length <5 µm) high aspect ratio materials have been tested after deposition of three doses in the lungs. There was no evidence of genotoxic effects in livers and spleens, or acute phase response in plasma. There was no evidence of MWCNT fibrogenicity. Remarkably nanofibrillated celluloses were rather inflammogenic and persistent in mouse lung. The inflammatory responses in mice and in rats were strongly correlated. Some HARN materials were more inflammogenic and genotoxic than others. High specific surface area (BET) and low diameter was identified as a predictor of increased pulmonary inflammation. In addition, length significantly predicted pulmonary inflammation, whereas surface oxidation (-OH and -COOH) was a predictor of lowered inflammation. BET surface area, and therefore diameter size, significantly predicted genotoxicity in BAL fluid cells and lung tissue.

In inhalation experiments with 2 pristine MWCNT, "long and thick" NM-401 and "short and thin" NM-403, NM-403 was more inflammogenic than NM-401. Since NM-403 had a 10-fold higher specific surface area than NM-401, these results were in agreement with those obtained by pulmonary instillation. Despite the persistent presence of carbon nanotubes in lung tissues, no significant histopathological changes were observed. The results may be helpful for the development of safer HARN materials.

A repeated-dose 90-day oral toxicity study in rat with amorphous silica (SiO_2) on the basis of OECD guideline 408 did not find marked and clearly dose-dependent effects after oral doses of maximally 50 mg/kg bw per day.

In prenatal toxicity studies with cerium dioxide, amorphous silicon dioxide and multi-walled carbon nanotubes, no overt toxicity in terms of miscarriage or malformations was found.

PBPK models for inert NPs (non-pegylated polyacrylamide, gold, titanium dioxide) and a PBPK model for inhalation exposure to cerium dioxide NPs were developed. Further development is needed especially with respect to regulatory use.

The performed ecotoxicity studies addressed methodological limitations in the standardisation of manufactured nanomaterials (MNMs) ecotoxicity testing and attempted to address the reproducibility of such studies through round robin-style testing. Within NANoREG, the existing OECD and ISO standard methods for ecotoxicity assessment using three priority species repre-

senting different trophic levels have been adapted specifically for MNM testing and developed into defined Standard Operational Procedures (SOPs). Silver nanoparticles showed a high ecotoxic potency. A lower ecotoxicity was generally observed in the test systems for MWCNTs and titanium, cerium, silicon and zinc oxide MNMs. In general, comparable findings were observed for the same MNM types with the same species by different partners. Despite a standardised MNM dispersion method and careful attention to the SOPs, some variation in EC50 values was determined between partners conducting the same tests, indicating the difficulty in achieving fully reproducible ecotoxicity data with MNMs.

Immunotoxic and genotoxic effects of biopersistent nanofibrillated celluloses differed among the four materials studied. Effects were also seen with the bulk-sized cellulose studied. The outcome of the in vivo toxicity tests was not consistently predicted by in vitro toxicity studies performed with the same materials.

2.2 Background of the task

See respective chapters in DOW or respective task reports

2.3 Description of the work carried out

See respective specific deliverable reports or task reports

2.4 Results

2.4.1 Task 4.1 Inhalation: Nano in vivo: chronic and carcinogenicity study testing GBP nanomaterials.

A combined chronic/carcinogenicity whole body inhalation study was performed according to OECD TG 453 with several protocol extensions. Female rats (n=100/group) were exposed to cerium dioxide (NM-212, 0.1; 0.3; 1; 3 mg/m³) and barium sulfate (NM-220; 50 mg/m³) for 24 months. 50/100 animals were kept a further 6 months exposure-free until terminal sacrifice. A control (n=100) was exposed to clean, filtered air in parallel. The aim is to investigate lung carcinogenicity and putative systemic effects of low-dose exposures to biopersistent nanoparticles.

2.4.2 Task 4.2 Long term effects of nanomaterials: systemic toxicity (histopathological evaluation).

The histological examinations are ongoing, results are available from the 12-months interim section. There was some evidence that an inflammation-mediated secondary local genotoxicity in the lung could not be excluded at the higher exposure concentrations. On the other hand, CeO_2 inhalation exposure did not induce any significant effect on the analysed systemic genotoxicity endpoints, irrespective of dose and time. CeO_2 exposure-related histopathological findings were exclusively observed in the respiratory tract but not systemically. In the nasal cavity, the incidence of age-related intra-epithelial eosinophilic globules was increased in the 3 mg/m³ high-dose CeO_2 exposure group as compared to the control group and associated with minimal inflammatory cell infiltration. Adverse effects in the lung included dose-dependent alveolar/interstitial inflammatory cell infiltration, alveolar/interstitial granulomatous inflammation and interstitial fibrosis. Alveolar lipoproteinosis was observed in the 3 mg/m³ high-dose CeO_2 exposure group only and cholesterol granulomas occurred in a single female each CeO_2 the 1 and 3 mg/m³ CeO_2 exposure groups. After 12 month of inhalation exposure neither neoplastic nor pre-neoplastic treatment-related findings were seen in the lungs of CeO_2-exposed animals. No macroscopically visible tumours were detected at the 24 months and 30 months sacrifices.

Although statistically not significant, some adverse effects such as alveolar/interstitial inflammatory cell infiltration, alveolar/interstitial granulomatous inflammation, and interstitial fibrosis have already been observed in the 0.1 mg/m³ low-dose CeO_2 exposure group. Thus, a NOAEL (no observed adverse effect level) could not be established for the lung after 12 months of exposure to the present CeO_2 nanoparticle concentrations. Independent on the exposure concentration the CeO_2 the lung burden increased from 3 to 24 months with a factor of ~5-7 including the concentrations below overload despite accumulation over exposure time.

There was no evidence for systemic toxicity in the interim section after 12 months including the lung-associated lymph nodes although the cerium levels were relatively high in this tissue.

2.4.3 Task 4.3 Organ burden quantification.

Summary of work

The CeO_2 (NM-212) burdens in peripheral organs and faeces out of the 2 year chronic inhalation study (see task 4.1) was investigated.

Overview of main results

The lung burdens were maximally 3500 µg/g lung at the highest exposure concentration of 3 mg/m³. Independent on the exposure concentration the CeO_2 the lung burden increased from 3 to 24 months in a linear manner with a factor of ~5-7 including the concentrations below overload. The CeO_2 burden of liver, kidney, spleen, brain, heart, bone, and olfactory bulb was generally very low (far less than 1 µg/g organ). The highest CeO_2 burdens in organs remote to the exposed organ lung was bone, liver and spleen with maximally 1.9, 1.4, and 1 µg/g tissue. In brain, maximum CeO_2 levels were 0.005 µg/g tissue which is a factor of 700000 below the lung burden. Only in tracheobronchial and mediastinal lymph nodes, high levels of CeO_2 were found with maximally 25 mg/g tissue. This may be due to the fact that these lymph nodes drain the lung. CeO_2 concentrations in blood were on a very low level of < 0.1 ng/ml. As expected, increasing burden in lymph nodes was detected from month 3 to month 12 and from month 12 to month 24.

2.4.4 Task 4.4 Pattern of particle distribution in organs.

Summary of work

Intracellular uptake and distribution pattern of CeO_2 nanoparticles (NPs) were monitored in rat lung tissues following chronic exposure of Wistar rats to aerosolized CeO_2 by the following label-free molecule and element based imaging techniques: Ion beam microscopy (IBM), Confocal Raman microscopy (CRM) and Time-of-Flight secondary ion mass spectrometry (ToF-SIMS).

Ion beam microscopy, an element based imaging technique, comprises two methods: micro-Rutherford backscattering (µRBS) and micro-Proton induced X-ray emission (µPIXE). These methods were simultaneously applied to each pixel of tissue to visualize the NPs in their biological environment and to quantify NP uptake at single cell level. Additionally, CRM and ToF-SIMS were used to monitor translocation and co-localization of NP with different cell types and biomolecules in rat lung.

Translocation pattern of NPs, their co-localization with different types of cells and biomolecules at submicron resolution were analysed by CRM. The average concentration of NPs in the alveolar septum of single alveoli was quantified and compared with that from a 28 days study.

Overview of main results

The tissues studies stemmed from the chronic study on inhalation toxicity and carcinogenicity of CeO_2.

Lung tissue of Wistar rats showed an inhomogeneous distribution of NPs following 2 years of chronic CeO_2 inhalation. The CeO_2 particles were found mainly in macrophages and the alveolar septum. Some NPs were also detected in the endothelium of blood vessels and in close vicinity of the nucleus in pneumocytes. Some NPs were able to cross epithelial and endothelial barriers of alveoli and showed translocation into other organs.

The average concentration of NPs in the alveolar septum was measured in lung tissue from animals out of the second lowest dose group (0.3 mg/m^3 CeO_2). A value of ca. 400 ppm was achieved, which is by a factor of 3 below the average concentration of NPs in lung tissue out of

the highest dose group (25 mg/m^3) of a subacute 28 day study. A long-term accumulation of NP agglomerates in macrophages, alveolar septum, epithelial and endothelial layers reflects a reduced lung clearance over the study duration of two years. The intracellular intrinsic Fe concentration in the alveolar septum was increased remarkably in cells which have taken NPs up. The intracellular content of Fe is known as a marker for inflammation.

2.4.5 Task 4.5 Other Biokinetic and oral, dermal, inhalation, toxicity studies in vivo.

Task 4.5.1 Biological effects of pulmonary deposition of HARN in rats.

Task 4.5.2 Biological effects of inhalation of HARN in rats.

Summary of work

10 different commercial non-rigid short (average length< 5 µm) HARN materials have been tested after deposition of three doses in the lungs. C57BL/6J female mice by pulmonary instillation and different biological effects are studied 1 day, 28 days and 3 months after instillation. For a subset of HARN mice have been followed for a year.
Rats were exposed during 28 days with a 6 month follow-up. They were exposed by nose only inhalation, 2x3h/day; 5 days/week for 4 weeks to filtered air or 2 concentrations of 0.5 and 1.5 mg/m^3 of pristine MWCNT, "long and thick" NM-401 and "short and thin" NM-403.

Overview of main results

All high aspect ratio nanomaterials caused pulmonary inflammation. There was no significant increase in tumours, serious morbidity or mortality. Histopathological examination demonstrated effects in lungs such as increase in lymphocytic and macrophagic infiltration compared to controls. There was no evidence of genotoxic effects in livers and spleens, or acute phase response in plasma. Nanofibrillated celluloses (NFCs) were inflammatory also at lower doses and they persist in the lung for a relatively long time. The inflammatory responses in mice and in rats were strongly correlated. Some HARN materials are more inflammogenic and genotoxic than others. Remarkably cellulose nanomaterials are rather inflammogenic and persistent in mouse lung.

Inflammation persisted on day 92 for the thin (14-17 nm) MWCNT. Using adjusted, multiple regression analyses, specific surface area (BET) was identified as a predictor of increased pulmonary inflammation on all post-exposure days. In addition, length significantly predicted pulmonary inflammation, whereas surface oxidation (-OH and -COOH) was predictor of lowered inflammation on day 28. BET surface area, and therefore diameter size, significantly predicted genotoxicity in BAL fluid cells and lung tissue. After one year a few focal fibrotic lesions,and not progressive fibrosis was observed. Inflammatory activity in the lungs after exposure to MWCNT was persistent up to one year by lymphocytic aggregates. This study provides information on possible toxicity driving physicochemical properties of MWCNT. The results may contribute to safe-by-design manufacturing of MWCNT, thereby minimizing adverse effects (Poulsen et al. Nanotoxicology 2016; 10(9): 1263–1275).

After one year a few focal fibrotic lesions, and not progressive fibrosis was observed. Inflammatory activity in the lungs after exposure to MWCNT was persistent up to one year by lymphocytic aggregates.

In the inhalation experiments, NM-403 was more inflammogenic than NM-401. Since NM-403 had a 10-fold higher specific surface area than NM-401, these results were in agreement with those obtained by pulmonary instillation. It is unclear whether NM-401 and NM-403 are able to induce significant DNA strand breaks in the lung or other organs such as spleen or liver. Despite the persistent presence of carbon nanotubes in lung tissues, no significant histopathological changes were observed.

2.4.5.3 Task 4.5.3 Escalation dose pharmacokinetic study.

Task has been discontinued.

2.4.5.4 Task 4.5.4 In vivo genotoxicity of MNM.

Task has been integrated into task 4.5.5.

2.4.5.4.1 Task 4.5.4.1 Pattern of particle distribution in vitro and in vivo.

The task underlying the deliverable initially intended to serve as to correlate the results of the in vivo subtask 4.5.4 (initially in vivo genotoxicity testing on titanium dioxide) to respective in vitro experiments. The aim was to assess the relevance of systemic genotoxicity and particle distribution. These structural investigations aimed to relate particle occurrence and genotoxicity. The in vivo genotoxicity testing was later included in the 90-day oral study with amorphous silicon dioxide. Due to high background of silica, particle occurrence could not be investigated using the in vivo samples. Thus, the task switched to perform in vitro studies with TiO_2. In vitro exposure has shown that nanoparticles can accumulate in intestinal Caco2 cells remaining even after a recovery period. The TiO_2 nanoparticles are mostly segregated in vesicles without detection inside the nucleus at any time point and concentration investigated.

2.4.5.5 Task 4.5.5 Repeated dose 90-day oral toxicity study.

Summary of work

A repeated-dose 90-day oral toxicity study in rat with amorphous silica (SiO_2, SAS NM-203) was performed on the basis of the OECD guideline 408. Additional parameters were included for the hazard characterization (as in OECD TG 407). In particular, the following parameters have been evaluated: biodistribution in target tissues, histopathological analysis serum biomarkers, genotoxicity including germ cell genotoxicity, reproductive toxicity, and immunotoxicity. The results from the study were used to identify a Benchmark Dose lower confidence limit (BMDL). The traditional NOAEL approach is also used for regulatory purposes.

Overview of main results

The administration of SAS NM-203 via oral gavage in rats at the following dose levels – 2, 5 10, 20 and 50 mg/kg bw per day – caused no signs of overt toxicity in both sexes, at any dose levels during the treatment period. For all the endpoints evaluated up to now, it is evident the difficulty of defining a clear dose-response relationship. Although the dose-responses were not linear for most parameters, BMDs were calculated which showed high confidence limits. The BMD of 0.468 mg/kg bw per day for decreased lymphocytes in blood count in male rats and for female rats the BMD of 0,079 mg/kg bw per day for intralobular lymphoid infiltration in liver was suggested for deriving a Reference Point.

2.4.5.6 Task 4.5.6. Prenatal toxicity study.

Summary of work

Pregnant mice have been exposed to different concentrations of three materials. Cerium dioxide (CeO_2 JRCNM02102a 5 and 20 mg/kg), multiwalled carbon nanotubes (MWCNT JRCNM04001a 200 and 800 mg/kg oral gavage), and amorphous silicon dioxide were applied

through two different routes: via the oral route for carbon nanotubes and amorphous silicon dioxide, and via the pulmonary route for cerium dioxide.

Overview of main results

Maternal effects, characterized by a lower weight gain during gestation were observed in mice exposed to multi-walled carbon nanotubes at the highest dose. No overt toxicity in terms of miscarriage or malformations was found. The data suggest a lack of relevant embryo toxicity.

2.4.5.7 Task 4.5.7 Acute and repeated nose-only inhalation toxicity study.

Summary of work

Rats were exposed with one single intratracheal instillation to 0; 0.5; 50 or 500 µg/rat and sacrificed 3h, 24h, 35 days and 90 days after the treatment. Test materials used were TiO_2 (NM105: 21 nm, NM101: 6 nm, NM100: 100 nm) and CeO_2 (NM212: 28 nm). Initial lung burden and pulmonary clearance were evaluated by measuring the Ti or Ce content in lungs by ICP-MS. Several endpoints linked to cytotoxicity, inflammation, oxidative stress and genotoxicity were assessed on lungs. Histopathology of the lungs was performed on rats sacrificed one and three months after exposure (results available only for TiO_2 NM100 and 101).

Overview of main results

For the highest dose (500 µg/rat), all NM were detected in the tracheobronchial lymph nodes after 35 and 90 d. There was no significant systemic distribution in liver, kidneys and spleen. No marked effects were seen for all tested NM regarding ROS production. Considering overall pro-inflammatory effects (assessed by BAL cellularity and cytokine dosage in BALF), lung inflammation was more pronounced for TiO_2 NM105 (21 nm), TiO_2 NM101 (8 nm) and CeO_2 NM212 (29 nm) than for TiO_2 NM100 (100 nm). To conclude, considering the overall short-term toxicity, the relative ranking was NM105~NM101~NM212>NM100. Pulmonary instillation of all tested NM did not induce the formation of micronuclei in blood polychromatic (immature) erythrocytes. Histopathological evaluation is on-going for TiO_2 NM100 and 101.

After 24h exposure, for the titanium materials but not for CeO_2, there was some indication that pro-inflammatory effects seemed to show a potency ranking similar to *in vitro* testing (air-liquid interface technique, monoculture (A549) or co-culture with alveolar macrophages (A549 + THP-1)) based on comparing mass concentration/surface area.

2.4.5.8 Task 4.5.8 Development of PBPK models.

Summary of work

A PBPK model originally developed for intravenously injected pegylated polyacrylamide (PAA-PEG) NPs in rats (Li et al., 2015) was expanded to other inert NPs (non-pegylated PAA, gold, titanium dioxide). In addition, a PBPK model for inhalation exposure to cerium dioxide NPs has been developed. These models are useful to understand and predict the relation between the exposure to nanoparticles and their fate in the body, including how body burden and accumulation may relate to a various exposure scenarios.

Overview of main results

The PBPK model for intravenously injected nanoparticles adequately describes the biodistribution of four different types of nanoparticles (pegylated PAA, PAA, gold and titanium dioxide). The model is a step forward towards a general nano-PBPK model for intravenously exposed nanoparticles. The results from simulation of a biodistribution study in rats exposed nose-only to cerium dioxide for 4-6 h seem to adequately describe the biodistribution kinetics. The model includes mucociliary clearance, olfactory uptake, phagocytosis, and entry into the systemic circulation by alveolar wall penetration. The PBPK model described the biodistribution well and again suggested phagocytosis to be very important. The findings make clear that nanoparticles require new PBPK models based on different physiological parameterization than known for molecular equivalents of these particles. It stresses that kinetics of (nano)particles is handled differently by the body than molecular forms. Before application in the regulatory setting, the physiological parameterization needs to be elaborated and validated.

2.4.6 Task 4.6 Biokinetics and toxicity in aquatic organisms.

Task 4.6.1 Toxicity studies in aquatic systems.

Task 4.6.2 Biokinetic studies in aquatic systems.

Summary of work

The performed ecotoxicity studies addressed the methodological limitations in the standardisation of manufactured nanomaterials (MNMs) ecotoxicity testing, developed modified SOPs, and attempted to address the reproducibility of such studies through round robin-style testing.

Overview of main results

Significant advances have been made which are described below. Three priority species representing different trophic levels have been selected for testing with NANoREG core MNMs: the unicellular green algae *Pseudokirchneriella subcapitata*, the crustacean *Daphnia magna* and the soil nematode *Caenorhabditis elegans*. Existing OECD and ISO standard methods for ecotoxicity assessment have been adapted specifically for MNM testing and developed into defined Standard Operational Procedures (SOPs). One of the key issues in ecotoxicity testing of MNMs is their reproducible dispersion in the different culturing media required for each organism. A specific ecotoxicity dispersion SOP has been prepared in collaboration with WP2, which includes the optional addition of environmentally representative concentrations (10 mg/L) of Suwannee river natural organic matter (SR-NOM) to the culturing media where MNM dispersions are unstable. An increase in the dispersion stability of several MNMs (MWCNTs, CeO_2 NPs and TiO_2 NPs) has been observed after this addition of SR-NOM, leading to a corresponding improvement in reliability and reproducibility of the ecotoxicity test results.

Silver nanoparticles showed a high ecotoxic potency, and lower ecotoxicity was generally observed in the test systems for MWCNTs and titanium, cerium, silicon and zinc oxide MNMs.

2.4.7 Task 4.7 Acute immunotoxic and genotoxic effects of fibrous nanomaterials.

Summary of work

The main aim of this task is to evaluate the acute immunotoxic and genotoxic effects of nanofibrillar cellulose (NFC) samples provided by two industrial partners, Stora Enso and UPM-Kymmene. Altogether four gel-form NFC samples (fibril diameter 2-15 nm; length several micrometers) and a bulk-sized cellulose were included in the study. As inhalation was considered to be the most relevant route of exposure, the experiments focused on pulmonary effects in mice *in vivo* using pharyngeal aspiration. Biological samples were collected 24 h after the exposure for acute effects and 28 days later for subacute effects.

Overview of main results

NFC distribution in the lungs appeared to depend on the size of the material, but all materials studied were biopersistent for the 28-day observation period. The toxic effects observed differed among the four NFCs studied, but effects were also seen with the bulk-sized cellulose. However, one NFC was not genotoxic. The outcome of the *in vivo* toxicity tests was not consistently predicted by *in vitro* toxicity studies performed with the same materials in WP5.

2.5 Data management

See respective task deliverable reports

3 Deviations from the work plan

See respective task deliverable reports