Keywords: Titanium dioxide nanoparticles; Toxicokinetics; Inhalation; Rats

Introduction

Titanium dioxide (TiO₂) nanoparticles (NPs) are widely used in industrial and consumer products because of their higher catalytic activity compared to their larger analogues [1]. They improve the performance of energy production technologies (new generation of photovoltaic cells for solar panels, production of batteries) and are also used as bleaching agents, UV filters (in sunscreens, cosmetics, paints, etc.), in self-cleaning and anti-fogging materials, and for water pollution reduction owing to their photocatalytic properties.

A growing number of studies indicate that exposure to TiO₂ NPs may present health risks [2-6]. Nonetheless, the limited knowledge of their biological behavior and reactivity complicates the establishment of clear safety guidelines to be adopted, such as the choice of size and form of the safest NPs [7]. For these reasons, TiO₂ NPs have been included in the list of nanomaterials monitored by the OECD [8].

Human exposure to TiO₂ NPs can occur during their manufacture, use, integration into manufactured products, and during the destruction and recycling of these products. Exposure of workers is possible during handling process, where TiO₂ NPs can be found as aerosols, suspensions or emulsions. In the environment, NPs can easily form nanoaerosols. Therefore, after human exposure to these aerosols, NPs can deposit in the respiratory tract, the main route-of-entry of particles in the body [9-11].

Recent in vitro and in vivo studies tend to show that the biological behavior and reactivity of inhaled NPs could be related to parameters such as their size, number, concentration, specific surface area and agglomeration state [12,13]. These parameters could determine the site of deposition in the respiratory tract, their retention time in the lungs and their ability to translocate to the systemic circulation. Thus, the assessment of inhalation exposure to NPs under precise and controlled conditions is of great importance [14,15].

Inhalation exposures have been conducted to determine the cytotoxicity of TiO₂ NPs [16-18] and there have been attempts to investigate pulmonary effects induced by inhaled TiO₂ NPs in rodents [18-21]. However, the various parameters tested during the generation of aerosols (exposure time, concentration, NP size and agglomeration state) make it difficult to compare experimental data.

To date, a few in vivo studies have also been performed to determine the deposition of TiO₂ in the lungs and its biodistribution after specific inhalation exposure conditions [22-25]. However, the impact of exposure conditions such as the primary nanometric size (<100 nm), agglomeration state of NPs, and mass concentration of inhaled TiO₂...
NPs under controlled and stable conditions – on the pulmonary retention, absorption and extrapulmonary tissue distribution as a function of time has not been assessed within a same experimental protocol. Recently, a high-performance inhalation device has been set up in our laboratory, allowing the generation of nanoaerosols under controlled conditions and nose-only exposures of small rodents [26]. This device was used to assess the kinetic times courses of titanium (Ti) elements following a 6-h inhalation to 15 mg/m³ of the mostly used 20 nm particle size TiO₂ [25].

The overall objective of this study was to conduct a more systematic assessment of the impact of variations in the inhalation conditions of TiO₂ NP aerosols on pulmonary retention and systemic absorption in animals. The specific objectives were thus to evaluate the time courses of lung retention and bioanalytics of Ti elements after nasal inhalation of TiO₂ NPs of: 1) three different primary sizes, 2) two different agglomeration states and 3) three different exposure concentrations.

Methods

Nanomaterial characterization

TiO₂ nanopowder of 20 nm particle size (surface area 200-220 m²/g) and crystal anatase form was purchased from Nanostructured and Amorphous Materials, Inc. (Houston, TX, USA). TiO₂ NPs of 7 and 50 nm and in the anatase form were purchased from MK Impex Corp., (Mississauga, ON, CA). Characterization of TiO₂ nanomaterials in terms of size, shape, structure and agglomeration was carried out using conventional and high resolution transmission electron microscopy (TEM), scanning electron microscopy (SEM), Raman spectroscopy and X-ray diffraction (XRD) analysis. Moreover, lung sections collected after animal exposure to TiO₂ aerosols were analyzed in Pujalté et al. [26]: time-integrated mass concentration of the aerosol was also used in order to produce aerosol of larger agglomerates. A full description of the inhalation system is presented in Pujalté et al. [26] and optimization of generation of parameters was conducted prior to this study (purified airflow rate, dilution air flow rate, pressure in exposure chamber, temperature, relative humidity, internal pressure, O₂ and CO₂ rates). Exposure conditions were in accordance with OECD 403 guideline [8].

Measurements of the ambient conditions, concentrations, particle size distribution and aerodynamic diameter in the aerosol was conducted in real time. A DustTrak Aerosol Monitor was used to estimate the mass concentrations (mg/m³) through time (Model 8520, TSI Inc., USA); values were recorded on line every 10 seconds and means, and max were calculated over the total generation period. The time-integrated mass concentrations of TiO₂ in the aerosol were calibrated by gravimetric analysis of a borosilicate glass filter of 50 mm (700-800-FI, TSE Systems GmbH, Germany) sampled at a flow rate of 2 L/min; a micro-balance (XP2U, Mettler-Toledo Inc., USA) was used to weigh the filter pre- and post-generation. The size distribution (nm) of the aerosol in the exposure chamber was also monitored on-line with a nanospectrometer (NanoSpectroPan, TSE Systems GmbH, Germany) equipped with 2 measurement units. The NP measuring unit uses an electrical sensor to monitor size in the electric mobility range of 10 to 193 nm and the optical aerosol measuring unit counts particles in the optical equivalence diameter range of 0.250 to 35.2 µm. This device requires 1 minute per scan at a sample flow rate of 1.2 L/min. The geometric mean diameter, dₐ, and geometric standard deviation, σₐ, were used to determine the size distribution of particles in the aerosol based on the number of particles (particles/cm³) [27].

A full description of monitored exposure conditions is provided in Pujalté et al. [26]: time-integrated mass concentration of the aerosols; on-line estimation of mass concentration; size distribution of the aerosol in the exposure chamber; stability of particle numbers and mass concentrations with time.

Experimental design: Rats were divided in 6 exposure groups (Table 1) and exposed nose only to different primary sizes (7, 20 and 50 nm) of TiO₂ NPs at a concentration of 15 mg/m³ for 6 h. The effect of exposure concentrations (1, 7 and 15 mg/m³) and agglomeration state (larger and smaller agglomerates of >100 nm mean aerodynamic diameter and less than 100 nm, respectively) was also tested in rats exposed to the most commonly used NP size (20 nm). The different exposure conditions, as well as the mean mass concentration (mg/m³), mean number of particles (particles/cm³), geometric mean size of agglomerates (nm) in the aerosols (geometric standard deviation), are summarized in Table 1. Supplementary materials also provides data confirming the stability of TiO₂ aerosols during the 6-h inhalation in rats of the 6 exposure groups and size distribution of TiO₂ particles in the aerosols as function of mass concentration for the 6 rat exposure groups. A group of control rats was also formed (Table 1) and exposed to compressed air.

Animals were sacrificed by CO₂ asphyxiation. Blood was withdrawn by cardiac puncture and tissues (kidney, liver, spleen, lungs, lymph nodes and olfactory bulbs) were excised at 0, 3, 6, 12, 24, 48, 72 h following the onset of inhalation. Twenty-four rats were used per exposure group and four rats were used per time of sacrifice. Urine and feces were collected at 0-12, 12-24, 24-48, 48-72 h following the onset of inhalation. Blood was buffered with an equal volume of PBS buffer with 1 mM of EDTA (trace metal grade, 60-00-4, Sigma-Aldrich, Oakville, Canada) and 0.01% of butylated hydroxytoluene (128-37-0, Sigma-Aldrich, Oakville, Ontario, Canada), flash frozen in liquid nitrogen and kept at -80°C until analysis.

Exposure to titanium dioxide nanoparticles, aerodynamic characterization and biological sampling

Animals and acclimatization: Male Sprague-Dawley rats weighing 150-175 g (5-6 weeks old) were purchased from Charles River (Charles River Canada Inc., St-Constant, Canada). Lighting was maintained on a 12-h light–dark cycle and room temperature was kept at 22 ± 5°C. Prior to inhalation, rats were placed two per cage and had access to water and food ad libitum. On days 1 to 6 prior to inhalation exposure, animals were progressively acclimatized to the “nose-only” inhalation units, during 15, 30, 45, 60, 180 and 360 minutes per day, in order to limit stress during exposure period. Over the 3 days preceding inhalation, they were also gradually acclimatized to the metabolic cages during 1, 2 and 3 h per day, respectively. Following inhalation period, rats were cleaned with a damp cloth to avoid indirect exposure with NPs and placed in individual metabolic cages (for separate collection of urine and feces). The body weight gain for each animal was recorded daily. The experiment was approved by the Ethics Committee on Animal Experiments of the University of Montreal.

Generation and characterization of nanoaerosols: Two types of generators have been used in this study. Firstly, a “Collision 6-jet aerosilizer” (BGI Inc., Waltham, USA) was used for the generation of liquid aerosols [27]. A TiO₂ NP suspension was prepared from the nanopowder in ultrapure deionized water (18 mL) and a constant stirring was applied (120 rpm for a 125 mL aqueous suspension) [25,26]. A “Dust-jet generator” generating aerosols from dry nanopowders was also used in order to produce aerosol of larger agglomerates.
liquid nitrogen and stored at -80°C until analysis. Tissue samples were rinsed with saline at 0.9%, weighed, flash frozen in liquid nitrogen and stored at -80°C until analysis, except for lymph nodes and olfactory bulbs, which were stored at -20°C. Urine volume was measured and feces were weighed, and then stored at -20°C until analysis.

Quantification of titanium dioxide by inductively coupled plasma mass spectrometry (ICP-MS)

Sample preparation and digestion for elemental Ti analysis by ICP-MS was performed in a cleanroom (ISO2 standards 14644-1). The wet digestion method used was adapted from Mester et al. [28] and Subramanian [29] and is described in Pujalté et al. [25]. Briefly, as detailed in Pujalté et al. [25], tissues and feces collected were blended with a PBS buffer containing 1 mM of ETDA (trace metal grade, A463-5000, Fisher Scientific, Ottawa, Canada), and 2 mL of hydrogen peroxide (H2O2) at 30% (optima grade, P170-500, Fisher Scientific, Ottawa, Canada). The mixture was digested overnight in a heating block (HotBlock sc150, Environmental Express, Charleston, USA) set to a maximum temperature of 135°C. The solution was evaporated to a volume of less than 0.25 mL with the block heater, readjusted to a volume of 10 mL with ultrapure deionized water (18 mΩ) and shortly shaken.

Elemental analysis was then conducted with an Agilent ICP-MS 7700x (Agilent, Mississauga, Canada) in a cleanroom (ISO 3 standards 146442-1) [25]. Quantification was carried out using a six-point-calibration curve with internal standard correction. All samples and standards were measured with the ICP-MS system operating in the following conditions: RF Power at 1600 W, nebulizer gas flow rate at 0.65 L of Ar/min, dilution gas flow rate of 0.4 L Ar/min, and collision gas flow of 4.5 mL He/min. The isotope measured were \(^{48}\text{Ti}\) and \(^{100}\text{Sn}\) as internal standard. The limit of detection was in the order of 0.05 ppb, which is equivalent to 0.05 ng/g of tissue. Ti quantitation results are expressed as TiO2 equivalents.

Results

Pulmonary Ti retention profiles of inhaled TiO2 NPs

Figure 1 shows the effect of the exposure concentration (1, 7 or 15 mg/m³), primary NP size (7, 20 or 50 nm), and agglomeration state (smaller or larger agglomerates with mean aerodynamic diameter of < and >100 nm, respectively) on the time profile of Ti concentrations in lungs following the onset of a 6-h inhalation period in male Sprague-Dawley rats (Table 1 and Figure S1 for monitored parameter values for the 6 exposure groups). The exposure concentration (monitored mean of 1, 7, 15 mg/m³ and 31,000, 446,000, 786,000 particles/cm³) to 20 nm NPs of similar low agglomeration state (monitored average of 76-80 agglomerates (nm) in the aerosol (geometric standard deviation)) tested

| Exposure group\(^a\) | Inhalation exposure conditions of rats to aerosols of TiO2 NPs (for 6 h) | Effects of exposure conditions tested | Monitored mean concentrations (mg/m³) and mean (SSD) number of particles in the aerosol\(^b\) (particles/cm³) | Geometric mean size of agglomerates (nm) in the aerosol (geometric standard deviation)\(^c\) |
|-----------------------|---------------------------------------------------------------------------------|-----------------------------------|--------------------------------------------------|-----------------------------------------------|
| Control               | No exposure to aerosol                                                         | No effects                        | 0.008; 427                                       | ----                                           |
| Group 1               | 15 mg/m³, 20 nm anatase, low agglomeration state with Collision 6-jet (<100 nm) | Reference group for the effects of concentrations, initial sizes and agglomeration states | 14.9; 786,000 ± 54,000 | 76.3 (1.8)                                      |
| Group 2               | 1 mg/m³, 20 nm anatase, low agglomeration state with Collision 6-jet (<100 nm) | Effect of exposure concentration (Comparison with groups 1 and 3) | 1.06; 31,000 ± 1 893 | 79.6 (1.9)                                      |
| Group 3               | 7 mg/m³, 20 nm anatase, low agglomeration state with Collision 6-jet (<100 nm) | Effect of exposure concentration (Comparison with groups 1 and 2) | 7.09; 446,000 ± 24,000 | 77.3 (1.9)                                      |
| Group 4               | 15 mg/m³, 7 nm anatase, low agglomeration state with Collision 6-jet (<100 nm) | Effect of nanoscale size (Comparison with groups 1 and 5) | 14.7; 1 523,000 ± 250,000 | 74.4 (1.8)                                      |
| Group 5               | 15 mg/m³, 50 nm anatase, low agglomeration state with Collision 6-jet (<100 nm) | Effect of nanoscale size (Comparison with groups 1 and 4) | 14.6; 1 716,000 ± 43,000 | 74.6 (1.8)                                      |
| Group 6               | 15 mg/m³, 20 nm anatase, high agglomeration state with Dust-jet (>100 nm)       | Effect of agglomeration state (Comparison with group 1) | 15.0; 14 400 ± 3 600 | 134.3 (3.2)                                     |

\(^a\) n = 24 rats per group; 4 rats per time point.

\(^b\) See Figure S1 for graphs of the stability of TiO2 aerosols during the 6-h inhalation in rats of the 6 exposure groups.

\(^c\) See Figure S1 for graphs of size distribution of TiO2 particles in the aerosols as function of mass concentration for rat exposure groups 1 to 6.

Table 1: Inhalation exposure conditions of rats to titanium dioxide nanoparticles.
This could suggest a greater pulmonary retention for the smaller agglomerates compared to the larger agglomerates under study.

**Biokinetic profiles of Ti elements in the body**

Translocation to blood appeared limited for all exposure conditions when Ti levels were compared to background levels of this element observed prior to exposure and in control rats, the latter resulting from Ti sources other than NP inhalation. The kinetic profiles of Ti elements in blood did not indicate any increase in blood levels proportional to TiO₂ exposure concentrations in the aerosols (Figure 2A). Thus, at the end of the 6-h inhalation, mean concentrations (±SD) found in blood of rats exposed to 1, 7 and 15 mg/m³ were 0.027 ± 0.008, 0.034 ± 0.020 and 0.030 ± 0.003 µg TiO₂ equivalents/mL of blood, respectively. Blood concentrations at subsequent times were similar for the different exposure concentrations.

Similarly, the primary size of the NPs in the aerosol had no obvious impact on Ti blood concentrations as a function of time, again when generating similar size distribution of agglomerates of 20 nm NPs time points were quite large. For these three exposure conditions, Ti concentration values did not return to background levels at the end of the 72 h follow-up period, indicating a pulmonary retention persisting beyond this period.

The agglomeration state of NPs in the aerosol was also found to have an impact on pulmonary retention when Ti lung concentrations were compared in rats exposed to two agglomeration states of TiO₂ of 20 nm primary particle size, at a concentration of 15 mg/m³. Figure 1C shows a similar pulmonary deposition for the two agglomeration states during the first 6 h (monitored mean size of agglomerates of 20 nm NPs of 134 versus 76 nm, mean particle number of 14 400 versus 786,000 p/cm³ for a monitored mean concentration of 15 mg/m³). Mean (±SD) concentrations found in the lungs were 1.74 ± 0.62 and 1.63 ± 1.48 μg TiO₂ equivalents/g tissue for rats exposed to less and more agglomerated aerosols, respectively. However, after the end of inhalation, Ti concentrations in lungs as a function of time remained higher for rats exposed to the aerosol of NPs less agglomerated compared to those exposed to the aerosol of NPs more agglomerated.

**Figure 1:** Effect of the exposure concentration (A), primary particle size (B) and agglomeration state (C) on the time courses of TiO₂ concentrations (mean ± SD) in the lungs following the onset of a 6-h inhalation in male Sprague-Dawley rats (n=4 in each group).

**Figure 2:** Effect of the exposure concentration (A), primary particle size (B) and agglomeration state (C) on the time courses of TiO₂ concentrations (mean ± SD) in blood following the onset of a 6-h inhalation in male Sprague-Dawley rats (n=4 in each group).
(mean of 74 to 76 nm) for a monitored mean exposure concentration of 15 mg/m³ (Figure 2B). However, mean blood profiles tended to show a slightly higher translocation in the blood stream of the smallest nanometric size of 7 nm compared to the 20 and 50 nm sizes. Nevertheless, for these three exposure conditions, time profiles were variable and standard deviations on blood concentration values at the different time points were quite large.

On the other hand, compatible with observations in lungs, the agglomeration state of NPs in the aerosol appeared to influence Ti blood concentrations as a function of time and thus to have an apparent impact on translocation – although limited – into the systemic circulation (Figure 2C). At the end of the 6-h inhalation, mean blood concentrations (±SD) appeared similar, with 0.030 ± 0.003 and 0.035 ± 0.015 μg of TiO₂ equivalents/mL of blood for rats exposed to less and more agglomerated aerosols, respectively. However, after the end of the inhalation and in particular between 24 and 72 h following the onset of inhalation, Ti concentrations in blood remained on average circa two times higher for rats exposed to larger agglomerates as compared to smaller agglomerates.

Translocation to the lymphatic systems also appeared limited for all exposure conditions when comparing Ti levels after the onset of inhalation to background Ti levels observed prior to exposure and in control rats. In lymph nodes, there was no observed effect of the exposure concentration and the primary nanoscale size on Ti levels over time, despite a slight tendency for exposure concentrations at early time points (before 12 h following onset of inhalation) (Figure 3A and 3B). Ti concentrations in lymph nodes at subsequent time points were similar for the different exposure concentrations and primary particle size. For these exposure conditions, Ti concentration-time profiles in lymph nodes were variable and standard deviations at the different time points were quite large.

The agglomeration state of the NPs in the aerosol appeared to slightly influence the time course of Ti concentrations in lymph nodes (Figure 3C). Overall, Ti concentrations in lymph nodes – although reflecting limited transfer to the lymphatic system – were higher in rats exposed to smaller agglomerates of TiO₂ NPs as compared to those exposed to larger agglomerates. Specifically, at the end of the 6-h inhalation, Ti concentrations found in lymph nodes were on average two times higher for rats exposed to less agglomerated NPs as compared to more agglomerated particles, i.e., average (±SD) of 0.50 ± 0.32 and 0.25 ± 0.12 μg TiO₂ equivalents/g tissue, respectively. The impact of the agglomeration state was thus inverted compared to what was observed in blood. Baseline Ti levels of in lymph nodes, as assessed by elemental concentrations observed in control rats, were 20 times higher than those observed in blood of controls. Moreover, standard deviations on lymphatic concentration values at the different time points were quite significant.

The impact of exposure concentrations, primary NP size and agglomeration state in the aerosol on Ti levels in olfactory bulbs was also evaluated. Although a slight increase in concentrations was apparent during the 6-h inhalation period, there was no clear effect of the different variations in exposure conditions on Ti time profiles observed in this tissue. For the other internal organs, i.e. the kidneys, spleen and liver, no obvious differences in Ti temporal profiles were observed between the various exposure conditions (Table 2).

The kinetic profiles of Ti elements in urine and faeces were compared in rats subjected to different inhalation exposure conditions. However, the excretion values were highly variable making it difficult to observe a clear pattern of excretion (data not shown).

**Discussion**

**Generations of NP aerosols**

Using a NP aerosol generation device allowing to perform inhalation exposures of NPs in rats, it was possible to generate different aerosols of TiO₂ NPs under the conditions recommended by the OECD [8]. The "Collision 6-jet" aerosol generator used for the first five exposure conditions was able to produce aerosols with a small particle size distribution with geometric averages below 100 nm, i.e. aerosols of less agglomerated NPs as targeted (mean values of 76.3, 79.5, 77.3, 74.4 and 74.6 nm for groups 1 to 5, respectively, as described in Table 1). Optimization of velocity and airflow as well as a separation compartment also made it possible to meet the desired generation conditions [18,19,30]. A "Dust-jet" generator was used for the sixth exposure condition. It was able to generate aerosols of more
agglomerated NPs (agglomerates with average diameter of >100 nm or on average 134 nm) [19].

Exposure groups 4 and 5 compared to the first exposure group (Table 1) were also used to assess the impact of the primary size of NPs on their lung deposition and their ability to cross biological barriers. The same average mass exposure concentration of 15 mg/m3 (Table 1) were also used to assess the impact of the primary size of NPs on average 134 nm) [19].

Using the various generation conditions of NP aerosols, it was possible to better define the toxicokinetics of Ti elements after different exposure conditions to TiO2 NPs and thus the pulmonary retention and systemic disposition profiles of Ti elements.

Using the various generation conditions of NP aerosols, it was possible to better define the toxicokinetics of Ti elements after different exposure conditions to TiO2 NPs and thus the pulmonary retention and systemic disposition profiles of Ti elements. It has been documented that NPs, in general, can be retained in the lungs following inhalation exposure. Pulmonary retention of NPs may arise from the fact that small NP agglomerates are more poorly taken up by phagocytes and thus less prolonged interaction small particles with epithelial and endothelial pulmonary interstitial tissues [15,33,34]. According to the literature, prolonged interaction small particles with epithelial and endothelial cells of the respiratory tract can favor translocation and in turn passage to the lymphatic system and blood circulation by different mechanisms, including phagocytosis, endocytosis, pinocytosis and transcytosis, and ensuing exocytosis, or deposition in the interstitium [9,10,15,35]. This high retention of particles in the lungs can contribute to their continuous release into the lymphatic and blood system long after a single exposure. In the systemic circulation, particles can interact with plasma protein components and circulating blood cells (erythrocytes, leukocytes, thrombocytes) [36], and then reach the different extrapulmonary organs where they can accumulate. In terms of clearance, NP agglomerates in the alveolar space have been reported to be predominantly eliminated by alveolar macrophages and mucociliary transport to the upper respiratory tract where they can be ingested and excreted in faeces [37-40]. The gastrointestinal absorption of TiO2 particles of different sizes after ingestion is considered negligible [41]. Wang et al. [4] also showed that translocation across the epithelium of the gastrointestinal tract represented less than 0.005% of the ingested dose.

For all exposure conditions tested, this study showed that Ti elements were retained in lungs and that the residence time in this tissue exceeded the 72-h monitoring period. For most exposure conditions tested, peak levels of Ti in the lungs were also reached after the end of 6-h inhalation exposure to NP aerosols in rats in line with Tsuda et al. [42] indicating that particles retained in the upper respiratory tract could migrate to lower regions, and in turn the lung several hours after the end of the inhalation period. Furthermore, for all exposure conditions in our study, lymph node levels of Ti elements were higher than blood levels, which is consistent with previous studies [43,44]; this phenomenon was also observed after repeated exposure in rats [22]. Translocation rate of inhaled TiO2 NPs to the lymphatic

| Tissue          | Time of sampling (h) | Controls | Group 1* | Group 2* | Group 3* | Group 4* | Group 5* | Group 6* |
|-----------------|----------------------|----------|----------|----------|----------|----------|----------|----------|
| Kidneys         | 3                    | 51.7 ± 9.2 | 82.1 ± 35.6 | 57.1 ± 17.5 | 57.6 ± 3.7 | 38.2 ± 12.6 | 57.6 ± 3.7 |
|                 | 6                    | 52.9 ± 10.4 | 48.5 ± 11.8 | 42.4 ± 1.5 | 62.4 ± 17.4 | 57.7 ± 51.7 | 62.4 ± 17.4 |
|                 | 12                   | 70.8 ± 16.6 | 52.5 ± 7.2 | 42.3 ± 5.9 | 72.7 ± 31.8 | 31.4 ± 7.4 | 72.7 ± 31.8 |
|                 | 24                   | 50.8 ± 7.1 | 59.4 ± 18.7 | 45.8 ± 10.4 | 51.9 ± 9.2 | 27.7 ± 3.5 | 51.9 ± 9.2 |
|                 | 48                   | 40.2 ± 5.3 | 56.1 ± 16.1 | 54.5 ± 5.3 | 86.1 ± 29.2 | 23.2 ± 5.3 | 86.1 ± 29.2 |
|                 | 72                   | 54.4 ± 15.7 | 86.8 ± 32.6 | 48.6 ± 9.1 | 85.2 ± 24.4 | 30.8 ± 6.5 | 85.2 ± 24.4 |
| Spleen          | 56.8 ± 25.1          | 36.8 ± 7.1 | 70.1 ± 17.8 | 42.3 ± 5.9 | 57.6 ± 3.7 | 38.2 ± 12.6 | 57.6 ± 3.7 |
|                 |                      | 17.2 ± 7.8 | 27.1 ± 8.9 | 10.1 ± 6.3 | 43.3 ± 14.6 | 36.2 ± 14.4 | 25.1 ± 13.5 |
|                 | 6                    | 87.4 ± 80.8 | 122.5 ± 165.6 | 85.8 ± 91.9 | 87.9 ± 76.9 | 43.4 ± 17.5 | 39.4 ± 5.7 |
|                 | 12                   | 44.7 ± 23.0 | 140.1 ± 213.7 | 70.1 ± 59.3 | 39.5 ± 7.7 | 34.9 ± 8.0 | 28.7 ± 16.9 |
|                 | 24                   | 33.5 ± 14.4 | 26.4 ± 9.2 | 25.1 ± 5.2 | 32.2 ± 7.5 | 41.0 ± 8.4 | 52.3 ± 19.7 |
|                 | 48                   | 76.4 ± 34.8 | 109.8 ± 58.7 | 60.4 ± 46.0 | 46.4 ± 15.4 | 33.5 ± 8.1 | 56.5 ± 51.6 |
|                 | 72                   | 38.3 ± 9.2 | 68.6 ± 23.4 | 56.5 ± 37.5 | 52.3 ± 12.7 | 69.6 ± 11.9 | 36.5 ± 10.1 |
| Liver           |                      | 17.2 ± 7.8 | 27.1 ± 8.9 | 10.1 ± 6.3 | 43.3 ± 14.6 | 36.2 ± 14.4 | 25.1 ± 13.5 |
|                 | 3                    | 27.1 ± 8.9 | 10.1 ± 6.3 | 43.3 ± 14.6 | 36.2 ± 14.4 | 25.1 ± 13.5 | 13.5 ± 6.4 |
|                 | 6                    | 38.5 ± 40.3 | 11.3 ± 9.3 | 26.2 ± 20.4 | 33.3 ± 8.9 | 16.3 ± 4.5 | 13.9 ± 1.3 |
|                 | 12                   | 32.0 ± 11.7 | 10.1 ± 4.3 | 42.1 ± 22.8 | 43.9 ± 32.2 | 17.6 ± 4.1 | 13.0 ± 1.7 |
|                 | 24                   | 25.7 ± 5.3 | 7.7 ± 3.2 | 27.4 ± 21.3 | 23.3 ± 6.5 | 19.8 ± 4.4 | 17.6 ± 9.4 |
|                 | 48                   | 51.7 ± 29.6 | 10.4 ± 3.4 | 21.9 ± 3.2 | 29.8 ± 14.8 | 11.1 ± 3.5 | 22.2 ± 14.8 |
|                 | 72                   | 30.4 ± 20.9 | 13.9 ± 5.3 | 21.5 ± 9.5 | 25.7 ± 3.0 | 13.3 ± 0.7 | 8.6 ± 1.1 |
| Offactory bulb  |                      | 19.8 ± 8.0 | 25.0 ± 10.2 | 11.3 ± 3.3 | 18.4 ± 2.3 | 36.2 ± 12.2 | 32.4 ± 13.7 | 13.9 ± 9.6 |
|                 | 3                    | 22.9 ± 6.3 | 9.7 ± 6.5 | 35.8 ± 24.3 | 33.1 ± 10.5 | 27.8 ± 15.1 | 27.6 ± 15.7 |
|                 | 6                    | 12.2 ± 7.3 | 13.7 ± 2.2 | 26.7 ± 29.5 | 24.9 ± 3.7 | 29.1 ± 17.0 | 20.4 ± 12.6 |
|                 | 12                   | 16.4 ± 1.0 | 7.6 ± 2.1 | 12.7 ± 3.5 | 23.8 ± 13.5 | 26.9 ± 8.4 | 21.1 ± 11.3 |
|                 | 24                   | 11.3 ± 2.7 | 17.4 ± 5.3 | 15.8 ± 15.4 | 22.7 ± 9.4 | 10.0 ± 8.1 | 13.7 ± 6.6 |
|                 | 48                   | 9.9 ± 3.1 | 17.3 ± 13.7 | 22.5 ± 11.7 | 24.0 ± 15.5 | 16.7 ± 10.2 | 21.1 ± 13.8 |

*Group description is provided in Table 1.

Table 2: Concentrations of TiO2 in tissues (ng/g) of rats following different inhalation exposure conditions to TiO2 nanoparticles.
system and blood circulation, as assessed from elemental analysis, was very low compared to total amounts retained in the lungs over time. According to the literature, main elimination pathway of NPs from the lungs appears to be through phagocytosis of NP agglomerates by alveolar macrophages, which migrate to the tracheobronchial region for mucociliary clearance to the gastrointestinal tract and subsequent ingestion [37,40,45].

**Impact of inhalation exposure conditions on the toxicokinetic profiles of Ti elements**

This study showed that exposure concentrations of TiO$_2$ NPs in the aerosols had an effect on Ti lung concentrations and retention. Nevertheless, this was not clearly reflected by a translation of Ti elements in blood and lymphatic system proportional to the exposure concentration. Also, as expected, the primary size of the NPs had no obvious effect on Ti lung concentrations and retention or on translocation to blood and lymphatic system, when particles in the aerosol had similar agglomeration states. In contrast, the agglomeration state of NPs in the aerosol had an apparent impact on pulmonary retention and – although limited – on blood and lymph translocation. Indeed, a lower lung retention and higher blood translocation during 72-h monitoring period were observed for the more agglomerated NPs. In contrast to blood levels, lymphatic concentrations as a function of time were lower for the more agglomerated NPs.

Our results on the impact of the agglomeration state on pulmonary retention are in agreement with data published in the literature, indicating that the reactivity of the NPs and the resulting size of the agglomerates can have an impact on their kinetic behavior [18]. Sedimentation and impaction have been described as basic mechanisms explaining the sites of deposition of particles in the respiratory system. Indeed, several studies report the importance of particle size on their deposition in deep alveolar regions and retention [9,46,47]. An aerosol composed of micrometric particles and large NP agglomerates have been documented to deposit in the upper airways (extrathoracic: nasal cavities, oral cavity, pharynx, larynx) while particles of nanometric size are deposited in the lower airways (intrathoracic: trachea, bronchi, bronchioles) and deep alveolar regions, with an extremely large surface area (about 130-140 m² in humans) [9,42]. Some authors reported that particles with a size between 10 and 20 nm have a deposition rate in the alveolar regions of about 50% [9,48]. In the present study, temporal follow-up of retention of Ti in total lung was performed, including bronchi and alveolar regions, without the upper air regions. In our study, the size of the agglomerates had an apparent impact on lung retention of Ti elements and on translocation into the lymphatic system and blood circulation, but not the primary nanometric particle size. However, all primary sizes were less than 100 nm of diameter and therefore in the nanometric scale.

The different mechanisms by which NPs can be internalized – phagocytosis/endocytosis/pinocytosis/transcytosis and exocytosis across epithelial cells or passage through cellular interstices – have been reported to depend on the size of particles and agglomerates [49-52]. In addition, some studies have hypothesized that deagglomeration may occur after pulmonary deposition [53]. Inflammation may also result in an increased leakage of particles to the lymph nodes [54]. Furthermore, dissolution of NPs retained in the respiratory tract by phagocytic cells – leading to the formation of ionic species more easily absorbed – has been shown for some metallic NPs, such as nanoceria and zinc oxide NPs [55,56]; dissolution of TiO$_2$ NPs, which would be dependent on size of agglomerates, cannot be excluded. These mechanisms may partly explain differences in lymph and blood levels observed in our study between the two agglomeration states. More research is needed to understand this mechanism.

In addition, for a given NP primary size, our study showed that the mass concentration of NPs in the aerosol (mg/m³) and numerical particle count (particles/cm³) had a direct impact on pulmonary concentration but not on systemic translocation. The more an aerosol is concentrated in mass or number of particles, the greater the amounts deposited in the lungs.

With regards to our results on the kinetic profiles of Ti elements observed in internal organs, i.e. liver, kidneys, spleen and olfactory bulbs, it was possible to detect Ti in all tissues and at all times, at concentrations of the same order of magnitude as those observed in controls. These results confirm the poor transfer to extrapulmonary tissues for all exposure conditions tested. Levels were variable over time in a given tissue and no clear impact of the exposure conditions on the temporal profiles or recovered amounts was detected. On the other hand, the French team of Gâte et al. [22] showed that there was a translocation in extrapulmonary organs that was significant at day 90 in rats after a repeated exposure to 10 mg/m³ of a nanostructured TiO$_2$ aerosol 6 h/day, 5 days/week for 4 weeks. In addition, it has been reported that NPs can also reach olfactory bulbs by translocation along axons of olfactory nerves [57]. Although a slight increase in Ti concentrations was apparent in olfactory bulbs during the 6-h inhalation, no clear impact of the various exposure conditions could be revealed. Further studies are needed to understand translocation mechanisms in sub-regions of the brain.

**Conclusion**

Overall, following the 6 tested inhalation exposure conditions in rats, it was observed that exposure concentrations and agglomeration state had an impact on pulmonary tissue levels of Ti elements. Translocation into the systemic circulation and lymphatic system appeared limited for all tested exposure conditions, but there seemed to be an influence of the agglomeration state of the NPs in the aerosols on the concentration-time profiles of Ti in blood and lymph nodes. Size of agglomerates was found to be the most important factor determining the site-of-entry retention and – although limited – transfer of particles into the systemic circulation.

**Disclosure Statement**

The authors declare no conflicts of interest. The authors alone are responsible for the contents and writing of the paper.

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