**Supplementary Material for IT**

**Quantitative biokinetics of titanium dioxide nanoparticles after intratracheal instillation in rats (Part 3)**

# Wolfgang G. Kreyling§\*#, Uwe Holzwarth+, Nadine Haberl\*, Jan Kozempel+1, Alexander Wenk\*2, Stephanie Hirn\*, Carsten Schleh\*3, Martin Schäffler\*, Jens Lipka\*, Manuela Semmler-Behnke\*4 and Neil Gibson+

# \* Helmholtz Center Munich – German Research Center for Environmental Health, Comprehensive Pneumology Center, Institute of Lung Biology and Disease, Helmholtz Centre Munich, Ingolstaedter Landstrasse 1, D-85764 Neuherberg / Munich, Germany,

#  Helmholtz Center Munich – German Research Center for Environmental Health, Institute of Epidemiology 2, Ingolstaedter Landstrasse 1,D-85764 Neuherberg / Munich, Germany

# + European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Via E. Fermi 2749, I-21027 Ispra (VA), Italy

# §Corresponding author

Wolfgang G. Kreyling

Institute of Epidemiology 2

Helmholtz Centre Munich,

Ingolstaedter Landstrasse 1

D-85764 Neuherberg / Munich

Germany

# Tel.: +49 89 2351 4817

E-mail address: [Kreyling@helmholtz-muenchen.de](mailto:Kreyling@helmholtz-muenchen.de)

1 Current address:Czech Technical University in Prague, Faculty of Nuclear Sciences and Physical Engineering, Břehová 7, CZ-11519 Prague 1, Czech Republic

2 Current address: Dept. Infrastructure, Safety, Occupational Protection, German Research Center for Environmental Health, D-85764 Neuherberg / Munich, Germany

3 Current address: Abteilung Gesundheitsschutz, Berufsgenossenschaft Holz und Metall, D-81241 München, Germany

4 Current address: Bavarian Health and Food Safety Authority, D-85764Oberschleissheim, Germany

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**Radiolabeling of titanium dioxide (TiO2) nanoparticles**

Pure titanium dioxide (TiO2, type ST-01 from Ishihara Sangyo Kaisha, Ltd, Osaka, Japan) with spherically-shaped primary anatase particles of 7-10 nm crystal size, was proton irradiated using the MC40 Cyclotron at the Joint Research Centre (Ispra, Italy) in a specially designed thin target capsule that allows efficient cooling of the sample from both sides as described earlier ([Kreyling, 2011](#_ENREF_9), [Holzwarth, 2012](#_ENREF_7)) ([Hildebrand, 2015](#_ENREF_5)).

The ion beam energy was set so that, after passage through the beamline, capsule windows and the cooling water, the ion energy incident on the nanoparticles was about 13.5 MeV (1 eV ≈ 1,602 × 10-19 J), suitable for efficient creation of 48V *via* the (p,n) nuclear reaction from Ti. Two batches of 20 mg ST-01 TiO2NP were irradiated with a proton beam current of 5 μA; one with an 48V-activity concentration of 1.0 MBq•mg-1 (48V-activity per TiO2 mass) was used for the 1h, 4h and 24h retention experiments. The second one was irradiated on five consecutive days yielding an activity concentration of 2.35 MBq•mg-1 and was used for the 7d and 28d retention experiments in order to preserve sufficient sensitivity in spite of longer radioactive decay and to detect any minor redistribution and clearing processes. At these radioactivity concentrations the atomic ratio of 48V:Ti in the nanoparticles is about 2.6 × 10-7 and 6.2 × 10-7, respectively. Hence, statistically, from 1000 primary TiO2 particles only a few will contain a 48V radiolabel.

The radioactive 48V decays back to 48Ti *via* electron capture or positron emission, with a half-life of 15.97 days, emitting γ-rays with an energy of 0.99 MeV and 1.3 MeV in the process, as well as γ-rays of 511 keV that result from electron-positron annihilation.

During the activation process, each 48V-radiolabel created will recoil due to conservation of momentum and be implanted into another nanoparticle several hundred nm from its original position. Since this process, together with the chemical difference, may result in some cases in non-perfect integration of the 48V within the nanoparticle, with the risk of subsequent radiolabel leaching, the [48V]TiO2NP were carefully washed, as described below, and free 48V was removed. Due to diffusion processes in the tiny particles brining 48V-ions to the nanoparticle surface and possibly due to a very slow dissolution of the nanoparticles themselves in aqueous media (Vogelsberger, 2003, Vogelsberger et al. 2008) this leaching is likely to be a continuous process. Thus, washed suspensions of radiolabelled [48V]TiO2NP may develop new 48V-ion impurities, which may amount to 2% of the total activity within 28 days at low pH-values (Hildebrand et al., 2015).

The temperature at the centre of the nanoparticle sample during irradiation was raised by approximately 150°C but this is below the threshold temperature for the onset of structural changes ([Holzwarth, 2012](#_ENREF_7), [Inagaki, 2009](#_ENREF_8)). Radiation damage due to collision processes was calculated, indicating that only a small fraction of the NP atoms would be displaced from their lattice positions. An XRD scan of a sample of [48V]TiO2NP treated to a similar irradiation treatment showed that neither the crystalline structure (anatase) nor the average crystallite size had measurably changed during the activation process ([Gibson, 2011](#_ENREF_4), [Holzwarth, 2012](#_ENREF_7)).

**Nanoparticle preparation for administration and nanoparticle characterization**

After proton irradiation, the nanoparticles were recovered from the irradiation capsule and subjected to a procedure to disperse them, remove free 48V and size-select a ‘nano-fraction’ of the material for the subsequent experiments by removing larger aggregates/agglomerates. Thus, a size selected ‘nano-fraction’ of radiolabeled [48V]TiO2NP of aggregated/agglomerated ST-01 TiO2NP was used for the experiments.

The [48V]TiO2NP were recovered from the irradiation capsule, suspended in 150 μL sodium pyrophosphate solution (0.5 M), used as a surfactant and filled up with double-distilled water (dd H2O) to 3 mL. This suspension was ultrasonicated for 1 min and filtered through a 0.22 μm disposable membrane syringe filter to remove large agglomerates and then sonicated again in a water-bath for 1 min (Branson 15010 Bath Sonicator, 42 kHz, 70W). It was then maintained for 24 hours at 70°C. Afterwards, soluble 48V was separated from the nanoparticles by centrifugation (1 min; 4000•*g*). The nanoparticle pellet was resuspended in 2 mL of 0.1 mM sodium pyrophosphate solution and sonicated in a water-bath (30 min). Afterwards, the nanoparticle sodium pyrophosphate suspension was incubated for another 30 min at 70°C, the suspension was centrifuged again (1 min; 4000•*g*), the pellet recovered and resuspended in 2 mL dd H2O, sonicated (1 min) and filtered through a 0.22 μm syringe filter. The filter was discarded and the suspension was washed using a 3 kDa Amicon centrifugation filter (50 min, 4000 × *g*). Up to this preparation step the total loss of 48V activity due to ion release and activiy retained in large aggregates or agglomerates was about 15%. The nanoparticles were recovered from the Amicon filter cup with 250 µL dd H2O and then topped up to a volume of 2 mL with dd H2O. The suspension was incubated for 12 hours at 70 °C and afterwards sonicated in a water bath (60 min). The resulting suspension was centrifuged (6 min, 4000•*g*) and the supernatant containing the smallest particle fraction was taken for the experiments.

While this procedure contains only one washing step by centrifugal ultrafiltration, keeping track of the various resuspension steps of the recovered pellet, the sodium pyrophosphate that might still be present in a single dose of [48V]TiO2NP is of the order of 1 pmol.

For each of the five individual biodistribution studies at different retention time points we prepared new [48V]TiO2NP suspensions, starting from the same TiO2 anatase ST-01 stock and using the protocol given above. A simultaneous start of [48V]TiO2NP application to all rats (four rats per group, five retention time points, three application routes) was not possible due to the rather short radioactive decay half-life of 48V (15.97 d) and the large number of samples (typically more than 20 for each rat, resulting in more than 1200 for the whole experimental series) to be sequentially quantified by -ray spectrometry with sufficient counting statistics over measuring times of up to four hours. As a result, the experiments investigating the five different retention time points had to be staggered. However, each [48V]TiO2NP suspension was used for all three biodistribution studies reported in the three relevant articles in this volume of Nanotoxicology – either after intravenous injection (IV) ([Kreyling et al., 2017 Part 1](#_ENREF_11)) or after intra-esophageal instillation (gavage) ([Kreyling et al., 2017 Part 2](#_ENREF_13)) or after intratracheal instillation (IT) ([Kreyling et al., 2017 Part 3](#_ENREF_12)). Thus each retention time point was analyzed for all three exposure routes starting from the same [48V]TiO2NP suspension batch with the same nanoparticle properties.



**Figure S1:** Hydrodynamic diameter of the five separately prepared [48V]TiO2NP suspensions measured directly before administration in biodistribution studies at five different retention time points (1h, 4h, 24h, 7d and 28d).

The hydrodynamic diameter of the nanoparticles and the zeta potential were measured in triplicates several times during preparation for control purposes using dynamic light scattering (DLS) by a Malvern Zetasizer (Malvern, Herrenberg, Germany). Finally, prior to each *in vivo* application, the size distribution was measured again by DLS. Figure S1 shows the hydrodynamic size distributions of the separately prepared [48V]TiO2NP suspensions measured by DLS immediately prior to the *in vivo* application. The size distributions are plotted as intensity frequencies. While there was some variability in the size distributions after the dispersion and size selection procedure they overlap very well. Only the suspension for the 4h time point appeared to have a particle size somewhat smaller than the others. Mean data of the numeric evaluation are compiled in Table S1.

**Table S1:** Physicochemical characteristics of the [48V]TiO2NP suspensions used for 5 different retention times. For each retention time point the same suspension was applied by three different exposure routes: intravenous injection (IV), intra-esophageal instillation (GAV) or intratracheal instillation (IT).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Retention time |  | 1h | 4h | 24h | 7d | 28d |
| Zeta Potential\* | [mV] | -38.9 ± 4.2 | -33.2 ± 2.4 | -29.9 ± 8.1 | -42.7 ± 9.2 | -35.2 ± 7.6 |
| Z-average\* | [nm] | 93 | 72 | 93 | 82 | 101 |
| PDI\* |  | 0.157 | 0.228 | 0.160 | 0.197 | 0.135 |
| Crystal structure§ |  | anatase | anatase | anatase | anatase | anatase |
| Primary part. diameter§ | [nm] | 7 - 10 | 7 - 10 | 7 - 10 | 7 - 10 | 7 - 10 |
| Specific surface area (BET) & | m2•g-1 | 316 | 316 | 316 | 316 | 316 |

\* measured with a Malvern Zetasizer;

PDI – polydispersity index;

§ manufacturer dara confirmed by XRD measurements;

&BET – [Brunauer](http://de.wikipedia.org/wiki/Stephen_Brunauer), [Emmett](http://de.wikipedia.org/wiki/Paul_Hugh_Emmett), [Teller](http://de.wikipedia.org/wiki/Edward_Teller) Method to measure specific surface area. (own measurements; Autosorb-1, Quantachrome Instruments, Florida, USA).

The mean ± STD of Z-averages and PDI of all five distributions (given in Table S1) are 88 ± 11 nm and 0.18 ± 0.04, respectively. The PDI indicates that the size distributions are polydisperse but with a rather narrow size distribution. Whether the sizes were plotted as intensity or volume or number frequency distributions only one single peak was detected. Therefore, the volume and number distributions may be considered as reliable. The mean ± STD of the volume and number distribution maxima of all five spectra are 65 ± 12 nm and 48 ± 11 nm, respectively. We conclude therefore that we successfully selected a reasonably narrowly distributed ‘nano-fraction’ of the original ST-01 TiO2 particles aggregates/agglomerates by applying the multiple step size selection procedure described above. This was supported by TEM investigations (presented in Figure S2) which revealed approximately spherical aggregates/agglomerates of roughly 50 nm in diameter after the size selection and dispersion process. Samples for transmission electron microscopy were prepared from the aqueous suspensions on glow discharged 300 mesh Formvar®-coated copper grids and investigated with a Philips 300 TEM at 60 kV acceleration voltage.

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**Figure S2:** Transmission electron micrograph of size-selected TiO2NP sampled immediately after the preparation procedure described above. TEM sample preparation leads to ‘clumping’ together of aggregates/agglomerates on the support grid.

From the known activity concentration of the proton irradiated batches of TiO2 nanoparticles (1.0 MBq•mg-1 and 2.35 MBq•mg-1) and the 48V-radioactivity of the applied [48V]TiO2NP, the applied nanoparticle mass was calculated for each application route and at each of the five retention time points as reported in Table S2. While the aggregate/agglomerate numbers applied to each rat were not possible to calculate, since it was impossible to determine the apparent specific density of the [48V]TiO2NP due to their porous, chain-like structure shown in Figure S2 the number of primary nanoparticles administered can be estimated if necessary.

**Animals**

Healthy, female Wistar-Kyoto rats (WKY/Kyo@Rj rats, Janvier, Le Genest Saint Isle, France), 8–10 weeks of age (270 ± 9.2 g mean (± SD) body weight) were housed in pairs in relative-humidity and temperature controlled ventilated cages (VentiRack Bioscreen TM, Biozone, Margate, UK) on a 12-hr day/night cycle. Rodent diet and water were provided *ad libitum*. After purchase the rats were adapted for at least two weeks and then randomly attributed to the experimental groups. All experiments were conducted under German federal guidelines for the use and care of laboratory animals and were approved by the Regierung von Oberbayern (Government of District of Upper Bavaria, Approval No. 211-2531-94/04) and by the Institutional Animal Care and Use Committee of Helmholtz Centre Munich.

**Nanoparticle application and animal maintenance in metabolic cages**

Although we aimed to obtain the same [48V]TiO2NP concentration in each of the five suspension preparations, in fact the [48V]TiO2NP concentrations varied by a factor of two due to the multiple step preparation process. In addition, we found pronounced differences in [48V]TiO2NP retention in the syringes used for each application even though we had minimized the time between finalizing the preparation of the [48V]TiO2NP suspension, filling the 1-mL minimal-dead-space-insulin syringe (Omnican 100, Braun, Melsungen, Germany, specified dead space 0.4 µL) and application to each rat to less than two minutes. These material losses in the syringe can be determined for each rat by measuring the residual radioactivity of the [48V]TiO2NP left in the syringe and flexible cannula after application of the [48V]TiO2NP suspension. Actually, the losses in the syringe varied from 10% up to 50% and were, indeed, the most prominent cause of the variability in the applied dose (see data compiled in Table S2). These losses that occurred during the application procedure are matched by the difference between the total radioactivity loaded into the syringe and the total balance of the [48V]TiO2NP activity determined in each rat during the experiments. Similar observations were not made in the auxiliary studies where radioactivity was applied in ionic form.

Such significant losses may result from electrostatic [48V]TiO2NP adhesion to the plastic walls of the syringe and may occur not only with the nanoparticle suspensions we were using but as well with those used by other investigators using the same or similar application methods. Since the use of radiolabelled nanoparticles requires much less cumbersome specimen preparation as required for other analytical techniques when striving for a complete, balanced biodistribution of nanoparticles in laboratory animals, such detailed measurements are usually not performed with non-radioactively labeled nanoparticles. Consequently such losses will usually remain undiscovered and might have contributed substantially to unresolved variability of the nanoparticle doses applied to experimental animals in other similar experimental studies reported in scientific literature.

**Table S2:** Application of [48V]TiO2NP suspensions *via* IT-instillation. The effectively instilled 48V activity into the rats (first row) was determined from the balance over all tissue samples (including the carcass), organs, urine and feces collected from each rat. It was frequently much smaller than the 48V activity loaded into the into the 1mL insulin-syringes (Omnican® 100, Braun, Melsungen, Germany; specified dead volume < 0.4µL) due to retention of nanoparticles in the syringe and flexible cannula. The last row presents the percentages of the 48V-activities retained in the syringe and flexible cannula. The applied nanoparticle mass refers to the mass effectively received by the rats.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Retention time |  | 1h | 4h | 24h | 7d | 28d |
| Effective  48V radioactivity received by rats# | [kBq] | 11.7 ± 1.6 | 10.9 ± 1.0 | 11.1 ± 1.3 | 145.8 ± 44.1 | 47.8 ± 9.2 |
| Applied [48V]TiO2NP mass& | [µg] | 11.7 ± 1.6 | 10.9 ± 1.0 | 11.1 ± 1.3 | 62.1 ± 18.7 | 20.3 ± 3.9 |
| Percentage of [48V]TiO2NP retained in the syringe after application | % | 51 ± 14 | 38 ± 6 | 13 ± 4 | n.d. | n.d. |

#Mean ± SD of integrally measured 48V radioactivity of each rat, n = 4 for each group

&Mean ± SD of [48V]TiO2NP mass calculated from the radioactivity concentration (1.0 MBq⋅mg-1 for 1h, 4, 24, and 2.35 MBq⋅mg-1 for 7d and 28d) and the integrally measured 48V-radioactivity of each rat, n = 4 for each group

n.d. = not determined

Nanoparticle suspensions were administered immediately after preparation to non-fasted animals as described earlier ([Kreyling, 2014](#_ENREF_10), [Schleh, 2012](#_ENREF_18), [Hirn, 2011](#_ENREF_6), [Semmler, 2004](#_ENREF_20), [Kreyling, 2002](#_ENREF_14)). Firstly, the rats were anesthetized by inhalation of 5% isoflurane in oxygen until muscular tonus relaxed. For IT-instillation the anesthetized rat was fixed with its incisors to a rubber band on a board at an angle of 60° to the lab bench in a supine position. A flexible cannula (2.7 x 50 mm, B. Braun, Melsungen, Germany) was placed into the upper third of the trachea. For control of erroneous placement, breathing airflow through the cannula was controlled with a pneumotachograph connected to the cannula. The suspension (60 µl) which contained [48V]TiO2NP was gently instilled followed by 300-400 µl of air using a 1-ml insulin-syringe (Omnican 100, Braun. Melsungen, Germany, specified dead space 0.4µL) to improve increased deposition in the peripheral lungs. IT-instillation was aligned with the rat’s inspired breath. After removal of the tracheal flexible cannula rats were kept vertical for a few minutes and controlled for regular breathing. The syringe and cannula used for the IT-instillation were collected for measurements of the residual retained [48V]TiO2NP activity.

After application of the nanoparticle suspensions, rats were kept individually in metabolism cages (Tecniplast, Hohenpreissenberg, Germany) for quantitative but separate collection of urine and feces. For ethical reasons the 28-day groups of rats were maintained individually on cotton cloths in normal cages starting immediately after [48V]TiO2NP application. The cloth was replaced by a new cloth every 3-4 days, and from the collected cloth fecal droppings were separated quantitatively. After separation, the cloth contained only 48V originating from urine which had soaked and dried.

Five [48V]TiO2NP suspensions were prepared for each of the five retention time points to be studies and the same suspension was used for all three application routes. Table S3 shows the effectively applied [48V]TiO2NPmassand activitydoses after IT-instillation, after intra-esophageal instillation (gavage) ([Kreyling et al., 2017 Part 2](#_ENREF_13)) and after intravenous (IV) injection ([Kreyling et al., 2017 Part 1](#_ENREF_11)) used to study the 24h retention time point.

**Table S3:** 48V-TiO2 NP mass and 48V radioactivity *via* three routes of application. Effective dose is the sum of radioactivity of all organs, tissues and urinary and fecal excretion of a given rat averaged over four rats; 24-hour groups, mean ± SD, n = 4.

|  |  |  |  |
| --- | --- | --- | --- |
| 24 hours | IT-instillation | Gavage | IV-injection |
| Effective 48V radioactivity [kBq] | 11.7 ± 0.6 | 12.2 ± 1.3 | 16.6 ± 0.2 |
| Applied 48V-TiO2 NP mass [µg] | 11.7 ± 0.6 | 12.2 ± 1.3 | 16.6 ± 0.2 |

**Sample preparation for radiometric analysis**

At the chosen retention time points of 1h, 4h, 24h, 7d and 28d after intratracheal [48V]TiO2NP instillation rats were anesthetized (by 5 % isoflurane inhalation) and euthanized by exsanguination *via* the abdominal aorta as described earlier ([Kreyling, 2014](#_ENREF_10), [Schleh, 2012](#_ENREF_18), [Hirn, 2011](#_ENREF_6), [Semmler, 2004](#_ENREF_20), [Kreyling, 2002](#_ENREF_14)). In this way approximately 60-70% of the total blood volume could be recovered. Organs, tissues, the remaining carcass and excretions as specified in Table S4 were collected for radiometric analysis . During dissection, none of the organs were cut and all fluids were cannulated (where necessary) in order to avoid any cross contamination. Additionally , in each rat a broncho-alveolar lavage (BAL) was performed and the lavaged lungs, the lavaged cells (BALC) and the lavage fluid (BALF), separated by centrifugation, were analysed separately. Organs, tissues, remaining carcass and excretions were collected for radio-analysis (see Table S4).

**Table S4:** Organ, tissue and other samples prepared for radiometric analysis:

|  |  |  |  |
| --- | --- | --- | --- |
| Lavaged lungs\* | Trachea + main bronchi | BAL cells\* | BAL fluid\* |
| liver | spleen(2nd) | kidneys(2nd) |  |
| brain(2nd) | heart(2nd) | uterus(2nd) |  |
| GIT: gastro-intestinal tract, comprising oesophagus, stomach, small and large intestine | | | |
| total skin(2nd) | muscle sample(2nd)& | head: skinned head(2nd)  without brain | |
| exsanguinated blood sample(2nd) | | | |
| bone sample(2nd)  (humerus or femur carefully cleaned from muscles and fat) | | | |
| skeleton: estimated from measured bone sample activity times 10% BW (derived in Eqn. (S8)) | | | |
| carcass(2nd) : total remaining carcass beyond the above listed organs and tissues, consists of skeleton and soft tissue | | | |
| soft tissue: non-osseous tissues of the carcass calculated to be the difference between carcass and skeleton activities; it includes muscles, fat, skin, connective tissue, paws | | | |
| Secondary organs: sum of all organs(2nd) indexed and listed above | | | |
| excretion: total daily urine and feces, collected separately | | | |

\* Lavaged lungs; broncho-alveolar lavage (BAL); separation of BAL cells from BAL fluid supernatant by centrifugation as described in ([Kreyling, 2014](#_ENREF_10))

& muscles were collected from both hind legs

(2nd) The index “(2nd)“ indicates those secondary organs and tissues which may accumulate [48V]TiO2NP after translocation across the air-blood-barrier (ABB) into blood circulation.

**Radiometric and statistical analysis**

The 48V radioactivity of all samples was measured by γ-ray spectrometry without any further physico-chemical preparation in either a lead-shielded 10-mL or a lead-shielded 1-L well type NaI(Tl) scintillation detector as previously described ([Kreyling, 2014](#_ENREF_10), [Schleh, 2012](#_ENREF_18), [Hirn, 2011](#_ENREF_6), [Semmler, 2004](#_ENREF_20)). For radiometric analysis the 511keV γ-ray emission – resulting from electron-positron annihilation - was used. The count rates were corrected for physical decay and background radiation. Additionally, count rates were calibrated to a 48V reference source in order to correlate 48V-radioactivity to the mass of the TiO2NP. Samples yielding net counts (*i.e.*, background-corrected counts) in the 511keV region-of-interest of the 48V γ-ray spectrum were defined to be below the detection limit (< 0.2 Bq) when they were less than three standard deviations of the background count rate in this region-of-interest.

For a complete balance of the administered 48V-radioactivity within each rat, the 48V activities of all individual samples were summed up for each rat and used as a denominator for the calculation of the 48V-activity fraction in each sample. These fractions were averaged over the four rats of each group and are reported with the standard error of the mean (SEM) as described earlier ([Kreyling, 2014](#_ENREF_10), [Schleh, 2012](#_ENREF_18), [Hirn, 2011](#_ENREF_6), [Semmler, 2004](#_ENREF_20), [Kreyling, 2002](#_ENREF_14)).

All calculated significances are based on a One-Way-ANOVA test and a *post hoc* Tukey Test. In case of an individual two group comparison, the unpaired t-test was used. p ≤ 0.05 was considered significant.

**Blood correction and total blood volume**

In order to obtain the true value of 48V-activity in the organs and tissues of interest the radioactivity contributed by the residual blood retained after exsanguination had to be subtracted. In the case of the carcass, the difference between the estimated total blood volume of the animal and the sum of all organ blood contents and the collected blood sample was calculated to be the blood volume of the carcass.

**Table S5:** Organ specific weight factors  for the residual blood in the organ tissue after exsanguination given as residual blood weight per organ weight according to ([Oeff, 1955](#_ENREF_17)).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| lung (g•g-1) | liver (g•g-1) | spleen (g•g-1) | kidneys (g•g-1) | brain (g•g-1) |
| 0.28 ± 0.10 | 0.14 ± 0.03 | 0.16 ± 0.04 | 0.22 ± 0.04 | 0.018 ± 0.001 |
| heart (g•g-1) | GIT§  (g•g-1) | muscle (g•g-1) | fat (g•g-1) | thyroid\* (g/organ) |
| 0.15 ± 0.02 | 0.020 ± 0.006 | 0.016 ± 0.002 | 0.012 ± 0.002 | 0.008 ± 0.001 |

\* thyroid given for complete organ; § intestine and stomach

The blood contents of organs and tissues were calculated according to the findings of Oeff and Konig ([Oeff, 1955](#_ENREF_17)) and the 48V-radioactivities of the organs were corrected for these values as follows:

|  |  |  |
| --- | --- | --- |
|  |  | (S1) |

where denotes the 48V activity measured in the organ "i" at the time point in Bq which is corrected for the residual blood content by subtracting calculated according to

|  |  |  |
| --- | --- | --- |
|  |  | (S2) |

making use of the mass and the activity measured for the blood recovered from exsanguination at the time point , the mass of the organ  and the organ specific weight factor  compiled in Table S5 for the residual blood in the organ or tissue according to ([Oeff, 1955](#_ENREF_17)). The total blood volume *BV* in mL was estimated to be

|  |  |  |
| --- | --- | --- |
|  |  | (S3) |

according to the work of Lee and Blaufox ([Lee, 1985](#_ENREF_15)), where *BW* denotes the body weight in g.

To determine the 48V-activity in the residual blood of the remaining carcass or skeleton for each rat the following procedure was applied. Firstly, the mass of the residual blood volume in the carcass or skeleton (tissue = carcass or skeleton) was calculated by subtracting from the mass of the total blood volume  the mass of the sampled blood volume  and the sum of the masses of the residual blood volumes of all organs  which had been calculated according to the findings of Oeff and Konig ([Oeff, 1955](#_ENREF_17))

|  |  |  |
| --- | --- | --- |
|  |  | (S4) |

For each rat the 48V-activity in the residual blood of the remaining carcass or skeleton (tissue = carcass or skeleton) is then given by

|  |  |  |
| --- | --- | --- |
|  |  | (S5) |

as the 48V-activity concentration determined from the blood sample times the mass of the residual blood in carcass or skeleton times the mass fraction of carcass or skeleton with respect to the rat’s body weight. This estimate assumes that the residual blood volume is proportional to the mass of either the carcass or skeleton. Since the remaining carcass consists of the skeleton and soft tissue, the 48V-activity in the residual blood of the soft tissue is the difference between 48V-activities of carcass minus skeleton:

|  |  |  |
| --- | --- | --- |
|  | . | (S6) |

To determine the contribution of the 48V-activity in the residual blood to the total 48V activity retained in all organs and tissues, the ratio

|  |  |  |
| --- | --- | --- |
|  |  | (S7) |

is defined, where the 48V-activity of the residual blood retained in each organ,, is calculated according to Eqn (S2). As the retention of 48V-activity in blood, organs and tissues depends on time and follows different patterns the ratio  itself depends on time. This is shown in Figure S3 for all organs, carcass, skeleton and soft tissue.



**Figure S3:** Ratio  of the 48V-activity in the residual blood over the measured organ or tissue activity. Panel A: liver, spleen, lungs; panel B: kidneys, heart, uterus; panel C: carcass, skeleton, soft tissue. Mean ± SEM, n = 4.

**48V-activity determination of skeleton and soft tissue**

The 48V-activity in the whole skeleton of each rat was extrapolated from the activity of a bone sample and its mass,), assuming the estimated weight of the skeleton to be 10% of the total body weight (Charkes et al., 1979)

|  |  |  |
| --- | --- | --- |
|  | . | (S8) |

For this purpose the bone sample was carefully cleaned from other tissue. The 48V-activity to be assigned to the soft tissue  of each rat was calculated from the difference of the 48V-radioactivity content of the total remaining carcass  (including soft tissue, muscle sample, skeleton, bone sample) and the activity in the skeleton as determined in Eqn (S8):

|  |  |  |
| --- | --- | --- |
|  |  | (S9) |

**Biokinetics of soluble, ionic 48V after intratracheal instillation**

Ideally, the determined activity of 48V is directly proportional to the mass of the [48V]TiO2NP. However, 48V ions that might become detached from the [48V]TiO2NP could compromise the accuracy of the determined biodistribution. Therefore, additional experiments were performed to investigate the translocation and biodistribution of soluble, ionic 48V at 24 hours and 7 days after IT-instillation. These data were used for correction of ionic 48V-release from the [48V]TiO2NP when exposed to body fluids. In order to mimic 48V release from the TiO2NP we also added to the carrier-free ionic 48V 0.33 µg•µL-1 ionic Ti(NO3)4 in 60 µL of distilled water to obtain a nitrate solution of sufficient ionic strength stably maintaining the 48V-ions and adjusted the pH value to 5. Hence, 27 kBq of ionic 48V and 20 µg of ionic Ti were instilled during inspiration into the trachea of each rat and followed by 300 – 400 µL of air. No losses of ionic 48V were found in the syringe and flexible cannula used for IT-instillation. The biokinetics were measured after 24h and 7d and the corresponding biodistributions are presented in Figure S4.



**Figure S4:** Biokinetics of soluble ionic 48V-radioisotope at 24 hours and 7 days after IT-instillation of a volume 60 µL of carrier free 48V in 0.33 µg•µL-1 ionic Ti(NO3)4 aqueous solution. Mean ± SEM, n = 4. Levels of significances: \*\* p<0.01; \*\*\* p<0.001.

The biodistribution after both time points was generally very similar to that found after IV injection with the highest amounts in urinary excretion followed by retention in the remaining carcass (including skeleton and soft tissues). However, in contrast to IV-injection ([Kreyling et al., 2017 Part 1](#_ENREF_11)), retention in the lungs (7.8% and 3.3% after 24h and 7d, respectively) is higher than in the liver (2.8% and 2.9% after 24h and 7d, respectively), while blood levels are very similar after both routes of application including the decline from day 1 to day 7.

48V found in the GIT and feces may have been originated from both mucociliary action towards the larynx and subsequent swallowing (fast lung clearance, MCC) and hepatobiliary clearance originating from the liver. Therefore, 48V-activity found in GIT and cumulative fecal excretion during the first 48 hours was excluded from the normalization and the analysis as it was not available for translocation through the air blood barrier.

**[48V]TiO2NP accumulation and retention in secondary organs and tissues: Data evaluation and correction for release of ionic 48V from [48V]TiO2NP**

[48V]TiO2NP cleared from the thoracic airways *via* mucociliary clearance (MCC) will not be available for the translocation across the air-blood-barrier (ABB). Therefore, for calculations of [48V]TiO2NP translocation across the ABB, the fraction cleared by MCC was excluded from the complete balance by subtracting the 48V-radioactivity contributions of the head (without brain), trachea, gastro-intestinal tract (GIT), and feces obtained during the first two days after IT-instillation from the overall radioactivity balance of each animal and normalizing the activities determined in all other organs and tissues to the new (reduced) balance. Thus, the amount of IT-instilled material at time  that is available for translocation through the ABB is reduced due to MCC within the first 48h after IT-instillation by

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| --- | --- | --- |
|  |  | (S10) |

where  denotes the 48V-activity determined for the head without the brain,  the 48V-activity in the trachea,  the one in the gastro-intestinal tract and  the activity of the feces collected during the first 48h. In order to determine the amount of [48V]TiO2NP that is translocated through the ABB as a fraction of [48V]TiO2NP that are available for translocation, we have to normalize all activity values measured for all organs and tissues () to a value which is smaller than the value  (also defined as initially instilled dose, ID) that has been administered by IT-instillation at time . Thus, the normalization is done with this reduced activity, defined as the *initial peripheral lung dose* (IPLD), , calculated as

|  |  |  |
| --- | --- | --- |
|  | . | (S11) |

The IPLD is a specific value for each rat used in the five retention time groups. The fraction of material accumulated in organs and tissues after passing the ABB can now be determined for each rat by normalizing the activities  according to

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| --- | --- | --- |
|  |  | (S12) |

All urinary samples and the slow long-term, macrophage-mediated [48V]TiO2NP clearance (LT-MC) fractions from days > 2 during the remaining retention time are considered as a part of the initial peripheral lung dose, IPLD, since all 48V-activity in urinary excretion must have passed the ABB and the activity included in the long-term macrophage-mediated clearance (LT-MC) was present for sufficient time in the lungs that it could have contributed to translocation through the ABB.

However, when the fast mucociliary [48V]TiO2NP fraction (MCC) cleared from the conducting airways was determined in order to determine the absorbed [48V]TiO2NP through the gut wall, MCC data are given as fractions  of the IT-instilled dose ID of ionic 48V or [48V]TiO2NP-radioactivity in the entire rat ()

|  |  |  |
| --- | --- | --- |
|  |  | (S13) |

Thus, in this case the normalization is done for each rat to  and not to the IPLD value determined in Eqn. (S11). A similar equation holds for the auxiliary study. In order to correct the determined activity data in the main study for contributions of free 48V-ions we make the conservative assumption that all urinary 48V-excretion at any time is only of ionic origin and no [48V]TiO2NP were excreted in urine because they are too large to pass renal filtration (e.g. ([Choi, 2007](#_ENREF_2))).

For the auxiliary biokinetics study using soluble, ionic 48V, the remaining fraction of ionic 48V+ in the body  at any time  can be written as

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| --- | --- | --- |
|  | , | (S14) |

where the 48V-activity fraction measured in the GIT comprises the activity due to 48V-ions in the stomach, the small and large intestine, and the oesophagus, while and  denote the fraction of applied activity that has been accumulated in fecal and urinary excretion, respectively, up to the retention time .

After having determined  and the cumulative urinary excretions in the auxiliary and the main study,  and , the ion content in the body of the rats in the main study  can be estimated from

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| --- | --- | --- |
|  |  | (S15) |

under the assumption that the urinary excretion of activity is entirely due to 48V-ions. While this calculation is done for each rat of the main study,  and  indicate that the mean values are used that are obtained from four rats for a given retention time in the auxiliary study.

In order to be able to estimate  data throughout the study period of 28 days, the data up to 7 days from the auxiliary study were extrapolated by a least square fit with two exponential terms up to 28 days as shown in Figure S5.

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**Figure S5:** (A) Experimentally determined cumulative urinary excretion data up to time  in the auxiliary study () and main study (). (B) 48V-ion content  in the body experimentally determined in the auxiliary study and the total 48V-activity fraction  determined in the main study; *i.e.* the sum of 48V-ions and [48V]TiO2NP activities beyond lungs. (C) With the data from (A) and (B) the fraction of ions in the body can be calculated applying Eqn (S15); subsequently the  beyond lungs can be calculated by subtracting the 48V-ion contribution from the total activity. The extrapolations of the data from the auxiliary study beyond day 7 were done using least square fits with two exponential terms. For this purpose normalisation was done to the total applied dose ID. Data are mean SEM, n = 4.

The total fraction of 48V-activity in the organism at retention time  is determined using cumulative fractions ɑfeces(t) and ɑurine(t) (without feces collected during the first 48h) as

|  |  |  |
| --- | --- | --- |
|  | . | (S16) |

This equation holds for the main and the auxiliary study. The fraction of [48V]TiO2NP in the body beyond the lungs is calculated by the difference between and  and the contemporary total lung dose at any time according to

|  |  |  |
| --- | --- | --- |
|  |  | (S17) |

An analogous equation holds for the auxiliary study. The contemporary lung dose  is derived from an exponential fit (least square method; Solver, Frontline Systems, Inc., Incline Village, USA) using two term

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| --- | --- | --- |
|  |  | (S18) |

as shown in Figure S6.



**Figure S6:** Retention of total lungs (contemporary total lung dose, including BAL) and a double exponential fit (least square method); fit parameters are given (least square sum is 0.00369).

Having derived an estimate for the fraction of 48V-ions in the body we can further estimate the distribution of these ions among the various organs with help of the data obtained in the auxiliary study, which yields

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| --- | --- | --- |
|  | . | (S19) |

In order to determine the [48V]TiO2NP fraction for each organ in the main study, the ionic 48V-fraction in organ “i”  is subtracted from the total measured 48V-activity of that organ  at each time point as

|  |  |  |
| --- | --- | --- |
|  |  | (S20) |

The [48V]TiO2NP data at the retention time points 1h, 4h and 24h were corrected with the 24h data of the auxiliary study, and those at 7d and 28d were corrected with the auxiliary data from day 7. For the correction of the 48V-ion contribution beyond day 7 the data from the auxiliary study were extrapolated as shown in Figure S5. The effect of the corrections can be assessed from the tabulated data (Table 3, main document).

**[48V]TiO2NP accumulation and retention in secondary organs and tissues relative to the entire translocated fraction through ABB**

In order to compare [48V]TiO2NP-fractions after IT-instillation directly to those fractions obtained from the intravenous injection study ([Kreyling et al., 2017 Part 1](#_ENREF_11)), retention kinetics of [48V]TiO2NPin secondary organs, remaining carcass, blood and urine are given as fractions of [48V]TiO2NP which had crossed the ABB into the blood circulation. For this purpose the 48V-activities of each secondary organ or tissue  are normalized to the sum of all 48V-activities of all secondary organs and tissues (labeled 2nd) as compiled in Table S4 according to

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| --- | --- | --- |
|  | , | (S21) |

where the index i extends over all secondary organs and tissues (cf. Table S4).

**Determination of fast mucociliary nanoparticle clearance from airways and macrophage-mediated long-term nanoparticle clearance from peripheral lungs**

The mucociliary cleared [48V]TiO2NP-fraction is determined according to Eqn (S13). It turned out that fast [48V]TiO2NPclearance  into the GIT is a rather large fraction and additionally showing a high intersubject variability (see Table 2, main document). Similarly, cumulative long-term macrophage-mediated nanoparticle clearance is considered as

|  |  |  |
| --- | --- | --- |
|  | for t > 48h | (S22) |

From and we estimated the fraction of [48V]TiO2NP and ionic 48V absorbed through the gut wall which adds to the accumulation in secondary organs and tissues. This estimate is based on the results of [48V]TiO2NP and ionic 48V absorption,  (=1h, 4h, 24h) (i = secondary organs or tissues), of which the biokinetics of the same [48V]TiO2NP was determined after intra-esophageal instillation (gavage) ([Kreyling et al., 2017 Part 2](#_ENREF_13)). Hence, the GIT-absorbed fast cleared nanoparticle fractions of each organ or tissue i becomes

|  |  |  |
| --- | --- | --- |
|  | for *t* < 48 h, | (S23) |

and the absorbed fraction due to long-term macrophage-mediated clearance becomes for each organ or tissue:

|  |  |  |
| --- | --- | --- |
|  | for *t* ≥ 48 h. | (S24) |

In the study after intra-esophageal [48V]TiO2NP instillation (gavage) ([Kreyling et al., 2017 Part 2](#_ENREF_13)) we have found that after 2 days roughly half of the absorbed fractions of accumulated 48V-activities in secondary organs and tissues originated from [48V]TiO2NP absorption across the gut walls and the other half from released 48V-ions. Therefore, we can introduce

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|  |  | (S25) |

as a reasonable approximation for the translocation of ions and nanoparticles through the gut epithelium. Hence, the absorbed [48V]TiO2NP fractions become

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| --- | --- | --- |
|  |  | (S26) |

**Differentiation of [48V]TiO2NP translocated across the ABB *versus* [48V]TiO2NP absorbed across the GIT walls**

According to the above, [48V]TiO2NP accumulation and retention in each organ and tissue results from two particle clearance pathways out of the lungs: (a) [48V]TiO2NP translocation across the ABB into blood circulation  and (b) [48V]TiO2NP absorption across the GIT walls  which were eliminated from the lungs towards the larynx and swallowed into the GIT. In addition, not only particulate [48V]TiO2NP but also 48V-ions are translocated *via* both pathways giving rise to the contributions and  accordingly. Thus, four contributions sum up to the total 48V-activity measured in each organ and tissue (indexed with i):

|  |  |  |
| --- | --- | --- |
|  |  | (S27) |

which can be derived using the combined knowledge obtained in the IT-instillation and the gavage study (GAV) ([Kreyling et al., 2017 Part 2](#_ENREF_13)) according to

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| --- | --- | --- |
|  |  | (S28) |

where we have made use of Eqn.s (S23) to (S27).

Since the ionic fraction  is determined by Eqn (S19) both particulate fractions can be calculated – the translocated [48V]TiO2NP across the ABB,  from Eqn (S28) and the absorbed [48V]TiO2NP fraction across the GIT walls,  from Eqn (S26). To better illustrate the contribution of gut absorbed and accumulated [48V]TiO2NP in the various organs and tissues a ratio

|  |  |  |
| --- | --- | --- |
|  |  | (S29) |

is calculated of the gut absorbed [48V]TiO2NP over the total accumulation (sum of gut absorbed and ABB translocated fractions).

**[48V]TiO2NP relocation within the lungs**

Serial broncho-alveolar lavages (BAL) of rat lungs were performed at each time point of sacrifice, to assess [48V]TiO2NP retention on the lung epithelium as free [48V]TiO2NP or associated with freely moving lung surface macrophages *versus* [48V]TiO2NP relocation from the epithelial surface either bound and/or taken up by cells of the epithelial barrier and beyond. BALs were performed by applying 6 x 5 ml of phosphate-buffered-saline (PBS) without Ca2+ or Mg2+ under gentle massage of the thorax. The recovered BAL fluid (BALF) (about 80-90% of instilled PBS) was centrifuged at 500 × *g* for 20 min at room temperature to separate the lavaged cells from the supernatant and [48V]TiO2NP content was determined by -ray-spectrometry. At each BAL the total number of lavaged cells was acquired with a hemocytometer by a dilution of the spun-down cells and trypan-blue staining. For cell differentiation cyto-centrifuged slide preparations of the lavaged cells were Wright-Giemsa stained for each sacrificed animal.

By -ray-spectrometry the fractions of free [48V]TiO2NP in the BALF *versus* [48V]TiO2NP in BAL cells and in the lavaged lungs were determined. Applying this BAL procedure we obtained about 4 × 106 macrophages per BALF on average very similar to our previous studies ([Semmler, 2004](#_ENREF_20), [Semmler-Behnke, 2007](#_ENREF_19)). Normalizing the number of totally recovered macrophages of each BAL by the mean number of the total surface macrophage population of 12.5 ± 0.8 × 106 previously determined in the lungs of WKY rats ([Semmler-Behnke, 2007](#_ENREF_19)) we estimated the total fraction of [48V]TiO2NP associated with lung surface macrophages.

Figure S7 shows the kinetics of free [48V]TiO2NP in BALF which is about 30% of total [48V]TiO2NP in lungs one hour after IT-instillation. However, it drops very rapidly to 0.1% at days 7 and 28. In contrast, the macrophage-associated [48V]TiO2NP fraction is about 40% of total [48V]TiO2NP in the lungs starting from 4 hours until the end of the study. Since the lavaged AM in BALC are less than the entire pool of all lung surface macrophages (AM-pool), the kinetics of the estimated total number of all [48V]TiO2NP in the AM-pool ([Semmler-Behnke, 2007](#_ENREF_19)) is plotted in Figure S7 showing a slowly increasing [48V]TiO2NP fraction of about 60%. This curve is compared to the average of 20 nm iridium NP (IrNP) within the AM pool (represented by a dashed line) taken from our previous data ([Semmler, 2004](#_ENREF_20), [Semmler-Behnke, 2007](#_ENREF_19)). Similarly the average of 2.1 µm polystyrene latex (PSL) particles is indicated by the dashed line which was determined by ([Lehnert, 1989](#_ENREF_16)). In our previous report we concluded that only a small fraction (≈20%) of 20 nm [192Ir]IrNP was internalized in the total AM pool while about a fraction of 80% was already relocated and bound and/or taken up by cells of the epithelial barrier or underlying interstitial spaces. This pattern was quite the opposite to that of 2.1 µm PSL particles which remained predominantly (≈80%) in the total AM pool on the epithelial surface throughout the entire time of six months (([Lehnert, 1989](#_ENREF_16)); ([Semmler-Behnke, 2007](#_ENREF_19))). Note, particles of any size in the AM pool were continuously eliminated *via* AM migration towards the mucociliary escalator and out of the lungs; yet, relative to the contemporary lung burden the 20% [192Ir]IrNP fraction in the AM pool and the 80% fraction of 2.1 µm PSL in the AM pool remained constant. The same retention and relocation pattern as obtained for 2.1 µm PSL in rats was confirmed for 1.4 µm fused alumino-silicate particles in hamsters ([Ellender, 1992](#_ENREF_3)). Comparing these data sets with our 70 nm [48V]TiO2NP it appears that the [48V]TiO2NP were less bound and/or taken up by cells of the epithelium and/or interstitium compared to the 20 nm [192Ir]IrNP but a 60% fraction resided in the AM pool almost like the 2.1 µm PSL particles. Hence, slightly bigger sized 70 nm [48V]TiO2NP seemed to be more effectively phagocytized than the small 20 nm[192Ir]IrNP resulting in a smaller fraction of [48V]TiO2NP relocated into the epithelium / interstitium. Yet, material differences (*i.e.* Ir versus Ti) may play an additional role, but note both nanoparticles are aggregates/agglomerates. This needs further investigation which will follow in our next reports on the biokinetics after inhalation of 20 nm TiO2NP and AuNP.



**Figure S7**: Kinetics of lavageable [48V]TiO2NP fractions based on measured BALC and those of free [48V]TiO2NP in BALF normalized to the contemporary lung burden as derived in Eqn. (S18). Additionally, estimated [48V]TiO2NP fractions in the AM pool. These data are compared to averaged fractions of 20 nm 192IrNP in the total AM pool ([Semmler, 2004](#_ENREF_20), [Semmler-Behnke, 2007](#_ENREF_19)) and to those of 2.1 µm PSL particles in the total AM pool (Lehnert et al. 1989).

**[48V]TiO2NP in the trachea and main bronchi**

Efficient particle retention and accumulation in the hilar lymph nodes at the first bifurcation and along the trachea has been described in many inhalation studies on various species over decades for micron-sized particles. Therefore, we analyzed the [48V]TiO2NP content in the trachea and both main bronchi at each retention time point, see Table S6.

**Table S6:** [48V]TiO2NP retention in the trachea and both main bronchi at each retention time point in % IPLD. This sample includes hilar lymph nodes around the first bifurcation and lymph nodes along the trachea. Fractions are mean ± SD; n = 4 for each time point.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| retent. time | 1h | 4h | 24h | 7d | 28d |
| trachea | 0.0206 ± 0.0152 | 0.0206 ± 0.0214 | 0.0219 ± 0.0136 | 0.0058 ± 0.0069 | 0.0038 ± 0.00032 |

In fact, [48V]TiO2NP retention in the trachea and main bronchi may be caused by two mechanisms: (a) gradual [48V]TiO2NP accumulation in the hilar lymph nodes and along the trachea and (b) [48V]TiO2NP on the airway epithelium in transit towards the larynx as a result of mucociliary clearance first by MCC and followed by LT-MC. During the first 24h, fractions are rather constant as would be expected for transit particles due to MCC. At d7, fractions are five-fold lower and at 28d they are one order of magnitude lower. This decrease is congruent with the declining LT-MC pattern. Hence, any super-imposed lymph node accumulation expected to increase with time is not detectable within the data variation.

**Estimated hepato-biliary clearance (HBC) of [48V]TiO2NP**

When estimating the translocated [48V]TiO2NP fraction across the ABB, as shown in Figure 3B (main document), the [48V]TiO2NP which were cleared *via* hepato-biliary clearance from the liver need to be included to the totally translocated fraction. However, we were not able to experimentally determine HBC in the present IT-instillation study because of the large contribution of [48V]TiO2NP clearance from the lungs *via* the tracheo-bronchial tree and larynx into the GIT. Yet, using the HBC data from ([Kreyling et al., 2017 Part 1](#_ENREF_11)) after IV injection we can estimate the [48V]TiO2NP fraction *via* HBC. Using the ratios of HBC over liver content from ([Kreyling et al., 2017 Part 1](#_ENREF_11)) at various time points we multiply these ratios with the liver content after IT-instillation

|  |  |  |
| --- | --- | --- |
|  |  | (S30) |

The results are presented in Table S7.

**Table S7:** Estimated HBC after [48V]TiO2NP IT-instillation between 1 to 28 days based on measured HBC after IV-injection taken from ([Kreyling et al., 2017 Part 1](#_ENREF_11)). Fractions are given relative to the initial peripheral lung deposition. Data are mean, n = 4 rats for each time point.

|  |  |  |  |
| --- | --- | --- | --- |
| time | 24h | 7d | 28d |
| HBC (IV) | 0.0043 | 0.0120 | 0.027 |
| liver (IV) | 0.95 | 0.94 | 0.92 |
| liver (IT) | 0.0014 | 0.00095 | 0.00075 |
| HBC (IT) | 6.3 × 10-6 | 1.2 × 10-5 | 2.2 × 10-5 |

Due to the very small [48V]TiO2NP accumulation in the liver after IT-instillation HBC is extremely small. Hence the total [48V]TiO2NP translocation increases in Figure 3B (main document) only marginally. Note, however, that [48V]TiO2NP which had crossed the ABB were most likely covered with a different protein corona , compared to [48V]TiO2NP after IV injection. As a result values of HBC (IT) may deviate from those estimated in Table S7. Similarly, due to the large differences in the dose rates of IV-injected *versus* ABB crossing there also may be different retention sites in the liver which could also affect the HBC kinetics ([Bachler, 2014](#_ENREF_1)). Interestingly, estimating [48V]TiO2NP HBC normalized to the [48V]TiO2NP which had crossed the ABB, HBC values are a factor of about 30 higher than relative to IPLD which is still well below 1%.

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