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NANoREG

Grant Agreement Number 310584

Deliverable D 4.05

Lung burden and particle detection and quantification in olfactory bulbs - chronic exposure

Due date of deliverable: *2015/12/15*

Actual submission date: *2015/12/15*

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Work package/task:	WP4 / Tasks 4.3 and 4.4
Document status:	draft / final
Confidentiality:	confidential / restricted / public
Key words:	CeO_2, ICP-MS, ToF-SIMS

DOCUMENT HISTORY

Version	*Date*	*Reason of change*
1	2015/12/08	Completion of the document
2	2016/02/23	Revision of the document
3	2017/02/21	Project Office harmonized lay-out
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: Bundesinstitut für Risikobewertung, BfR, partner no. 6

Owner(s) of this document	
Owner of the content	BfR, partner no. 6
Co-Owner 1	ULEI, partner no. 45

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1 Description of task

A 2 year inhalation study with cerium dioxide (NM-212, Ø 28 nm) according to OECD TG 453 has been performed. Female Wistar rats were divided into five dose groups: 0 mg/m^3 (control group), 0.1 mg/m^3 (group 1), 0.3 mg/m^3 (group 2), 1 mg/m^3 (group 3) and 3 mg/m³ (group 4). After 3 and 12 months of exposure three animals per dose group were sacrificed. After 24 months of exposure another four animals per dose group (except control group: 2 animals) were sacrificed. The concentration of CeO_2 in lungs and olfactory bulb was determined after 3, 12 and 24 months of exposure.

Clearance and accumulation of CeO_2 nanoparticles were investigated after 3, 12 and 24 months inhalation of low (group 1 and 2) and mid (group 3 and 4) concentrations.

2 Description of work & main achievements

2.1 Summary

For this study we examined organ burden of CeO_2 nanoparticles in lungs and olfactory bulb at termination time points of 3, 12 and 24 months.

The amount of ceria found in the lungs was dose group dependent. Lowest and highest concentrations of CeO_2 were found in groups 1 and 4, respectively. For each dose group the particle retention in the lungs was found to be higher than the clearance.

Concentrations of CeO_2 detected in olfactory bulb increased from month 3 to month 24 in each dose group. Animals of group 1 showed the lowest ceria amount in olfactory bulb, whereas concentration in group 4 was highest.

CeO_2 burden of lungs and olfactory bulb ranged as follows: group 1 < group 2 < group 3 < group 4 and 3 months < 12 months < 24 months.

In the previous deliverable 4.4 we reported the organ burden achieved within a subacute 28 day inhalation study according to OECD TG 412. The detected CeO_2 concentrations in lungs and olfactory bulb were considered taken as a benchmark for the currently examined organs out of the 2 year inhalation study (OECD TG 412). The amounts of ceria in lungs and olfactory bulb were in the expected range.

An in-house validation of an analytical method for the sample preparation and quantification of cerium in tissues of small intestine, olfactory bulb, lymph nodes and bone marrow has been performed. All specified parameters and analytical conditions were met. The validation was successful and the method was used for examination of samples out of the 2 year inhalation study.

Studies on particle detection by Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) and Ion Beam Microscopy (IBM) were not performed so far, since the sample preparation of lungs by partner no. 13 in cooperation with ITEM Hannover is still in progress and will require until March 2016.

Since olfactory bulb showed very low amounts of CeO_2 in ICP-MS analysis, it was excluded from studies on particle distribution according to the specification in the DOW.

Particle detection pattern in extrapulmonary organs after chronic low-dose exposure (OECD TG no. 453) will be reported in the next deliverable 4.6.

2.2 Background of the task

In a 2 year inhalation study, conducted according to OECD TG 453, CeO_2 nanoparticles (NM-212, Ø 28 nm) were used as a representative of poorly soluble, respirable granular biodurable particles without known significant specific toxicity (GBP). The objective was to investigate potential low dose effects caused by chronic inhalation and also to compare the particle distribution in lung tissue by the use of imaging

techniques. In order to correlate particle distribution with potential effects of CeO_2 nanoparticles, slices for ToF-SIMS and IBM studies are being taken adjacent to those for histopathological investigations.

2.3 Description of the work carried out

2.3.1 *Organ burden*

2.3.1.1 Analytical task

The task within the deliverable was to determine the concentration of cerium in tissues of lungs and olfactory bulb.

2.3.1.2 Method development

We developed and validated proactively a method for sample preparation and analysis of cerium from CeO_2 nanoparticles (NM-212, Ø 28 nm) in tissues of small intestine, olfactory bulb, lymph nodes and bone marrow.

2.3.1.3 Freeze-drying

The whole lymph nodes, olfactory bulb, bone marrow and 10 cm of the small intestine were sampled into 2 ml Eppendorf tubes. The individual tissue samples were homogenised, freeze-dried under vacuum and stored at -80 °C.

2.3.1.3.1 Microwave wet-chemical digestion

The freeze-dried tissue samples were digested in separate vessels each with an appropriate mixture of acid and oxidizing agent (2.5 mL H_2O, 2 mL HNO_3 (69 %), 1 mL H_2O_2 (30 %)). A suitable temperature and energy controlled digestion program was used to break down the tissue and to dissolve the CeO_2 nanoparticles.

The gathered solutions were diluted further with ultrapure water including the addition of the two internal standards indium (^{115}In) and lutetium (^{175}Lu). These standards served as internal standards at either side of the molecular mass of the two ^{140}Ce and ^{142}Ce isotopes.

2.3.1.3.2 ICP-MS analysis

Quantification was carried out with a quadrupole Thermo Fisher X Series II instrument. For analysis, ^{140}Ce and ^{142}Ce were selected out of the five naturally occurring cerium isotopes for quantification.

2.3.1.4 Validation

The method above was in-house validated for all the four peripheral organs. Therefore the tissue samples were spiked with three[1] (small intestine, olfactory bulb and lymph nodes) and two[2] (bone marrow) different concentrations (0.2 ppb[1] 2 ppb[1,2] and 20 ppb[1,2]) The NANOGENOTOX protocol was used to prepare the CeO_2-spiking dispersions.

2.3.1.4.1 Validation

The tissue samples were tested in line with DIN ISO/IEC 17025 “General requirements for the competence of testing and calibration laboratories” and the resulting SOP for the validation of analytical methods. The criteria specificity/selectivity, stability, accuracy and precision as well as recovery were tested for all sample matrices and media. Additionally, appropriate system suitability criteria and a suitable certified reference material for cerium (BCR-670) were selected to receive a high confidence and excellence in the gathered test values. Thus the validation passed and the method was considered suitable for the purpose.

2.3.1.5 Quantification of cerium in lung and olfactory bulb

An appropriate analytical method for the quantification of cerium in lung (see deliverable 4.4) and olfactory bulb tissue has been developed.

Thus the determination of CeO_2 concentrations in lung and olfactory bulb was carried out with the method previously established, comprising the steps of sampling, freeze drying, wet chemical microwave digestion and determination of cerium ions with ICP-MS.

The values of ^{140}Ce and ^{142}Ce isotopes are calculated as [µg CeO_2 / lung] and [ng CeO_2 / olfactory bulb], respectively. The analytical range of this method is 0.1 ppb - 20 ppb.

2.3.2 *Particle distribution*

The sample preparation of lungs for ToF-SIMS and IBM will be done by partner no. 13 in cooperation with ITEM Hannover and is still in progress. Therefore no results on particle distribution can be reported so far.

The olfactory bulb showed very low amounts of CeO_2 in ICP-MS analysis. Therefore it was excluded from the detection of particle distribution according to the specification in the DOW.

2.4 Results

In the following, results of the method validation for the tissues specified above and for organ burden of lung and olfactory bulb are presented. Both organs were used for determination of organ burden within the 2 year inhalation study with CeO_2 nanoparticles.

The sample preparation of lungs for ToF-SIMS will be done by partner no. 13 in cooperation with ITEM Hannover and is still in progress.

The olfactory bulb showed very low amounts of CeO_2 in ICP-MS analysis. Therefore it was excluded from the detection of particle distribution according to the specification in the DOW.

2.4.1 *Organ burden*

2.4.1.1 Validation

An in-house validation for sample preparation and analysis of CeO_2 (NM-212) was performed using three independently prepared organ tissues of small intestine, lymph nodes, olfactory bulb and bone marrow. Each tissue sample was measured in triplicate by ICP-MS. Cerium amounts were calculated using the internal standard ^{115}Indium. Recovery rates are reported in table 1. Results are given for ^{140}Ce in figure 1 and for ^{142}Ce in figure 2.

Table 1: Recovery of CeO_2 NM-212 presented as ^{140}Ce in different organ tissues

	Recovery [%]		**Relative Standard Deviation [%]**	
Organ tissue spiked with CeO_2	**^{140}Ce**	**^{142}Ce**	**^{140}Ce**	**^{142}Ce**
olfactory bulb 0.2 ppb	101.2	106.0	5.4	10.3
lymph nodes 0.2 ppb	96.0	100.3	6.7	9.2
small intestine 0.2 ppb	118.8	136.4	14.3	34.4
olfactory bulb 2 ppb	97.8	100.4	0.7	0.2
lymph nodes 2 ppb	96.0	98.0	5.9	5.7
small intestine 2 ppb	96.9	99.6	1.6	1.5

bone marrow 2 ppb	100.3	103.2	5.1	5.3
olfactory bulb 20 ppb	95.2	95.6	2.5	2.5
lymph nodes 20 ppb	93.9	94.6	1.6	1.7
small intestine 20 ppb	97.3	97.6	1.8	1.5
bone marrow 20 ppb	105.7	105.5	1.5	1.8

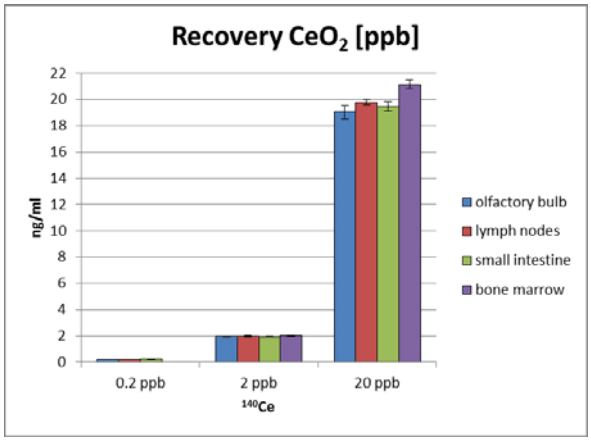

Figure 1: Recovery of CeO_2 NM-212 presented as ^{140}Ce in different organ tissues

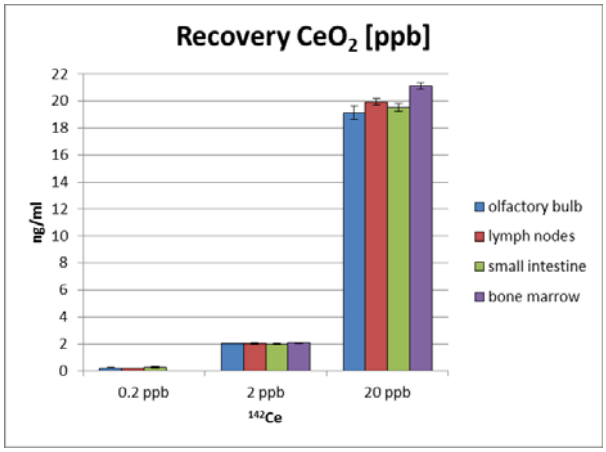

Figure 2: Recovery of CeO_2 NM-212 presented as ^{142}Ce in different organ tissues

Experiments on recovery in three independently prepared tissue samples (unspiked and spiked with 0.2 ppb, 2 ppb, 20 ppb CeO_2) revealed an average RSD < 6 %. For small intestine tissue samples, spiked with 0.2 ppb CeO_2, a RSD of 14.3 % and 34.4 % for ^{140}Ce and ^{142}Ce, respectively was observed. At low concentrations, adhering faeces in the small intestine apparently caused increased recovery rates, especially for ^{142}Ce and the internal standard ^{175}Lu (data not shown), caused by isobaric interferences. Therefore it is recommended to use the isotope ^{140}Ce referred to the internal standard ^{115}In for analysis of small intestine. Due to the low amount of available bone marrow samples, two concentrations (2 ppb and 20 ppb) were spiked only.

Specificity of the analytical method was demonstrated for the isotopes ^{140}Ce, ^{142}Ce, ^{115}In and ^{175}Lu, linearity R was > 0.999. Sample stability was confirmed for up to 14 days for prepared tissue samples.

2.4.1.2 Analytical task

All concentrations measured with ICP-MS are mean values based on three (n = 3). For lung and olfactory bulb three animals per dose group were examined after 3 and 12 months. After 24 months four animals were examined each per dose group. The mean values of three and four organs each were again calculated as mean value for lung and olfactory bulb, respectively. The results for all organs are shown in table 2.

Note:

Dose group 0: 0 mg/m³ (control group)

Dose group 1: 0.1 mg/m³

Dose group 2: 0.3 mg/m³

Dose group 3: 1 mg/m³

Dose group 4: 3 mg/m³

Table 2: Overview of animals examined on CeO_2 organ burden after chronic exposure

Animal no.	Dose group	Exposure time	Exposure concentration	Examined organs
601	10	3 months	0 mg/m³	lung, olfactory bulb
602	10	3 months	0 mg/m³	lung, olfactory bulb
603	10	3 months	0 mg/m³	lung, olfactory bulb
611	11	3 months	0.1 mg/m³	lung, olfactory bulb
612	11	3 months	0.1 mg/m³	lung, olfactory bulb
613	11	3 months	0.1 mg/m³	lung, olfactory bulb
621	12	3 months	0.3 mg/m³	lung, olfactory bulb
622	12	3 months	0.3 mg/m³	lung, olfactory bulb
623	12	3 months	0.3 mg/m³	lung, olfactory bulb
631	13	3 months	1 mg/m³	lung, olfactory bulb
632	13	3 months	1 mg/m³	lung, olfactory bulb
633	13	3 months	1 mg/m³	lung, olfactory bulb
641	14	3 months	3 mg/m³	lung, olfactory bulb
642	14	3 months	3 mg/m³	lung, olfactory bulb
643	14	3 months	3 mg/m³	lung, olfactory bulb
604	20	12 months	0 mg/m³	lung, olfactory bulb
605	20	12 months	0 mg/m³	lung, olfactory bulb
606	20	12 months	0 mg/m³	lung, olfactory bulb
614	21	12 months	0.1 mg/m³	lung, olfactory bulb
615	21	12 months	0.1 mg/m³	lung, olfactory bulb

Animal no.	Dose group	Exposure time	Exposure concentration	Examined organs
616	21	12 months	0.1 mg/m³	lung, olfactory bulb
624	22	12 months	0.3 mg/m³	lung, olfactory bulb
625	22	12 months	0.3 mg/m³	lung, olfactory bulb
626	22	12 months	0.3 mg/m³	lung, olfactory bulb
634	23	12 months	1 mg/m³	lung, olfactory bulb
635	23	12 months	1 mg/m³	lung, olfactory bulb
636	23	12 months	1 mg/m³	lung, olfactory bulb
644	24	12 months	3 mg/m³	lung, olfactory bulb
645	24	12 months	3 mg/m³	lung, olfactory bulb
646	24	12 months	3 mg/m³	lung, olfactory bulb
607	30	24 months	0 mg/m³	lung, olfactory bulb
608	30	24 months	0 mg/m³	lung, olfactory bulb
617	31	24 months	0.1 mg/m³	lung, olfactory bulb
199	31	24 months	0.1 mg/m³	lung, olfactory bulb
619	31	24 months	0.1 mg/m³	lung, olfactory bulb
200	31	24 months	0.1 mg/m³	lung, olfactory bulb
627	32	24 months	0.3 mg/m³	lung, olfactory bulb
300	32	24 months	0.3 mg/m³	lung, olfactory bulb
629	32	24 months	0.3 mg/m³	lung, olfactory bulb
630	32	24 months	0.3 mg/m³	lung, olfactory bulb
637	33	24 months	1 mg/m³	lung, olfactory bulb
638	33	24 months	1 mg/m³	lung, olfactory bulb
639	33	24 months	1 mg/m³	lung, olfactory bulb
400	33	24 months	1 mg/m³	lung, olfactory bulb
647	34	24 months	3 mg/m³	lung, olfactory bulb
648	34	24 months	3 mg/m³	lung, olfactory bulb
649	34	24 months	3 mg/m³	lung, olfactory bulb
650	34	24 months	3 mg/m³	lung, olfactory bulb

Statistical analysis with DUNNETT's test and GRUBB`s test were performed on the results of lung and olfactory burden in the frame of a combined chronic toxicity/carcinogenicity inhalation study (OECD TG 453). Detected lung burden in the groups of 1-4 was significantly different from the CeO_2 concentrations in the lungs of control groups.

Concentrations in olfactory bulb were on a very low level of < 15 ng/organ. However CeO_2 concentrations were significantly different from those in the control groups (with exception of group 1 after 24 months). As expected, increasing organ burden was detected from month 3 to month 12 and from month 12 to month 24 for both organs in each dose group. No statistical outliers were detected in measured values of lung and olfactory bulb using GRUBBs test.

Table 2 shows the results achieved with organs examined after 3, 12 and 24 months of inhalation. Figures 3 and 4 and tables 3 and 4 show the calculated organ burden for CeO_2 after 3, 12 and 24 months of exposure according to table 2. The given amount of CeO_2 refers to the isotope ^{140}Ce, which was quantified based on the internal standard ^{115}In. Similar results were achieved using the isotope ^{142}Ce and the internal standard ^{175}Lu, respectively.

Table 3: CeO_2 lung burden [µg CeO_2/lung]

		Cerium dioxide lung burden				
Exposure time		control	0.1 mg/m³	0.3 mg/m³	1 mg/m³	3 mg/m³
3 months	animal 1	0.72	15.97	44.83	246.93	626.21
	animal 2	1.93	15.38	45.61	286.72	838.63
	animal 3	1.03	19.33	54.98	240.77	851.26
	mean	**1.23**	**16.89**	**48.47**	**258.14**	**772.03**
	± SD	0.63	0.002	0.01	0.03	0.13
12 months	animal 1	2.22	45.79	224.12	829.37	2492.75
	animal 2	1.49	39.88	257.20	915.08	2334.97
	animal 3	0.97	62.01	202.17	1028.64	2981.34
	mean	**1.56**	**49.22**	**227.83**	**924.36**	**2603.02**
	± SD	0.63	0.01	0.03	0.10	0.34
24 months	animal 1	1.60	48.45	292.19	1197.64	4378.98
	animal 2	1.09	127.04	347.40	1573.97	3435.07
	animal 3		78.81	332.10	1778.52	3975.96
	animal 4		58.60	421.91	1250.14	5857.74
	mean	**1.35**	**78.23**	**348.40**	**1450.01**	**4411.94**
	± SD	0.36	0.03	0.05	0.28	1.04

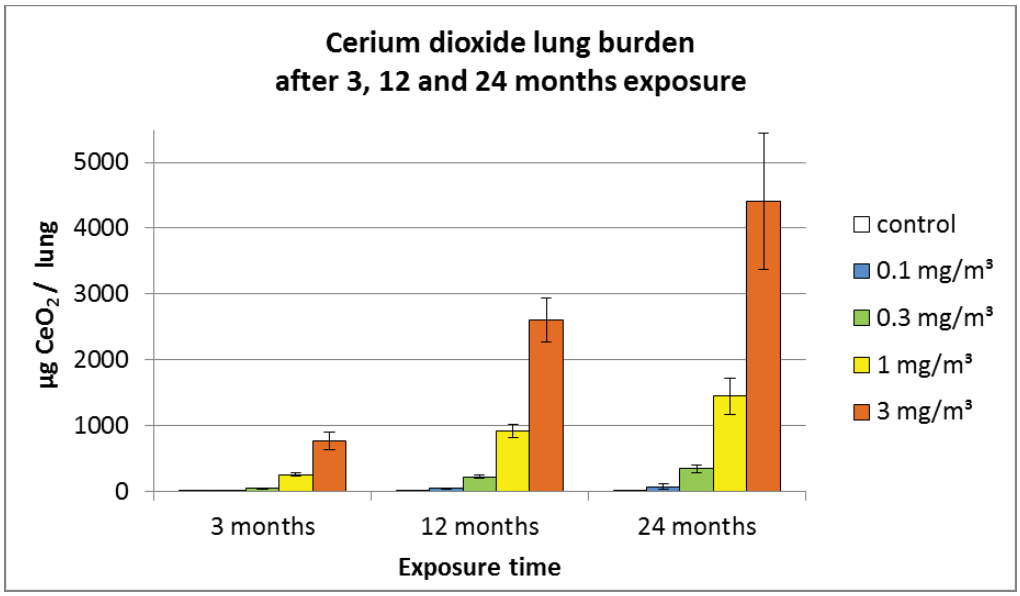

Figure 3: CeO_2 lung burden

Table 4: CeO_2 olfactory bulb burden [ng CeO_2/olfactory bulb]

		Cerium dioxide olfactory bulb burden				
Exposure time	Value	control	0.1 mg/m³	0.3 mg/m³	1 mg/m³	3 mg/m³
3 months	animal 1	0.02	0.35	0.20	0.44	4.44
	animal 2	0.01	0.08	0.17	0.33	2.20
	animal 3	0.02	0.06	0.17	0.43	0.92
	mean	**0.2**	**0.16**	**0.18**	**0.40**	**2.52**
	± SD	0.01	0.16	0.02	0.06	1.78
12 months	animal 1	0.23	0.23	0.40	0.64	1.94
	animal 2	0.16	0.16	0.41	1.41	2.01
	animal 3	0.26	0.26	0.43	0.94	1.42
	mean	**0.22**	**0.22**	**0.42**	**1.00**	**1.79**
	± SD	0.05	0.05	0.02	0.39	0.32
24 months	animal 1	0.59	0.38^{+}	1.50	2.01	11.68
	animal 2	0.43	0.35^{+}	1.15	1.20	14.49
	animal 3		0.59^{+}	1.17	2.20	3.18
	animal 4		0.35^{+}	1.33	3.03	6.59
	mean	**0.51**	**0.42**$^{+}$	**1.29**	**2.11**	**8.98**
	± SD	0.11	0.11	0.16	0.75	5.07

$^{+}$: not significant compared to control levels

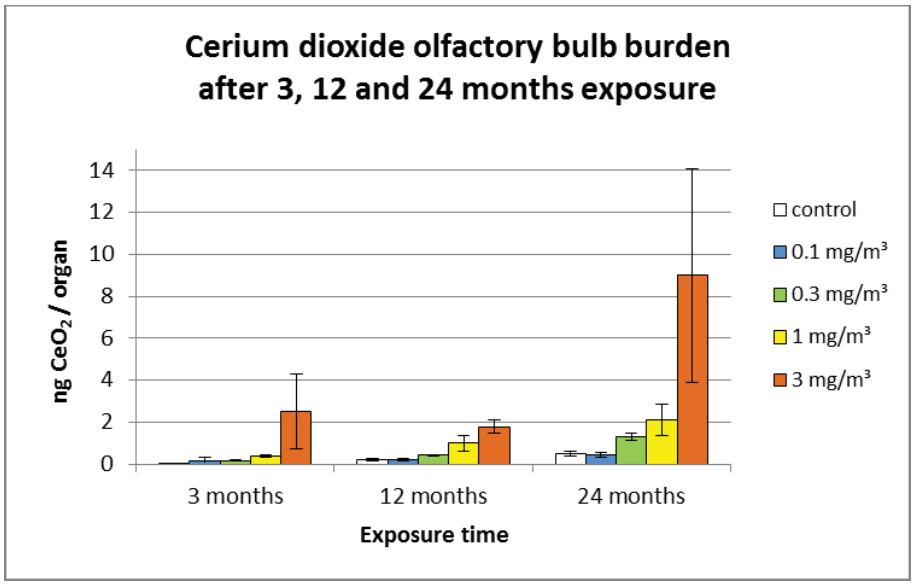

Figure 4: CeO_2 olfactory bulb burden

2.4.2 *Particle distribution*

The sample preparation of lungs for ToF-SIMS will be done by partner no. 13 in cooperation with ITEM Hannover and is still in progress.

The olfactory bulb showed very low amounts of CeO_2 in ICP-MS analysis. Therefore it was excluded from the studies on particle distribution according to the specification in the DOW.

2.5 Evaluation and conclusions

While studies on organ burden quantification (Task 4.3) are progressing according to the project planning, there is a delay of the results on particle distribution pattern (Task 4.4) due to a delayed availability of samples. Further attention needs to be given to the close cooperation with histopathological and histochemical tasks in order to identify molecular changes that are relevant for toxic or carcinogenic effects.

2.5.1 *Organ burden*

Within D 4.5, lung and olfactory bulb burden of rats following to 2 years inhalation of CeO_2 nanoparticles (NM-212) is reported. There is a significant correlation between aerosol concentration and organ burden. In each dose group the organ burden was constantly increasing with time of exposure, although lung clearance is expected to occur likewise. However lung clearance was impaired in each dose group due to the CeO_2 concentrations rising over the course of the exposure period. Thus overload is already to be expected if low doses (0.1 and 0.3 mg/m³) and mid doses (1 and 3 mg/m³) are inhaled chronically. Lowest and highest CeO_2 amounts in lung were 78.22 µg and 4411.94 µg achieved after 24 months of inhalation of 0.3 g/m³ and 3 mg/m³ CeO_2, respectively. Notably, the lung burden after 28 days of exposure to 25 mg/m³ (see deliverable 4.4) and 12 months to 3 mg/m³ was about the same.

Calculation of the linear regression revealed a linear increase of lung burden in each dose group with a correlation of 0.999. While the influence of the dose is highly significant ($p < 0.001$), this is not the case for time.

Also, investigation of the olfactory bulb showed increasing CeO_2 concentrations over time in all dose groups. It is assumed, that nanoparticles are taken up by the nerve endings of the olfactory bulb from where they might be further translocated. Lowest and highest CeO_2 amounts in olfactory bulb after 24 months of exposure were measured with 0.42 ng and 8.98 ng.

The amounts of ceria detected in olfactory bulb are equivalent to 2.47*10-4 % of the corresponding lung burden. However, the data show a significant organ burden (p-value < 0.05) of olfactory bulb in all dose groups compared to the control.

It is assumed that also secondary organs like lymph nodes, liver, spleen and kidney will show significant CeO_2 concentrations compared to control groups. Out of the secondary organs, tracheobronchial and mediastinal lymph nodes are anticipated to possess highest amounts of CeO_2 due to their lung draining functions. This was already shown by the results on organ burden within the 28 day study (OECD TG 412) performed previously. Furthermore, liver is expected to be a target organ for particle clearance due to its metabolic characteristics.

Concerning excretion, low amounts of CeO_2 are expected in urine while most of the material should be eliminated via faeces.

Results on particle distribution and elimination will be reported in the next deliverable.

2.5.2 *Particle distribution*

The sample preparation of lungs for ToF-SIMS will be done by partner no. 13 in cooperation with ITEM Hannover and is still in progress.

Olfactory bulb showed very low amounts of CeO_2 in ICP-MS analysis. Therefore it was excluded from the detection of particle distribution according to the specification in the DOW.

3 Deviations from the work plan

Due to a delay in sample preparation within the 2 year inhalation study (OECD TG no. 453), studies on particle distribution pattern were not started so far. Results on particle distribution pattern in lungs out of the 2 year inhalation study are expected for May 2016.

4 References / Selected sources of information (optional)

The methods and endpoints addressed within this deliverable 4.5 (ICP-MS analysis and ToF-SIMS analysis) were reported to CIRABC and are available as ISA-TAB-Nano templates online.

5 List of abbreviations (optional)

none

6 Annexes (optional)

none