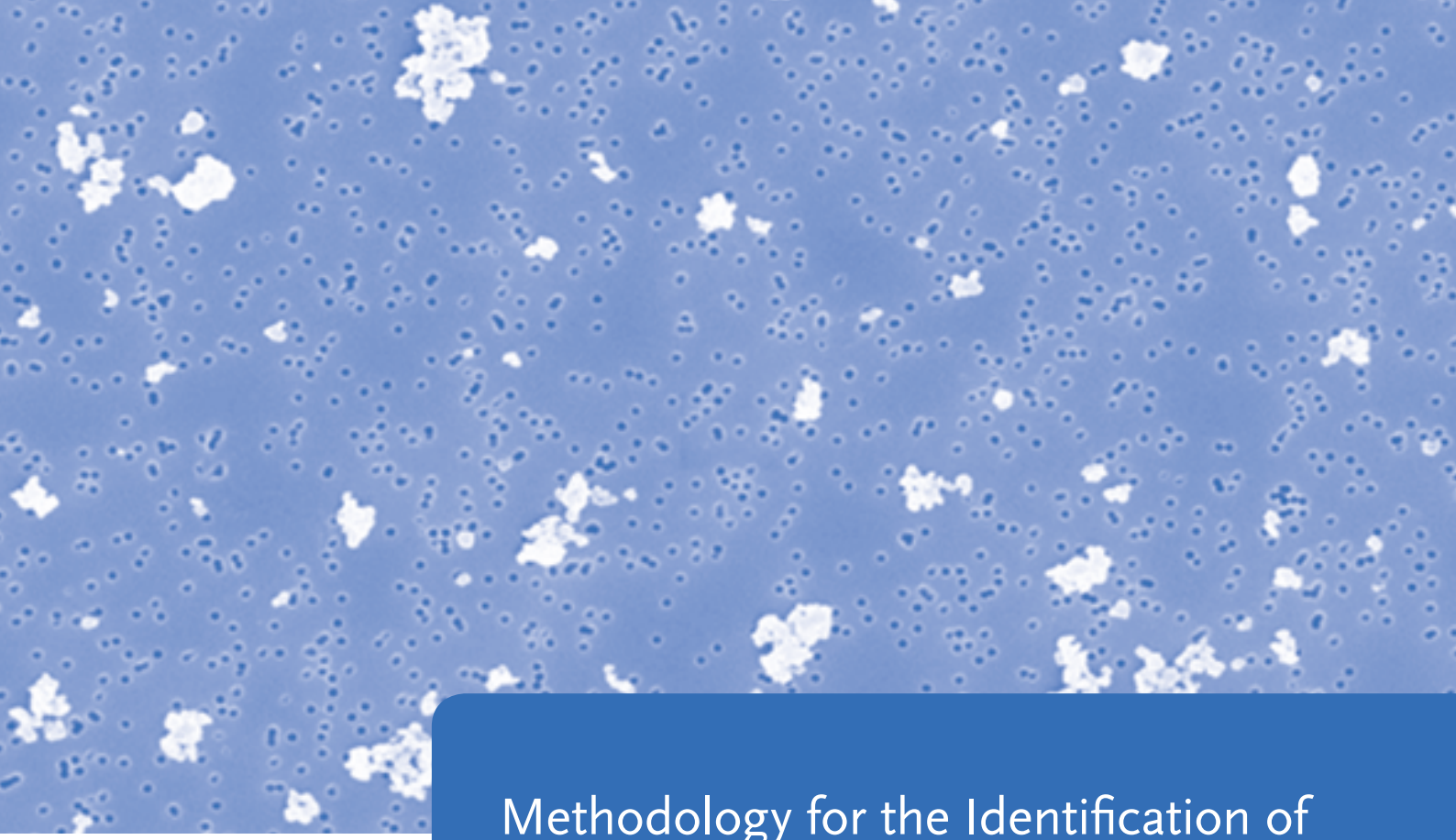

Methodology for the Identification of Granular Biopersistent Particles (GBP) at Workplaces

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The responsibility for the contents of this publication lies with the authors.

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Methodology for the Identification of Granular Biopersistent Particles (GBP) at Workplaces

Abstract

The so-called GBP category was formed by definition, i.e. includes **respirable Granular Biopersistent Particles without known significant specific toxicity**. The category comprises various materials such as minerals, metals, metal oxides or polymers that show a negligible solubility in lung fluids (lung lining fluid, lysosomal fluid).

Module 1: In vivo study - Analysis of the bronchoalveolar lavage fluid (BALF) following intratracheal instillation

The analysis of the inflammatory potency of 6 GBP candidates included µ-TiO_2 "Bayertitan T", nano-TiO_2 P25, µ-Eu_2O_3, µ-$BaSO_4$, µ-ZrO_2 and nano-SiO_2, each at a low (0.5 µl per rat) and high (1.5 µl) volumetric dose. In the differential cell count, µ-TiO_2 did not induce statistically significant polymorphonuclear neutrophil (PMN) levels on day 3 post-treatment in the low dose group. The GBP requirement of a very low inflammogenicity was confirmed. PMN levels of approx. 12% in the high dose group indicated a bolus effect. µ-$BaSO_4$ showed a behavior similar to µ-TiO_2. In contrast, nano-TiO_2 P25, µ-Eu_2O_3, µ-ZrO_2 and nano-SiO_2 did not meet this GBP criterion.

Module 1: In vivo study - Chemical analysis of the lung burdens

On day 3, an average retention of approx. 70% as compared to the administered total dose was detected; approx. 1/3 of the dose is eliminated from lungs by rapid clearance mechanisms (coughing, ciliae-mediated processes, etc). The clearance half-time showed a value close to the physiological rat lung clearance of approx. 60 days in the µ-TiO_2 "Bayertitan T" low dose group. In the high dose group a doubled half-time was observed (overload effect). In the µ-$BaSO_4$ and amorphous silica groups, smaller values in the range of 25-40 days were calculated indicating an additional dissolution effect. In the µ-Eu_2O_3 and µ-ZrO_2 groups increased half-times, i.e. 4- to 5-fold and 2- to 4-fold, respectively, were calculated indicating a clear surface chemistry-related contribution to the toxic and clearance-retardative outcome.

At µ-TiO_2 "Bayertitan T" and nano-TiO_2 P25 very low ionic moieties regarding the total lung burden were detected (≤0.1weight-% in the 0.5 µl doses), the other dusts showed higher (0.2-0.7 weight-%) or very high (30 weight-%; Eu_2O_3) moieties.

Module 2: In vitro assays

The plasmid scission (PSA) and the cytotoxicity assay towards THP-1 cells indicated effects matching to the in vivo results in the Eu_2O_3 dust group. Overall, in vitro assays under investigation did not mirror the in vivo results with statistically significant power.

Module 3: Acellular solubility of test materials

On the basis of the acellular solubility results in artificial lung fluids a threshold value of ≤ 1 mg/l could be agreed on to define the category of "low soluble particles".

Outlook

The inhalation exposure pathway will show lower effects as compared to intratracheal instillation as no bolus effects will occur. Therefore, the final setting of maximum tolerable clearance $t_{1/2}$ and PMN levels to define the GBP category should await the outcome of the inhalation validation study.

Key words:

GBP, PMN, retention, biosolubility

Methodik zur Identifizierung von granulären, biopersistenten Stäuben (GBS) an Arbeitsplätzen

Kurzreferat

Die sogenannte Kategorie der GBS wird per definitionem von **respirablen, granulären und biopersistenten Stäuben** gebildet, die **keine signifikante und spezifische Toxizität** aufweisen. Diese Kategorie umfaßt verschiedene Materialien wie Mineralien, Metalle, Metalloxide oder Polymere, die eine vernachlässigbar kleine Löslichkeit in Lungenflüssigkeit zeigen (Lungenflüssigkeitsfilm, lysosomale Flüssigkeit).

Modul 1: In vivo Studie - Analyse der bronchoalveolären Lavageflüssigkeit (BALF) nach intratrachealer Instillation

Sechs GBS-Kandidaten, d.h. µ-TiO_2 "Bayertitan T", nano-TiO_2 P25, µ-Eu_2O_3, µ-$BaSO_4$, µ-ZrO_2 und nano-SiO_2 wurden auf ihr inflammatorisches Potential analysiert, jeweils in einer niedrigen und einer hohen Dosis (0,5 µl bzw. 1,5 µl volumetrische Dosis pro Ratte). µ-TiO_2 induzierte bei 0,5 µl keinen statistisch signifikanten Titer von Granulozyten an Tag 3. Das GBS-Kriterium einer sehr geringen Entzündungswirkung wurde erfüllt. 12% Granulozyten in der hohen Dosis wiesen auf einen Partikel-Bolus-Effekt hin. µ-$BaSO_4$ zeigte ähnliche Werte wie µ-TiO_2. - Dagegen erfüllten nano-TiO_2 P25, µ-Eu_2O_3, µ-ZrO_2 und nano-SiO_2 dieses GBS-Kriterium nicht.

Modul 1: In vivo Studie – Chemische Analyse der Lungenbeladung

An Tag 3 nach Behandlung wurde eine Retention von ca. 70% der Gesamtdosis gemessen; ca. 1/3 der Gesamtdosis wurde durch schnelle Clearance aus der Lunge entfernt (Husten, Zilienclearance). In der TiO_2 "Bayertitan T"-Niedrigdosisgruppe wurde eine Clearance-$t_{1/2}$ von ca. 60 Tagen (physiologischer Wert) ermittelt. In der hohen Dosis war die $t_{1/2}$ verdoppelt (Überladungseffekt). In den µ-$BaSO_4$- und nano-SiO_2-Gruppen wurden niedrigere Werte (25-40 Tage) berechnet, die auf einen zusätzlichen Löslichkeitseffekt hinweisen. In den µ-Eu_2O_3 und µ-ZrO_2-Gruppen ergaben sich erhöhte $t_{1/2}$-Werte (um den Faktor 4-5 und 2-4), die auf einen Beitrag der Partikel-Oberflächenchemie zur Toxizität und Clearancereduktion hinwiesen. Bei µ-TiO_2 und nano-TiO_2 P25 ergaben sich in den 0,5 µl-Dosisgruppen sehr geringe ionische Anteile an der Gesamtlungenbeladung (≤0,1 Gewichts-%), bei den anderen Stäuben lagen diese höher (0,2-0,7 Gewichts-%) bzw. sehr hoch (30 Gewichts -%; Eu_2O_3).

Modul 2: In vitro Tests

Der Plasmid-Scission-Assay (PSA) und ein Zytotoxizitätstest an THP-1-Zellen zeigten mit Eu_2O_3-Partikeln signifikante Effekte; Eu_2O_3 war auch bei den in vivo Untersuchungen am toxischsten. Insgesamt spiegelten sich in den benutzten in vitro Tests die beobachteten in vivo Effekte nicht mit statistischer Signifikanz wider.

Modul 3: Azelluläre Löslichkeit der Teststäube

Auf der Basis der Löslichkeitsdaten in künstlichen Lungenflüssigkeiten kann die für GBS geforderte "Schwerlöslichkeit" bei ≤ 1 mg/l angesetzt werden.

Ausblick

Da die Instillation gegenüber der Inhalation eine erhöhte Toxizität (Bolus-Effekt) verursacht, sollte vor der Festsetzung für GBS tolerierbarer Clearance-$t_{1/2}$ und Granulozyten-Werte die inhalative Validierung (BAuA-Projekt F2364) abgewartet werden.

Schlagwörter:

GBS, PMN, Retention, Biolöslichkeit

1 Information on the Study

Fraunhofer ITEM Study No :	02N 14 534 (non-GLP): BAL Analysis 02N 14 535 (non-GLP): Chemical Analysis
Research Facility:	Fraunhofer ITEM
Fraunhofer ITEM Project Manager: in vivo Toxicology	Dr. Otto Creutzenberg
Aerosol Physics:	Prof. Dr. Wolfgang Koch
Clinical Chemistry:	Dr. Tanja Hansen
In vitro Toxicology:	Dr. Jan Knebel
Chemical Analysis:	Dr. Sven Schuchardt
Sponsor's Study Manager:	Dr. Bruno Orthen
Projekt Initiation Date:	February 1, 2014
Project Completion Date:	April 30, 2016

2 Introduction

Fine fractions of dusts occurring in occupational settings are of high relevance for the safety at workplaces and therefore are strictly regulated by authorities. Appropriate threshold values should guarantee that lung diseases are not induced in chronically exposed workers exposed up to long periods. Respirable **Granular Biopersistent Particles (GBP) without known significant specific toxicity** show a negligible solubility in physiological lung fluid (extracellular lung lining fluid, intracellular lysosomal fluid) and do not exhibit a specific chemistry-related toxicity at volumetric non-overload conditions.

The theory of volumetric overload has been pioneered by Morrow and was experimentally comprehensively investigated by various groups in the 80/90ies of the last century. Dust-laden alveolar macrophages gradually loose their motility due to excessive particle-cell, cell-cell chemotactic interactions and migratory inhibition factors. The inability of macrophages to translocate to the mucociliary escalator is correlated to an average composite particle volume per alveolar macrophage in the lung. According to Morrow, the clearance impairment by the volumetric overload with dust particles starts at approx. 6% of the alveolar macrophage (MORROW, 1988).

The physiological rat lung clearance and its overloading has been documented precisely in subchronic and chronic inhalation toxicity tests with an experimental toner powder (i.e. without surface coating) using low soluble radioactively tagged particles, e.g. ^{85}Sr-polystyrene particles, as tracer. These polymer particles did not exhibit a chemical-based toxicity and represented well an inert dust. The studies resulted in typical physiological half-times of approx. 60 days that increased up to more than 1000 days depending on the level of dust load in the lung (clearance retardation or even collapse; MUHLE et al., 1990; BELLMANN et al., 1991). According to Morrow volumetric lung overload in the rat lung starts at approx. 0.3 µl particle load per gram rat lung, at 1 µl/g lung an evident overload is observed resulting in lung clearance half-times of approx. 300-400 days.

Some granular particles, e.g. certain quartz varieties are classified as human lung carcinogen. The majority of dusts for that lung tumours have been reported in rat inhalation studies showed these findings only at lung burdens having caused impaired particle clearance. The development of tumours is probably due to an overload-driven perpetuating inflammatory reaction and oxidative stress. This is the secondary genotoxicity type and a threshold dose for the adverse chronic effects can be postulated which is defined by a lung particle burden not exceeding the physiological lung clearance capacity. Thus, the lung tumors observed in chronic rat studies at very high particulate exposure concentrations may not be relevant for human extrapolation to low-exposure concentrations (OBERDÖRSTER, 1995).

In 2000, a workshop of the German Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK Commission) concluded that “the lung tumors observed in chronic rat inhalation studies with high dose GBP … are due to a secondary genotoxicity”, which in rats “operates only at high doses and high levels of neutrophils” and for “GBP, pathology in rodents indicates that if there is no inflammation there is no fibrosis, and if there is no fibrosis, there is no cancer” (BORM et al., 2015). Besides volumetric lung load also the surface area of GBP is an important dose-metric. Especially for agglomerates of nanoparticles the specific surface area is a basic determinant of the toxic potential and should be analysed as well as the agglomerate density; the latter is smaller than the

material density, thus leading to higher volumetric loads at same mass loads (nanoscaled vs. microscaled). The particularities (higher specific surface and specific volume) of nanoparticle agglomerates vs. microsized bulk material have to be observed while checking nano-dusts for their applicability as a GBP. The volumetric overload approach of Morrow can explain very well the effects observed for microscaled dusts, however, needs some adaptation for nanodusts due to the different physical properties. This means that for a nano-dust, in addition to the volumetric issue, an increased specific surface contributes to the observed toxic effect that is regularly higher than that observed for the µ-bulk material. - Experimentally, dust samples that fulfill the GBP criteria are regularly microscaled dusts. Up to now there is no nanoscaled variety of a known µ-GBP that could be experimentally demonstrated to fulfill the GBP criteria, too.
A convincing criterion for defining a GBP is the half-time of lung clearance. In the rat model, a half-time of approx. 60 days measured at volumetric non-overload conditions stands for a toxicologically inert dust.
In this study the rats were administered with identical volumetric of GBP candidates, i.e. 0.5 and 1.5 µl per rat. It was assumed that the physiological clearance was not impaired at 0.5 µl whereas 1.5 µl show induce clear overload effects (increased half-times, inflammatory reactions). For the microscaled dusts the material densities (e.g. $\rho_{TiO2\ =}$ 4.3), for the nanoscaled dusts a lower agglomerate density (e.g. $\rho_{TiO2\ P25}$ = 3.8) were used.

GBP can be regulated with the same threshold limit value (in Germany currently 1.25 mg/m^3 for the respirable fraction; TRGS 900). In 2012, the MAK Commission derived a new threshold value of 0.3 mg/m^3 for GBP with density = 1, recognizing that in concentrations exceeding the physiological lung clearance capacity GBP can cause chronic inflammation and increase the lung cancer risk in laboratory animal experiments. The GBP category will include dusts that following occupational long-term exposure to concentrations not higher than 0.3 mg/m^3 should not induce adverse effects in the respiratory tract of workers.

3 Objectives

Using 6 granular dust candidates the solubility and non-inflammogenicity criteria for GBP dusts were experimentally determined. Two well-characterised inert dusts, i.e. µ-TiO_2 Bayertitan T and µ-$BaSO_4$ and 4 other candidates should be analysed at the same volumetric lung burdens in the rat model and the basic data recorded for GBP dusts defining “low solubility” and “non-adverse inflammation”.

A. The experimental modules were
- To perform an intratracheal instillation study using equivalent dust volume doses;
- To measure the inflammatory response in lung lavage fluid (BALF) at days 3 and 28;
- To derive a general value for the term ‘low soluble’ (e.g. a solubility in lung fluid of approx. 1 mg/l H_2O) → Chemical analysis of dust biopersistence in lungs at days 3, 28 and 90;
  Rationale:
  Which granular dusts show a negligible biosolubility in the range ≤1 mg/l and do not show a specific chemistry-related toxicity?

B. To establish an in vitro assay with lung cells; proof on predictability of in vitro vs. in vivo results.

4 Selection and Basic Data of “6 Dusts Group”

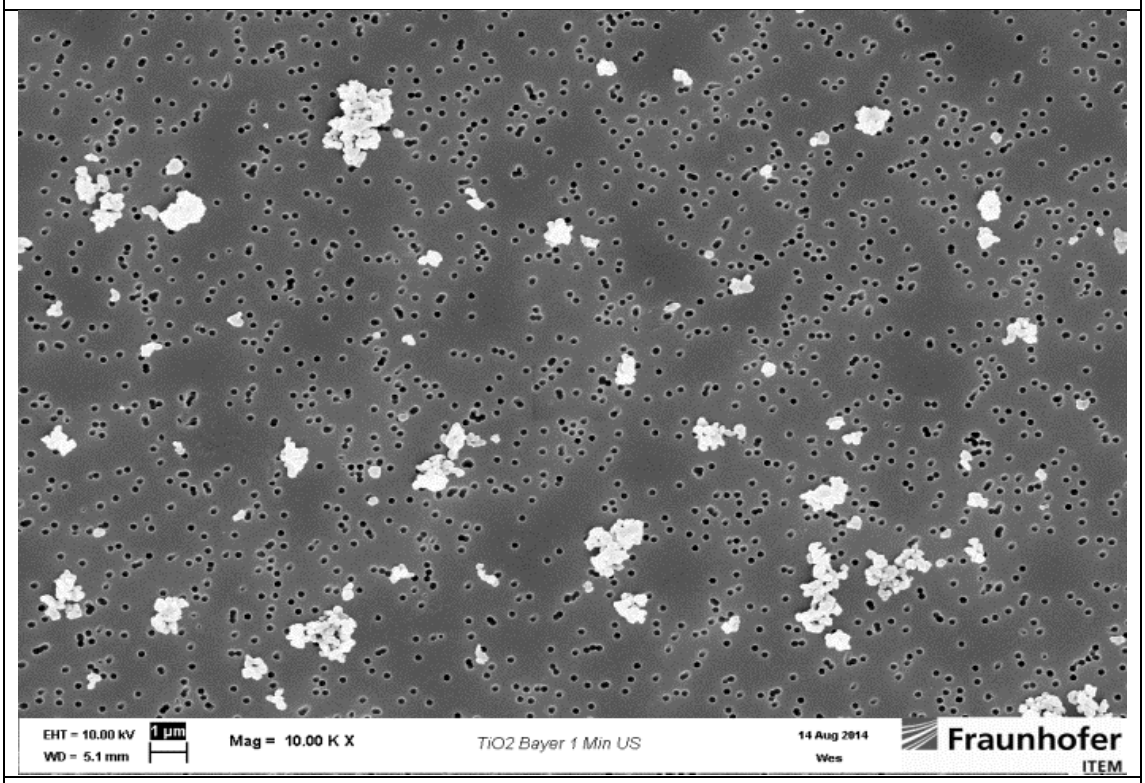
A telephone conference was held on March 4, 2014 to agree on 6 dusts as “GBP candidates” of the study. Participating parties on this discussion were IFA, St. Augustin (Mattenklott), University of Gießen (Walter), BAuA, Dortmund as sponsor (Gebel, Orthen) and Fraunhofer ITEM, Hannover (Creutzenberg), the latter as executing research institute. The physico-chemical data of the finally agreed 6-dust group is presented in Table 4.1.

Table 4.1 Selection oft the 6 dusts to be used in the GBP project

Dust	Origin	Properties
1 **µ-TiO_2** **Bayertitan T**	Bayer Produced in 1985	ρ: 4.3 98,17% TiO_2 99,5% Rutile BET: 1,9 m^2/g; EGME: 21,7 m^2/g µ-TiO_2; MMGD: 1,8 µm (GSD: 1,9) ζ-Potential: pH=4 -11,47 mV; pH=6 -35,08 mV; pH=8 -43,03 mV Toxicity profile: Prototype of an inert dust for in vivo tests at Fraunhofer ITEM **Literature:** CREUTZENBERG et al. (2008), Inhalation Tox 20, 995
2 **nano-TiO_2** **TiO_2 P25** Commercial sample, purchased and characterised by EU/JRC	Evonik	ρ: 3.8 BET: 60 m^2/g Anatase/Rutile 80%/20% Widely used „Standard titanium dioxide dust“ for in vivo toxicity testing Comm.: This TiO_2 type (JRC code: NM-105), though consisting of an anatase/rutile 80%/20% mixture, exhibits a smaller toxic potential in lungs than the surface-modified pure rutile TiO_2 types NM-103 and NM-104
3 **µ-Eu_2O_3**	American Elements	ρ: 7.4 A microsized dust with very small nanosized moiety.
4 **µ-$BaSO_4$**	Sigma-Riedel	ρ: 4.5 A microsized $BaSO_4$ was selected CAS # 7727-43-7 Lot # SZBD0080V **Literature:** CULLEN et al., 2000
5 **µ-ZrO_2** Y-stabilised Zirconia (YSZ)	American Elements	ρ: 5.7 Stabilized zirconia or zirconium oxide Standard powder particle sizes average in the range of - 325 mesh, - 100 mesh, 10-50 microns and submicron (< 1 µm) White high surface area particles available fully stabilized (8 mol%) or partially stabilized (3 mol%) or doped with yttria (yttrium oxide). Nanoscale yttria is typically 5 - 100 nanometers (nm) with specific surface area (SSA) of 25 - 50 m^2/g. Application: High temperature ceramics, technical ceramics, prostheses Solubility in water is given with 1 mg/l (20 °C).
6 **nano-SiO_2** Amorphous SiO_2, “nano-structured”	JRC, Ispra	ρ: 2.2 (gas pycnometry) JRC code: NM-200; precipitated amorphous SiO_2. The solubility of SiO_2 in water is dependent on its modification or its grade of crytalline order. In case of quartz (crystalline SiO_2) the solubility at 25 °C is approx. 10 mg SiO_2 per litre water (solubility equilibrium may be reached very slowly only). In contrary, amorphous silica shows at 25 °C with approx. 120 mg/l water an evidently increased solubility. With increasing temperature solubility becomes higher (quartz: 60 mg/l water at 100 °C; amorphous silica: 1100 mg/l water at 75 °C. - Data by Fraunhofer IKTS: In water up to 14 days at a concentration of 5 g/l → approx. 5% SiO_2 dissolves (also determined in RPMI w/ horse serum and FBS as well as in DMEM w/ FBS (up to 48h))

ρ: Material densities
“nano-structured”: formed by primary nanoparticles; agglomerated and aggregated/sintered following aging

μ-TiO_2 Bayertitan T



nano-TiO_2 P25 NM-105

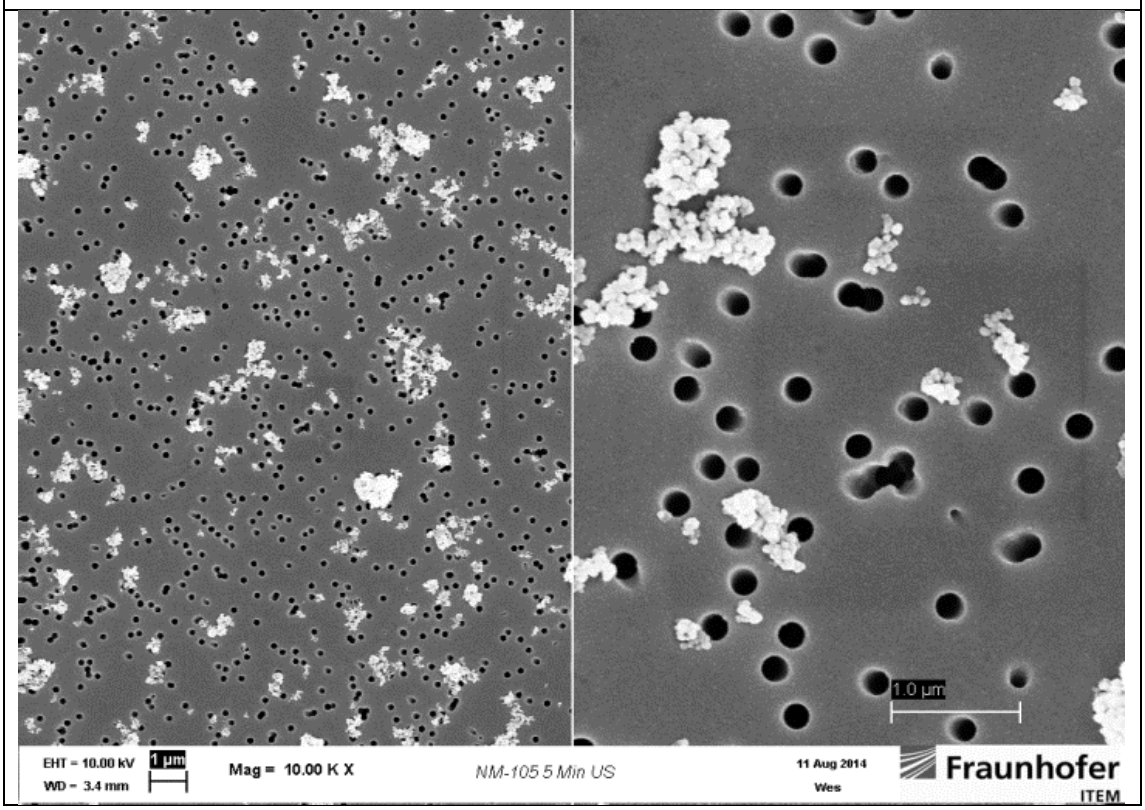
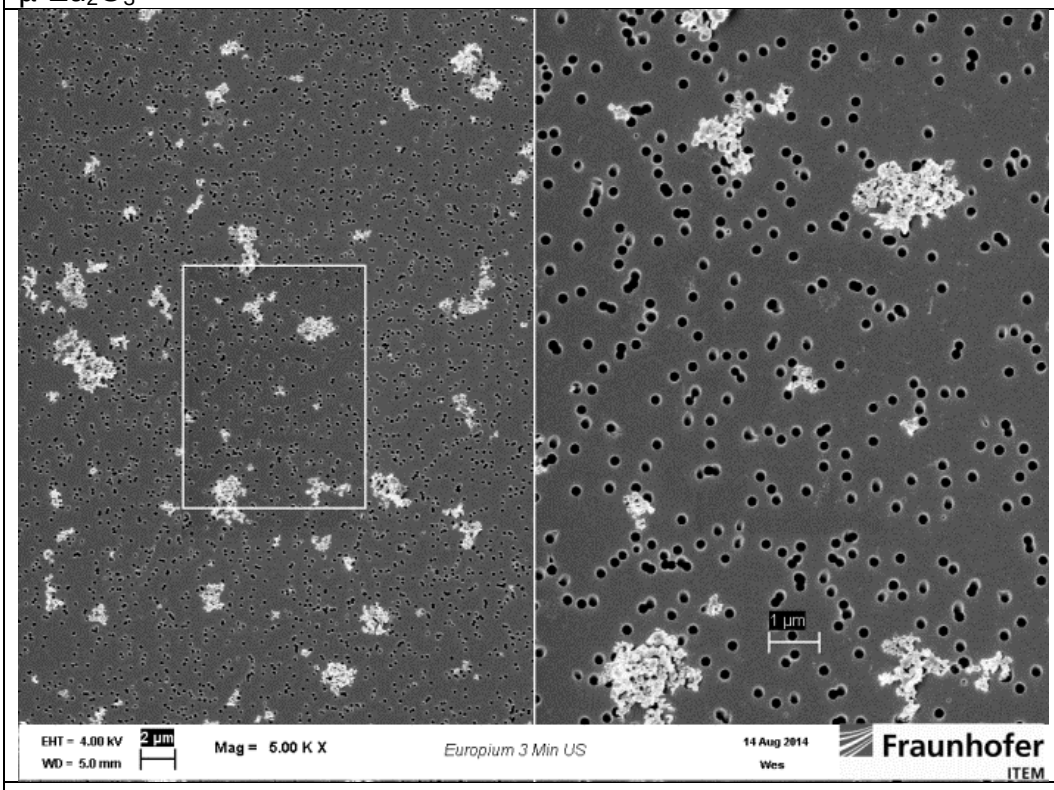


Fig. 4.1 SEM photographs of the “6-dust group”

μ-Eu_2O_3



μ-$BaSO_4$

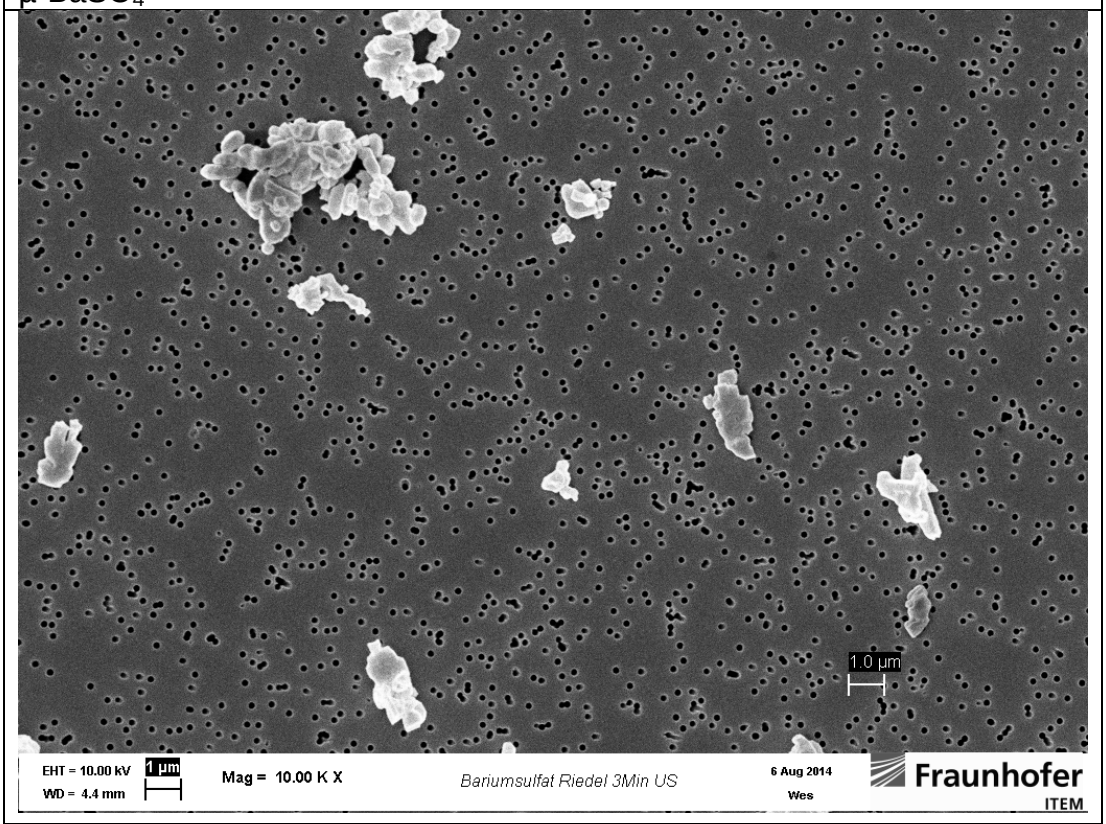
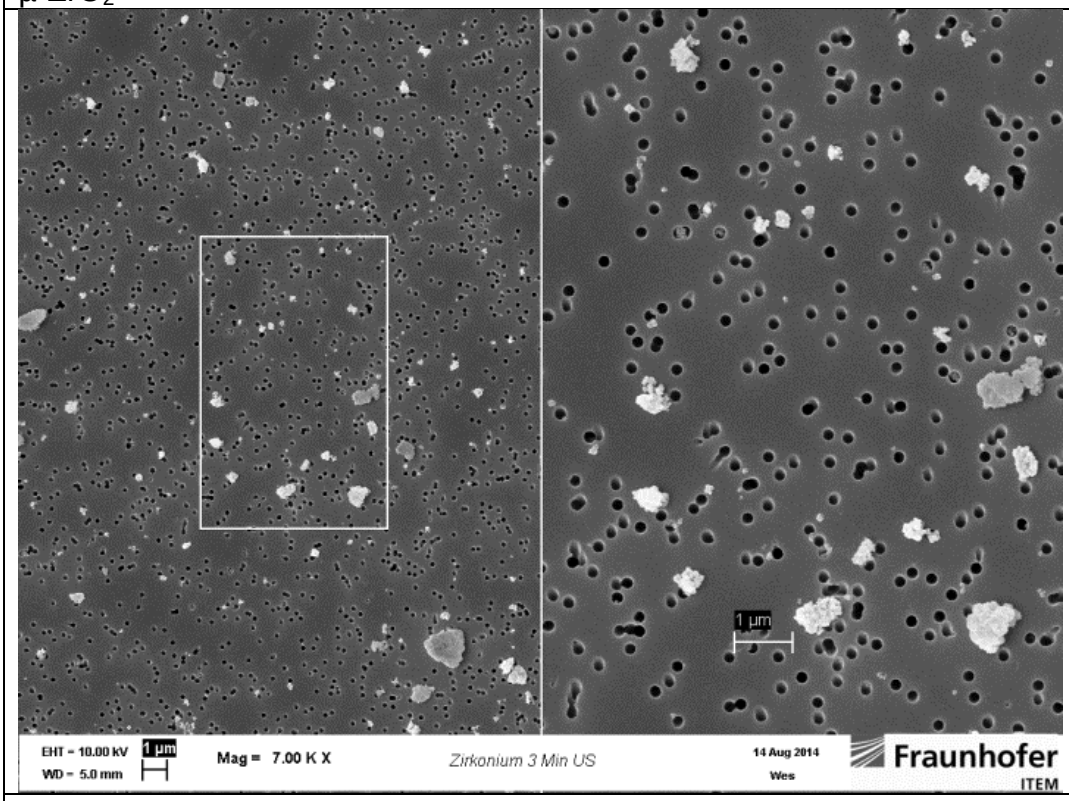


Fig. 4.2 SEM photographs of the “6-dust group”- cont’d

μ-ZrO_2



nano-SiO_2 NM-200

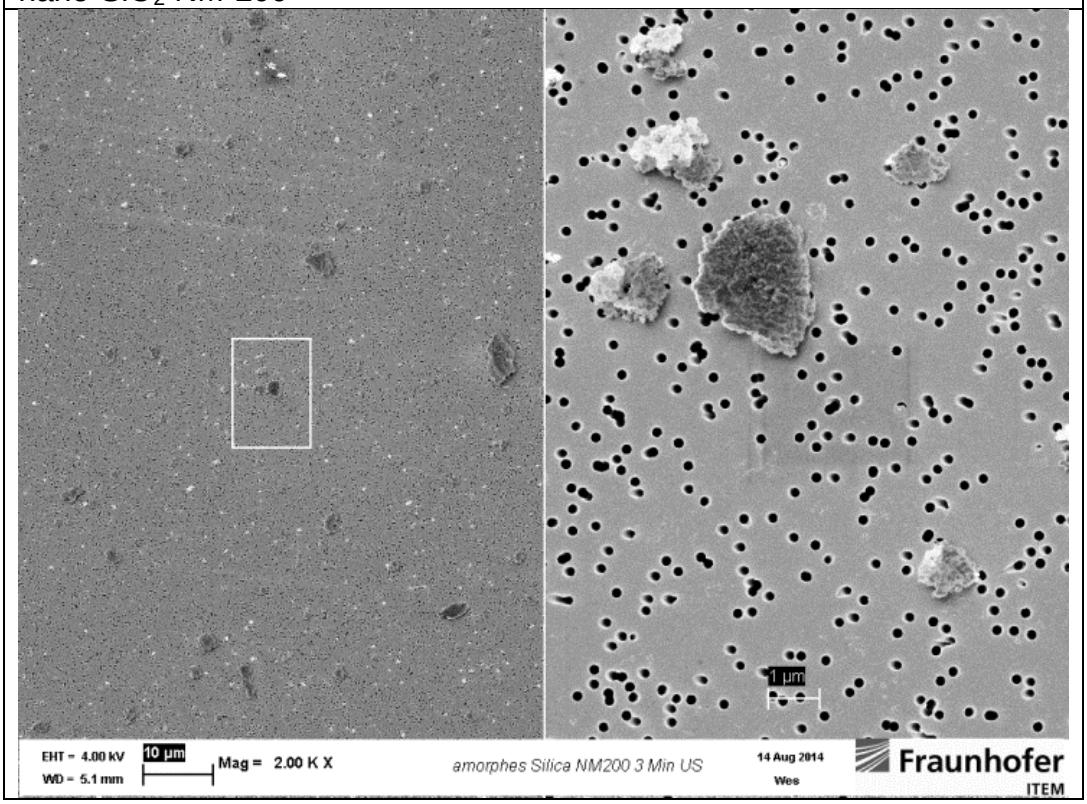


Fig. 4.3 SEM photographs of the “6-dust group” – cont’d

5 Conduct of the Study

5.1 Staff

Project Management	Dr. rer. nat. Otto Creutzenberg
Module 1: in vivo study	
Veterinarian:	Dr. med. vet. Thomas Tillmann
Clinical Chemistry:	Dr. rer. nat. Tanja Hansen
Chemical Analytics:	Dr. rer.nat. Sven Schuchardt Dipl.-Ing. Heiko Kock
Module 2: in vitro study	
In vitro Toxicology:	Dr. rer. nat. Jan Knebel Dr. rer. nat. Tanja Hansen
Module 3: Acellular solubility tests	
In vitro Toxicology:	Dr. rer.nat. Sven Schuchardt Dipl.-Ing. Heiko Kock

5.2 Project Modules

Start of project:	February 1, 2014
End of project:	April 30, 2016

Module 1
Intratracheal instillation test with subsequent analysis of the BAL fluid
Fraunhofer ITEM study no. 02 N 14 534

Intratracheal instillation test with subsequent chemical analysis of the test item lung loads
Fraunhofer ITEM study no. 02 N 14 535

Module 2: In vitro toxicity of the “GBP dusts” in acellular/cellular assays

Module 3: Acellular solubility tests for definition of a threshold value “low solubility”

6 Materials and Methods - in vivo Study

6.1 Body Weight Data

Body weights were recorded weekly using electronical balances and the PROVANTIS system (data management system for laboratory studies).

6.2 BALF Analysis after i.tr. Instillation

Animal model
Male Wistar rats, strain Crl:WI(Han) were used as in vivo model. The 156 rats were 9 weeks aged at intratracheal treatment.

Animal treatment
Before starting the main test, it was checked in a pre-test (see Appendix 1 for experimental design) whether rats would tolerate the high dose of 1.5 µl/rat for all 6 test dusts. The pre-test confirmed the tolerability.
In the main test, rats were treated by intratracheal instillation on two consecutive days (two ½ aliquots amounting to the total dose) administering 0.5 and 1.5 µl/rat in each group. For conversion of the dose volumes to dose masses material densities or agglomerate densities* were used (see table 6.1 and section 9.2). Rats were sacrificed 3 and 28 days after instillation (see Appendix 2).

Table 6.1 Densities of the test substances

Groups	Test substances	Material density ρ
2 + 3	µ-TiO_2 - Bayertitan T	4.3
4 + 5	nano-TiO_2 - TiO_2 P25 EU/JRC	3.8*
6 + 7	µ-Eu_2O_3	7.4
8 + 9	µ-$BaSO_4$	4.5
10 + 11	µ-ZrO_2 - Y-stabilised (YSZ)	5.7
12 + 13	nano-SiO_2 - NM-200	2.2*

*For conversion of the dose volumes to dose masses material densities were used. This is the regular approach for microscaled dusts because a void volume is negligible. For the nanoscaled dusts TiO_2 P25 (material density TiO_2 = 4.3) and nano-SiO_2 (material density TiO_2 = 2.65) reduced material densities were used. For TiO_2 P25 an agglomerate density of 3.8 g/cm^3 was estimated to take into account the higher void volumes of nanomaterials. For nano-SiO_2 an experimental value of 2.2 g/cm^3 (determined by gas pycnometry; see Table 4.1) was available.

Analysis of the bronchoalveolar lavage fluid (BALF)
Bronchoalveolar lavage was performed in 6 males per group after end of treatment (day 3 and 28). The method of HENDERSON et al. (1987) was used with minor modifications.
Following preparation, the lungs were lavaged with saline using two lavages of 5 ml each. The lavage fluid was collected in calibrated tubes and the harvested volume was recorded. Until processing the BALF was kept on ice. Leukocyte concentration of the lavagate was determined using a counting chamber and two cytospots were

prepared with a cytocentrifuge (Shandon Co., Frankfurt, Germany) for differential cell count (macrophages, neutrophils, lymphocytes).

Endpoints
After centrifugation of the BALF, biochemical indicators relevant for diagnosis of lung damage were determined in the supernatant (lactic dehydrogenase - LDH, β-glucuronidase, total protein). These parameters were analysed according to routine clinical chemistry protocols using a Cobas Fara device (Roche Co., Grenzach, Germany).

The justification of the parameters is given below:

Cytological parameters
- total cell count (recruitment of lung leukocytes)
- differential cell count (inflammatory (PMNs) or immunological (lymphocytes) reactions; a total of 400 leukocytes per rat were evaluated)

Biochemical parameters
- lactic dehydrogenase (LDH = cytosolic marker enzyme; increased permeability of membranes, cell damage and lysis)
- β-glucuronidase (measure of phagocytic activity of macrophages; lysis of macrophages)
- total protein (marker of transsudation; damage of epithelial cells)

6.3 Chemical Analysis of Lung Loads after i.tr. Instillation

Animal model
Male Wistar rats, strain Crl:WI(Han) were used as in vivo model. The 234 rats were 9 weeks aged at intratracheal treatment.

Animal treatment
Rats were treated by intratracheal instillation on two consecutive days (two ½ aliquots amounting to the total dose) administering 0.5 and 1.5 µl/rat in each group. Rats were sacrificed 3, 28 and 90 days after instillation (see Appendix 2).

Retention analysis of “6-dust group” in lungs
After sacrifice the right lung lobes were subjected to lyophilisation and subsequent low-temperature ashing and the test items retained in lung tissue were determined using ion-coupled plasma mass spectroscopy (ICP-MS). The soluble und insoluble moiety, the latter being the moiety gainable by filtration (0.2 µm nuclepore filters; Whatman) were separately analysed. In addition, particle retention was determined in exemplary organs such as liver and brain; spleen and kidneys were preserved for potential analysis.

7 Materials and Methods - Acellular/cellular in vitro Assays

7.1 Plasmid Scission Assay

The ROS-generating capacity of the test dusts was characterized using the plasmid scission assay. This cell-free assay is based on the direct bioreactivity of particles in aqueous solution against plasmid DNA. A plasmid ΦX174 RF DNA molecule susceptible to be damaged by ROS was used. Undamaged DNA exists in the supercoiled form, whereas damaged DNA is linearized.
Four concentrations were analyzed for each dust. Carbon Black (Printex®90, 0.1 mg/ml) was used as positive control. Plasmid DNA was diluted in molecular grade water to yield a concentration of 200 µg/ml. 1 µl DNA solution were combined with 19 µl sample and the samples were gently agitated for 6 h and subsequently electrophoresed on a 0.6% agarose gel. Gels were stained with ethidium bromide and imaged using a Gel Doc 2000 station (BioRad) and the software package Quantity One (Version 4.6.9).

7.2 ESR Measurements

Cell-free
For ESR measurements the spin trapping reagents 4-hydroxy-TEMPO (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) was used to investigate the ROS generating potential of the compounds. TEMPO itself can be measured by ESR-spectrometry. It is a stable radical which is neutralized when reacting with other reactive species resulting in a loss of ESR signal intensity and was used for the cellular and cell free ESR measurements.
For cell free ESR measurements the respective concentrations of the test compounds were the two doses used in the in vivo experiments (recalculated to in vitro cell growth areas) and the high in vivo dose multiplied by 5 (Table 7.1). The final artificial alveolar fluid (AAF) concentration in each sample was adjusted to 15% by dilution with a HBSS/AAF mixture. The formulation of the reaction mixture is shown in Table 7.2.

Table 7.1 In vitro concentrations (µg/ml) for the respective substance (calculated from 2950 cm² lung surface in vivo with 2/3 deposition)

Substance	1st Concentration	2nd Concentration	3rd Concentration
µ-TiO_2	0.98	2.92	14.60
nano-TiO_2	0.86	2.58	12.88
µ-Eu_2O_3	1.43	5.02	25.08
µ-$BaSO_4$	1.02	3.06	15.28
µ-ZrO_2	1.29	3.87	19.34
nano-SiO_2	0.50	1.49	7.46

Table 7.2 Formulation of the reaction mixture for the cell free ESR measurements.

Substance	Stock concentration	Final concentration	Volume
H_2O_2 ($Aqua_{bidest}$)	250 mM	50 mM	100 µl
Test-solution (AAF)	5x	1x	100 µl
Tempo ($Aqua_{bidest}$)	150 µM	22.5 µM	75 µl
HBSS/AAF	33.33% AAF	15% AAF	225 µl
Total volume	--	--	500 µl

The reaction mixtures were pipetted in Eppendorf vials, placed on a mixer (Thermomixer compact, Eppendorf, Germany) and shaken at 1250 RPM and 25 °C. The reaction was started by addition of the test item-suspensions (suspended in HBSS containing 15% AAF). ESR spectra were recorded after 10 minutes with Tempo as spin trapping reagent. For ESR measurements, 50 µl of the respective solution was sucked into a glass capillary and analyzed in a Bruker ESR-Spectrometer using the following parameters: Center Field 3460G; Microwave Power 13 mW; Modulation Amplitude 1.8G; Max. receiver gain; Number of Scans 10; Scan width 200G.

Cellular measurements
Cultured cells (THP-1 cells; see 7.3) were exposed to the test substances as described above at EC_{20} concentration and a spin trapping reagent was added to the basal compartment of each well. After exposure the cells were washed and mechanically detached by scraping. The resulting cell suspension was transferred to an Eppendorf vial and resuspended. Small samples of approximately 50 µl were then sucked into glass capillaries and analyzed in a Bruker ESR-Spectrometer.

7.3 Cytotoxicity towards THP-1 Cells

Routine culture of THP-1 cells
The human monocytic cell line THP-1 (DSMZ, ACC-16) was routinely grown in suspension in DMEM medium supplemented with 10% heat-inactivated FBS and 0.01% gentamycin (growth medium). THP-1 cells were splitted three times a week, so that the cell concentration never exceeded $1x10^6$ cells/ml. On Fridays, cells were taken from the routine culture for macrophage differentiation.

Macrophage differentiation

On the previous day the corresponding number of transwells (BD; 0.4 µm pore size; Polyester; 12-well; 353180) were pre-conditioned in growth medium (1.5 ml bottom compartment, 0.5 ml upper compartment) in a companion plate (BD; 12-well; 353503) at 37 °C, 90% humidity, 5% CO_2 in the incubator until seeding on the next day. Before the actual seeding of the cells was performed an appropriate volume of 50 nM PMA (Phobol 12-myristate 13-acetate; Sigma; P8139; stock solution: 50,000 nM in DMSO) containing growth medium was prepared (0,1% DMSO). 1 ml of the solution was placed in a fresh companion plate and the empty pre-conditioned transwells were transferred to this PMA-containing medium (no medium should be lacking through the membrane of the transwells). The so prepared transwells were placed back into the incubator. To prepare the cell suspension, an appropriate amount of cells was taken from the routine culture and transferred to a falcon. The cells were pelleted at 800 rpm, 4 °C for 5 min and the supernatant was discarded. The cell pellet was resuspendet in medium so that a concentration of $0.375x10^6$ cells/ml was reached. The appropriate amount of PMA-stock solution was added to the cell suspension to prepare a concentration of 50 nM PMA. After gently mixing, 200 µl of cell solution (75,000 cells) were placed into the empty upper compartment of the transwell. The cells were then incubated for 72 hours in the incubator, during which the cells should adhere at the membrane and undergo a morphological change. Thereafter a medium change was performed into fresh growth medium and the cells are ‘rested’ in this PMA-free medium for another 24 h. Subsequently the differentiation process should be completed and the cells were ready for exposure.

Experimental design with THP-1 monocytes

Per day of experiment the substances were tested w/ and w/o AAF in parallel. Therefore a 48-well plate were set up with two wells per condition (medium control, AAF control, five particle concentrations w/ and w/o AAF, triton x-100 control). Therefore $0.75x10^6$ cells/ml were transferred to a Falcon® tube and pelleted at 800 rpm, 4 °C for 10 min. The supernatant was discarded and replaced by the corresponding treatment solution in exposure medium (DMEM + 2% heat-inactivated serum + 0,01% gentamycin) so that a cell concentration of $0.6x10^6$ cells/ml was prepared. 0.5 ml of the cell suspension with respective treatment (300,000 cells) were then placed in a fresh 48-well plate with a row of pure exposure medium at the outer wells to prohibit evaporation of the fluids. After 24 h exposure in the incubator 50 µl of WST-8 solution were added to each well and the cells were incubated for another 30 min at 37 °C. Per well three technical replicates of 100 µl were transferred in a fresh 96-well and the absorbance of the colored solution was measured at 450 nm.

Experimental design with THP-macrophages

Per day of experiment two substances were tested in parallel w/ AAF. Therefore two companion plates were set up with two transwells per condition (AAF control, three concentrations w/ AAF per particle, triton x-100 control). 1.5 ml of fresh exposure medium with 5% AAF was placed in the bottom compartment of the companion plate. The medium from the rested cells in the transwells was discarded and the macrophages-containing transwells were placed in the prepared companion plate. Uninterrupted 0.5 ml of the respective treatment solution was placed in the transwells on top of the cells. After 24 h exposure 300 µl medium from the bottom compartment was collected for IL-8 measurement and stored at -80 °C. Thereafter 50 µl of WST-8 solution were added to each transwell and the cells were incubated for another 2 h at

37 °C. Per well three technical replicates of 100 µl were transferred in a fresh 96-well and the absorbance of the colored solution was measured at 450 nm.
For the IL-8 determination the Human CXCL8/IL-8 ELISA Kit was used (DuoSet®ELISA; R&D Systems; DY208). The supernatant was measured undiluted and in two technical replicates.

8 Materials and Methods – Acellular Solubility of Test Items

Artificial lung fluids are widely used to get information on probable biosolubility of unknown particles and can well supplement the database because mostly the provided data of particles refer to the solvent water. While artificial alveolar fluid (AAF) and Gamble's solution (GS) mirror the composition of the lung lining fluid (surfactant; pH=7.4), artificial lysosomal fluid (ALF) simulates the ambience in the lysosomes of alveolar macrophages (pH=4.5). The composition of the artificial lung fluids used in this study is given in Table 8.1.

Method

- 100 mg of the test item were shaken in 50 ml artificial lung fluid solution in darkness
- Temperature 37 +/-2 °C
- pH: 4.5 – 7.4
- Measurements after 0.5 - 1 - 2 - 3 - 4 - 6 - 8 - 24 - 48 - (72) hrs
- Centrifugation: 4000 rpm, 15 min
- Filtration of supernatant with 0.1 µm pore size filter

Table 8.1 Composition of Gamble's solution (GS), artificial alveolar (AAF) and artificial lysosomal fluid (ALF)

	GS	**AAF**	**ALF**	Remarks
	(g/l)	(g/l)	(g/l)	
$MgCl_2$ x 6 H_2O	0.2033	0.2033	0.106	
NaCl	6.0193	6.0193	3.210	
KCl	0.2982	0.2982		
Na_2HPO_4	0.1420	0.1420	0.095	
Na_2SO_4	0.0710	0.0710	0.039	
$CaCl_2$ x 2 H_2O	0.3676	0.3676	0.128	
NaAc x 3 H_2O	0.9526	0.9526		
$NaHCO_3$	2.6043	2.6043		
Na-citrate x 2 H_2O	0.0970	0.0970	0.077	
Phosphatidyl-choline		0.1000		Choline: low solubility in H_2O; in dissolution tests pre-solution with EtOH
NaOH			6.000	
Citric acid			20.800	
Glycine			0.059	weighing
Na-tartrate x 2 H_2O			0.090	
Na-lactate			0.085	
Na-pyruvate			0.086	7.83 ml / 100 mM-solution
Formaldehyde			2.300	ml, 16%
pH	**7.4**	**7.4**	**4.5**	adjusted with H_3PO_4 / NaOH

Acellular Test on Solubility

The solubility was analysed at the two basic pH values existing in lungs:

pH = 7.4 Surfactant lung lining fluid	Artificial alveolar fluid (AAF)
pH = 7.4	Gamble's solution (GS)
pH = 4.5 Lysosomal fluid in macrophages	Artificial lysosomal fluid (ALF)

In this approach, the i.) particle deposition at the surfactant of alveoli and ii.) the uptake of particles in the lysosomes of the alveolar macrophages are simulated. The soluble (ionic) moiety was quantified by using ICP-MS analysis.The solubility of test items was derived from the ratio ionic moiety/start mass of test item.

Recommendations given by TG Guideline OECD 105

"Low soluble" particles should be analysed using a high start mass in small solvent volume: e.g. 0.25 g/50 ml; analysis to be done in triple measurements.
Alternatively, 3 different start masses can be chosen (e.g. 0.1/0.25/0.5 g/50 ml).
Justification: High start masses result (in case of "not soluble" compounds) i.) more easily in significantly detectable ionic fractions and ii.) avoid errors while determining the start masses.

9 Results – in vivo Study

9.1 Body Weight Development

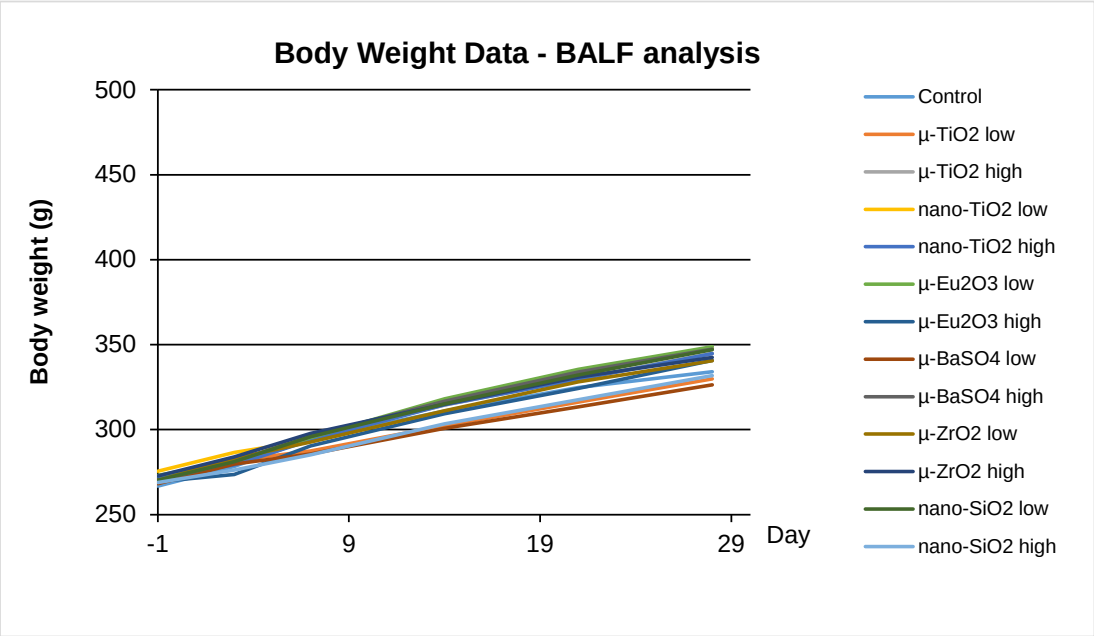


Fig. 9.1 Body weight development I

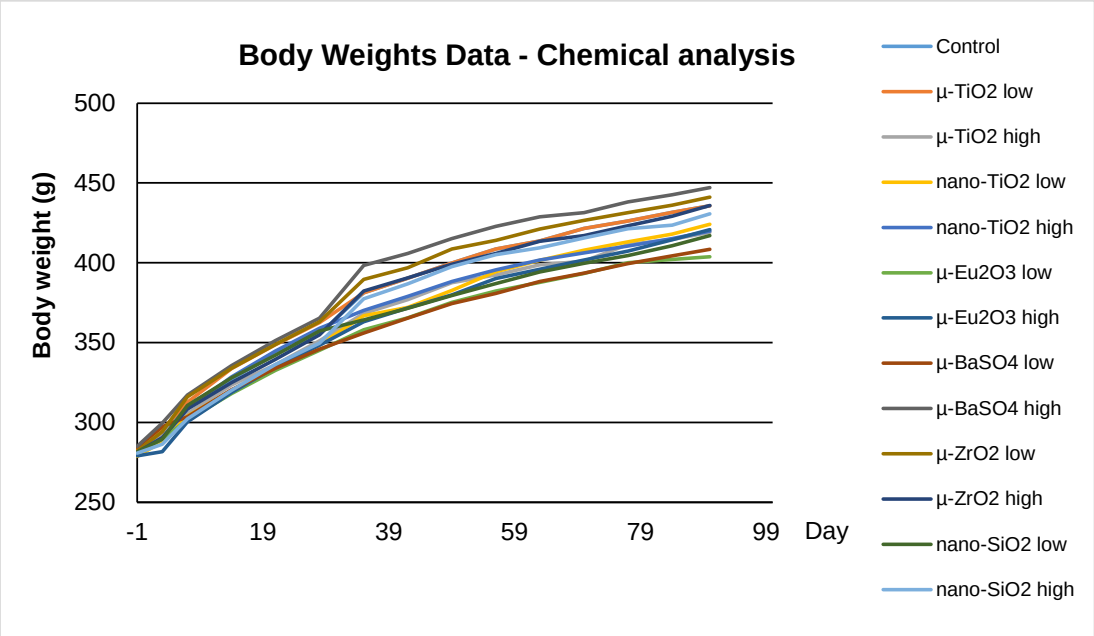


Fig. 9.2 Body weight development II

Body weights of rats used for BAL or chemical analysis did not show statistically significant changes as compared to vehicle controls.

9.2 Intratracheal Instillation: BALF Analysis

Results of PMN analysis in BALF are presented in Figures 9.3/9.4 (percentual values) and in Figures 9.5/9.6 (absolute values). Mean data are shown in a table in Appendix 3.
The differential cell count showed a slight increase in inflammatory cell (polymorphonuclear neutrophils - PMN) levels after treatment with µ-TiO_2 (rutile) and µ-$BaSO_4$: < 5% after 3 days in the low dose group; < 18% in the high dose group; full recovery after 28 days. In contrast, the nano-TiO_2 (anatase) showed a stronger response (PMN > 30% after 3 and 28 days). The rare earth µ-Eu_2O_3 dust showed the strongest effect (approx. 50-60 and 40% PMN) with exhibiting a red-coloured lung lavage fluid (hemolytic effect), after 3 and 28 days. µ-ZrO_2 and nano-SiO_2 exhibited a strong acute response after 3 days, however, mostly complete recovery after 28 days.
In absolute numbers, the PMN concentration in BALF was in the range of 2000 PMN/ml (vehicle control) up to >300,000 PMN/ml in the Eu_2O_3 high dose group (day3).

Results of biochemical parameters in the BALF supernatant mirror the outcome of the differential cell count (Table 9.1; data are presented in normalized mode; control= 100%). The means as absolute data are shown in a table in Appendix 3.

Overall, µ-TiO_2 (rutile; Bayertitan T) and µ-$BaSO_4$ (Sigma-Riedel) met the “not inflammatogenic” criterion (PMN influx) at volumetrically non-overload dose, the other ones did not. µ-Eu_2O_3, µ-ZrO_2 and nano-SiO_2 showed a strong acute PMN response (day 3) that persisted up to day 28. For nano-TiO_2 (anatase) a strong PMN response was observed at day 3 and 28 after instillation.

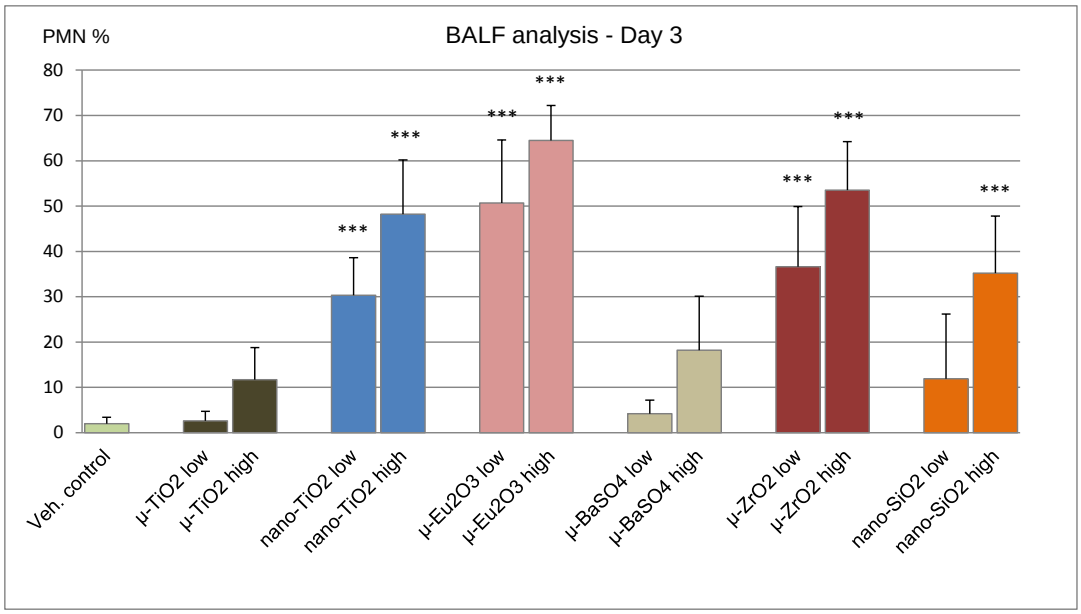


Fig. 9.3 Polymorphonuclear neutrophils (PMN) levels in BALF at day 3, percentual values

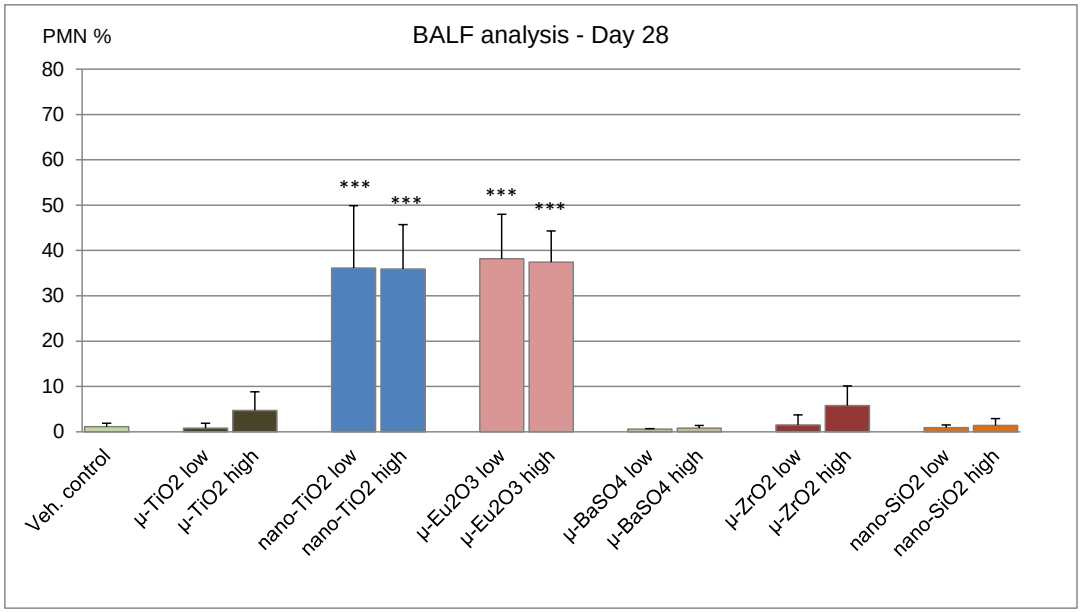


Fig. 9.4 Polymorphonuclear neutrophils (PMN) levels in BALF at day 28, percentual values

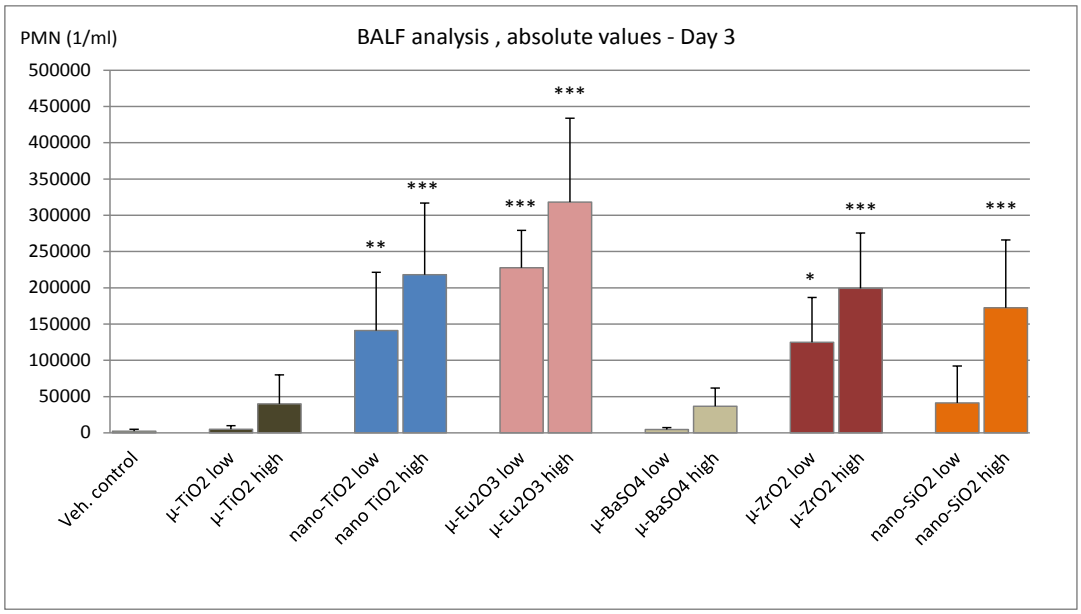


Fig. 9.5 Polymorphonuclear neutrophils (PMN) levels in BALF at day 3, absolute values

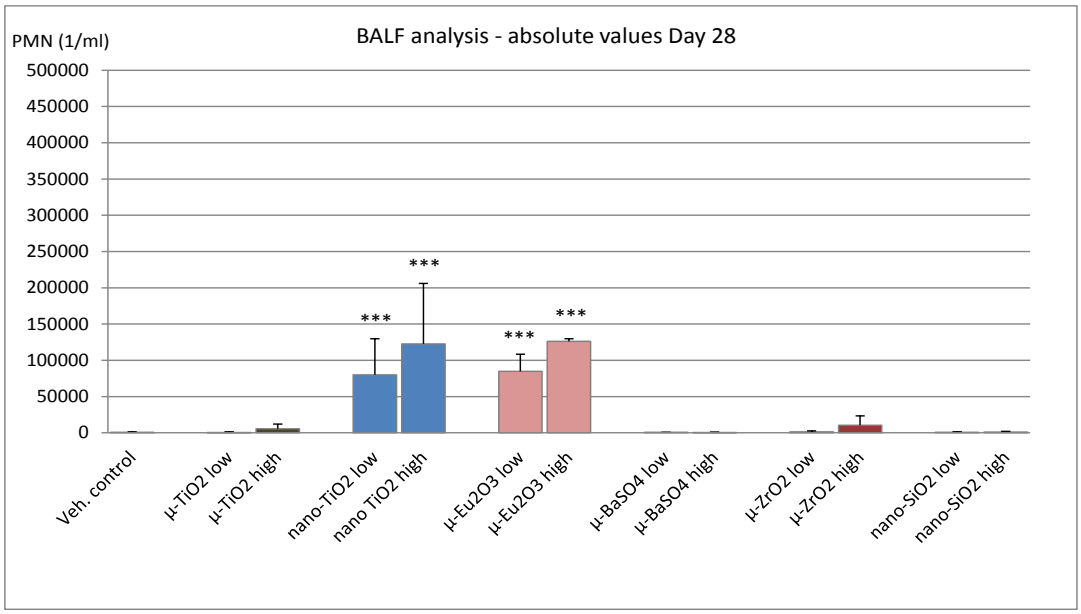


Fig. 9.6 Polymorphonuclear neutrophils (PMN) levels in BALF at day 28, absolute values

Table 9.1 Normalised data of analytes in the BALF

Day 3 (normalized data: vehicle control = 100%)

Males		LDH U/L	GLU U/L	TP mg/L
1m	Mean	100	100	100
2m	Mean	146	103	117
3m	Mean	199	138	167
4m	Mean	415	476	318
5m	Mean	765**	829	471
6m	Mean	1601**	4068**	2023**
7m	Mean	2041**	5191**	2057**
8m	Mean	121	74	113
9m	Mean	203	126	157
10m	Mean	536	1676**	1159**
11m	Mean	1058**	2397**	1294**
12m	Mean	432	288	294
13m	Mean	733**	588	468

Day 28

Males		LDH U/L	GLU U/L	TP mg/L
1m	Mean	100	100	100
2m	Mean	98	85	90
3m	Mean	160	185	117
4m	Mean	330	500	1934
5m	Mean	509**	1050	2845
6m	Mean	673**	2660**	687**
7m	Mean	1399**	8150**	1589**
8m	Mean	118	125	96
9m	Mean	126	100	102
10m	Mean	116	110	91
11m	Mean	263	265	159
12m	Mean	137	125	109
13m	Mean	110	85	107

Statistics Test: Dunnett Test: * - 5% significance level; ** - 1% significance level

Group 1 - Vehicle Control | Group 2 - μ-TiO_2 low | Group 3 - μ-TiO_2 high
Group 4 - nano-TiO_2 low | Group 5 - nano-TiO_2 high | Group 6 - μ-Eu_2O_3 low
Group 7 - μ-Eu_2O_3 high | Group 8 - μ-$BaSO_4$ low | Group 9 - μ-$BaSO_4$ high
Group 10 - μ-ZrO_2 low | Group 11 - μ-ZrO_2 high | Group 12 - nano-SiO_2 low
Group 13 - nano-SiO_2 high

LDH Lactic dehydrogenase - GLU β-Glucuronidase - TP Total protein

9.3 Intratracheal Instillation: Chemical Analysis of the Test Items

In Table 9.2 the actual volumetric and gravimetric doses determined by chemical analysis are given in absolute numbers as well as in percentages of the nominal doses instilled. The results of the chemical analysis at 3, 28 and 90 days post-treatment are given in Tables 9.3 – 9.8 (means). Individual data is given in Appendix 4. Means are illustrated also in Figures 1-6 (see Appendix 5).
The retention values analysed at day 3 after intratracheal instillation showed an average retention of approx. 66% as compared to the administered total dose in the low dose groups and of 72% in the high dose groups, i.e. approx. 1/3 of the dose has been eliminated from lungs by rapid clearance mechanisms (coughing, ciliae-mediated processes, etc) within the first 3 days (Table 9.2). This value is in accordance with historical data found at the same range (e.g. various preceding Fraunhofer studies).

Table 9.2 Volumetric and gravimetric doses detected at day 3 post-treatment

Dust #	Dust sample	Relative density	Low doses: 0.5 µl day 0 equivalent to µg:	Low doses: Determinedat day 3 % of administered dose	High doses: 1.5 µl day 0 equivalent to µg:	High doses: Determinedat day 3 % of administered dose
1	**µ-TiO_2 Bayertitan T**	4.3	2150	74.6	6450	87.9
2	**nano-TiO_2 TiO_2 P25**	3.8	1900	71.8	5700	67.3
3	**µ-Eu_2O_3 low „nano-structured“ fraction**	7.4	3700	75.7	11100	77.7
4	**µ-$BaSO_4$**	4.5	2250	57.2	6750	65.8
5	**µ-ZrO_2 Y-stabilised**	5.7	2850	51.8	8550	61.5
6	**Amorphous nano-SiO_2 „nano-structured“**	2.2	1100	18.8	3300	16.3
Day 3-retention: Average value of groups 1-5				66%		72%

For conversion of the dose volumes to dose masses material densities were used. This is the regular approach for microscaled dusts because a void volume is negligible. For nanoscaled dusts, experimental densities are often not available, the German MAK commission (MAK, 2015) recommends in this case to use a default value of 50% of the material density. For the nanoscaled dusts TiO_2 P25 (material density TiO_2 = 4.3) and nano-SiO_2 (material density TiO_2 = 2.65) reduced material densities were used. For TiO_2 P25 an agglomerate density of 3.8 g/cm^3 was estimated to take

into account the higher void volumes of nanomaterials. For nano-SiO_2 an experimental value of 2.2 g/cm^3 (determined by gas pycnometry; see Table 4.1) was available.

Table 9.3 Total burden – Dose 0.5 µl

Dust #			(µg/lung)				(% of total dose)		
			Day 3	Day 28	Day 90		Day 3	Day 28	Day 90
1	**µ-TiO_2**	Mean	1604	752	416		74.3	34.8	19.3
		SD	242	169	126				
2	**nano-TiO_2**	Mean	1365	1050	855		71.8	55.3	45.0
		SD	123	259	144				
3	**µ-Eu_2O_3**	Mean	2802	2628	2255		75.7	71.0	60.9
		SD	413	143	294				
4	**µ-$BaSO_4$**	Mean	1286	369	111		56.9	16.3	4.9
		SD	164	143	44				
5	**µ-ZrO_2**	Mean	1475	1527	998		51.6	53.4	34.9
		SD	159	251	200				
6	**nano-SiO_2**	Mean	207	52	16		18.8	4.7	1.5
		SD	15	14	4.6				

Table 9.4 Total burden – Dose 1.5 µl

Dust #			(µg/lung)				(% of total dose)		
			Day 3	Day 28	Day 90		Day 3	Day 28	Day 90
1	**µ-TiO_2**	Mean	5667	3200	2614		87.7	49.5	40.1
		SD	1724	1006	725				
2	**nano-TiO_2**	Mean	3834	3690	3577		67.3	64.7	62.8
		SD	420	439	565				
3	**µ-Eu_2O_3**	Mean	8630	8285	7249		77.7	74.6	65.3
		SD	903	633	525				
4	**µ-$BaSO_4$**	Mean	4442	2074	908		65.7	30.7	13.4
		SD	1068	572	339				
5	**µ-ZrO_2**	Mean	5255	4903	4215		61.4	57.3	49.2
		SD	665	694	1005				
6	**nano-SiO_2**	Mean	537	139	47		16.3	4.2	1.4
		SD	92	20	5.4				

Total dose = administered dose (see Table 9.2)

Table 9.5 Particulate burden – Dose 0.5 µl

Dust #			(µg/lung)				(% of total dose)		
			Day 3	Day 28	Day 90		Day 3	Day 28	Day 90
1	**µ-TiO_2**	Mean	1603	752	415		74.2	34.8	19.2
		SD	242	169	126				
2	**nano-TiO_2**	Mean	1359	1046	851		71.5	55.0	44.8
		SD	123	259	143				
3	**µ-Eu_2O_3**	Mean	2114	1536	1629		57.1	41.5	44.0
		SD	480	304	488				
4	**µ-$BaSO_4$**	Mean	1270	360	110		56.2	15.9	4.9
		SD	175	143	44				
5	**µ-ZrO_2**	Mean	1463	1520	994		51.2	53.1	34.8
		SD	160	250	202				
6	**nano-SiO_2**	Mean	205	50	15		18.6	4.6	1.4
		SD	15	14	4.7				

Table 9.6 Particulate burden – Dose 1.5 µl

Dust #			(µg/lung)				(% of total dose)		
			Day 3	Day 28	Day 90		Day 3	Day 28	Day 90
1	**µ-TiO_2**	Mean	5662	3196	2609		87.6	49.5	40.4
		SD	1723	1007	727				
2	**nano-TiO_2**	Mean	3822	3678	3564		67.1	64.5	62.5
		SD	420	440	560				
3	**µ-Eu_2O_3**	Mean	7246	5793	5696		65.3	52.2	51.3
		SD	1046	592	834				
4	**µ-$BaSO_4$**	Mean	4269	2028	838		63.2	30.0	12.4
		SD	955	552	353				
5	**µ-ZrO_2**	Mean	5234	4884	4205		61.1	57.1	49.1
		SD	664	697	1010				
6	**nano-SiO_2**	Mean	534	138	46		16.2	4.2	1.4
		SD	93	20	5.3				

Total dose = administered dose (see Table 9.2)

Table 9.7 Ionic burden – Dose 0.5 µl

Dust #			(µg/lung)				(% of total dose)		
			Day 3	Day 28	Day 90		Day 3	Day 28	Day 90
1	**µ-TiO_2**	Mean	1.3	0.7	0.4		0.060	0.032	0.019
		SD	0.3	0.3	0.2				
2	**nano-TiO_2**	Mean	5.8	4.1	4.1		0.031	0.022	0.02
		SD	1.9	2.1	0.9				
3	**µ-Eu_2O_3**	Mean	688	1093	625		18.6	29.5	16.9
		SD	145	280	287				
4	**µ-$BaSO_4$**	Mean	16	9.3	1.0		0.71	0.41	0.044
		SD	13	6.8	0.6				
5	**µ-ZrO_2**	Mean	11	6.9	4.4		0.39	0.24	0.15
		SD	4.5	3.7	3.3				
6	**nano-SiO_2**	Mean	2.1	1.6	1.1		0.19	0.15	0.10
		SD	0.5	0.3	0.4				

Table 9.8 Ionic burden – Dose 1.5 µl

Dust #			(µg/lung)				(% of total dose)		
			Day 3	Day 28	Day 90		Day 3	Day 28	Day 90
1	**µ-TiO_2**	Mean	5.1	3.5	2.1		0.079	0.054	0.033
		SD	2.7	2.1	0.2				
2	**nano-TiO_2**	Mean	12.8	12.0	13.3		0.22	0.21	0.23
		SD	4.1	4.0	4.7				
3	**µ-Eu_2O_3**	Mean	1384	2491	1553		12.5	22.4	14.0
		SD	344	406	606				
4	**µ-$BaSO_4$**	Mean	205	46	53		3.0	0.68	0.78
		SD	131	34	28				
5	**µ-ZrO_2**	Mean	21	19	10		0.24	0.22	0.11
		SD	5.5	16	8.5				
6	**nano-SiO_2**	Mean	1.9	1.5	1.0		0.06	0.05	0.03
		SD	0.3	0.2	0.2				

Total dose = administered dose (see Table 9.2)

Information on biokinetic behavior of nano-$BaSO_4$

To exclude the cleared mass underlying rapid clearance mechanisms from half-time calculation for the deep lung, only the retained masses at day 3, 28 and 90 (not the administered dose at day 0) were included into this calculation (see Table 9.9).

The clearance half-times (see Appendix 6) showed a value close to the physiological rat lung clearance of approx. 60 days in the µ-TiO_2 "Bayertitan T" low dose group. In the high dose group a slightly increased half-time (89 vs. 60 days) was observed (overload effect). In the nano-TiO_2 P25 group a doubled half-time was calculated in the low dose group (clearance retardation).

In the µ-$BaSO_4$ and nano-SiO_2 groups (either the low and high dose groups), smaller values in the range of 25-40 days were calculated indicating an additional dissolution effect. .

In the µ-Eu_2O_3 and µ-ZrO_2 groups increased half-times, i.e. 4- to 5-fold (low and high dose) and 2- to 4-fold, respectively, were calculated indicating a clear surface-chemistry-related contribution to the toxic and clearance-retardative outcome.

µ-TiO_2 "Bayertitan T" and nano-TiO_2 P25 showed very low ionic moieties regarding the total lung burden. Levels in lungs at all 3 time-points did not exceed the 0.1% percentage except TiO_2 P25 in the high dose → ≥ 0.2%.

Table 9.9 Calculated half-time values (absolute values and percentual values vs. 60 days of physiological rat lung clearance)

	Nominal dose (µl/rat)	Clearance half-time (days)	Clearance half-time (in % vs. the physiological half-time in rat lungs; 60 days = 100%)
µ-TiO_2	0.5	47	78
	1.5	89	148
nano-TiO_2	0.5	141	235
	1.5	866	1443
µ-Eu_2O_3	0.5	277	462
	1.5	347	578
µ-$BaSO_4$	0.5	26	43
	1.5	39	65
µ-ZrO_2	0.5	133	222
	1.5	257	428
nano-SiO_2	0.5	25	42
	1.5	27	45

The calculation of the clearance half-times is presented in Appendix 6.

µ-Eu_2O_3 resulted in the highest ionic percentages of all 6 dusts amounting to a range of 17-30% of the total mass in lungs.
µ-$BaSO_4$ showed low ionic moieties of 0.8% or lower regarding the total lung burden (at all 3 time-points). **µ-ZrO_2** showed low ionic moieties regarding the total lung burden. Levels in lungs at all 3 time-points did not exceed the 0.4% percentage.
Nano-SiO_2 showed low ionic moieties of 0.2% or lower regarding the total lung burden (at all 3 time-points).
Comparison **µ-Eu_2O_3 vs. nano-SiO_2**: Both show high solubility, however, the ionic moiety of Eu_2O_3 is eliminated from lungs more slowly than that of amorphous SiO_2. The difference may be caused by different transport mechanisms of the ions.

To reveal the mechanistic reason for the very specific behavior of $BaSO_4$ a comprehensive toxicokinetic investigation was conducted by KONDURU et al., 2014. A high biosolubility was found for a nano-$BaSO_4$ after lung instillation. The same was observed in this BAuA project using a µ-type of $BaSO_4$ particles.

The physiological rat lung clearance has been precisely investigated using low soluble radioactively tagged particles, e.g. 85Sr-polystyrene particles resulting in half-times of 50-70 days; therefore, a mean of approx. 60 days is a well-documented experimental value (MORROW, 1988; MUHLE et al., 1990; BELLMANN et al., 1991). The clearance half-time showed a value close to the physiological rat lung clearance of approx. 60 days in the **TiO_2 "Bayertitan T"** low dose group. In the high dose group a slight increase was observed (overload effect). For comparison: In the **nano-TiO_2 P25** low dose group the clearance was doubled as compared to physiological conditions (89 vs. 60 days clearance half-time); however, a rather high volumetric dose was used for this nanosized dust: The material density is 4.3 g/cm^3; in this GBP experiment, 3.8 g/cm^3 was chosen as agglomerate density → 500 µl. For comparison, 4.3 x ½ = 2.2 g/cm^3 is the value recommended by the MAK commission (MAK, 2015) to estimate unknown agglomerate densities. The dose of 1900 µg would correspond to 860 nl administered volume (MAK density), equivalent to 570 nl actual dose in the deep lung after 3 days. Overall, in the low dose group no lung overload was existing, independently on the chosen agglomeration density.
In the µ-ZrO_2 and µ-Eu_2O_3 low dose groups the half-times were doubled or even higher (factor: 4-5). Lower half-times than 60 days observed in the µ-$BaSO_4$ and nano-SiO_2 groups indicate an additive half-time effect due to an evident biosolubility. Correspondingly, in the high dose groups increased values were detected indicating a clear overload effects in the nano-TiO_2 P25 and µ-ZrO_2 high dose groups.

10 Results – Acellular/cellular in vitro Assays

10.1 Plasmid Scission Assay

The results of the Plasmid Scission Assay (PSA) are shown in Appendix 8, Fig. 1-6.
The PSA was negative for µ-TiO_2 Bayertitan (rutile), nano-TiO_2 P25 (anatase), µ-ZrO_2 and amorphous SiO_2.
The rare earth µ-Eu_2O_3 dust showed a strong effect at the highest concentration tested.
µ-$BaSO_4$ was effective at the two highest concentrations.
The results obtained with µ-Eu_2O_3 and µ-$BaSO_4$ were confirmed in an additional independent experiment.

10.2 Cell-free ESR Measurements

The results of the cell-free ESR measurements are shown in Appendix 8, Fig. 7-12. Relevant effects of the test dusts were not observed.

10.3 Cellular ESR Measurement

Results of the cellular ESR-experiments are shown in Appendix 8, Figures 13-18. Treatment related effects were not observed.

10.4 Cytotoxicity towards THP-1 Cells

With undifferentiated, monocytic THP-1 cells, no treatment related effects were observed (see Appendix 8, Figures 19-24). However, when differentiated THP-1 macrophages were challenged with the dusts using the same concentration, a dose-dependent decrease in viability was observed with the rare earth µ-Eu_2O_3 dust (Appendix 8, Figures 25-30).

10.5 IL-8 Secretion in THP-1 Macrophages

THP-1 cells were differentiated towards the macrophage phenotype and challenges with the dusts at three concentrations for 24 h. IL-8 was then quantified in the supernatant by ELISA. Significant treatment-related effects were not observed (Appendix 8, Figure 31).

11 Results – Acellular Solubility of Test Items

Results of solubility analysis are presented in Tables 11.1-11.6 and Figures 11.1-11.4.

Table 11.1 Solubility of µ-TiO_2 Bayertitan T in AAF, GS and ALF

µ-TiO_2 Bayertitan T		Concentration of soluble moiety vs. total			Solubility			Solubility
		Mean	ASD	RSD	Mean	ASD	RSD	
Time (hrs)	Lung fluid simulant	(mg/g)		(%)	(%)			(mg/l lung fluid simulant)
0.5	AAF Gamble ALF	<0.004 <0.004 0.001	0.0001	6.4	<0.0004 <0.0004 0.0001	0.00001	6.4	<0.004 <0.004 0.002
1	AAF Gamble ALF	<0.004 <0.004 0.003	0.0001	3.2	<0.0004 <0.0004 0.0003	0.00001	3.2	<0.004 <0.004 0.006
2	AAF Gamble ALF	<0.004 <0.004 0.007	0.0007	9.2	<0.0004 <0.0004 0.0007	0.00007	9.2	<0.004 <0.004 0.014
3	AAF Gamble ALF	<0.004 <0.004 0.011	0.0000	0.2	<0.0004 <0.0004 0.0011	0.00000	0.2	<0.004 <0.004 0.022
4	AAF Gamble ALF	<0.004 <0.004 0.017	0.0010	6.2	<0.0004 <0.0004 0.0017	0.00010	6.2	<0.004 <0.004 0.034
6	AAF Gamble ALF	<0.004 <0.004 0.026	0.0011	4.1	<0.0004 <0.0004 0.0026	0.00011	4.1	<0.004 <0.004 0.052
8	AAF Gamble ALF	<0.004 <0.004 0.028	0.0004	1.3	<0.0004 <0.0004 0.0028	0.00004	1.3	<0.004 <0.004 0.056
24	AAF Gamble ALF	<0.004 <0.004 0.056	0.0005	0.9	<0.0004 <0.0004 0.0056	0.00005	0.9	<0.004 <0.004 0.112
48	AAF Gamble ALF	<0.004 <0.004 0.084	0.0017	2.0	<0.0004 <0.0004 0.0084	0.00017	2.0	<0.004 <0.004 0.168
72	AAF Gamble ALF	<0.004 <0.004 0.102	0.0030	2.9	<0.0004 <0.0004 0.0102	0.00030	2.9	<0.004 <0.004 0.204
120	AAF Gamble ALF	<0.004 <0.004 0.126	0.0028	2.3	<0.0004 <0.0004 0.0126	0.00028	2.3	<0.004 <0.004 0.252

ASD: Absolute standard deviation - RSD: Relative standard deviation
N=3 (for each lung fluid simulant)
For comparison: Solubility in pure water: practically insoluble
Reference: https://de.wikipedia.org/wiki/Titan%28IV%29-oxid

Table 11.2 Solubility of nano-TiO_2 P25 in AAF, GS and ALF

nano-TiO_2 P25		Concentration of soluble moiety vs. total			Solubility			Solubility
		Mean	ASD	RSD	Mean	ASD	RSD	
Time (hrs)	Lung fluid simulant	(mg/g)		(%)	(%)			(mg/l lung fluid simulant)
0.5	AAF Gamble ALF	<0.004 <0.004 0.003	0.002	7.4	<0.0004 <0.0004 0.003	0.0002	7.4	<0.004 <0.004 0.006
1	AAF Gamble ALF	<0.004 <0.004 0.004	0.003	8.7	<0.0004 <0.0004 0.004	0.0003	8.7	<0.004 <0.004 0.008
2	AAF Gamble ALF	<0.004 <0.004 0.007	0.006	9.2	<0.0004 <0.0004 0.007	0.0005	9.2	<0.004 <0.004 0.014
3	AAF Gamble ALF	<0.004 <0.004 0.008	0.005	6.4	<0.0004 <0.0004 0.008	0.0005	6.4	<0.004 <0.004 0.016
4	AAF Gamble ALF	<0.004 <0.004 0.010	0.002	1.9	<0.0004 <0.0004 0.010	0.0002	1.9	<0.004 <0.004 0.020
6	AAF Gamble ALF	<0.004 <0.004 0.013	0.012	9.6	<0.0004 <0.0004 0.013	0.0012	9.6	<0.004 <0.004 0.026
8	AAF Gamble ALF	<0.004 <0.004 0.015	0.006	3.7	<0.0004 <0.0004 0.015	0.0006	3.7	<0.004 <0.004 0.030
24	AAF Gamble ALF	<0.004 <0.004 0.024	0.007	2.8	<0.0004 <0.0004 0.024	0.0007	2.8	<0.004 <0.004 0.048
48	AAF Gamble ALF	<0.004 <0.004 0.031	0.012	3.8	<0.0004 <0.0004 0.031	0.0012	3.8	<0.004 <0.004 0.062
72	AAF Gamble ALF	<0.004 <0.004 0.038	0.028	7.4	<0.0004 <0.0004 0.038	0.0028	7.4	<0.004 <0.004 0.076
96	AAF Gamble ALF	<0.004 <0.004 0.034	0.021	6.0	<0.0004 <0.0004 0.034	0.0021	6.0	<0.004 <0.004 0.068

ASD: Absolute standard deviation - RSD: Relative standard deviation
N=3 (for each lung fluid simulant)
For comparison: Solubility in pure water: practically insoluble
Reference: https://de.wikipedia.org/wiki/Titan%28IV%29-oxid

Table 11.3 Solubility of μ-Eu_2O_3 in AAF, GS and ALF

μ-Eu_2O_3		Concentration of soluble moiety vs. total			Solubility			Solubility
		Mean	ASD	RSD	Mean	ASD	RSD	
Time (hrs)	Lung fluid simulant	(mg/g)		(%)	(%)			(mg/l lung fluid simulant)
0.5	AAF Gamble ALF	<0.0005 0.01 703	0.000 7	5.3 1.0	<0.0005 0.001 70.3	0.0000 0.7	5.3 1.0	<0.0005 0.02 1406
1	AAF Gamble ALF	0.001 0.04 777	0.000 0.002 7	12.8 4.8 0.9	0.0001 0.004 77.7	0.0000 0.0002 0.7	12.8 4.8 0.9	0.002 0.08 1554
2	AAF Gamble ALF	0.065 0.13 883	0.017 0.003 4	26.6 2.4 0.5	0.006 0.013 88.3	0.0017 0.0003 0.4	26.6 2.4 0.5	0.130 0.26 1766
3	AAF Gamble ALF	0.183 0.18 909	0.011 0.008 8	5.8 4.3 0.9	0.018 0.018 90.9	0.0011 0.0008 0.8	5.8 4.3 0.9	0.366 0.36 1818
4	AAF Gamble ALF	0.265 0.22 888	0.007 0.005 8	2.7 2.3 0.9	0.026 0.022 88.8	0.0007 0.0005 0.8	2.7 2.3 0.9	0.53 0.44 1776
6	AAF Gamble ALF	0.328 0.28 896	0.018 0.003 8	5.5 0.9 0.8	0.032 0.028 89.6	0.0018 0.0003 0.8	5.5 0.9 0.8	0.656 0.56 1792
8	AAF Gamble ALF	0.383 0.33 909	0.016 0.009 21	4.3 2.8 2.4	0.038 0.033 90.9	0.0016 0.0009 2.1	4.3 2.8 2.4	0.766 0.66 1818
24	AAF Gamble ALF	0.574 0.20 994	0.018 0.014 53	3.2 6.9 5.4	0.057 0.020 99.4	0.0018 0.0014 5.3	3.2 6.9 5.4	1.148 0.40 1988
48	AAF Gamble ALF	0.712 0.28 945	0.065 0.017 5	9.1 5.8 0.5	0.071 0.028 94.5	0.0065 0.0017 0.5	9.1 5.8 0.5	1.424 0.56 1890
72	AAF Gamble ALF	0.813 0.43 898	0.015 0.022 13	1.9 5.2 1.4	0.081 0.043 89.8	0.0015 0.0022 1.3	1.9 5.2 1.4	1.626 0.86 1796
96	AAF Gamble ALF	n.m. n.m. 843	n.m. n.m. 14	n.m. n.m. 1.7	n.m. n.m. 84.3	n.m. n.m. 1.4	n.m. n.m. 1.7	n.m. n.m. 1686

n.m. = not measured
ASD: Absolute standard deviation - RSD: Relative standard deviation
N=3 (for each lung fluid simulant)
For comparison: Solubility in pure water: practically insoluble
Reference: https://de.wikipedia.org/wiki/Europium%28III%29-oxid

Table 11.4 Solubility of µ-$BaSO_4$ in AAF, GS and ALF

µ-$BaSO_4$		Concentration of soluble moiety vs. total			Solubility			Solubility
		Mean	ASD	RSD	Mean	ASD	RSD	
Time (hrs)	Lung fluid simulant	(mg/g)		(%)	(%)			(mg/l lung fluid simulant)
0.5	AAF	0.14	0.001	0.8	0.014	0.0001	0.8	0.28
	Gamble	0.25	0.011	4.4	0.025	0.0011	4.4	0.50
	ALF	3.73	0.07	1.8	0.373	0.01	1.8	7.46
1	AAF	0.16	0.002	1.4	0.016	0.0002	1.4	0.32
	Gamble	0.25	0.004	1.6	0.025	0.0004	1.6	0.50
	ALF	4.07	0.07	1.7	0.407	0.01	1.7	8.14
2	AAF	0.18	0.003	1.4	0.018	0.0003	1.4	0.36
	Gamble	0.28	0.006	2.3	0.028	0.0006	2.3	0.56
	ALF	4.69	0.07	1.5	0.469	0.01	1.5	9.38
3	AAF	0.20	0.004	1.8	0.020	0.0004	1.8	0.40
	Gamble	0.28	0.005	1.6	0.028	0.0005	1.6	0.56
	ALF	5.02	0.12	2.4	0.502	0.01	2.4	10.04
4	AAF	0.21	0.005	2.3	0.021	0.0005	2.3	0.42
	Gamble	0.29	0.002	0.6	0.029	0.0002	0.6	0.58
	ALF	5.19	0.09	1.8	0.519	0.01	1.8	10.38
6	AAF	0.22	0.003	1.6	0.022	0.0003	1.6	0.44
	Gamble	0.29	0.008	2.9	0.029	0.0008	2.9	0.58
	ALF	5.34	0.12	2.2	0.534	0.01	2.2	10.68
8	AAF	0.24	0.003	1.2	0.024	0.0003	1.2	0.48
	Gamble	0.30	0.006	2.1	0.030	0.0006	2.1	0.60
	ALF	5.45	0.12	2.1	0.545	0.01	2.1	10.90
24	AAF	0.26	0.002	0.9	0.026	0.0002	0.9	0.52
	Gamble	0.36	0.010	3.7	0.036	0.0010	3.7	0.72
	ALF	5.55	0.10	1.8	0.555	0.01	1.8	11.10
48	AAF	0.27	0.010	3.7	0.027	0.0010	3.7	0.54
	Gamble	0.38	0.005	1.4	0.038	0.0005	1.4	0.76
	ALF	5.59	0.09	1.6	0.559	0.01	1.6	11.18
72	AAF	0.29	0.010	3.6	0.029	0.0010	3.6	0.58
	Gamble	0.40	0.003	0.7	0.040	0.0003	0.7	0.80
	ALF	5.65	0.12	2.2	0.565	0.0123	2.2	11.30
96	AAF	0.28	0.012	4.4	0.028	0.0012	4.4	0.56
	Gamble	0.40	0.003	0.8	0.040	0.0003	0.8	0.80
	ALF	5.62	0.12	2.2	0.562	0.0125	2.2	11.24

ASD: Absolute standard deviation - RSD: Relative standard deviation
N=3 (for each lung fluid simulant)
For comparison: Solubility in pure water: 2.5 mg/l

Reference: BDI (2013). Bundesverband der Deutschen Industrie. Der GBS-Grenzwert aus Sicht der deutschen Industrie
http://www.google.de/url?sa=t&rct=j&q=&esrc=s&source=web&cd=3&ved=0CDsQFjAC&url=http%3A%2F%2Fwww.baua.de%2Fde%2FThemen-von-A-Z%2FGefahrstoffe%2FAGS%2Fpdf%2FAGS-publik-2013-4.pdf%3F__blob%3DpublicationFile%26v%3D2&ei=FWk5UuDnC4GWtQbD9YFQ&usg=AFQjCNGOMH2b6bOupu0aeKRj5HUBM-dlcg&bvm=bv.52288139,d.bGE

Table 11.5 Solubility of µ-ZrO_2 in AAF, GS and ALF

µ-ZrO_2		Concentration of soluble moiety vs. total			Solubility			Solubility
		Mean	ASD	RSD	Mean	ASD	RSD	
Time (hrs)	Lung fluid simulant	(mg/g)		(%)	(%)			(mg/l lung fluid simulant)
0.5	AAF Gamble ALF	<0.010 <0.015 0.05	0.001	1.3	<0.0010 <0.0015 0.005	0.0001	1.3	<0.010 <0.015 0.1
1	AAF Gamble ALF	<0.010 <0.015 0.08	0.003	3.4	<0.0010 <0.0015 0.008	0.0003	3.4	<0.010 <0.015 0.16
2	AAF Gamble ALF	<0.010 <0.015 0.12	0.003	2.6	<0.0010 <0.0015 0.012	0.0003	2.6	<0.010 <0.015 0.24
3	AAF Gamble ALF	<0.010 <0.015 0.15	0.001	0.6	<0.0010 <0.0015 0.015	0.0001	0.6	<0.010 <0.015 0.3
4	AAF Gamble ALF	<0.010 <0.015 0.17	0.005	2.6	<0.0010 <0.0015 0.017	0.0005	2.6	<0.010 <0.015 0.34
6	AAF Gamble ALF	<0.010 <0.015 0.22	0.003	1.3	<0.0010 <0.0015 0.022	0.0003	1.3	<0.010 <0.015 0.44
8	AAF Gamble ALF	<0.010 <0.015 0.24	0.003	1.4	<0.0010 <0.0015 0.024	0.0003	1.4	<0.010 <0.015 0.48
24	AAF Gamble ALF	<0.010 <0.015 0.47	0.006	1.3	<0.0010 <0.0015 0.047	0.0006	1.3	<0.010 <0.015 0.94
48	AAF Gamble ALF	<0.010 <0.015 0.58	0.038	6.5	<0.0010 <0.0015 0.058	0.0038	6.5	<0.010 <0.015 1.16
72	AAF Gamble ALF	<0.010 <0.015 0.66	0.008	1.2	<0.0010 <0.0015 0.066	0.0008	1.2	<0.010 <0.015 1.32
96	AAF Gamble ALF	<0.010 <0.015 0.67	0.012	1.8	<0.0010 <0.0015 0.067	0.0012	1.8	<0.010 <0.015 1.34

ASD: Absolute standard deviation - RSD: Relative standard deviation
N=3 (for each lung fluid simulant)
For comparison: Solubility in pure water: 1 mg/l
Reference: https://de.wikipedia.org/wiki/Zirconium%28IV%29-oxid

Table 11.6 Solubility of nano-SiO_2 NM-200 in AAF, GS and ALF

nano-SiO_2		Concentration of soluble moiety vs. total			Solubility			Solubility
		Mean	ASD	RSD	Mean	ASD	RSD	
Time (hrs)	Lung fluid simulant	(mg/g)		(%)	(%)			(mg/l lung fluid simulant)
0.25	AAF	30.3	0.8	2.5	3.0	0.1	2.5	60.6
	Gamble	32.5	1.1	3.4	3.2	0.1	3.4	65.0
	ALF	6.3	0.3	4.1	0.6	0.0	4.1	12.6
0.5	AAF	41.9	0.9	2.2	4.2	0.1	2.2	83.8
	Gamble	40.4	1.5	3.8	4.0	0.2	3.8	80.8
	ALF	10.4	0.3	2.9	1.0	0.0	2.9	20.8
1	AAF	53.0	1.1	2.1	5.3	0.1	2.1	106.0
	Gamble	49.2	1.4	2.9	4.9	0.1	2.9	98.4
	ALF	13.1	0.4	3.0	1.3	0.0	3.0	26.2
1.5	AAF	52.9	1.0	1.9	5.3	0.1	1.9	105.8
	Gamble	53.1	1.0	1.9	5.3	0.1	1.9	106.2
	ALF	14.2	0.4	2.6	1.4	0.0	2.6	28.4
2	AAF	56.0	0.9	1.7	5.6	0.1	1.7	112.0
	Gamble	53.3	1.3	2.4	5.3	0.1	2.4	106.6
	ALF	18.3	0.5	2.7	1.8	0.0	2.7	36.6
3	AAF	60.0	1.3	2.1	6.0	0.1	2.1	120.0
	Gamble	57.6	1.1	1.9	5.8	0.1	1.9	115.2
	ALF	24.6	0.7	2.9	2.5	0.1	2.9	49.2
4	AAF	64.3	1.1	1.8	6.4	0.1	1.8	128.6
	Gamble	53.7	1.4	2.5	5.4	0.1	2.5	107.4
	ALF	25.4	0.3	1.2	2.5	0.0	1.2	50.8
5	AAF	59.5	1.2	2.0	6.0	0.1	2.0	119.0
	Gamble	56.8	1.1	1.9	5.7	0.1	1.9	113.6
	ALF	28.1	0.3	1.0	2.8	0.0	1.0	56.2
6	AAF	61.1	1.0	1.6	6.1	0.1	1.6	122.1
	Gamble	54.5	1.2	2.2	5.4	0.1	2.2	109.0
	ALF	34.7	0.9	2.6	3.5	0.1	2.6	69.4
8	AAF	65.1	1.1	1.7	6.5	0.1	1.7	130.2
	Gamble	56.3	1.0	1.7	5.6	0.1	1.7	112.6
	ALF	38.9	0.4	1.0	3.9	0.0	1.0	77.8
24	AAF	64.7	0.9	1.4	6.5	0.1	1.4	129.4
	Gamble	51.6	0.4	0.8	5.2	0.0	0.8	103.2
	ALF	52.4	1.3	2.5	5.2	0.1	2.5	104.8

ASD: Absolute standard deviation - RSD: Relative standard deviation
N=3 (for each lung fluid simulant)
For comparison: Solubility in pure water: practically insoluble
Reference: https://de.wikipedia.org/wiki/Siliciumdioxid

The solubility of µ-TiO_2 Bayertitan T, nano-TiO_2 P25 and µ-ZrO_2 is negligible in AAF and GS. In ALF, it is measurable, however, at very low levels (maximum at approx. 1 mg/l).

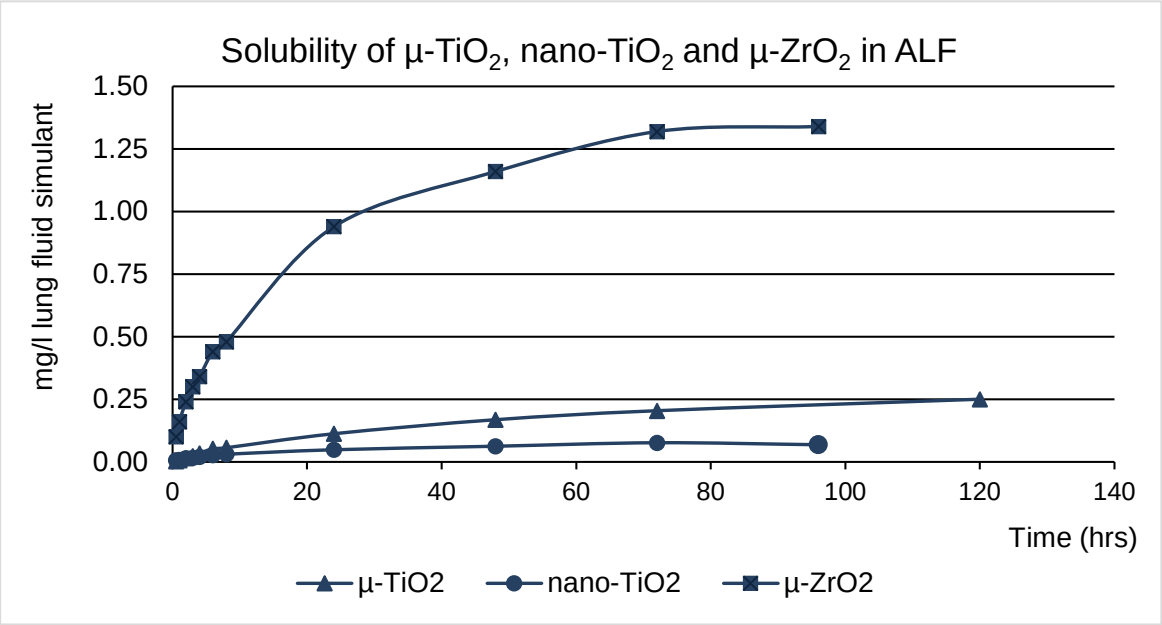


Fig. 11.1 Solubility of µ-TiO_2 Bayertitan T, nano-TiO_2 P25 and µ-ZrO_2 in ALF

The solubility of µ-$BaSO_4$ is negligible in AAF and GS. In ALF, values reach at maximum approx. 11 mg/l.

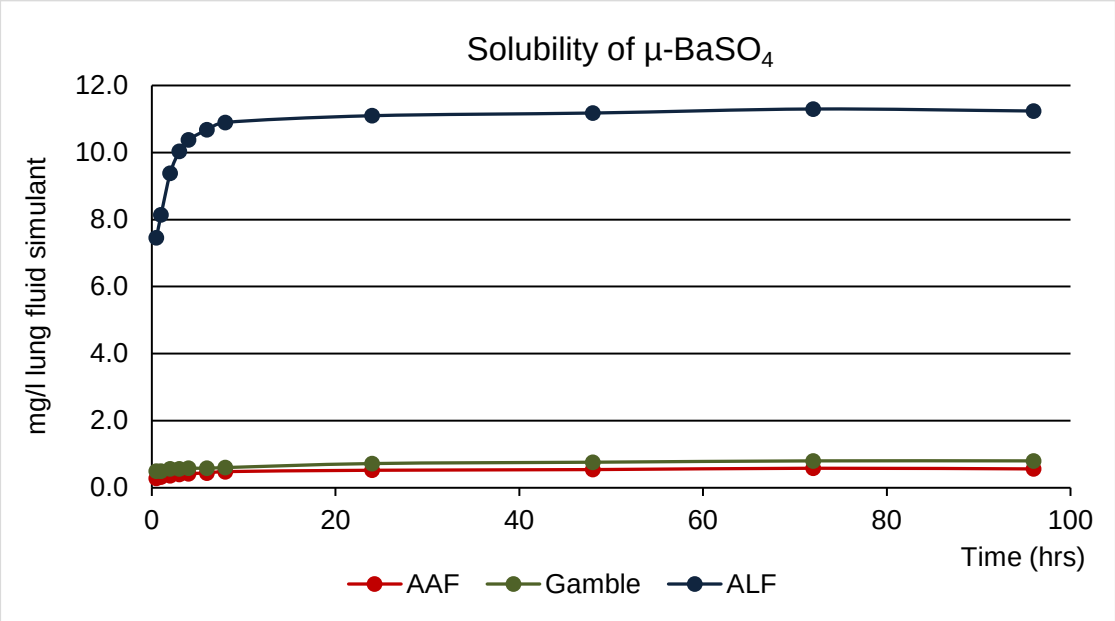


Fig. 11.2 Solubility of µ-$BaSO_4$ in AAF, GS and ALF

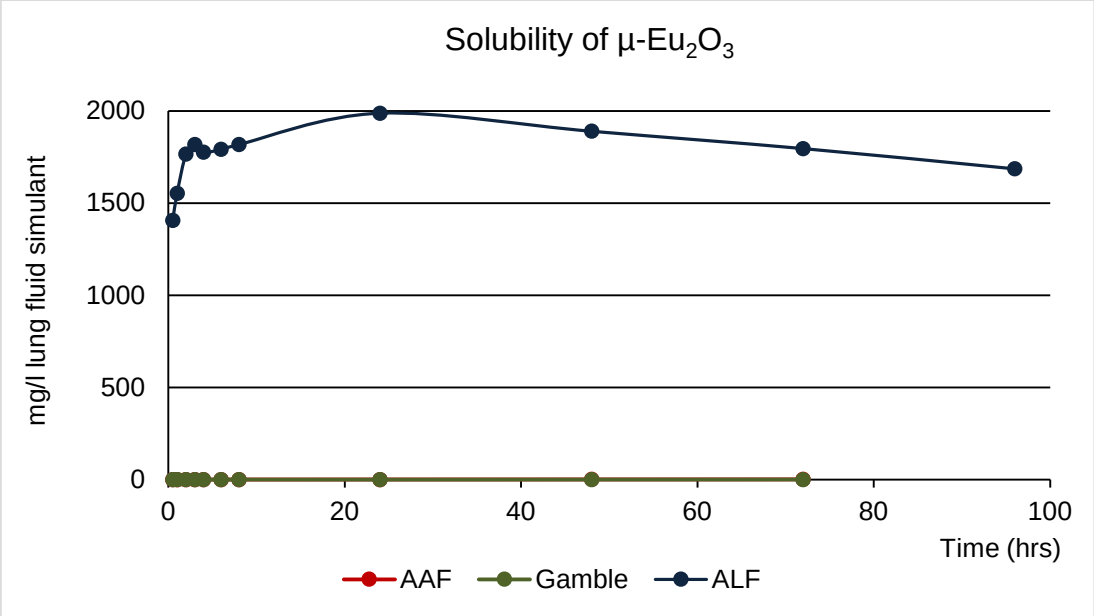


Fig. 11.3 Solubility of µ-Eu_2O_3 in AAF, GS and ALF (AAF curve covered by GS curve)

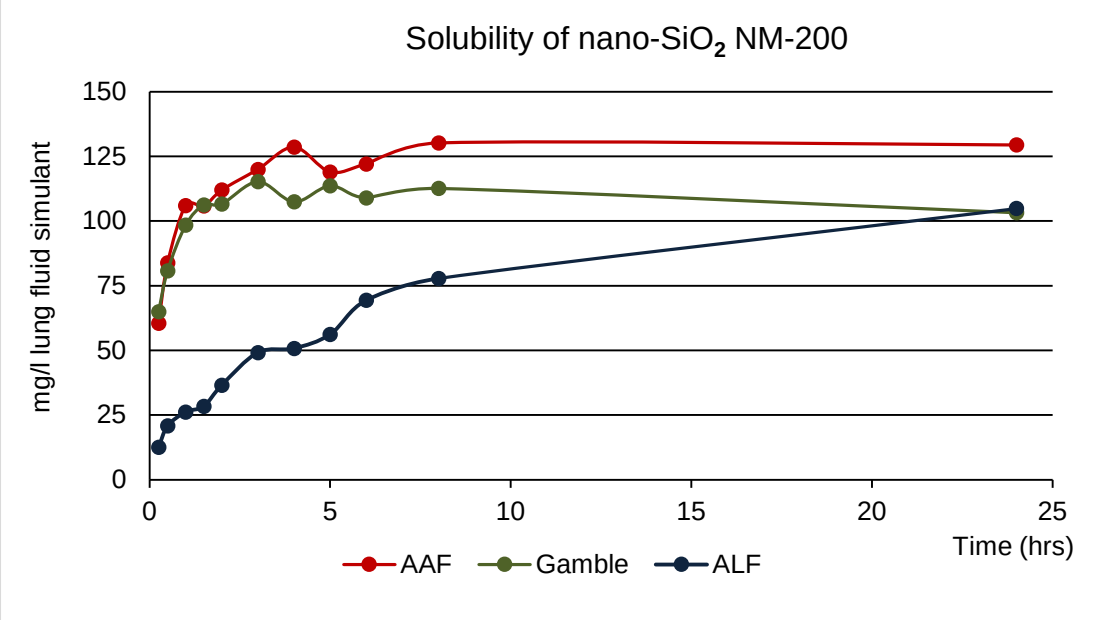


Fig. 11.4 Solubility of nano-SiO_2 in AAF, GS and ALF

In this study, for µ-TiO_2 Bayertitan T, nano-TiO_2 P25 and µ-ZrO_2 very low solubility data were detected in simulated lung fluids. In AAF and GS the values of solubility were below the margins of detection limit, in ALF they reached 0.25 and 0.08 mg/l for TiO_2 Bayertitan T and TiO_2 P25, respectively. For ZrO_2 the threshold value of 1 mg/l was only exceeded after 2, 3 and 4 days of treatment in ALF (maximum: 1.34 mg/l). ZrO_2 is doped with yttria. The latter showed a 10-fold higher solubility than ZrO_2 in ALF.
The solubility of $BaSO_4$ reached 0.8 mg/l at maximum in AAF and GS and 11.3 mg/l in ALF. In contrary, under in vivo conditions the solubility is higher caused by special

transport mechanisms. Particle dissolution is a likely. Injected nano-$BaSO_4$ were localized in the reticuloendothelial organs and redistributed to the bone over time. These experiments have been described in detail by KONDURU et al., 2014.
Eu_2O_3 showed low solubility in AAF and GS (1.63 mg/l at maximum). In contrary, in ALF a very high solubility was analysed reaching 2000 mg/l after 24 hours.
Amorphous SiO_2 showed a high solubility in all three media reaching up to 130, 115 and 100 mg/l in AAF, GS and ALF, respectively.

AAF - artificial alveolar fluid, pH=7.4; GS – Gamble’s solution, pH=7.4; ALF – artificial lysosomal fluid, pH=4.5

12 Summary

Introduction
The so-called GBP category was formed by definition, i.e. includes **respirable Granular Biopersistent Particles (GBP) without known significant specific toxicity**. This category comprises various materials such as minerals, metals, metal oxides or polymers that show a negligible solubility in lung fluids (extracellular lung lining fluid, intracellular lysosomal fluid). These particles can be regulated with the same threshold limit value (in Germany currently 1.25 mg/m^3 for the respirable fraction). They exhibit their toxicity and toxicokinetic behaviour after uptake in lungs predominantly based on the surface and volume.

Module 1: In vivo study
Analysis of the bronchoalveolar lavage fluid (BALF)
In the 6-dust group under analysis, **TiO_2 type "Bayertitan T"**, a microscaled powder with low solubility was included that by experimental experience was known to fulfill the conditions of GBP dusts according to the criteria suggested by Morrow (1989). A second dust, i.e. microscaled **$BaSO_4$ "Sigma Riedel"**, was also considered as a nuisance dust with regard to its inflammogenicity potential, however, suggested to exhibit a considerable biosolubility as reported by KONDURU et al. for a nano-$BaSO_4$ (2014). These two dusts, both of them in the low (0.5 µl per rat) and high (1.5 µl) dose groups, did not induce statistically significant polymorphonuclear neutrophil (PMN) levels in the differential cell count on day 3 post-treatment. Albeit PMN levels of approx. 12% and 18%, respectively, were observed in the high dose groups, these findings were caused rather due to a bolus effect after instillation than due to a specific surface chemistry-based toxicity. In the low dose groups simulating the non-overload situation PMN levels were < 5%. After 28 days of recovery the PMN levels returned mostly to values typically observed in control animals (≤ 2%).
In contrast to TiO_2 type Bayertitan T, the nanostructured **TiO_2 P25** exhibited a PMN recruitment of 30-45% that after 28 days still persisted at a moderate level of approx. 35%. **Europium oxide** (Eu_2O_3) induced the strongest response (50-60% PMN detected on day 3) with a mostly persisting reaction after 28 days of approx. 40% PMN. For this dust a clear surface chemistry-related inflammogenicity was observed.
Zirconium oxide (ZrO_2) showed a moderate to strong acute reaction on day 3, i.e. 35-50% PMN in BALF, however, on day 28 a practically complete normalisation was observed. In this case, a bolus effect due to instillation may lead to an overestimation of the inflammatory potential. Following inhalation uptake the strength of acute effects might be lessened.
Amorphous SiO_2 type NM-200 showed a slight inflammatory effect in the low dose group (approx. 10% PMN). On day 28, both the low and high dose showed a full normalization. The result in the low dose group may allow an inclusion of NM-200 in the GBP category so far as inflammatory effects are addressed. On the other hand the high biosolubility of many amorphous silicas is well-known. These particles do not meet the "low soluble" criterion.

Chemical analysis of the lung burdens
In this study the rats were administered with identical volumetric of GBP candidates, i.e. 0.5 and 1.5 µl per rat. It was assumed that the physiological clearance was not impaired at 0.5 µl whereas 1.5 µl show induce clear overload effects (increased half-

times, inflammatory reactions). For the microscaled dusts the material densities (e.g. $\rho_{TiO2 =}$ 4.3), for the nanoscaled dusts a lower agglomerate density ($\rho_{TiO2\ P25}$ = 3.8) or a pycnometrically determined vaue (ρ_{SiO2} = 2.2) were used.
Retention of test materials: On day 3, an average retention of approx. 66% as compared to the administered total dose was found in the low dose groups and of 72% in the high dose groups; approx. one third of the dose is eliminated from lungs by rapid clearance mechanisms (coughing, ciliae-mediated processes, etc.).
The clearance half-time $t_{1/2}$ showed a value close to the physiological rat lung clearance of approx. 60 days in the **µ-TiO_2 "Bayertitan T"** low dose group and of 90 days in the high dose group (at lung overload). For **nano-TiO_2 P25** $t_{1/2}$ was 141 and 866 days in the low and high dose groups, respectively. This is a doubling or even >10-fold increase as compared to the physiological value (clear overload in the high dose).
In the **µ-$BaSO_4$** and **amorphous silica** groups (either the low and high dose groups), smaller values in the range of 25-40 days were calculated indicating an additional dissolution effect.
In the **Europium oxide** and **Zirconium oxide** groups increased half-times, i.e. 4- to 5-fold (low and high dose) and 2- to 4-fold, respectively, were calculated indicating a clear surface-chemistry-related contribution to the toxic and clearance-retardative outcome.
TiO_2 "Bayertitan T" and TiO_2 P25 showed very low ionic moieties regarding the total lung burden. Levels in lungs at all 3 time-points did not exceed the 0.1% percentage except TiO_2 P25 in the high dose → ≥ 0.2%.
Europium oxide resulted in the highest ionic percentages of all 6 dusts amounting to a range of 17-30% of the total mass in lungs.
µ-$BaSO_4$ showed low ionic moieties of 0.8% or lower regarding the total lung burden (at all 3 time-points). At **ZrO_2** low ionic moieties were observed, levels in lungs at all 3 time-points did not exceed the 0.4% percentage.
Amorphous silica showed low ionic moieties of 0.2% or lower (at all 3 time-points).
Comparison **Eu_2O_3 vs. amorphous silica**: Both show high solubility, however, the ionic moiety of Eu_2O_3 is eliminated from lungs more slowly than that of amorphous SiO_2. The difference may be caused by different transport mechanisms of the ions.

Module 2: In vitro assays
Plasmid Scission Assay (PSA)
µ-Eu_2O_3 dust showed a strong effect at the highest concentration tested; µ-$BaSO_4$ was also effective at the second highest concentration.

Cell-free ESR measurements
No effects observed with any test material

Table 12.1 Instillation study: Clearance half-times and results of BAL analysis

Dust	Dose (µl/rat)	effective dose d 3 (µl/rat)	Clearance half-time (d)[2]	BAL PMN (%) d 3	BAL PMN (%) d 28
Vehicle control	0	0	approx. 60	2.0	1.1
µ-TiO_2 Bayertitan T	0.5	0.37 (75%)	47	2.6	0.8
	1.5[1]	1.32 (88%)	89	11.7	4.7
nano-TiO_2 P25	0.5	0.36 (72%)	141	30.3	36.1
	1.5	1.01 (67%)	866	48.2	35.9
µ-Eu_2O_3	0.5	0.38 (76%)	277	50.7	38.2
	1.5	1.17 (78%)	347	64.5	37.4
µ-$BaSO_4$	0.5	0.29 (57%)	26	4.2	0.6
	1.5	0.99 (66%)	39	18.2	0.8
µ-ZrO_2	0.5	0.26 (52%)	133	36.6	1.5
	1.5	0.92 (62%)	257	53.5	5.8
nano-SiO_2 NM-200	0.5	0.094 (19%)	25	11.9	0.9
	1.5	0.24 (16%)	27	35.2	1.4

[1] 1.5 µl/rat is considered as overload dose
[2] based on total burden (particulate + ionic) in the period day 3 to day 90; standard value rat: 60 d

Table 12.2 In vitro assays

Dust (max. dose)	Plasmid Scission Assay	ESR Cell free	ESR THP-1 monocytes	Cytotoxicity THP-1 monocytes	Cytotoxicity THP-1 macrophages	IL-8 secretion THP-1 macrophages
µ-TiO_2 Bayertitan T (max. 14.6 µg/ml)	-	-	-	-	-	-
nano-TiO_2 P25 (max. 12.9 µg/ml)	-	-	-	-	-	-
µ-Eu_2O_3 (max. 25.1 µg/ml)	positive at 25.1 µg/ml	-	-	-	positive at 25.1 µg/ml	-
µ-$BaSO_4$ (max. 15.3 µg/ml)	positive at 3.1 and 15.3 µg/ml	-	-	-	-	-
µ-ZrO_2 (max. 19.3 µg/ml)	-	-	-	-	-	-
nano-SiO_2 NM-200 (max. 7.5 µg/ml)	-	-	-	-	-	-

-: negative

Cellular ESR measurements towards THP-1 monocytes
No effects observed with any test material

Cytotoxicity towards THP-1 Cells
No effects were observed in THP-1 monocytes with any test material. When differentiated THP-1 macrophages were challenged with the test dusts using the same con-

centration, a dose-dependent decrease in viability was observed with the rare earth µ-Eu_2O_3 dust.

IL-8 Secretion in THP-1 Macrophages
No effects observed with any test material

Module 3: Acellular solubility of test materials
In this study, for µ-TiO_2 Bayertitan T, nano-TiO_2 P25 and µ-ZrO_2 very low solubility data were detected in simulated lung fluids. In artificial alveolar fluid (AAF) and Gamble's solution (GS) the values of solubility were below the margins of detection limit, in artificial lysosomal fluid (ALF) they reached 0.25 and 0.08 mg/l for µ-TiO_2 Bayertitan T and nano-TiO_2 P25, respectively. For µ-ZrO_2 the threshold value of 1 mg/l was only exceeded after 2, 3 and 4 days of treatment (maximum: 1.34 mg/l).
The solubility of $BaSO_4$ reached 0.8 mg/l at maximum in AAF and GS and 11.3 mg/l in ALF. In contrary, under in vivo conditions the solubility is higher caused by special transport mechanisms. Injected $BaSO_4$ nanoparticles were localized in the reticuloendothelial organs and redistributed to the bone over time. These experiments have been described in detail by KONDURU et al., 2014.
Eu_2O_3 showed low solubility in AAF and GS (1.63 mg/l at maximum). In contrary, in ALF a very high solubility was analysed reaching 2000 mg/l after 24 hours. This is in accordance with a preceding study published by CREUTZENBERG et al., 2015.
Amorphous SiO_2 showed a high solubility in all three media reaching up to 130, 115 and 100 mg/l in AAF, GS and ALF, respectively.
AAF - artificial alveolar fluid, pH=7.4; GS – Gamble's solution, pH=7.4; ALF – artificial lysosomal fluid, pH=4.5

Table 12.3 Acellular solubility of dusts in artificial lung fluids

Dust	After time (hrs)	AAF pH 7.4 (mg/l)	Gamble pH 7.4 (mg/l)	ALF pH 4.5 (mg/l)
µ-TiO_2 Bayertitan T	120	<0.004	<0.004	0.252
nano-TiO_2 P25	96	<0.004	<0.004	0.068
µ-Eu_2O_3	72	1.63	0.86	1796
µ-$BaSO_4$	96	0.56	0.80	11.24
µ-ZrO_2	96	<0.010	<0.015	1.34
nano-SiO_2 NM-200	24	129.4	103.2	104.8

Conclusion
In vivo study
µ-TiO_2 (rutile; Bayertitan T) was the only dust that was approved as a typical GBP (without impaired lung clearance; no evidence of a significant PMN recruitment). µ-$BaSO_4$ (Sigma-Riedel), albeit with negligible inflammatory potency (PMN influx), showed a high biosolubility with barium transfer to the bones. The observed high solubility is based on special mechanisms that have been characterised in the meanwhile for a nano-$BaSO_4$ by KONDURU et al., 2014.
Nano-TiO_2 P25 (anatase) does not fulfill the GBP criteria as it induced a slightly increased clearance half-time; in addition, it showed significant PMN levels in the low dose group. µ-Eu_2O_3 and µ-ZrO_2 failed to meet GBP criteria because of strongly increase clearance half-times and each inducing a strong acute inflammatory response.

Nano-SiO_2 met the GBP criteria in the low dose in terms of inflammogenicity, however, not the low solubility criterion.

In vitro assays
The plasmid scission assay (PSA) and the cytotoxicity assay towards THP-1 cells indicated effects corresponding to the in vivo results in the Eu_2O_3 dust group. Overall, in vitro assays under investigation did not mirror the in vivo results with statistically significant power.

Acellular solubility of test materials
On the basis of the acellular solubility data analysed in this study using artificial lung fluids a threshold value of ≤ 1 mg/l is recommended to define the category of so-called “low soluble dusts”. This value should not be exceeded in one of the three lung fluid simulants.

Outlook
The inhalation path may show lower inflammatory effects in lungs as compared to intratracheal instillation as no bolus effects will occur. Therefore, the final setting of maximum PMN levels and particle solubility to define the GBP category should await the outcome of the parallel inhalation validation study.

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Appendix 1 Pre-test to check the tolerability of doses in rats

Intratracheal instillation test in the rat → BAL
Crl:WI(Han)-Rats: 12 animals

Group	Treatment	Dose (µl/rat)	Dose (mg/rat)	Animal number per group Day 3	Cell concentration in BALF (cells/ml)
3	µ-TiO_2 Bayertitan T	1.5	2 x 3.23	2	388,125
5	nano-TiO_2	1.5	2 x 2.85	2	637,500
7	µ-Eu_2O_3	1.5	2 x 5.55	2	710,000
9	µ-$BaSO_4$	1.5	2 x 3.38	2	370,625
11	µ-ZrO_2	1.5	2 x 4.28	2	677,500
13	nano-SiO_2	1.5	2 x 1.65	2	608,750
				Total of rats: 12	

BALF: Bronchoalveolar lavage fluid

Material densities	**ρ**
µ-TiO_2 Bayertitan T	4.3
nano-TiO_2 P25 NM-105	3.8
µ-Eu_2O_3	7.4
µ-$BaSO_4$	4.5
µ-ZrO_2	5.7
nano-SiO_2 NM-200	2.2

Action

Instillation on 6-Oct-2014 and 7-Oct-2014 → Total dose given in two consecutive halves

BAL on 10-Oct-2014 → Determination of cell concentration in BALF

Conclusion

- ➔ All rats showed a satisfactory general health status up to day 3 post-treatment
- ➔ µ-TiO_2 Bayertitan T and µ-$BaSO_4$ showed the lowest leukocyte recruitment (approx. 388,125 cells/ml), µ-Eu_2O_3 the highest value (710,000 cells/ml)
- ➔ The planned high dose is acceptable for the main test

Appendix 2 Experimental Design of the Instillation Study

Male Wistar rats, Crl:WI (Han)
Intratracheal instillation test in the rat
Administration of total dose in 2 aliquots (halves) on consecutive days
BAL: sacrifice at day 3 and 28 following instillative administration
Chemical analysis: sacrifice at day 3, 28 and 90 following instillative administration

Group	Treatment	Dose* (µl/rat)	Dose* (mg/rat)
1	0,9% NaCl	0.3 ml	
2	µ-TiO_2 Bayertitan T	0.5	2 x 1.08
3		1.5	2 x 3.23
4	nano-TiO_2	0.5	2 x 0.95
5		1.5	2 x 2.85
6	µ-Eu_2O_3	0.5	2 x 1.85
7		1.5	2 x 5.55
8	µ-$BaSO_4$	0.5	2 x 1.13
9		1.5	2 x 3.38
10	µ-ZrO_2	0.5	2 x 1.43
11		1.5	2 x 4.28
12	nano-SiO_2	0.5	2 x 0.55
13		1.5	2 x 1.65
		Total of rats (BAL study):	156
		Total of rats (Chemical analysis):	234

* Dosis scheme below or beyond the threshold of volumetric overload

Material densities	**ρ**
µ-TiO_2 Bayertitan T	4.3
nano-TiO_2 P25 NM-105	3.8
µ-Eu_2O_3	7.4
µ-$BaSO_4$	4.5
µ-ZrO_2	5.7
nano-SiO_2 NM-200	2.2

Appendix 3 Analysis of Bronchoalveolar Lavage Fluid (BALF)

Sacrifice at day 3 following instillative administration
Differential cell count – means, percentual

Wistar Han		Leucocyte conc.(1/ml)	MPh (%)	PMN (%)	Lympho (%)
GROUP					
1	Mean	145625	98.2	2.0	0.2
	Std	91865	1.3	1.4	0.3
	N	#5	6	6	6
2	Mean	183924	96.8	2.6	0.5
	Std	84782	2.3	2.1	0.4
	N	6	6	6	6
3	Mean	320729	87.2	11.7	1.4
	Std	178884	7.4	7.1	1.0
	N	6	6	6	6
4	Mean	481042	***67.5	***30.3	***2.3
	Std	69370	19.5	18.3	2.2
	N	6	6	6	6
5	Mean	486667	***50.8	***48.2	1.0
	Std	243521	11.6	12.0	0.8
	N	6	6	6	6
6	Mean	471542	***47.2	***50.7	***2.3
	Std	138488	***14.1	13.9	1.1
	N	6	6	6	6
7	Mean	483958	35.0	***64.5	0.7
	Std	142597	7.6	7.7	0.5
	N	6	6	6	6
8	Mean	129896	94.5	4.2	1.4
	Std	38065	3.7	3.0	0.7
	N	6	6	6	6

[#] Lungs of rat# 1104 were perforated due to technical error; sample not analysed

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level
Group 1 - Control Group 2 - μ-TiO_2 low Group 3 - μ-TiO_2 high
Group 4 - nano-TiO_2 low Group 5 - nano-TiO_2 high Group 6 - μ-Eu_2O_3 low
Group 7 - μ-Eu_2O_3 high Group 8 - μ-$BaSO_4$ low Group 9 - μ-$BaSO_4$ high
Group 10 - μ-ZrO_2 low Group 11 - μ-ZrO_2 high Group 12 - nano-SiO_2 low
Group 13 - nano-SiO_2 high
MPh – Macrophages; PMN – Polymorphonuclear cells; Lympho - Lymphocytes

Sacrifice at day 3 following instillative administration
Differential cell count – means, percentual (Continued)

Wistar Han		Leucocyte conc.(1/ml)	MPh (%)	PMN (%)	Lympho (%)
GROUP					
9	Mean	220200	81.2	18.2	0.9
	Std	96395	12.2	11.9	0.6
	N	#5	5	5	5
10	Mean	339167	***62.2	***36.6	1.3
	Std	114419	13.2	13.3	0.6
	N	6	6	6	6
11	Mean	395208	***46.0	***53.5	0.6
	Std	202892	10.3	10.7	0.3
	N	6	6	6	6
12	Mean	337292	87.8	11.9	0.4
	Std	122220	15.0	14.3	0.5
	N	6	6	6	6
13	Mean	485833	***64.0	***35.2	1.1
	Std	174083	13.2	12.6	0.6
	N	6	6	6	6

[#] Lungs of rat# 9101 not analysed; reason: no particles detected in lungs

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

Group 1 - Control
Group 2 - µ-TiO_2 low
Group 3 - µ-TiO_2 high
Group 4 - nano-TiO_2 low
Group 5 - nano-TiO_2 high
Group 6 - µ-Eu_2O_3 low
Group 7 - µ-Eu_2O_3 high
Group 8 - µ-$BaSO_4$ low
Group 9 - µ-$BaSO_4$ high
Group 10 - µ-ZrO_2 low
Group 11 - µ-ZrO_2 high
Group 12 - nano-SiO_2 low
Group 13 - nano-SiO_2 high

MPh – Macrophages; PMN – Polymorphonuclear cells; Lympho – Lymphocytes

Sacrifice at day 3 following instillative administration
Differential cell count – means, absolute

Wistar Han		Leucocyte conc.(1/ml)	MPh (1/ml)	PMN (1/ml)	Lympho (1/ml)
GROUP		145625	14368	2233	153
1	Mean				
	Std	91865	91774	2613	225
	N	#5	#5	#5	#5
2	Mean	183924	178202	4982	809
	Std	84782	82517	5008	567
	N	6	6	6	6
3	Mean	320729	277123	40005	4433
	Std	178884	143052	39813	4397
	N	6	6	6	6
4	Mean	481042	**329677	141098	***10821
	Std	69370	118035	80173	10025
	N	6	6	6	6
5	Mean	486667	***263588	217983	4803
	Std	243521	155290	98803	3386
	N	6	6	6	6
6	Mean	471542	***233945	227489	***10989
	Std	138488	135507	51844	6513
	N	6	6	6	6
7	Mean	483958	***163417	318245	2994
	Std	142597	42317	115599	2676
	N	6	6	6	6
8	Mean	129896	123776	4653	1663
	Std	38065	39579	2644	672
	N	6	6	6	6

[#] Lungs of rat# 1104 were perforated due to technical error; sample not analysed
Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

Sacrifice at day 3 following instillative administration
Differential cell count – means, absolute (Continued)

Wistar Han		Leucocyte conc.(1/ml)	MPh (1/ml)	PMN (1/ml)	Lympho (1/ml)
GROUP					
9	Mean	220200	182461	36738	1508
	Std	96395	95758	25122	1073
	N	#5	5	5	5
10	Mean	339167	*210179	124975	4071
	Std	114419	83791	61559	2226
	N	6	6	6	6
11	Mean	395208	***193396	199373	2618
	Std	202892	139733	76188	2452
	N	6	6	6	6
12	Mean	337292	295296	41147	1187
	Std	122220	120067	50930	1298
	N	6	6	6	6
13	Mean	485833	***309875	172360	5042
	Std	174083	109304	93774	2596
	N	6	6	6	6

[#] Lungs of rat# 9101 not analysed; reason: no particles detected in lungs

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

Group 1 - Control
Group 2 - µ-TiO_2 low
Group 3 - µ-TiO_2 high
Group 4 - nano-TiO_2 low
Group 5 - nano-TiO_2 high
Group 6 - µ-Eu_2O_3 low
Group 7 - µ-Eu_2O_3 high
Group 8 - µ-$BaSO_4$ low
Group 9 - µ-$BaSO_4$ high
Group 10 - µ-ZrO_2 low
Group 11 - µ-ZrO_2 high
Group 12 - nano-SiO_2 low
Group 13 - nano-SiO_2 high

MPh – Macrophages; PMN – Polymorphonuclear cells; Lympho – Lymphocytes

Sacrifice at day 28 following instillative administration
Differential cell count – means, percentual

Wistar Han		Leucocyte conc.(1/ml)	MPh (%)	PMN (%)	Lympho (%)
GROUP					
1	Mean	82188	98.3	1.1	0.6
	Std	25623	1.7	0.8	1.0
	N	6	6	6	6
2	Mean	87917	98.9	0.8	0.3
	Std	43284	1.2	1.1	0.2
	N	6	6	6	6
3	Mean	105313	94.9	4.7	0.4
	Std	41314	4.4	4.1	0.5
	N	6	6	6	6
4	Mean	214750	***62.1	***36.1	*1.8
	Std	83994	13.2	13.8	0.7
	N	6	6	6	6
5	Mean	326167	***62.8	***35.9	1.3
	Std	190891	9.8	9.8	1.0
	N	6	6	6	6
6	Mean	222188	***60.5	***38.2	1.3
	Std	22803	9.6	9.8	0.7
	N	6	6	6	6
7	Mean	342813	***61.8	***37.4	0.8
	Std	98962	7.1	6.9	0.6
	N	6	6	6	6
8	Mean	106042	99.2	0.6	0.2
	Std	42130	0.2	0.1	0.3
	N	6	6	6	6

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

Group 1 - Control | Group 2 - µ-TiO_2 low | Group 3 - µ-TiO_2 high
Group 4 - nano-TiO_2 low | Group 5 - nano-TiO_2 high | Group 6 - µ-Eu_2O_3 low
Group 7 - µ-Eu_2O_3 high | Group 8 - µ-$BaSO_4$ low | Group 9 - µ-$BaSO_4$ high
Group 10 - µ-ZrO_2 low | Group 11 - µ-ZrO_2 high | Group 12 - nano-SiO_2 low
Group 13 - nano-SiO_2 high

MPh – Macrophages; PMN – Polymorphonuclear cells; Lympho - Lymphocytes

Sacrifice at day 28 following instillative administration
Differential cell count – means, percentual (Continued)

Wistar Han		Leucocyte conc.(1/ml)	MPh (%)	PMN (%)	Lympho (%)
GROUP					
9	Mean	91354	98.8	0.8	0.4
	Std	21259	0.6	0.6	0.3
	N	6	6	6	6
10	Mean	123813	98.0	1.5	0.5
	Std	64640	3.0	2.2	0.9
	N	6	6	6	6
11	Mean	145208	93.5	5.8	0.6
	Std	132002	4.6	4.3	0.6
	N	6	6	6	6
12	Mean	107709	98.0	0.9	1.1
	Std	45960	1.1	0.6	0.6
	N	6	6	6	6
13	Mean	97188	97.8	1.4	0.9
	Std	54725	1.9	1.5	0.6
	N	6	6	6	6

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

Group 1 - Control
Group 2 - µ-TiO_2 low
Group 3 - µ-TiO_2 high
Group 4 - nano-TiO_2 low
Group 5 - nano-TiO_2 high
Group 6 - µ-Eu_2O_3 low
Group 7 - µ-Eu_2O_3 high
Group 8 - µ-$BaSO_4$ low
Group 9 - µ-$BaSO_4$ high
Group 10 - µ-ZrO_2 low
Group 11 - µ-ZrO_2 high
Group 12 - nano-SiO_2 low
Group 13 - nano-SiO_2 high

MPh – Macrophages; PMN – Polymorphonuclear cells; Lympho – Lymphocytes

Sacrifice at day 28 following instillative administration
Differential cell count – means, absolute

Wistar Han		Leucocyte conc.(1/ml)	MPh (1/ml)	PMN (1/ml)	Lympho (1/ml)
GROUP					
1	Mean	82188	80874	853	460
	Std	25623	25742	544	679
	N	6	6	6	6
2	Mean	87917	87097	571	249
	Std	43284	43389	887	200
	N	6	6	6	6
3	Mean	105313	99168	5740	404
	Std	41314	36662	6276	537
	N	6	6	6	6
4	Mean	214750	130920	***79986	**3844
	Std	83994	59068	49657	2734
	N	6	6	6	6
5	Mean	326167	*199661	***122517	**3989
	Std	190891	109886	83454	3403
	N	6	6	6	6
6	Mean	222188	134566	***84921	2700
	Std	22803	26917	23534	1653
	N	6	6	6	6
7	Mean	342813	**213992	***126353	2467
	Std	98962	76722	32926	1736
	N	6	6	6	6
8	Mean	106042	105176	648	218
	Std	42130	41696	247	464
	N	6	6	6	6

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

Group 1 - Control
Group 2 - μ-TiO_2 low
Group 3 - μ-TiO_2 high
Group 4 - nano-TiO_2 low
Group 5 - nano-TiO_2 high
Group 6 - μ-Eu_2O_3 low
Group 7 - μ-Eu_2O_3 high
Group 8 - μ-$BaSO_4$ low
Group 9 - μ-$BaSO_4$ high
Group 10 - μ-ZrO_2 low
Group 11 - μ-ZrO_2 high
Group 12 - nano-SiO_2 low
Group 13 - nano-SiO_2 high

MPh – Macrophages; PMN – Polymorphonuclear cells; Lympho – Lymphocytes

Sacrifice at day 28 following instillative administration
Differential cell count – means, absolute (Continued)

Wistar Han		Leucocyte conc.(1/ml)	MPh (1/ml)	PMN (1/ml)	Lympho (1/ml)
GROUP					
9	Mean	91354	90332	623	400
	Std	21259	21200	503	298
	N	6	6	6	6
10	Mean	123813	122113	1329	370
	Std	64640	65408	1551	588
	N	6	6	6	6
11	Mean	145208	133510	10603	1096
	Std	132002	118769	12780	1368
	N	6	6	6	6
12	Mean	107709	105589	901	1218
	Std	45960	45126	609	979
	N	6	6	6	6
13	Mean	97188	95292	1140	755
	Std	54725	54276	964	501
	N	6	6	6	6

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

Group 1 - Control
Group 2 - μ-TiO_2 low
Group 3 - μ-TiO_2 high
Group 4 - nano-TiO_2 low
Group 5 - nano-TiO_2 high
Group 6 - μ-Eu_2O_3 low
Group 7 - μ-Eu_2O_3 high
Group 8 - μ-$BaSO_4$ low
Group 9 - μ-$BaSO_4$ high
Group 10 - μ-ZrO_2 low
Group 11 - μ-ZrO_2 high
Group 12 - nano-SiO_2 low
Group 13 - nano-SiO_2 high

MPh – Macrophages; PMN – Polymorphonuclear cells; Lympho – Lymphocytes

Sacrifice at day 3 following instillative administration
Biochemical parameters in the BALF supernatant – means

Males		LDH U/L	GLU U/L	TP mg/L
1m	Mean	40.0	0.34	112.6
	S.D.	13.9	0.09	37.9
	N	5	5	5
2m	Mean	58.3	0.35	131.5
	S.D.	17.6	0.05	26.8
	N	6	6	6
3m	Mean	79.5	0.47	188.5
	S.D.	25.1	0.05	34.8
	N	6	6	6
4m	Mean	165.8	1.62	358.0
	S.D.	36.1	0.83	97.5
	N	6	6	6
5m	Mean	306.0**	2.82	530.8
	S.D.	65.5	0.92	79.9
	N	6	6	6
6m	Mean	640.2**	13.83**	2278.2**
	S.D.	150.4	4.19	1125.4
	N	6	6	6
7m	Mean	816.5**	17.65**	2315.8**
	S.D.	218.7	7.00	844.2
	N	6	6	6
8m	Mean	48.2	0.25	126.7
	S.D.	11.0	0.08	23.8
	N	6	6	6
9m	Mean	81.2	0.43	176.8
	S.D.	23.5	0.14	41.8
	N	6	6	6
10m	Mean	214.2	5.70**	1305.5**
	S.D.	56.5	1.66	585.5
	N	6	6	6
11m	Mean	423.3**	8.15**	1457.0**
	S.D.	146.5	2.12	638.1
	N	6	6	6
12m	Mean	172.8	0.98	330.7
	S.D.	27.7	0.40	22.0
	N	6	6	6
13m	Mean	293.0**	2.00	527.0
	S.D.	185.1	1.03	175.2
	N	6	6	6

Statistics Test: Dunnett Test: * - 5% significance level; ** - 1% significance level

Group 1 - Control | Group 2 - μ-TiO_2 low | Group 3 - μ-TiO_2 high
Group 4 - nano-TiO_2 low | Group 5 - nano-TiO_2 high | Group 6 - μ-Eu_2O_3 low
Group 7 - μ-Eu_2O_3 high | Group 8 - μ-$BaSO_4$ low | Group 9 - μ-$BaSO_4$ high
Group 10 - μ-ZrO_2 low | Group 11 - μ-ZrO_2 high | Group 12 - nano-SiO_2 low
Group 13 - nano-SiO_2 high

LDH: Lactic Dehydrogenase GLU: β-Glucuronidase TP: Total Protein

Sacrifice at day 28 following instillative administration
Biochemical parameters in the BALF supernatant – means

Males		LDH U/L	GLU U/L	TP mg/L
1m	Mean	24.5	0.20	106.8
	S.D.	5.2	0.06	27.9
	N	6	6	6
2m	Mean	24.0	0.17	96.5
	S.D.	4.9	0.05	22.8
	N	6	6	6
3m	Mean	39.3	0.37	124.5
	S.D.	12.8	0.12	20.4
	N	6	6	6
4m	Mean	80.8	1.00	206.5
	S.D.	15.0	0.33	29.7
	N	6	6	6
5m	Mean	123.7**	2.10	303.8
	S.D.	36.7	1.16	73.7
	N	6	6	6
6m	Mean	164.8**	5.32**	733.8**
	S.D.	44.8	2.74	291.2
	N	6	6	6
7m	Mean	342.7**	16.30**	1696.7**
	S.D.	131.4	7.68	548.1
	N	6	6	6
8m	Mean	29.0	0.25	102.0
	S.D.	7.2	0.05	14.2
	N	6	6	6
9m	Mean	30.8	0.20	108.8
	S.D.	12.4	0.09	31.9
	N	6	6	6
10m	Mean	28.3	0.22	97.2
	S.D.	7.9	0.10	17.2
	N	6	6	6
11m	Mean	64.5	0.53	170.3
	S.D.	21.6	0.21	37.0
	N	6	6	6
12m	Mean	33.5	0.25	113.0
	S.D.	7.5	0.10	12.8
	N	6	6	6
13m	Mean	27.0	0.17	114.5
	S.D.	6.7	0.08	12.5
	N	6	6	6

Statistics Test: Dunnett Test: * - 5% significance level; ** - 1% significance level

Group 1 - Control | Group 2 - μ-TiO_2 low | Group 3 - μ-TiO_2 high
Group 4 - nano-TiO_2 low | Group 5 - nano-TiO_2 high | Group 6 - μ-Eu_2O_3 low
Group 7 - μ-Eu_2O_3 high | Group 8 - μ-$BaSO_4$ low | Group 9 - μ-$BaSO_4$ high
Group 10 - μ-ZrO_2 low | Group 11 - μ-ZrO_2 high | Group 12 - nano-SiO_2 low
Group 13 - nano-SiO_2 high

LDH: Lactic Dehydrogenase GLU: β-Glucuronidase TP: Total Protein

Appendix 4 Chemical analysis of lung loads (individual data)

Group 2: **Titanium dioxide; µ-TiO_2 Bayertitan T** **Dose: 2,16 mg or 0,5 µl**

Day 3 post instillation (d3+)

Group	Animal	TiO_2 - soluble (ionic)			TiO_2 - unsoluble (particulate)			TiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
2 d3+	113	0,6	0,03	5,1	1736	45	2,6	1737	45	2,6
	114	1,1	0,01	1,3	1911	18	0,9	1912	18	0,9
	115	1,3	0,03	2,7	1801	3	0,1	1802	3	0,1
	116	1,1	0,02	1,4	1430	1	0,1	1431	1	0,1
	117	1,7	0,06	3,4	1326	2	0,2	1328	2	0,2
	118	1,6	0,02	1,4	1414	2	0,1	1416	2	0,1
	Mean	**1,3**	0,3	21,8	**1603**	242	15,1	**1604**	242	15,1

Day 28 post instillation (d28+)

Group	Animal	TiO_2 - soluble (ionic)			TiO_2 - unsoluble (particulate)			TiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
2 d28+	119	0,7	0,03	4,6	779	15	1,9	780	15	1,9
	120	0,4	0,05	11,4	760	4	0,6	760	4	0,6
	121	1,1	0,11	9,8	789	3	0,3	790	3	0,3
	122	0,6	0,07	11,0	655	2	0,4	656	2	0,4
	123	1,2	0,04	3,8	1019	1	0,1	1020	2	0,1
	124	0,4	0,01	1,3	508	2	0,4	508	2	0,4
	Mean	**0,7**	0,3	44,4	**752**	169	22,5	**752**	169	22,5

Day 90 post instillation (d90+)

Group	Animal	TiO_2 - soluble (ionic)			TiO_2 - unsoluble (particulate)			TiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
2 d90+	125	0,3	0,02	4,6	294	4	1,5	294	4	1,5
	126	0,2	0,00	1,5	303	2	0,7	303	2	0,7
	127	0,4	0,00	1,2	617	2	0,4	618	2	0,4
	128	*1,5*	*0,06*	*4,4*	445	5	1,2	447	5	1,2
	129	0,6	0,07	11,4	489	11	2,3	490	11	2,3
	130	0,4	0,03	8,5	344	10	2,9	345	10	2,9
	Mean	**0,4**	0,2	46,2	**415**	126	30,4	**416**	126	30,4

Data in italics: Not included for mean value
ASD: Absolute standard deviation - RSD: Relative standard deviation

Group 3: **Titanium dioxide; µ-TiO_2 Bayertitan T** **Dose: 6,46 mg or 1,5 µl**

Day 3 post instillation (d3+)

Group	Animal	TiO_2 - soluble (ionic)			TiO_2 - unsoluble (particulate)			TiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
3 d3+	113	4,7	0,4	8,9	8571	25	0,3	8575	26	0,3
	114	8,1	1,8	22,7	6296	31	0,5	6304	33	0,5
	115	1,1	0,0	0,1	3402	2	0,1	3403	2	0,1
	116	8,2	1,8	21,8	5490	11	0,2	5498	13	0,2
	117	3,9	0,2	5,7	5439	15	0,3	5443	15	0,3
	118	4,6	0,3	6,6	4771	9	0,2	4776	9	0,2
	Mean	**5,1**	2,7	53,2	**5662**	1723	30,4	**5667**	1724	30,4

Day 28 post instillation (d28+)

Group	Animal	TiO_2 - soluble (ionic)			TiO_2 - unsoluble (particulate)			TiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
3 d28+	119	1,7	0,1	4,0	5197	16	0,3	5199	16	0,3
	120	1,0	0,1	6,8	2851	2	0,1	2852	2	0,1
	121	4,2	0,7	17,6	2391	5	0,2	2395	5	0,2
	122	2,6	0,5	20,8	2858	5	0,2	2861	6	0,2
	123	4,7	0,5	11,2	2777	5	0,2	2782	5	0,2
	124	6,7	1,4	20,5	3104	29	0,9	3110	30	1,0
	Mean	**3,5**	2,1	60,4	**3196**	1007	31,5	**3200**	1006	31,4

Day 90 post instillation (d90+)

Group	Animal	TiO_2 - soluble (ionic)			TiO_2 - unsoluble (particulate)			TiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
3 d90+	125	1,3	0,1	7,8	3078	1	0,0	3079	1	0,0
	126	*15,8*	*0,5*	*2,9*	1889	4	0,2	1905	5	0,2
	127	5,3	0,4	8,2	3237	8	0,3	3243	9	0,3
	128	0,8	0,1	12,7	2527	3	0,1	2528	3	0,1
	129	1,6	0,1	8,3	3316	5	0,2	3318	5	0,2
	130	1,6	0,4	21,7	1609	7	0,4	1610	7	0,4
	Mean	**2,1**	0,2	7,5	**2609**	727	27,9	**2614**	725	27,7

Data in italics: Not included for mean value
ASD: Absolute standard deviation - RSD: Relative standard deviation

Group 4: **nano-Titanium dioxide;TiO_2 P25 (EU/JRC)** **Dose: 1,9 mg or 0,5 µl**

Day 3 post instillation (d3+)

Group	Animal	TiO_2 - soluble (ionic)			TiO_2 - unsoluble (particulate)			TiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
4 d3+	113	6,3	0,2	3,9	1252	2	0,2	1258	3	0,2
	114	5,8	0,3	5,0	1223	6	0,5	1229	6	0,5
	115	4,0	0,1	3,6	1389	2	0,1	1393	2	0,1
	116	3,4	0,3	7,6	*867*	*5*	*0,6*	*871*	*6*	*0,6*
	117	8,7	0,3	3,5	1406	7	0,5	1415	7	0,5
	118	6,5	0,3	3,9	1524	5	0,3	1531	6	0,4
	Mean	**5,8**	1,9	33,2	**1359**	123	9,0	**1365**	123	9,0

Day 28 post instillation (d28+)

Group	Animal	TiO_2 - soluble (ionic)			TiO_2 - unsoluble (particular)			TiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
4 d28+	119	5,9	0,9	15,8	607	2	0,4	613	3	0,5
	120	2,3	0,2	6,8	853	3	0,3	855	3	0,4
	121	2,8	0,0	1,1	1253	5	0,4	1256	5	0,4
	122	3,0	0,1	3,8	1197	7	0,6	1200	7	0,6
	123	7,5	0,1	0,7	1211	19	1,5	1219	19	1,5
	124	3,1	0,1	3,4	1156	13	1,1	1159	13	1,2
	Mean	**4,1**	2,1	50,9	**1046**	259	24,8	**1050**	259	24,6

Day 90 post instillation (d90+)

Group	Animal	TiO_2 - soluble (ionic)			TiO_2 - unsoluble (particulate)			TiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
4 d90+	125	3,2	0,3	9,4	758	6	0,7	761	6	0,8
	126	4,4	0,5	10,8	920	8	0,8	925	8	0,9
	127	4,6	0,2	4,8	1111	14	1,2	1115	14	1,2
	128	2,6	0,2	7,4	746	1	0,1	749	1	0,1
	129	4,7	0,2	3,8	751	5	0,6	755	5	0,7
	130	5,0	0,4	7,8	822	3	0,3	827	3	0,3
	Mean	**4,1**	0,9	23,2	**851**	143	16,8	**855**	144	16,8

ASD: Absolute standard deviation - RSD: Relative standard deviation

Group 5: **nano-Titanium dioxide;TiO_2 P25 (EU/JRC)** **Dose: 5,7 mg or 1,5 µl**

Day 3 post instillation (d3+)

Group	Animal	TiO_2 - soluble (ionic)			TiO_2 - unsoluble (particulate)			TiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
5 d3+	113	10,8	0,1	0,6	3615	49	1,4	3626	49	1,4
	114	16,5	1,0	6,0	3798	63	1,6	3814	64	1,7
	115	10,1	0,6	5,6	3571	37	1,0	3581	38	1,1
	116	7,1	0,0	0,3	3939	38	1,0	3946	38	1,0
	117	17,2	1,8	10,7	3414	24	0,7	3431	26	0,8
	118	15,3	1,0	6,4	4594	25	0,5	4609	26	0,6
	Mean	**12,8**	4,1	31,8	**3822**	420	11,0	**3834**	420	11,0

Day 28 post instillation (d28+)

Group	Animal	TiO_2 - soluble (ionic)			TiO_2 - unsoluble (particulate)			TiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
5 d28+	119	10,0	0,1	1,3	3874	9	0,2	3884	10	0,2
	120	14,5	0,8	5,3	2979	30	1,0	2994	31	1,0
	121	11,8	0,8	6,9	3333	31	0,9	3345	31	0,9
	122	6,0	0,0	0,6	3913	72	1,8	3919	72	1,8
	123	17,9	0,4	2,0	4181	1	0,0	4199	1	0,0
	124	12,1	0,5	4,5	3788	1	0,0	3800	1	0,0
	Mean	**12,0**	4,0	33,5	**3678**	440	12,0	**3690**	439	11,9

Day 90 post instillation (d90+)

Group	Animal	TiO_2 - soluble (ionic)			TiO_2 - unsoluble (particulate)			TiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
5 d90+	125	13,6	0,4	2,8	3866	14	0,4	3880	14	0,4
	126	17,0	0,9	5,3	3843	26	0,7	3860	27	0,7
	127	19,5	1,3	6,8	4323	19	0,4	4343	20	0,5
	128	5,9	0,2	3,3	2784	13	0,5	2790	13	0,5
	129	11,2	0,8	6,9	3451	28	0,8	3462	28	0,8
	130	12,4	0,8	6,8	3114	37	1,2	3126	37	1,2
	Mean	**13,3**	4,7	35,6	**3564**	560	15,7	**3577**	565	15,8

ASD: Absolute standard deviation - RSD: Relative standard deviation

Group 6: **Europium oxide (μ-Eu_2O_3)** **Dose: 3,7 mg or 0,5 μl**

Day 3 post instillation (d3+)

Group	Animal	Eu_2O_3 - soluble (ionic)			Eu_2O_3 - unsoluble (particulate)			Eu_2O_3 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(μg/Organ)		(%)	(μg/Organ)		(%)	(μg/Organ)		(%)
6 d3+	113	681	3	0,5	1611	< 1	< 0,1	2292	3	0,1
	114	863	2	0,2	1864	< 1	< 0,1	2727	2	0,1
	115	430	17	4,0	2609	4	0,1	3039	21	0,7
	116	758	7	0,9	1589	< 1	< 0,1	2347	7	0,3
	117	737	3	0,4	2440	2	0,1	3176	6	0,2
	118	657	2	0,4	2573	2	0,1	3231	5	0,1
	Mean	**688**	145	21,1	**2114**	480	22,7	**2802**	413	14,7

Day 28 post instillation (d28+)

Group	Animal	Eu_2O_3 - soluble (ionic)			Eu_2O_3 - unsoluble (particulate)			Eu_2O_3 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(μg/Organ)		(%)	(μg/Organ)		(%)	(μg/Organ)		(%)
6 d28+	119	1107	21	1,9	1380	2	0,1	2487	23	0,9
	120	1072	2	0,2	1721	1	< 0,1	2793	3	0,1
	121	1197	15	1,2	1541	1	0,1	2738	16	0,6
	122	1494	23	1,5	1176	1	0,1	2670	24	0,9
	123	624	0	0,0	2032	2	0,1	2656	3	0,1
	124	1063	16	1,5	1364	3	0,2	2427	19	0,8
	Mean	**1093**	280	25,6	**1536**	304	19,8	**2628**	143	5,4

Day 90 post instillation (d90+)

Group	Animal	Eu_2O_3 - soluble (ionic)			Eu_2O_3 - unsoluble (particulate)			Eu_2O_3 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(μg/Organ)		(%)	(μg/Organ)		(%)	(μg/Organ)		(%)
6 d90+	125	901	18	2,0	1390	1	0,1	2292	19	0,8
	126	630	2	0,3	2016	< 1	< 0,1	2646	2	0,1
	127	998	6	0,6	761	2	0,2	1758	7	0,4
	128	571	1	0,1	1684	1	0,1	2255	2	0,1
	129	240	3	1,2	1930	2	0,1	2170	5	0,2
	130	413	1	0,2	1996	1	0,0	2408	2	0,1
	Mean	**625**	287	45,9	**1629**	488	29,9	**2255**	294	13,0

ASD: Absolute standard deviation - RSD: Relative standard deviation

Group 7: **Europium oxide (µ-Eu_2O_3)** **Dose: 11,1 mg or 1,5 µl**

Day 3 post instillation (d3+)

Group	Animal	Eu_2O_3 - soluble (ionic)			Eu_2O_3 - unsoluble (particulate)			Eu_2O_3 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
7 d3+	113	1409	23	1,6	6668	4	0,1	8077	27	0,3
	114	1719	2	0,1	7151	5	0,1	8870	8	0,1
	115	921	3	0,3	7380	8	0,1	8302	11	0,1
	116	1585	4	0,3	5823	0	0,0	7408	4	0,1
	117	1666	7	0,4	7467	9	0,1	9133	16	0,2
	118	1005	8	0,8	8988	7	0,1	9993	15	0,1
	Mean	**1384**	344	24,8	**7246**	1046	14,4	**8630**	903	10,5

Day 28 post instillation (d28+)

Group	Animal	Eu_2O_3 - soluble (ionic)			Eu_2O_3 - unsoluble (particulate)			Eu_2O_3 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
7 d28+	119	2244	64	2,9	6181	7	0,1	8425	71	0,8
	120	2428	21	0,8	6167	5	0,1	8595	26	0,3
	121	2384	6	0,3	6478	1	< 0,1	8862	7	0,1
	122	2972	20	0,7	4995	2	< 0,1	7967	22	0,3
	123	1953	6	0,3	5201	2	< 0,1	7153	8	0,1
	124	2967	5	0,2	5738	2	< 0,1	8705	7	0,1
	Mean	**2491**	406	16,3	**5793**	592	10,2	**8285**	633	7,6

Day 90 post instillation (d90+)

Group	Animal	Eu_2O_3 - soluble (ionic)			Eu_2O_3 - unsoluble (particulate)			Eu_2O_3 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
7 d90+	125	1976	36	1,8	4682	7	0,2	6658	43	0,6
	126	2386	21	0,9	5486	11	0,2	7872	32	0,4
	127	1148	1	0,1	6211	6	0,1	7358	7	0,1
	128	1212	17	1,4	5558	8	0,1	6770	25	0,4
	129	772	9	1,2	7059	16	0,2	7831	25	0,3
	130	1825	15	0,8	5180	9	0,2	7005	24	0,3
	Mean	**1553**	606	39,0	**5696**	834	14,6	**7249**	525	7,2

ASD: Absolute standard deviation - RSD: Relative standard deviation

Group 8: **Barium sulfate (µ-$BaSO_4$)** **Dose: 2,26 mg or 0,5 µl**

Day 3 post instillation (d3+)

Group	Animal	$BaSO_4$ - soluble (ionic)			$BaSO_4$ - unsoluble (particulate)			$BaSO_4$ - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
8 d3+	113	10,4	0,1	0,7	1195	5	0,4	1206	5	0,4
	114	9,03	0,21	2,3	1481	6	0,4	1490	7	0,4
	115	40,8	3,3	8,1	995	12	1,2	1036	15	1,5
	116	17,6	0,3	1,8	1227	12	1,0	1245	12	1,0
	117	8,72	0,07	0,8	1292	9	0,7	1301	9	0,7
	118	9,21	0,09	1,0	1428	6	0,4	1437	6	0,4
	Mean	**16,0**	12,6	79,1	**1270**	175	13,8	**1286**	164	12,8

Day 28 post instillation (d28+)

Group	Animal	$BaSO_4$ - soluble (ionic)			$BaSO_4$ - unsoluble (particulate)			$BaSO_4$ - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
8 d28+	119	5,96	0,03	0,6	275	17	6,1	281	17	6,0
	120	4,01	0,04	0,9	387	8	2,0	391	8	2,0
	121	20,2	0,8	4,2	280	10	3,4	300	10	3,4
	122	14,1	0,2	1,5	608	3	0,5	622	3	0,5
	123	2,48	0,11	4,5	405	11	2,7	408	11	2,7
	124	8,96	0,13	1,5	205	2	0,9	214	2	0,9
	Mean	**9,3**	6,8	72,7	**360**	143	39,6	**369**	143	38,7

Day 90 post instillation (d90+)

Group	Animal	$BaSO_4$ - soluble (ionic)			$BaSO_4$ - unsoluble (particulate)			$BaSO_4$ - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
8 d90+	125	0,56	0,01	2,7	93,2	4,2	4,5	93,8	4,2	4,5
	126	1,35	0,03	2,6	173	6	3,6	174	6	3,6
	127	2,11	0,11	5,4	148	1	0,4	150	1	0,5
	128	0,70	0,02	3,0	64,3	0,1	0,2	65,0	0,1	0,2
	129	0,64	0,01	1,9	116	6	5,3	117	6	5,3
	130	0,59	0,07	11,4	67,2	0,0	0,0	67,8	0,1	0,1
	Mean	**0,99**	0,62	62,7	**110**	44	39,8	**111**	44,4	39,9

ASD: Absolute standard deviation - RSD: Relative standard deviation

Group 9: **Barium sulfate (µ-$BaSO_4$)** **Dose: 6,76 mg or 1,5 µl**

Day 3 post instillation (d3+)

Group	Animal	$BaSO_4$ - soluble (ionic)			$BaSO_4$ - unsoluble (particulate)			$BaSO_4$ - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
9 d3+	*113*	*10,2*	*0,4*	*4,2*	3159	0	0,0	3169	0	0,0
	114	340	1	0,3	5464	6	0,1	5804	7	0,1
	115	77,9	1,7	2,1	4566	18	0,4	4644	19	0,4
	116	155	4	2,7	3195	11	0,4	3350	16	0,5
	117	352	1	0,2	5071	8	0,2	5423	9	0,2
	118	102	2	2,0	4158	6	0,1	4260	8	0,2
	Mean	**205**	131	64,0	**4269**	955	22,4	**4442**	1068	24,0

Day 28 post instillation (d28+)

Group	Animal	$BaSO_4$ - soluble (ionic)			$BaSO_4$ - unsoluble (particulate)			$BaSO_4$ - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
9 d28+	119	112	2	1,4	2807	5	0,2	2919	6	0,2
	120	22,6	0,9	4,0	1481	6	0,4	1503	7	0,5
	121	38,9	1,1	2,8	2336	10	0,4	2374	11	0,5
	122	42,5	1,4	3,2	1532	10	0,6	1574	11	0,7
	123	16,3	0,6	3,5	2376	17	0,7	2392	17	0,7
	124	46,2	0,7	1,5	1636	10	0,6	1682	11	0,7
	Mean	**46,4**	34,2	73,8	**2028**	552	27,2	**2074**	572	27,6

Day 90 post instillation (d90+)

Group	Animal	$BaSO_4$ - soluble (ionic)			$BaSO_4$ - unsoluble (particulate)			$BaSO_4$ - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
9 d90+	*125*	*201*	*3*	*1,7*	588	6	1,0	789	9	1,2
	126	86,0	1,6	1,8	579	2	0,4	665	4	0,6
	127	31,9	0,2	0,5	1144	23	2,0	1176	23	2,0
	128	26,6	0,2	0,7	524	6	1,2	550	6	1,1
	129	65,7	0,8	1,2	1385	34	2,4	1450	34	2,4
	130	*8,88*	*0,09*	*1,0*	807	7	0,8	816	7	0,8
	Mean	**52,5**	28,2	53,7	**838**	353	42,1	**908**	339	37,4

Data *in italics*: Not included for mean value

ASD: Absolute standard deviation - RSD: Relative standard deviation

Group 10:	**Y-stabil. Zirconium oxide; YSZ (μ-ZrO_2)**	**Dose: 2,86 mg**	thereof	**0,39 mg Y_2O_3 or 0,5 µl**

Day 3 post instillation (d3+)

Group	Animal	Y_2O_3 - soluble (ionic)			Y_2O_3 - unsoluble (particulate)			Y_2O_3 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
10 d3+	113	22,8	0,2	0,9	234	1	0,5	257	1	0,5
	114	34,7	0,2	0,5	242	1	0,3	277	1	0,3
	115	35,1	0,1	0,1	210	2	1,0	245	2	0,8
	116	21,4	0,3	1,5	186	1	0,3	207	1	0,4
	117	*7,5*	*0,9*	*12,4*	*49,4*	*0,2*	*0,5*	*56,9*	*1,2*	*2,1*
	118	29,0	0,3	1,0	238	2	0,7	267	2	0,7
	Mean	**28,6**	6,4	22,4	**222**	24	10,8	**251**	27	10,8

Day 28 post instillation (d28+)

Group	Animal	Y_2O_3 - soluble (ionic)			Y_2O_3 - unsoluble (particulate)			Y_2O_3 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
10 d28+	119	16,0	0,0	0,3	245	2	0,9	261	2	0,8
	120	19,0	0,1	0,3	232	0	0,1	251	0	0,1
	121	11,9	0,1	0,6	186	1	0,6	198	1	0,6
	122	18,3	0,1	0,5	251	0	0,1	270	0	0,1
	123	19,3	0,0	0,2	203	2	0,8	222	2	0,7
	124	19,3	0,1	0,4	294	1	0,4	313	1	0,4
	Mean	**17,3**	2,9	16,8	**235**	38	16,3	**252**	40	15,9

Day 90 post instillation (d90+)

Group	Animal	Y_2O_3 - soluble (ionic)			Y_2O_3 - unsoluble (particulate)			Y_2O_3 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
10 d90+	125	9,5	0,1	0,9	211	1	0,5	220	1	0,5
	126	19,2	0,2	0,8	127	1	0,6	146	1	0,7
	127	16,5	0,1	0,4	134	0	0,3	151	0	0,3
	128	4,9	0,2	4,5	130	0	0,3	135	1	0,5
	129	6,4	0,0	0,3	146	0	0,1	153	0	0,1
	130	12,9	0,0	0,4	169	1	0,6	182	1	0,6
	Mean	**11,6**	5,7	48,8	**153**	32	21,1	**164**	31	19,2

Data *in italics*: Not included for mean value
ASD: Absolute standard deviation - RSD: Relative standard deviation

Group 11:	**Y-stabil. Zirconium oxide; YSZ (μ-ZrO_2)**	**Dose: 8,56 mg**	thereof	**1,18 mg Y_2O_3 or 1,5 µl**

Day 3 post instillation (d3+)

Group	Animal	Y_2O_3 - soluble (ionic)			Y_2O_3 - unsoluble (particulate)			Y_2O_3 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
11 d3+	113	60,0	0,8	1,4	652	4	0,6	712	5	0,7
	114	65,2	1,3	2,0	836	4	0,5	901	5	0,6
	115	46,4	0,6	1,3	900	3	0,4	947	4	0,4
	116	80,4	1,6	1,9	877	6	0,7	957	8	0,8
	117	67,2	1,5	2,2	729	5	0,7	796	6	0,8
	118	99,0	2,4	2,4	899	7	0,7	998	9	0,9
	Mean	**69,7**	18,1	26,0	**816**	102	12,6	**885**	109	12,3

Day 28 post instillation (d28+)

Group	Animal	Y_2O_3 - soluble (ionic)			Y_2O_3 - unsoluble (particulate)			Y_2O_3 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
11 d28+	119	40,0	0,7	1,8	757	3	0,4	797	4	0,5
	120	34,8	0,8	2,4	852	6	0,7	887	7	0,8
	121	31,2	0,6	1,9	800	0	0,1	832	1	0,1
	122	55,2	0,6	1,1	582	1	0,2	637	2	0,3
	123	64,2	1,5	2,4	781	2	0,3	846	4	0,4
	124	61,5	0,9	1,5	898	1	0,1	959	2	0,2
	Mean	**47,8**	14,3	29,8	**778**	109	14,0	**826**	108	13,1

Day 90 post instillation (d90+)

Group	Animal	Y_2O_3 - soluble (ionic)			Y_2O_3 - unsoluble (particulate)			Y_2O_3 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
11 d90+	125	49,3	0,5	1,0	469	5	1,1	518	6	1,1
	126	63,4	1,4	2,3	779	2	0,3	842	4	0,4
	127	39,7	0,9	2,3	472	0	0,0	512	1	0,2
	128	34,2	0,7	2,0	617	0	0,1	651	1	0,2
	129	26,6	0,3	1,2	776	1	0,1	802	1	0,2
	130	33,9	0,8	2,2	846	4	0,5	880	5	0,6
	Mean	**41,2**	13,2	32,1	**660**	165	25,0	**701**	164	23,4

ASD: Absolute standard deviation - RSD: Relative standard deviation

Group 12: **amorphous Silicon dioxide (nano-SiO_2 NM-200)** **Dose: 1,1 mg or 0,5 µl**

Day 3 post instillation (d3+)

Group	Animal	SiO_2- soluble (ionic)			SiO_2 - unsoluble (particulate)			SiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
12 d3+	113	2,26	0,16	7,2	179	5	2,6	181	5	2,7
	114	1,97	0,05	2,4	222	1	0,3	224	1	0,3
	115	2,68	0,14	5,0	202	3	1,6	205	3	1,7
	116	2,64	0,04	1,6	205	3	1,3	207	3	1,3
	117	1,61	0,06	3,8	214	5	2,5	216	5	2,5
	118	1,73	0,03	2,0	208	5	2,5	210	5	2,5
	Mean	**2,1**	0,5	21,2	**205**	15	7,2	**207**	15	7,0

Day 28 post instillation (d28+)

Group	Animal	SiO_2- soluble (ionic)			SiO_2 - unsoluble (particulate)			SiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
12 d28+	119	1,66	0,13	7,8	59,4	2,1	3,5	61,1	2,2	3,6
	120	1,67	0,07	3,9	26,5	1,7	6,4	28,2	1,8	6,3
	121	1,58	0,10	6,4	49,8	1,4	2,7	51,4	1,5	2,9
	122	2,25	0,04	1,7	44,3	1,0	2,2	46,5	1,0	2,2
	123	1,31	0,07	5,4	68,3	2,4	3,6	69,6	2,5	3,6
	124	1,37	0,09	6,7	52,3	2,4	4,6	53,7	2,5	4,7
	Mean	**1,6**	0,3	20,4	**50,1**	14,2	28,4	**51,7**	14,1	27,2

Day 90 post instillation (d90+)

Group	Animal	SiO_2- soluble (ionic)			SiO_2 - unsoluble (particulate)			SiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
12 d90+	125	1,15	0,04	3,8	15,8	0,3	1,9	16,9	0,3	2,1
	126	1,00	0,06	6,2	15,3	1,7	11,3	16,3	1,8	11,0
	127	1,61	0,05	3,1	14,8	0,5	3,6	16,4	0,6	3,6
	128	1,33	0,04	2,8	9,9	0,6	6,4	11,2	0,7	6,0
	129	0,69	0,09	12,7	23,3	0,2	1,0	24,0	0,3	1,4
	130	0,64	0,13	20,2	11,2	0,7	6,2	11,8	0,8	6,9
	Mean	**1,1**	0,4	35,1	**15,0**	4,7	31,2	**16,1**	4,6	28,4

ASD: Absolute standard deviation - RSD: Relative standard deviation

Group 13: **amorphous Silicon dioxide (nano-SiO_2 NM-200)** **Dose: 3,3 mg or 1,5 µl**

Day 3 post instillation (d3+)

Group	Animal	SiO_2- soluble (ionic)			SiO_2 - unsoluble (particulate)			SiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
13 d3+	113	2,12	0,15	7,0	661	22	3,4	663	22	3,4
	114	1,47	0,09	6,4	539	7	1,3	541	7	1,3
	115	2,13	0,04	2,0	565	2	0,4	567	2	0,4
	116	2,10	0,06	3,1	568	5	0,9	570	5	0,9
	117	*5,82*	*0,05*	*0,8*	383	4	1,0	388	4	1,0
	118	1,66	0,03	1,8	489	6	1,2	490	6	1,2
	Mean	**1,9**	0,3	16,3	**534**	93	17,4	**537**	92	17,1

Day 28 post instillation (d28+)

Group	Animal	SiO_2- soluble (ionic)			SiO_2 - unsoluble (particulate)			SiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
13 d28+	119	1,41	0,05	3,2	149	5	3,4	150	5	3,4
	120	1,29	0,11	8,3	100	8	7,6	101	8	7,6
	121	1,84	0,03	1,8	151	7	4,4	153	7	4,4
	122	1,53	0,01	0,8	154	4	2,3	155	4	2,3
	123	1,33	0,05	3,5	133	1	1,1	134	1	1,1
	124	1,36	0,03	2,3	139	4	3,1	141	4	3,1
	Mean	**1,5**	0,2	14,0	**138**	20	14,5	**139**	20	14,5

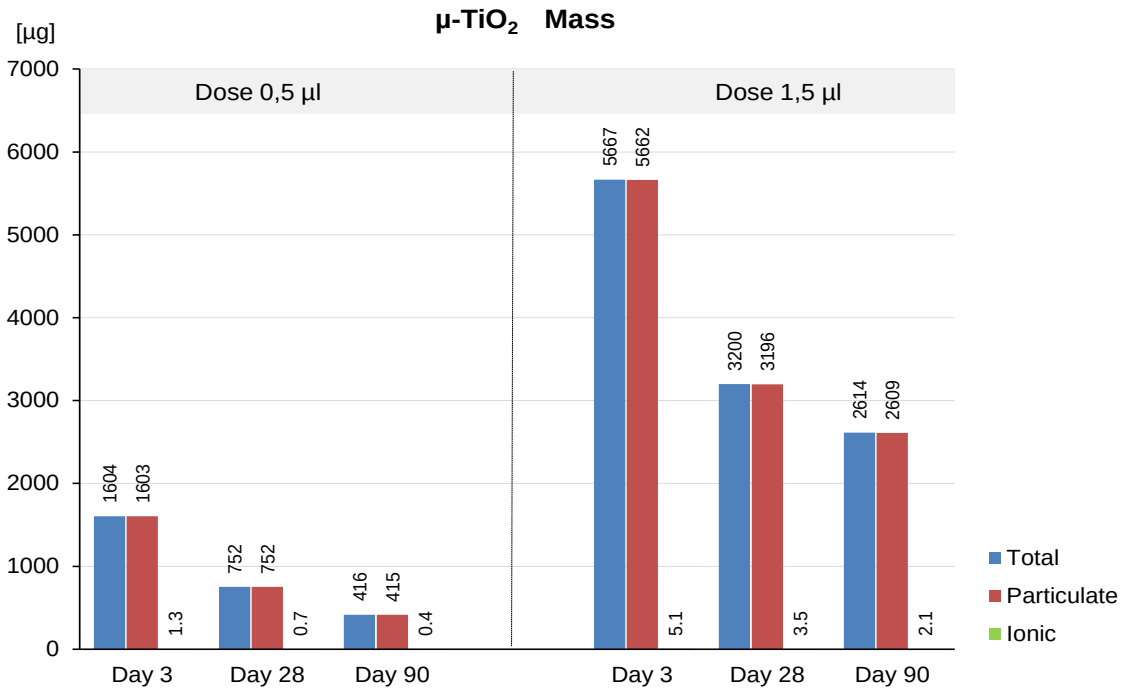
Day 90 post instillation (d90+)

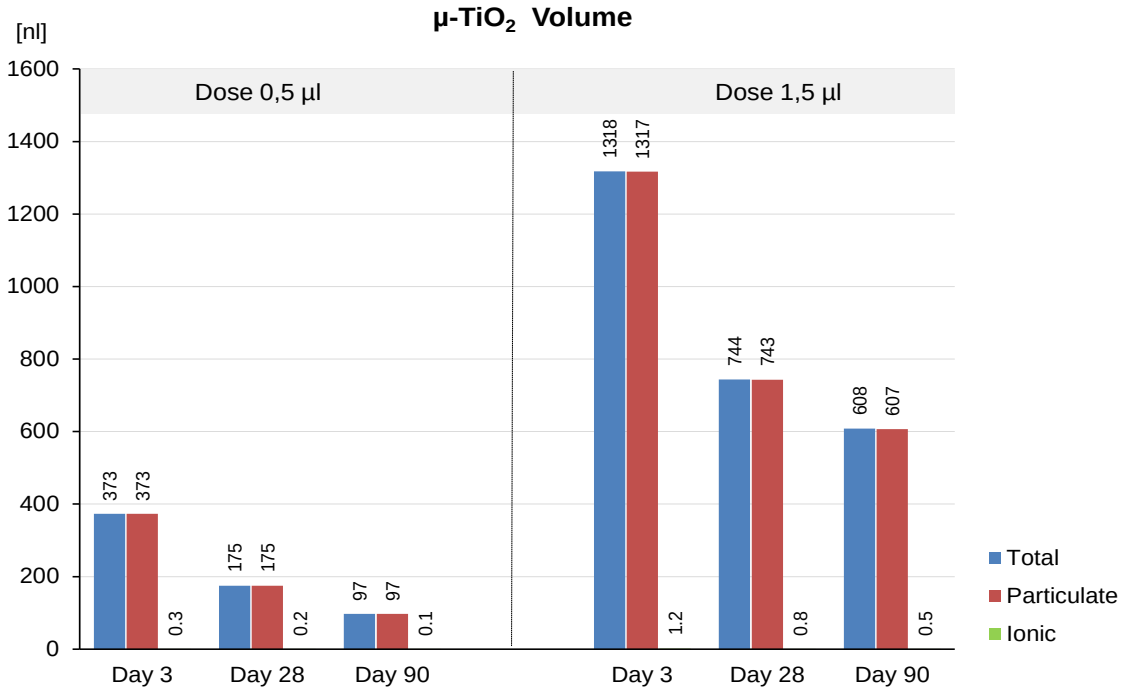
Group	Animal	SiO_2- soluble (ionic)			SiO_2 - unsoluble (particulate)			SiO_2 - Total		
		Mean	ASD	RSD	**Mean**	ASD	RSD	**Mean**	ASD	RSD
		(µg/Organ)		(%)	(µg/Organ)		(%)	(µg/Organ)		(%)
13 d90+	125	1,20	0,15	12,5	51,8	0,7	1,4	53,0	0,9	1,7
	126	0,78	0,04	5,4	49,2	0,4	0,9	50,0	0,5	1,0
	127	0,99	0,04	4,0	45,9	0,6	1,2	46,9	0,6	1,3
	128	1,39	0,02	1,1	49,8	0,8	1,5	51,2	0,8	1,5
	129	0,93	0,09	9,1	38,8	0,5	1,2	39,7	0,5	1,4
	130	0,80	0,07	8,7	40,7	0,9	2,3	41,5	1,0	2,4
	Mean	**1,0**	0,2	23,5	**46,0**	5,3	11,4	**47,1**	5,4	11,5

Data *in italics*: Not included for mean value

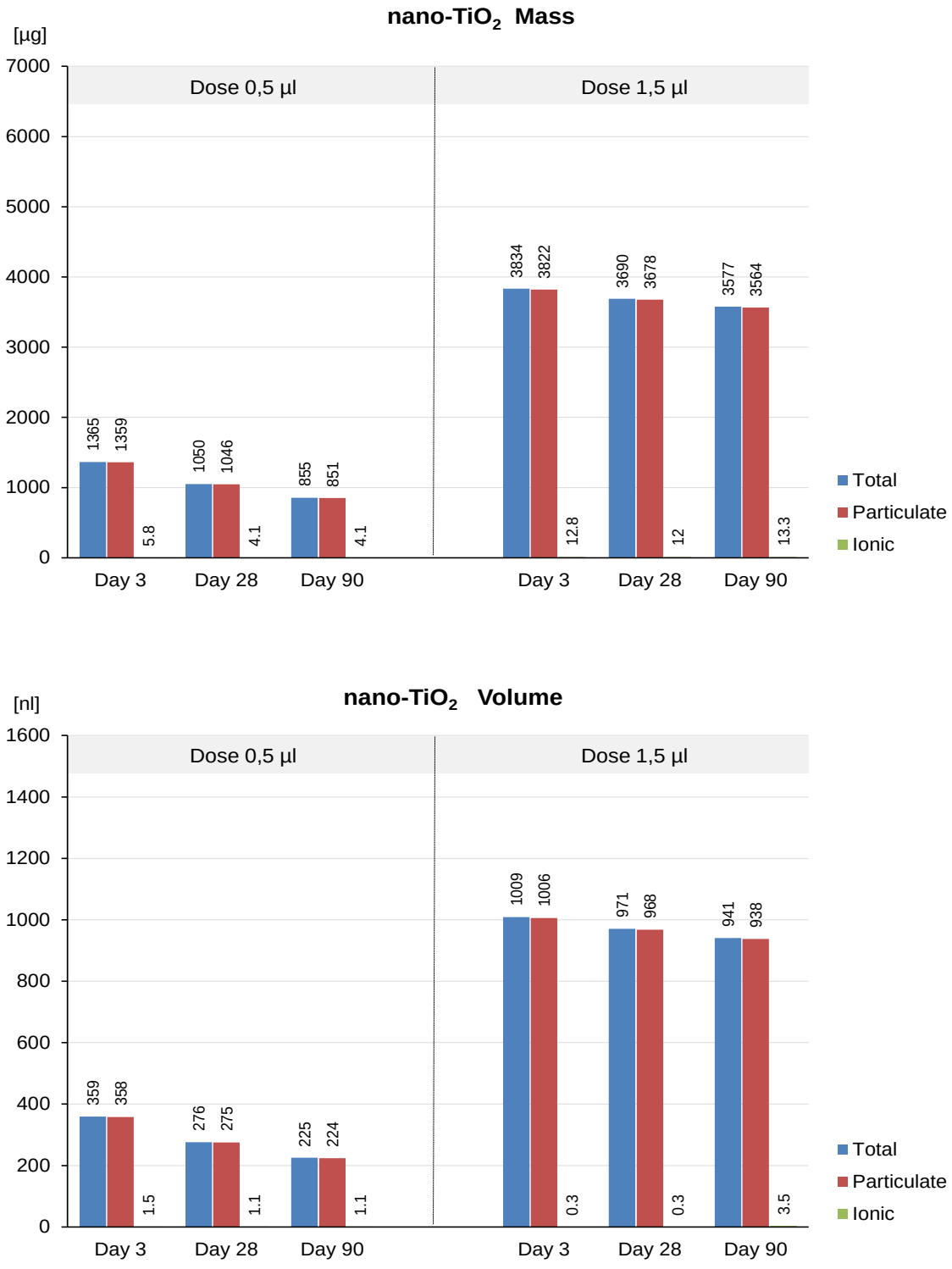
ASD: Absolute standard deviation - RSD: Relative standard deviation

Appendix 5 Chemical analysis of lung loads - means (figures)

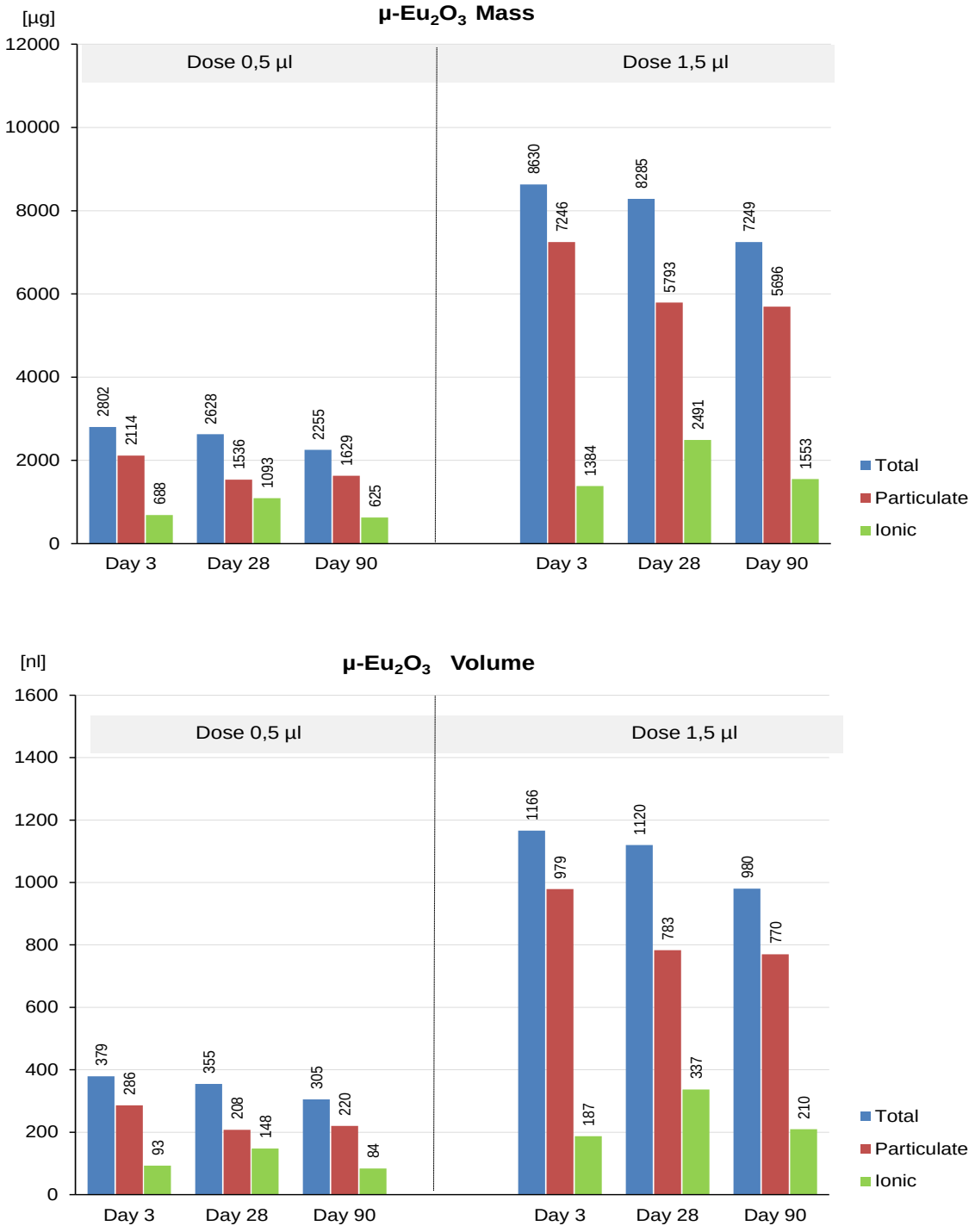




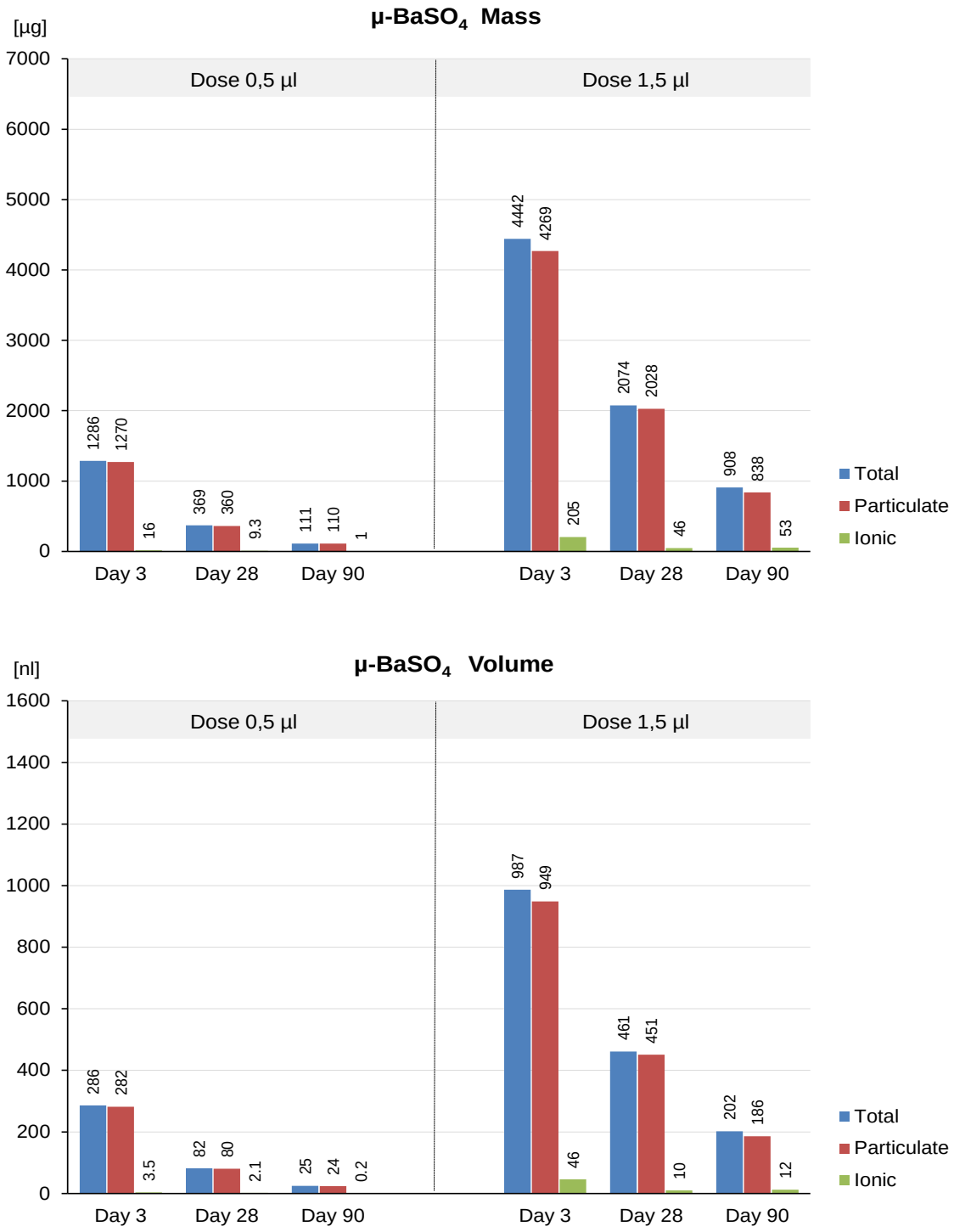
App. 5, Fig. 1a/b Titanium dioxide Bayertitan T - Total, particulate and ionic mass in lungs (µg/lung or nl/lung) → 0.5 µl: non-overload - 1.5 µl overload (volumetric)



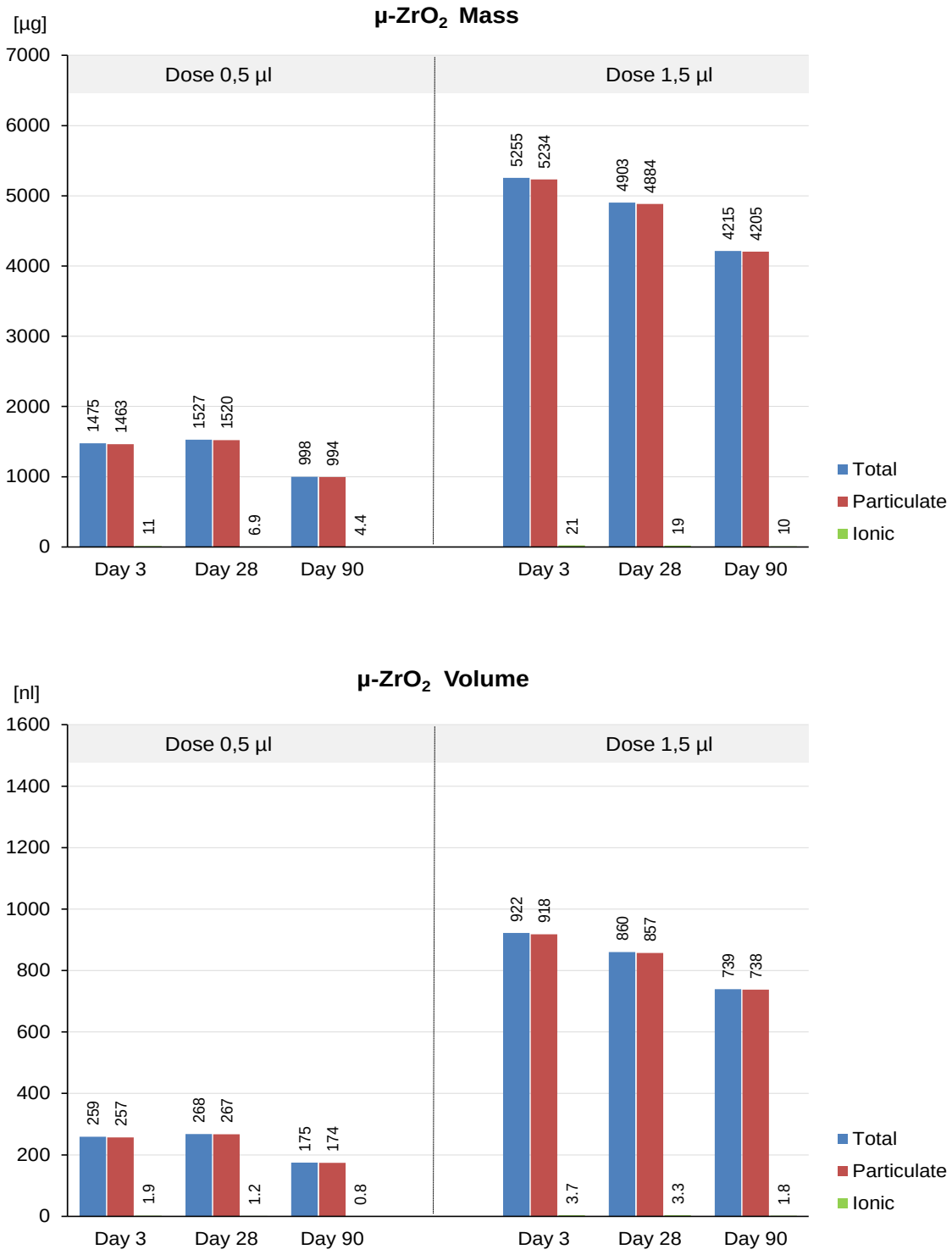
App. 5, Fig. 2a/b Titanium dioxide P25 - Total, particulate and ionic mass in lungs (µg/lung or nl/lung) → 0.5 µl: non-overload - 1.5 µl overload (volumetric)



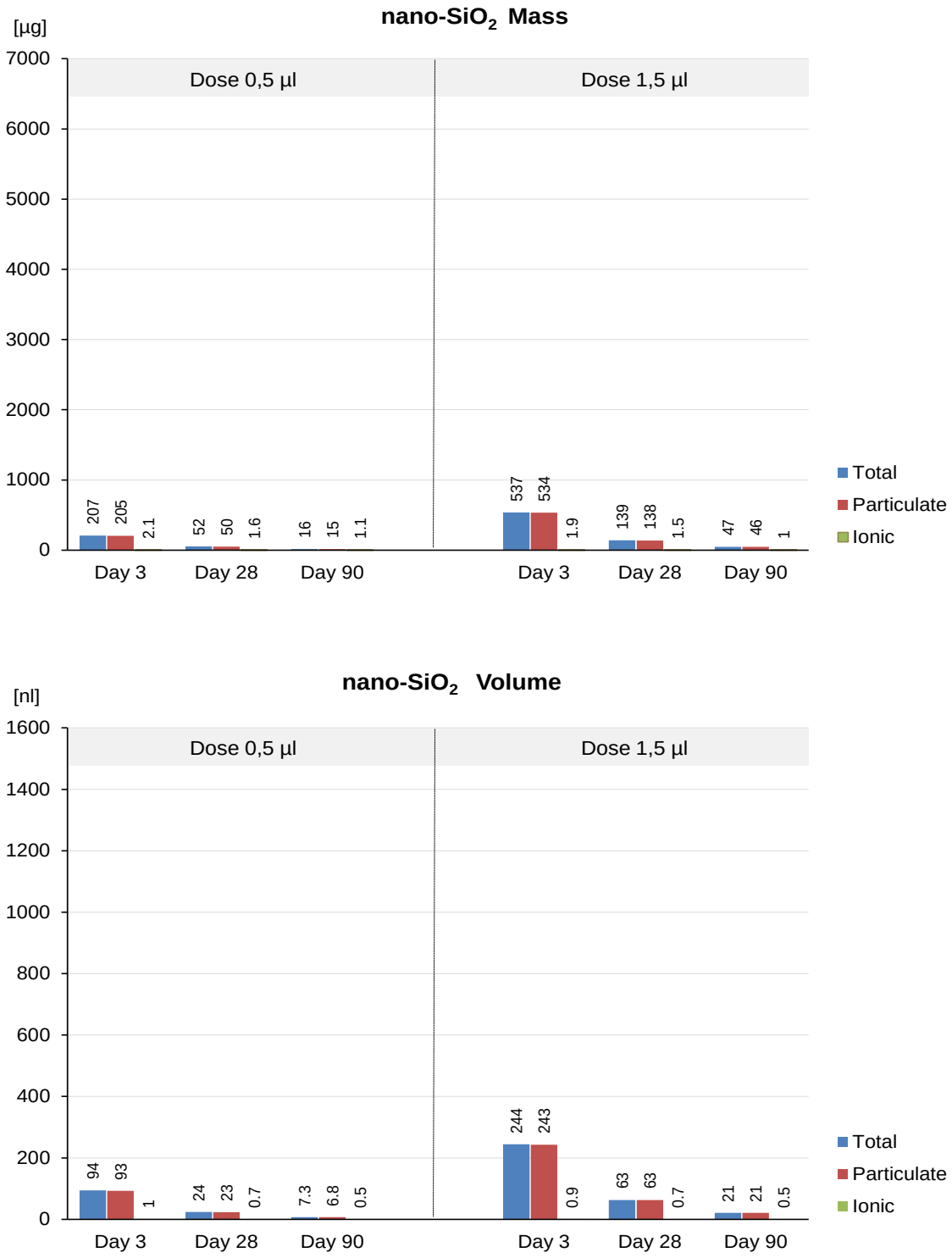
App. 5, Fig. 3a/b Europium oxide -Total, particulate and ionic mass in lungs (µg/lung or nl/lung) → 0.5 µl: non-overload - 1.5 µl overload (volumetric)



App. 5, Fig. 4a/b Barium sulfate - Total, particulate and ionic mass in lungs (µg/lung or nl/lung) → 0.5 µl: non-overload - 1.5 µl overload (volumetric)



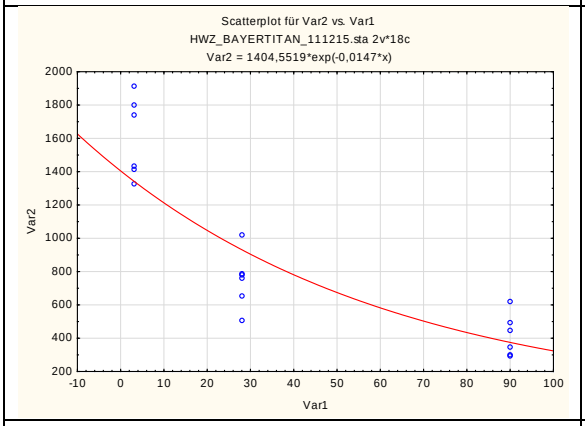
App. 5, Fig. 5a/b Zirconium oxide - Total, particulate and ionic mass in lungs (µg/lung or nl/lung) → 0.5 µl: non-overload - 1.5 µl overload (volumetric)



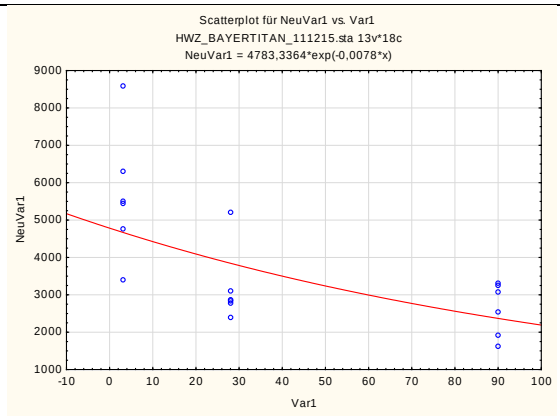
App. 5, Fig. 6a/b Amorphous silica - Total, particulate and ionic mass in lungs (µg/lung or nl/lung) → 0.5 µl: non-overload - 1.5 µl overload (volumetric)

Appendix 6 Calculation of half-times $t_{1/2}$ (first order kinetics)

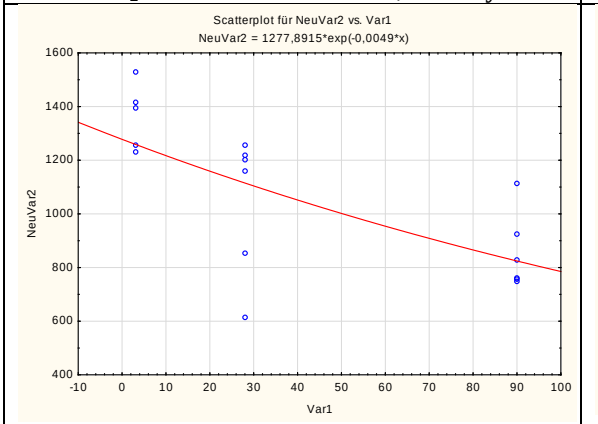
μ-TiO_2 Bayertitan T - low dose – k = 0,0147day^{-1}



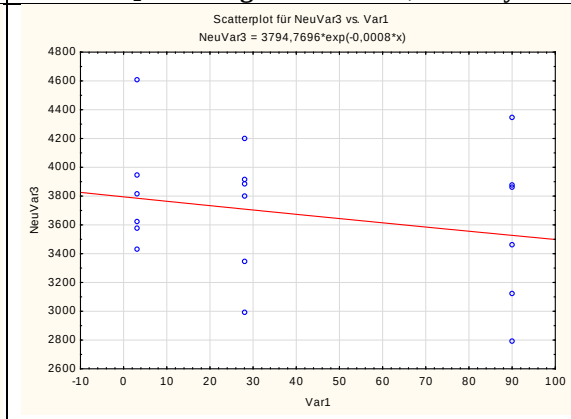
μ-TiO_2 Bayertitan T - high dose – k = 0,0078 day^{-1}



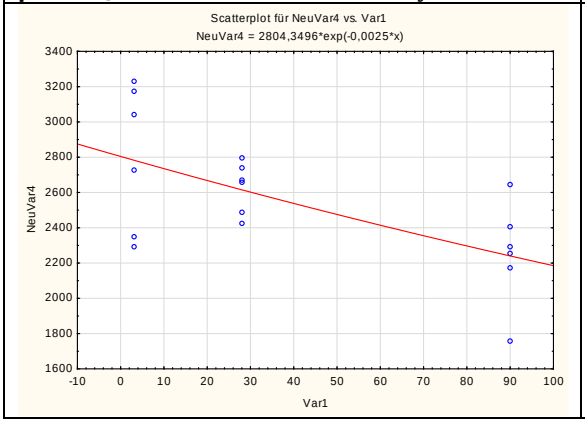
nano-TiO_2 P25 - low dose – k = 0,0049day^{-1}



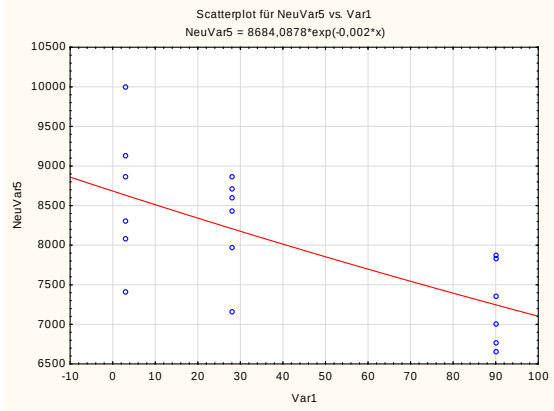
nano-TiO_2 P25 - high dose – k = 0,0008 day^{-1}



μ-Eu_2O_3 - low dose – k = 0,0025 day^{-1}

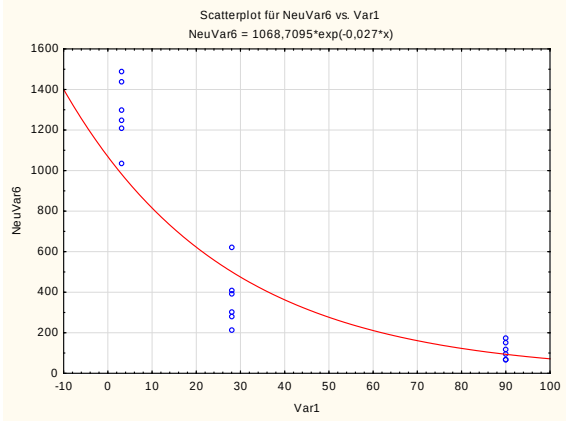


μ-Eu_2O_3 - high dose – k = 0,0020 day^{-1}

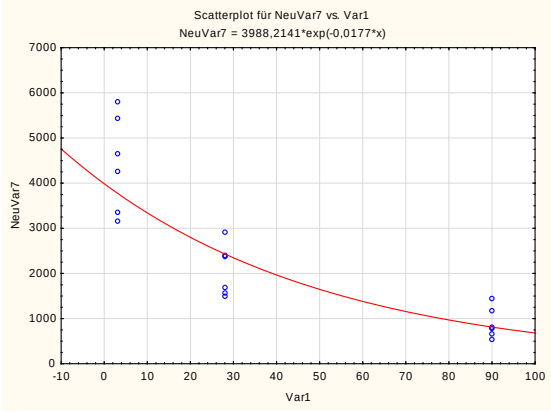


x axis: time (days)
y axis: µg/lung

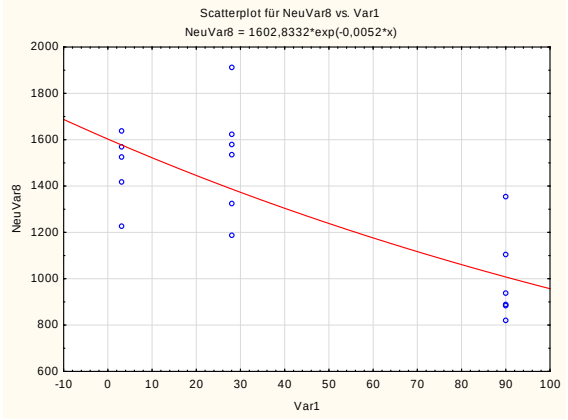
µ-$BaSO_4$ - low dose – k = 0,0270day^{-1}



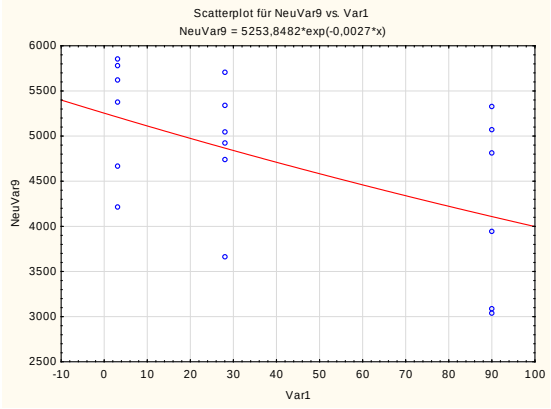
µ-$BaSO_4$ - high dose – k = 0,0177 day^{-1}



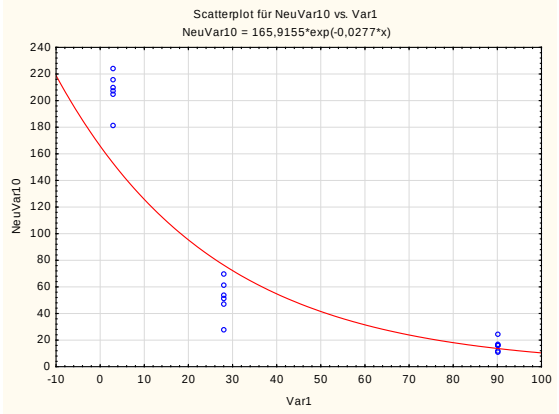
µ-ZrO_2 - low dose – k = 0,0052day^{-1}



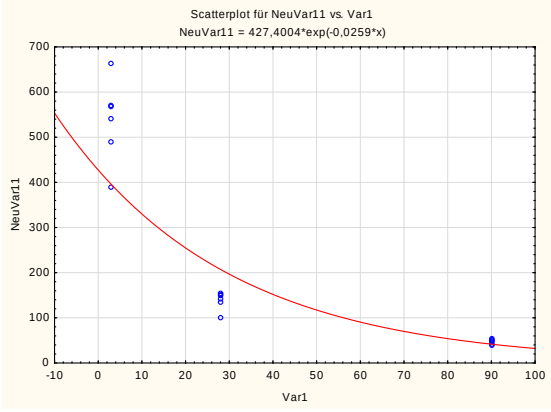
µ-ZrO_2 - high dose – k = 0,0027 day^{-1}



nano-SiO_2 - low dose – k = 0,0277day^{-1}



nano-SiO_2 - high dose – k = 0,0259 day^{-1}



x axis: time (days)
y axis: µg/lung

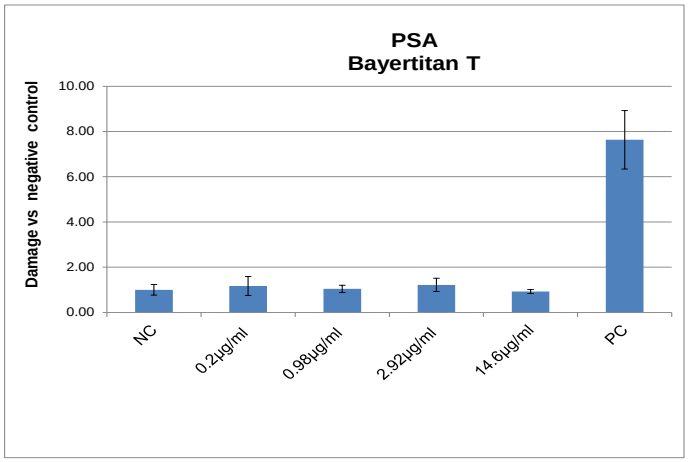
Appendix 7 Yttrium Oxide in µ-Zirconium Oxide (additional analysis)

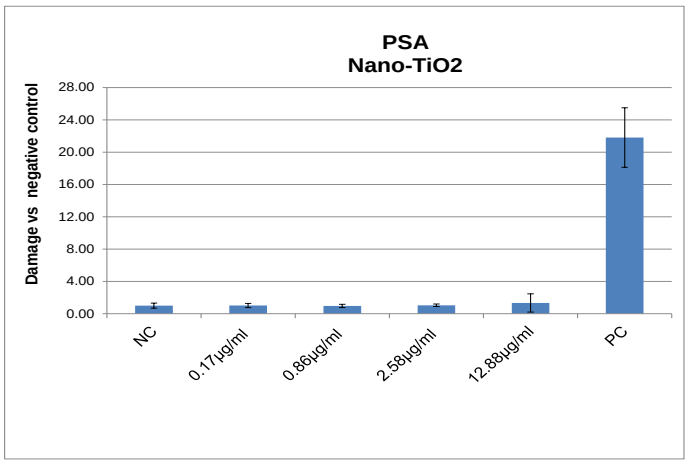
Y_2O_3 in µ-ZrO_2		Concentration of soluble moiety vs. total			Solubility			Solubility
		Mean	ASD	RSD	Mean	ASD	RSD	
Time (hrs)	Lung fluid simulant	(mg/g)		(%)	(%)			(mg/l lung fluid simulant)
0.5	AAF Gamble ALF	<0.003 <0.003 3.7	0.03	0.7	<0.0003 <0.0003 0.37	0.003	0.7	<0.003 <0.003 7.4
1	AAF Gamble ALF	<0.003 <0.003 3.9	0.01	0.2	<0.0003 <0.0003 0.39	0.001	0.2	<0.003 <0.003 7.8
2	AAF Gamble ALF	<0.003 <0.003 4.1	0.04	1.0	<0.0003 <0.0003 0.41	0.004	1.0	<0.003 <0.003 8.2
3	AAF Gamble ALF	<0.003 <0.003 4.2	0.01	0.2	<0.0003 <0.0003 0.42	0.001	0.2	<0.003 <0.003 8.4
4	AAF Gamble ALF	<0.003 <0.003 4.2	0.04	0.9	<0.0003 <0.0003 0.42	0.004	0.9	<0.003 <0.003 8.4
6	AAF Gamble ALF	<0.003 <0.003 4.4	0.05	1.2	<0.0003 <0.0003 0.44	0.005	1.2	<0.003 <0.003 8.8
8	AAF Gamble ALF	<0.003 <0.003 4.4	0.04	0.9	<0.0003 <0.0003 0.44	0.004	0.9	<0.003 <0.003 8.8
24	AAF Gamble ALF	<0.003 <0.003 5.1	0.20	3.9	<0.0003 <0.0003 0.51	0.020	3.9	<0.003 <0.003 10.2
48	AAF Gamble ALF	<0.003 <0.003 5.3	0.03	0.5	<0.0003 <0.0003 0.53	0.003	0.5	<0.003 <0.003 10.6
72	AAF Gamble ALF	<0.003 <0.003 5.2	0.01	0.3	<0.0003 <0.0003 0.52	0.001	0.3	<0.003 <0.003 10.4
96	AAF Gamble ALF	<0.003 <0.003 5.1	0.09	1.7	<0.0003 <0.0003 0.51	0.009	1.7	<0.003 <0.003 10.2

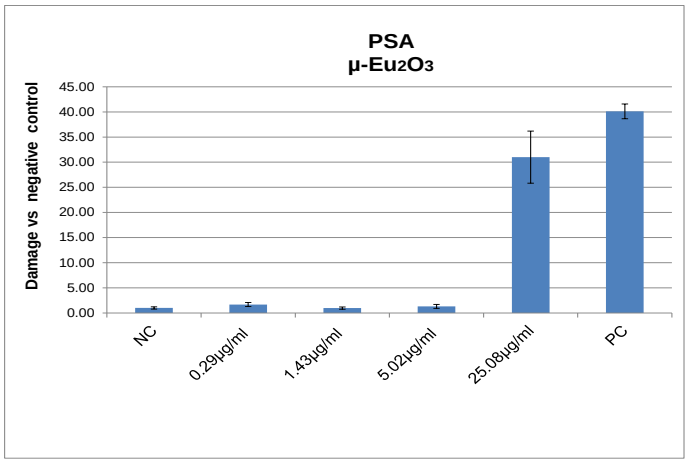
ASD: Absolute standard deviation - RSD: Relative standard deviation
N=3 (for each lung fluid simulant)

In ALF, the solubility of yttria is 10-fold higher than the value measured for zirconia.

Appendix 8 Results of acellular/cellular in vitro assays (figures)

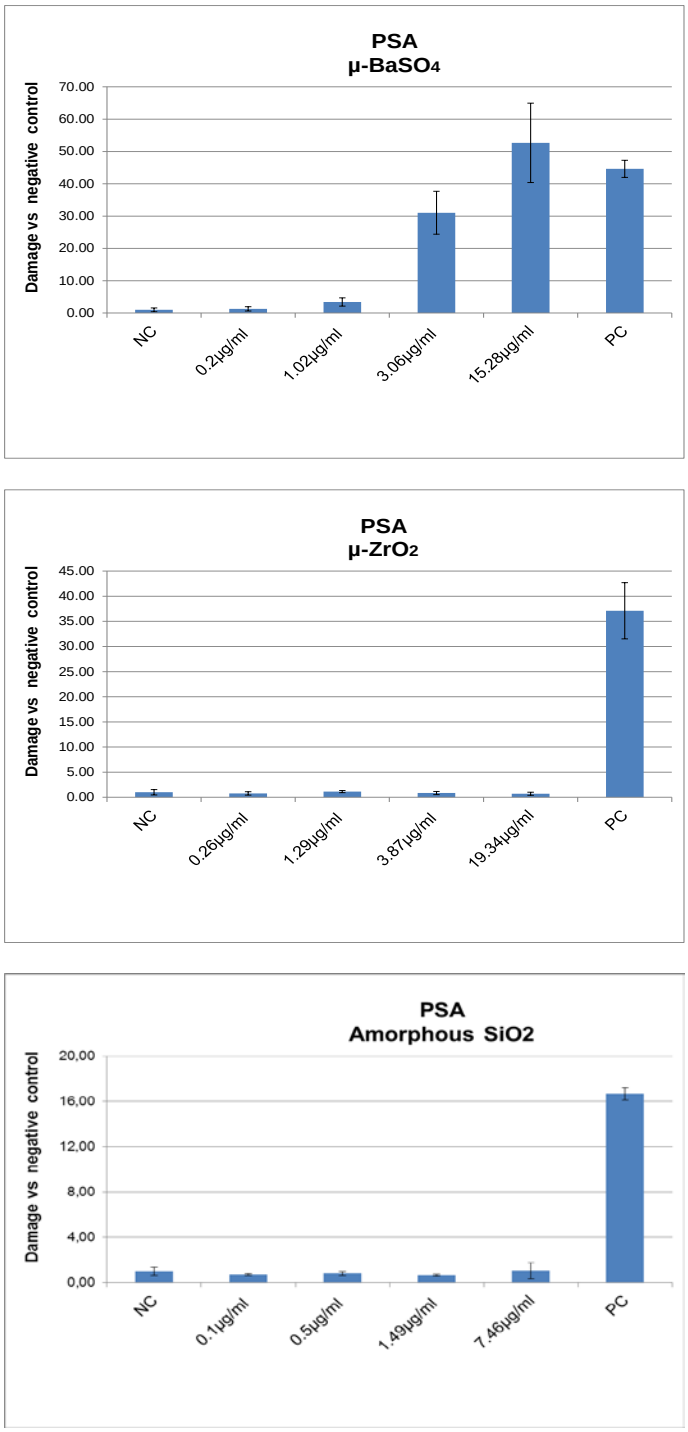






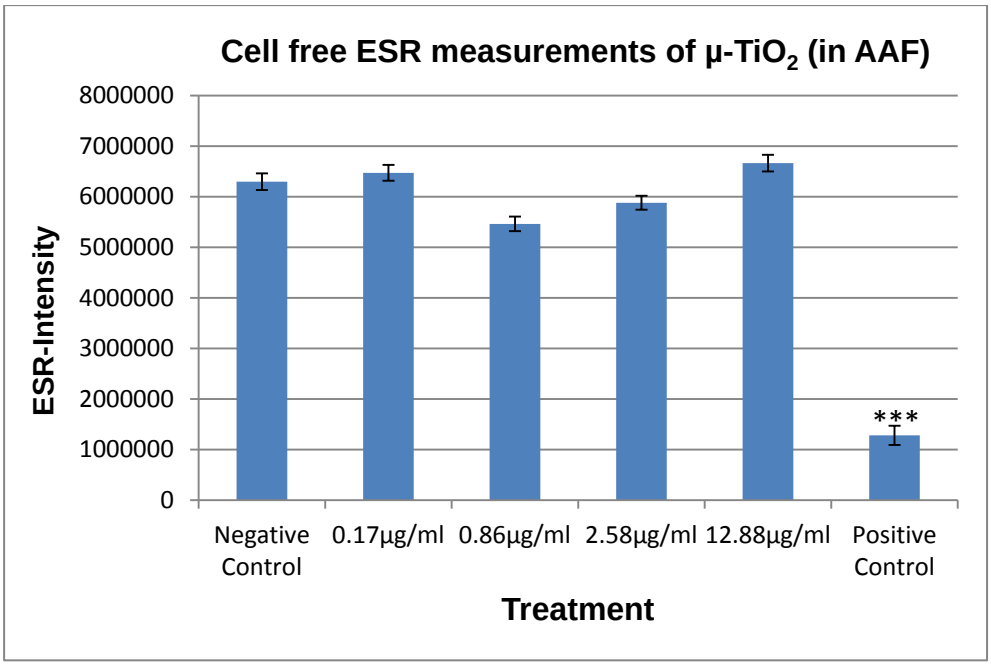
PC: positive control, NC: negative control

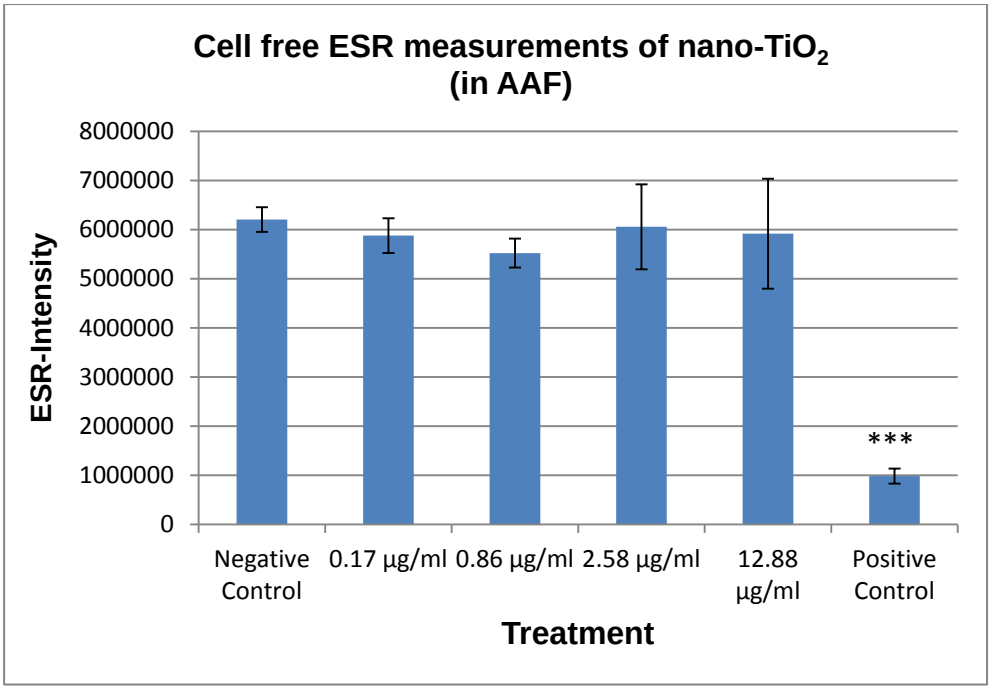
App. 8, Fig. 1-3 Results of the PSA.



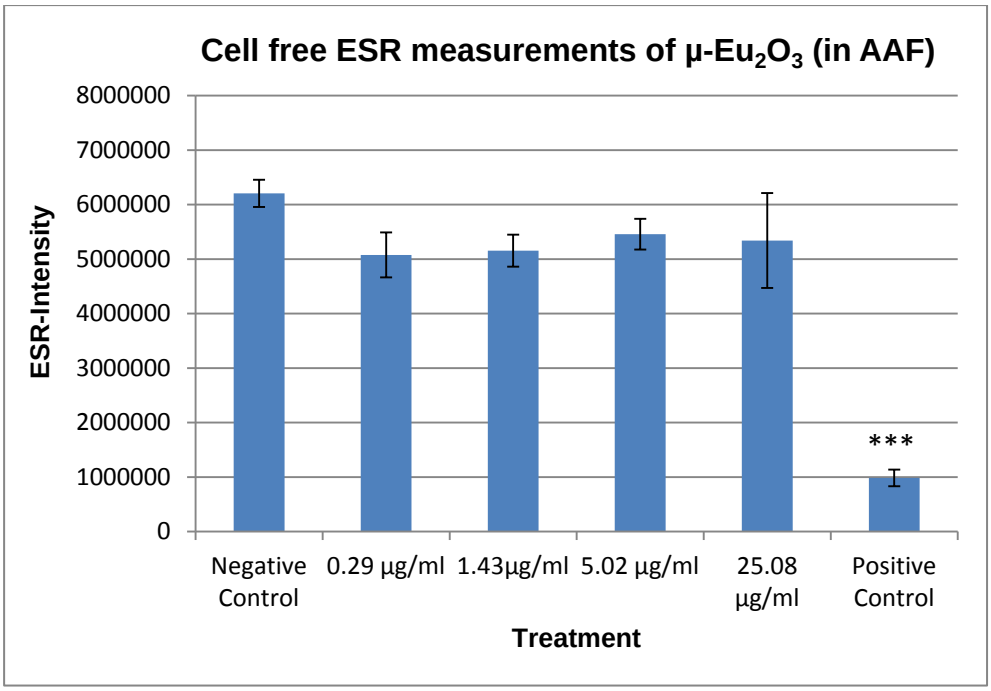
PC: positive control, NC: negative control

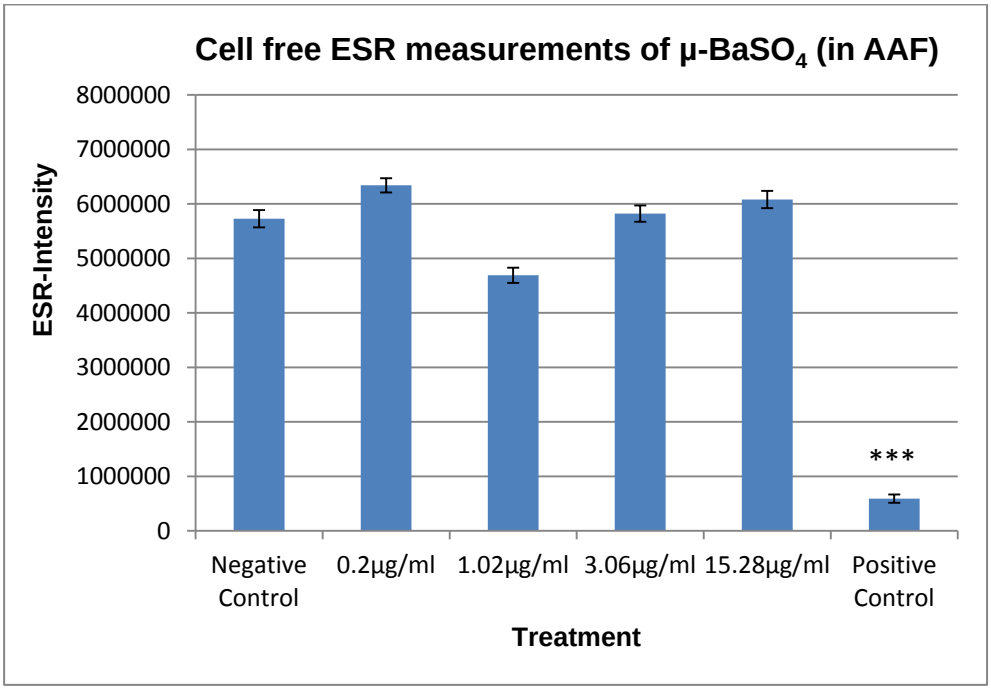
App. 8, Fig. 4-6 Results of the PSA – cont'd



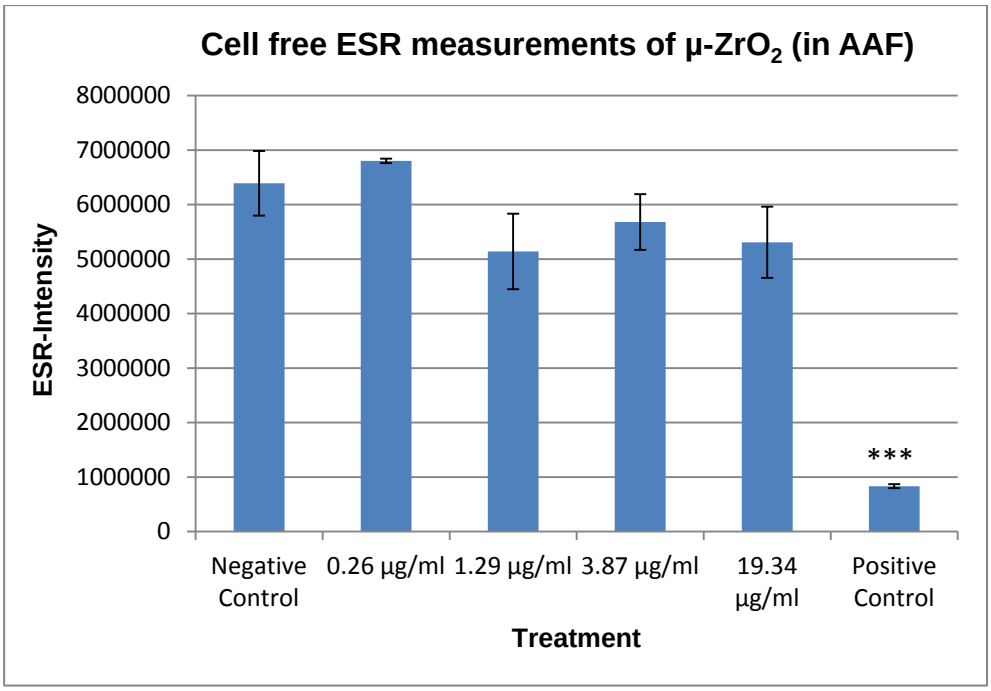


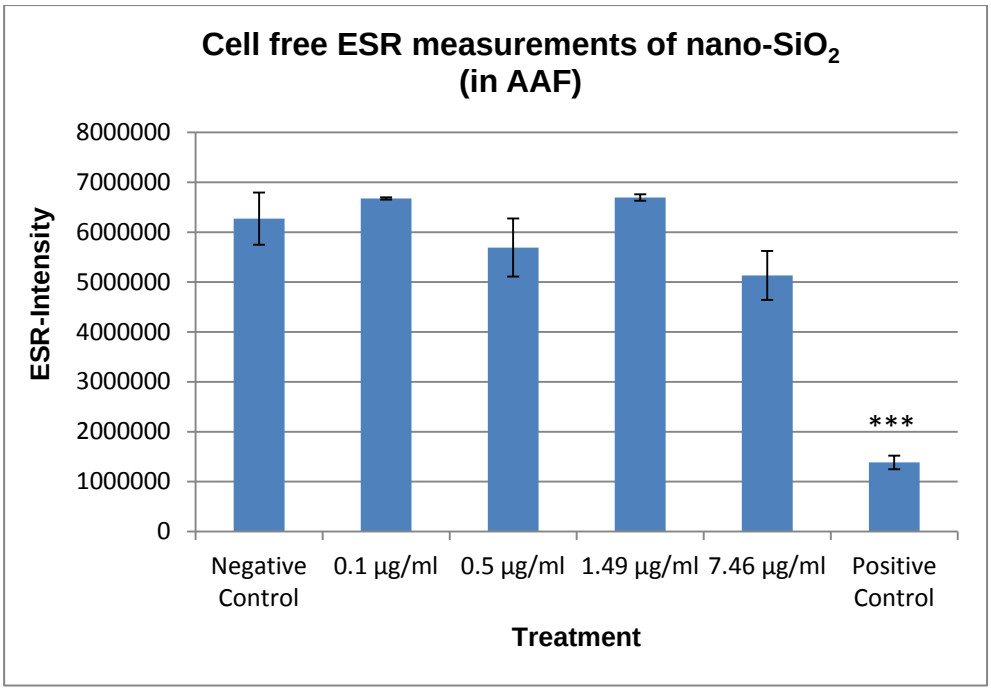
App. 8, Fig. 7-8 Results of the cell-free ESR assay.



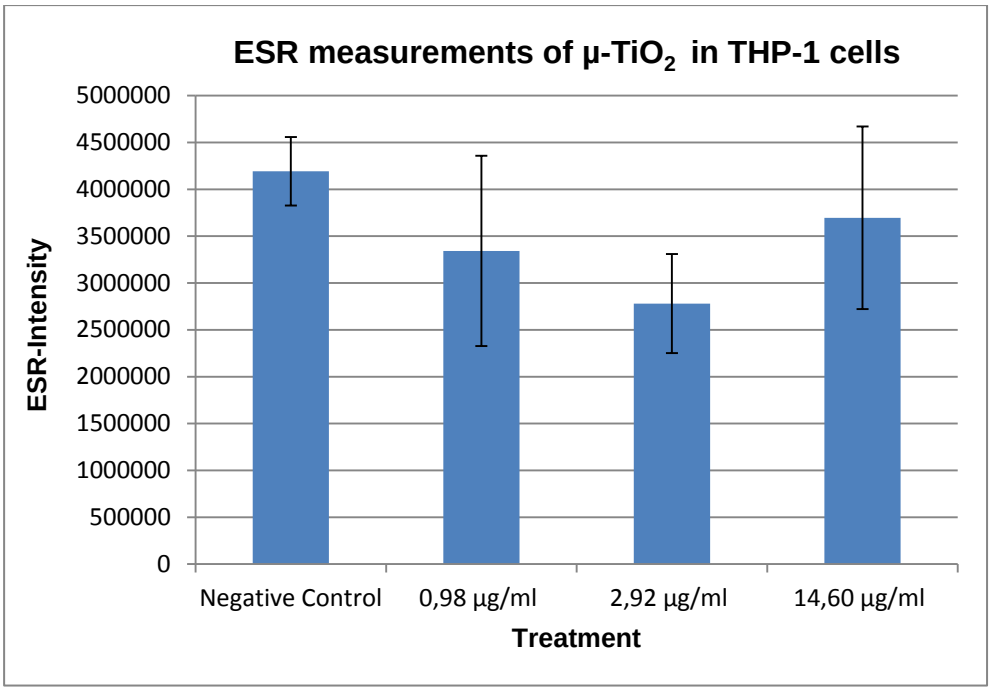


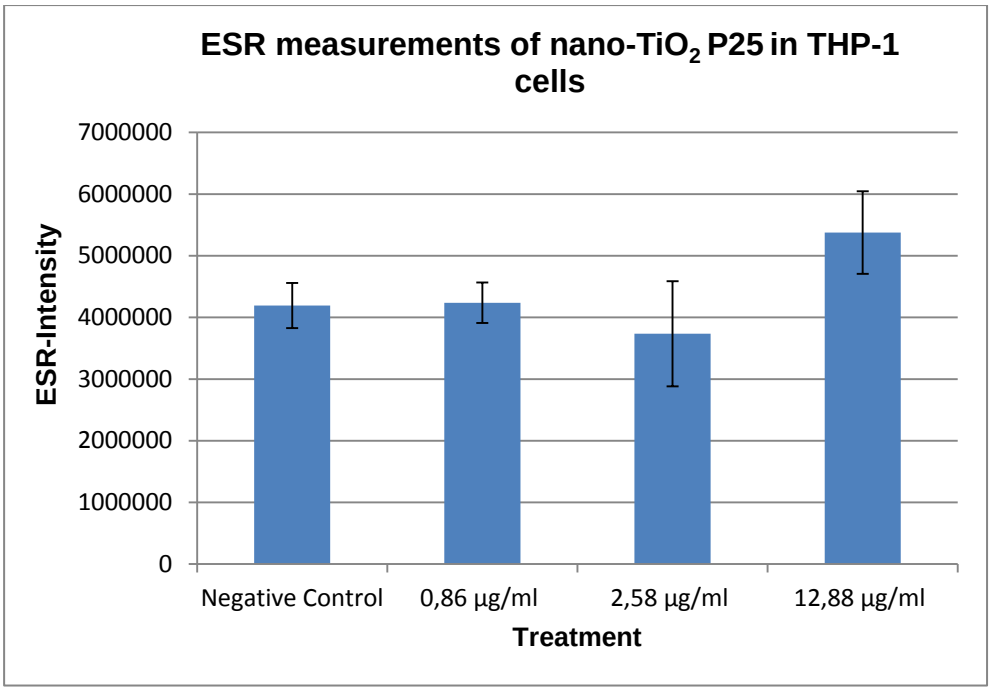
App. 8, Fig. 9-10 Results of the cell-free ESR assay.



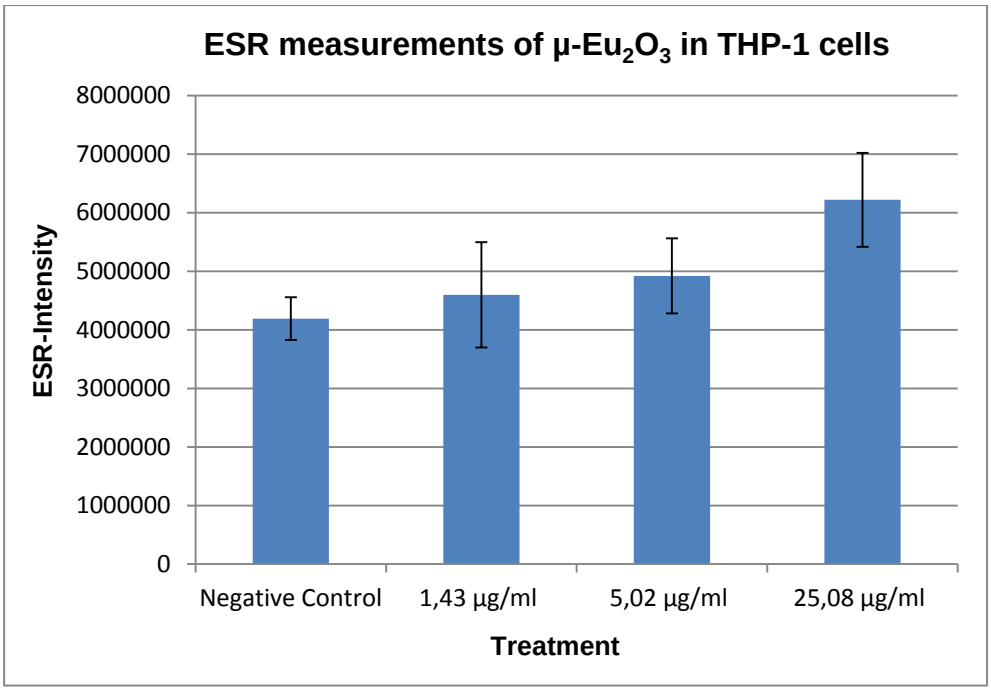


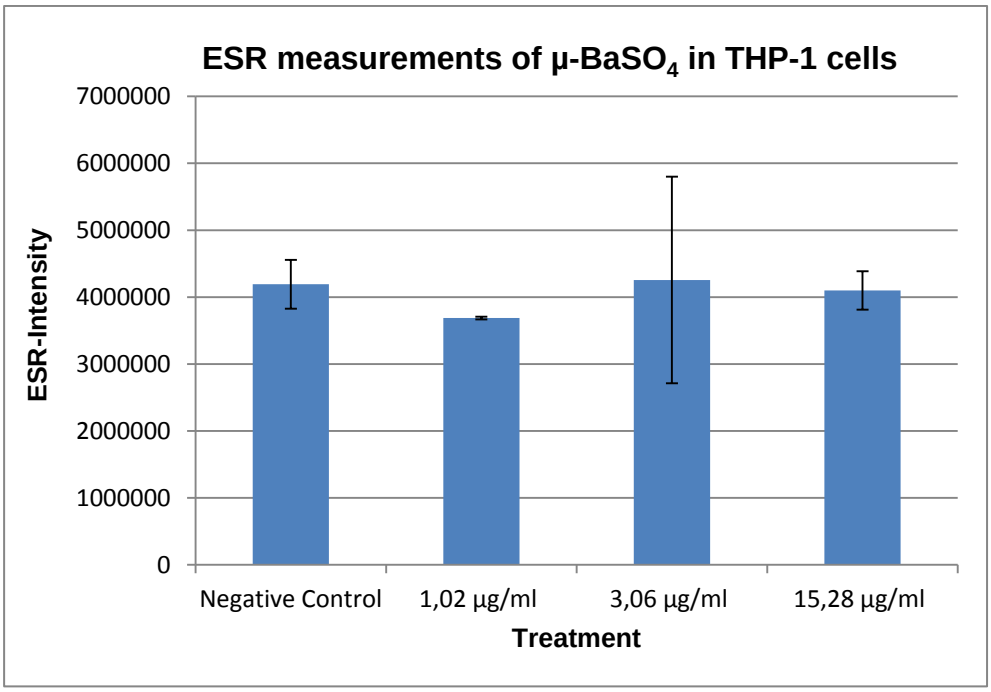
App. 8, Fig. 11-12 Results of the cell-free ESR assay – cont'd



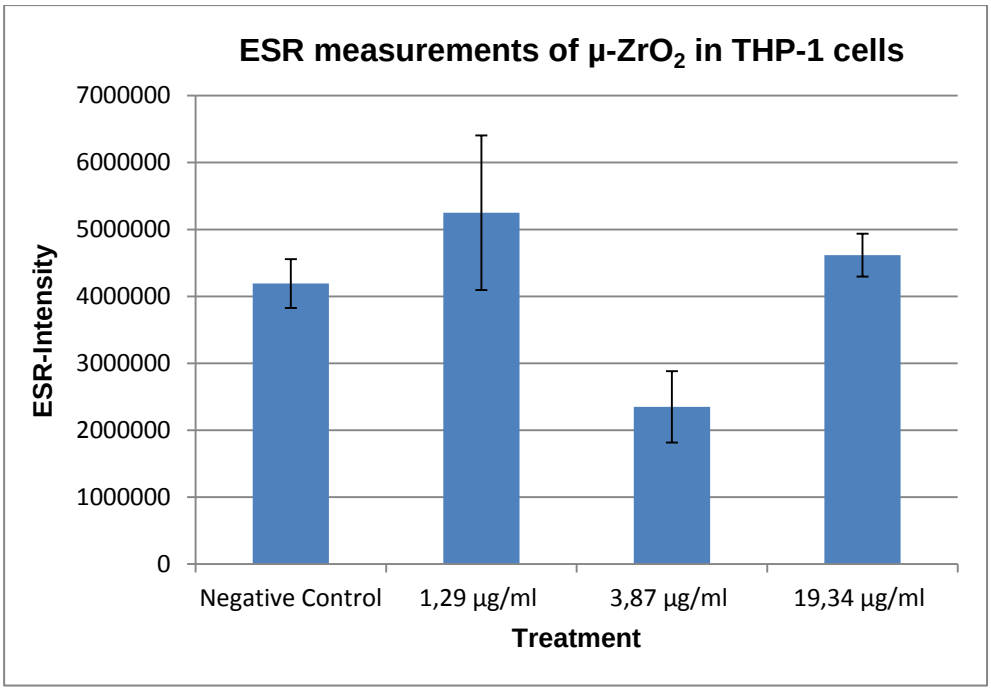


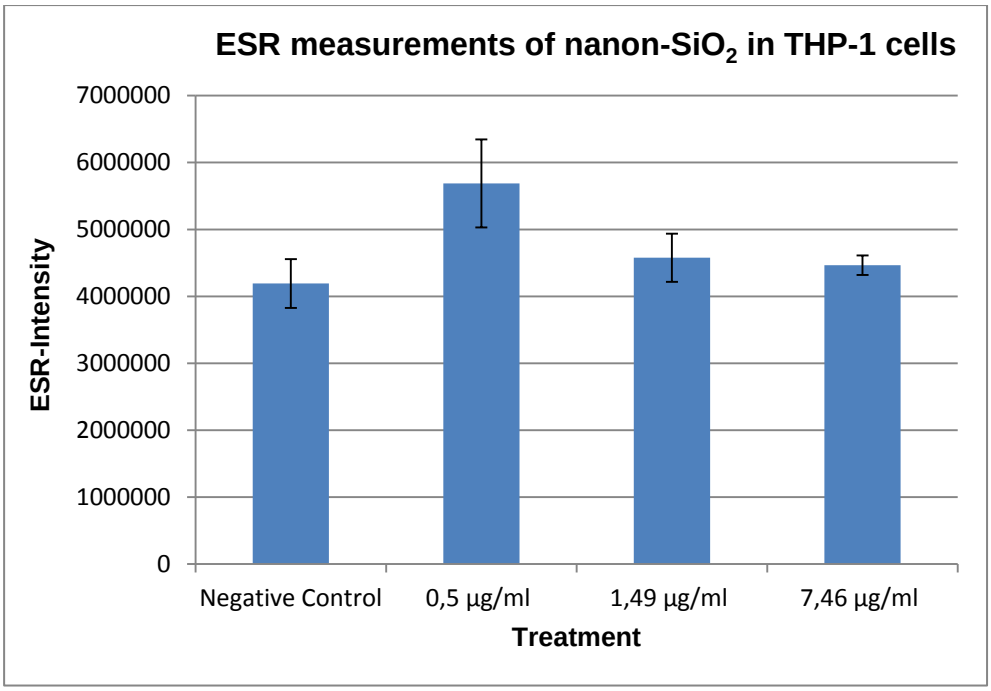
App. 8, Fig. 13-14 Results of the cellular ESR assay with THP-1 monocytes.



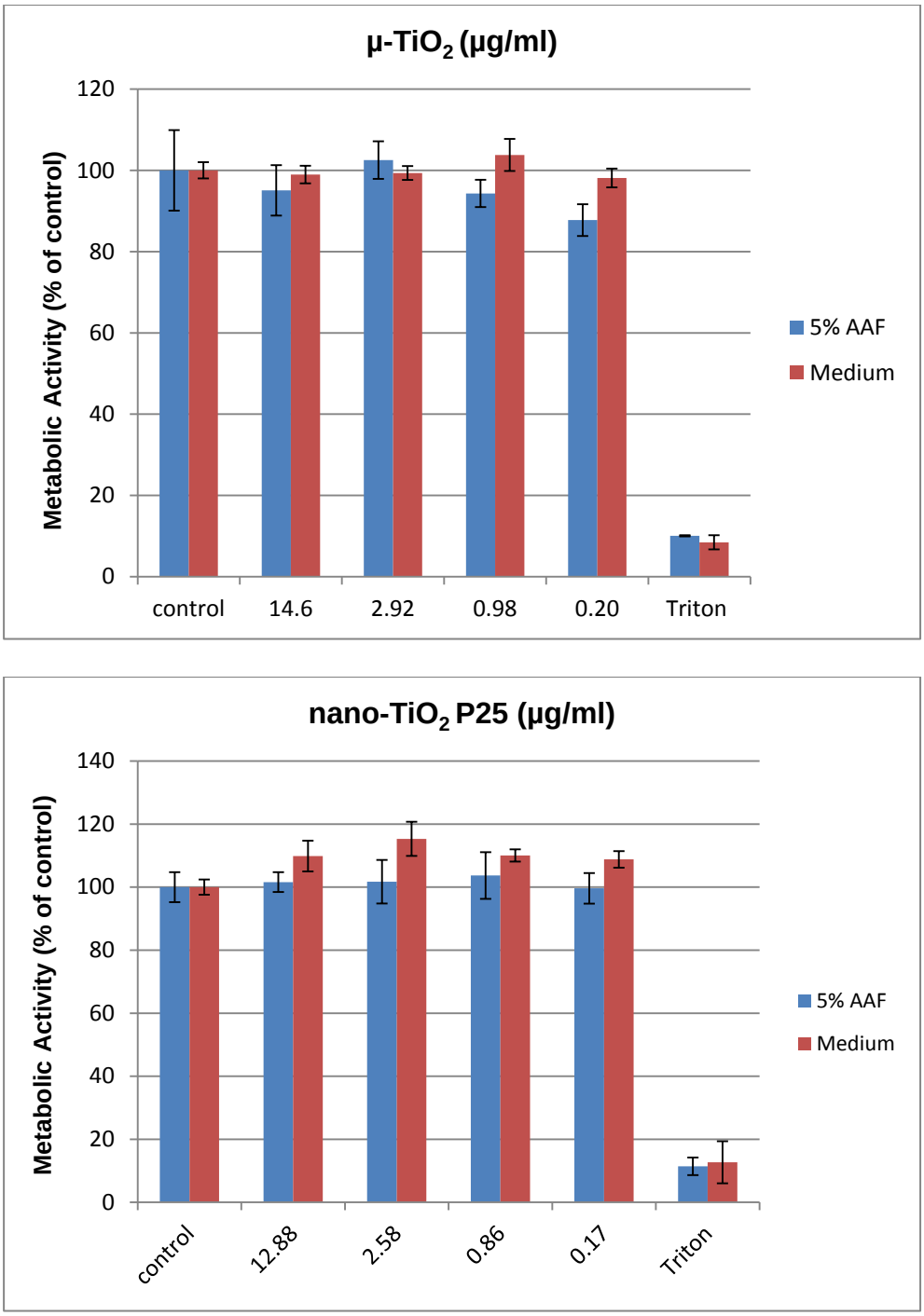


App. 8, Fig. 15-16 Results of the cellular ESR assay with THP-1 monocytes – cont'd

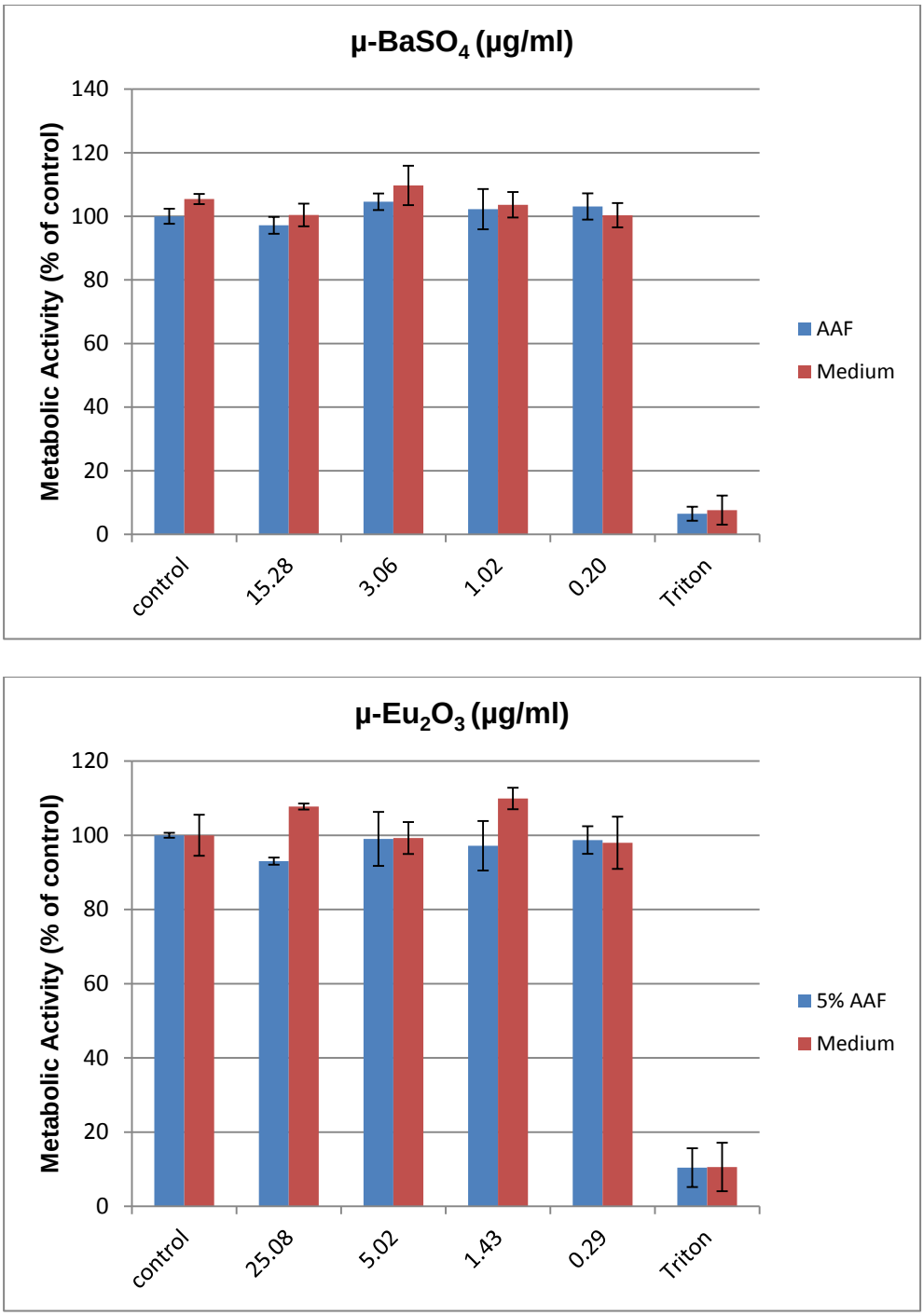




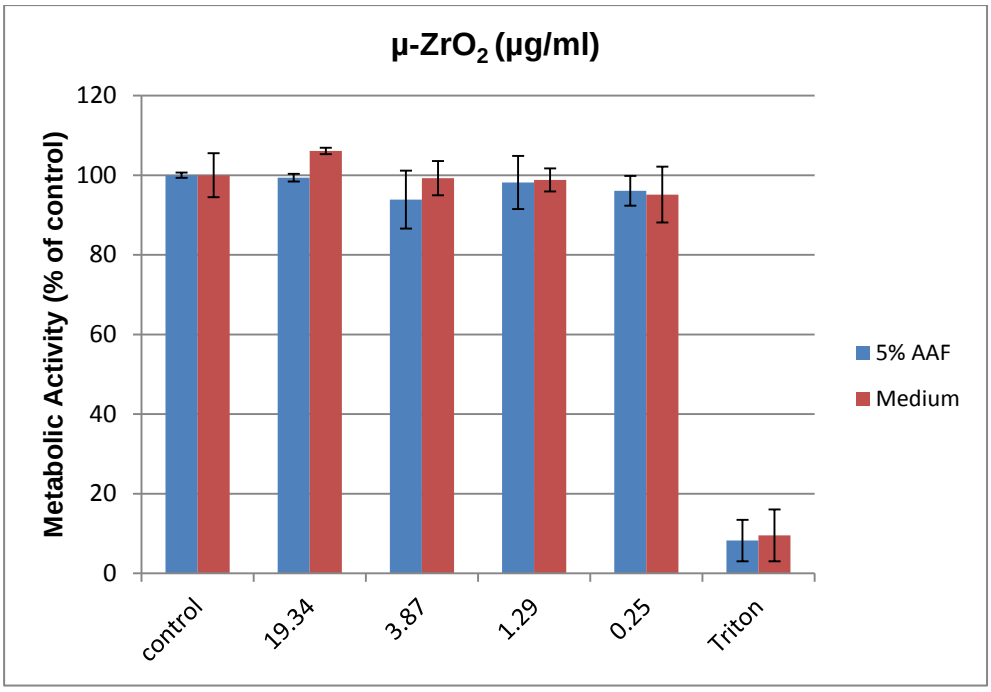
App. 8, Fig. 17-18 Results of the cellular ESR assay with THP-1 monocytes – cont'd

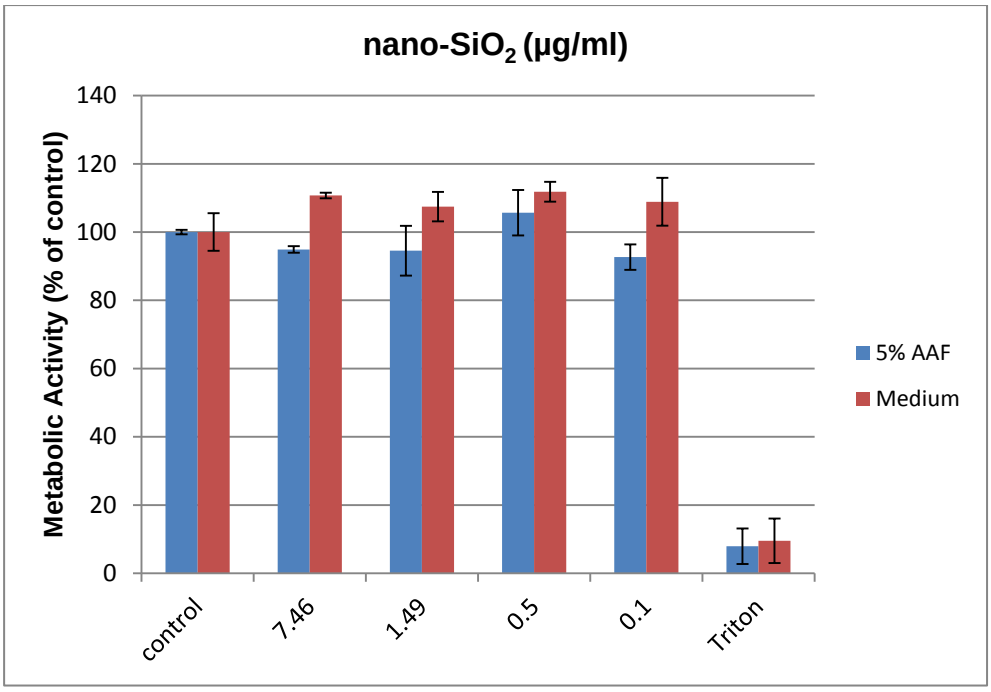


App. 8, Fig. 19-20 Cytotoxicity of dusts towards THP-1 monocytes. Metabolic activity was analyzed using the WST-assay.

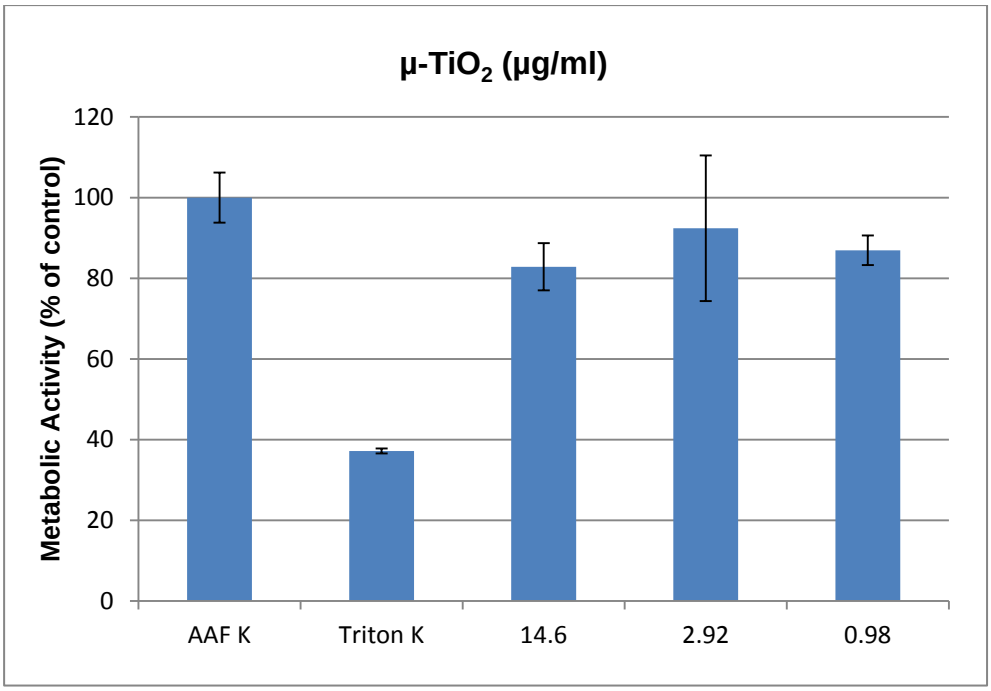


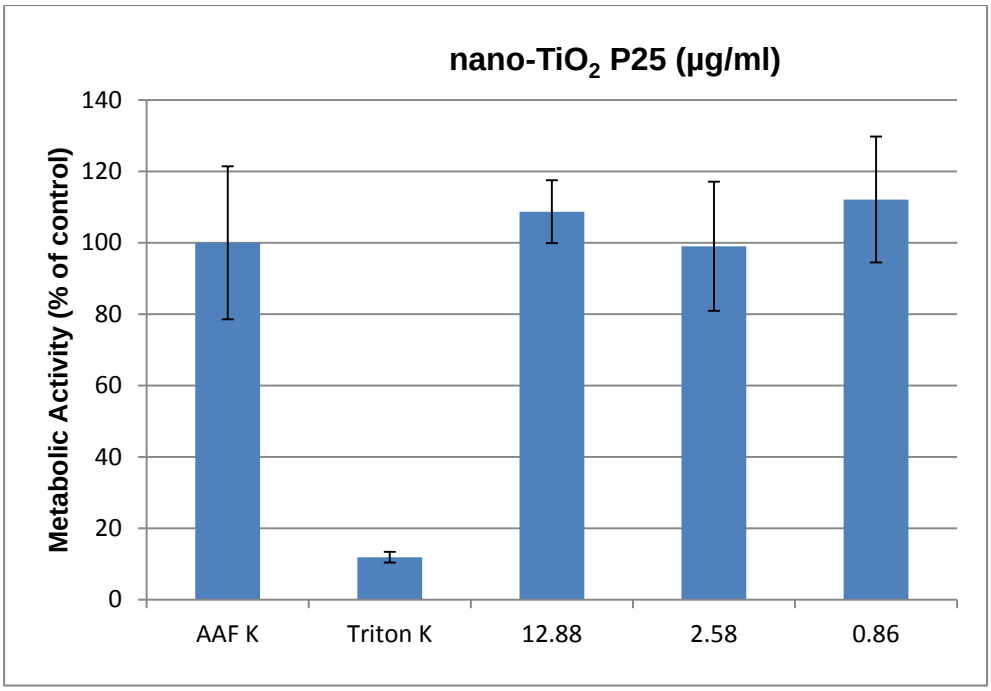
App. 8, Fig. 21-22 Cytotoxicity of dusts towards THP-1 monocytes.
Metabolic activity was analyzed using the WST-assay – cont'd.



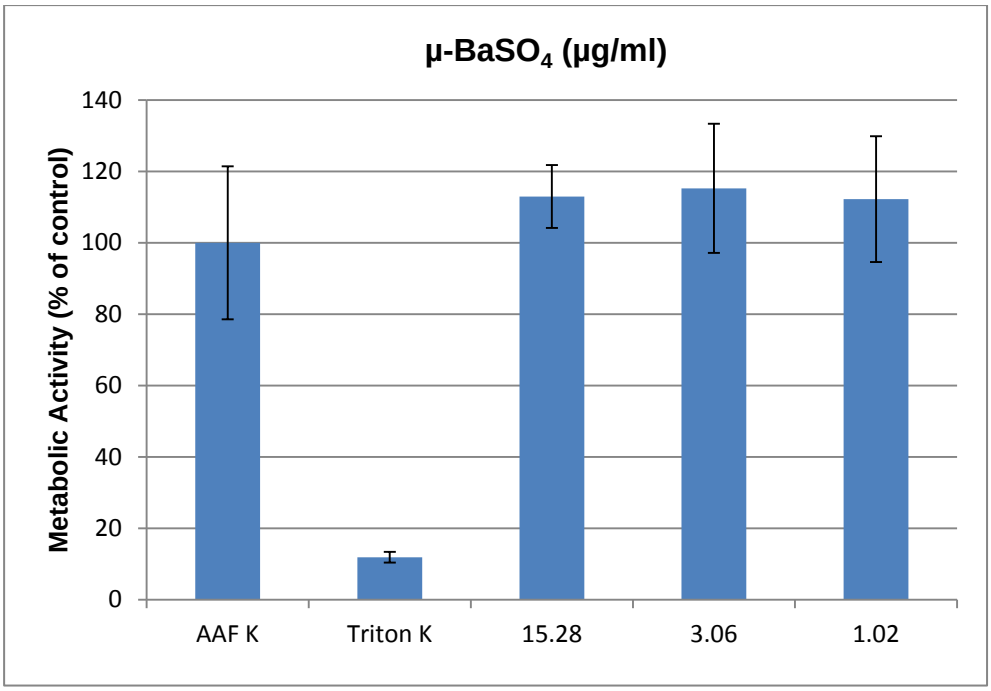


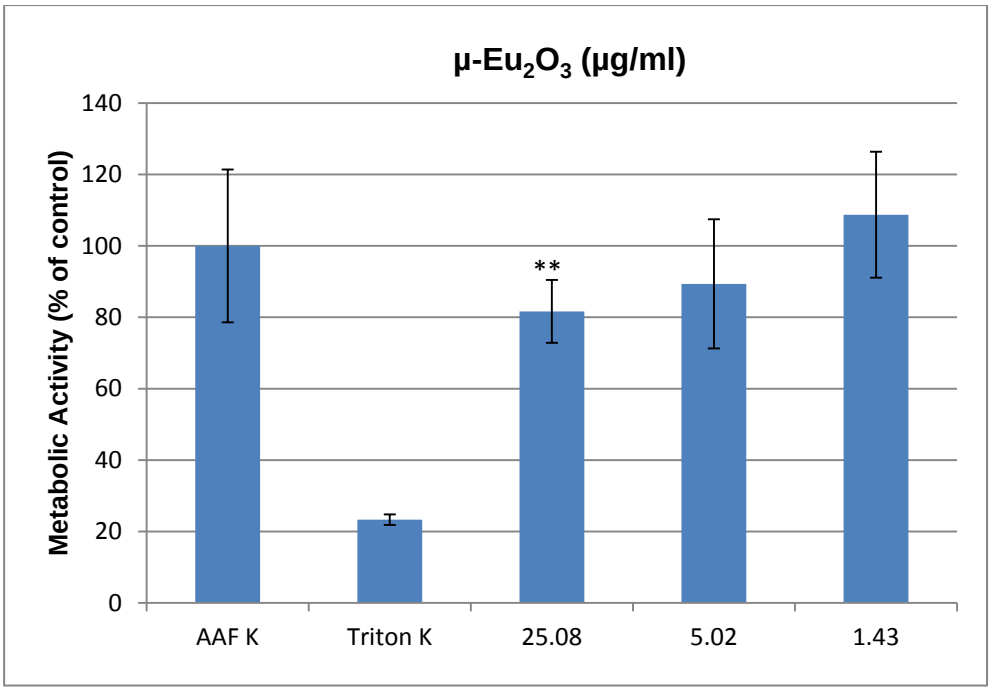
App. 8, Fig. 23-24 Cytotoxicity of dusts towards THP-1 monocytes.
Metabolic activity was analyzed using the WST-assay – cont'd.



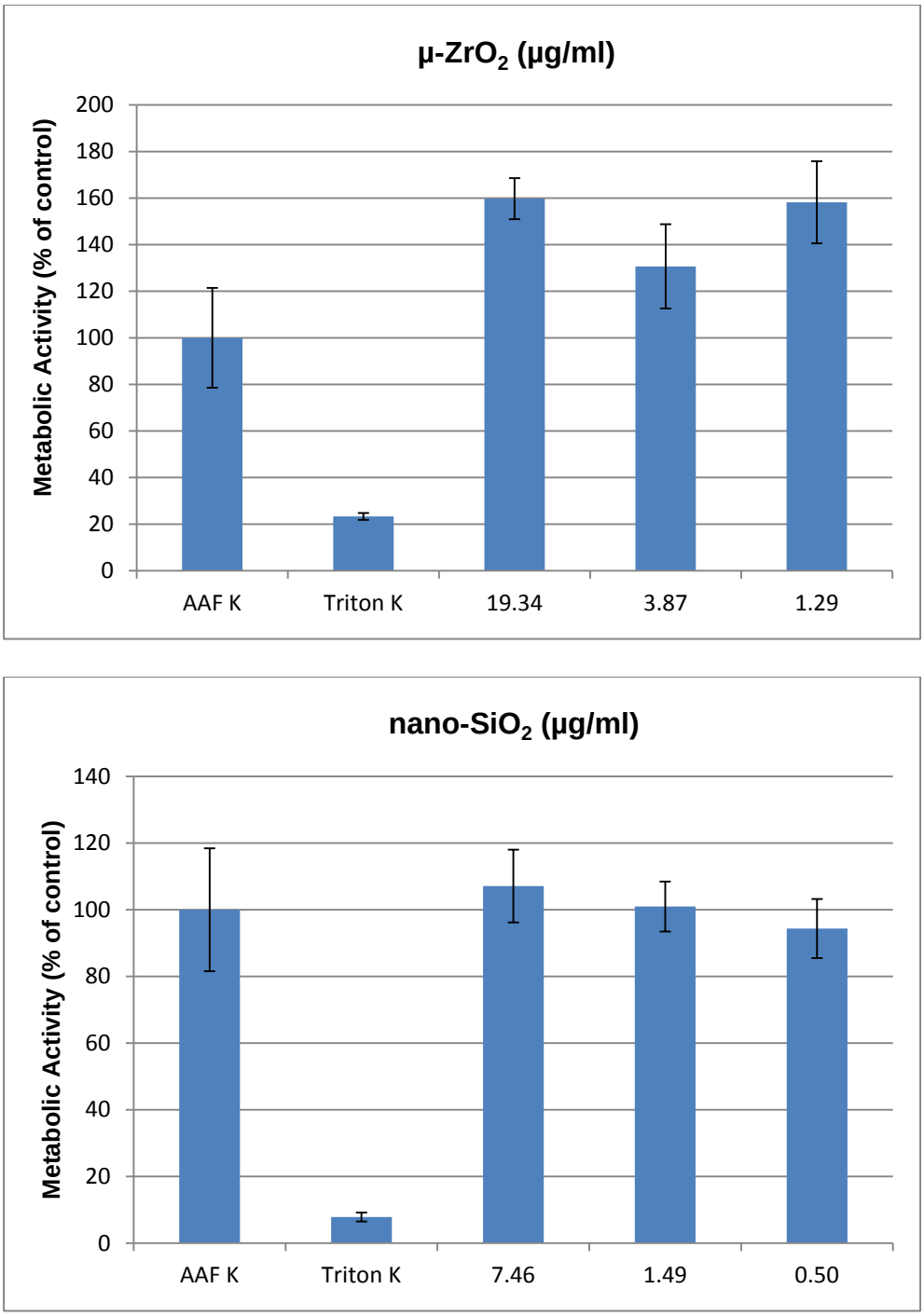


App. 8, Fig. 25-26 Cytotoxicity of dusts towards THP-1 macrophages. Metabolic activity was analyzed using the WST-assay.

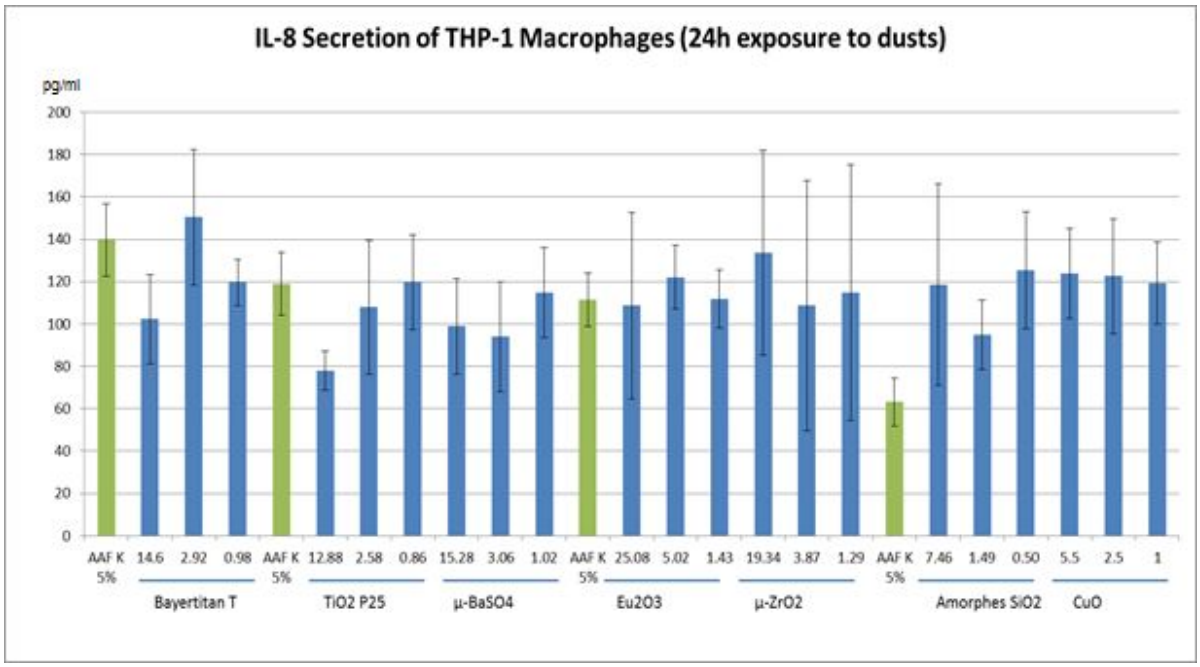




App. 8, Fig. 27-28 Cytotoxicity of dusts towards THP-1 macrophages. Metabolic activity was analyzed using the WST-assay – cont'd.



App. 8, Fig. 29-30 Cytotoxicity of dusts towards THP-1 macrophages. Metabolic activity was analyzed using the WST-assay – cont'd.



App. 8, Fig. 31 IL-8 secretion of THP-1 macrophages following 24 h exposure to test dusts. IL-8 was quantified by ELISA.

Appendix 9 Body Weights – means

BALF analysis		Day relative to start date					
Group		-1	3	7	14	21	28
Control	Mean	266.70	278.41	294.58	310.85	324.78	334.15
	SD	16.64	17.34	22.66	25.37	28.53	33.43
	N	12	12	6	6	6	6
µ-TiO_2 low	Mean	270.35	281.79	287.50	302.42	316.22	329.78
	SD	14.12	16.18	12.60	13.07	15.82	15.09
	N	12	12	6	6	6	6
µ-TiO_2 high	Mean	269.49	281.20	295.38	314.42	329.88	344.70
	SD	13.96	14.78	12.16	15.03	15.91	16.15
	N	12	12	6	6	6	6
nano-TiO_2 low	Mean	275.44	286.58	293.67	315.78	332.43	347.67
	SD	13.21	14.49	13.56	12.86	14.48	18.56
	N	12	12	6	6	6	6
nano-TiO_2 high	Mean	272.32	279.13	293.72	314.65	329.52	344.68
	SD	11.40	11.43	17.01	21.14	23.59	24.18
	N	12	12	6	6	6	6
µ-Eu_2O_3 low	Mean	273.05	281.45	295.33	318.12	335.52	348.72
	SD	13.02	12.01	14.45	19.31	21.67	23.27
	N	12	12	6	6	6	6
µ-Eu_2O_3 high	Mean	269.36	273.55	290.40	309.28	324.17	340.65
	SD	9.32	11.49	14.54	18.85	17.22	20.05
	N	12	12	6	6	6	6
µ-$BaSO_4$ low	Mean	268.23	279.78	285.62	300.82	313.32	326.32
	SD	15.35	17.45	8.34	10.57	14.27	17.77
	N	12	12	6	6	6	6
µ-$BaSO_4$ high	Mean	269.86	281.28	295.97	316.78	333.87	347.37
	SD	16.74	19.00	23.01	26.89	30.82	30.86
	N	12	12	6	6	6	6
µ-ZrO_2 low	Mean	270.13	283.09	292.70	311.10	328.07	340.48
	SD	16.07	18.17	20.99	18.49	17.75	19.76
	N	12	12	6	6	6	6
µ-ZrO_2 high	Mean	272.74	283.99	297.87	315.05	330.85	342.50
	SD	20.15	21.79	15.31	17.80	20.50	20.86
	N	12	12	6	6	6	6
nano-SiO_2 low	Mean	270.33	281.09	295.93	315.20	332.22	347.03
	SD	13.45	14.48	6.82	8.48	8.57	10.16
	N	12	12	6	6	6	6
nano-SiO_2 high	Mean	268.91	276.21	285.25	303.32	317.57	331.72
	SD	17.60	18.47	29.37	36.07	39.42	39.96
	N	12	12	6	6	6	6

Chemical analysis		Day relative to start date					
Group		-1	3	7	14	21	28
Control	Mean	282.04	294.80	310.58	327.23	342.85	355.21
	SD	23.85	22.77	24.33	24.47	23.47	22.45
	N	18	18	12	12	12	12
µ-TiO_2 low	Mean	282.93	295.39	312.62	333.74	348.64	362.49
	SD	22.99	21.83	19.76	17.45	16.94	16.67
	N	18	18	12	12	12	12
µ-TiO_2 high	Mean	280.01	292.92	305.62	323.48	336.28	351.08
	SD	24.04	23.46	27.36	27.92	29.44	31.85
	N	18	18	12	12	12	12
nano-TiO_2 low	Mean	278.70	288.09	303.83	320.09	335.18	348.63
	SD	21.34	20.58	20.77	23.08	24.71	25.73
	N	18	18	12	12	12	12
nano-TiO_2 high	Mean	281.57	289.69	308.51	328.61	344.80	359.07
	SD	21.82	21.25	22.09	23.58	25.18	29.65
	N	18	18	12	12	12	12
µ-Eu_2O_3 low	Mean	281.17	289.71	301.41	318.18	332.69	345.12
	SD	17.50	17.30	20.20	18.38	19.79	21.70
	N	18	18	12	12	12	12
µ-Eu_2O_3 high	Mean	279.07	281.73	300.33	318.86	334.81	348.26
	SD	22.74	22.67	23.08	24.32	25.25	26.79
	N	18	18	12	12	12	12
µ-$BaSO_4$ low	Mean	280.15	292.51	303.45	320.53	334.04	345.90
	SD	17.41	17.73	21.21	24.19	26.55	28.81
	N	18	18	12	12	12	12
µ-$BaSO_4$ high	Mean	284.52	297.37	317.09	335.63	351.34	365.41
	SD	32.12	30.29	34.13	35.61	37.30	38.31
	N	18	18	12	12	12	12
µ-ZrO_2 low	Mean	282.06	294.14	316.02	334.03	348.96	363.01
	SD	31.31	29.29	28.26	29.68	30.78	32.50
	N	18	18	12	12	12	12
µ-ZrO_2 high	Mean	281.26	290.49	308.46	324.92	339.52	354.97
	SD	24.53	24.24	27.24	26.93	28.57	29.25
	N	18	18	12	12	12	12
nano-SiO_2 low	Mean	281.52	289.58	310.71	328.08	342.13	357.33
	SD	25.10	23.68	23.94	26.44	28.11	29.33
	N	18	18	12	12	12	12
nano-SiO_2 high	Mean	280.81	286.40	302.29	320.17	336.34	349.62
	SD	23.65	22.97	28.34	30.01	30.06	29.51
	N	18	18	12	12	12	12

Chemical analysis		Day relative to start date								
Group		**35**	**42**	**49**	**56**	**63**	**70**	**77**	**84**	**90**
Control	Mean	381.18	389.22	399.47	406.27	410.65	417.95	420.60	425.97	429.08
	SD	23.69	25.23	26.47	24.50	27.07	26.18	28.22	27.19	26.85
	N	6	6	6	6	6	6	6	6	6
μ-TiO_2 low	Mean	381.15	390.38	399.82	408.67	413.65	421.45	426.18	431.63	435.75
	SD	12.26	11.23	11.78	10.12	11.72	13.17	12.81	13.45	15.85
	N	6	6	6	6	6	6	6	6	6
μ-TiO_2 high	Mean	368.23	376.87	387.48	392.43	398.68	400.92	411.45	414.67	419.00
	SD	33.30	36.70	38.18	39.01	39.30	41.71	42.48	43.12	43.66
	N	6	6	6	6	6	6	6	6	6
nano-TiO_2 low	Mean	366.90	372.08	382.73	394.43	401.50	407.88	413.07	417.93	424.18
	SD	11.67	11.72	11.38	11.65	12.63	13.13	15.34	13.82	14.74
	N	6	6	6	6	6	6	6	6	6
nano-TiO_2 high	Mean	370.22	378.92	388.25	395.52	401.73	406.17	410.50	415.25	419.88
	SD	25.09	23.91	23.15	22.13	25.28	24.05	25.96	24.55	24.71
	N	6	6	6	6	6	6	6	6	6
μ-Eu_2O_3 low	Mean	358.00	365.60	375.17	382.38	387.75	393.25	399.98	402.12	403.78
	SD	22.87	21.83	22.67	24.79	24.61	24.37	25.96	23.29	23.93
	N	6	6	6	6	6	6	6	6	6
μ-Eu_2O_3 high	Mean	363.00	371.60	379.85	390.15	395.98	401.68	407.42	414.45	420.85
	SD	30.77	31.02	34.38	36.19	38.00	37.24	33.68	36.51	35.14
	N	6	6	6	6	6	6	6	6	6
μ-$BaSO_4$ low	Mean	355.97	365.38	374.38	380.73	388.25	393.60	399.52	404.37	408.52
	SD	31.77	35.28	36.68	39.39	40.07	42.62	44.49	42.45	45.93
	N	6	6	6	6	6	6	6	6	6
μ-$BaSO_4$ high	Mean	398.38	405.92	415.08	422.87	428.72	431.43	438.12	442.55	447.17
	SD	38.57	38.69	39.86	39.35	39.36	38.72	38.03	39.28	39.97
	N	6	6	6	6	6	6	6	6	6
μ-ZrO_2 low	Mean	389.63	396.95	408.68	414.02	421.05	426.63	431.43	436.12	441.12
	SD	18.51	18.49	19.54	22.44	21.86	20.84	22.21	21.04	22.57
	N	6	6	6	6	6	6	6	6	6
μ-ZrO_2 high	Mean	382.35	390.48	399.03	405.82	413.52	416.95	423.22	429.10	435.85
	SD	30.02	31.98	33.84	35.59	36.25	33.62	33.21	31.87	36.43
	N	6	6	6	6	6	6	6	6	6
nano-SiO_2 low	Mean	364.27	371.50	379.52	387.03	394.33	399.62	404.45	410.62	416.97
	SD	37.73	39.04	40.00	39.88	38.62	38.13	35.32	35.16	37.34
	N	6	6	6	6	6	6	6	6	6
nano-SiO_2 high	Mean	377.42	386.88	397.58	405.05	409.40	415.47	421.25	423.57	430.72
	SD	32.18	34.24	31.19	34.60	33.91	33.76	38.16	35.95	36.25
	N	6	6	6	6	6	6	6	6	6