**Supplementary Material for GAV**

**Quantitative biokinetics of titanium dioxide nanoparticles after oral instillation in rats (Part 2)**

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

\* Helmholtz Center Munich – German Research Center for Environmental Health, Comprehensive Pneumology Center, Institute of Lung Biology and Disease, 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:

Dr. Wolfgang G. Kreyling

Institute of Epidemiology 2

Helmholtz Centre Munich, German Research Center for Environmental Health

D-85764 Neuherberg / Munich

Germany

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

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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) in a specially designed thin target capsule that allows efficient cooling of the sample from both sides as described earlier (Abbas et al.. 2010, [Kreyling et al., 2011](#_ENREF_5), [Holzwarth et al., 2012](#_ENREF_4)) ([Hildebrand et al., 2015](#_ENREF_2)).

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 nanoparticle 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 a 48V-activity concentration of 1.0 MBq/mg (48V-activity per TiO2 mass) was used for the 1h, 4h and 24h retention experiments, the second one was irradiated on five consecutive days yielded an activity concentration of 2.35 MBq/mg and was used for the 7d retention experiment 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 nanoparticles, with subsequent radiolabel leaching, the 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 (Inagaki et al., 2009; Holzwarth et al., 2012). Radiation damage due to collision processes was calculated, indicating that only a small fraction of the nanoparticles' atoms would be displaced from their lattice positions. An XRD scan of a sample of 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 et al., 2011; Holzwarth et al., 2012).

**Nanoparticle preparation for application 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 to with double-distilled water (dd H2O) to 3 mL. This suspension was fi ultrasonicated for 1min and filtered through a 0.22 μm 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 filter 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 should be less than 1 nmol.

For each of the four individual retention time points we prepared new [48V]TiO2NP suspensions, starting from one of two TiO2 anatase ST-01 stock suspensions mentioned above and using the protocol given above. A simultaneous start of [48V]TiO2NP administration to all rats (four rats per group, five 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 four different periods of retention time had to be staggered. However, each [48V]TiO2NP suspension was used for all three biokinetics studies reported in the three relevant articles in this volume – either after intravenous injection (IV) ([Kreyling et al., 2017 Part 1](#_ENREF_7)), after intra-esophageal instillation (gavage, GAV) ([Kreyling et al., 2017 Part 2](#_ENREF_9)) and after intratracheal instillation (IT) ([Kreyling et al., 2017 Part 3](#_ENREF_9)) for each period of retention time. Thus the same retention time points were analyzed for all three exposure routes starting 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 times (1h, 4h, 24h, 7d and 28d). – Please note that in the gavage study the retention time 28d was not investigated.

The hydrodynamic diameter of the particles 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 the same suspension was applied in three different exposure routes *via* intravenous injection (IV), intra-esophageal instillation (GAV) and intratracheal instillation (IT) – The 28d study was not performed for gavage.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| 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 | 316 | 316 | 316 | 316 | 316 |

\* measured with a Malvern Zetasizer;

PDI – polydispersity index;

§ manufacturer data 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 narrow distributed ‘nano-fraction’ of the original ST-01 TiO2 particles 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 300mesh 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 conecentration of the proton irradiated batches of TiO2 nanoparticles (1.0 MBq/mg and 2.35 MBq/mg) the 48V-radioactivity and the determined 48V-activity of the administered [48V]TiO2NP, the applied nanoparticle mass was calculated for each application route and each of the five retention times as reported in Table S2. While the numbers of aggregates/agglomerates 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 applied can be estimated if necessary.

**Table S2:** Application of [48V]TiO2NP suspensions by gavage. The effectively applied 48V-activity to 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 1mL insulin-syringes (Omnican® 100, Braun, Melsungen, Germany; specified dead volume < 0.4µL) due to retention of nanoparticles in the syringe and cannula. The last row presents the 48V-activity fraction retained in the syringe and cannula. The applied nanoparticle mass refers to the mass effectively received by the rats.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Retention time | Unit | 1h | 4h | 24h | 7d |
| Effective 48V radioactivity received by rats# | [kBq] | 13.07 ± 1.22 | 8.53 ± 0.34 | 12.22 ± 1.34 | 67.24 ± 6.38 |
| applied [48V]TiO2NP mass& | [µg] | 13.07 ± 1.22 | 8.53 ± 0.34 | 12.22 ± 1.34 | 28.61 ± 2.71 |
| Percentage of [48V]TiO2NP retained in the syringe after administration+ | % | 51 ± 14 | 38 ± 6 | 12 ± 4 | 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 kBq/µg for 1h, 4h, 24h studies and 2.35 kBq/µg for 7d study) and the integrally measured 48V-radioactivity of each rat, n = 4 for each group

+ fractional loss [48V]TiO2NP during gavage that was detected in the syringe after application

n.d. = not determined

**Influence of the acidic and basic environment of the GIT on possible de-agglomeration of TiO2NP**

[48V]TiO2NP suspensions were prepared as described above and two samples were taken, one immediately after preparation and a second one after three hours. With the first sample the hydrodynamic diameter of the untreated control suspension was assessed by DLS measurements and then the stomach passage of the [48V]TiO2NP was simulated. For this purpose the [48V]TiO2NP suspension was incubated at a pH value of 2 for 30 minutes. In this period the suspension was vortexed every 5 min for 1 min. Then the hydrodynamic diameter of the suspension was determined. In a next step, using the same sample, the passage through the small intestine was simulated by setting the pH value of the suspension to 9 and incubating it for 2 hours and vortexing it every 15 min for 1 min. After this simulation the hydrodynamic diameter was determined again by DLS. The data given below show that no de-agglomeration or gradual dissolution was detected, but instead further agglomeration occurred under acidic as well as under basic conditions (Figure S3). Z-averages were 77nm and 76 nm and PDI were 0.19 and 0.18 of controls before and after three hours, respectively. The simulation of the GIT passage yielded a Z-average value of 112nm (PDI 0.14) after simulated stomach passage at a pH value of 2 and a Z-average value of 275 nm (PDI 0.45) after additional simulated passage through the small intestine at a pH value of 9.

**Figure S3:** The influence of acidic conditions in the stomach and basic conditions in the small intestine on the hydrodynamic diameter of [48V]TiO2NP suspensions. Shown are the DLS measurements of the [48V]TiO2NP suspension: after 30 min incubation at pH 2; and after additional 2h incubation at a pH of 9; controls at and after .

**Animals**

Healthy, female Wistar-Kyoto rats (WKY/Kyo@Rj rats, Janvier, Le Genest Saint Isle, France), 8–10 weeks of age (261 ± 10 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 four 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 very significant differences in [48V]TiO2NP retention in the 1-mL insulin, minimal-dead-space syringes (Omnican® 100, Braun, Melsungen, Germany; specified dead volume 0.4 µL) used for each application even though we had minimized the time between finalizing the preparation of the [48V]TiO2NP suspension, filling the syringe 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 cannula after application of the [48V]TiO2NP suspension. Actually, the losses in the syringe varied from 10% up to 60% and were 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.

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 radiolabeled nanoparticles and -spectrometry requires much less cumbersome specimen preparation than do other analytical techniques suitable for determining 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 may have contributed substantially to unresolved variability and errors of the nanoparticle doses applied to experimental animals in other similar experimental studies reported in the scientific literature.

Nanoparticle suspensions were applied to non-fasted animals as described earlier ([Kreyling et al., 2014](#_ENREF_6), [Schleh et al., 2012](#_ENREF_13), [Hirn et al., 2011](#_ENREF_3), [Semmler et al., 2004](#_ENREF_14), [Kreyling et al., 2002](#_ENREF_10)). Firstly, the rats were anesthetized by inhalation of 5% isoflurane in oxygen until muscular tonus relaxed. For intra-esophageal instillation (gavage, GAV), 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 × 50 mm, B. Braun, Melsungen, Germany) was placed into the upper third of the esophagus and the [48V]TiO2NP suspension (60 µL) was gently instilled followed by 100 µL of air using a 1-mL insulin syringe (Omnican® 100, Braun, Melsungen, Germany; specified dead volume 0.4 µL) to accelerate the suspension into the stomach. After removal of the esophageal catheter rats were kept vertical for a few minutes and controlled for regular breathing. The syringe and cannula used for the instillation were retained for measurements of the residual [48V]TiO2NP due to wall losses. 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.

**Sample preparation for radiometric analysis**

At the chosen retention time periods of 1h, 4h, 24h, and 7d after [48V]TiO2NP applied by gavage rats were anesthetized (by 5% isoflurane inhalation) and euthanized by exsanguination *via* the abdominal aorta as described earlier ([Kreyling et al., 2014](#_ENREF_6), [Schleh et al., 2012](#_ENREF_13), [Hirn et al., 2011](#_ENREF_3), [Semmler et al., 2004](#_ENREF_14), [Kreyling et al., 2002](#_ENREF_10)). In this way approximately 60-70% of the total blood volume could be recovered. Organs, tissues, carcass and excretions as specified in Table S3 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.

**Table S3:** Organ, tissue and other samples prepared for radiometric analysis.

|  |  |  |
| --- | --- | --- |
| lungs | liver | spleen |
| kidneys | brain | heart |
| uterus | | |
| GIT: gastro-intestinal tract, comprising oesophagus, stomach, small and large intestine | | |
| total skin | muscle sample& | exsanguinated blood |
| head: skinned head without brain | | |
| bone sample (humerus or femur carefully cleaned from muscles and fat) | | |
| skeleton: estimated from measured bone sample activity times 10% BW (derived in Eqn (S8)) | | |
| carcass: 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 listed | | |
| excretion: total daily urine and faeces, collected separately | | |

& muscles were collected from both hind legs

**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 et al., 2014](#_ENREF_6), [Schleh et al., 2012](#_ENREF_13), [Hirn et al., 2011](#_ENREF_3), [Semmler et al., 2004](#_ENREF_14)). For radiometric analysis the 511 keV 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 511 eV 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 applied 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 fractional 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 et al., 2014](#_ENREF_6), [Schleh et al., 2012](#_ENREF_13), [Hirn et al., 2011](#_ENREF_3), [Semmler et al., 2004](#_ENREF_14), [Kreyling et al., 2002](#_ENREF_10)).

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.

**Distinction between gut walls and content**

In order to distinct between the content of the gut (chime) and its walls, the stomach, small intestine and colon were collected from three additional sets of four rats intra-esophageally instilled with a similar amount of [48V]TiO2NP suspensions and studied after 1h, 4h and 24h. All samples of the gut were cut open alongside and the chime was gently scraped out and rinsed off the gut walls. All samples underwent the usual -ray spectrometry analysis.

**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 S4:** 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 and Konig, 1955](#_ENREF_13)).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Lungs (g•g-1) | liver (g•g-1) | spleen (g•g-1) | kidney (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 and Konig, 1955](#_ENREF_12)) 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 time  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, the mass of the organ  and the organ specific weight factor  for the residual blood in the organ tissue according to the work of Oeff and Konig ([Oeff and Konig, 1955](#_ENREF_12)). The total blood volume *BV* in mL was estimated to be

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

|  |  |  |
| --- | --- | --- |
|  |  |  |

according to the work of Lee and Blaufox ([Lee and Blaufox, 1985](#_ENREF_11)), 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 from Eqn (S2) according to the findings of Oeff and Konig ([Oeff and Konig, 1955](#_ENREF_12))

|  |  |  |
| --- | --- | --- |
|  |  | (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 taken 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 (S5). 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 S4 for all organs, remaining carcass, skeleton and soft tissue.



**Figure S4:** 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, brain, uterus; panel C: carcass and 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 48V in ionic form after intra-esophageal instillation / gavage**

Additional experiments were performed to investigate the absorption of soluble, ionic 48V through the gut epithelium and its biodistribution at 24 hours and 7 days after oral gavage. These data were used to correct for ionic 48V-release from the [48V]TiO2NP mainly during the preparation of the suspension to be administered and in the GIT. In order to mimic such ionic 48V-release from the TiO2NP we added to the carrier-free ionic 48V-radionuclide 0.33 µg/µL ionic Ti(NO3)4 in 60 µL of distilled water in order to obtain a nitrate solution of a 48V : Ti ratio similar to that in the [48V]TiO2NP matrix and also a sufficient ionic strength to stably maintain the ions in solution. Additionally the pH value was adjusted to 5 at which no spontaneous precipitation occurred. Hence, 27 kBq of ionic 48V and 20 µg of ionic Ti in an aqueous volume of 60 µL were instilled into the esophagus of each rat and the biokinetics were measured after 24h and 7d (Figure S5).



**Figure S5:** Biokinetics of ionic 48V 24 hours and 7 days after oral application by gavage of carrier free 48V in 0.33 µg/µL ionic Ti(NO3)4 aqueous solution. Mean absorbed fractions were 0.87% ± 0.44% and 0.69% ± 0.43%, respectively. Mean ± SEM, n = 4. Levels of significances: \*\* p<0.01; \*\*\* p<0.0001. (<DL ≡ below detection limit)

At both time points, 24h and 7d after application, almost all 48V-ions were either in the GIT or were excreted in feces, namely (99.4%, including fecal fraction of 84.8%) and (99.3%, including fecal fraction of 99.3%), respectively (see Figure S5). Only about 0.87% and 0.69% were absorbed through the gut wall at 24h and 7d, respectively. At both time points about half of the absorbed 48V-ions were excreted in urine (0.44% and 0.34%, respectively).

In the carcass (soft tissue and skeleton) the 48V-fraction was low and increased from 0.32% at 24 hours to 0.24% at 7 days. Blood levels and those in secondary organs were well below 0.1%. The predominant fecal excretion suggests that the ionic 48V did not stay as ions in the gut content but became metabolized and the metabolite was excreted in feces.

**Correction of the biokinetics assigned to [48V]TiO2NP for the effect of free 48V-ions**

The mass of the nanoparticles is quantified by the measurement of the γ-ray emissions of the radiolabel 48V using the known activity concentration (in Bq/ng) of 48V in the [48V]TiO2NP.

Since the nanoparticles may be prone to release of radiolabels from the surface, and because there are indications in literature for a very slow but presumably not negligible nanoparticle dissolution ([Vogelsberger, 2003](#_ENREF_15), [Vogelsberger et al., 2008](#_ENREF_16)) a small fraction of the radiolabels may be released into the suspension and later inside the body of the animals as ions. Moreover, we have to consider that a certain amount of 48V may be released from the [48V]TiO2NP in the GIT due to chemical attack at the pH values found there ([Hildebrand et al., 2015](#_ENREF_2)). Since ionic 48V and radiolabeled [48V]TiO2NP exhibit distinctly different excretion behaviors and biokinetics, the activity contribution from 48V-ions has to be taken into account in order to determine the biodistribution of the [48V]TiO2NP. The auxiliary study described above was therefore used to correct the [48V]TiO2NP biokinetics for the presence of 48V in ionic form.

Assuming that urinary excretion of radioactivity is only due to 48V-ions and not [48V]TiO2NP, which are theoretically too large to pass renal filtration (e.g. Choi et al., 2007), with the auxiliary study results we may deduce the total fraction of activity in the form of 48V-ions which has passed from the GIT into the body after oral administration of [48V]TiO2NP . Firstly, we have to determine the activity fractions of ionic 48V in the body in the auxiliary study () and in the main study () at any time *t.*

In the auxiliary study the activity of 48V-ions in the body after passage of the epithelial barrier is equal to the total activity  of 48V-ions administered by intra-esophageal instillation, the activity still present in the GIT, , and the cumulative activities that were excreted from the body in form of feces () and urine () up to time :

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

Dividing Eqn (S10) by the total administered 48V-activity gives us for the corresponding 48V-activity fractions:

|  |  |  |
| --- | --- | --- |
|  | , | (S11) |

All the 48V-activity that crossed the epithelial barrier is distributed to the blood, organs, skeleton and soft tissue:

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

where the sum extends over all organs and tissues “i” listed in Table S3 except the GIT. Due to the low fraction of activity that passes the epithelial barrier and the errors involved in measuring the activities in Eqn (S10), is calculated from Eqn (S12) rather than Eqn (S11). The same balance for the 48V-activities and activity fractions can be set up for the main study taking into account that each organ or compartment “i” now contains a component made up of activity due to free 48V-ions and a second one due to [48V]TiO2NP. Thus, the total activities and the corresponding total activity fractions , obtained by normalizing the determined total 48V activities to  are now represented by

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

This relation holds for all specimens in Table S3 except for the urine since we assumed that the [48V]TiO2NP are too large to pass renal filtration. Therefore we may experimentally determine the absorbed fraction of 48V-ions in the body beyond the GIT in the main study at any time point with help of the auxiliary study calculating the ratio of the urinary excreted activity fraction up to time  according to the equation

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

where the average values  and  obtained from four rats in the auxiliary study are used. The quantities on the right-hand side of Eqn (S14) refer to individual rats in the main study. Figure S6 shows the evolution of the daily urinary excreted activity fraction and of the cumulative urinary excretion in the auxiliary study with ionic 48V and in the main study with [48V]TiO2NP for the duration of the experiments. In the main study the cumulative urinary excretion is about one order of magnitude lower than in the auxiliary study which according to Eqn (S10) implies that in the main study a correspondingly lower fraction of the total applied activity is retained in the body in form of free 48V-ions.



**Figure S6:** Evolution of the daily urinary excreted 48V-activity fractions (A) and the cumulative urinary excretion (B) of 48V-ions in the auxiliary and the main study during the 24h and 7d experiments; (C) presents the daily fecal excretion of 48V activity the auxiliary and the main study. The 48V-activity fractions are given with respect to the dose applied by intra-esophageal instillation. Mean ± STD, n = 4.

Having determined the activity fraction of 48V-ions retained in the body  in the auxiliary study from Eqn (S12) it is possible to calculate the corresponding value  in the main study applying Eqn (S14). Applying Eqn (S12) analogously for the total activity in the main study yields , which is presented in Figure S7. Dissolving Eqn (13) allows to determine the activity  that can be attributed to the [48V]TiO2NP (see Figure S7).

|  |  |  |
| --- | --- | --- |
|  |  |  |
| A | B | C |
| **Figure S7:** A: Experimental data for the activity fraction of 48V-ions retained in the body  in the auxiliary study. B: The experimental data for the total retained activity fraction in the body in the main study and C: decomposition of  into contributions of ions  and nanoparticles. After three days both fractions are similar and rather constant over time, suggesting that about half of the absorbed 48V-activity is ionic and the other half is due to [48V]TiO2NP. | | |

The activity fraction of free 48V-ions in the main study  is distributed among all organs and tissues according to the biodistribution of 48V-ions in the auxiliary study applying the relation

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

where  and  refer to average values obtained from four rats in the auxiliary study. Combining Eqns (S13) to (S15) we can derive for the activity fraction of [48V]TiO2NP in any organ or tissue “i”

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

In this way the nanoparticle content in the organs of individual rats in the main study is obtained by subtracting a fraction of the 48V-activity attributed to 48V-ions according to their (averaged) biodistribution in the auxiliary study.

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

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

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

   4 Current address: Bavarian Health and Food Safety Authority, D-85764 Oberschleissheim, Germany [↑](#footnote-ref-1)