A common European approach to the regulatory testing of nanomaterials

NANoREG

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Deliverable D4.1

Initiate and perform in-life long term inhalation study

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1 Description of task

Task 4.1 of the NANoREG project serves to experimentally study hypotheses on the assumed mode of action of selected nanomaterials. It aims at clarifying essential questions in the risk assessment of granular biopersistent particulate nanomaterials (GBP). As inhalation is considered to be the most relevant route of exposure, a chronic study was performed using inhalation. An OECD TG 453 compliant study, conducted with OECD depository materials (NM-212 CeO_2 and NM-220 $BaSO_4$) under GLP, focuses on investigating a putative inhalation carcinogenicity of GBP nanomaterials in low dose exposures. This study offers relevant information not only for occupational but also for environmental and consumer health. Moreover, systemic distribution and systemic toxicity will be studied as well.

2 Description of work & main achievements

2.1 Summary

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. A control (n=100) was exposed to clean, filtered air in parallel. The 24-month exposure period was successfully terminated and 50 animals per dose group sacrificed. The remaining animals are kept exposure-free for maximally 6 additional months.

Up to 24 months exposure to both nanoparticles did not lead to body weight reduction. The mortality rates were in an acceptable range. Macroscopically evident tumours were not detected after 24 months of exposure.

This deliverable describes the background, design and performance of the inhalation test. Analyses regarding particle deposition and their systemic distribution, Lung carcinogenicity and putative systemic effects will be studied in 2016 and 2017. Further results will be reported in deliverables D4.5 [M36], D4.6 [M36], and D4.7 [M42]

2.2 Background of the task

In the last years, there has been an emphasis on the experimental testing of nanomaterials using in vitro and in vivo approaches. However, several essential questions on nanomaterial toxicology cannot yet be clarified by such approaches. One of these questions is a putative carcinogenicity of nanomaterials. Due to reasons of feasibility it is not possible to test each single nanomaterial for carcinogenicity. Grouping approaches for safety testing can be chosen in case a common mode of action is known.

A relevant group of nanomaterials are likely to share a common mode of carcinogenic action. These nanomaterials belong to a group of materials named poorly soluble, low toxicity particles (PSLT) (Dankovic et al. 2007), poorly soluble particles of low cytotoxicity (PSP) (Oberdorster 2002) or respirable granular biodurable particles without known significant specific toxicity (GBP) (Roller and Pott 2006). All terms describe the same type of materials. Industrial-relevant nanomaterials like carbon black or titanium dioxide belong to this group. There is a current scientific controversy, whether the lung tumours detected in chronic rat inhalation studies induced by PSLT only appear at high exposure concentrations (i.e. so-called dust ‘overloading’ of the lungs) associated with inflammation. According to the overload hypothesis, in lower (and real-life) exposure levels there is no dust overloading and no inflammation in the lung and consequentially no tumour risk in case an exposure threshold is not exceeded. Several authors (e.g. (Morrow 1992) describe that dust overloading in the rat becomes evident in respirable dust concentrations higher than 1 mg/m³ in a chronic study.

Further up to now unclarified aspects with respect to putative health hazards of nanomaterials will be studied. This comprises the systemic distribution of particles after chronic inhalation exposure and a putative

accumulation in tissues like brain or the cardiovascular system and putative adverse effects associated with this chronic accumulation.

Long-term exposure to biopersistent, poorly soluble nanomaterials and possible carcinogenicity induced thereof, has been identified as one of the major data gaps for regulatory decision process in the field of nanomaterials (Becker *et al.* 2011). While an increasing number of short-term data becomes available, long-term inhalation studies according to GLP and OECD TG guidelines in rodents are technically demanding and the resources needed require a high investment. Only two nanomaterials, titanium dioxide and carbon black, have been tested so far in rodent inhalation carcinogenicity studies. In these studies tumours in rats were obtained at higher doses (Heinrich *et al.* 1994; Heinrich *et al.* 1995b; Nikula *et al.* 1995).

The limited data as well as the uncertainty of the role of inflammation and overload (ILSI 2000) in the process of tumour development when exposed to poorly soluble nanomaterials, hinder any regulatory decision on how to evaluate the long-term risk of nanomaterials. Uncertainty on mode of action in the respiratory tract and the possibility of translocation to extra-pulmonary organs do not allow a final evaluation using short-term studies only. Insight into mechanistic hypothesis, i.e. the role of inflammation and / or overload has to be addressed with a long-term study to gain regulatory insight into model substances and allow a proper future risk evaluation. Well characterized material and study design selection is crucial for the success of the project to serve as basis for regulatory decisions. Furthermore, based on the rare incidence of lung tumours the study design has to be adapted to gain sufficient statistical power. An OECD TG 453 compliant study conducted with OECD depository material under GLP was carried out and will provide insight into general principles and allow an exemplary risk assessment to be conducted as well as evaluation of the role that inflammation and overload play in the formation of tumours in the lung.

The study serves to experimentally verify the ‘overload’ hypothesis. It aims to clarify an essential question in the risk assessment of an industrial relevant group of particulate nanomaterials. Only few representative materials have to be tested and the results can be cross-read to other PSLT nanomaterials. For the first time, relevant results for low dose exposures will be available for nanomaterials for which the mode of possible carcinogenic action is determined exclusively by dust toxicity. As the study focuses on investigating a putative inhalation carcinogenicity of PSLT nanomaterials in low dose exposures, this study offers relevant information not only for occupational but also for environmental and consumer health.

The chosen approach will help to clarify additional aspects with respect to putative health hazards of nanomaterials, i.e. the systemic distribution of particles after chronic inhalation exposure and a putative accumulation in tissues like brain or the cardiovascular system and putative adverse effects associated with this chronic accumulation.

2.3 Description of the work carried out

2.3.1 *Study objectives*

The objective of this combined chronic inhalation toxicity and carcinogenicity study is to determine the effects of two nanoparticles NM-212 CeO_2 and NM-220 $BaSO_4$ in female Wistar rats following prolonged and repeated whole-body inhalation exposure. The application of the OECD TG 453 guideline should generate data which identify the majority of chronic and carcinogenicity effects and determines concentration-response relationships. The design and conduct should allow determination of the carcinogenic potential as well as general toxicity, including physiological, biochemical, and hematological effects and exposure-related morphological (pathology) effects after chronic exposure (12 month).

CeO_2 was selected as model substance at low, mid and high dose level based on its effects in kinetic and short-term studies, its inert properties and its commercial relevance (Keller *et al.* 2014; Konduru *et al.* 2014; Molina *et al.* 2014). $BaSO_4$ was initially selected as negative control at one high dose level. Data for $BaSO_4$ were available from the NanoCare project using a short-term inhalation study (NanoCare, 2009 (Landsiedel *et al.* 2014)). $BaSO_4$ is considered to serve as example substance for low inflammation effects and positive control for overload, based on preliminary data, which indicate no or very low inflammation (Landsiedel *et al.* 2014); Cullen et al. 2000; Tran et al. 2000) even at overload concentrations.

2.3.2 *Study protocols*

The conduct of inhalation exposures will be performed according to the following test guideline concerning repeated dose inhalation toxicity studies:

- Organization for Economic Cooperation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, No. 413 "Sub-Chronic Inhalation Toxicity: 90-day Study" adopted 07 September 2009.

In addition the study was carried out taking into account the following guidelines:

- Organization for Economic Cooperation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Method 453 "Combined Chronic Toxicity/Carcinogenicity Study in Rodents" adopted 07 Sep 2009.
- Commission Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), Part B.33.: Combined Chronic Toxicity/Carcinogenicity Test
- US Environmental Protection Agency (EPA), Health Effects Test Guidelines OPPTS870.4300, Combined Chronic Toxicity/Carcinogenicity, EPA 712-C-98-212, August 1998

In deviation to the guidelines, only females were exposed, because female rats are considered to be slightly more sensitive concerning carcinogenicity after inhalation exposure to dust aerosols (Nikula et al. 2000).

The chronic study was started with 100 rats per dose group. 50 animals per dose group were sacrificed after 24 months. The remaining animals are currently kept exposure-free till natural death or till month 30. The intention for this extension is to enhance study sensitivity. It is known that a relevant portion of particle induced tumours become detectable first rather late in rats. The study sensitivity to detect lung tumours will be further enhanced by an extended lung histopathology as 60 instead of 6 slices will be studied per lung.

Satellite groups were sacrificed after 12 months (chronic group with 10 animals per dose for histopathology) and after 3 months, 12 months, and 24 months (for kinetic/organ burden evaluations). Results for 3 months have been reported in deliverables .4.3 and 4.4.

Main exposure groups are used for histopathology examinations (carcinogenicity groups). Post-exposure animals are sacrificed and examined after 30 months or if the only 25% or less animals are still alive. Animals of each group which die during the exposure or post-exposure period are examined as well.

2.4 Results

2.4.1 *Pre-studies (subacute exposure)*

A 28 day range-finding study with nanoscaled CeO_2 (NM212) and $BaSO_4$ (NM220) was performed as technical pre-study (Keller *et al.* 2014; Konduru *et al.* 2014). In this 28 day study, lung burdens (content of test materials in the lung) of CeO_2 were measured over 129 days post-exposure period at almost seven time points. Retention half-life times were calculated based on deposition and clearance of the test materials out of the lungs. For nanoscaled CeO_2, the lung burden decreased only very slowly over 129 days of the post-exposure period, indicating a very slow clearance of the test material out of the lung.

Nanoscaled $BaSO_4$ was almost removed out of the lungs already after 63 days of post-exposure period. Based on these results, following concentrations were selected for the long-term inhalation study:

3 mg/m³: as high concentration with expected toxic effects

1 mg/m³ : as mid concentration

0.3 mg/m³: as a second low concentration

0.1 mg/m³: as low concentration and expected NOAEL

$BaSO_4$ nanoparticle neither caused any adverse effect in short-term (5-day) and in the 28-day inhalation study. Thus, a high concentration of 50 mg/m³ was selected.

These concentrations were selected after discussion with an expert advisory team and the German authorities in spring 2013.

2.4.2 *Chronic study (24 months exposure)*

Figures 1 and 2 show exemplary SEM picture of CeO_2 and $BaSO_4$, respectively, sampled for the exposure atmosphere of the chronic study.

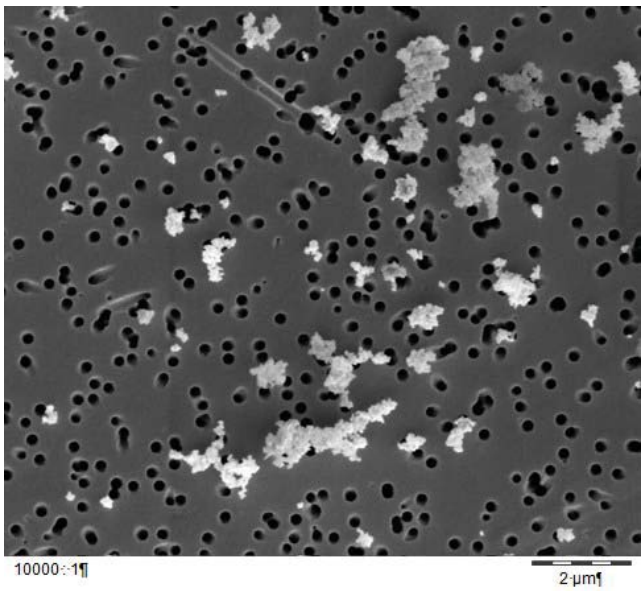

Figure 1 SEM picture of CeO_2 particles obtained from the exposure atmosphere, exposure 0.1 mg/m³

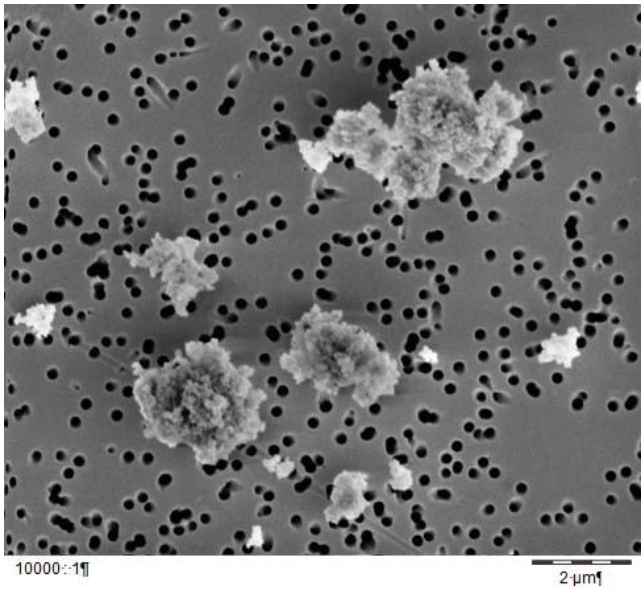

Figure 2 SEM picture of $BaSO_4$ particles obtained from the exposure atmosphere, exposure 50 mg/m³

Aerosol characterization showed a homogenous particle distribution in the inhalation chambers and the generated particles were in the respirable range for rats over the whole 24-month exposure period. Mass median aerodynamic diameters (MMAD) ranged between 1.4.-2.3 µm, the geometric standard deviations (GSDs) ranged between 2.0-2.4 µm) (Table 1). The animals were exposed 6 h/d and 5 d/week (working days only).

The interim sacrifice after 12 months (75 animals) was successful. The sacrifice for the 24 months animals (carcinogenicity group, 300 animals) in June 2015 was also successful.

Table 1 Characterization of the test atmospheres

	CeO_2				$BaSO_4$
Target concentration (mg/m³)	0.1	0.3	1	3	50
Measured concentration (mg/m³)	0.11 ± 0.02	0.31 ± 0.1	1.01 ± 0.1	3.01 ± 0.4	50.3 ± 5.8
MMAD (µm)	2.3	1.7	1.5	1.4	2.0
GSD	2.4	2.2	2.2	2.1	2.0

It becomes evident from figure 2 that the exposure concentrations were stable over the whole exposure period for all test groups.

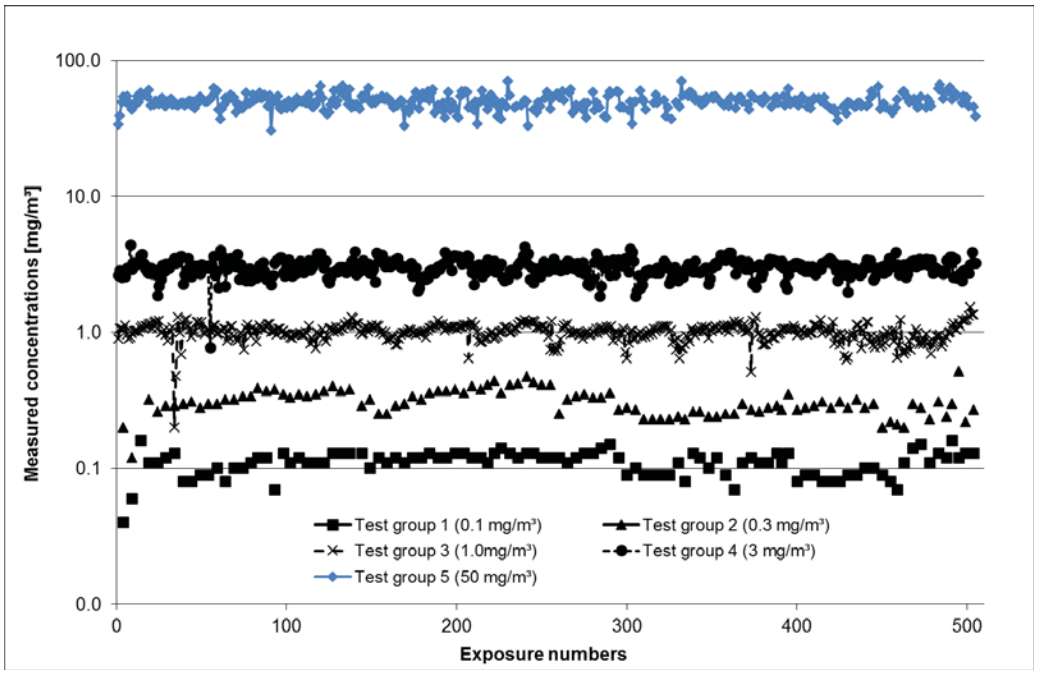

Figure 2 Stability of the exposure concentrations during the 24-month exposure phase

In the exposed groups, the body weight development was not impaired by substance treatment (Figure 3) indicating the absence of excess treatment mediated toxicity.

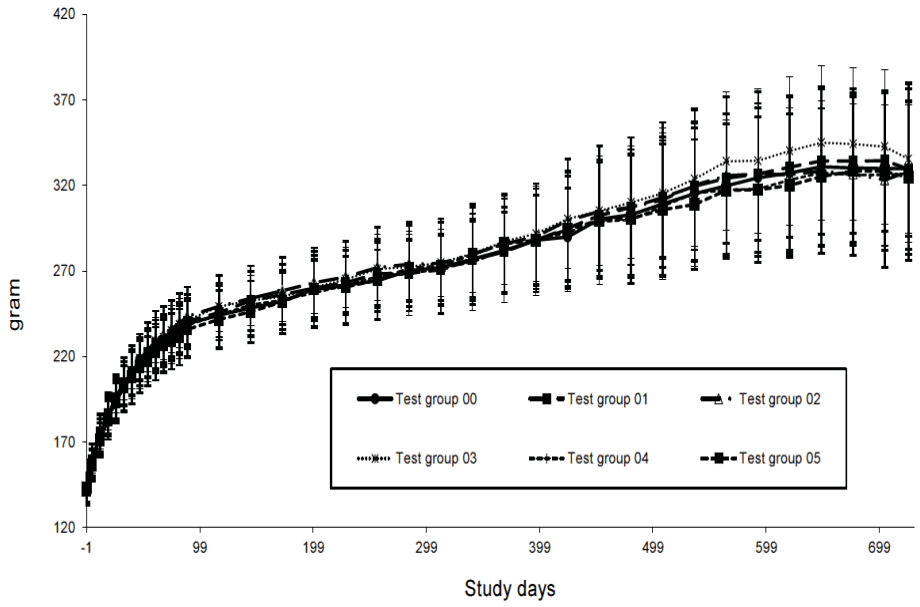

Figure 3 Body weight development during the 24-month exposure phase

The in-life part of the long-term study was finalized in time. The exposure period has started on June 11, 2013 and continues till June, 10 2015 with a subsequent post-exposure period of 6 months (December 2015).

Up to study day 734, the maximum mortality rate of all test groups was below 30 % (see Figure 4). In average over all test groups, it was 25.5% which is slightly higher than the average BASF historical control (21.5% which is, however, obtained with feeding studies).

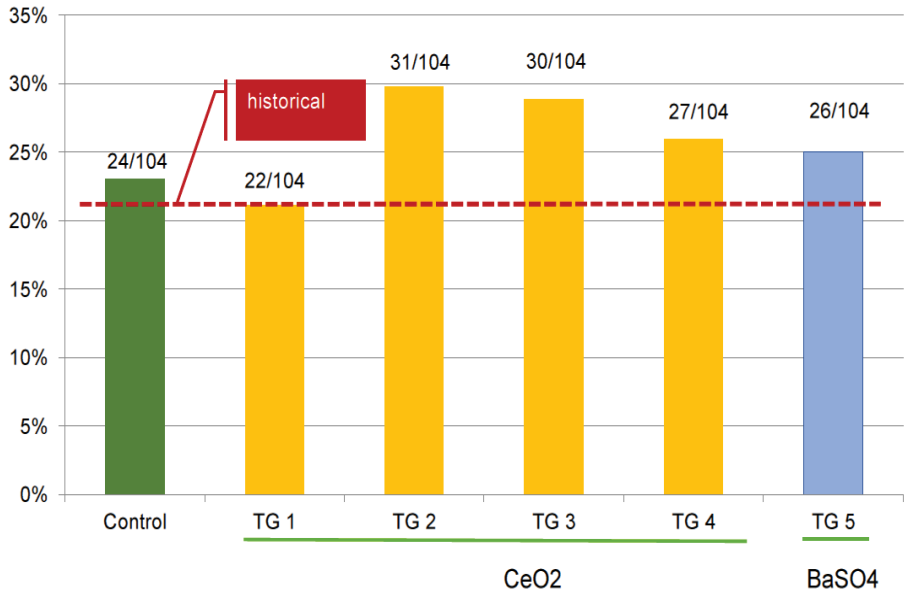

Figure 4 Lethality rate in the different study groups up to study day 734
TG1-4, test groups 1-4 in increasing CeO_2 exposure; historical, average mortality rate of chronic rat studies in BASF historical controls

No macroscopically visible tumour was detected in any animal in the 24-month sections.

After exposure termination, a post-exposure period of 6 months started for the remaining animals. The in-life part of the study will end in December 2015.

Evaluation and conclusions

The exposure phase of the chronic study has been successfully terminated. Exposure concentrations were found lying in the targeted levels. The main phase to generate results from the study samples has begun but will take a considerable effort and time. Thus, results crucial to NANoREG are not yet available. Currently, final or even interim evaluations cannot yet be made nor can conclusions be drawn at this moment.

Further results will become available in deliverables 4.5 [M34], 4.6 [M36], and 4.7 [M42].

3 Deviations from the work plan

No major or relevant deviations from the work plan were necessary. Thus, there is nothing to report.

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