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Quantitative biokinetic analysis of radioactively labelled, inhaled Titanium dioxide Nanoparticles in a rat model

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Quantitative biokinetic analysis of radioactively labelled, inhaled Titanium dioxide Nanoparticles in a rat model

by

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16. Abstract

The aim of this project was the determination of the biokinetics of TiO_2 nanoparticles (NP) in the whole body of healthy adult rats after NP administration to the respiratory tract – either via inhalation or instillation. We developed an own methodology to freshly synthesize and aerosolize TiO_2-NP in our lab for the use of inhalation studies. These NP underwent a detailed physical and chemical characterization providing pure polycrystalline anatase TiO_2-NP of about 20 nm (geometric standard deviation 1.6) and a specific surface area of 270 m²/g. In addition, we developed techniques for sufficiently stable radioactive ^{48}V labelling of the TiO_2 NP. The kinetics of solubility of ^{48}V was thoroughly determined.
The methodology of quantitative biokinetics allows for a quantitative balance of the retained and excreted NP in control of the administered NP dose and provides a much more precise determination of NP fractions and concentrations of NP in organs and tissues of interest as compared to spotting biokinetics studies.
Small fractions of TiO_2-NP translocate across the air-blood-barrier and accumulate in secondary target organs, soft tissue and skeleton. The amount of translocated TiO_2-NP is approximately 2% of TiO_2-NP deposited in the lungs. A prominent fraction of these translocated TiO_2-NP was found in the remainder. Smaller amounts of TiO_2-NP accumulate in secondary
organs following particular kinetics. TiO_2-NP translocation was grossly accomplished within the first 2-4 hours after inhalation followed by retention in all organs and tissues studied without any detectable clearance of these biopersistent TiO_2-NP within 28 days.
Therefore, our data suggest crossing of the air-blood-barrier of the lungs and subsequent accumulation in secondary organs and tissues depends on the NP material and its physico-chemical properties. Furthermore, we extrapolate that during repeated or chronic exposure to insoluble NP the translocated fraction of NP will accumulate in secondary target organs. When these NP are biopersistent as the materials we have studied, much higher NP doses are expected to accumulate in secondary target organs than after our two-hours inhalation studies. In this case accumulated NP doses may well reach levels of the initiation or the modulation of adverse health effects. Hence, further studies are necessary investigating the biokinetics and toxicologically relevant responses and the underlying mechanisms of TiO_2 NP in secondary target organs after chronic exposure.

17. Keywords
Titanium dioxide nanoparticles, anatase, inhalation, biokinetics study, secondary organs and tissues, radioactive label ^{48}V

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16. Kurzfassung

Ziel dieser Studie war das biokinetische Verhalten von Nanopartikeln (NP) im unteren Nanometerbereich (< 50 nm) zu untersuchen, nachdem sie vorher entweder inhaliert oder per Instillation in die Lunge von gesunden erwachsenen weiblichen Ratten appliziert worden sind. Weil die Anwendung von kommerziellen nanostrukturierten TiO_2 Partikeln im unteren Nanometerbereich zurzeit technisch nicht realisierbar ist, haben wir die Partikel selbst frisch generiert und Ratten mit diesem NP Aerosol beatmet. Wir haben diese TiO_2 NP physikalisch und chemisch sehr genau als polykristalline Anatase TiO_2 NP mit einer spezifischen Oberfläche von 270 m²/g und mit einem medianen Durchmesser von 20 nm charakterisiert. Wir haben die TiO_2 NP für unsere quantitativen Biokinetikstudien radioaktiv mit ^{48}V markiert.
Das von uns entwickelte Verfahren der quantitativen biokinetischen Analyse radioaktiv markierter Nanopartikel erlaubt eine makroskopische Bestimmung der retinierten Fraktion von Nanopartikeln (NP) in allen ausgesuchten Organen und Geweben zum Zeitpunkt der Gewinnung der Organe. Obwohl die angereicherten Fraktionen nach der 2-stündigen Inhalation sehr gering sind, konnten wir zu allen 5 Zeitpunkten in der 28-Tage Studie zeigen, dass die NP in den sekundären Zielorganen langzeitlich akkumuliert und retiniert werden. Die Menge der TiO_2-NP, die die Blut-Luft-Schranke der Lunge überwinden können, liegt in etwa bei 2% der in der Lunge vorhandenen NP und befinden sich fast vor allem im Restkörper. Nur geringe Mengen an TiO_2-NP werden in die anderen Organe umverteilt. Die Umverteilung der NP erfolgt in einer für jedes einzelne Organ eigenen Zeitkinetik. Die Translokation von NP über die Blut-Luft-Schranke der Lunge in diverse Organe und Körpergewebe ist offensichtlich abhängig vom appliziertem Material, so dass von unterschiedlichen Toxizitäten der einzelnen Materialen ausgegangen werden muss.
Im Fall von biopersistenten NP kann man nicht ausschließen, dass bei chronischer Exposition die akkumulierten NP Dosen zu gesundheitlichen Effekten nicht nur in den Aufnahmeorganen sondern auch in den sekundären Organen führen können.

17. Schlagwörter
Titandioxid Nanopartikel, Anatase, Inhalation, Biokinetik, sekundäre Organe und Gewebe, Radioaktive Markierung mit ^{48}V

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Projekt: Quantitative Biokinetik-Analyse radioaktiv markierter inhalierter Titandioxid Nanopartikel in einem Rattenmodell

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Final Report

1. **Tasks of the project**; *Aufgabenstellung*,

The aim of this project was the determination of the biokinetics of TiO_2 nanoparticles (NP) in the whole body of healthy adult rats after NP administration to the respiratory tract – either via inhalation or instillation. Here we offered applying our previously developed methodology of quantitative biokinetics using radio-labelled NP. Quantitative biokinetics means that not only the NP absolute contents and concentrations of NP in primary and secondary organs and tissues of interest are determined but also those of the remaining body (carcass) and those in faecal and urinary excretion. This methodology of quantitative biokinetics allows for a quantitative balance of the retained and excreted NP in control of the administered NP dose and provides a much more precise determination of NP fractions and concentrations of NP in organs and tissues of interest. In addition, the radio-analysis provides a dynamics of radioactive quantities ranging over more than four orders of magnitude which in turn allows for a precise determination of low NP contents in organs of highest interest like brain, heart, genital organs, etc. Another advantage is the absence of any physical-chemical preparatory step prior to the radio-analysis which frequently will lead to artificial losses when NP quantities are very low. A major challenge is the firmly bound radio-labelling to the NP matrix which requires careful development.

2. **Premises of this R&D project**; *Voraussetzungen, unter denen das FE-Vorhaben durchgeführt wurde,*

We already performed inhalation studies followed by quantitative biokinetics analysis using radio-labelled iridium NP aerosols of 20 and 80 nm median diameter (Kreyling et al., 2002; Semmler et al., 2004 + 2007) and meanwhile also inhalation of 20 nm radio-labelled elemental carbon NP (Kreyling et al., 2009). We also performed instillation studies by quantitative biokinetics analysis using radio-labelled gold NP (Semmler-Behnke et all., 2008). For these previous studies we have characterized the various NP very carefully as detailed in the papers and (Szymczak et al., 2007).
In addition, we have already performed inhalation studies followed by morphometric analyses of the lungs using non-radioactive TiO_2 aerosols of 20 nm median diameter (Kapp et al., 2004, Geiser et al., 2005, Geiser et al., 2008)

1. Use of our inhalation apparatuses ranging from whole-body-exposure, to nose-only-exposure to intubation / ventilation inhalation.
2. development of NP radio-labelling
    a. Development of radio-labelling of available industrial TiO_2 NP (e.g. P25) using proton irradiation at a cyclotron and leaching tests for the confirmation of the firmly bound radiolabel in the NP matrix.
    b. Development of the modification and adaptation of our existing spark-ignition NP aerosol generator using radio-labelled titanium electrodes
3. application of our well established instillation protocol
4. application of our well established analytical methodology of quantitative biokinetics
5. application of our well established dissection protocol with an emphasis on the minimization of cross contamination
6. application of our well established gamma spectrometers for radio analysis
7. application of our well established computer routines for data evaluation

3. **Design and procedure of the project**; *Planung und Ablauf des Vorhabens,*

The design of the project is based on the previously developed methodology on quantitative biokinetics after the administration of radio-labelled NP.

Quantitative biokinetics

Briefly, quantitative biokinetics means that not only the NP absolute contents and concentrations of NP in primary and secondary organs and tissues of interest are determined but also those of the remaining body (carcass) and those in faecal and urinary excretion. This methodology of quantitative biokinetics allows for a quantitative balance of the retained and excreted NP in control of the administered NP dose and provides a much more precise determination of NP fractions and concentrations of NP in organs and tissues of interest. In addition, the radio-analysis allows for a dynamics of radioactive quantities over more than four orders of magnitude. No any physical-chemical preparatory step prior to the radio-analysis is required, which frequently will lead to artificial losses when NP quantities are very low.

Radio-labelling of NP

In a first attempt proton irradiation of a powder of available industrial TiO_2 (P25, Degussa / Evonik) was performed using a cyclotron accelerator operated by ZAG GmbH in Karlsruhe. This allowed ^{48}V radio-labelling of the TiO_2 NP. Tests at ZAG Karlsruhe were performed to optimize irradiation conditions to achieve sufficiently a high specific radioactivity of the NP. Subsequently, we tested the radio-labelled NP for sufficiently low leaching rates of the radio-label ^{48}V off the TiO_2 NP.

In a second attempt we developed a methodology proton irradiating titanium electrodes selectively at their tip providing sufficiently high specific ^{48}V radioactivity solely at the one tip of the electrodes. These were then used in our spark-ignition NP aerosol generator adapted to the use of highly radioactive electrodes under the conditions of safe operation and the regulations of radiation protection. In pilot studies we tested the radio-labelled NP for sufficiently low leaching rates of the radio-label ^{48}V off the TiO_2 NP. The NP aerosols generation was based on protocols derived from our previous studies using non-radioactive TiO_2 NP aerosols. It turned out that further engineering of the NP aerosol production was required to minimize ^{48}V radio-label leaching off the TiO_2 NP. This was achieved by introducing a heat degradation unit in the downstream NP aerosol line to further stabilize

the tested the radio-labelled NP for sufficiently low leaching rates of the radio-label ^{48}V within the TiO_2 NP.

Inhalation of freshly produced ^{48}V radio-labelled TiO_2 NP
Inhalation of freshly produced ^{48}V radio-labelled TiO_2 NP using the modified and adapted spark ignition NP aerosol generator was based on the previously developed inhalation protocols used for the previous NP, see Top 2 above.

Instillation of suspensions of ^{48}V radio-labelled TiO_2 NP
Protocols for the instillation of suspensions of ^{48}V radio-labelled TiO_2 NP were based on those derived previously (see Refs. in Top 2) and adapted to the requirements resulting from the physico-chemical properties of the TiO_2 NP, e.g. dispersion protocols.

Dissection, radio-analysis and data evaluation
Protocols for dissection, radio-analysis and data evaluation were based on those derived previously (see Refs. in Top 2) and adapted accordingly.

4. **Scientific and technical status to be based on**; *wissenschaftlichem und technischem Stand, an den angeknüpft wurde, insbesondere*

- list of known constructions, protocols and IPR to be used in the project

Angabe bekannter Konstruktionen, Verfahren und Schutzrechte, die für die Durchführung des FE-Vorhabens benutzt wurden,

1. Adaptation and use of our inhalation apparatuses ranging from whole-body-exposure, to nose-only-exposure to intubation / ventilation inhalation.
2. experimental experience from methods of previous NP radio-labelling; (Kreyling et al., 2002; Semmler et al., 2004 + 2007; Kreyling et al., 2009; Semmler-Behnke et all., 2008; Szymczak et al., 2007)
3. application and adaptation of our well established instillation protocol
4. application and adaptation of our well established analytical methodology of quantitative biokinetics
5. application and adaptation of our well established dissection protocol with an emphasis on the minimization of cross contamination
6. application and adaptation of our well established gamma spectrometers for radio analysis
7. application and adaptation of our well established computer routines for data evaluation

- list of literature references and literature data bases; *Angabe der verwendeten Fachliteratur sowie der benutzten Informations- und Dokumentationsdienste,*

- List of Endnote based literature data bases of more than 10 000 entries collected over the last three decades
- Libraries at the Helmholtz Center Munich, Ludwig-Maximillian-University, Technical University Munich
- Intensive use of international search tools like Pubmed, Medline, Web of Sciences, Google, etc.

5. **Cooperation with other Partners** *Zusammenarbeit mit anderen Stellen.*

For the radio-labelling of the TiO_2 NP or Ti electrodes close cooperation and scientific exchange with ZAG GmbH, Karlsruhe, was mandatory.
In addition, for design and concept of the radio-labelling of the TiO_2 NP or Ti electrodes we frequently consulted:

1. Prof. Dr. Tilman Butz, Director of the Institute of Nuclear Solid State Physics, University of Leipzig, Linnéstr. 5, 04103 Leipzig
2. Dr. Neil Gibson, Institute for Health and Consumer Protection, Joint Research Centre TP 500, Via E. Fermi, 2749, I-21027 Ispra (VA), Italy

For the characterization of the own taylor-made TiO_2 NP we consulted several colleagues highly acknowledged in their scientific community and sourced out various characterization methods to them:

1. EELS-TEM, EFTEM: Prof. Dr. Marianne Geiser,
2. HRTEM + XEDS: Prof. Dr. Knut Deppert, Univeristy of Lund, Institute of Solid State Physics, Box 118, 22100 Lund, Sweden
3. XRD: Dr. Neil Gibson, Institute for Health and Consumer Protection, Joint Research Centre TP 500, Via E. Fermi, 2749, I-21027 Ispra (VA), Italy
4. XRD + BET: Prof. Dr. Pratim Biswas, Washington University in St Louis, Department of Energy, Environmental & Chemical Engineering, Campus Box 1180, One Brookings Drive, St. Louis,, MO 63130, USA
5. Consultation of international experts in nanoparticle toxicology:
   a. Prof. Dr. Günter Oberdörster, University of Rochester, USA;
   b. Ken Donaldson, University of Edinburgh, UK;
   c. Vicki Stone, Napier University of Edinburgh, UK;
   d. Prof. Dr. Paul Borm, University of Maastricht, Netherlands;
   e. Prof. Dr. Kenneth Dawson, University College Dublin, Ireland
6. Consultation of infrastructural expertise provided within the Helmholtz Center Munich and particularly within our Institute of Lung Biology & Disease (iLBD).

II. Comprehensive presentation of results *Eingehende Darstellung*

1. **Achieved goals;** *des erzielten Ergebnisses,*

II.1.1. Background and Introduction

In the present study, the biokinetics of small nanoparticles (NP) with a diameter of less than 50 nm was investigated using healthy adult rats. The particles were aerosolized and inhaled by the rats as well as instilled. Since most of the already existing studies investigated the kinetics and toxicology of larger TiO_2 particle-aggregates (> 200 nm), we decided to focus on these relatively small TiO_2 nanoparticles with a diameter less than 50 nm. Considering this, our study differs significantly from the BMBF project NanoCare. In detail, NanoCare focuses on upgrade and partially validation of already existing data. Furthermore, in the

NanoCare studies as well as the already existing literature, the retention of TiO_2 particles was mainly studied in the respiratory tract. In addition, selected organs as well as the whole body were analysed with an accuracy of 1 %.

Approximately 100 000 tons of TiO_2 nanoparticles and microparticles are produced per year. An extensive exposure of the whole population, special groups of people and especially susceptible individuals are the consequence (Lomer et al., 2004). Therefore, to study the biokinetic behaviour of these particles following to inhalation is indispensable.

Previously, we developed a method to quantitatively determine radioactive labeled nanoparticles. Thereby, a macroscopic analysis of the retained fraction of nanoparticles in selected organs is warranted (Kreyling et al., 2002; Semmler et al., 2004). These fractions can be relatively calculated in proportion to the whole amount of particles in the body as well as the excrement. This quantitative dosimetric method warrants a very effective analysis of the nanoparticles within 3-5 size ranges, which is a substantial precondition, since the translocation of nanoparticles to the bloodstream via primary target organs as well as the accumulation in secondary target organs is very low (Kreyling et al., 2002; Semmler et al., 2004; Semmler-Behnke et al., 2007; Wiebert et al., 2006; Wiebert et al., 2006; Moller et al., 2008).

In the present project, we analysed the toxikokinetics of applied nanoparticles at several timepoints.

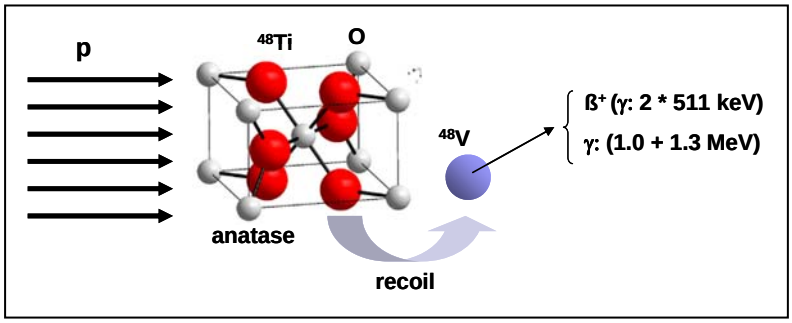
II.1.2. Materials and Methods

II.1.2.1 Materials

- TiO_2 P25 NP (Degussa / Evonik, Marl)
- PEG-TiO_2 NP of the former company Nanosolutions, Hamburg
- Our own tailor-made TiO_2 NP aerosols generated by a spark generator

II.1.2.1.1 Radiolabeling of commercial TiO_2 NP

For the radioactive labeling of titanium dioxide NP (TiO_2 NP) we use the proton irradiation in a cyclotron, figure on the left. During this process a nuclear reaction inside of the Ti-atom occurs, so that radioactive vanadium ^{48}V originates. ^{48}V is generated by the collision of the ^{48}Ti with a proton. Thereby the ordinal of 22 (Ti) increases 23 (V), because the proton is integrated in the nucleus and a neutron from the nucleus of the titanium is emitted during the nuclear reaction: ^{48}Ti (p, n) ^{48}V. The radioactive ^{48}V decays with a half-life of 16 days. This process leads to the formation of a positron and gamma rays with energy values of 0.99 and 1.3 MeV. The positron (anti-material) recombines immediately with an electron. The whole energy is emitted in the form of 2 gamma rays with 511 keV which emit in opposite directions (180°). The kinetic energy of the incident proton may lead to a recoil pulse of the transformed ^{48}V-atom. Therefore, the transformed ^{48}V-atom can be knocked out



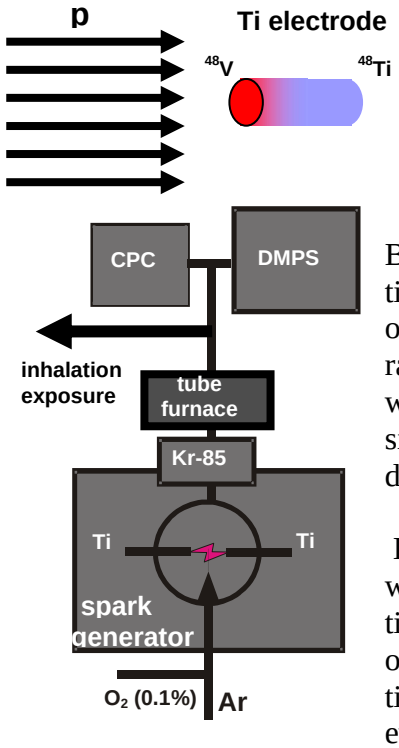
of the TiO_2 lattice to be replaced at another place of the lattice. If this happens on the surface of the primary TiO_2 NP, the ^{48}V is not tightly integrated into the lattice. Thereby it can e.g. be dissolved as an ion from the particle matrix in aqueous solutions. After the optimization of the radiation parameters, TiO_2 NP samples were irradiated with the following parameters:

Sample	high	low
Specific activity (MBq / mg)	1	0.1
Atomic ratio ^{48}V : TiO_2	$1.7\ 10^{-8}$	$1,7\ 10^{-9}$

That means, for instance, at a specific activity of 1 MBq/mg statistically not more than one radioactive ^{48}V-atom can be found per single irradiated 20 nm TiO_2 NP. Furthermore, from more than 1000 NP of this size only one NP statistically contains a radioactive ^{48}V atom. These ratio data show that the lattice like structure and other physical properties of the TiO_2 NP have not been damaged or significantly changed by the irradiation process.

1. The proton irradiations were made successfully in the Zyklotron AG (ZAG), Karlsruhe. ZAG irradiated TiO_2 in powder form as well as titanium electrode rods. We have covered the costs for irradiation and transport within this project.
   a. Proton irradiations of Degussa TiO_2 NP (P25) were successfully made for test purposes. The NP returned from the irradiation process as a white powder as sent in. However the particles were as strongly agglomerated as the original un-irradiated P25 NP powder. Therefore this nano-structured material did not fulfill our demands for nanoparticles to be smaller than 100 nm.
   b. The proton irradiation of 4.5 nm PEG-TiO_2 NP was not successful; after this irradiation the powder was not white anymore but black. This indicated that the PEG molecules have been oxidized and ashed during the proton irradiation. It was not possible to disperse the black powder.

2. Within the scope of the cooperation with the European Joint Research Center (JRC) in Ispra, Italy, a solution of dissolved ^{48}V was produced and provided. Thereby we could make our control tests, as described below.



II.1.2.1.2 Production of ^{48}V radioactive labeled TiO_2 NP by means of a spark generator

Besides the irradiation of the NP powder we also irradiated titanium rods with a diameter of 3 mm a length of 4 mm on one circular side, to be used for the production of ^{48}V radiolabeled TiO_2 NP aerosols, figure on the left. This warranted a high activity density of ^{48}V on the circular front side used for generating NP in the spark generator as described below.

In the spark generator (GFG100 Palas, Karlsruhe) sparks were ignited by a high voltage discharge between the adjacent titanium rod electrodes in an argon gas stream with 0.1% of oxygen, picture on the left. In this spark ignition process a tiny amount of titanium and ^{48}V of the electrode surface evaporates and condenses very quickly forming primary

particles of the size of 2-5 nm. Because of the O_2 content in the gas stream these particles already are made up of TiO_2 with ^{48}V labeling. These primary particles occur at very high concentrations (>> 10^8 cm^{-3}), such that rapid coagulation occurs during the cooling. According to the initially high temperature, aggregates with firm chemical bonds will be formed. During further cooling agglomerates will be formed by weak physical forces like van-der-Waals-forces. The highly charged aerosol is quasi-neutralized by an inline radioactive 85Kr source 10 cm^3 downstream the spark generation chamber. Further coagulation is subsequently stopped by dilution of the aerosol with oxygen and nitrogen.
Subsequently, a thermal treatment in a tube furnace with 950°C improves the firm integration of the ^{48}V-atoms into the matrix of the NP aggregates/-agglomerates. Using a condensation particle counter (CPC 3022, Aachen TSI) and a differential particle size spectrometer (DMPS 3071 + CPC 3010, TSI, Aachen) the concentration and electrical mobility size distribution of the produced NP are continuously measured online. The resulting ^{48}V-TiO_2 NP aerosol is oxygenated at 20-22% and humidified to 75% relative humidity. Hence, a physiological aerosol is provided to the animals for inhalation within 5 seconds.

II.1.2.2 Characterization

1. P25 TiO_2 NP (Degussa)
   - For this industrial NP CAS certifications were investigated.
   - From every used sample the hydrodynamic diameter was measured by dynamic light scattering (DLS, Malvern HPPS, Malvern, Herrenberg).
   - We have checked the electo-negative surface charge (zeta potential) in water using a Zetasizer and we were able to confirm the values cited in the literature.
   - The specific surface was determined to be 52 m^2/g and corresponds with in the literature shown values.

2. PEG- TiO_2 NP
   - Unfortunately, these NP made by the former company Nanosolutions in Hamburg have no certification by the manufacturer and cannot be reordered since the company does not exist any longer. However, the following data are available:
     - Material: titaniumdioxid
     - Occurrence: the NPs are existent as a powder
     - Crystalline structure: 100% anatase
     - Size distribution: mean diameter of 4.5 nm; standard deviation 0.1
     - Surface modification: polyethylene glycol (PEG)
     - Hydrodynamic diameter (own measurement): 20 nm, standard deviation 0.2
     - Aggregation / agglomeration: the original particles dispersed completely with a minimum of agglomeration; the increased hydrodynamic size arises from the PEG surface modification
     - Specific surface: amounts approx. 400 m^2/g of the pure TiO_2 NP (as calculated), from PEG-TiO_2 NP approx. 200 m^2/g
     - Zeta potential (own measurement): -22 mV
     - Solubility: no detectable solubility in water.

3. Our own tailor-made TiO_2 NP aerosols generated with a spark generator
   i. Size distribution and concentration: Using a condensation particle counter (CPC 3022, Aachen TSI) and a differential mobility particle size spectrometer (DMPS 3071 + in 3010, TSI, Aachen) the concentration and size distribution of the generated NP were continuously measured online during the exposure.

ii. For the electron-microscopic analysis applying high-resolution transmission electron microscopy (HRTEM, University of Lund, Sweden) NP samples were collected on copper grids with a nanometer aerosol sampler (TSI 3089, Aachen).
iii. A crystallographic analysis with an X-ray diffractometer (XRD, JRC Ispra, Italy) was made.
iv. The specific surface of the TiO_2 NP was determined with the BET method which is based on a measurement of an absorped monolayer of nitrogen molecules on the NP surface.

II.1.2.3 In vitro solubility of ^{48}V radio-labeled TiO_2 NP

Solubility tests:
P25 ^{48}V-TiO_2 NP: NP samples with known mass were either suspended in distilled water or in 0.05 M sodiumpyrophosphate and dispersed in an ultrasonic bath for a period of 15 sec.
Separation of particular or suspended fractions was either performed by centrifugation at 15 x10^3 g for a period of 40 min or ultra-filtered at 4x 10^3 g for 30 min through an Amicon ultrafiltration tube with a molecular weight cut off at 5 kDa (Millipore, Amicon Ultra – 15; 5000 MWCO). Subsequently the two fractions were analyzed by gamma-spectroscopy.

^{48}V-TiO_2 NP aerosol: Aerosols of ^{48}V-TiO_2 NP were collected on an absolute filter and measured gamma-spectrometrically. Subsequently the filter was mounted in a filter holder with a maximum supernatant volume of 200 mL. 50 mL distilled water were added and after 5 minutes, 1 hour and 24 hours the water was pressed through the filter by compressed air (1.5 bar) and the ^{48}V activity in the filtrate was measured.

II.1.2.4 Laboratory animals

Healthy, adult (3-months old) female WKY-rats were used as laboratory animals. The experiments were carried out in line with the ethical principles of the responsible authorities, the government of district of Upper Bavaria and the ethical committee of the Helmholtz-Center-Munich. The number of the governmental approval was: Az: 209.1/211-2531-94/04.
The rats were adapted to our animal facilities at a temperature of 22 $\pm$ 3°C and 50 $\pm$ 5% relative humidity with a 12 hour dark - 12 hour light cycle for a minimum of 10 days after delivery. Standard rodent chow and water were available ad libitum.

II.1.2.5 Concept of quantitative biokinetic studies

In order to give reliable statements on the distribution of the applied TiO_2 NP it is essential that the examination is quantitatively evaluated. At time t=0 radioactive material is administered into the rats. At different points of time t_1, t_2, t_n… the animals are sacrificed. We take the blood,

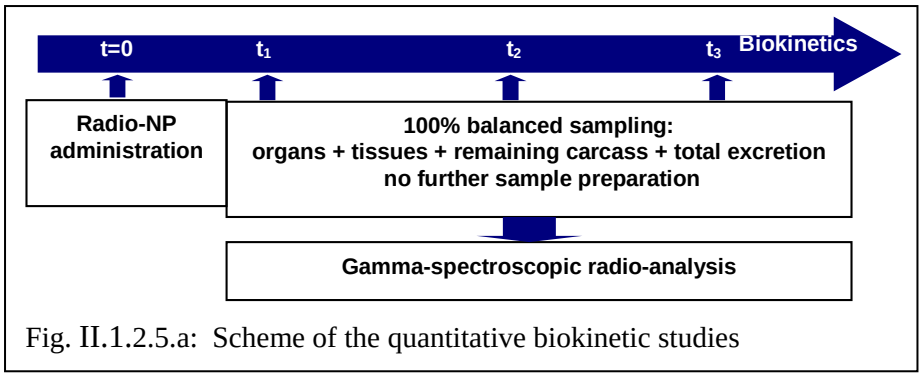


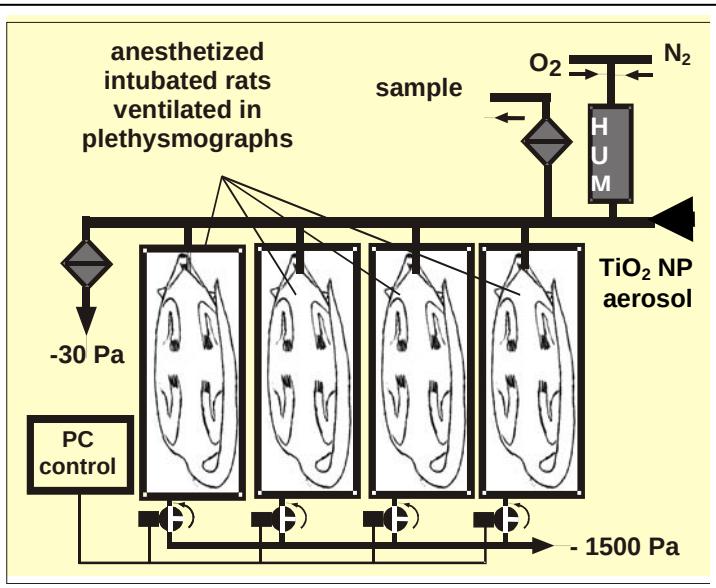
Fig. II.1.2.5.a: Scheme of the quantitative biokinetic studies

all organs in toto, tissue samples, the complete remainder (i.e. the whole animal tissue which is not examined in the before-mentioned individual samples) and all excrements (separated according to feces and urine). Only thus a 100% balance of biokinetics of each individual animal is possible, Fig. II.1.2.5.a.
In all subsequently described studies 4 rats were used for each application method and each time point. This number of animals was sufficient for a statistical evaluation as demonstrated in our former biokinetic studies (Kreyling et al., 2002, Semmler et al., 2004, Semmler-Behnke et al., 2007 + 2008), and at the same time the limited number satisfies the reduction principle in animal testing.

II.1.2.6 ^{48}V labeled TiO2 NP applications:

II.1.2.6.1 Inhalation:

Four whole-body-plethysmographs which are operated intermittendly at low pressure are connected to the aerosol line. Each anaesthetized and intubated rat is connected through an endotracheal tubus to the aerosol line in each whole-body-plethysmograph; the tubus is inserted into the trachea and tightened at the larynx. Afterwards the plethysmographs are closed, Fig. II.1.2.6.a. At periodic intervals (in these studies 40/minute) a low pressure of -1500 Pa is applied to the plethysmographs by computer-control, which causes an inspiration of the animals. After one second the negative pressure is removed and the animals expire again. During these investigations the animals were ventilated with a defined ^{48}V labeled TiO_2-aerosol for a period of 2h. Using this inhalation apparatus an optimized deposition can be achieved; inhaled NP are rather homogeneously deposited in all lung regions, see planar gamma-camera picture in Fig. II.1.2.6.b. Additionally, NP contamination of extrathoracic airways in the head and the fur is avoided. This inhalation apparatus for controlled inhalation of radioactive labeled NP by intubated animals is worldwide unique and is in our use since more than six years.



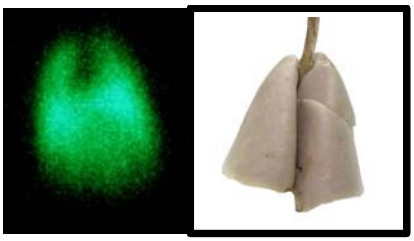


Fig. II.1.2.6.a: Computer-controlled ventilation of intubated and anaesthetized rats in plethysmograph boxes.

Fig. II.1.2.6.b: NP distribution in the lungs measured with gamma camera (left) and for comparison a dried rat lung at complete inspiration.

2.6.2 Intratracheal instillation:

For this application the anaesthetized animals were positioned onto an inclined board (60°) in a dorsal position. The animals are fixed with the front teeth to an elastic band. After that the larynx was exposed to a cold-light-source from the outside (extrinsically). The tongue was brought forward with forceps and the tongue root is slightly pressed towards the mandible. Now, catheter is gently inserted into the trachea under visual control.
The correct position of the catheter in the trachea is controlled with a mirror right at the end of the catheter for a visual condensate on the mirror during expiration. Now, a syringe filled with the NP suspension for instillation is connected to catheter and the NP suspension is injected during the inspiration phase of the animal. Instillations of 50µl of NP suspension of a concentration of 1mg/ml were applied to each animal.

II.1.2.7 Supporting studies

II.1.2.7.1 Determination of biokinetic distribution of intravenously injected ^{48}V-TiO_2 NP-suspension

For a better understanding and interpretation of the biokinetic distribution of ^{48}V labeled TiO_2-NP administered to the lungs, it is necessary to also intravenously inject the NP suspensions as well. Based on these additional studies differences can be evaluated between NP directly injected into blood and those NP entering the blood circulation after having crossed the air-blood-barrier of the lungs. From our biokinetic studies using gold NP (Semmler-Behnke et al., 2008) we have evidence that the subsequent accumulation pattern in different secondary target organs change drastically between the two routes of administration.
At first the animals are anaesthetized with isoflurane, and then the tail is dipped into warm water in order to widen the tail vein. Then the blood flow is blocked by a tourniquet at the tail root; and a flexible cannula (24G ¾“) is distally placed in the tail vein. The tourniquet is released and the correct position of the cannula in the vein is checked injecting a small amount of PBS without any hemorrhage. Then the TiO_2 NP-suspension is applied through the cannula. If necessary, the bleeding is stopped with a cotton swap in order to avoid secondary hemorrhage.

II.1.2.7.2 Determining the biokinetic distribution of intra-esophageal applied ^{48}V- TiO_2 NP-suspension (i.e. gavage)

A further set of studies was also necessary administering an NP suspension into the gastrointestinal tract. As stated above, the conducting airways in the lungs possess a very effective mechanism eliminating NP deposited on the airways epithelium, the “mucociliary clearance”, which continuously transports material along the airways towards the larynx, where it is subsequently swallowed. To estimate the resorbed fraction of ^{48}V-labelled TiO_2 NP across the gastrointestinal well a well-defined TiO_2 NP-suspension was given directly into the esophagus (gavage) and subsequently the distribution in the organs and in the excretion was measured as described above.
Animals previously anaesthetized with isoflurane, were located in the same way on the tilted board as during instillation (see above). The tongue was pulled forward with forceps. A catheter (in this case a flexible cannula 16G 2“) was inserted into the esophagus under visual inspection using the cold-light-source. The correct position of the tubus was checked using a mirror as described above. However, proper placement of the cannula in the esophagus was assured when no condensate appeared visible on the mirror during exspiration.

II.1.2.7.3 Determining biokinetic distribution of dissolved ^{48}V radio labeling

To determine biokinetic distribution of non-particulate, ionic ^{48}V radio label which eventually may have leached off the TiO_2 NP matrix, an aqueous solution of ionic ^{48}V was either instilled intratracheally as described above or the solution was injected intravenously into the tail vein. These data are needed to correct for solubilized ^{48}V radio label as described in 2.9.1. see below.

II.1.2.8 Retention and clearance of TiO_2 NP

After the application of TiO_2 NP each rat was kept individually in a metabolic cage in order to collect the whole urine and feces quantitatively and separately, see picture to the left. In cases where the retention times lasted more than 7 days, the animals were kept in normal cages in which fleece mats were laid out to absorb the urine. Thereby, in these cages the daily urine and daily feces were collected quantitatively and separately for the first 7 days and afterwards urinary and fecal excretions were integrated over three and four days until the end of the 28 days period.

II.1.2.9 Radioanalytical measurement and analysis

In our studies we usually use γ-emitting radio-isotopes such as ^{48}V. Using this γ-emitters the amount of radioactivity is measured without further sample preparation after the dissection of the animals so that no artifactual losses occurred due to preparatory steps .
By means of a massively lead-shielded NaI well-type-scintillation detectors including sample changers the individual samples were measured γ-spectrometrically. The lead shield reduces background radiation and the well geometry and the applied γ-spectrometry software further increases the sensitivity by measuring the photo peaks of the gamma emission line. The amount of the ^{48}V radioactivity is calculated in units of Bq at a refernce time point after correction of background radiation and physical decay of the ^{48}V isotope in each individual sample. The measured radioactivity is directly proportional to the applied TiO_2 retained in the sample.
In vivo retention of the retained ^{48}V activity in the rats of the 28-days study was measured gamma-spectrometrically twice a week with an additional collimated NaJ scintillation-detector in order to determine the retention kinetics for every single animal, Fig. II.1.2.9.a.

Fig. II.1.2.9.a: Left: Scheme of gamma-spectroscopic measurements with an automated sample changer. Center: Measuring station for large samples of a volume up to 1 liter. Right: Measuring station for small samples up to a volume of 10 mL.

For evaluation of the data the following parameters are significant:

A_{rat} = entire ^{48}V radioactivity in the animal after application of the radioactive materials

$A_{rat} = \sum_{i=0}^{n} A_i$ A_i = the ^{48}V radioactivity of sample i

In addition to the ^{48}V radioactivity of each sample the fraction is then normalized to the entire activity

(2) $f_i = \frac{A_i}{A_{rat}}$

in this equation note that the activity (A) is directly proportionate to the mass (m) of the applied TiO_2 NP material.

(3) $A(^{48}V) \sim m\ (TiO_2)$

Several corrections have to be taken into account when processing the data:

II.1.2.9.1 Correction on „fast clearance" after inhalation or intratracheal instillation

The term „fast clearance" covers the cleaning mechanism of the conducting airways to the larynx within 24 hours after application of TiO_2 NP into the lung. By this process NP deposited in the airways are transported by the mucociliary action to the larynx, where they are swallowed passing through the GI-tract and eventually being excreted with the feces. To correct the fractions of (2) they are divided by the correction factor k_{fc}, and the amended data are referred to as f_i'.

(4) $k_{fc} = 1 - (f_{feces} + f_{GIT} + f_{trachea/larynx})$

f_{feces} = Fraction feces

f_{GIT} = Fraction gastrointestinal tract

$f_{trachea/larynx}$ = Fraction trachea/larynx

(5) $f_i' = f_i / k_{fc}$

II.1.2.9.1 Correction for dissolved ^{48}V eventually leached off the administered TiO_2 NP

Assessing the in vivo ^{48}V solubility of the administered TiO_2 NP, supporting studies using soluble ^{48}V are required, to determine the distribution of soluble ^{48}V within the body and excretion.
The according correction factor (k_{sol} (t)) at the time t is calculated for each sample (g_{i_sol} (t)) individually after application of dissolved ^{48}V:

(6) $k_{i,sol}(t) = \frac{g_{i\_sol}(t)}{g_{urine\_sol}(t)}$

g_{i_sol} (t) = Fraction of the sample i after application of dissolved ^{48}V
g_{urine_sol} (t) = Fraction sample at application of dissolved ^{48}V

We presume very conservatively that no ^{48}V labeled TiO_2 NP are excreted in the urine but only dissolved ^{48}V. Then the correction factor $k_{i,sol}$ (t) for each sample is applied to estimate

the dissolved ^{48}V fraction f_{i_sol} in each sample using the urinary fraction after TiO_2 NP administration as an indicator of the potential dissolution of ^{48}V .

(7) $f_{i\_sol} = k_{i,sol} * f_{urine}$

Then the particulate fraction f_{i_part} of each samples is:

(8) $f_{i\_part} = f_i - f_{i\_sol}$

f_{i_sol} (t) = dissolved fraction of a sample after NP application
f_{i_part} (t) = particulate fraction of a sample after NP application

After analysis of the data for each single animal the standardized values of all animals of a group were averaged, and thus the biokinetic was determined using all data at all time points.

II.1.2.10 Statistics

To test significant differences the Student t-test was used. A significant difference was assumed if $p < 0.05$.

II.1.3. Results and discussion

II.1.3.1 Dispersion of available TiO_2 nano-particles (NP) in aqueous media

1. TiO_2 NP (Degussa) P25
   It is well known that P25 NP disperse rather poorly. To receive NP as small as possible the following methods of dispersion were applied:
   - ultrasonic treatment in distilled water (ultrasonic bath + ultrasonic probe)
   - ultrasonic treatment in rat's albumin solution (ultrasonic bath + ultrasonic probe)
   - ultrasonic treatment in rat's serum solution (ultrasonic bath + ultrasonic probe)
   - ultrasonic treatment in Tween-80 detergent solution (ultrasonic bath + ultrasonic probe)
   - ultrasonic treatment in CTAB detergent solution (ultrasonic bath + ultrasonic probe)
   - ultrasonic treatment in DPPC (di-palmytoyl phosphate...) (ultrasonic bath + ultrasonic probe)
   - ultrasonic treatment using phosphine-modified surface P25 TiO_2 NP in distilled water (ultrasonic bath + ultrasonic probe)

   In all these tests the median hydrodynamic diameter was >> 100 nm. Yet, our aim of a median hydrodynamic diameter < 100 nm was missed.

2. Selection of a nano-fraction from P25 TiO_2 particles
   To meet our demands of a nano-scaled NP-fraction, we have selected a size distribution with a median diameter of 100 nm measured by DLS (dynamic light scattering) starting from an original P25 TiO_2 NP-suspension by centrifugation. The selection parameters were determined with non-radioactive original P25 TiO_2 NP
   Solvent: 0.005 M sodium pyrophosphate in distilled water
   Centrifugation: 20 min at 2000 g; afterwards extraction of the size-selected supernatant
   Mass fraction of the size-selected NP: 5%

Next an original P25 TiO_2 NP-sample was proton-irradiated first and then dispersed according to the above mentioned protocol. A size-selected dispersion of the irradiated TiO_2 NP-suspension was subsequently compared with an inactive size-selected NP-suspension using the same solvent. As shown in figure II.1.3.1.a, the size distributions differ only slightly.

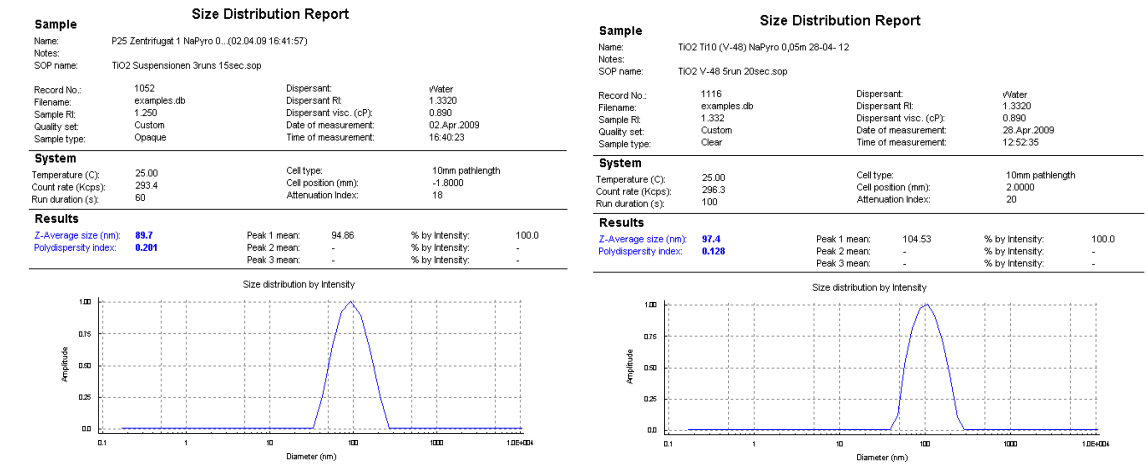


Figure II.1.3.1.a: DLS measurement of the size distribution inactive (on the left) and a radioactive labeled size fraction (on the right) of P25 TiO_2 NP.

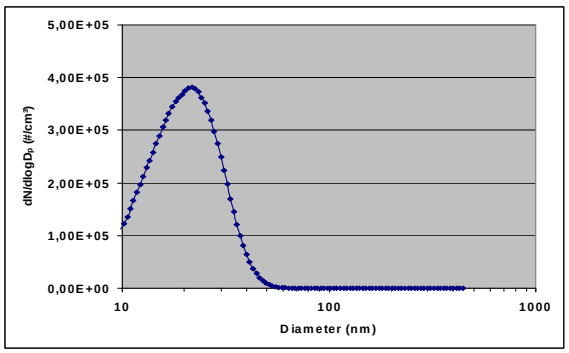
However, the in vitro solubility of the size-selected P25 ^{48}V-TiO_2 NP with 0.005 M aqueous sodium pyrophosphate was 37% separating the dissolved from the particulate fraction by centrifugation and 25% using the Amicon-ultrafiltration method. A slightly lower solubility was measured for distilled water.

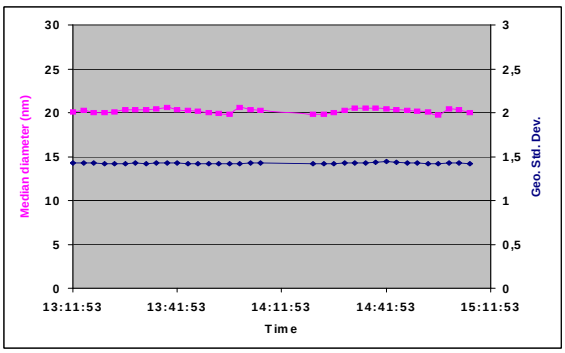
3. Monodisperse 4.5 nm TiO_2 NP with a polyethylene-glycol (PEG) surface modification
These TiO_2 NP disperse very well in water and in a NaCl (0.1%) solution. By means of dynamic light scattering they have a hydrodynamic diameter of 20 nm with a very small distribution (SD 0.15). Unfortunately, these PEG-surface-modified TiO_2 NP are not suitable for the proton irradiation, because the PEG coating of the white PEG-TiO_2 NP reduces to black elementary carbon ash and it is impossible to disperse the NP again.

II.1.3.2 Characterization of our own tailor-made ^{48}V-TiO_2 NP-aerosols generated with a spark generator

a. *Aerosol generation:* For the aerosol generation the addition of oxygen into the spark chamber was minimized in order to provide production of TiO2 NP and minimize irritant O_3 in the aerosol which would irritate the airways of the exposed rodents. An O_2 flow rate of 0.1% of the Ar flow rate was chosen since NP turned out to be TiO_2 and the O_3 concentration was below 20 ppm.

b. *Aerosol-concentration:* The aerosol was usually at a concentration of 3-6 10^6 NP/cm³ and constant for the whole exposure, Figure II.1.3.2.a.





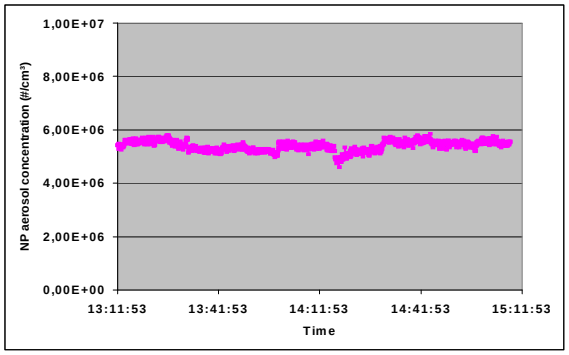


Figure II.1.3.2.a: Aerosol data from the inhalation study for the 24-hours retention study. Top left: mean NP aerosol spectrum during the whole inhalation period. Top right: Median NP electrical mobility diameter (red) and geometric standard deviation (blue) of the aerosol during the inhalation measured with SMPS. Left: NP aerosol concentration during the inhalation, measured with a CPC3022.

c. *Size and size distribution*: The median electrical mobility diameter of the thermally untreated NP was about 25 nm, the diameter of the thermally treated NP was approx. 10% smaller, Figures II.1.3.2.a and II.1.3.2.b.

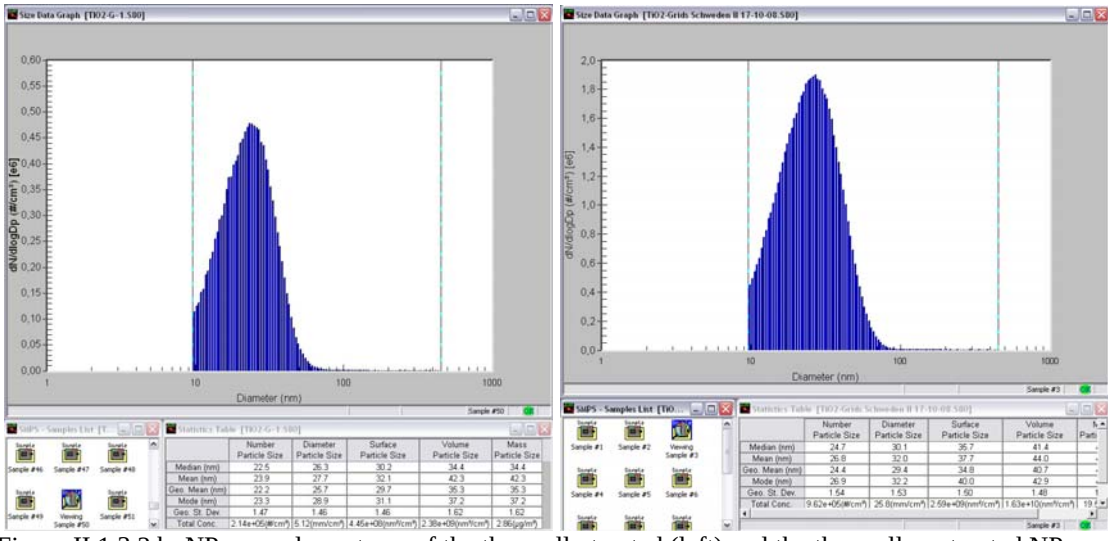


Figure II.1.3.2.b: NP aerosol spectrum of the thermally treated (left) and the thermally untreated NP (right)

d. *TiO_2 NP morphology*: In the HRTEM the coagulated structure of the 3-5 nm primary particles in aggregated / agglomerated NP is visible Figures II.1.3.2.c and II.1.3.2.d.

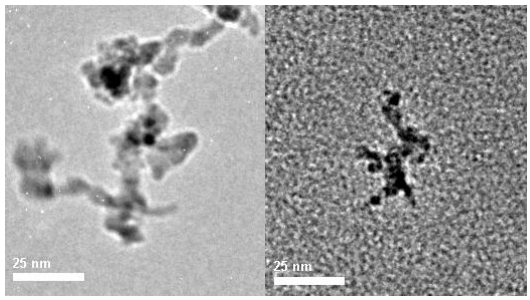


Figure II.1.3.2.c: left: thermally aftertreated crystalline TiO_2 NP; 39170x magnification. Right: thermally untreated amorphous TiO_2 NP; 39170x magnification

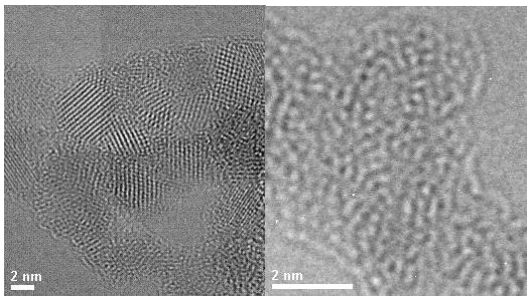


Figure II.1.3.2.d: left: thermally aftertreated crystalline TiO2 NP; 1090000x magnification. On the right: thermally untreated amorphous TiO2 NP; 559000x magnification.

But differences between the thermally untreated and the thermally treated TiO_2 NP are not visible. This means that the thermal treatment results in a slight sintering of the primary particles and stable fixation of the ^{48}V radio-label – which is reflected by the slightly smaller NP size – but no massive melting of the primary particles was found. The melting point of both crystal forms (anatase and rutil) amounts to 1500°C. This follows also from the measured specific BET surface – see next below.
Furthermore the high-resolution TEM pictures show that the thermally treated NP have purely crystalline structures, but the thermally untreated NP have purely amorphous structures (Geiser et al., in 2005).

e. *Chemical composition of the TiO_2 NP*: The XEDS analysis in the HRTEM confirmed the chemical compounding of the TiO_2, see Figure II.1.3.2.e. This was also confirmed by electron energy loss spectroscopy (EELS) in TEM.

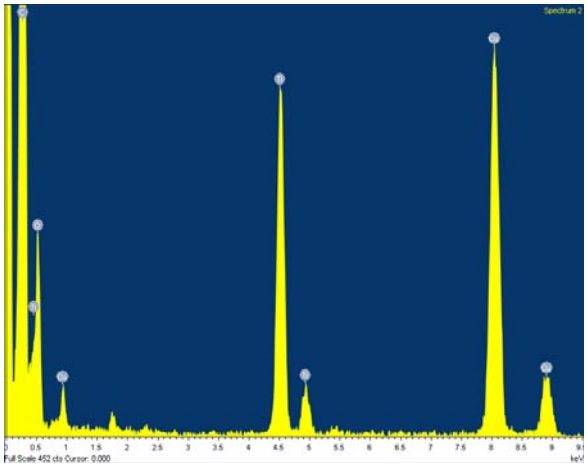


Figure II.1.3.2.e: XEDS analysis in the HRTEM confirms that the NP are chemically composed of TiO_2. Beside the peaks for Ti and O_2 the copper lines of the EM substrate are recognised.

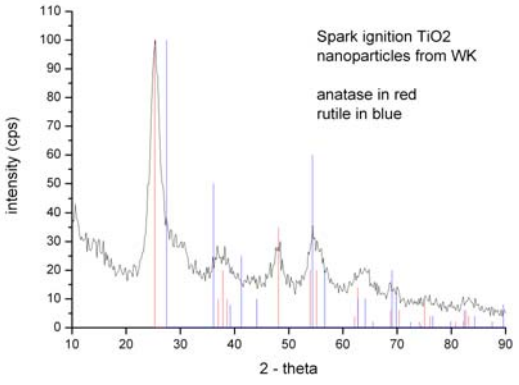


Figure II.1.3.2.f: X-ray diffraction analysis (XRD) of a TiO_2 NP sample on a silicone wafer demonstrating dominance of anatase.

f. C*rystallinity of the TiO_2 NP*: The X-ray diffraction analysis (XRD) of a TiO_2 NP sample on a silicon wafer showed that the TiO_2 NP primarily consists of anatase with a very small amount of rutil (<5%), Figure II.1.3.2.f.

g. *Specific surface of the TiO_2 NP*: The analysis of the specific surface (BET) of the thermally treated TiO_2 NP amounted to 270 m²/g and was slightly smaller than the thermally untreated TiO_2 NP of 330 m²/g.

h. *In vitro solubility of ^{48}V radio-labeled TiO_2 NP:* The in vitro dissolution tests of untreated ^{48}V-TiO_2 NP generated by spark ignition the production in a tube furnace, showed a considerable ^{48}V-solubility up to 25% after 24 hours. In contrast, when the NP-aerosols had been heated up to 950°C in a tube furnace immediately after their generation, the dissolved ^{48}V-fraction of the thermally treated TiO_2 NP amounted to only 2% after 24 hours.

II.1.3.3 Results of in vivo studies

II.1.3.3.1 Biokinetics auf dissolved ^{48}V following intratracheal or intravenous applicationin adult rats

These investigations were indispensable for the correction of the instilled and inhaled ^{48}V-TiO_2-NP. Therefore, these results are described at first in this report.
As shown in Figure II.1.3.3.1.a, 50 % of the dissolved ^{48}V is eliminated by urine within the first 24 hours after application. However, some ^{48}V fractions remain in single organs as well as the remainder. The fraction of dissolved ^{48}V, remaining in single organs as well as the remainder is similar for both applications after 7 and 28 days. Admittedly, the amount of dissolved ^{48}V in the blood is decreasing drastically from day 1 to day 7.
As described in chapter 2.9.2 and shown in the equations 6 - 8, the particulate fractions after ^{48}V-TiO_2-NP inhalation or instillation will be calculated correcting for dissolved ^{48}V fractions.

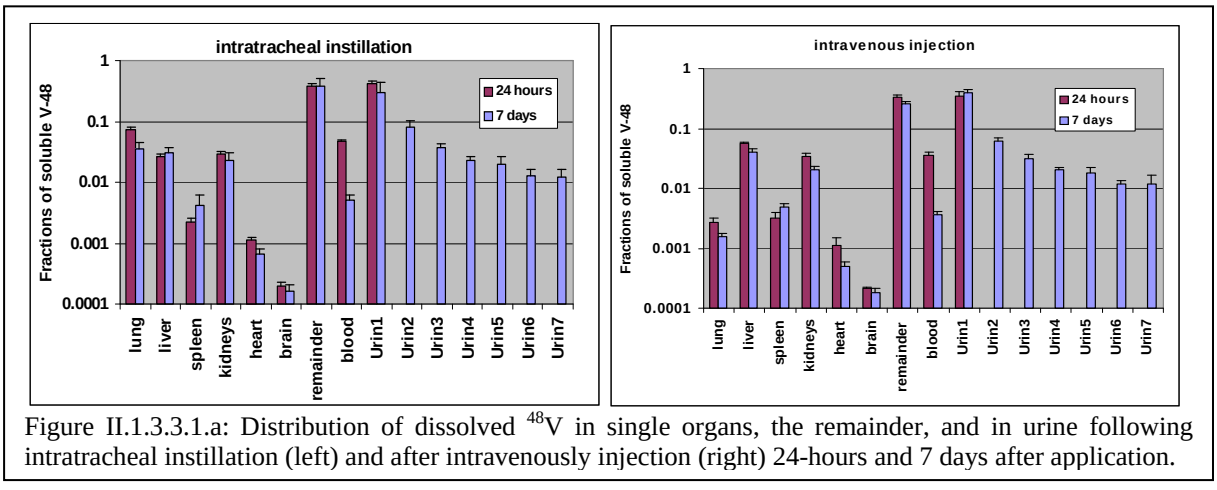


Figure II.1.3.3.1.a: Distribution of dissolved ^{48}V in single organs, the remainder, and in urine following intratracheal instillation (left) and after intravenously injection (right) 24-hours and 7 days after application.

II.1.3.3.2 Inhalation of 20 nm ^{48}V radioactive labelled TiO_2-NP by computer-controlled-breathing of intratracheal intubated adult rats

Some parameters of the ^{48}V-TiO_2-NP aerosol used for the 24-hours retention study, are shown in Figure II.1.3.2.a and II.1.3.2.b. After the 2-hours-inhalation-period of ^{48}V-TiO_2-NP, the rats were sacrificed after several time points after: a) 0 hours; b) 4 hours; c) 24 hours; d) 7 days; e) 28 days. The intratracheal intubation leads to a deposition fraction in the thoracic airways of about 0.15 – 0.2. In the first 24 hours following airway deposition, most ^{48}V-TiO_2-NP are transported to the larynx by the mucociliary escalator and are subsequently swallowed. This fraction of ^{48}V-TiO_2-NP can be measured by gamma

spectrometry in the gastro-intestinal tract and in the feces. As described in chapter 2.9.1 and in equation 4 these measured data were subtracted from the total ^{48}V activity and re-normalized. Since the fraction of ^{48}V, found in urine (presumed to represent the dissolved ^{48}V) was not more than approximately 0.05, the corrections in the respective organs did not lead to ^{48}V-TiO_2-NP fractions below the detection limit of the γ-spectrometers. Note however, due to this conservative assumption that all ^{48}V radioactivity found in urine results from ^{48}V dissolution we were not able to distinguish whether ^{48}V-TiO_2-NP were excreted in urine. According to the corrections of the raw data regarding the fast clearance of NP from the airways and the dissolved ^{48}V fraction, more than 90 % of the ^{48}V-TiO_2-NP remained in the lungs during the whole observation period (Figure II.1.3.3.2.a.). Interestingly, immediately after the two-hours inhalation the ^{48}V-TiO_2-NP fractions in the secondary organs and blood are lower than at later time points indicating that the process of translocation across the air-blood barrier is not yet finished. After four hours a maximum of translocated ^{48}V-TiO_2-NP was observed in liver, kidneys, heart, and blood. For these organs, elimination of the ^{48}V-TiO_2-NP was found starting after day 7. In contrast, the maximum of ^{48}V-TiO_2-NP in the brain was observed seven days after inhalation. In case of the remainder, consisting of head, skin, muscles, bones, and adipose tissue, a NP translocation fraction of about 2-7 % of the inhaled ^{48}V-TiO_2-NP was detected during the whole time. In the remainder the accumulation of ^{48}V-TiO_2-NP was higher than in the sum of all secondary organs. Importantly, the NP translocation to the spleen was different compared to the other organs. A consistent increase of the translocated NP fraction was observed during the whole time of the measurement and no decline was detected during 28 days. However, a later elimination of nanoparticles can not be excluded and should be a matter of further studies.

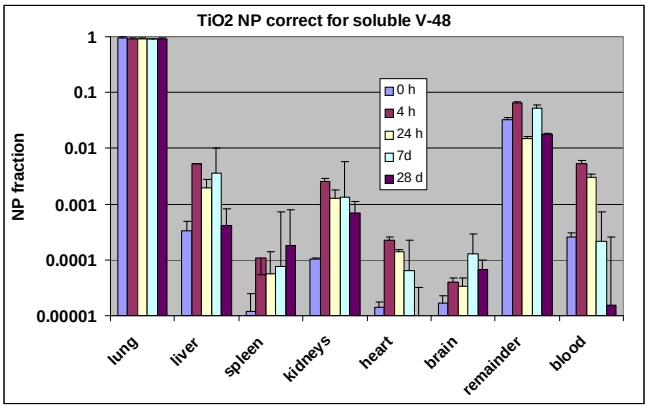


Figure II.1.3.3.2.a: Biokinetics of TiO_2-NP following to a 2-hours inhalation period and various autopsy time points (0h, 4h, 24h, 7d und 28d).

In addition to secondary target organs, the lungs as the primary organ of intake were investigated distinguishing between NP in the lavaged lungs and in the broncho-alveolar lavage fluid (BALF). Importantly, about 60 % of the ^{48}V-TiO_2-NP were retained in the lungs and not accessible in BALF (Figure II.1.3.3.2.b). After 24 hours the lavageable NP fraction accounted for about 20%. This agrees very well with previous studies which describe higher contents of micron-sized particles in the BALF and similar fractions of NP in BALF (Semmler-Behnke et al., 2007; Takenaka et al., 2006; Lehnert et al., 1989). Interestingly, no free nanoparticles were detected in the BALF supernatant later than 24 hours after

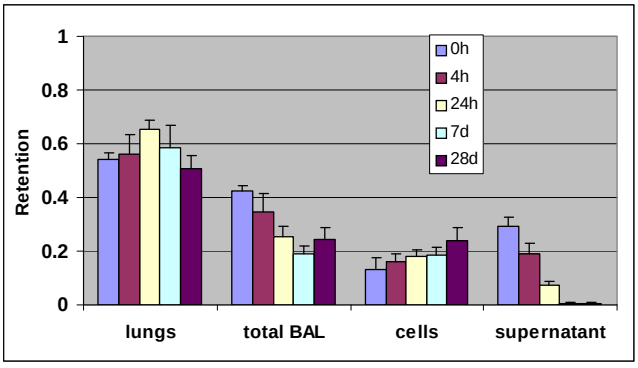


Figure II.1.3.3.2.b: Fractions of NP found in lavaged lungs, broncho alveolar lavage fluid, and in the BAL cells and supernatant. Note, the sum of "lungs" and "total BAL" is smaller than unity because of the translocated fraction.

inhalation, indicating either an uptake of the NP by macrophages or by the epithelium (Figure II.1.3.3.2.b).

II.1.3.3.2. Intratracheal Instillation and intravenous injection of P25 ^{48}V-TiO$_2$-NP (<100 nm) in adult rats.

In this study a suspension of proton activated ^{48}V-TiO$_2$-NP (P25; Evonik) was intratracheally instilled. Approximately a fraction of 0.2 – 0.3 of P25 ^{48}V-TiO$_2$-NP was deposited in the thoracic airways. In the first 24 hours, almost all of these NP were transported to the larynx by the mucociliary escalator and subsequently swallowed. The fraction of swallowed particles was measured for each individual animal and was thereupon subtracted from the entire activity as it is described in chapter 2.9.1 and equation 4.
After 24 hours, a broad distribution of the ^{48}V-radioactivity was observed in the body (Figure II.1.3.3.3.a). Importantly, approximately 20 % of the instilled ^{48}V activity was detected in urine. Since we assume dissolution of the ^{48}V radio-label out of the matrix of ^{48}V-TiO$_2$-NP, we corrected these activities as we corrected the observed ^{48}V activities following to TiO$_2$ NP-inhalation. Thereby we assume that the whole activity in the urine results from dissolved ^{48}V. Based on this estimate approximately 40 % of the ^{48}V-activity was dissolved from the P-25 ^{48}V-TiO$_2$-NP (Figure II.1.3.3.3.a). Hence, particulate ^{48}V-TiO$_2$-NP were only detected in lungs, liver, and spleen. Activities in all other organs were below the detection limit (Figure II.1.3.3.b.). This was true for both, P-25 ^{48}V-TiO$_2$-NP intratracheal instillation and intravenous injection.

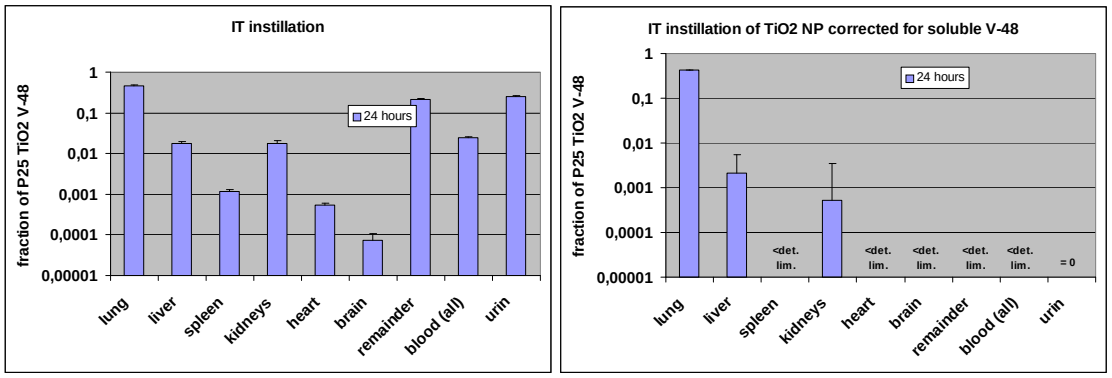


Figure II.1.3.3.3.a: Raw data of distribution of ^{48}V-TiO$_2$-NP investigated 24 hours after intratracheal instillation (left figure) and after the correction regarding the dissolved fraction of ^{48}V (right figure)

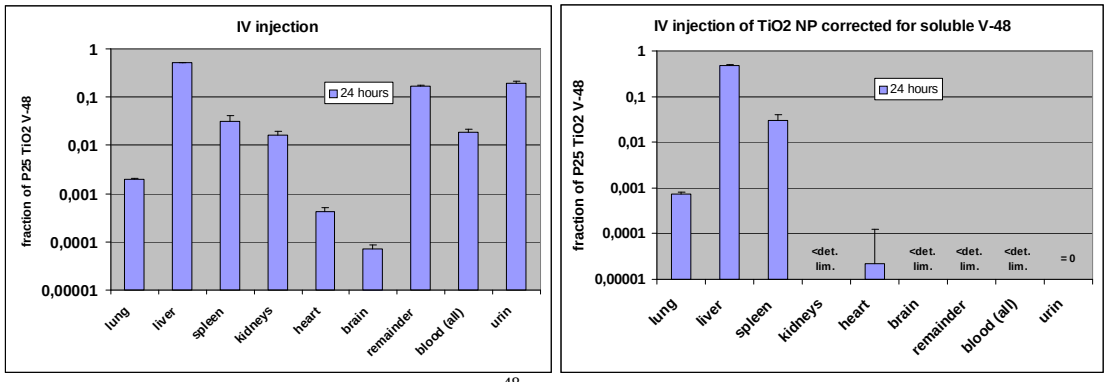


Figure II.1.3.3.3.b: Raw data of distribution of ^{48}V-TiO$_2$-NP, investigated 24h after intravenous injection (left figure) and after the correction regarding the dissolved ^{48}V (right figure)

II.1.3.3.3. Influence of the material of the particles regarding the translocation via the blood-air-barrier of the lung 24 hours after inhalation

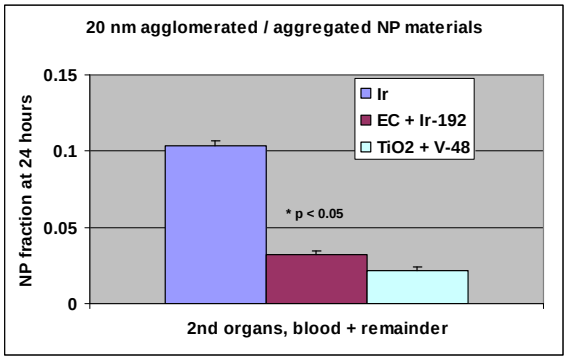


Figure II.1.3.3.3.c: Translocation of inhaled NP composed of different materials via the blood-air-barrier, 24 hours after inhalation

Previous studies using 20 nm similarly aggregated / agglomerated nanoparticles composed of Iridium (Ir) as well as elemental carbon (EC) have shown that translocation via the air-blood-barrier of lungs strongly depends on the NP material during the first 24 hours (Kreyling et al., 2002; Semmler et al., 2004; Semmler-Behnke et al., 2007; Kreyling et al., 2009). Fractions of Ir-NP, EC-NP, and TiO_2-NP translocate of approximately 10 %, 3 %, and 2 %, respectively; the Ir fraction differs significantly from both other NP fractions. The importance of the respective material is further confirmed by NP accumulation in spleen, heart, brain and the remainder (Figure II.1.3.3.3.c and II.1.3.3.3.d). In contrast, during the first 24 hours following to application no material-specific translocation could be detected for the liver and kidneys. The liver is an important filter organ taking up particles by Kupffer cells; this happens independently on the respective NP material, this could be an explanation for the observed phenomenon. Interestingly, the large part of all three translocated NP (TiO_2-NP, Ir-NP, and EC-NP) were found in the remainder, consisting of muscles, adipose tissue, skin, and skeleton. In addition, more TiO_2-NP than EC-NP were found in kidneys and blood. A further study, investigating the translocation of 18 nm spherical gold nanoparticles, showed interesting results (Semmler-Behnke et al., 2008). Translocation of these perfectly spherical gold-NP is approximately one dimension smaller compared to EC-NP. However, in this study the gold nanoparticles were not inhaled by the rats but administered by instillation. Therefore a direct comparison with the results from the studies with Ir, TiO_2, and EC is hardly possible. Since the gold nanoparticles were spherical, NP morphology may be another reason for the observed differences.

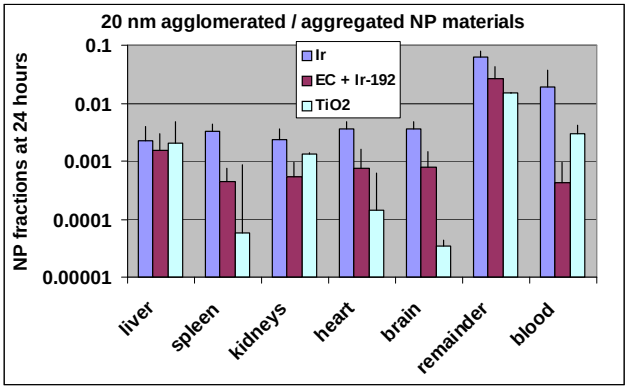


Figure 3.3.3.d: Translocation and accumulation of NP composed of different materials 24 hours after inhalation.

II.1.4. Summary and Evaluation of the Results

The prominent aim of these studies was the determination of the biokinetics of inhaled TiO_2-NP in the lower nanometer size range (approximately 20 nm). Therefore, the translocation of inhaled TiO_2-NP into blood and subsequent accumulation in secondary target organs was quantified during 28 days. Unfortunately, we were not able to disperse commercially available TiO_2 NP to the anticipated extent even though their nanostructural size was as low as about 20 nm. Therefore we decided to develop an own methodology to freshly synthesize TiO_2-NP in our lab for the use of inhalation studies. Using these NP the

aims of this project could be realized and inhalation studies were accomplished with TiO_2-NP of about 20 nm size. These NP underwent a detailed physical and chemical characterization providing pure polycrystalline anatase TiO_2-NP of about 20 nm (geometric standard deviation 1.6) and a specific surface area of 270 m^2/g. In addition, we developed techniques for sufficiently stable radioactive ^{48}V labelling of the TiO_2 NP. The kinetics of the solubility of ^{48}V was thoroughly determined. Due to the low solubility of the radioactive ^{48}V radiolabel finally achieved, our biokinetic data were thoroughly evaluated providing the biokinetics of 20 nm ^{48}V-TiO_2-NP after a controlled short-term inhalation.
Unfortunately, the use of commercially available TiO_2 NP (P25, Evonik) was not possible although these NP would have been more relevance because of their wide spread use in sciences, technologies, medicine and by the population at large. The poor dispersibility of the TiO_2-NP not below a median diameter of 150 nm and the pronounced dissolution of the radioactive ^{48}V label were the limitations. Therefore the results can not be used without reservation and may only depict a trend at best. However, the results are not in conflict with the results from the 20 nm TiO_2 NP inhalation study. Developing solutions for the described problems appears to be an urgent need for the future.
In summary, our experiments have shown that most of the inhaled TiO_2-NP were retained in the lungs as observed earlier for other NP materials iridium and elemental carbon of same same size and morphology. Small fractions of TiO_2-NP translocate across the air-blood-barrier and accumulate in secondary target organs, soft tissue and skeleton. The amount of translocated TiO_2-NP is approximately 2-7% of TiO_2-NP deposited in the lungs. A prominent fraction of these translocated TiO_2-NP was found in the remainder. Smaller amounts of TiO_2-NP accumulate in secondary organs following particular kinetics. TiO_2-NP translocation was grossly accomplished within the first 2-4 hours after inhalation followed by retention in all organs and tissues studied without any detectable clearance of these biopersistent TiO_2-NP within 28 days. Previously we have investigated similar long-term biokinetics of iridium NP up to six months after a single short-term inhalation which also showed persistent long-term retention not only in the lungs but also in secondary organs, the skeleton and soft tissue (Kreyling et al., 2002; Semmler et al., 2004; Semmler-Behnke et al., 2007). Therefore, our data suggest that during repeated or chronic exposure to insoluble NP the translocated fraction of particles will accumulate in secondary target organs. Because of the biopersistence much higher NP doses are expected to accumulate in secondary target organs than after our two-hours inhalation studies. According to our biokinetic data and their extrapolation, further studies are necessary investigating the biokinetics and toxicologically relevant responses and the underlying mechanisms of TiO_2 NP in secondary target organs after chronic exposure. In addition, TiO_2-NP uptake via other routes like the gastro-intestinal-tract after oral administration needs further investigation as there are only very few data available.
Regarding our experiments, we conclude that translocation of NP via the air-blood-barrier of the lungs depends on the respective material and its physico-chemical properties like size etc. For a better estimation of the toxic potential of the TiO_2 NP a thorough estimate of the exposure of the total population, specific groups and susceptible individuals appears to be necessary. The need of thorough exposure studies including all possible routes of uptake into the body results from the wide spread use of nanostructured TiO_2 materials in large quantities under very many different conditions.

II.1.5 Literatur

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2. **Expected benefit and exploitation of the results;** *des voraussichtlichen Nutzens, insbesondere der Verwertbarkeit des Ergebnisses,*

The aim of this study was the biokinetics of inhaled anatase 20 nm TiO_2 NP and not any submicron and micron sized agglomerates / aggregates of nanoscaled TiO_2 NP. This was clearly achieved by using our own taylor-made anatase TiO_2 NP which we very comprehensively characterized. However, contribution had to be paid that these are no

industrial NP. Yet, we are working on the design of according inhalation studies. We are not aware of any break-through inhalation studies with an inhaled median size of less than 50 nm. Hence our study provides unique and yet unmatched data.
The exploitation of toxicological studies including biokinetics studies is rather limited as the developed concept and design, the applied methodology and the scientific progress of knowledge were obtained with limited options for exploitation. The development of market-close or market-ready products was not the intention and goal of this study.

3. **Other R&D related projects and their progress;** *des während der Durchführung des FE-Vorhabens dem AN bekannt gewordenen Fortschritts auf dem Gebiet des Vorhabens bei anderen Stellen,*

There are no efforts underway nor any publications on our unmatched and unique quantitative biokinetics of inhaled 20 nm TiO_2 NP. However, there are publications on the biokinetics and effects of submicron and micron sized agglomerates / aggregates of industrial TiO_2 NP. These biokinetics studies do not provide a consolidated balance of the whole of the retained and excreted NP in the experimental animals:

1: van Ravenzwaay B, Landsiedel R, Fabian E, Burkhardt S, Strauss V, Ma-Hock L. Comparing fate and effects of three particles of different surface properties: nano-TiO(2), pigmentary TiO(2) and quartz. Toxicol Lett. 2009 May 8;186(3):152-9. Epub 2008 Dec 7. PubMed PMID: 19114093.
2: Ma-Hock L, Burkhardt S, Strauss V, Gamer AO, Wiench K, van Ravenzwaay B, Landsiedel R. Development of a short-term inhalation test in the rat using nano-titanium dioxide as a model substance. Inhal Toxicol. 2009 Feb;21(2):102-18. PubMed PMID: 18800274.
3: Fabian E, Landsiedel R, Ma-Hock L, Wiench K, Wohlleben W, van Ravenzwaay B. Tissue distribution and toxicity of intravenously administered titanium dioxide nanoparticles in rats. Arch Toxicol. 2008 Mar;82(3):151-7. Epub 2007 Nov 14. PubMed PMID: 18000654.

Furthermore there are numerous publications on TiO2 NP characterization and health effects – many of them are in vitro studies – which we are carefully monitoring.

Radio-labelling of TiO_2 NP efforts are underway at the Institute for Health and Consumer Protection, of the Joint Research Centre in Ispra, Italy, with whom we are closely collaborating.

4. **Anticipated and already available publications of the results;** *der erfolgten oder geplanten Veröffentlichungen des FE-Ergebnisses nach § 20.*
Wenn zur Wahrung berechtigter Interessen des AN oder Dritter oder aus anderen sachlichen Gesichtspunkten bestimmte Einzelheiten aus dem Bericht vertraulich zu behandeln sind (z. B. Wahrung der Priorität bei Schutzrechtsanmeldungen), so hat der AN den AG ausdrücklich darauf hinzuweisen.

According to the rather limited period of the project (13 months + 5 months extension) and its complexity no publications in the international journals have been published. However, two publications in international peer-reviewed journals are anticipated:

1. Generation and characterization of radio-labelled TiO_2 NP produced by spark ignition
2. Biokinetics of inhaled ^{48}V radio-labelled TiO_2 NP during 28 days after short-term inhalation