Towards a method for detecting the potential genotoxicity of nanomaterials

Deliverable 7: Identification of target organs and biodistribution including ADME parameters

Final Report

Identification of target organs and biodistribution including ADME parameters
March, 2013

This document arises from the NANOGENOTOX Joint Action which has received funding from the European Union, in the framework of the Health Programme under Grant Agreement n°2009 21.
This publication reflects only the author's views and the Community is not liable for any use that may be made of the information contained therein.

Co-funded by
the Health Programme
of the European Union

NANOGENOTOX

Grant Agreement n° 2009 21 01

WP 7: Toxicokinetics and tissue distribution of MNs for specification of organs at risk for genotoxicity testing

Deliverable 7: Identification of target organs and biodistribution including ADME parameters

Deliverable leader: RIVM

Wim H de Jong

National Institute for Public Health and Environment (RIVM),

Rijksinstituut voor Volksgezondheid en Milieu - 3720 BA Bilthoven, THE NETHERLANDS

rivm
National Institute
for Public Health
and the Environment

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by
the Health Programme
of the European Union

Workflow	
Author(s) Nicklas Raun Jacobsen, Håkan Wallin (NRCWE) Wim de Jong, Agnes Oomen, Esther Brandon (RIVM) Petra Krystek (Philips Innovation Services; former MiPlaza) Margarita Apostolova, Irina Karadjova (IMB-BAS) Francesco Cubadda, Federica Aureli, Francesca Maranghi (ISS) Vincent Dive, Frédéric Taran, Bertrand Czarny (CEA)	**Reviewer(s)** WP Leader: Wim de Jong (RIVM) Michel Laurentie (ANSES) Coordinator: Nathalie Thieriet, Florence Etore (ANSES)

Document status: Final report
Creation date: 17/09/2012
Completion date: 26/03/2013

Confidentiality level of the deliverable		
PU	Public	**PU**
CO	Confidential, only for members of the consortium (including the Commission Services)	

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Contents

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

1. Introduction

This report summarizes the results of the toxicokinetics studies. The aim of the WP7 was to identify relevant organs for genotoxicity testing based on the determination of organ exposure to nanomaterials. The distribution of the nanomaterials into the various organs is an indication for the organs at risk for nanomaterial toxicity and thus also genotoxicity, based on the interaction and activity of the nanomaterials with the cells of the organ. In WP7, the kinetic parameters and tissue distribution were determined for selected nanomaterials: titanium dioxide (TiO_2), silicon dioxide (SiO_2) as synthetic amorphous silica (SAS), carbon nanotubes (CNTs) after oral and intravenous administration.

The oral route of exposure was chosen as this is a common route of exposure for consumers. However, after oral exposure the absorption may vary greatly, although also indications for systemic toxicity have been noticed (reviewed by Frölich and Roblegg, 2012). After inhalation and dermal exposure in general the distribution and absorption was demonstrated to be low if any. Low absorption after oral intake was calculated for ZnO nanoparticles but was mainly ascribed to the soluble Zn fraction present in the nanomaterial (Baek et al., 2012). For oral exposure to TiO_2 at high doses (5g/kg body weight) uptake from the GI-tract and distribution to organs was observed, although also uptake from Ti originating from other sources (e.g. feed) may be suggested as also Ti was detected in organs of control animals (Wang et al., 2007). In addition to the oral route also the intravenous route of exposure was investigated as this route of administration circumvents the biological barriers present and results in direct systemic availability of the nanomaterials in the blood circulation and thus the internal organs.
Organ and blood samples were collected and evaluated for the presence of Ti, Si, and CNTs. For SAS and TiO_2 nanomaterials, not the nanomaterials themselves but the elements Si and Ti were determined using inductively coupled plasma-mass spectrometry (ICP-MS) as the analytical technique. For the carbon nanotubes the determination of C was not an option to study the tissue distribution as C is present in all tissues. Therefore, CNTs were radiolabeled with ^{14}C atoms to allow detection in the body.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by
the Health Programme
of the European Union

2. Nanomaterials

2.1 Nanomaterials studied

Not all nanomaterials as investigated in the NANOGENOTOX project were investigated for their toxicokinetics and tissue distribution. The nanomaterials used in the experiments for tissue distribution after intravenous (IV) and oral administration are presented in Table 2-1.

Table 2-1. Nanomaterials studied for their tissue distribution

Nanomaterials	Ref.	Route	Partner
TiO_2	NM-100	IV	RIVM
	NM-101	oral	NRCWE
	NM-102	oral, IV	NRCWE, RIVM
	NM-103	oral, IV	NRCWE, RIVM
	NM-104	oral, IV	NRCWE, RIVM
	NM-105	oral, IV	NRCWE, IMB-BAS
SAS	NM-200	oral, IV	ISS
	NM-203	oral, IV	
CNTs	NM-400	oral, IV	CEA
	NM-401	oral, IV	
	NM-402	oral, IV	
	NRCWE-006	oral, IV	

2.2 Nanomaterial characterisation

A full characterisation of these nanomaterials is presented in the reports of WP4. For the *in vivo* studies to the tissue distribution of the nanomaterials the nanomaterials were evaluated for their size distribution as an indication for the quality of the used dispersions. The methods used for the evaluation of the dispersions, and the results of these evaluations are presented in Annex 1.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

3. General Principles

3.1 Study schedule

Rats were treated by the treatment schedule presented in Figure 3-1. The rats received a single (day 1) or repeated (on 5 consecutive days, day 1-5) administrations via the oral or intravenous (IV) route. Tissue samples were obtained at various time points after intravenous administration. Samples were collected at day 2 (24 hour after a single IV administration at day 1), and at day 90 after the single administration. After the five times repeated administrations at days 1-5 samples were collected at day 6 (24 hour after the last of the administrations days 1 to 5), and at day 14, day 30 and day 90. Blood samples were obtained to evaluate the elimination of Ti after the IV administration at day 1 (single dose) and day 5 (repeated doses) at t=5, t=10, t=20, t=30 minutes, and t=1, t=2, t=4, and t=8 hours. Also at autopsy at day 2, day 6, day 14, day 30 and day 90 after administration blood was collected.

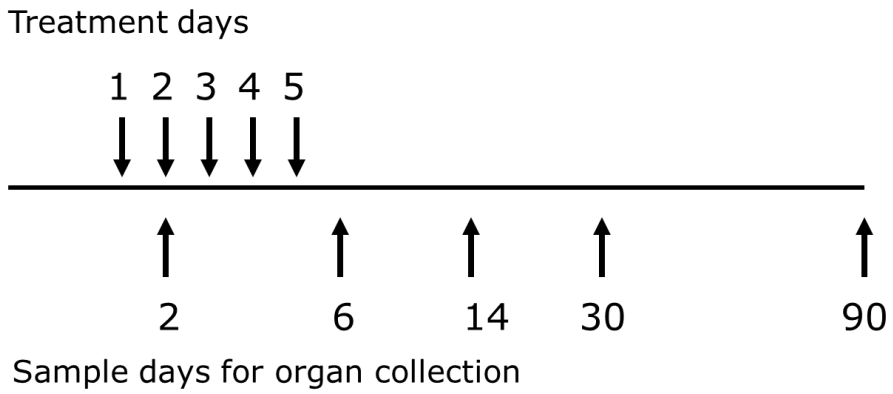


Figure 3-1. Treatment schedule for determination of tissue levels of various nanomaterials. The upper part of the schedule shows the number of administrations, while the lower part shows the days after start of the treatment (=day 1) when organs were collected and investigated for nanomaterial content. In addition, blood was collected on day 1 and day 5 to determine elimination from blood.

For each nanomaterial eighteen male (n=18) and 12 female (n=12) rats were IV injected with nanomaterial. Six (n=6) male and three (n=3) female animals were IV injected with the vehicle used for the dispersion of the nanomaterial. At each time point investigated (see Figure 3-1) n=3 male or female animals were evaluated for the tissue distribution of the nanomaterial. After single IV treatment, tissue levels were evaluated at days 2 and 90, whereas after five times repeated IV treatments tissue levels were evaluated for male animal at days 6, 14, 30 and 90, and for female animals at days 6 and 90. Vehicle treated animals were investigated as follows; males n=2 at days 2, 6, and 90, and females n=1 at days 2, 6 and 90. Blood was collected at days 1 and 6 after a single or five times repeated

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

www.nanogenotox.eu

nanomaterial administration, n=2 animals for each time point, to evaluate blood kinetics and clearance. For oral administration a similar schedule was used for the repeated oral administrations resulting in n=3 animals either males or females for evaluation of Ti tissue levels. The treatment schedule is presented in Table 3-1.

Table 3-1. Design of toxicokinetic study for determination of tissue distribution of nanomaterials.

Treatment	Autopsy				
	Day 2	Day 6	Day 14	Day 30	Day 90
MN (1x)	3M+3F	-	-	-	3M+3F
MN (5x)	-	3M+3F	3M	3M	3M+3F
Vehicle	2M+1F	2M+1F	-	-	2M+1F

MN= Manufactured Nanomaterial, MNs were administered either by oral or intravenous route.

3.2 Ethical issues

Prior to start all the protocols for the various animal, experiments were evaluated and approved by the animal ethical and/or welfare committees at the Institutes at which the animal experiments were performed (CEA, IMB-BAS, ISS, NRCWE and RIVM). All experiments were performed according to European and/or national laws regulating the use of laboratory animals.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

4. Titanium dioxide (TiO_2)

4.1 Methods

4.1.1 Animals

Wistar male and female rats were used at NRCWE, RIVM and IMB-BAS for the oral studies with NM-101, NM-102, NM-103, NM-104, and NM-105, and the IV studies with NM-100, NM-102, NM-103, NM-104, and NM-105; (see also Table 2-1). The study schedule is described in section 3.1. Animals were bred under specific pathogen-free (SPF) conditions and barrier maintained during the entire experiment in Macrolon cages at a room temperature of 21 ± 2°C (IMB-BAS, NRCWE) and 23 ± 1°C (RIVM), a relative humidity of 50 ± 5% and a 12-h light/dark cycle. Standard feeding chow diet and water were provided *ad libitum*. Rats were randomly divided into groups of 1-3/cage. The rats were kept under pathogen-limited conditions and were allowed to acclimatize for 1-2 weeks before they entered the experimental protocol. All rats were 9-10 weeks at exposure start.

4.1.2 NM Sample preparation

For TiO_2 samples were prepared according to the dispersion protocol from WP4. In short, a 2.56 mg/ml stock suspension was prepared by prewetting the TiO_2 powder in 0.5 vol % ethanol followed by dispersion in 0.05 wt% Rat Serum Albumin (RSA) in ultrapure water. The sonication of the suspensions was performed on ice (Branson Sonifier S-450D, Branson Ultrasonics Corp., Danbury, CT, USA, equipped with a disruptor horn Model number: 101-147-037) 10% power (RIVM, NCRWE) and a Cell Disruptor Sonicator W-375 (Ultrasonic Inc.) at IMB-BAS for 16 minutes. The stock suspensions were diluted (9:1 vol/vol) with 10x concentrated phosphate buffer pH 7.4 (702 mg NaH_2PO_4 x$2H_2O$, 4155 mg Na_2HPO_4 x$7H_2O$, dissolved in 1L) and used immediately.

Endotoxin levels of NM-100, NM-102, NM-103, and NM-104 were investigated for the vials as received from JRC. The NM-100, NM-102, NM-103, and NM-104 preparations were investigated in the Limulus Amebocyte Lysate (LAL) test (Endosafe®, Charles River Laboratories International, Wilmington, MA, USA). All preparations investigated showed endotoxin levels below 20 IU (international units). Presence of endotoxin could not be confirmed by fatty acid analysis.
Endotoxin concentrations of NM-105 as dispersed in phosphate buffer and in several dilutions were quantified with the PyroGene Recombinant Factor C (Endotoxin detection assay, Lonza GmbH). The fluorescence was continuously monitored using the DTX-880 microplate reader (Beckman Coulter, USA). Four endotoxin standards (10 to 0.01 endotoxin units /ml) were used to generate a standard curve to calculate endotoxin concentrations, according to the manufacturer's instruction. Two different tests were performed- the

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Routine and the Inhibition/Enhancement. The different Lot numbers of NM-105 used for iv administration were found free of endotoxins.

4.1.3 Doses administered

4.1.3.1 Oral administration of NM-101, NM-102, NM-103, NM-104, NM-105

For the oral route a dispersion of the various TiO_2 nanomaterials (NM-101, NM-102, NM-103, NM-104, NM-105) was used containing 2.3 mg TiO_2 per ml. The single dose groups received a dose per animal of 2.3 mg resulting in a dose 10.2– 11.4 mg/kg b.w. for male animals, and 13.1 – 15.2 mg/kg for female animals depending on the actual weight of the animal. The repeated dose groups received five consecutive daily (day 1-5) administrations of 2.3 mg in one ml, resulting in a cumulative dose ranging between 51 – 57 mg/kg b.w. for male animals, and 65.5 – 76 mg/kg b.w. for female animals depending on the actual weight of the animal. All animal procedures followed the guidelines for the care and handling of laboratory animals established by the Danish government, and the Animal Experiment Inspectorate under the Ministry of Justice, approved the study (Case number: 2010/561-1779).

4.1.3.2 IV administration of NM-100, NM-102, NM-103, and NM-104

The TiO_2 doses for the oral and IV route were administered using a TiO_2 dispersion containing 2.30 mg TiO_2 per ml. For the IV route the single dose groups received a dose per animal of 2.3 mg per animal resulting in a dose between 8.7 – 9.7 mg/kg body weight (b.w.) for male animals, and 12.4 – 13.7 mg/kg b.w. for female animals depending on the actual weight of the animal (weight range males 238 g – 265 g, females 168 g – 186 g at the start of the experiment).

The repeated dose groups received total cumulative dose per rat between 43.5 – 48.5 mg/kg b.w. for male rats, and 62 – 68.5 mg/kg b.w. for female rats depending on the actual weight of the animal. All experiments were approved by an independent Ethical Committee on Animal Experimentation and were conducted in compliance with all applicable provisions of the national laws of the Netherlands i.e., the Experiments on Animal Decree and the Experiments on Animal Act. Study numbers were: DPA registration 201000255, 201000287, and 201100309.

4.1.3.3 IV administration of NM-105

For the IV dosing of NM-105 the single dose groups received a dose of approximately 10.5 TiO_2 mg/kg b.w. in 1 ml, whereas the repeated dose groups after five days treatment received a total cumulative dose of about 52.5 mg/kg b.w. These experiments were approved by the Animal Research Committee of the Institute of Neuroscience – BAS (Protocol N 27/02.06.2011) prior to the study.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

4.1.4 Organs sampled

4.1.4.1 Oral study

After the oral administration of TiO_2 nanomaterials (NM-101, NM-102, NM-103, NM-104, NM-105) the rats were anaesthetised using Hypnorm® (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml from Janssen Pharma) and Dormicum® (Midazolam 5 mg/ml from Roche). Both were mixed with equal volume of sterile water. The rats were killed by exsanguination via intracardiac puncture and the following organs were collected: blood (450 µl weighing ~500mg), spleen, liver, lungs, kidneys, heart, skin, muscle, bone, bone marrow and mesenteric lymph nodes (with mesenteria). In a follow up study performed under identical conditions, 6 rats were exposed for either vehicle or NM-102. The small (duodenum, jejunum, ileum) and large intestines (caecum, colon, rectum) including content were collected. All organs were snap frozen on dry ice and stored at -20°C. In view of the results obtained after repeated dosing (see below) tissues from animals after a single administration were not evaluated.

4.1.4.2 IV study

After IV administration of NM-100, NM-102, NM-103 and NM-104 the rats were anaesthetized by inhalation of isoflurane (Isoflu®, AST Pharma, Oudewater, The Netherlands) in oxygen and subsequently euthanized by drawing blood from the abdominal aorta. Next to blood (0.5-1.0 ml) the following organs were collected: liver, spleen, kidneys, thymus, heart, lungs, lymph nodes (mesenteric and popliteal), brain, bone including bone marrow (femur), testes/ovaries, skin, muscle. Urine and faeces where collected when feasible. All organs were stored at -20°C. Blood (100-200 µl) was collected via orbital puncture at day 1 and day 5 to evaluate Ti elimination from the blood circulation.

Following the IV injection with NM 105 (day 1 or 5) approximately 250 µl blood was taken from the tail vein at 5, 10, 20, 30, 60 min and 2 h (5x IV), and also at 4, and 8 h (1x IV) for Ti kinetics distribution study. On day 1, 6, 14, 30, and 90 of treatment, the animals were anaesthetised with ketamine (100 mg/kg b.wt) and euthanized. Blood samples were collected by intracardiac puncture together with liver, lung, spleen, kidney, heart, testes, abdominal aorta and brain and were frozen at -80 °C until further analysis. The injections were well tolerated and no adverse effects were observed during the entire experiment. During all housing period a drinking water and conventional animal chow were provided *ad libitum*.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

www.nanogenotox.eu

4.1.5 Determination of Ti

Four different methods for Ti determination were developed within WP7. Two out of four investigated methods were used for the Ti determination (HR-ICP-MS for studies using NM-100, NM-102, NM-103 and NM-104, and ICP-OES for studies with NM-105). The two other methods evaluated in WP7 were not used for the determination of Ti in the animal tissue samples, but are shortly described in Annex 2 of this report.

4.1.5.1 Sample pretreatment by digestion and measurement by High-Resolution Inductively Coupled Plasma Mass Spectrometry (HR-ICP-MS)

The tissue distribution of the TiO_2 nanomaterials administered to the animals was evaluated by determining the Ti content in blood and organs at various time points after administration. A tissue sample was weighed (approximately 0.5 g) into 15 ml polypropylene tubes and 0.5 ml ultrapure water, 1 ml nitric acid conc. ($cHNO_3$) and 0.75 ml hydrofluoric acid conc. (HF) were added. The tubes were placed on a block heater (type: Stuart SBH200D; supplied by Omnilabo, Breda, The Netherlands) and the mixture was slowly heated to a final temperature of 90°C. The mixture was left for two days at this temperature. Afterwards, ultrapure water was added to a total volume of 15 ml. With the applied procedure, it was possible to achieve a good digestion of the original MNs as well as of the biological materials. This procedure was used in the experiments for Ti determination of oral and intravenous studies performed with NM-100, NM-102, NM-103, and NM-104, and for the oral studies with NM-105.

The Ti measurements were performed by Philips Innovation Services (former MiPlaza, Eindhoven, The Netherlands). The measurements were performed with a HR-ICP-MS (High-Resolution Inductively Coupled Plasma Mass Spectrometry, ELEMENT XR, Thermo Fisher, Germany), using a set-up with on-line addition of an internal standard. The measurement of Ti was carried on two Ti-isotopes in the medium resolution (MR) mode: ^{47}Ti(MR) and ^{49}Ti(MR). The accurate masses and abundances are: ^{47}Ti (46.95176 atm): 7.3% resp. ^{49}Ti (48.94787 atm) 5.5%. The element Ti consists in total of five naturally abundant isotopes. Nevertheless, the other three isotopes cannot be used for quantification by HR-ICPMS due isobaric and polyatomic interferences. The abundances as well as the not resolvable, isobaric interferences are:

^{46}Ti with an abundance of 8.00% is interfered by calcium (^{46}Ca);

^{48}Ti with an abundance of 73.80% is interfered by calcium (^{48}Ca);

^{50}Ti with an abundance of 5.40% is interfered by chromium (^{50}Cr) and vanadium (^{50}V).

The MR mode was chosen for ^{47}Ti and ^{49}Ti as the following possible polyatomic interferences which can be resolved by using this MR mode, do not interfere the measurement of Ti:

^{47}Ti: $^{31}P^{16}O$, $^{11}B^{36}Ar$, $^{7}Li^{40}Ar$, $^{15}N^{16}O_2$, $^{14}N^{16}O_2{}^{1}H$, $^{12}C^{18}O^{16}O^{1}H$

^{49}Ti: $^{33}S^{16}O$, $^{13}C^{36}Ar$, $^{31}P^{18}O$, $^{9}Be^{40}Ar$, $^{14}N^{18}O^{16}O^{1}H$

The solutions were measured against an external calibration with internal standard correction. Two different internal standards (IS) were tested: ^{69}Ga resp. ^{115}In. Both measured

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

isotopes of Ti are interference free, lead to comparable results and they can be used for the quantification of Ti. Both internal standards (Ga respectively In) can be used for this application. For evaluation of the method used and quality control the general principles as described in the Eurochem Guide "The fitness for purpose of analytical methods (1998)" were applied.

The used sample introduction system and instrumental operating conditions of HR-ICP-MS (ELEMENT XR) set-up is summarized in Table 4-1 and the methodical parameters are given in Table 4-2. The measurements of Ti were performed on both the ^{47}Ti and ^{49}Ti isotopes. As shown in the simulation results (Figure 4-1) for the quantification by external calibration with internal standard correction ^{47}Ti was used with the HR-ICP-MS in medium resolution. There is a good separation for ^{47}Ti from possible polyatomic interference on this mass. So, ^{47}Ti (accurate mass 46.95176) was chosen for the measurements as it allows for Ti detection without interference, while ^{49}Ti was used as general control isotope. In addition, ^{47} Ti is present to a higher degree (7.3%) than ^{49}Ti (5.5%). A commercial reference material as obtained from Sero AS (Seronorm™ Trace Elements Whole Blood, Sero AS, Billingstad, Norway) was analysed according to the same procedure.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 4-1. Sample introduction system and instrumental operating conditions of HR-ICP-MS (ELEMENT XR).

Sample introduction system	
Nebulizer	MicroMist Nebulizer 0.2 ml/min; from Glass Expansion, Australia
Operation mode	Pumped with on-line mixing of internal standard, 1:1
Spray chamber	Twister Spray Chamber with Helix; from Glass Expansion, Australia
Cones	Nickel, type H
Instrumental operating conditions	
RF power	1225 W
Cool gas flow	16 L/min Ar
Auxilary gas flow	0.9 L/min Ar
Sample gas flow	0.99 L/min Ar

Table 4-2. Method and measuring parameters using HR-ICP-MS (ELEMENT XR).

Medium resolution mode (R=4000)	
Isotope (internal standard)	Used for quantification: ^{47}Ti (^{115}In) Used for control: ^{49}Ti (^{115}In)
Acquisition/'mass' window	125%
Search window	50%
Integration window	60%
Samples per peak	20
General parameters	
Acquisition mode	E-scan

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

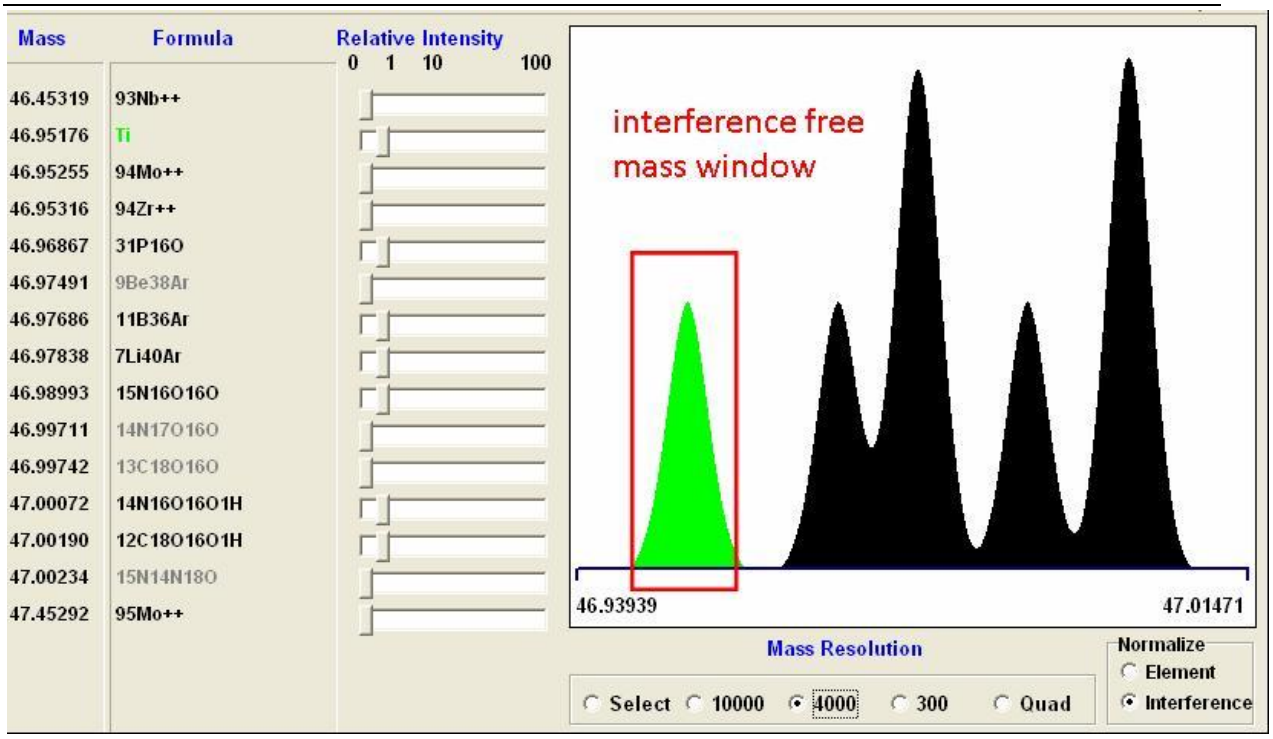


Figure 4-1. Simulation of ^{47}Ti determination in medium resolution (MR) mode of HR-ICP-MS as used for Ti determination.

Due to the fact that Ti can be also abundant in polypropylene tubes and in the used chemicals (acids and water), it is of great relevance to determine the blank concentration of Ti and to correct the raw analytical data for this concentration of Ti.

The blank concentration of Ti was determined for 15 ml polypropylene tubes from three suppliers and these results are given in Table 4-3.

Table 4-3. Blank concentration of Ti concentrations from 15 ml polypropylene tubes and the used chemical.

Supplier	c(Ti) [µg/L]
Sarstedt	(n=3): 1.6 ± 0.1
Corning	(n=3): (1.6 ± 0.1)
Greiner	(n=3): (2.3 ± 0.3)

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

During this study, in all series tubes from Sarstedt (Sarstedt BV, Etten-Leur, The Netherlands) were used and regularly controlled. The average of (n=41) is (1.4 ± 0.4) µg/L Ti. Within each series, the related blank concentration was used for correction.

The limit of detection (LOD; as 3xSD (3σ), n=20) is estimated on the results obtained for approximately 0.5 g tissue material from control animals of this study after applying the complete procedure of digestion and measurement with HR-ICPMS. The limit of detection (LOD) was determined as the analyte concentration corresponding to a 3σ value while the limit of quantification (LOQ) was determined as the analyte concentration corresponding to a 10σ value. The LOD is 0.05 µg Ti /g tissue and the LOQ 0.15 µg Ti /g tissue. For the tissue series evaluated for Ti contents after oral administration (for five consecutive days) the LOD was 0.03 µg Ti /g tissue, and the LOQ 0.09 µg Ti /g tissue.

As no certified reference material exists, two control materials (Seronorm™ Trace Elements Whole Blood, Sero AS, Billingstad, Norway) were pretreated and analyzed according to the same procedure in various series. Representative control measurements perfomed during Ti determination of the various tissue samples is shown in Table 4-4. Although the mean levels were observed in the same range as provided by the manufacturer for the two control materials, the variation in the measurements seems high as indicated by the standard deviation being approximately 31% of the mean value. No explanation can be presented. However, also for the levels provided by the manufacturer the standard deviation varied considerably being, 28%, 16% and 3% for the data provided and in the same range as our result for LOT 1003192 (Table 4-4).

Table 4-4. Measurement of Ti in two commercially available control materials both Seronorm™ Trace Elements Whole Blood (analysis by MiPlaza)

Sample	Reference concentration provided as "additional analytical values"	Measurement results
L-2 Ref 210205 LOT 1003192	18 ± 5 µg/L Ti* 19.5 ± 3.1 µg/L Ti	19 ± 6 µg/L Ti (n=11)
L-3 Ref 210305 LOT 1112691	12.8 ± 0.4 µg/L Ti	13 ± 4 µg/L Ti (n=9)

* data as provided by manufacturer Sero AS, Billingstad, Norway.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

4.1.5.2. Sample pretreatment by digestion and measurement by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

For the IV studies with NM-105 the following procedure was use for Ti determination: The excised tissues were homogenized with Teflon/glass homogenizer and approximately 1 g of each was mixed with 3 ml concentrated HNO_3 + 0.2 ml HF. The mixtures were digested in microwave (MW) oven and after cooling the samples were diluted to 5 ml with MilliQ water.

ICP-OES [Inductively Coupled Plasma Optical Emission Spectroscopy (ULTIMA 2, HORIBA Jobin Yvon, France)] was used for the determination of titanium concentration in the samples solutions.

Reliable and accurate determination of TiO_2 in biological samples typically consists of two steps: quantitative and complete sample and TiO_2 dissolution and interference free instrumental measurement of Ti. As a first step experiments were performed for the optimization of the acid mixture used for digestion, and conditions for complete dissolution of TiO_2. Model solutions containing TiO_2 at different concentration levels were obtained using stock solution of TiO_2 particles (2.56 mg/ml TiO_2 pre-wetted with ethanol, and dispersed in the presence of 0.05% BSA) prepared according to the proposed procedure developed in WP4. From one side it is well known that quantitative dissolution of TiO_2 requires HF, however, it is also known that the complex of Ti with F^- is very volatile. This means that sample dissolution should be performed in closed vessels, preferably under pressure. From the other side the presence of high concentrations of HF acid in sample solution is dangerous for quartz or glass instrumental parts e.g. nebulizer, chamber etcetera of ICP-OES or ICP-MS instruments. Therefore careful optimization of the amount of HF added is important in order to avoid any acid evaporation at the end of dissolution step. Evidently MW digestion with acid mixture consisting of HNO_3 conc. and a minimum amount of HF will be most useful for the dissolution of TiO_2, while in the same time under optimal conditions full digestion of sample organic matrix could be achieved. Experiments with various amounts of HNO_3 conc. and HF were performed with liver samples obtained from control animals which were spiked before dissolution at desired concentration levels with TiO_2. Recoveries obtained for different procedures are presented in Table 4-5.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 4-5. Recoveries achieved for different digestion mixtures and procedures (sample amount – 1 g liver from control animal, three parallel determinations, instrumental measurement by ICP OES).

Procedure/mixture		Recoveries (Measured Ti / Added Ti (%))	
		Spike concentration (110 µg)	Spike concentration (1100 µg)
1	3 ml HNO_3 (MW digestion)*	78±7	38±8
2	3 ml HNO_3 + 0.1 ml HF (MW digestion)	88±5	82±7
3	3 ml HNO_3 + 0.2 ml HF (MW digestion)	98±3	97±3
4	3 ml HNO_3 (open vessel)	48±7	25±8
5	3 ml HNO_3 +0.2 ml HF (open vessel)	84±6	73±6
6	3 ml HNO_3 +0.2 ml HF (90 °C, closed vessel, two days, recommended procedure)	93±5	92±5

* microwave assisted digestion system (CEM MARS, USA) using the program: step 1: 5 min at 300W; step 2: 5 min at 600W.

The procedure N3 (Table 4-5) was adopted for all tissues analyzed for the presence of NM-105 as indicated by Ti measurement, under the optimal instrumental parameters presented in Table 4-6.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 4-6. Optimal instrumental parameters for ICP-OES measurements

Parameters	
Power	1.1 kW
Argon flow:	
Plasma	12 L/min
Sheat	0.5 L/min
Carrier	0.5 L/min
Nebulizer	Concentric
Sample uptake	1.1 ml/min
Integration time	2 s
Replicates	3
Wavelengths, nm	336.121; 334.941

Both wavelengths could be used for Ti measurement however sensitivity achieved at 334.941 nm is higher and this wavelength is recommended and used here. Experiments performed with model solutions and added/found method showed that ICP-OES is very suitable instrumental methods for Ti determination in biological samples characterized with long calibration range and low matrix interferences.

The Limit of Detection and Limit of Quantification calculated as 3σ and 10σ criteria (σ being the standard deviation of measurements), respectively were obtained by parallel analysis of organs from control animals. The results are presented in Table 4-7.

Table 4-7. Limit of Detection and Limit of Quantification determined by ICP-OES for NM-105

Organs	Limit of Detection (LOD), µg Ti/g	Limit of Quantification (LOQ), µg Ti/g	RSD range, % for TiO_2 content between 0.15-200 µg/g
Liver , Lung, Spleen, Kidney (1 g in 5 ml final solution)	0.05	0.15	3-8

LOD (Limit of Detection) LOQ (Limit of Quantification), RSD (Relative Standard Deviation).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

4.1.6 Calculation of the recovery as µg Ti.

For the calculation of Ti recovery in biological samples the following formula was used:

$$\% \text{ recovery} = \{[\text{Ti } (\mu g/g) \text{ X Tissue weight } (g)] \div 0.6 \text{ x Dose } (TiO_2 \text{ in } \mu g)\} \text{ x } 100$$

4.1.7 Microscopy

At the time of death, liver samples were fixed in formalin, paraffin embedded, and sectioned for light microscopy using routine hematoxylin and eosin (H & E) staining. For transmission electron microscopy the samples were fixed at the time of death in 2.5% glutaraldehyde for 24h, washed with PBS, post-fixed with 1% osmium tetraoxide and embedded in Durcupan (Fluka, Buchs, Switzerland) for preparation of ultrathin sections for transmission electron microscopy. Ultrathin sections (70 nm) were prepared using LKB Reichert ultramicrotome. Tissue sections were stained and contrasted with 2.5% uranyl acetate and 1% lead acetate. Electron microscope Hitachi-500 was used for examination.

4.1.8 Statistical analysis

Results were expressed as mean ± standard deviation. For NM-105 multigroup comparisons of the means were carried out by one-way analysis of variance (ANOVA) followed by Bonferroni's test to compare the differences between the groups (PASW Statistics 18.0, IBM, USA). The statistical significance for all tests was set at $p < 0.05$.

4.2 Results

4.2.1 Tissue distribution after oral administration

For the oral administration (NM-101, NM-102, NM-103, NM-104, NM-105) mesenteric lymph nodes, liver and spleen were selected as priority target organs. The GI-tract was selected as a Ti positive sample. All liver and spleen tissue samples contained very low amounts of Ti, only incidentally Ti levels at or above the Limit of Detection (0.03 µg/g tissue) were observed (Table 4-8). Concentrations were either close to the Limit of Detection (n=4), at Limit of Detection (n=3) or below the Limit of Detection (n=47) of 0.03 µg Ti / g tissue. Of the 4 samples with concentrations above the Limit of Detection 3 samples were spleens of NM-105 exposed rats.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

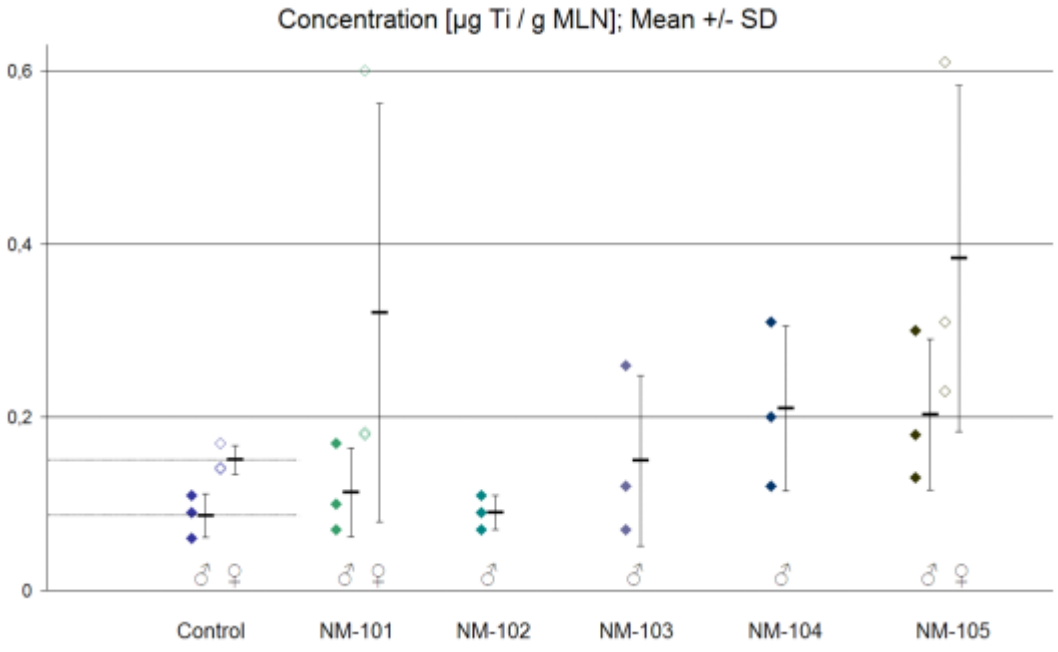
Co-funded by the Health Programme of the European Union

Table 4-8. Determinations of Ti in liver and spleen tissue samples.

		Liver			Spleen		
		Animal 1	Animal 2	Animal 3	Animal 1	Animal 2	Animal 3
Control	5 x 0 mg ♂	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
NM-101	5 x 2.304 mg ♂	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
NM-102	5 x 2.304 mg ♂	< 0.03	**0,03**	< 0.03	< 0.03	< 0.03	< 0.03
NM-103	5 x 2.304 mg ♂	**0,08**	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
NM-104	5 x 2.304 mg ♂	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
NM-105	5 x 2.304 mg ♂	< 0.03	< 0.03	< 0.03	< 0.03	**0,12**	< 0.03
Control	5 x 0 mg ♀	< 0.03	< 0.03	**0,03**	< 0.03	< 0.03	**0,03**
NM-101	5 x 2.304 mg ♀	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
NM-105	5 x 2.304 mg ♀	< 0.03	< 0.03	< 0.03	**0,21**	< 0.03	**0,13**

All concentration listed in [µg Ti / g tissue]

Mesenteric Lymph Nodes with mesenteria (MLN): All sub samples of MLN contained amounts above the Limit of Detection (Figure 4-2). Lowest detected concentration was 0.07 µg Ti / g MLN or 0.087 µg Ti for the whole MLN.



The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

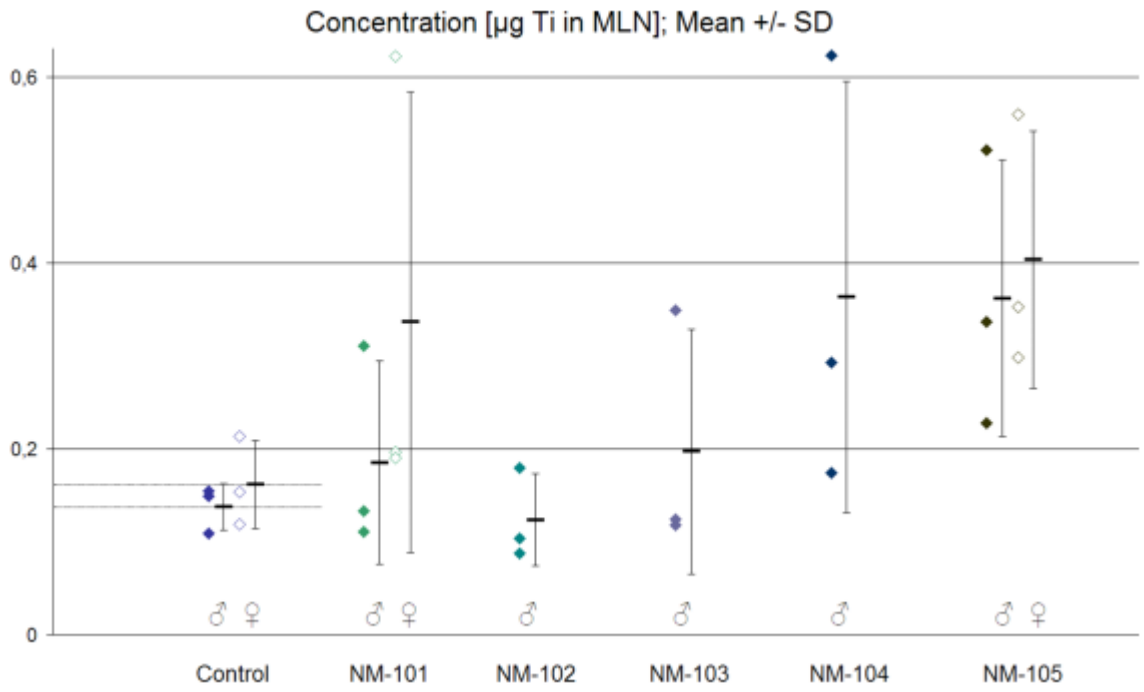


Figure 4-2. Concentration of Ti per gram Mesenteric Lymph Node tissue (top) or for the whole Mesenteric Lymph Node tissue (bottom) in control or TiO2 exposed male and female rats. The translocation of five different TiO_2 nanomaterials (NM-101-102-103-104-105) was examined.

There is no evidence for uptake of NM-101, NM-102 and NM-103. It therefore appears that there is no or a very low uptake of these particles. Slightly higher uptake was observed with materials NM-104 and NM-105. Due to the limited number of animals this gives borderline significant differences.

The Ti content within the GI tract from rats exposed to NM-102 for five consecutive days was also analysed. There were only marginally higher concentrations in the GI-tract of exposed rats compared to unexposed controls (Table 4-9). An explanation for Ti being detected in the control animals is that Ti is one of the most abundant elements in nature and it appears also to be present in the laboratory environment of the rats (e.g. feed). That Ti levels in exposed animals were only slightly elevated compared to non-treated animals can only be explained by a fast and almost complete elimination via defecation.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 4-9. Determinations of Ti in the GI Tract tissue samples following standard and extended digestion.

		GI tract standard digestion mg Ti / GI tract			GI tract extended digestion mg Ti / GI tract		
		Animal 1	Animal 2	Animal 3	Animal 1	Animal 2	Animal 3
Control	5 x 0 mg	0.6	0.4	0.3	0.7	0.5	0.4
NM-102	5 x 2.304 mg	0.8	0.4	0.4	0.7	0.6	0.6

It has previously been shown that standard transit time through a rat GI tract (female Wistar rat from oral uptake to excretion) has a mean time of 19.5h. The time was divided into a mean transit time of 1.97h for stomach, 2.07h for small and large intestines including the caecum and 15.5h for colon. When the authors stressed the animals by applying ½L of 30ºC water to the cage the mean transit time was significantly reduced to 5.39h. The time was divided into a mean transit time of 2.66h for stomach, 1.44h for small and large intestines including caecum and 1.29h for colon (Enck et al., 1989, Gut 30:455-459).

If oral dosing is considered a milder form of stress it is possible to observe reduced mean transit time in our rats. This hypothesis is likely the major cause for the small difference observed in Ti content of the GI tract of exposed and unexposed animals. However, also the animal diet may contain Ti as indicated by the Ti levels in control rats. It has also been shown that an increased content of cereal fibres in the feed reduces the GI-tract transit time in male Wistar rats (Flemming and Lee 1983). The diet used in this study of Flemming and Lee (1983) contained 16.8 and 11.8% of wheat and corn bran, respectively. Rats on a diet containing wheat or corn fibres excreted 84-85% of their intake within 24h. This means that if these rats on a diet as in the study of Flemming and Lee (1983) were exposed for 2.3 mg NM-102 then about 0.35mg would still remain in the GI tract (likely colon) 24 h after exposure. The difference between exposed and unexposed rats in Tables 4-8 and 4-9 is about 0.1 mg Ti / GI tract. The authors of the above mentioned study (Enck et al., 1989) do not mention the fibre content of the rat's diet. The Altromin 1324 feed used for the rats in the study contains 6 % raw fibres.

In conclusion, after oral administration of various TiO_2 nanomaterials none or very low uptake of TiO_2 was detected after five times repeated oral dosing of the animals. Only occasionally, some Ti could be detected in only 7 out of 54 samples of liver and spleen, although the levels measured were near the Limit of Detection of the ICP-MS used. For NM-104 and NM-105 also indications were present for some minimal distribution to the mesenteric lymph node. It is noteworthy that in the mesenteric lymph nodes and mesenteria (MLN) the detected difference in uptake between male and females (Female [Ti] - Male [Ti]) was larger when described per gram MLN. The difference between females and

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

males in Ti uptake of the MLN per gram tissue was 0.064 µg Ti/g tissue for controls, 0.207 µg Ti/g tissue for NM-101 and 0.180 µg Ti/g tissue for NM-105. When the difference was compared for the total MLN investigated, i.e. adjusted for the lower weight of female MLN, the difference between females and males was decreased. The difference based on Ti per MLN was 0.024 for controls, 0.151 for NM-101 and 0.042 µg Ti in MLN tissue for NM-105. When considering the data the higher values for NM-101 and NM-105 are mainly caused by one animal resulting in a high standard deviation of the mean (Figure 4-2). This suggests a similar Ti uptake for both genders and can be attributed to the fact that the amount of Ti is distributed in females in a smaller organ. The Ti level in the tissues of the GI-tract was not increased after treatment when compared to the Ti level in control animals, indicating a relatively high background in the GI-tract probably by ingestion of Ti via the feed. The oral exposure was initially evaluated only for the repeated dose schedule in order to obtain measurable levels in organs. As the results showed rather low levels in the main target organs like liver and spleen after the repeated administrations, samples obtained in the single dose experiment were not analysed for Ti content.

4.2.2 Tissue distribution after IV administration of NM-100, NM-102, NM-103 and NM-104

Background levels in animals used for the determination of the tissue distribution of TIO_2 nanomaterials NM-100, NM102, NM-103 and NM-104 were evaluated in vehicle treated animals after single and repeated administration of the vehicle. The results for the Ti content of organs of vehicle treated animals are presented in Table 4-10. For most organs only occasionally a value above the LOD (LOD = 0.05 µg Ti/g tissue) was noted. Only in the spleen in Exp 1 for all animals a concentration above the LOD was observed (Table 4-10).Additionally an estimation was made of mean values per group taking into consideration the incidental values above the LOD and the values below the LOD. In groups with rats with a detectable Ti level and rats with a measurement below the LOD, a simulant value was used for measurements below the LOD for estimation of the mean Ti for all animals investigated. By using a simulant value the information on animals with a measurement below the LOD was included in the estimation of the Ti concentration per gram organ for the group of animals. Using only the positive data would result in an overestimation of Ti content. Therefore, half the LOD values was used in the calculations as a simulant value for so called “non-detects” in order to be able to use the information on these tissues in the calculations. A value of 0 was considered to be too low a value introducing a bias to a low mean value, whereas using the LOD value itself (0.05 µg/g) was considered to result in a bias towards an overestimation of the organ Ti content (Brandon et al., 2012). Remarkably in the faeces of vehicle treated animals also clearly Ti levels were demonstrated well beyond the LOD which may be due to Ti present in the feed of the animals.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

www.nanogenotox.eu

Table 4-10 Presence of Ti in tissues of vehicle treated control rats.

	Ti µg/g organ[a]		Ti/ µg/g organ[b]	
Organ	Exp 1[c]	Exp 2[c]	Exp 1	Exp 2
Liver	< 0.05 (6)	0.37 ± 0.06 (3/9)	< 0.05 (6)	0.14 ± 0.17 (9)
Spleen	0.09 ± 0.02 (6)	0.09 (2/9)	0.09 ± 0.02 (6)	< 0.05 (6)
Kidney	0.06 (1/6)	nd	< 0.05 (6)	nd
Lung	0.08 (1/6)	nd	< 0.05 (6)	nd
Heart	< 0.05 (6)	nd	< 0.05 (6)	nd
Brain	0.05 (1/6)	nd	< 0.05 (6)	nd
Thymus	0.05 (1/6)	nd	< 0.05 (6)	nd
Testes	< 0.05 (6)	nd	< 0.05 (6)	nd
Blood	< 0.05 (4)	0.07 (1/9)	< 0.05 (4)	< 0.05 (9)
Faeces	6.33 ± 2.58 (6)	nd	6.33 ± 2.58 (6)	nd

a) Concentration of Ti in organs of vehicle treated control rats. When possible mean and standard deviation in µg/g tissue is presented. Within brackets number of animals investigated, or number of animals with value above LOD (=0.05 µg/g tissue) versus total number of animals investigated. In these organs all other animals had values below the LOD (<0.05 µg/g tissue). b) Estimation of mean tissue level taking into consideration all measurements including values of animals below LOD. When one animal in a group was found to contain Ti above the LOD of 0.05 µg/g tissue for the other animals in that same group half the value of the LOD being 0.025, was used for calculations. A tissue level presented as < 0.05 indicates an overall mean tissue level below the Limit of Detection (LOD=0.05 µg/g tissue). c) Exp. 1 shows results of 6 male rats, Exp. 2 shows results of 6 male and 3 female rats. nd, not done.

The Ti levels as µg/g tissue for male animals treated with the TiO_2 nanomaterials (NM-100, NM-102, NM-103, NM-104) are presented in Tables 4-11A-D.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 4-11A. Ti tissue distribution (µg/g tissue) in male and female rats after single intravenous TiO_2 (NM-100) administration.

NM-100	Male		Female	
Single Dose	Day 2	Day 90	Day 2	Day 90
Liver	97 ± 5	51 ± 6	175 ± 53	85 ± 8
Spleen	62 ± 3	72 ± 15	95 ± 16	122 ± 25
Kidney	0.48 ± 0.27	0.06 ± 0.01	0.35 ± 0.09	0.06 ± 0.01
Lung	16	5.3 ± 2.5	13.3 ± 5.1	12 ± 9
Heart	0.24 ± 0.06	0.10 ± 0.05	0.20 ± 0.03	0.13 ± 0.05
Brain	< 0.05	< 0.05	< 0.05	< 0.05 (2)
Thymus	0.05 ± 0.02	0.14 ± 0.08	< 0.05	0.17 ± 0.18
Testes	< 0.05	< 0.05	-	-
Ovaries	-	-	0.08 ± 0.03	0.10 ± 0.06
Skin	0.10 ± 0.06	nd	0.09 ± 0.06	nd
Blood	0.12 ± 0.04	< 0.05	0.10 ± 0.07	< 0.05

Mean and standard deviation in µg/g tissue, number of animals investigated n=3 unless otherwise indicated within brackets. nd, not done

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 4-11B. Ti tissue distribution (µg/g tissue) in male and female rats after single intravenous TiO_2 (NM-102) administration.

NM-102	Male		Female	
Single Dose	Day 2	Day 90	Day 2	Day 90
Liver	86 ± 12	46 ± 10	115 ± 13	85 ± 9
Spleen	44 ± 3	54 ± 7	47 ± 11	97 ± 43
Kidney	0.38 ± 0.07	0.05 ± 0.01	0.57 ± 0.43	0.09 ± 0.03
Lung	33 ± 21	27 ± 10	25 ± 23	19 ± 4
Heart	0.16 ± 0.02	0.10 ± 0.01	0.15 ± 0.04 (2)	0.14 ± 0.03
Brain	< 0.05	< 0.05	< 0.05	< 0.05 (2)
Thymus	< 0.05	0.23 ± 0.23	< 0.05	0.17 ± 0.08
Testes	< 0.05	< 0.05	-	-
Ovaries	-	-	0.12 ± 0.14	< 0.05 (2)
Skin	0.08 ± 0.02	nd	0.06 ± 0.04	nd
Blood	< 0.05	< 0.05	< 0.05	< 0.05

Mean and standard deviation in µg/g tissue, number of animals investigated n=3 for each time point unless otherwise indicated within brackets. Ti tissue levels were corrected for the initial body weight of the animals for comparison between male and female animals. nd, not done.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 4-11C. Ti tissue distribution (µg/g tissue) in male and female rats after single intravenous TiO_2 (NM-103) administration.

NM-103	Male		Female	
Single Dose	Day 2	Day 90	Day 2	Day 90
Liver	112 ± 20	88 ± 9 (2)	150 ± 1 (2)	178 ± 28
Spleen	56 ± 1	94 ± 10 (2)	87 ± 4 (2)	152 ± 13
Kidney	0.85 ± 0.11	0.17 (2)	0.86 ± 0.21 (2)	0.39 ± 0.20
Lung	22 ± 6	4.6 ± 4.9 (2)	28 ± 8 (2)	16 ± 4
Heart	0.57 ± 0.22	0.26 ± 0.20 (2)	0.40 ± 0.05 (2)	0.55 ± 0.24
Brain	0.08 ± 0.03	< 0.05 (2)	0.10 ± 0.03 (2)	< 0.05
Thymus	0.11 ± 0.05	0.80 ± 0.57 (2)	0.15 ± 0.05 (2)	0.83 ± 0.23
Testes	< 0.05	< 0.05 (2)	-	-
Ovaries	-	-	0.28 ± 0.01 (2)	0.54 ± 0.50
Skin	nd	nd	nd	nd
Blood	< 0.05	nd	< 0.05 (2)	nd

Mean and standard deviation in µg/g tissue, number of animals investigated n=3 for each time point unless otherwise indicated within brackets. nd, not done.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 4-11D. Ti tissue distribution (µg/g tissue) in male and female rats after single intravenous TiO_2 (NM-104) administration.

NM-104	Male		Female	
Single Dose	Day 2	Day 90	Day 2	Day 90
Liver	130 ± 36	111 ± 5	198 ± 24	166 ± 7
Spleen	67 ± 17	112 ± 19	98 ± 17 (2)	194 ± 28
Kidney	0.54 ± 0.05	0.14 ± 0.02	0.40	0.22 ± 0.12
Lung	7.3 ± 1.2	4.1 ± 1.0	6.3 ± 0.6	5.7 ± 1.5
Heart	0.17 ± 0.03	0.07 ± 0.01	0.24 ± 0.03	0.09 ± 0.01
Brain	< 0.05	< 0.05	< 0.05	< 0.05
Thymus	0.09 ± 0.02	0.07 ± 0.03 (2)	0.07 ± 0.02	0.08 ± 0.03
Testes	< 0.05	< 0.05	-	-
Ovaries	-	-	0.24 ± 0.03	0.16 ± 0.12
Skin	nd	nd	nd	nd
Blood	< 0.05	nd	< 0.05	nd

Mean and standard deviation in µg/g tissue, number of animals investigated n=3 for each time point unless otherwise indicated within brackets. nd, not done.

Major target organs are liver, spleen and lung, whereas low levels (<1 µg/g) were observed in most other organs investigated (heart, brain, thymus, testes, ovaries and skin). For most organs a decrease was noted between day 2 and day 90 with the exception being the Ti level in the liver after NM-103 administration in female animals. In the spleen for all four nanomaterials between day 2 and day 90, an increase in Ti level as µg Ti per gram organ was noted. Similar results were observed after repeated administration of NM-100, NM-102, NM-103, and NM-104. For liver and spleen in general higher Ti concentrations at day 2/day6 and day 90 after single and repeated administration, respectively, was observed in female animals when compared to male animals. For kidney, lung and other organs only incidentally Ti values in females were higher (single dose: NM-103 lung day 90; repeated dose NM-100 lung day 6, heart day 90; NM-102 lung day 90, NM-104 lung day 6). This can be attributed to the higher dose the females received per kg body weight as the female animals weighed less compared to the male rats, and both male and females received the same dose per animal. As the majority of the Ti ends in liver and spleen differences between male and females animals are most clearly observed in these two organs. The Ti levels per organ for repeated 5

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

times administration are presented in Tables 4-12A-D. After the repeated administration approximately a fivefold increase in Ti levels was noted compared to a single dose of TiO_2 (data not shown).

Table 4-12A Ti tissue distribution (µg/g tissue) in male and female rats after repeated intravenous TiO_2 (NM-100) administration.

NM-100		Day 6	Day 14	Day 30	Day 90
Liver	M	462 ± 35	391 ± 57	293 ± 99	294 ± 46
	F	576 ± 22(2)	nd	nd	470 ± 133
Spleen	M	327 ± 42	390 ± 51	380 ± 20	483 ± 57
	F	414 ± 18	nd	nd	827 ± 8(2)
Kidney	M	1.14 ± 0.16	0.59 ± 0.03	0.20 ± 0.04	0.28 ± 0.16
	F	0.98 ± 0.12	nd	nd	0.27 ± 0.07
Lung	M	43 ± 5	48 ± 11	38 ± 10	50 ± 19
	F	65 ± 9	nd	nd	55 ± 26
Heart	M	0.72 ± 0.14	0.51 ± 0.02	0.36 ± 0.02	0.33 ± 0.03
	F	0.66 ± 0.18	nd	nd	0.67 ± 0.13(2)
Brain	M	0.07 ± 0.02	0.06 ± 0.01(2)	<0.05(2)	0.08 ± 0.05
	F	0.06 ± 0.01	nd	nd	0.06 ± 0.01(2)
Thymus	M	0.26 ± 0.08	0.23 ± 0.16	0.35 ± 0.32	0.29 ± 0.18
	F	0.22 ± 0.24	nd	nd	1.24 ± 0.95
Testes	M	0.06 ± 0.01	0.04 ± 0.01	< 0.05(2)	< 0.05
Ovaries	F	0.41 ± 0.03	nd	nd	0.26 ± 0.19
Blood	M	0.27 ± 0.02	< 0.05	< 0.05	< 0.05
	F	0.15 ± 0.05	nd	nd	< 0.05

Animals were treated via intravenous administration on 5 consecutive days (days 1 - 5). Mean and standard deviation in µg/g tissue, number of animals investigated n=3 (3 males and 3 females) for each time point unless otherwise indicated within brackets. On day 14 and day 30 Ti levels were only determined in male animals (n=3).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 4-12B Ti tissue distribution (µg/g tissue) in male and female rats after repeated intravenous TiO_2 (NM-102) administration.

NM-102		Day 6	Day 14	Day 30	Day 90
Liver	M	437 ± 80	427 ± 32	375 ± 36	237 ± 7
	F	640 ± 40	nd	nd	402 ± 48
Spleen	M	228 ± 37	293 ± 48	343 ± 30	730 ± 65
	F	271 ± 17	nd	nd	730 ± 96
Kidney	M	1.31 ± 0.12	0.92 ± 0.07	0.53 ± 0.02	0.54 ± 0.08
	F	1.29 ± 0.23	nd	nd	0.62 ± 0.23
Lung	M	90 ± 14	110 ± 32	92 ± 23	69 ± 3
	F	105 ± 9	nd	nd	128 ± 31
Heart	M	0.67 ± 0.21	0.74 ± 0.20	0.44 ± 0.01	0.43 ± 0.16
	F	0.63 ± 0.08	nd	nd	0.62 ± 0.06
Brain	M	0.07 ± 0.02	0.08 ± 0.03	0.12 ± 0.07	<0.05
	F	0.11 ± 0.09	nd	nd	0.06 ± 0.03(2)
Thymus	M	0.11 ± 0.01	0.13 ± 0.04	0.19 ± 0.02(2)	1.25 ± 0.93(2)
	F	0.25 ± 0.19	nd	nd	0.77 ± 0.09(2)
Testes	M	0.06 ± 0.02	< 0.05	< 0.05	< 0.05
Ovaries	F	0.29 ± 0.04	nd	nd	0.25 ± 0.11
Blood	M	0.11 ± 0.03	< 0.05	< 0.05(2)	< 0.05
	F	0.10 ± 0.04	nd	nd	< 0.05

Animals were treated via intravenous administration on 5 consecutive days (days 1 - 5). Mean and standard deviation in µg/g tissue, number of animals investigated n=3 (3 males and 3 females) for each time point unless otherwise indicated within brackets. On day 14 and day 30 Ti levels were only determined in male animals (n=3).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 4-12C Ti tissue distribution (µg/g tissue) in male and female rats after repeated intravenous TiO_2 (NM-103) administration.

NM-103		Day 6	Day 14	Day 30	Day 90
Liver	M	612 ± 32	655 ± 102	519 ± 143	244 ± 41
	F	939 ± 294	nd	nd	663 ± 51
Spleen	M	337 ± 28	504 ± 26	864 ± 258	2162 ± 213(2)
	F	359 ± 23	nd	nd	1949 ± 376
Kidney	M	3.27 ± 0.23	1.87 ± 0.21	1.47 ± 0.15	2.30 ± 0.00(2)
	F	3.20 ± 0.46	nd	nd	1.40 ± 0.17
Lung	M	116 ± 5	71 ± 5	63 ± 7	99 ± 7(2)
	F	132 ± 36	nd	nd	104 ± 8
Heart	M	1.20 ± 0.10	0.66 ± 0.07	0.62 ± 0.12	1.60 ± 0.28(2)
	F	1.50 ± 0.35	nd	nd	1.15 ± 0.26
Brain	M	0.34 ± 0.04	0.19 ± 0.03	0.14 ± 0.03	0.14 ± 0.03(2)
	F	0.47 ± 0.28	nd	nd	0.10 ± 0.02
Thymus	M	0.80 ± 0.34	2.92 ± 2.86	2.48 ± 2.35	1.43 ± 0.49
	F	1.19 ± 0.47	nd	nd	0.55 ± 0.49(2)
Testes	M	0.08 ± 0.02	< 0.05	< 0.05	< 0.05
Ovaries	F	0.94 ± 0.23	nd	nd	4.47 ± 1.43
Blood	M	0.14 ± 0.05	0.07 ± 0.02	<0.05	nd
	F	0.08 ± 0.03	nd	nd	nd

Animals were treated via intravenous administration on 5 consecutive days (days 1 - 5). Mean and standard deviation in µg/g tissue, number of animals investigated n=3 (3 males and 3 females) for each time point unless otherwise indicated within brackets. On day 14 and day 30 Ti levels were only determined in male animals (n=3).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 4-12D Ti tissue distribution (µg/g tissue) in male and female rats after repeated intravenous TiO_2 (NM-104) administration.

NM-104		Day 6	Day 14	Day 30	Day 90
Liver	M	561 ± 83	600 ± 21	402 ± 23	317 ± 12
	F	968 ± 149	nd	nd	620 ± 52
Spleen	M	327 ± 102	752 ± 65	1132 ± 64	1918 ± 449
	F	404 ± 7	nd	nd	2699 ± 239
Kidney	M	2.13 ± 0.90	1.53 ± 0.21	1.50 ± 0.10	1.83 ± 0.60
	F	2.30 ± 0.26	nd	nd	1.50 ± 0.10
Lung	M	27 ± 12	29 ± 6	33 ± 4	48 ± 8
	F	53 ± 1	nd	nd	57 ± 3
Heart	M	0.47 ± 0.27	0.48 ± 0.03	0.52 ± 0.24	1.29 ± 0.38
	F	1.07 ± 0.55	nd	nd	1.19 ± 0.28
Brain	M	0.12 ± 0.05	0.07 ± 0.01	0.11 ± 0.05	0.14 ± 0.13
	F	0.12 ± 0.01	nd	nd	0.06 ± 0.01(2)
Thymus	M	0.71 ± 0.53	1.27 ± 0.80	2.54 ± 2.11	0.91 ± 0.11(2)
	F	0.57 ± 0.22	nd	nd	2.10 ± 0.28(2)
Testes	M	0.08 ± 0.06	0.06 ± 0.01	<0.05	< 0.05
Ovaries	F	1.10 ± 0.17	nd	nd	0.55 ± 0.48
Blood	M	0.12 ± 0.04	0.08 ± 0.03	< 0.05	nd
	F	0.08 ± 0.03	nd	nd	nd

Animals were treated via intravenous administration on 5 consecutive days (days 1 - 5). Mean and standard deviation in µg/g tissue, number of animals investigated n=3 (3 males and 3 females) for each time point unless otherwise indicated within brackets. On day 14 and day 30 Ti levels were only determined in male animals (n=3).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Similar to the single administration, from day 6 to day 90 after the repeated TiO_2 administrations in general a decrease is present in Ti level in the liver, kidney, testes, and blood, whereas the level in the spleen shows an increase. For lung, heart, brain, thymus and ovaries there was in general no decline in the Ti concentrations per gram tissue for four TiO_2 nanomaterials investigated (NM-100, NM-102, NM-103, NM-104).

Both after single and repeated TiO_2 nanomaterial administration Ti levels in the faeces were similar to those observed in vehicle control animals indicating a lack of excretion of the TiO_2 nanomaterials (Table 4-13). Most likely the Ti measured in the faeces is due to the presence of some Ti in the feed of the animals. However, Ti concentrations in the animal feed were not measured so other sources of Ti cannot be excluded. Urine samples were investigated in male and female animals after single (day 2) and repeated (day 6) NM-100 or NM-102 TiO_2 administration. In all of the urine samples investigated (n=24) the level of Ti was below the LOD (0.05 µg/g) (data not shown). As also after the repeated TiO_2 administration no Ti could be detected in the urine, urine samples were not further investigated.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 4-13 Presence of Ti (µg Ti/g) in faeces of vehicle treated control and TiO_2 treated animals

Males	Treatment				
	Vehicle	NM-100	NM-102	NM-103	NM-104
			Single dose		
Day 2	7.5 ± 2.1 (2)	6.0 ± 2.6 (3)	4.7 ± 0.6 (3)	4.7 ± 2.3 (3)	8.7 ± 2.9 (3)
Day 6	5.5 ± 4.9 (2)	-	-	-	-
Day 90	6.0 (2)	-	-	4.0 ± 1.7 (3)	6.7 ± 1.5 (3)
			Repeated dose		
Day 6	-	5.0 (3)	4.7 ± 1.5 (3)	5.0 ± 2.0 (3)	7.7 ± 5.8 (3)
Day 14	-	-	-	6.0 ± 1.7 (3)	9.0 ± 2.0 (3)
Day 30	-	-	-	5.7 ± 1.5 (3)	8.3 ± 3.0 (3)
Day 90	-	-	-	5.7 ± 1.5 (3)	6.3 ± 0.6 (3)
Females	Treatment				
	Vehicle	NM-100	NM-102	NM-103	NM-104
			Single dose		
Day 2	-	5.7 ± 1.5 (3)	5.0 ± 1.0 (3)	10.0 ± 1.0 (3)	9.0 ± 1.7 (3)
Day 90	-	-	-	7.7 ± 0.6 (3)	9.0 ± 2.6 (3)
			Repeated dose		
Day 6	-	7.3 ± 0.6 (3)	7.3 ± 1.2 (3)	6.7 ± 2.3 (3)	8.7 ± 1.2 (3)
Day 90	-	-	-	7.3 ± 1.5 (3)	7.7 ± 1.5 (3)

Mean and standard deviation in µg Ti/g faeces are presented, within brackets number of animals investigated.

The tissue distribution of the four nanomaterials as percentage of the dose is presented for the single administration of TiO_2 in Figure 4-3A-B, and for the repeated administrations in Figures 4-4A-D. Between the four nanomaterials some minor differences were present. For NM-100 and NM-

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

102 at day 2 no Ti could be recovered in the thymus after a single administration, whereas for NM-103 and NM-104 very low (<0.01% of the dose) levels of Ti were recovered. In the testes no Ti was recovered at day 2 after administration, whereas in the ovaries Ti was recovered at a low level (approximately 0.01% of the dose). At day 90 after the single dosing in blood (NM-100, NM-102), brain and testes no Ti was recovered, whereas in the ovaries still some Ti was observed in NM-100, NM-103, and NM-104. For NM-103 and NM-104 blood was not evaluated at day 90 after the single dosing. Most Ti was recovered in the liver followed by spleen and lung.

The difference in Ti content in spleen and liver of male and female animals, when the Ti concentration was expressed as µg Ti/g tissue, for most determinations disappears when we express the Ti concentration as Ti per organ (data not shown) or as % of the dose. For liver and spleen we see in general similar tissue levels when the Ti is expressed as % of the dose after single IV administration of NM-100, NM-102, NM-103 and NM-104. Of the other organs after single administration only the kidney of NM-104 shows a higher Ti content in male animals. After repeated administration we see a similar pattern. With some exceptions there is generally no difference in Ti content in organs of male and female animals. The exceptions are: NM-102 Ti in male spleen higher at day 90; NM-103 Ti in female liver higher at day 90, Ti in male spleen higher at day 90, Ti in male kidney higher at day 6 and day 90, Ti in male lung higher at day 90; NM-104 Ti in female liver higher at day 90. As most results for Ti content in organs of male and female animals are similar, and there is no clear pattern in these differences (e.g. the same organs in females or males always higher), the results for the organ distribution as % of the administered dose are presented including both male and female animals (Figures 4-3A-B and Figures 4-4A-D).

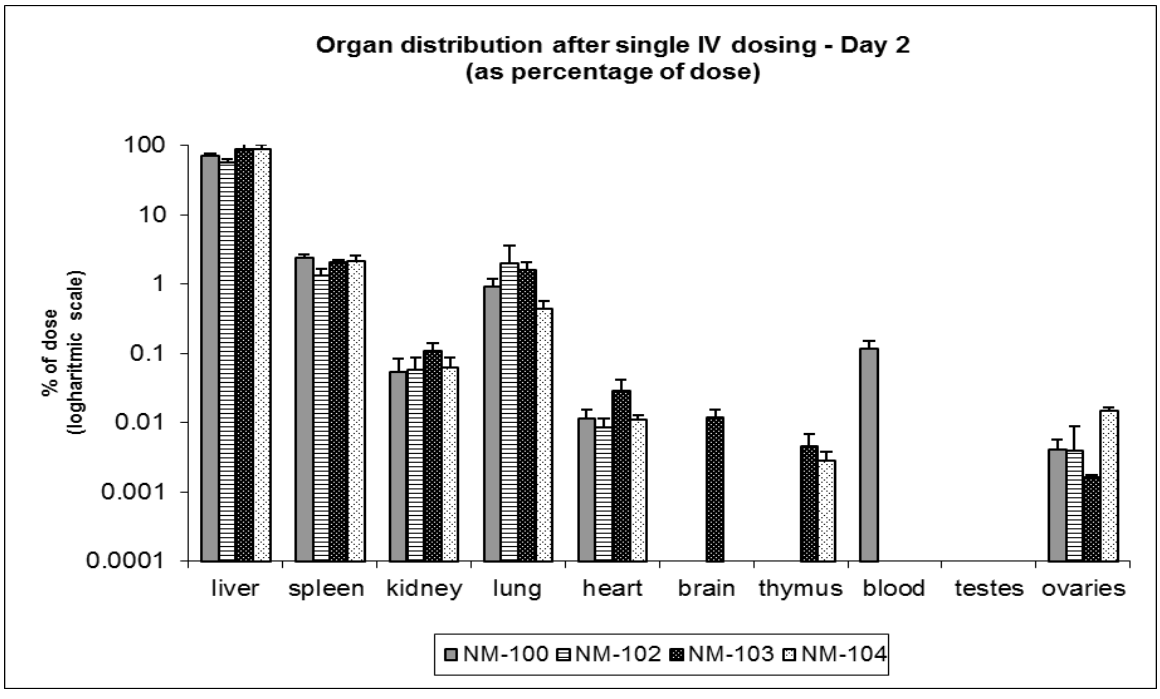


Figure 4-3A Tissue distribution of Ti for males and females at day 2 after single IV administration (24 hours after the IV administration) expressed as % of the Ti dose administered.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

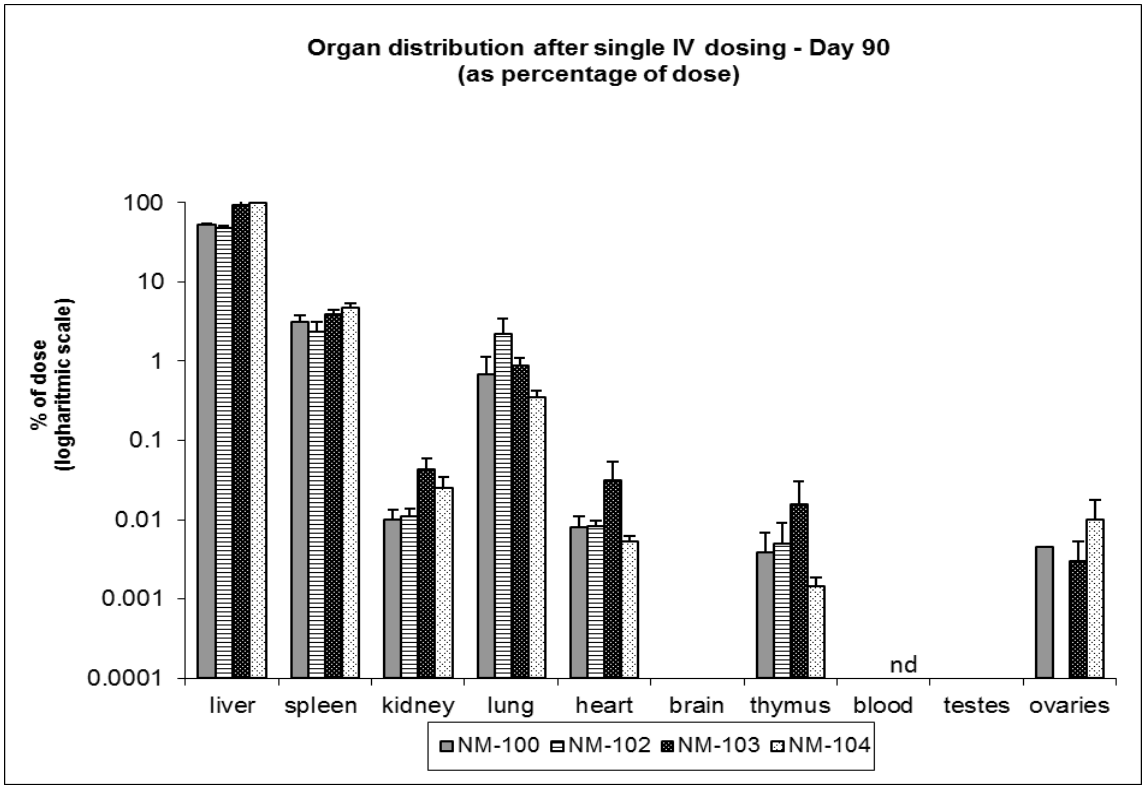


Figure 4-3B Tissue distribution of Ti for males and females at day 90 after single IV administration expressed as % of the Ti dose administered. nd, not done (NM-103, NM-104).

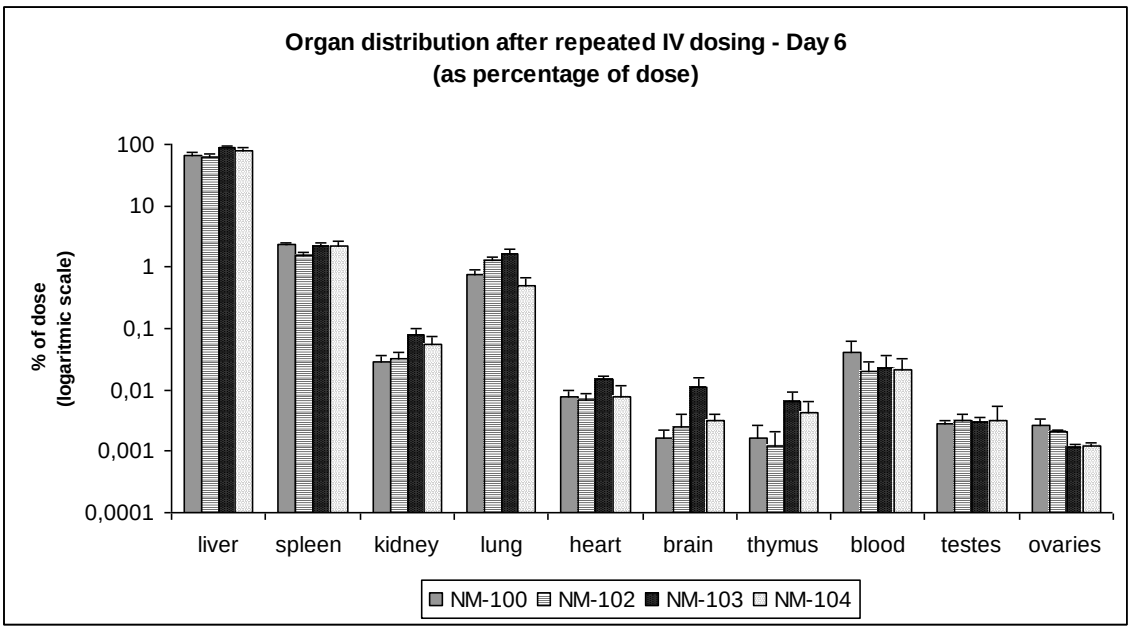


Figure 4-4A .Tissue distribution of Ti for males and females at day 6 (24 hours after the last IV administration) expressed as % of the total Ti dose administered during five consecutive days (days 1-5).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

www.nanogenotox.eu

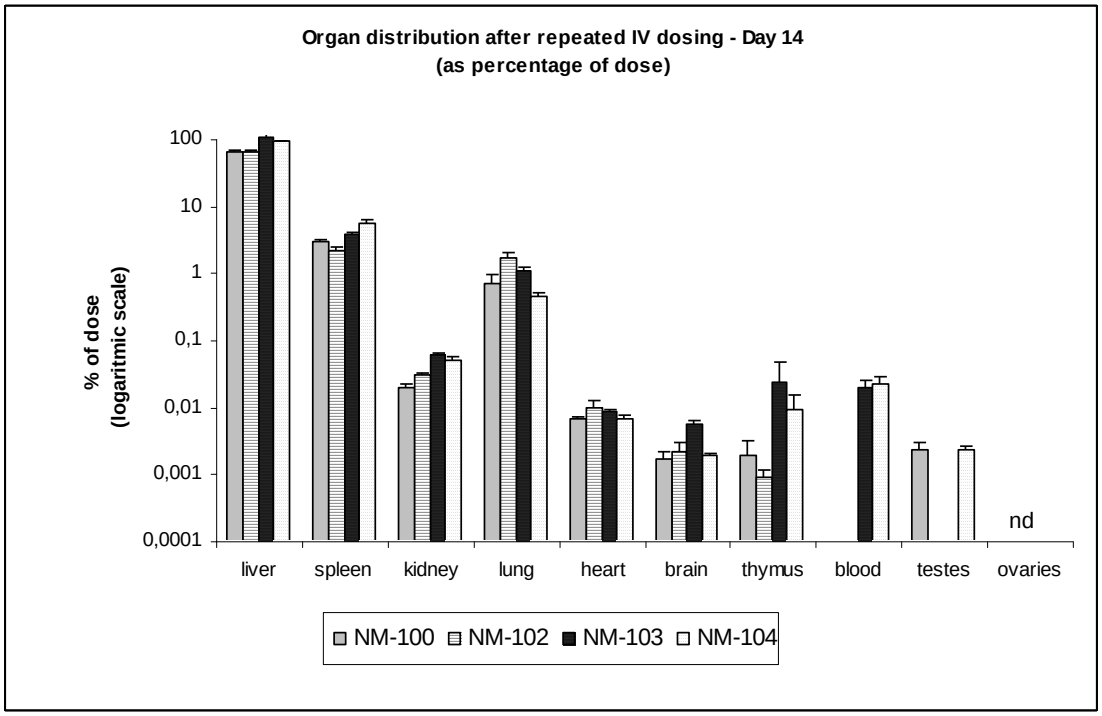


Figure 4-4B .Tissue distribution of Ti for males and females at day 14 expressed as % of the total Ti dose administered during five consecutive days (days 1-5). nd, not done

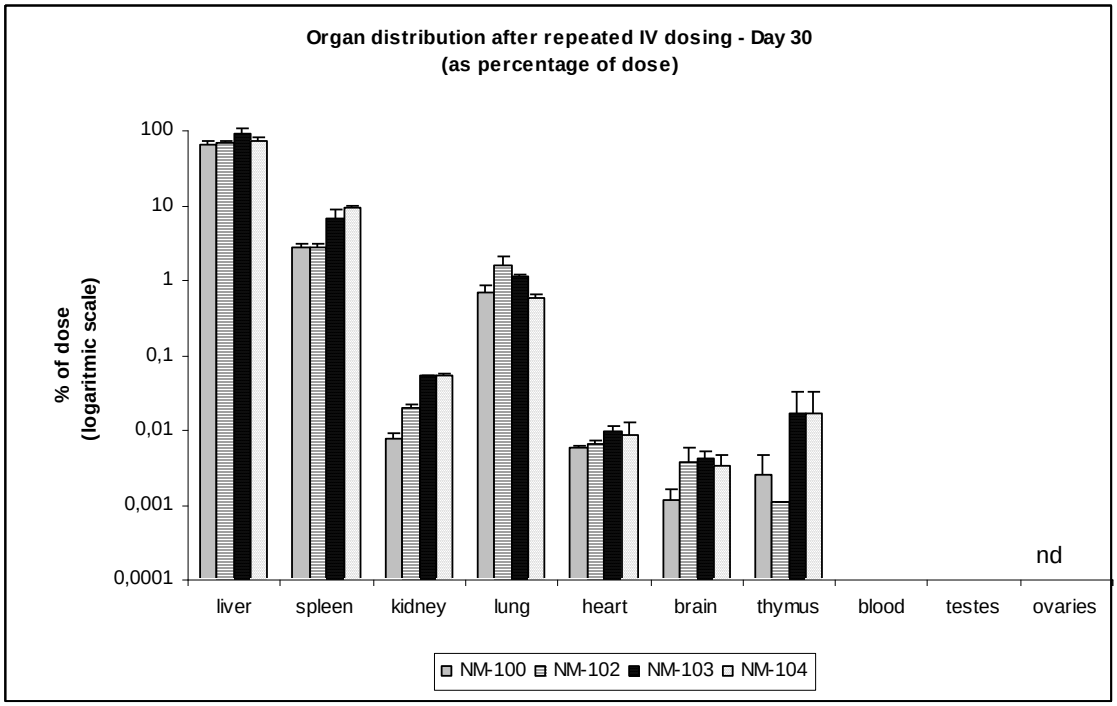


Figure 4-4C .Tissue distribution of Ti for males and females at day 30 expressed as % of the total Ti dose administered during five consecutive days (days 1-5). nd, not done.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

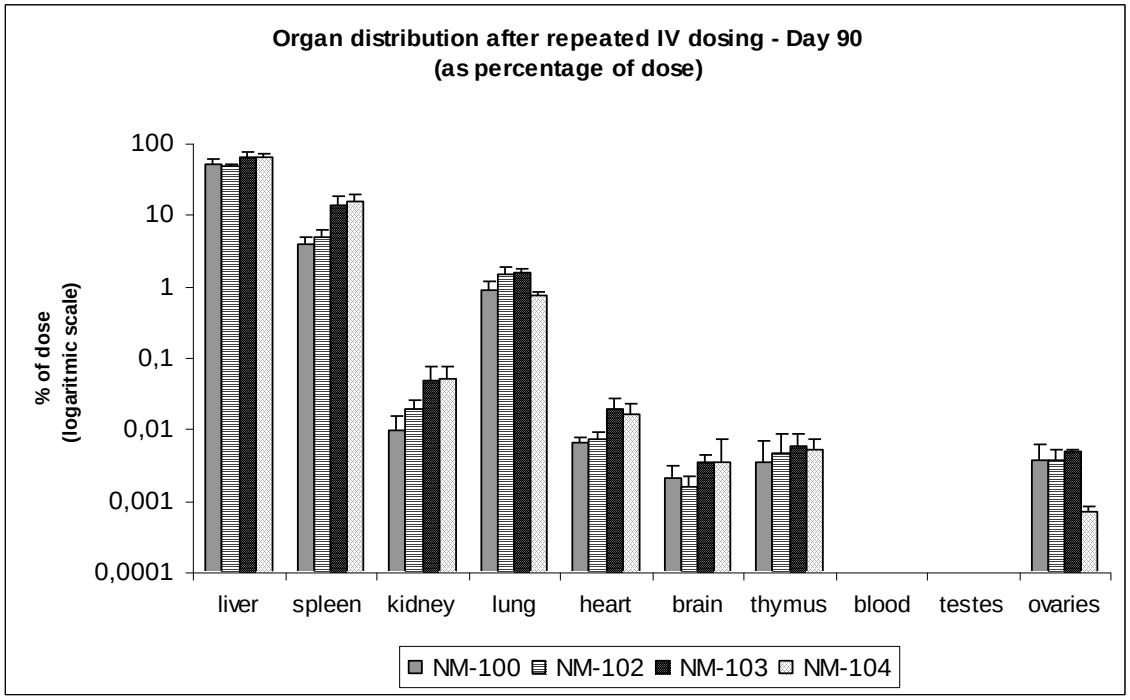


Figure 4-4D .Tissue distribution of Ti for males and females at day 90 expressed as % of the total Ti dose administered during five consecutive days (days 1-5).

On days 14 and 30 only male animals were investigated so there are no data for ovaries at those time points. NM-102 and NM-103 were not observed in the testes on day 14 and onwards whereas NM-100 and NM-104 were still present in the testes at day 14. From day 30 and onwards the level of Ti in the testes was below the Limit of Detection (Ti < 0.05 µg/g tissue). In contrast on day 90 after the repeated TiO_2 administrations Ti was still observed in the ovaries for all TiO_2 nanomaterials investigated (Figure 4-4D).

At 24 h after the administration still some Ti could be detected in the circulating blood (Figure 4-4A). NM-103 and NM-104 was still present in the blood at day 14 after start of the repeated IV administration (Figure 4-3B).

The recovery from the investigated tissues ranged from 52 to 110% of the dose (Table 4-14). Some decrease was observed for NM-100 and NM-102, and NM-103 whereas NM-104 remained stable over the time investigated, indicating that the excretion from the body is slow to not existing. NM-103 showed a decrease in recovery in contrast to NM-104. This decrease was mainly caused by a decrease in recovery in male animals (data not shown). The lack of excretion was confirmed by the data on Ti levels in urine and faeces. None of the four investigated TiO_2 nanomaterials induced an increase in Ti level in urine or faeces following repeated IV administration. The Ti level in the faeces was constant of the same magnitude and comparable to levels in vehicle control animals.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

www.nanogenotox.eu

Table 4-14 Recovery of Ti for males and females in the investigated organs as percentage of dose administered after single or repeated dosing.

Nanomaterial	Dosing	Day 2 Or Day 6	Day 14	Day 30	Day 90	Accumulation factor
NM-100	Single	74 ± 5	nd	nd	56 ± 2	0.8
	Repeated	72 ± 7	69 ± 4	69 ± 4	56 ± 10	0.8
NM-102	Single	62 ± 6	nd	nd	52 ± 3	0.8
	Repeated	65 ± 10	68 ± 6	70 ± 3	54 ± 1	0.8
NM-103	Single	95 ± 18	nd	nd	99 ± 12	1.0
	Repeated	92 ± 7	110 ± 21	96 ± 19	75 ± 13	0.8
NM-104	Single	92 ± 12	nd	nd	105 ± 7	1.1
	Repeated	82 ± 10	99 ± 3	81 ± 10	81 ± 7	1.0

nd, not determined.

All nanomaterials investigated were rapidly distributed from the bloodstream to the different organs (Figures 4-5A-D). Higher absolute concentrations were observed in blood after repeated dosing versus single dosing. NM-103 and NM-104 were detectable in blood until day 14 after the repeated administrations (Figure 4-4B).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

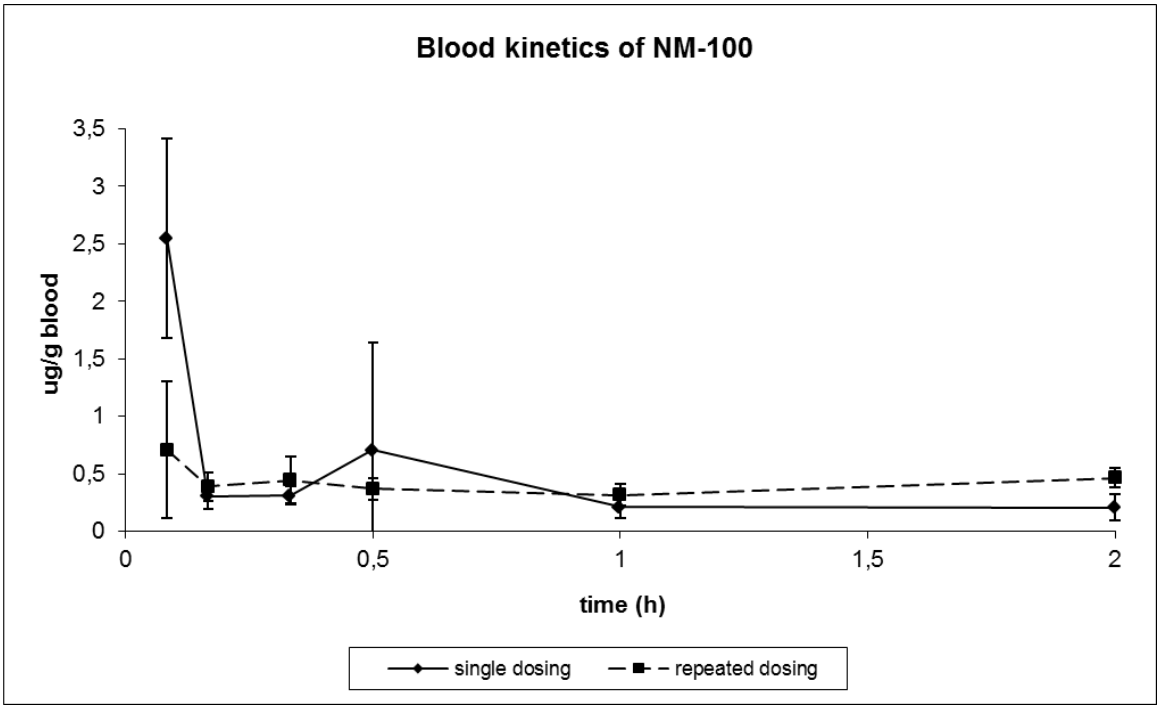


Figure 4-5A. Blood kinetics of NM-100 after single and repeated IV dosing.

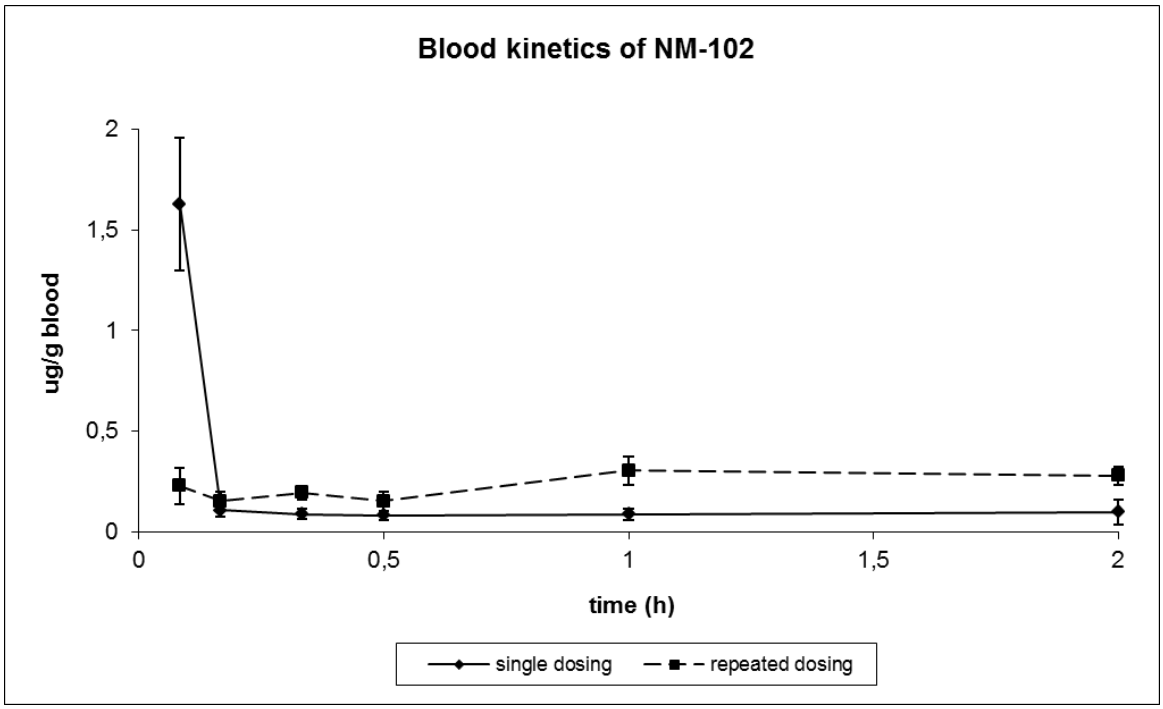


Figure 4-5B. Blood kinetics of NM-102 after single and repeated IV dosing.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

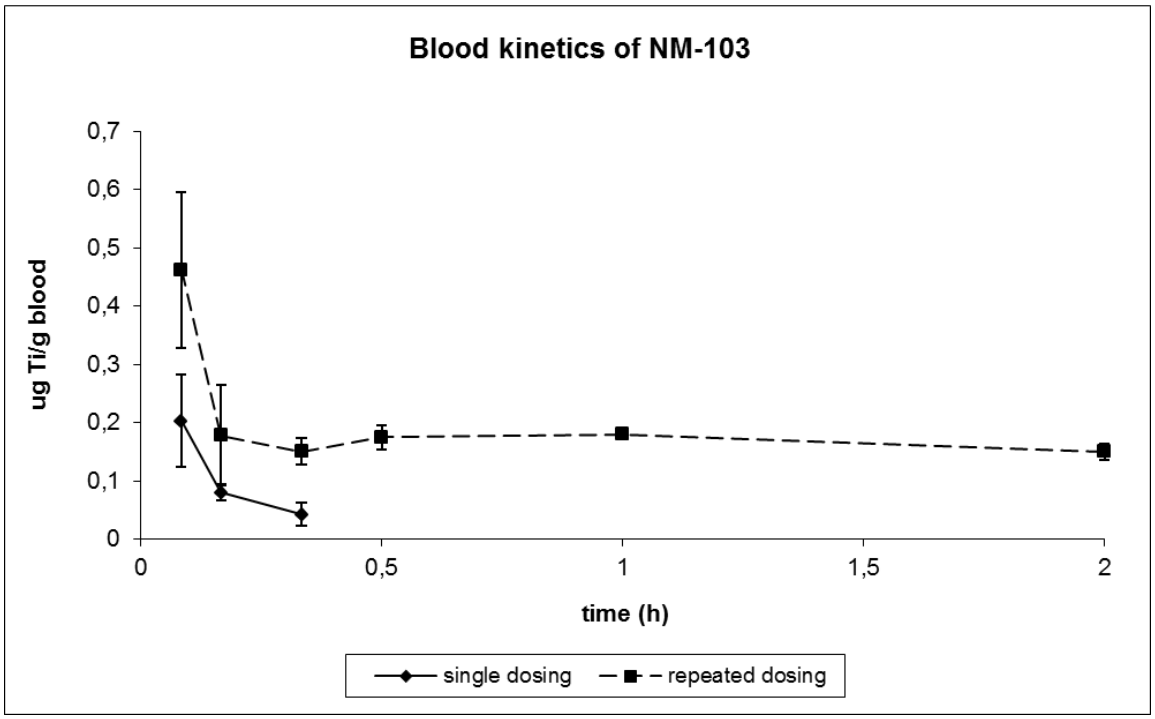


Figure 4-5C. Blood kinetics of NM-103 after single and repeated IV dosing.

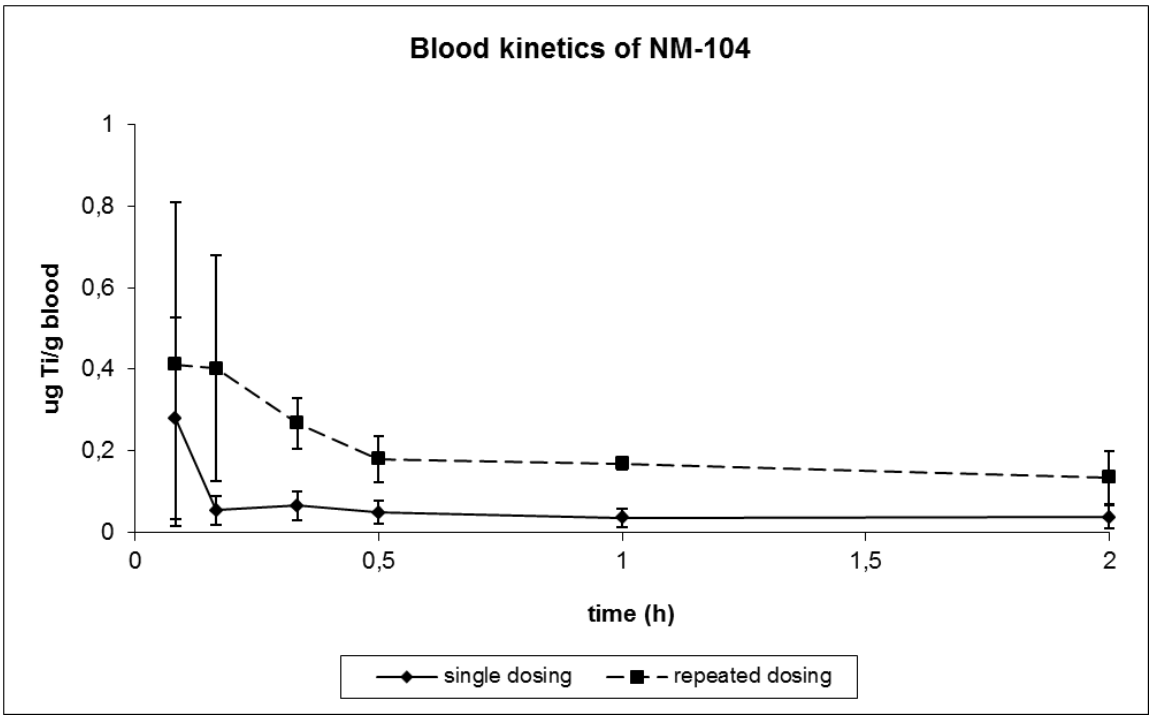


Figure 4-5D. Blood kinetics of NM-104 after single and repeated IV dosing.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

The major findings of the tissue distribution of NM-100, NM-102, NM-103 and NM-104 are summarised below.

Some minor differences were observed in organ distribution and degree of elimination from the organs between the different titanium dioxide nanomaterials (e.g. Figure 4-3A-B and Figures 4-4A-D). When expressed as µg per g tissue, the liver contained the highest concentration at the first day after the last dosing. The liver was the major target organ for the titanium nanomaterial studied with a % of the dose of 52%-105%, followed by the spleen with 2%-15%, lung with 0.5%-1.7% of the dose, while other organs contained less than 0.1% of the dose administered. The percentage of dose present decreased over time in the liver while the percentage of dose increased in the spleen, indicating a redistribution form the liver to the spleen. At day 90 the spleen contained a higher concentration per gram tissue when compared to the liver especially after repeated administration (Tables 4-11A-D and 4-12A-D). Liver and spleen are the major target organs after IV administration of TiO_2 with the liver containing the highest amount.

Some decrease but also a similar recovery was noted between day 2/day 6, and day 90 after administration of the TiO_2 nanomaterials, indicating that the excretion from the body is slow to not existing. The lack of excretion was confirmed by the data on Ti levels in urine (non detectable Ti levels) and levels of Ti in the faeces that were similar for both vehicle and TiO_2 treated animals, and did not change over time in the TiO_2 treated animals.

Higher absolute concentrations were observed in the organs after repeated dosing versus single dosing. However, no difference was observed when expressed as % of dose. Thus, the increase in organ concentration is dose proportional.

Based on Ti level in µg/g tissue it was found that females contained higher concentrations of nanomaterial in the organs. This can be explained by the fact that all animals received the same dose (2.3 mg TiO_2 in 1 ml). So, the female animals received a higher dose per kg body weight. However, when the data were evaluated as Ti per organ and as % of the dose administered in general no difference in organ distribution was observed between males and females. Only for NM-103 some differences between male and females remained.

The major difference between male and female animals was the disappearance of Ti in the reproductive organs. In male animals Ti was no more detectable in the testes on day 30 after administration, whereas on day 90 Ti was still detectable in the female ovaries. This difference can be explained by the differences in blood supply to the testes and ovaries. On day 6 a similar very low percentage of the dose was distributed to both testes and ovaries.

All nanomaterials investigated were rapidly distributed from the bloodstream to the different organs (see Figures 4-5A-D). Higher absolute concentrations were observed in blood after repeated dosing versus single dosing.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

The results indicate that once titanium (resulting from the administration of the investigated TiO_2 nanomaterials (NM-100, NM-102, NM-103, NM-104) is distributed to the organs from the bloodstream, it remains stored in the body for a period for at least 90 days, and probably longer. Elimination seems to be slow to almost absent and doses can be added. Some redistribution between tissues occurs notably between liver and spleen.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

4.2.3 Tissue distribution after IV administration of NM-105

4.2.3.1 Tissue distribution of NM-105

Following a single IV administration, the higher Ti concentrations were detected in liver, spleen and lung of male and female rats (Figure 4-6). The Ti concentration was decreased in the liver at day 90 compared to day 1 after administration, but still above the detection limit. In the kidney at day 90 Ti levels were in the range of the Limit of Detection/Limit of Quantification. At day 90, the rats administered once with NM-105, still show Ti presence in liver and kidney above the level of control samples, suggesting a longer time required for complete elimination of administered NM-105 from the body (Figure 4-6). Ti concentrations in the blood of the animals are presented in Figure 4-7.

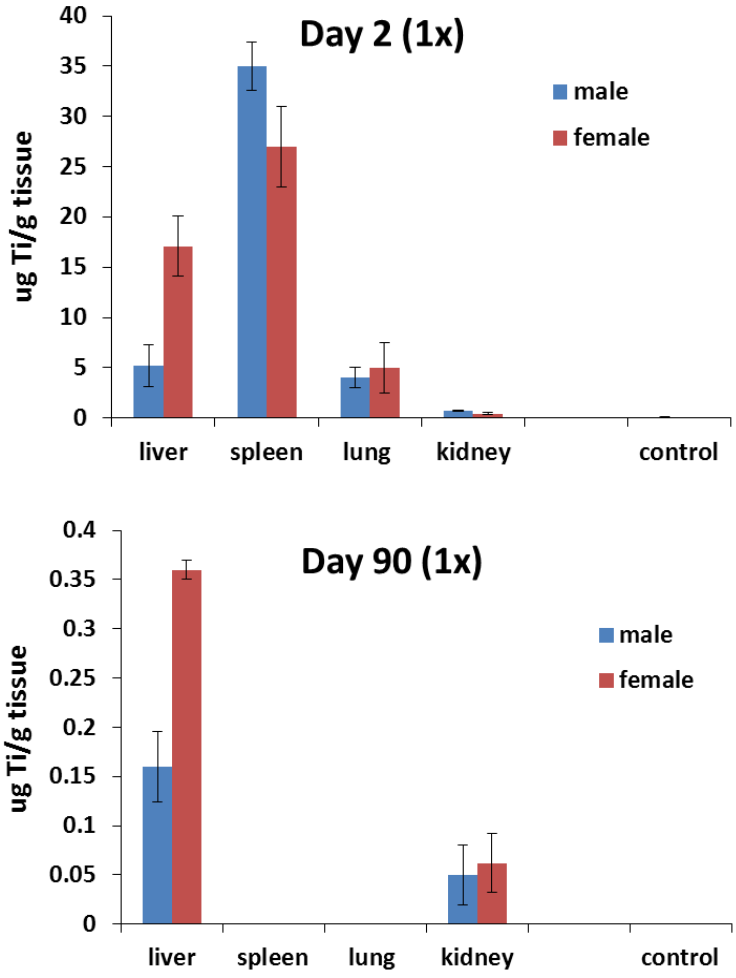


Figure 4-6. Distribution of Ti (µg/g tissue) in the organs of male and female Wistar rats following a single iv administration. Each bar represents the mean (n=3) for day 2 and 90.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.
www.nanogenotox.eu
Co-funded by the Health Programme of the European Union

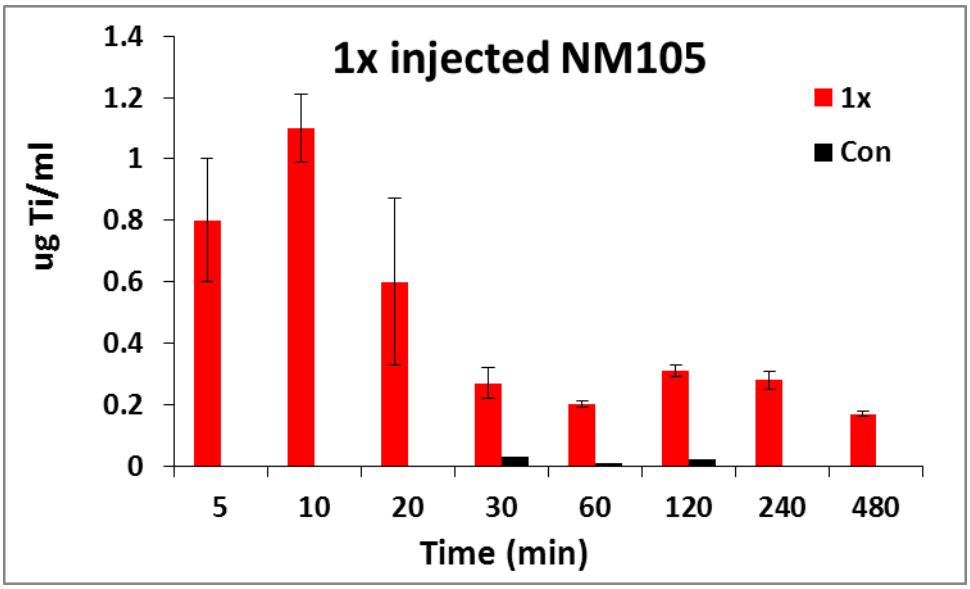
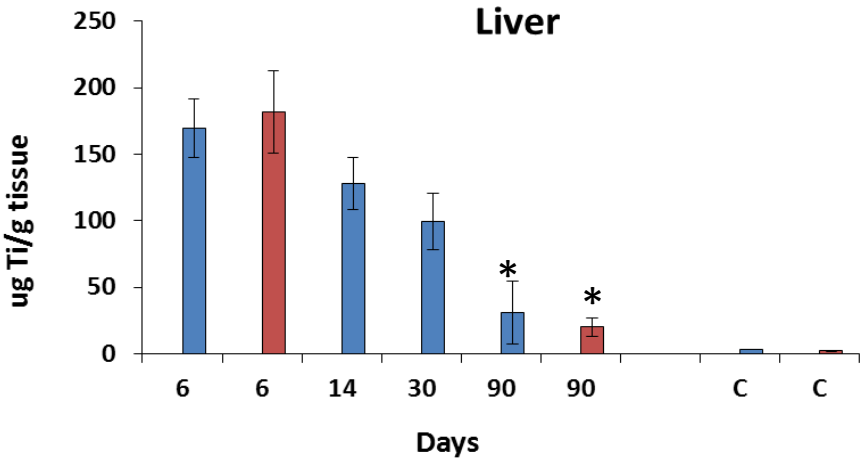
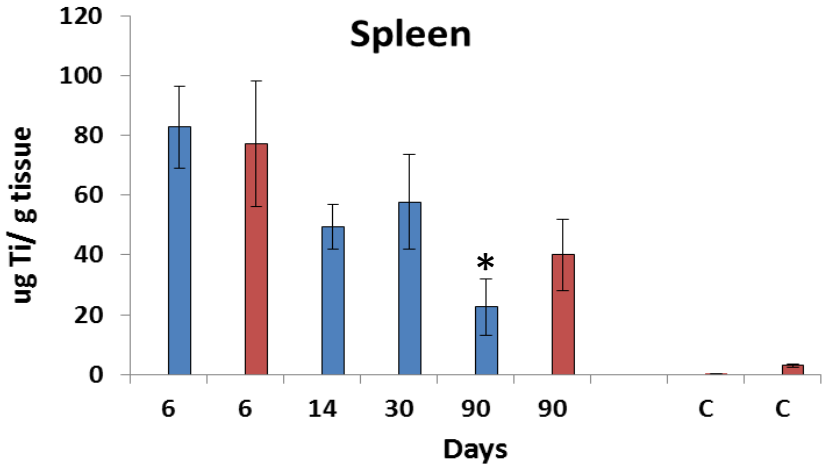


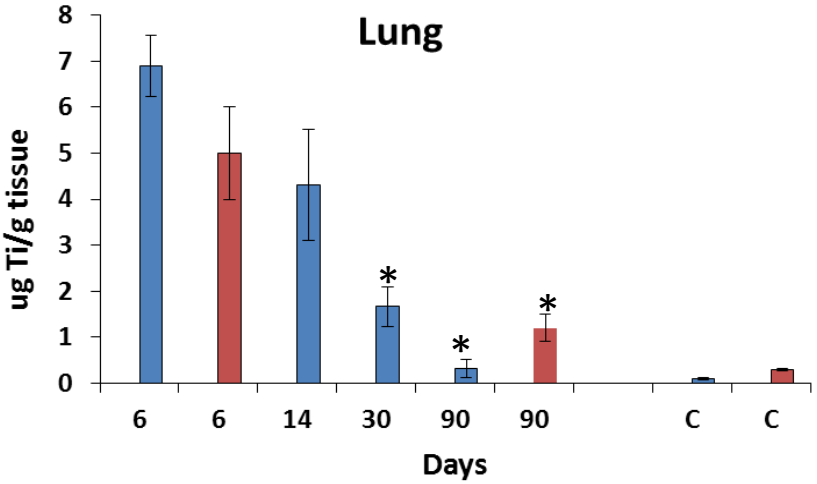
Figure 4-7. Kinetics of TiO_2 (shown as Ti) nanomaterial clearance from the blood in male (n=4) and female (n=2) Wistar rats following a single iv administration of NM-105.

All four organs investigated (liver, lung, kidney, and spleen) following repeated IV administration of NM-105 on 5 consecutive days, showed a gradual decrease in Ti content from day 6 to day 90 (Figure 4-8). At day 90 Ti could still be detected in the organs with the highest levels in the liver (51 µg/g tissue) and spleen (38 µg/g tissue), and much lower levels in lung (0.54 µg/g tissue) and kidney (0.07 µg/g tissue). The results for male (in blue bars) and female (in red bars) animals are summarized in Figure 4-8 and are presented in Table 4-15 as µg TiO_2/g tissue. The animals treated 5x with NM-105 also showed significant ($p<0.05$) accumulation of the MN in brain (0.57±0.12 µg Ti/g tissue) and heart (0.45±0.09 µg Ti/g tissue) compared with control animals. Ti kinetics in the blood is shown in Figure 4-9.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.
www.nanogenotox.eu
Co-funded by the Health Programme of the European Union







The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.
www.nanogenotox.eu
Co-funded by the Health Programme of the European Union

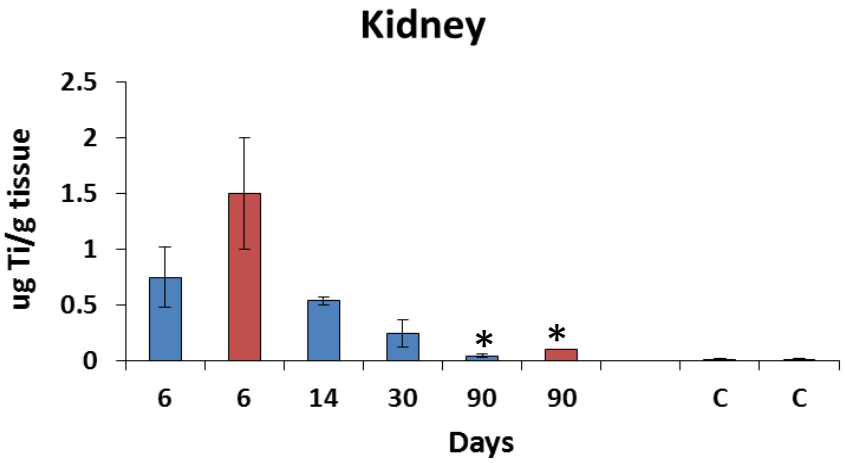


Figure 4-8 Distribution of Ti in the liver, spleen, lung and kidney. Each bar represents mean of the results from the test animals and controls (mean ± SD) for days 6, 14, 30 and 90. Blue bars – male rats (n=4); Red bars – female rats (n=3). *, $p < 0.05$ compared to day 6.

Table 4-15. TiO_2 distribution in the organs of male Wistar animals. The data are presented as average ± SD of 4 animals.

Tissue	Day 6	Day 14	Day 30	Day 90	Control*
µg TiO_2/g tissue					
Liver	283±36	213±33	166±35	51±39	0.08±0.05
Lung	11±1	7±2	2.78±0.72	0.54±0.35	<LOD
Kidney	1.2±0.4	0.90±0.06	0.41±0.20	0.07±0.03	< LOD
Spleen	138±23	82±12	96±26	37±9	0.16±0.08

*The data for the controls are presented as Ti (µg/g tissue). They are at the Limit of Detection and were not calculated as TiO_2.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.
www.nanogenotox.eu
Co-funded by the Health Programme of the European Union

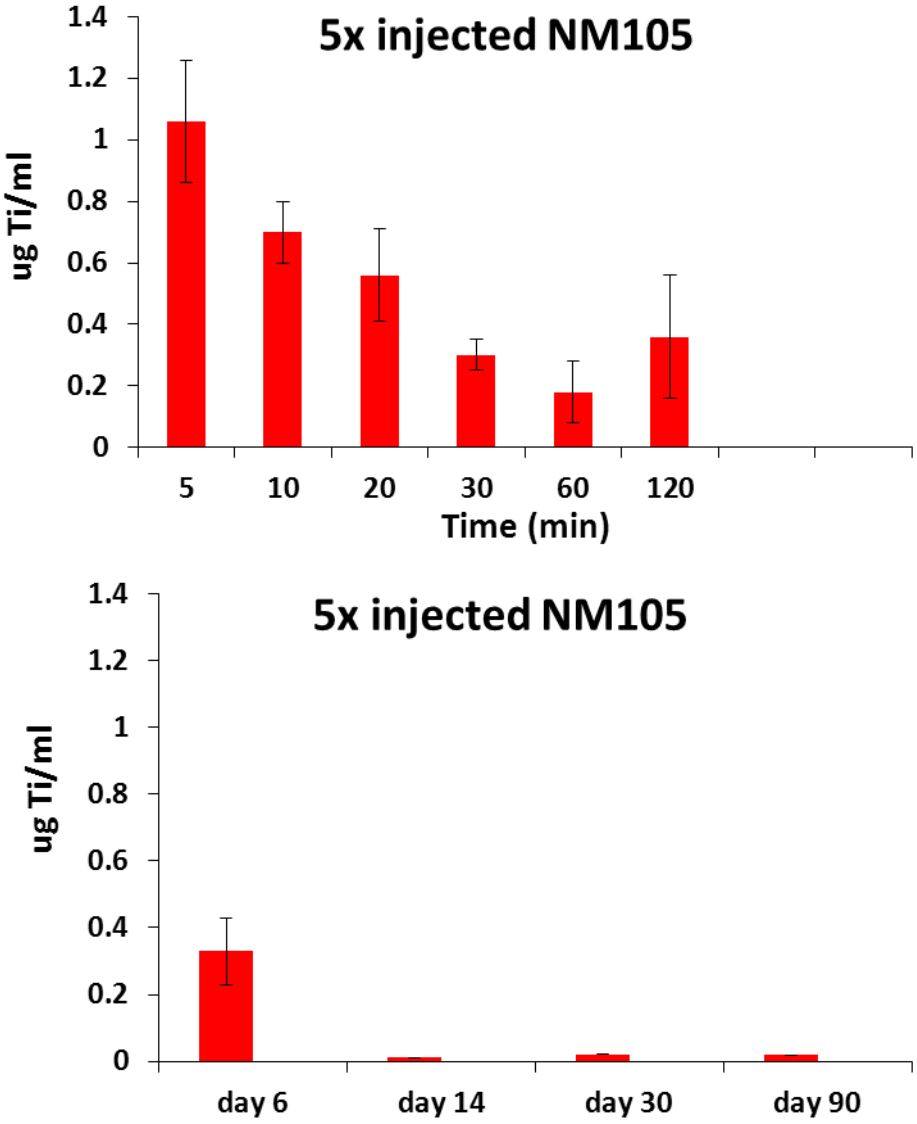


Figure 4-9. Kinetics of TiO_2 (shown as Ti) clearance from the blood in male (n=4) and female (n=3) Wistar rats following 5 days IV application of NM-105.

All rats treated 1x or 5x with NM-105 showed normal weight gain during the 90 days of the study (data not shown). Figure 4-10 represents the organ distribution of NM-105 after repeated IV dosing to male and female Wistar rats shown as percentage of dose. The recovery of Ti in the investigated organs as percentage of dose is shown in Figure 4-11.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.
www.nanogenotox.eu
Co-funded by the Health Programme of the European Union

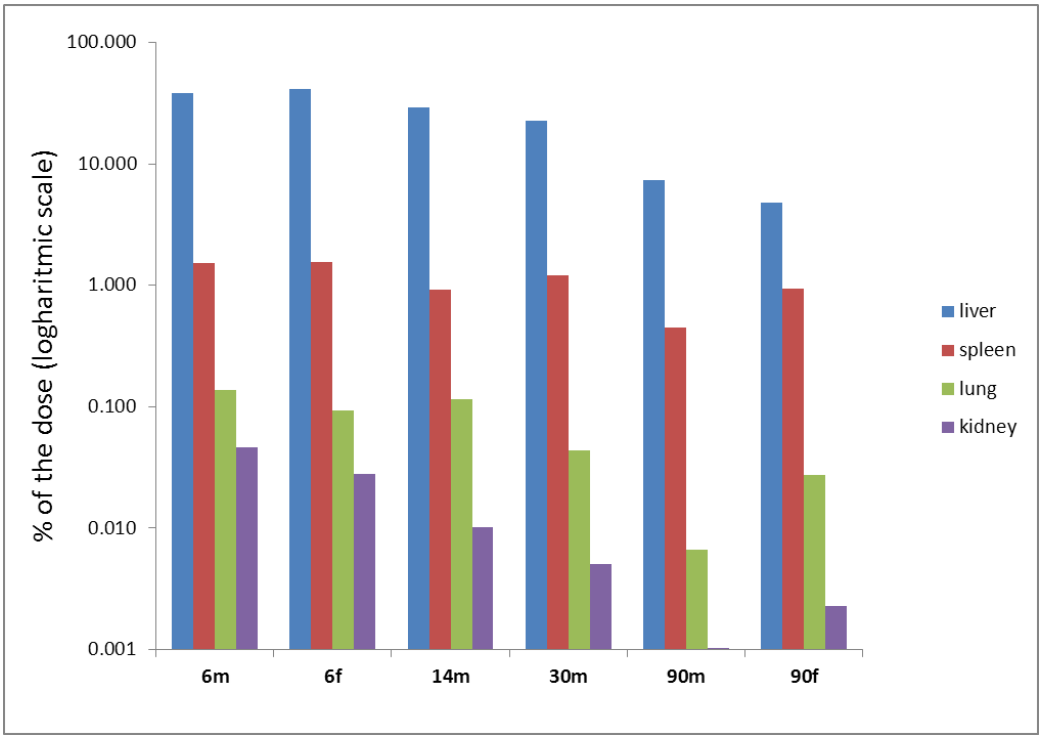


Figure 4-10. Organ distribution of NM-105 after 5 repeated IV dosing to male and female Wistar rats presented as percentage of dose measured on day 6, 14, 30, and 90. m: male rat; f: female rats.

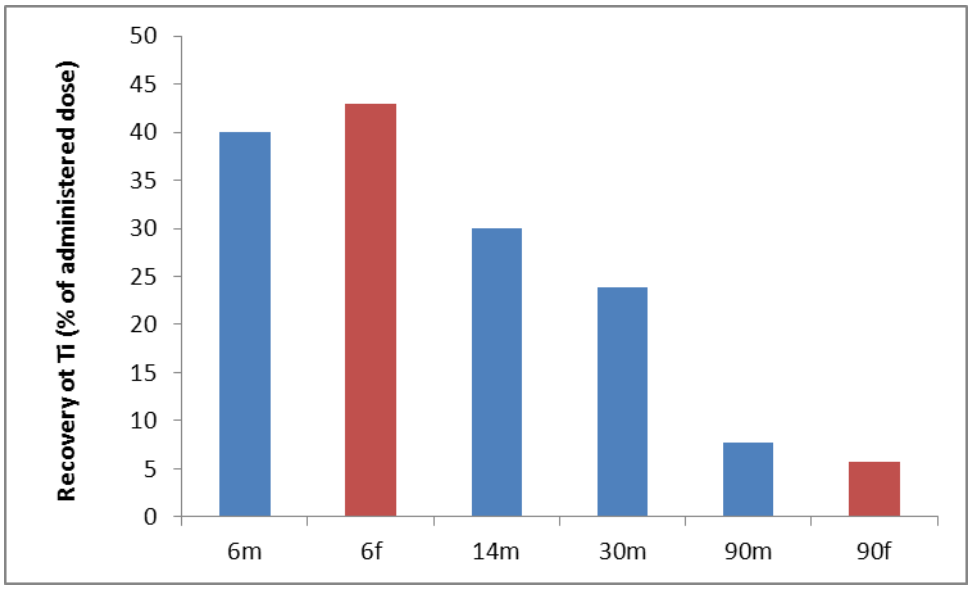


Figure 4-11. Recovery of Ti in the investigated organs (liver, spleen, lung, kidney) as percentage of dose following 5 consecutive administrations of NM-105 to male (m) and female (f) Wistar rats presented as percentage of dose measured on day 6, 14, 30, and 90.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

4.2.3.2 Histopathology of liver after IV administration of NM-105

The histopathology of liver sections of animals treated with NM-105 (5x) are presented in Figure 4-12. The liver tissue had no severe abnormal pathology changes compared with the control. The rats sacrificed on day 6 showed some areas with swelled hepatocytes (Figure 4-12b).

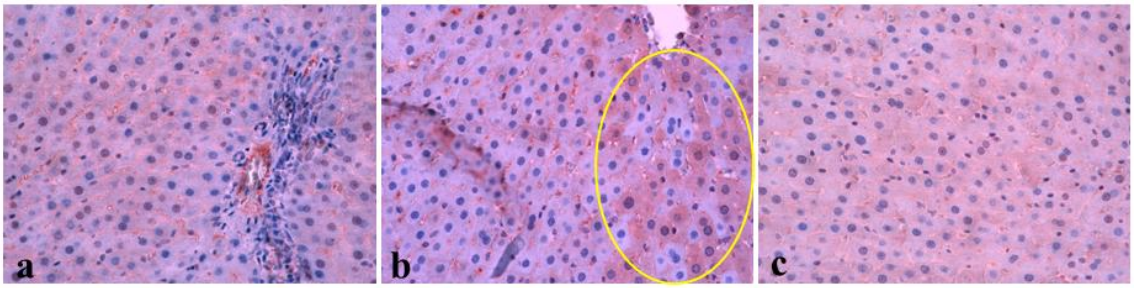


Figure 4-12. Histopathology of the liver tissue from male Wistar rats after IV injection with 11 mg/kg b.w. (total dose) NM-105 for consecutive 5 days. (a) control, phosphate injected animal; (b) animal sacrificed on day 6; (c) animal sacrificed on day 90. Yellow ellipse indicates an area with swelled hepatocytes. Magnification x100.

4.2.3.3 Transmission electron microscopy investigations

The ultrastructure of hepatocytes in control animals (Figure 4-13 a, b) showed a well-distributed chromatin, round or oval-shaped mitochondria, and compact nuclear membranes, whereas the animal groups treated IV with approximately 11 mg/kg b.w. of NM-105 for 5 consecutive days produce tunescent mitochondria, particle laden lysosomes, vacuolization in cytoplasm, and lipid droplets accumulation. The results suggest that NM-105 may damage the structure of hepatocytes in a mitochondria and lipid mediated pathways (Figure 4-13c–h). The NM-105 within rat hepatocytes was detectable even on day 90 (Figure 4-13g, h). The nanoparticles were found only in the cytoplasm and not in the nucleus.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

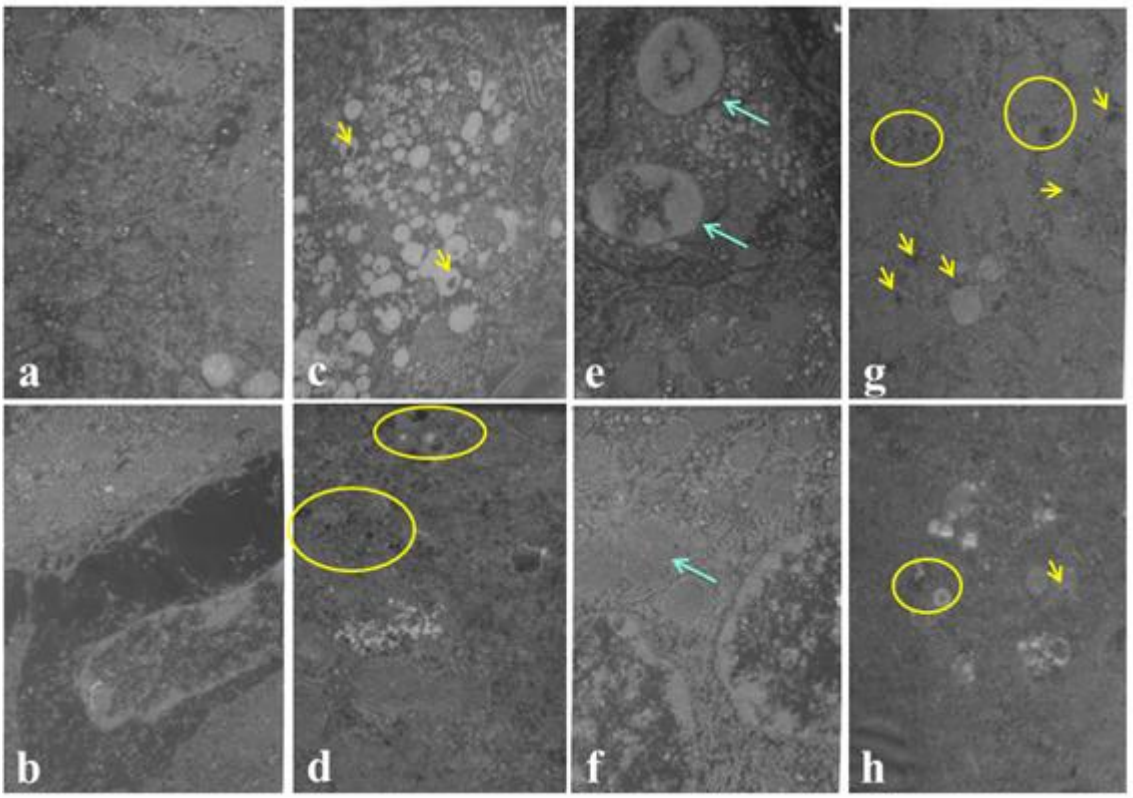


Figure 4-13. TEM images of liver of NM-105 treated male Wistar rats.
(a, b) Animal treated with phosphate buffer as control; (c-h) animal treated with 11 mg/kg b.w. NM-105 within 5 days; (c-f) liver samples from animals scarified on Day 6; (g-h) liver samples from animals scarified on day 90. Blue arrow indicates the tunescent mitochondria; On "e" is also visible a surrounding vacuolization; Yellow arrows show the interaction of NM-105 with cell organelles and particles engulfed in lysosomes. Yellow circles indicate presence of nanomaterial in the cytoplasm of hepatocytes. Magnifications: a-d, g, h 10 000x; e: 15 000x; f: 12 000x.

In conclusion, NM-105 was found to disappear from the blood circulation within 8 hours after single administration. After repeated administration low levels were still detected in blood on day 6 at 24 hours after the last administration. NM-105 mainly distributed to the liver, spleen and lung and to a lesser extent to the kidney. After repeated administration of NM-105 similar to the single administration the main target organs were liver, spleen, and to a lesser extent to lung and kidney. A gradual decrease in Ti content in the organs was observed between day 6 and day 90 after administration indicating excretion or redistribution of Ti to other (non-measured) organs.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

4.3 Conclusions

1. Ti levels in un-exposed and vehicle control exposed rats were in general below the Limit of Detection of 0.05 µg Ti/g tissue. The Ti content in the GI-tract was found to be similar in TiO_2 nanomaterial treated and control animals after oral administration of the various TiO_2 nanomaterials. Also after IV administration of NM-100, NM-102, NM-103, and NM-104, the Ti content in the faeces was similar in vehicle control and TiO_2 nanomaterial treated animals. These results indicate that the level of Ti in the GI-tract is most likely due to Ti present in the feed of the animals.

2. After oral administration only incidentally some Ti could be detected in liver and/or spleen, indicating a low uptake from the GI-tract. However, these low levels also demonstrated that uptake is in principle possible. For NM-104 and NM-105 indications were present for some minimal uptake in the mesenteric lymph nodes whereas NM-101, NM-102, and NM-103 showed no uptake in the mesenteric lymph nodes.

3. After oral exposure (vehicle, NM-101, and NM-105 female rats had a higher Ti concentration (in µg/g tissue) than male rats in the mesenteric lymph node and mesenteria. However, the difference largely disappeared when the data was corrected for the smaller weight of the female mesenteric lymph node and mesenteria.

4. The tissue distribution patterns for the NM-100, NM-102, NM-103, and NM-104 TiO_2 nanomaterials are similar. When injected IV the TiO_2 as determined by Ti content, rapidly disappears from the blood. Within 10 – 30 minutes a rapid decline in blood Ti occurs. However, low levels of Ti can be observed in blood until day 30 (NM-102 and NM-103) after repeated administration.

5. The major target organs were the liver followed by spleen and lung. Tissue distribution to other organs investigated (kidney, heart, thymus, reproductive organs) was very low (<0.1% of the dose). The Ti concentration decreased over time in the liver and increased in the spleen, indicating a redistribution form the liver to the spleen. After repeated administration at day 90 Ti levels in spleen were higher (Ti as µg/g tissue) when compared to liver.

6. Some decrease (NM-100, NM-102), but also a similar recovery (NM-103, NM-104) was noted between day 2/day 6, and day 90 after administration of the TiO_2 nanomaterials, indicating that the excretion from the body is slow or non-existent. The lack of excretion was confirmed by the data on Ti levels in urine (non detectable Ti levels) and levels of Ti in the faeces that were similar for both vehicle and TiO_2 treated animals, and did not change over time in the TiO_2 treated animals. Only for

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

NM-105 a considerable decrease in total recovery was observed at day 90 after IV administration.

7. For NM-100, NM-102, NM-103, and NM-104 female rats were found to have a higher tissue concentration than male rats when the Ti level was expressed as µg/g tissue. When the data were evaluated as Ti concentration per organ and as % of the dose administered in general no difference in organ distribution between male and female rats was noted for NM-100, NM-102, NM-103, and NM-104. Although, for some organs and nanomaterials, especially NM-103, differences were noted between male and female animals, the differences were minimal and without a clear pattern for females or males having higher concentrations.

8. For NM-100, NM-102, NM-103, and NM-104 the Ti levels in the organs after repeated administration (5x) were found to be a similar fraction of the dose when the Ti level was expressed as % of the dose, so the increase in organ concentration is proportional.

9. For NM-100 and NM-102 the excretion can be considered minimal in view of low to non-existing levels of Ti observed in urine. For NM-100, NM-102, NM-103 and NM-104 there are no indications for excretion via the faeces as the level of Ti in the faeces was relatively constant (between 3-10 µg/g faeces) in all animals in which faeces was investigated including vehicle treated control animals indicating that the Ti level in the faeces was not due to excretion but due to background levels in the feed.

10. Based on the results it can be concluded that once titanium (from the investigated titanium nanomaterials NM-100, NM-102, NM-103, and NM-104 was distributed to the organs from the bloodstream, it seemed to be trapped in the body for at least 90 days, and probably longer. For NM-100 and NM-102 some reduction in recovery was observed between day 2/day 6 and day 90 although still more than 50% of the administered dose was present in the organs investigated. This reduction may indicate either a redistribution of Ti in organs not measured, or excretion. For NM-103 and NM-104 only for NM-103 a decrease in recovery was observed after repeated administrations indicating some difference in kinetics between NM-103 and NM-104.

11. The results for the NM-100 and NM-102 (anatase), and NM-103 and NM-104 (both rutile) are in contrast with the results obtained for NM-105 an anatase/rutile mixture of TiO_2. For NM-105 a gradual decrease was seen in all organs investigated (liver, spleen, lung, kidney) resulting in a recovery of approximately 5% for NM-105 at day 90. For NM-100, NM-102, and NM-103 a reduction in recovery was observed but the recovery at day 90 after single and repeated doses remained well above 50% of the dose administered. For NM-104 no reduction in recovery was observed between day 2/day 6 and day 90.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

12. NM-105 TiO_2 disappeared from the blood following single administration within 8 hours. NM-105 mainly distributed to liver, spleen and lung and to a lesser extent to the kidney. At day 90 after the single IV administration Ti could still be detected in liver and kidney, whereas spleen and lung were negative. Compared to day 2 Ti levels were decreased considerably at day 90 after the single IV administration.

13. After repeated administrations of NM-105 for five consecutive days liver, spleen and lung were observed as major target organs, and low Ti levels were detected in the kidney. In all organs investigated (liver, spleen, lung and kidney) a gradual decrease in Ti level was observed from day 6 to day 90. Also after the repeated IV administrations the Ti concentration in the blood decreased rapidly but was still detectable at 24 hours after the last administration. At days 14, 30 and 90 Ti levels were below the Limit of Detection in the blood circulation.

14. For NM-105, Ti the recovery after repeated dosing decreased from approximately 40% at day 6 to approximately 5% at day 90, indicating removal of Ti from the major target organs or redistribution of Ti to organs not measured.

15. The animals treated with approximately 11 mg/kg of NM-105 for 5 consecutive days produce swollen mitochondria, particle laden lysosomes, vacuolization in cytoplasm, and lipid droplet accumulation in the liver. The results suggest that NM-105 may damage the structure of hepatocytes in a mitochondria and lipid mediated pathways.

16. The major difference observed between the various TiO_2 nanomaterials was that after NM-100, NM-102, NM-103, and NM-104 administration Ti persisted up until day 90 in the various organs and showed only a limited decline at day 90 compared to days 2 and day 6. In contrast, NM-105 clearly showed a decline in Ti concentration when determined at day 90 after administration. However, also for NM-105 some Ti was still present in liver and spleen above the concentrations measured in control rats.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

5. Synthetic Amorphous Silica (SAS, SiO_2)

5.1 Methods

5.1.1 Study schedule

Rats were treated by the schedule as presented in section 3.1.

5.1.2 Animals

For the IV and oral studies of SAS at ISS, which dealt with NM-200 and NM-203, Sprague-Dawley rats were used. The number of animals used per nanomaterial is presented in section 3.1 and Table 3-1. Animals were kept under standard laboratory conditions (room temperature of 22.0±0.5 °C, 50-60% relative humidity, and 12-h light/dark cycle with 12-14 air changes *per* hour). Water and food were available *ad libitum*. The rats were allowed to acclimatize for 2 weeks before they entered the experimental protocol. In order to avoid any possible contamination, all the instruments intended for the experimental procedures were sterilized before use and kept under a laminar flow cabinet.

One of the main issues in measuring the concentration of administered SAS (SiO_2) nanomaterials *via* Si determination is the high endogenous Si background in tissues and biological fluids. Si background concentration in tissues depends on the Si amount ingested via the diet. In order to find out whether the Si background in rat tissues could be reduced, different standard rat diets were analysed for their Si content. The diet with the lowest Si concentration was fed to the animals. As a result, Si background in rat tissues was reduced below the analytical limit of quantification (LOQ) in most cases.

5.1.3 Sample preparation

For SAS a suspension of 6.0 mg/ml was prepared in sterile normal saline (NaCl 0.9% w/v). The concentration of the suspension was selected on the basis of preliminary studies, which showed that in these conditions measurable Si concentrations could be detected in major organs after single dose IV administration. According to WP4, a suitable dispersion can be obtained using the above concentration and dispersion media.

Probe sonication of the suspensions was performed on ice (Bandelin Sonopuls Ultrasonic Homogenizer HD3200 series equipped with a SH 213 G booster horn and a sterile KE 76 tapered tip) for 16 minutes at 10% amplitude. The suspensions were used immediately for dosing animals.

5.1.4 Doses administered

The SAS doses for the oral and IV route were administered using a dispersion containing 6.0 mg/ml SAS for both NM-200 and NM-203. For the IV route, the single dose groups received a dose of 20 mg/kg b.w. The repeated dose groups received a total cumulative dose of 100 mg/kg b.w after 5 days treatment, for both oral and intravenous administration. About 2-2.5

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

ml/kg b.w. of the aqueous dispersion was administered in order to achieve a dose of 20 mg/kg b.w per day. Animals were orally treated by gavage. Controls were treated with vehicle only (sterile physiological solution) and 20 mg/kg b.w.

The animal protocols were approved under the following protocol numbers: Istituto Superiore di Sanità, Service for Biotechnology and Animal Welfare (D.M. 286, 31/01/92), Protocollo Per L'impiego Degli Animali In Sperimentazione (Protocol for the use of animal in experimental procedures), (D.L.vo 116 del 27/01/92), DM 218/1010 – B del 1/12/2010).

5.1.5 Organs sampled

Rats were anaesthetized with gaseous solution of isofluorane and blood samples were collected by intracardiac puncture and stored at -80 °C. Subsequently, animals were sacrificed by CO_2 inhalation and the following organs were collected and stored at -80°C: liver, spleen, kidneys, lungs, brain, hearth, uterus, ovaries, testes, adrenals, thyroid, thymus, GI tract, cutis, femur, muscle, urine, faeces, N. ischiadicus, popliteal and mesenteric lymph nodes.

5.1.6 Si determination

The use of quadrupole ICP-MS has been investigated as the first choice being a robust, high-throughput element-specific technique. However, Si determination by quadrupole ICP-MS presents several analytical challenges, the main being the Si background due to Si release from the quartz of the spectrometer sample introduction system and the spectral interferences on the potentially usable Si analytical masses. Therefore, state of the art technology for sample introduction and interference avoidance was used in order to overcome these problems and develop an entirely novel, fit-for-purpose method for Si determination (Aureli et al. 2012).

An Elan DRC II ICP mass spectrometer (Perkin Elmer, Norwalk, CT) was used as a Si specific detector. The problem of Si release from equipment was solved by completely eliminating quartz. For this purpose, the entire sample introduction system of the ICP mass spectrometer was substituted with non-quartz components. This entailed using a ceramic D-torch instead of the quartz torch, a sapphire injector instead of the quartz injector, a cyclonic spray chamber consisting of perfluoroalkoxy (PFA) material instead of the quartz spray chamber, and a PFA concentric nebulizer instead of the quartz nebulizer.

Si has three naturally occurring isotopes - i.e., ^{28}Si (abundance 92.2%), ^{29}Si (4.7%), and ^{30}Si (3.1%) - which suffer from severe polyatomic interferences in ICP-MS (Table 5-1).

In order to overcome polyatomic interferences, the dynamic reaction cell (DRC) technology has been used. This approach utilizes ion-molecule chemistry to eliminate interferences, thereby improving ICP-MS Limit of Detection (LOD). The DRC was pressurized with methane and the gas molecules converted the interfering ions into species that did not interfere with

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

the analyte during the subsequent mass separation in the analyzer quadrupole (Aureli et al. 2012). The ICP-DRC-MS method developed was used for interference free-detection of Si throughout. The instrumental operating conditions of ICP-DRC-MS are summarized in Table 5-2.

Table 5-1. Silicon naturally occurring isotopes and most abundant polyatomic interference in ICP-MS

Isotope	Interfering species
^{28}Si	$^{14}N^{14}N$, $^{12}C^{16}O$
^{29}Si	$^{14}N^{15}N$, $^{14}N^{14}NH^{+}$, $^{13}C^{16}O$, $^{12}C^{16}OH^{+}$
^{30}Si	$^{15}N^{15}N$, $^{14}N^{15}NH+$, $^{14}N^{16}O+$, $^{13}C^{17}O^{+}$, $^{12}C^{17}OH^{+}$

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 5-2. Operating conditions of the ICP-DRC-MS system

RF power	1.3 kW
Plasma Gas flow	15.5 l min^{-1}
Aux Gas flow	1.2 l min^{-1}
Carrier Gas	1.00 l min^{-1}
DRC	CH_4
	Cell gas flow 0.8 ml min^{-1}
	RP*q* 0.55
	CPV -17 V
	QRO -6 V
	CRO -8 V
Internal standard	^{74}Ge
Analytical mass	^{28}Si
Sample flow rate	1 ml min^{-1}
Measurement parameters	
Sweeps	20
Dwell time	200 ms
Reading	1
Replicates	3
Integration time	4s

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.
www.nanogenotox.eu
Co-funded by the Health Programme of the European Union

For sample preparation clean room conditions, ultrapure reagents and non-quartz materials were used throughout. Biological samples (tissues, blood) were handled under a clean bench to avoid Si contamination. All samples were stored in disposable polypropylene tubes, previously decontaminated through an acid treatment, and Teflon vessels were used in microwave digestion. Entire organs were digested in order to reduce sample handling and contamination risk with the exception of the largest organs, which were homogenised beforehand.

Tissue samples were submitted to closed-vessel digestion at high pressure with a microwave system equipped with vessel temperature control. Microwave irradiation cycles with a mixture containing HNO_3 (approx. 13 M):HF (approx. 4 M) were used for vessels cleaning. This allowed the reduction of the LOD to, e.g., 0.3 and 0.4 mg Si/kg on a fresh weight basis for liver and spleen, respectively.

Largest organs, namely liver, spleen and small intestine were digested at least in duplicate. The GI tract was subsampled for Si determination and submitted to a cleaning procedure consisting in the mechanical removal of digestion residues inside the tract using ultrapure water. Typical conditions for closed-vessel digestion were 2 g fresh sample added with 5 ml HNO_3 (13M) and 0.025 ml HF (4M), overnight pre-mineralization, and then addition of 2 ml of H_2O_2 (the following morning). The microwave programme used was as follows: 10 min 120 °C (ramp); 7 min 120 °C; 9 min 190 °C (ramp); 15 min 190 °C (maximum power 1000 W).

An approach enabling simultaneous microwave (MW) digestion of 60 samples in polypropylene tubes was developed for smaller organs. Lungs, kidneys, brain, heart, testes and ovaries, and mesenteric lymph nodes (pooled) were digested as whole organs in disposable Falcon™ tubes previously rinsed with a mixture containing HNO_3 2 M and HF 1M v/v by mean of microwave irradiation in a Multiprep apparatus. Tissue samples were weighed and left to stand overnight with 5 ml HNO_3 (13 M) and 0.025 ml HF(4 M) (pre-digestion step); the following morning, after addition of 2 ml H_2O_2, samples were irradiated using the following programme: 1 hour ramp to 90 °C; 7 hours 90 °C. Blood samples (1 ml) were digested with a mixture of 2 ml HNO_3 (13 M), 0.015 ml HF (4 M) and 1 ml H_2O_2 and microwave irradiated using the following programme: 1 hour ramp 90 °C; 5 hours 90 °C.

The Limits of Detection and Quantification were calculated as follows: twenty independent digestion blanks were measured and the standard deviation (*s*) was calculated. The Limit of Detection (LOD) was determined as the analyte concentration corresponding to a 3*s* value while the limit of quantification (LOQ) was determined as the analyte concentration corresponding to a 10*s* value. Both limits were expressed as mg Si/kg tissue (see below Table 5-11).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Quality control in Si determination by ICP-DRC-MS

There are few biological reference materials available to check accuracy of Si determination and all of them have indicative and not certified Si concentration values. Therefore, an internal quality control material (QC-ISS) was prepared starting from a fresh bovine liver sample.

The preparation of the QC-ISS was done as follows. After removing membranes, ligaments, and large blood vessels, the liver sample was thoroughly homogenized in an automatic blender. Following spiking with a known amount of Si, the sample was mixed again and then transferred into a disposable vessel for freeze drying. As the final step, the sample was homogenised and passed through a 125 µm sieve to obtain a fine powder, transferred in pre-scored vials and stored in a desiccator cabinet.

The Si concentration in QC-ISS was characterised by three analytical techniques, namely ICP-DRC-MS, ICP-optical emission spectroscopy (ICP-OES) and high resolution (HR)-ICP-MS. Analyses were carried out following oxidative digestion in the same conditions adopted for fresh organs. Results showed a very good agreement among techniques, as shown in Figure 5-1.

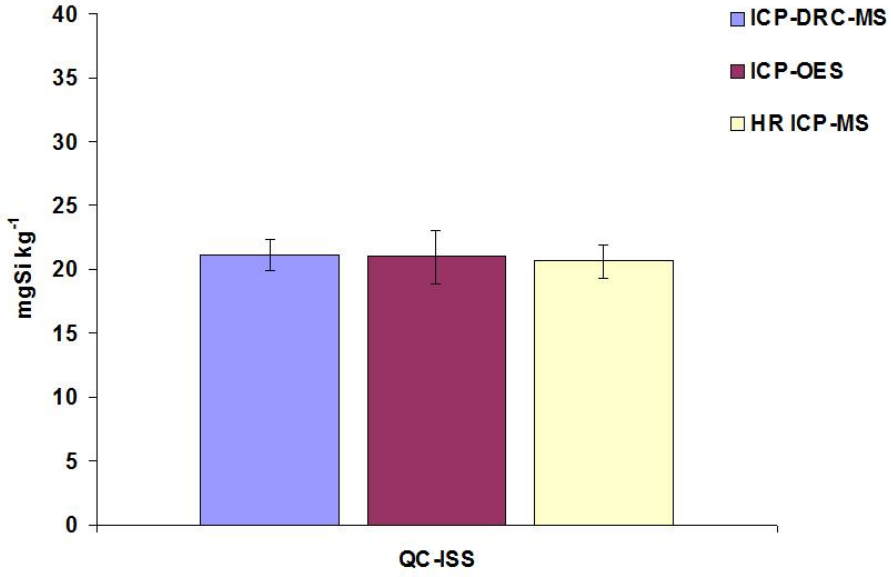


Figure 5-1. Analytical results of the interlaboratory comparison on Si concentration in the internal quality control sample prepared at ISS (QC-ISS).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

5.2 Statistical analysis

Analytical results are expressed as mean ± standard deviation. The concentrations are expressed in mg Si/kg (parts per million) on a fresh weight basis. The values that resulted to be ≤LOQ are highlighted in yellow. No numerical value is shown if the found concentration is ≤LOD, whereas a numerical value is shown if the found concentration is >LOD and ≤LOQ. However, it should be kept in mind that at levels >LOD and ≤LOQ, the accuracy of the measurement method is not optimal. Table 5-11 summarises the LOD and LOQ for the different organs and blood.

Differences among groups were analysed by one-way ANOVA using GraphPad Prism (GraphPad, US). Pair-wise comparisons of treated groups with control group were analysed by means of two-tailed Fisher Exact Test. Differences between groups were considered significant if the *p*-value was ≤ 0.05.

5.3 Results

5.3.1 SAS oral administration

5.3.1.1 Si tissue distribution after oral administration

The oral exposure was only investigated for the repeated dose schedule in order to obtain measurable levels in organs. As the results showed rather low levels in the main target organs like liver and spleen, the single dose experiment was not performed.

Tables 5-3 and 5-4 summarise the analytical data and give the picture of Si tissue distribution in rats after repeated oral dose of SAS nanoparticles. Each result is the mean of 3 animals. Tables 5-5 and 5-6 summarise the analytical data of Si concentrations in blood after repeated oral dose of SAS nanoparticles. Each result is the mean of 1-2 animals. The concentrations are expressed in mg Si/kg (parts per million) on a fresh weight basis.

The organ levels after repeated oral administration suggest minor differences both between male and female animals and the MN material (NM-200 and NM-203) investigated. However, the very low levels in the liver and spleen (< 2 mg/kg organ weight) near the LOQ and LOD indicate a very low absorption from the gastro-intestinal tract.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 5-3. Tissue distribution of Si in female rats after repeated oral dose of SAS nanoparticles (mg Si/kg fresh weight).

	Controls	**NM-200**		**NM-203**	
		Day 6	Day 14	Day 6	Day 14
Liver	0.6±0.1	1.3±0.3	1.3±0.2	0.8±0.2	0.3±0.1
Spleen	0.9±0.6	0.6±0.1	1.6±0.6	≤LOD	1.2 ±0.0
GI tract*	14.8±2.8	10.5±2.4	17.5±5.9	8.6±1.7	8.6 ±1.8
Mesenteric lymph nodes	≤LOD	≤LOD	≤LOD	≤LOD	≤LOD

* Small intestine. The values ≤LOQ are highlighted in yellow.

Table 5-4. Tissue distribution of Si in male rats after repeated oral dose of SAS nanoparticles (mg Si/kg fresh weight).

	Controls	NM-200		NM-203	
		Day 6	Day 14	Day 6	Day 14
Liver	0.5±0.1	≤LOD	0.4±0.1	≤LOD	≤LOD
Spleen	0.6±0.4	≤LOD	0.7 ±0.2	0.9±0.2	0.7±0.1
GI tract*	18.9±6.9	10.5±2.4	19.8 ±1.6	9.6±4.7	14.3±10.0
Mesenteric lymph nodes	≤LOD	≤LOD	≤LOD	≤LOD	≤LOD

* Small intestine. The values ≤LOQ are highlighted in yellow.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.
www.nanogenotox.eu
Co-funded by the Health Programme of the European Union

Table 5-5. Blood concentrations of Si in female rats after repeated oral dose of SAS nanoparticles (mg Si/kg fresh weight).

Females	30 min	60 min	2h	4h	8h	day 6*
Controls	NA	0.5	0.7	NA	NA	1.0
NM-200	0.4	0.3	≤LOD	0.5	NA	≤LOD
NM-203	0.5	0.4	0.5	0.5	0.9	≤LOD

*sacrifice.

Table 5-6. Blood concentrations of Si in male rats after repeated oral dose of SAS nanoparticles (mg Si/kg fresh weight).

Males	30 min	60 min	2h	4h	8h	day 6*
Controls	0.8	0.9	NA	NA	0.7	0.3
NM-200	0.8	0.7	0.6	0.5	NA	0.8
NM-203	0.4	0.5	0.5	0.6	0.6	≤LOD

*sacrifice.

5.3.1.2 Histopathology after SAS oral administration

In terms of effects, at sacrifice at day 6 altered organ weight (e.g. liver, lungs, uterus) was observed in both groups exposed to NM-200 and NM-203 (data not shown).

Preliminary histopathological examinations showed increased ratio between white and red pulp of the spleen and apoptosis in NM-203 treated male and female groups; increased ratio between white and red pulp of the spleen and altered vascularisation in NM-200 treated female group; altered vascularisation in liver in NM-203 treated female group (data not shown).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.
www.nanogenotox.eu
Co-funded by the Health Programme of the European Union

5.3.2 SAS IV administration

5.3.2.1 Si Tissue distribution after IV administration

Following IV single dose administration, a high bioaccumulation in liver, spleen, and lungs at day 2 was observed with both NM-200 and NM-203.

Marked particle- and gender-related differences in bioaccumulation and biodistribution were observed. It is notable the higher tendency of NM-203 to accumulate in spleen. At day 90, Si concentrations declined at levels close to (liver) or below the LOQ.

Tables 5-7 and 5-8 summarise the analytical data and give the picture of Si tissue distribution in rats after single IV dose of SAS nanoparticles. Each result is the mean of 3 animals.

Tables 5-9 and 5-10 summarise the analytical data of Si concentrations in blood after single IV dose of SAS nanoparticles. Each result is the mean of 1-2 animals.

Table 5-11 summarises the LOD and LOQ for the different organs and blood.

Table 5-7. Tissue distribution of Si in female rats (n=3) after single IV dose of SAS nanoparticles (mg Si/kg fresh weight).

	Controls	**NM-200**		**NM-203**	
		Day 2	Day 90	Day 2	Day 90
Liver	0.4 ±0.1	97 ± 15	1.6 ± 0.8	98 ± 19	1.2 ± 0.8
Spleen	≤LOD	40 ± 6	0.6 ± 0.1	79 ± 22	0.7 ± 0.5
Lungs	0.7 ± 0.3	42 ± 8	1.2 ± 0.1	11 ± 7	≤LOD
Heart	0.5 ± 0.4	0.9 ± 1.0	0.4 ± 0.2	0.4 ± 0.3	0.5 ± 0.4
Brain	0.4 ± 0.3	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.6 ± 0.2
Kidneys	0.5 ± 0.1	1.2 ± 0.3	0.9 ± 0.5	0.8 ± 0.2	0.6 ± 0.1
Ovaries	≤LOD	≤LOD	≤LOD	≤LOD	≤LOD

The values ≤LOQ are highlighted in yellow.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 5-8. Tissue distribution of Si in male rats (n=3) after single IV dose of SAS nanoparticles (mg Si/kg fresh weight).

	Controls	NM-200		NM-203	
		Day 2	Day 90	Day 2	Day 90
Liver	0.5 ±0.1	105 ± 10	4.1 ± 2.7	98 ± 20	1.6 ± 1.7
Spleen	≤LOD	39 ± 15	1.1 ± 1.5	237 ± 29	≤LOD
Lungs	0.7 ± 0.3	43 ± 10	≤LOD	24 ± 1	≤LOD
Heart	0.6 ± 0.2	0.8 ± 0.2	1.2 ± 0.4	2.1 ± 0.4	0.5 ± 0.1
Brain	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.8 ± 0.6
Kidneys	0.4 ± 0.1	1.1 ± 0.2	0.4 ± 0.1	1.4 ± 0.1	0.6 ± 0.3
Testes	1.6 ± 1.0	1.1 ± 0.2	0.9 ± 0.1	1.2 ± 0.2	0.8 ± 0.3

The values ≤LOQ are highlighted in yellow.

Table 5-9. Blood concentrations of Si in female rats (n=1-2) after single IV dose of SAS nanoparticles (mg Si/kg fresh weight).

Females	**5 min**	**10 min**	**20 min**	**30 min**	**60 min**	**2h**	**4h**	**8h**	**24h***
Controls	NA	NA	NA	0.4	0.4	0.7	NA	NA	0.6
NM-200	5.3	0.6	NA	0.6	0.6	1.2	1.7	2.1	0.4
NM-203	5.6	0.8	1.4	0.7	0.8	0.7	0.9	1.2	0.6

*sacrifice. The values ≤LOQ are highlighted in yellow.

Table 5-10. Blood concentrations of Si in male rats (n=1-2) after single IV dose of SAS nanoparticles (mg Si/kg fresh weight).

Males	5 min	10 min	20 min	30 min	60 min	2h	4h	8h	24h*
Controls	NA	NA	NA	0.5	0.7	0.6	NA	NA	0.4
NM-200	4.0	1.0	0.7	0.9	0.8	0.7	0.9	0.9	0.7
NM-203	4.3	0.6	0.7	0.8	0.7	1.2	1.1	0.8	0.7

*sacrifice. The values ≤LOQ are highlighted in yellow

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 5-11. Si Limit of Detection and Limit of Quantification of Si as determined by ICP-DRC-MS for the different organs and blood (mg Si/kg fresh weight).

	LOD	LOQ
Liver	0.3	0.9
Spleen	0.4	1.5
Lungs	0.6	1.8
Heart	0.4	1.2
Brain	0.2	0.8
Kidneys	0.3	0.9
Ovaries	1.8	6.1
Testes	0.2	0.8
GI tract*	0.3	1.1
Mesenteric lymph nodes	0.4	1.5
Blood	0.2	0.7

*small intestine

Tables 5-12 and 5-13 summarise the analytical data and give the picture of Si tissue distribution in rats after repeated IV dose of SAS nanoparticles. Each result is the mean of 3 animals. Tables 5-14 and 5-15 summarise the analytical data of Si concentrations in blood after repeated IV dose of SAS nanoparticles. Each result is the mean of 1-2 animals.

At day 6, a very high bioaccumulation in liver, spleen, and lungs was observed in both sexes and with both nanomaterials (Figures 5-2 to 5-6).

In males, Si concentrations at day 6 were above background in heart, kidneys and testes. At day 14 in male rats the level of Si in liver is still ≥100 mg/kg organ weight. Marked particle- and gender-related differences in bioaccumulation and biodistribution were observed. As for single dose administration, an higher tendency of NM-203 for accumulation in spleen at day 6 was noted (Table 5-12). At day 90, Si concentrations in spleen and liver were still distinctly higher than in controls, suggesting a longer time required for complete elimination of the administered nanomaterials from the body.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 5-12. Tissue distribution of Si in female rats (n=3) after repeated IV dose of SAS nanoparticles (mg Si/kg fresh weight).

	Controls	NM-200		NM-203	
		Day 6	Day 90	Day 6	Day 90
Liver	0.4 ±0.1	327 ± 32	38 ± 9	180 ± 78	20 ± 6
Spleen	≤LOD	96 ± 26	14 ± 1	140 ± 46	24 ± 4
Lungs	0.8 ± 0.3	71 ± 27	6.5 ± 2.8	21 ± 12	1.5 ± 0.1
Heart	0.5 ± 0.4	1.0 ± 0.7	≤LOD	1.3 ± 1.6	0.7 ± 0.3
Brain	0.4 ± 0.3	0.5 ± 0.1	0.4 ± 0.0	0.3 ± 0.2	0.4 ± 0.1
Kidneys	0.5 ± 0.1	2.4 ± 0.7	0.5 ± 0.1	3.3 ± 1.6	0.4 ± 0.2
Ovaries	≤LOD	≤LOD	≤LOD	≤LOD	≤LOD

The values ≤LOQ are highlighted in yellow.

Table 5-13. Tissue distribution of Si in male rats (n=3) after repeated IV dose of SAS nanoparticles (mg Si/kg fresh weight).

	Controls	NM-200				NM-203			
		Day 6	Day 14	Day 30	Day 90	Day 6	Day 14	Day 30	Day 90
Liver	0.4 ± 0.0	356 ± 62	159 ± 45	103 ± 15	14 ± 2	250 ± 29	100 ± 16	62 ± 10	11 ± 4
Spleen	≤LOD	105 ± 30	162 ± 61	480 ± 8	13 ± 1	357 ± 69	146 ± 27	77 ± 4	28 ± 9
Lungs	0.8 ± 0.3	82 ± 11	40 ± 2	16 ± 6	3.6 ± 0.8	66 ± 14	25 ± 3	11 ± 5	2.0 ± 0.4
Heart	0.6 ± 0.2	3.1 ± 1.2	2.7 ± 1.5	1.0 ± 0.3	0.9 ± 0.3	5.4 ± 3.5	3.7 ± 3.9	1.6 ± 1.3	1.8 ± 1.3
Brain	0.5 ± 0.2	0.5 ± 0.0	0.6 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.6 ± 0.2	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
Kidneys	0.4 ± 0.1	2.5 ± 0.4	0.7 ± 0.1	0.6 ± 0.0	0.5 ± 0.7	6.2 ± 1.2	3.3 ± 1.0	1.4 ± 0.4	0.6 ± 0.1
Testes	1.3 ± 0.3	1.4 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	1.7 ± 0.6	1.0 ± 0.1	0.7 ± 0.0	0.7 ± 0.1

The values ≤LOQ are highlighted in yellow.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.
www.nanogenotox.eu
Co-funded by the Health Programme of the European Union

Table 5-14. Blood concentrations of Si in female rats (n=1-2) after repeated IV dose of SAS nanoparticles (mg Si/kg fresh weight).

Females	**5 min**	**10 min**	**20 min**	**30 min**	**60 min**	**2h**	**4h**	**8h**	**24h***
Controls	NA	NA	NA	NA	0.4	0.7	NA	NA	0.3
NM-200	NA	NA	NA	1.0	1.0	0.8	NA	NA	0.3
NM-203	NA	NA	75.5	11.8	NA	12.3	14.2	1.6	0.3

*sacrifice. The values ≤LOQ are highlighted in yellow.

Table 5-15. Blood concentrations of Si in male rats (n=1-2) after repeated IV dose of SAS nanoparticles (mg Si/kg fresh weight).

Males	**5 min**	**10 min**	**20 min**	**30 min**	**60 min**	**2h**	**4h**	**8h**	**24h***
Controls	**NA**	**NA**	**NA**	**0.8**	**0.7**	**0.5**	**NA**	**NA**	**0.5**
NM-200	**1.5**	**1.1**	**3.5**	**15.2**	**1.0**	**NA**	**NA**	**NA**	**0.4**
NM-203	**NA**	**44**	**45**	**92**	**19**	**28**	**29**	**1.9**	**0.6**

*sacrifice. The values ≤LOQ are highlighted in yellow..

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

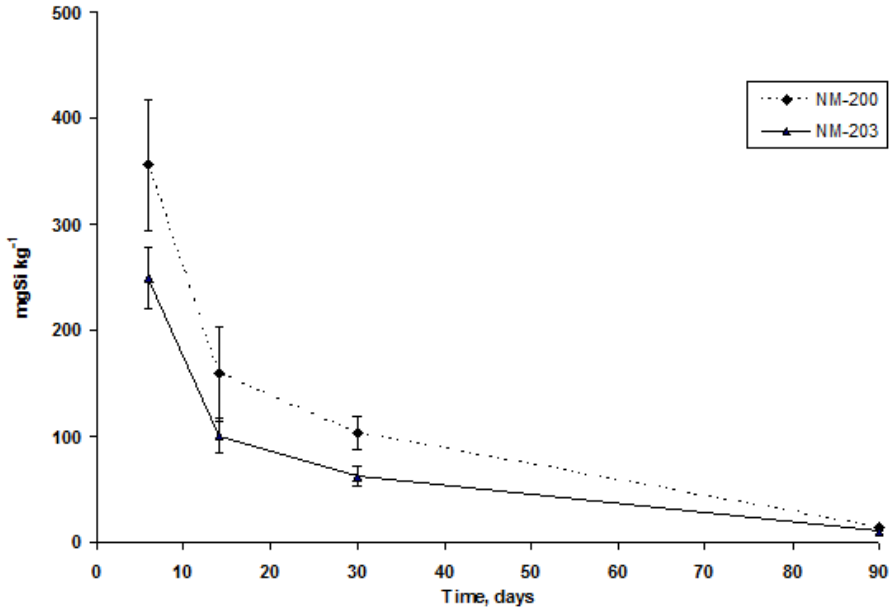


Figure 5-2. Si levels in liver after repeated IV dosing for 5 days to male Sprague-Dawley rats: NM-200 and NM-203.

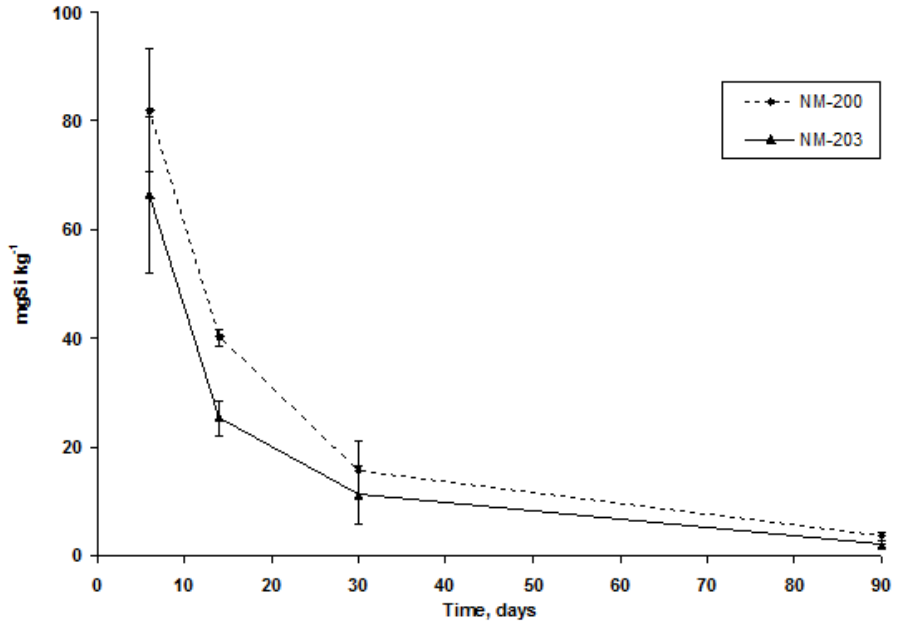


Figure 5-3. Si levels in lungs after repeated IV dosing for 5 days to male Sprague-Dawley rats: NM-200 and NM-203

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

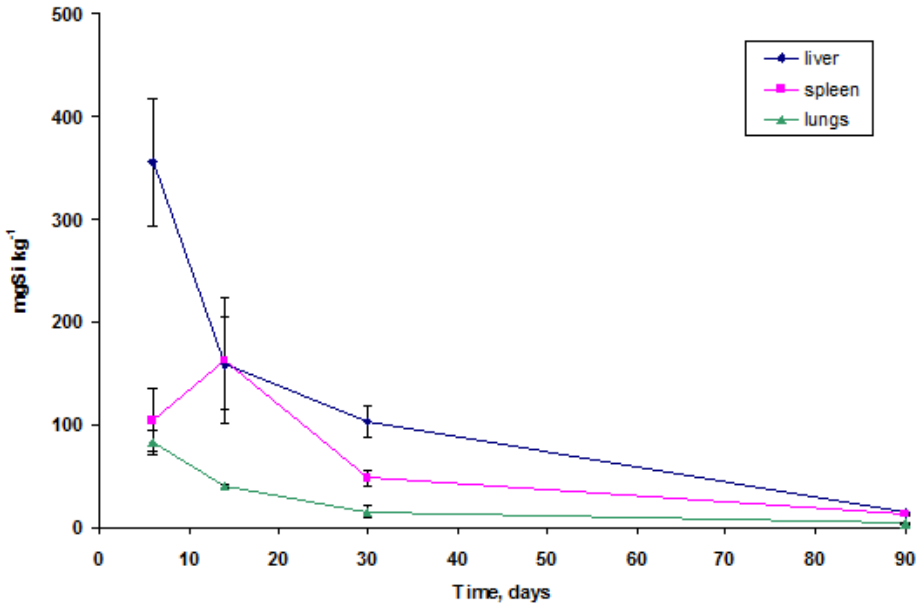


Figure 5-4. Organ distribution after IV repeated dosing of NM-200 for 5 days to male Sprague-Dawley rats. Si levels in major organs are shown.

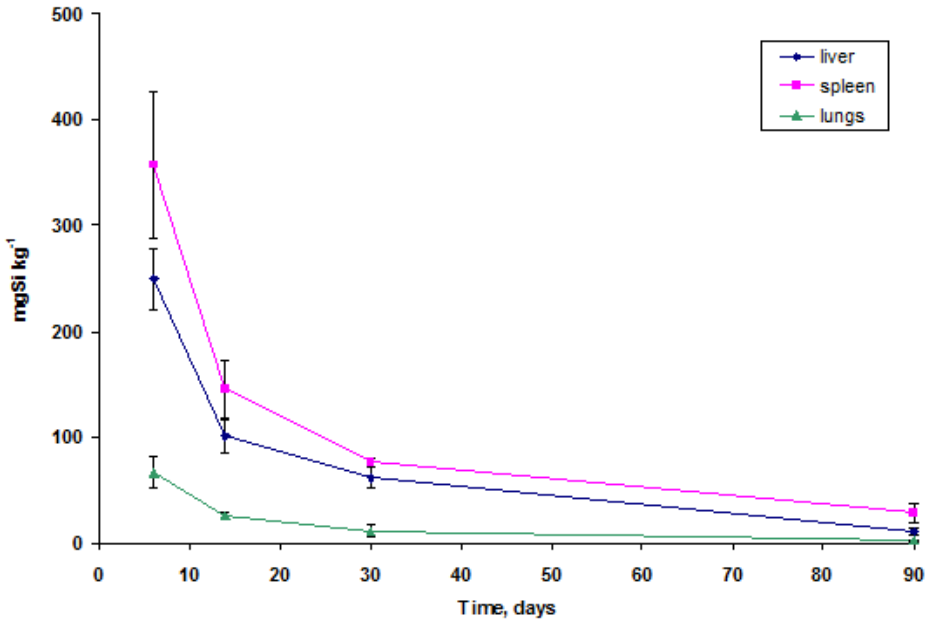


Figure 5-5. Organ distribution after IV repeated dosing of NM-203 for 5 days to male Sprague-Dawley rats. Si levels in major organs are shown.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.
www.nanogenotox.eu
Co-funded by the Health Programme of the European Union

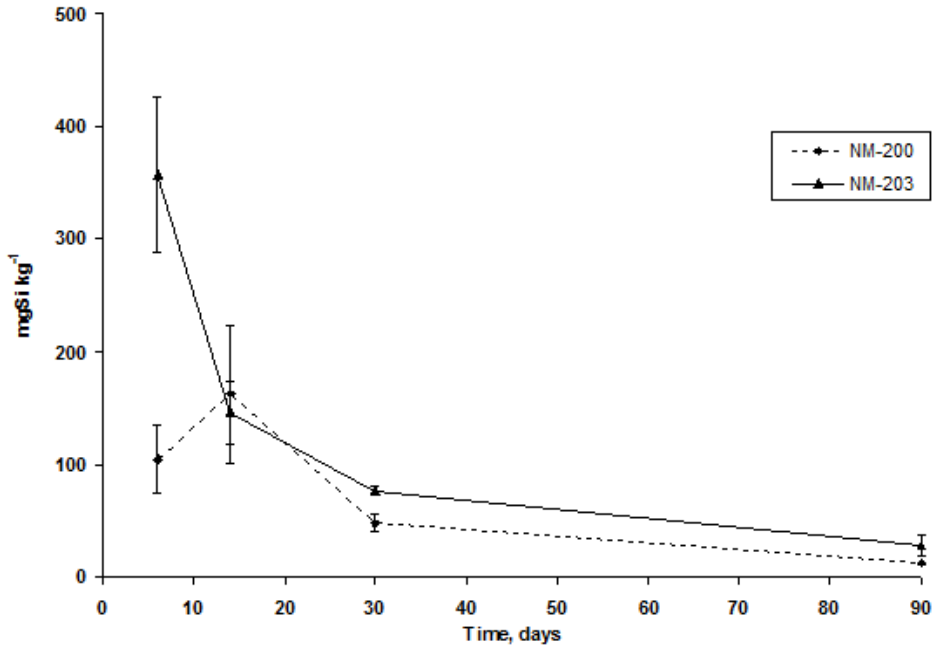


Figure 5-6. Si levels in spleen after repeated IV dosing for 5 days to male Sprague-Dawley rats: NM-200 and NM-203.

The Si levels per organ, obtained after correction of the data for the initial body weight, are shown in Tables 5-16 and 5-17.

Table 5-16. Si tissue distribution in male and female rats after repeated intravenous SiO_2 administration: NM-200. Control-corrected contents (µg/organ).

NM-200	Day 6 n=6 (M+F)	Day 14 n=3 (M)	Day 30 n=3 (M)	Day 90 n=6 (M+F)
Liver	3194 ± 926	2088 ± 809	1792 ±299	291 ± 96
Spleen	87 ± 28	299 ± 203	79 ± 7	14 ± 1
Lung	115 ± 30	53 ± 8	27 ± 8	7.8 ± 3.9
Heart	1.6 ± 0.8	2.1 ± 1.6	0.5 ± 0.4	0.6 ± 0.5
Kidney	2.9 ± 1.0	0.4 ± 0.1	0.7 ± 0.2	0.4 ± 0.4

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 5-17. Si tissue distribution in male and female rats after repeated intravenous SiO_2 administration: NM-203. Control-corrected contents (µg/organ).

NM-203	Day 6 n=6 (M+F)	Day 14 n=3 (M)	Day 30 n=3 (M)	Day 90 n=6 (M+F)
Liver	2322 ± 755	1806 ± 220	1269 ± 199	237 ± 62
Spleen	469 ± 294	263 ± 30	314 ± 75	90 ± 51
Lung	70 ± 46	40 ± 5	20 ± 12	2.4 ± 1.2
Heart	3.0 ± 2.5	2.9 ± 3.9	1.1 ± 1.2	1.4 ± 1.7
Brain	0.3 ± 0.4	-	-	0.1 ± 0.1
Kidney	7.3 ± 3.6	5.0 ± 2.3	2.2 ± 0.7	0.7 ± 0.5

The tissue distribution of the two nanomaterials as percentage of the dose is presented in Figures 5-7A-D after single and repeated IV dosing.

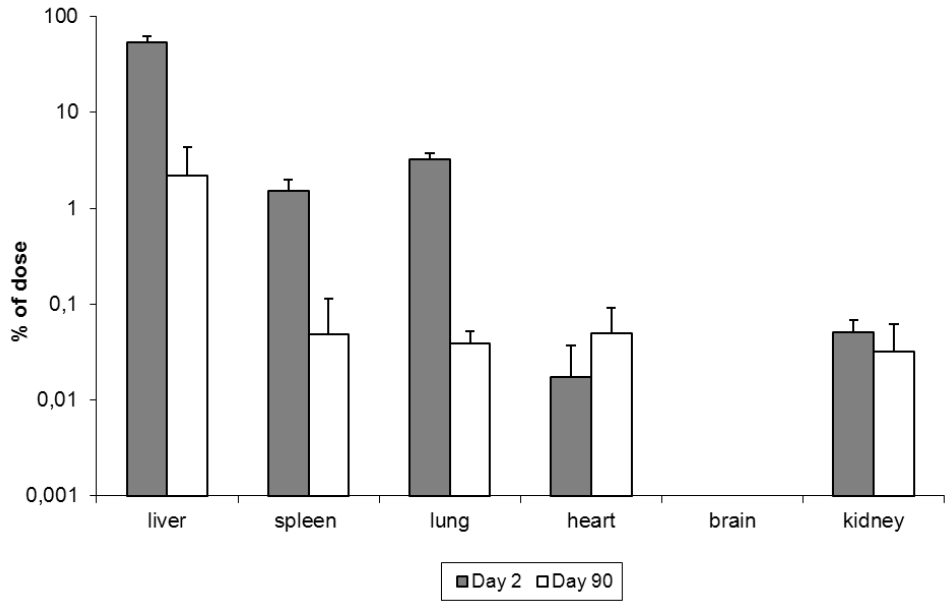


Figure 5-7A. Control-corrected Si tissue distribution expressed as percentage of the total Si administered as single dose (day 1) for NM-200.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.
www.nanogenotox.eu
Co-funded by the Health Programme of the European Union

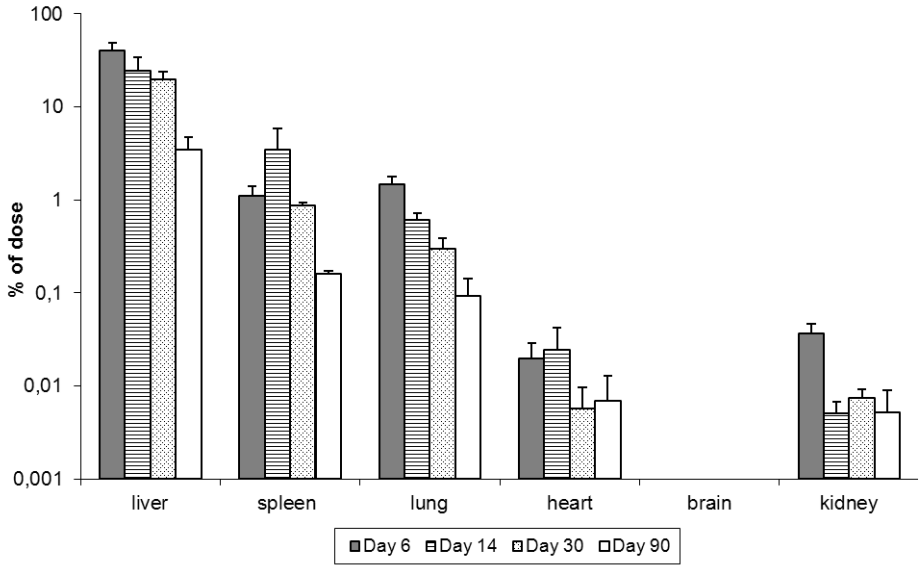


Figure 5-7B Control-corrected Si tissue distribution expressed as percentage of the total Si dose administered during five consecutive days (days 1-5) for NM-200.

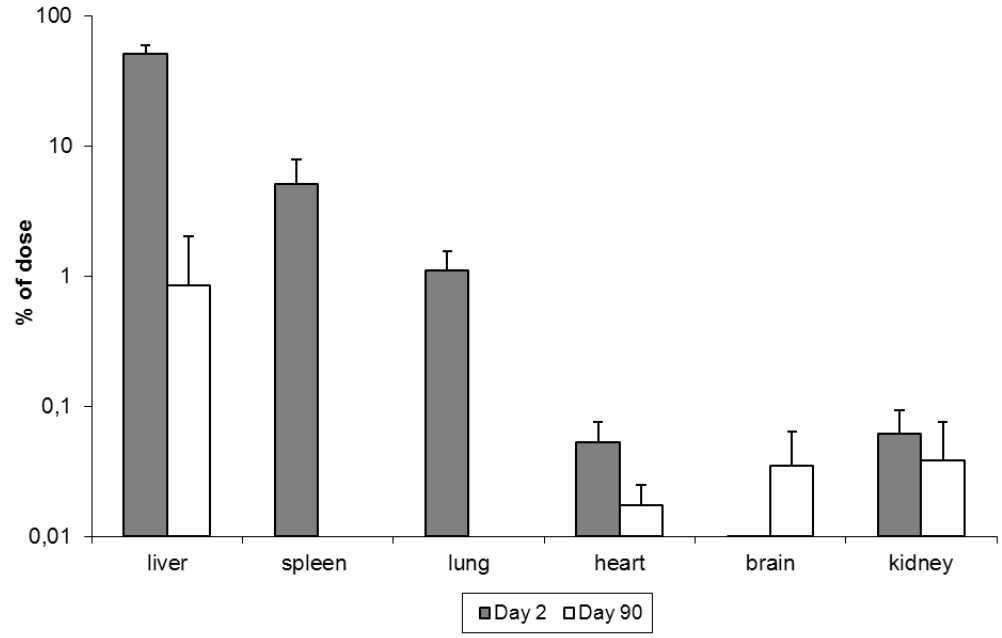


Figure 5-7C. Control-corrected Si tissue distribution expressed as percentage of the total Si administered as single dose (day 1) for NM-203.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

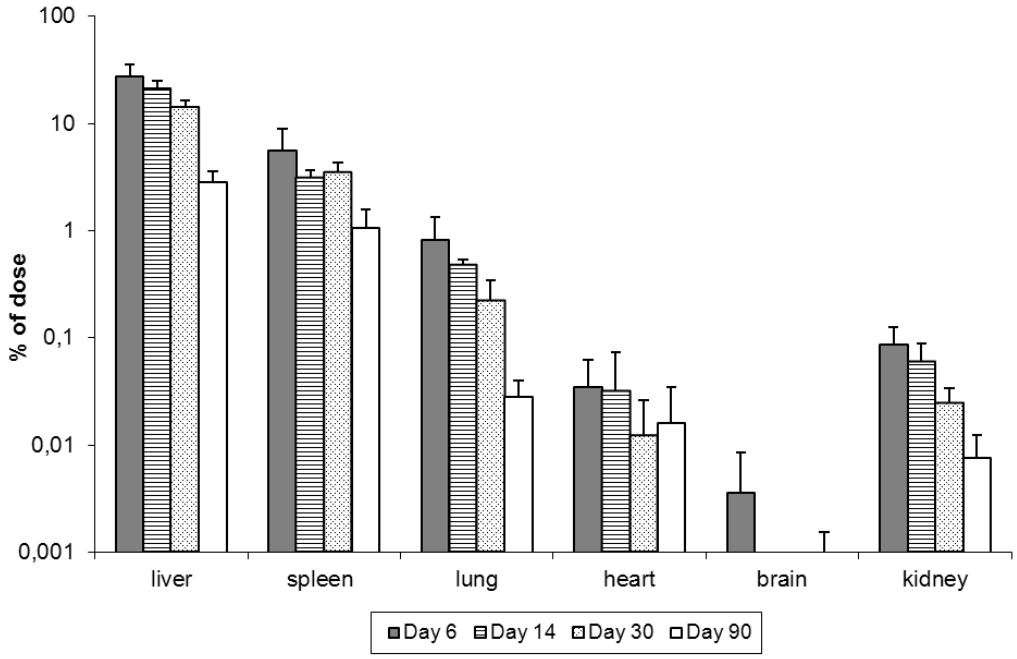


Figure 5-7D Control-corrected Si tissue distribution expressed as percentage of the total Si dose administered during five consecutive days (days 1-5) for NM-203.

The recovery of the dose calculated from the Si content in the investigated tissues ranged from 1 to 58% (Table 5-18). Some decrease was observed for both nanomaterials over time investigated, indicating that the excretion from the body is fast in the beginning but requires longer time to be complete.

Table 5-18 Recovery of Si in the investigated organs as percentage of dose administered after single or repeated dosing.

Nanomaterial	Dosing	Day 2/Day 6	Day 14	Day 30	Day 90
NM-200	Single	58 ± 10	nd	nd	2.3 ± 2.2
	Repeated	43 ± 8	28 ± 8	21 ± 4	3.7 ± 1.2
NM-203	Single	57 ± 8	nd	nd	0.9 ± 1.2
	Repeated	34 ± 11	25 ± 4	18 ± 3	3.9 ± 1.0

nd: not determined.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.
www.nanogenotox.eu
Co-funded by the Health Programme of the European Union

5.3.2.2 Histopathology

Following both single and repeated dose administration, gross observation at sacrifice at day 90 showed specific effects on liver and spleen.

Histopathological observations showed increase spleen and liver granuloma after single dose (+50%) and repeated dose (+100%) administration of NM-203. Furthermore after repeated IV exposure, NM-200 treated male animals showed a slight reduction in weight gain compared to control animals. For NM-203 treated rats, necrotic lesions at the site of injection were observed, accompanied by a decrease in body weight (males and females) (data not shown).

5.4 Conclusions

Tissue deposition following repeated oral administration to a total dose of 100 mg SAS /kg b.w. was negligible or absent. Slight differences in gender/material investigated are apparent, though the limited absorption makes it difficult to draw any conclusions.

In terms of effects, at sacrifice at day 6, altered organ weight (e.g. liver, lungs, uterus) was observed in both groups exposed to NM-200 and NM-203 (data not shown). Preliminary histopathological examinations showed effects on spleen in NM-203 treated male and female groups and NM-200 treated female group. Effects on the liver were observed in the NM-103 treated female group.

Tissue deposition was evident following both single and repeated dose IV administration.

Following single dose IV administration, high concentrations of Si were detected in liver, spleen and lungs, with marked particle- and gender-related differences. After 90 days, Si concentrations declined at concentrations near to or below the LOQ in all organs both for NM-200 and NM-203 and in male and female rats. NM-203 showed a markedly higher presence in the spleen of males compared to females and compared to NM-200 (both males and females); concentrations in liver were similar and apparently unrelated to particle- and gender. The highest concentration of NM-203 was observed in the spleen of male rats, while for NM-200 in male and female rats the highest concentration was noted in the liver. In female rats the concentration of NM-203 was similar for liver and spleen.

After repeated dose IV administration, Si peaked in blood at 20 min in females and at 30 min in males.

At days 6, Si concentrations were very high in liver, spleen and lungs, with marked particle- and gender-related differences, and above the LOQ in other organs as well. At day 14 and 30 considerable amounts of Si were still present in liver, spleen and lungs of male rats. In female rats, similar concentrations were observed in liver and spleen both at day 6 and day 90 for NM-203, while for NM-200 the highest concentration was present in the liver both at day 6 and day 90. In male rats NM-203 showed the highest concentration in the spleen,

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

while for NM-200 the highest concentration was in the liver. For NM-203 male rats a much higher distribution was noted compared to female rats at day 6 after the repeated administrations. At day 90 Si concentration in liver and spleen tissues of males and females were still distinctly higher than those in controls for both NM-200 and NM-203, suggesting a longer time required for complete elimination of administered NMs from the body.

Following both single and repeated dose administration, gross observation at sacrifice at day 90 showed specific effects on liver and spleen. Furthermore after repeated IV exposure, NM-200 treated male animals showed a slight reduction in weight gain compared to control animals. For NM-203 treated rats, necrotic lesions at the site of injection were observed, accompanied by a decrease in body weight (males and females).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

6. Carbon nanotubes (CNT)

6.1 Methods

6.1.1 Study schedule

Animals were treated with the schedule presented in Figure 3-1. For evaluation there were 6 animals per time/group (3 males and 3 females), except for day 14 and day 30 for repeated dosages for which only 3 males were investigated.

6.1.2 Animals

Wistar rats were obtained from Charles River, France. The rat's weight was between 226 and 234g for females and 256 and 266g for males.

6.1.3 Sample preparation

CNTs samples were prepared according to the dispersion protocol prepared by the WP4. A 2.56 mg/ml stock solution was prepared by prewetting the CNT powder in 0.5 vol % ethanol followed by dispersion in 0.05 wt% Rat Serum Albumin (RSA) in ultra pure water. The suspensions were sonicated for 15/16 minutes on ice. After preparation dispersed ^{14}C-multiwall carbon nanotubes (MWCNT) solutions were observed by optical microscopy to control sample homogeneity. According to the observations, depending on the MWCNT, the dispersion solutions were stable from one to several hours and therefore were immediately used after their preparation for IV injection in rats.

6.1.4 Doses administered

The CNT doses for the oral and IV route were administered using a suspension containing 2.56 mg CNT per ml. The single dose groups received a dose per animal between 9.6 – 10 mg/kg b.w. for male animals, and 10.9 – 11.3 mg/kg b.w. for female animals depending on the actual weight of the animal. The repeated dose groups received a total cumulative dose per animal between 48 – 50 mg/kg b.w after 5 days treatment, for male animals, and 54.5 – 56.5 mg/kg b.w. for female animals depending on the actual weight of the animal. The experiments were approved by the animal welfare committee as protocol N°12 104 prior to the study.

6.1.5 Organs sampled

Various organs and blood were collected including liver, lung, spleen, kidney, heart and testes. Organs were immediately removed and immersed in an - 80°C mixture of dry ice and isopentane to prevent MWCNT tissue redistribution.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

6.1.6 CNT determination

For the determination of the tissue distribution of the multi walled carbon nanotubes (MWCNT), the carbon nanotubes (CNTs,) NM-400, NM-401, NM-402 and NRCWE-006 were radiolabelled with ^{14}C.

^{14}C-labelling of carbon nanotubes was carried out using a three steps chemical process previously published (Georgin et al., 2009).[1] The first step of the process involves the formation of ^{14}C-labelled aroyl cyanides from a reaction of $Cu^{14}CN$ with aroyl chlorides previously generated on carbon nanotubes by treatment with oxalyl chloride. The second step corresponds to a palladium catalyzed decarbonylation of the aroyl cyanides into the corresponding ^{14}C-labelled nitriles. Hydrolysis of the so-formed labeled nitriles into carboxylic acids then afforded ^{14}C-MWCNTs that are identical to the MWCNT starting material (see Figure 6-1).

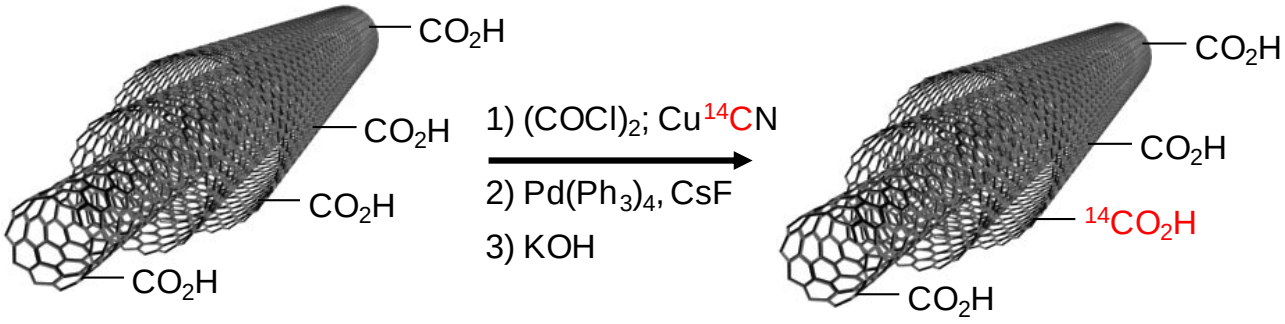


Figure 6-1. Radiolabelling of CNT batches.

Each step of the labelling process was followed by careful repeated washing in order to eliminate non-covalent radioactivity. Radioactive counting was used to estimate the efficiency of each step of the labelling procedure. The standard procedure[2] was applied to

[1] D. Georgin, B. Czarny, M. Botquin, M. Mayne-L'Hermite, M. Pinault, B. Bouchet-Fabre, M. Carriere, J-L. Poncy, Q. Chau, R. Maximilien, V. Dive and F. Taran. *J. Am. Chem. Soc.* **2009**, *131*, 14658-14659.

[2] Experimental procedure: *In a 500 ml flask, a mixture containing 500 mg of carbon nanotubes in 200 mlof thionyl chloride was stirred at 70°C for 18 hours. The reaction mixture was then concentrated under vacuum. 200 ml of acetonitrile and 250 mg of Cu14CN were added and this slurry was then stirred at room temperature for 18 hours. 200 mlof acetone were then added, the solution was centrifugated (5000 rd/min, 5 min) and the liquid phase was removed in order to take out excess of reagents. This operation was repeated 4 more times and the nanotubes were dried under vacuum. Radioactive counting of the acetone washing solutions showed no significant radioactivity after the second washing. Nanotubes were then suspended in 200 ml of dry toluene and 230 mg of tetrakis-(triphenylphosphine)-palladium and 320 mg of cesium fluoride were added. The reaction mixture was stirred at 110°C for 5 hours and several washing steps: with toluene, KOH 0.1 M, aqueous ammonia (20% v/v), HCl 0.01 M , ethanol and finally ether, were then applied. For each step of washing, 200 ml of solvent were added and sonication (ultrasonic bath) was applied during 15 min. Centrifugation (5000 rd/min, 5 min) was applied and the solvent was removed. Radioactivity measurements have been done on the washing solutions and indicated that ~50% of the total radioactivity was recovered in the washing phase following basic treatments; no significant radioactivity was detected after the washing with water. After these washing steps, the nanotubes were then heated in a mixture of 100 ml of potassium hydroxide 1M and 100 mlof ethanol during 18 hours. The liquid layer was removed, 200 ml of deionised water were added and HCl 0.1N was added until pH 5. The nanotubes were then washed two times with deionised water, ethanol, acetone and again with deionised water (same procedure as*

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

the four types of CNTs studied in Nanogenotox (NM-400, NM-401, NM-402 and NRCWE-006) and was successful on the NM-400 and NM-402 nanotubes. However, the labelling of NM-401 and NRCWE-006 carbon nanotubes required adjustments of the standard labelling procedure to reach reasonable level of labelling, probably due to low COOH content of these carbon nanotube batches. After numerous trials (change of solvents and reagents stoichiometry), the procedure was optimized and finally successful by increasing the Pd catalyst and CsF loading (optimized loading is three times higher than the standard procedure). Specific activities of each ^{14}C-labelled MWCNTs were directly measured with a liquid scintillation counter (Wallac 1409) and confirmed by $^{14}CO_2$ release counting after acidic decomposition treatment.[3]

The results, summarised in Table 6-1, showed specific activities ranging from 0.07 to 0.44 MBq/mg (2 to 12 µCi/mg) depending of the nanotubes nature. These levels of radiolabelling, which correspond to $^{14}C/^{12}C$ ratios ranging from 0.4 to 2/1000, are high enough to allow sensitive detection of MWCNT *in vivo*.

Table 6-1 Efficacy of ^{14}C labelling of MWCNTs

^{14}C-Carbon nanotube	Quantity	Specific activity	$^{14}C/^{12}C$ ratio
^{14}C-NM-400	79 mg	0.44 MBq/mg (12 µCi/mg)	~ 2/1000
^{14}C-NM- 401	225 mg	0.07 MBq/mg (2 µCi/mg)	~ 4/10000
^{14}C-NM-402	230 mg	0.36 MBq/mg (9.7 µCi/mg)	~ 2/1000
^{14}C-NRCWE-006	435 mg	0.12 MBq/mg (3.3 µCi/mg)	~ 7/10000

Blood samples were collected on abdominal aortic vein at days post administration of MWCNT. Quantitative determination of the radioactivity in blood was carried out by 1 µL blood deposition on glass slides and radioactivity counting using the b-imager™ 2000.

mentioned above). This procedure allowed us to obtain 79 to 435 mg of labeled nanotubes with a specific activity ranging from 0.07 to 0.44 MBq/mg (2 to 12 µCi/mg) depending of the nanotubes nature.

[3] Experimental procedure: *1 mg of the labelled nanotubes was introduced in a 25 mL flask containing 10 mL of a sulphuric acid / nitric acid (66/33) mixture. This flask was fitted with a reflux condenser and a system providing a slow flow of argon. At the end of the reflux condenser, 4 traps containing 10 mL of 1 M sodium hydroxide were linearly arranged. The acidic mixture was heated at 160°C under a flow of argon and the carbon nanotubes were transformed into carbon dioxide which was trapped by the sodium hydroxide traps. The counting of the radioactivity contained in the traps using a Ludlum model 2200 Bremstrahlung counter afforded the total activity of the nanotubes.*

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

For tissue collection rats were anesthetised with isoflurane 2% and sacrificed by exsanguination (abdominal aorta). Organs were immediately removed and immersed in an -80° C mixture of dry ice and isopentane to prevent MWCNT tissue redistribution. After blocking organs in mounting medium, 50 tissue sections (20 μm) were made at -20° C with a slicing microtome (LEICA Microsystems, France), selecting representative organ regions. After lay down on glass slides, these sections were left either in freezer for optical study, or were kept at room temperature for 1 day in the presence of silica gel to ensure complete drying, before radioimaging analysis. The quantitative determination of the radioactivity in dried tissue sections and imaging were carried out using a high performance autoradiography imager (b-imager^{TM} 2000, detection threshold of 0.01 cpm/mm^2 for ^{14}C Biospace Lab, Paris, France).

For each animal, the percentage (%) of the injected dose for whole organs was calculated by radioactivity integration to the whole organ volume, using the radioactivity level detected in the 50 tissue sections for each organ (integration procedure developed previously in our laboratories). The % of the injected dose for each analysed organ is reported.

6.2 Results

After oral administration none of the four MWCNT (NM-400, NM-401, NM-402, and NRCWE-006) showed translocation of the MWCNT from the GI-tract into the systemic circulation or any of the organs investigated (including spleen, liver, and lung) (data not shown).

In blood obtained at 24 hours after the administration of the ^{14}C labelled MWCNT very low to almost no radioactivity was detected whatever the protocol of MWCNT administration in either male or female rats for NM-401, NM-402, and NRCWE-006. However, for NM-400 at 24 hours after the IV administration both for the single and repeated dose a considerable level of ^{14}C was measured up to 10% of the injected dose (Tables 6-2 and 6-3). Table 6-2 shows the ^{14}C tissue distribution after a single IV MWCNT administration, and Table 6-3 shows the distribution after five repeated IV administrations.

NM-400. After a single IV dose for male rats most of the injected dose was observed in liver (24%) and lung (25%) at day 1, with a few percent present in spleen, kidneys, heart and testes. At day 90, 20% of injected dose is still observed in organs (cumulated data from all organs analysed), as compared to 65% observed at day 1, with liver and lung being the major targeted organs (Table 6-2). After the repeated IV dosing for male rats higher organ levels were observed when compared to the single dose (Table 6-3). In liver, the accumulated dose is 5-fold higher than the one observed in single injection. However, the percent of total injected dose observed at day 6 in all organs remains in a similar range to that observed by single dosage (ca. 39% instead 65% of the administered dose). Between day 6 and day 90, the percent of total injected dose measured decreased from 39% to 11% in the organs evaluated. As compared to single dose, for which both liver and lung were the main target

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

organs, repeated dosages involved a preferential accumulation in the liver. A decrease of organ loading is observed from day 1 to day 90, except for lung in which a 4% of the injected dose in observed at any time. Overall, a 60% reduction of the total injected dose was observed between day 1 and day 90.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Nanotubes	**Table 6-2A,** % of injected dose in organs in rat males (single dose)										
24h	Spleen	Liver	Lung	Kidney	Brain	Heart	Thymus	Testes	Pancreatic lymph node	Blood (total body)	TOTAL
IV NM 400	1,92 ± 0,33	23,79 ± 3,89	24,82 ± 1,76	1,89 ± 0,96	0,00	0,24 ± 0,04	0,00	0,22 ± 0,05		12,5 ± 2,5	65% ± 9%
IV NM 401	0,52 ± 0,42	15,11 ± 6	1,78 ± 1,5	0,37 ± 0,3	0,00	0,00	0,00	0,00	0,04 ± 0,01	0,00	17,8% ± 8%
IV NM 402	0,55 ± 0,14	6,98 ± 0,64	0,27 ± 0,13	0,07 ± 0,04	0,00	0,00	0,00	0,00		0,3 ± 0,3	8,2% ± 1,2%
IV NM NRCWE-006	1 ± 0,54	28,21 ± 8,37	1,2 ± 0,65	0,25 ± 0,04	0,00	0,00	0,00	0,16 ± 0,74	0,32 ± 0,19	0,00	31,1% ± 10,5%
Day 90	Spleen	Liver	Lung	Kidney	Brain	Heart	Thymus	Testes	Pancreatic lymph node	Blood (total body)	TOTAL
IV NM 400	0,39 ± 0,25	8 ± 1,83	11,4 ± 2,8	0,00	0,00	0,00	0,00	0,00	0,24 ± 0,15	0,00	20% ± 5%
IV NM 401	0,38 ± 0	3,46 ± 0,42	0,59 ± 0,17	0,13 ± 0,1	0,00	0,00	0,00	0,05 ± 0,04	0,09 ± 0,004	0,00	4,7% ± 0,7%
IV NM 402	0,23 ± 0,13	3,15 ± 2,2	0,3 ± 0,11	0,00	0,00	0,00	0,00	0,00		0,00	3,7% ± 2,4%
IV NM NRCWE-006	1 ± 0,83	16,40 ± 4,6	1 ± 0,73	0,22 ± 0,06	0,00	0,00	0,00	0,33 ± 0,21	2,12 ± 2	0,00	21% ± 8,4%

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

Nanotubes	**Table 6-2B**, % of injected dose in organs in rat females (single dose)										
24h	Spleen	Liver	Lung	Kidney	Brain	Heart	Thymus	Testes	Pancreatic lymph node	Blood (total body)	TOTAL
IV NM 400	0,69 ± 0,09	24,93 ± 14,6	1,15 ± 0,52	0,59 ± 0,35	0,00	0,00	0,00	0,00		10,8 ± 0,94	38,2% ± 16,6%
IV NM 401	0,22 ± 0,14	6,37 ± 3,87	1,03 ± 0,75	0,28 ± 0,02	0,00	0,00	0,00	0,02 ± 0,02	0,01 ± 0,6	0,00	7,9% ± 5,4%
IV NM 402	0,62 ± 0,33	23 ± 5	0,54 ± 0,19	0,08 ± 0,04	0,00	0,00	0,00	0,00		0,44 ± 0,03	24,6% ± 5,6%
IV NM NRCWE-006	0,85 ± 0,56	35,6 ± 18,2	2,17 ± 1,27	0,26 ± 0,21	0,00	0,00	0,05 ± 0,05	0,00	0,026 ± 0,02	2,73 ± 0,96	42% ± 21%
Day 90	Spleen	Liver	Lung	Kidney	Brain	Heart	Thymus	Ovaries	Pancreatic lymph node	Blood (total body)	TOTAL
IV NM 400	0,36 ± 0,24	3,4 ± 0,61	8,2 ± 0,56	0,00	0,00	0,00	0,00	0,00	0,09 ±0,04	0,00	12% ± 1,5%
IV NM 401	0,27 ± 0,11	2,5 ± 0,93	0,7 ± 0,28	0,18 ± 0,13	0,00	0,00	0,00	0,00	0,04 ± 0,15	0,00	3,7% ± 1,6%
IV NM 402	0,57 ± 0,14	5,5 ± 1,1	0,54 ± 0,25	0,00	0,00	0,00	0,00	0,00		0,00	6,6% ± 1,5%
IV NM NRCWE-006	1,32 ± 0,63	17,30 ± 7,08	1,83 ± 0,56	0,28 ± 0,17	0,00	0,00	0,00	0,00	0,11 ± 0,23	0,00	20,8% ± 8,7

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

www.nanogenotox.eu

Nanotubes	**Table 6-3A:** % of injected dose in organs in rat males (repeated doses)										
Day 6	Spleen	Liver	Lung	Kidney	Brain	Heart	Thymus	Testes	Pancreatic lymph node	Blood (total body)	TOTAL
IV NM 400	2,35 ± 0,39	25,42 ± 3,80	4,40 ± 4	1,30 ± 0,63	0,00	0,17 ± 0,10	0,07 ± 0,01	0,27 ± 0,09		5,17 ± 2,1	39% ± 10 %
IV NM 401	0,3 ± 0,04	3,96 ± 1,2	1,05 ± 1	0,10 ± 0,07	0,00	0,00	0,00	0,06 ± 0,03	0,10 ± 0,60	0,00	5,6% ± 3 %
IV NM 402	0,25 ± 0,06	4,78 ± 1,8	0,11 ± 0,03	0,02 ± 0,005	0,00	0,00	0,00	0,00		0,14 ± 0,03	5,3% ± 1,9%
IV NM NRCWE-006I	0,71 ± 0,5	22,96 ± 18,8	1,56 ±1,2	0,51 ± 0,21	0,00	0,07 ± 0,06	0,00	0,00	0,04 ± 0,06	0,25 ± 0,04	26,1% ± 20,5%
Day 14	Spleen	Liver	Lung	Kidney	Brain	Heart	Thymus	Testes	Pancreatic lymph node	Blood (total body)	TOTAL
IV NM 400	2,20 ± 1,22	35,21 ± 15,6	4,42 ± 3,26	0,43 ± 0,26	0,00	0,00	0,1 ± 0,04	0,09 ± 0,05		2,12 ± 0,95	44,6% ± 21%
IV NM 401	0,58 ± 0,22	5,82 ± 0,97	0,41 ± 0,14	0,07 ± 0,04	0,00	0,00	0,00	0,00	0,01 ± 0,007	0,00	6,9 % ± 1,5%
IV NM 402	0,30 ± 0,03	4,29 ± 0,4	0,1 ± 0,04	0,01 ± 0,006	0,00	0,00	0,00	0,02 ± 0,01		0,04± 0,02	4,7% ± 0,5%
IV NM NRCWE-006	1,57 ± 0,26	34,5 ± 5	1,9 ± 0,48	0,19 ± 0,03	0,00	0,00	0,00	0,00	0,22 ± 0,01	0,00	38,4% ± 5,8%

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

www.nanogenotox.eu

Nanotubes	**Table 6-3A:** % of injected dose in organs in rat males (repeated doses) continued										
Day 30	Spleen	Liver	Lung	Kidney	Brain	Heart	Thymus	Testes	Pancreatic lymph node	Blood (total body)	TOTAL
IV NM 400	0,58 ± 0,1	17,20 ± 6,04	2,32 ± 1,44	0,028 ± 0,01	0,00	0,00	0,00	0,00	0,089 ± 0,04	0,25± 0,11	20,4% ± 7,6%
IV NM 401	0,26 ± 0,02	3,79 ± 0,4	0,19 ± 0,03	0,1 ± 0,09	0,00	0,00	0,00	0,00	0,07 ± 0,02	0,00	4,4% ± 0,6%
IV NM 402	0,19 ± 0,02	3,9 ± 1,12	0,05 ± 0,03	0,00	0,00	0,00	0,00	0,00		0,00	4,1% ± 1,1%
IV NM NRCWE-006	1,65 ± 0,25	37,4 ± 8,5	1,83 ± 0,35	0,16 ± 0,08	0,00	0,00	0,00	0,00	0,57 ± 0,48	0,00	41,6% ± 90,7%
Day 90	Spleen	Liver	Lung	Kidney	Brain	Heart	Thymus	Testes	Pancreatic lymph node	Blood (total body)	TOTAL
IV NM 400	0,31 ± 0,04	6,23 ± 2,6	4,3 ± 1,2	0,05 ± 0,009	0,00	0,00	0,00	0,00	0,17 ± 0,06	0,00	11,1% ± 4%
IV NM 401	0,26 ± 0,05	2,93 ± 0,46	0,25 ± 0,04	0,03 ± 0,02	0,00	0,00	0,00	0,05 ± 0,04	0,08 ± 0,01	0,00	3,6% ± 0,6%
IV NM 402	0,43 ± 0,12	3,6 ± 1,83	0,13 ± 0,01	0,00	0,00	0,00	0,00	0,00		0,00	4,2% ± 1,9%
IV NM NRCWE-006	1,45 ± 0,35	26,89 ± 7,71	1,94 ± 0,65	0,21 ± 0,09	0,00	0,00	0,00	0,00	0,48 ± 0,36	0,00	31% ± 9,6%

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

www.nanogenotox.eu

Nanotubes	**Table 6-3B,** % of injected dose in organs in rat females (repeated doses)										
Day 6	Spleen	Liver	Lung	Kidney	Brain	heart	Thymus	Ovaries	Blood (total body)	Pancreatic lymph node	TOTAL
IV NM 400	2,26 ± 0,6	34,94 ± 16,02	32,78 ± 11,21	1,30 ± 0,46	0,00	0,28 ± 0,09	0,07 ± 0,02	0,02 ± 0,004	5,87 ± 0,40		77,3% ± 28,8%
IV NM 401	0,13 ± 0,1	4,1 ± 0,77	0,55 ± 0,026	0,09 ± 0,07	0,00	0,03 ± 0,02	0,00	0,006 ± 0,003	0,00	0,005 ± 0,002	5% ± 0,9%
IV NM 402	0,42 ± 0,19	10,59 ± 5	0,40 ± 0,4	0,04 ± 0,02	0,00	0,00	0,00	0,00	0,16 ± 0,30		11,6% ± 5,9 %
IV NM NRCWE-006	0,76 ± 0,4	34,76 ± 20,1	2,42 ± 2	0,19 ± 0,15	0,00	0,06 ± 0,03	0,00	0,01 ± 0,004	0,30 ± 0,09	0,08 ± 0,03	38,6% ± 22,7%
Day 90	Spleen	Liver	Lung	Kidney	Brain	Heart	Thymus	Ovaries	Blood (total body)	Pancreatic lymph node	TOTAL
IV NM 400	0,21 ± 0,13	7,92 ± 2,46	4,68 ± 0,97	0,04 ± 0,01	0,00	0,00	0,00	0,00	0,00	0,12 ± 0,06	13% ± 3,6%
IV NM 401	0,33 ± 0,05	2,7 ± 0,39	0,39 ± 0,06	0,05 ± 0,01	0,00	0,00	0,00	0,00	0,00	0,1 ± 0,1	3,6% ± 0,6%
IV NM 402	0,41 ± 0,02	3,21 ± 2,79	0,18 ± 0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,00	3,8% ± 2,9%
IV NM NRCWE-006	1,82 ± 0,55	22,02 ± 6,88	2,28 ± 0,95	0,31 ± 0,11	0,00	0,00	0,00	0,00	0,00	0,58 ± 0,34	27% ± 8,8%

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

www.nanogenotox.eu

NM-401: After a single IV dose for male rats, 18% of the injected dose of NM-401 was observed in the organs evaluated at day 1, with the liver being the main target organ (15%); at day 90 the total dose measured decreased to 5%, with liver being the major targeted organ (Table 6-2). After the repeated IV dosing for male rats, NM-401 elimination appeared more reduced, as compared to single dose, thus between day 1 and day 90, only a 35% reduction of the total injected dose was observed. For spleen, testes and pancreatic lymph nodes, the level of NM-401 in terms of percentage of the injected doses remained similar between day 1 and day 90. Compared to NM-400, a much lower percentage of the dose was recovered (NM-400 40% in males and 77% in females, NM-401 6% in males and 5% in females) at day 6 after five repeated administrations (Table 6-3).

NM-402: After a single IV dose for male rats for NM-402 only 8% of the injected dose was observed in organs at day 1, with the liver being the main target organ (7%). In females the recovery was 24% after a single dose (Table 6-2). At day 90 a 50% reduction of the level of NM-402 was observed when compared to day 1 in most organs, while for the lung a similar % of injected dose was observed both on day 1 after a single administration and at day 90 after repeated administration. After the repeated IV dosing for male rats compared to the data of day 6, almost no elimination of NM-402 was observed at day 90 after administration (Table 6-3). However, when comparing the elimination after repeated exposures and single exposure, NM-402 elimination appeared much more reduced. After a single exposure a 50% elimination was observed while almost no elimination was observed after repeated IV doses. A particular feature with this NM-402 was the observation that spleen was more loaded than lung at any time point.

NRCWE-006: After a single IV dose for male rats, 31% of the injected dose was observed in all organs at day 1, which reduced to 21% at day 90 (Table 6-2). A 50% reduction of the injected dose was observed in liver between day 1 and day 90, but in contrast no elimination was observed for lung, spleen and kidney. Of particular interest was the observation that the % of injected dose between day 1 and day 90 increased in testes and pancreatic lymph nodes, suggesting a possible translocation of particles from liver to testes and pancreatic lymph nodes. After the repeated IV dosing for male rats between day 6 and day 90, the percent of injected dose did not vary in most organs, with liver being the most loaded organ (Table 6-3). Over time, the percent of injected dose increased for pancreatic lymph nodes between day 6 and day 90 (from 0.04% to 0.5 %), possibly indicating particle translocation from liver to pancreatic lymph nodes.

Studies in females confirm in general the main results observed in males, with a slightly better clearance observed for NM-400. An extremely low level of radioactivity was observed in ovaries at day 6 after repeated administration. After IV injection of radiolabelled ^{14}C MWCNT (NM-400, NM-401, NM-402 and NRCWE-006) to rats, no radioactivity was observed

in bone marrow of the femur of the animals, as illustrated below in Figure 6-2 with ^{14}C radiolabelled NM-401.

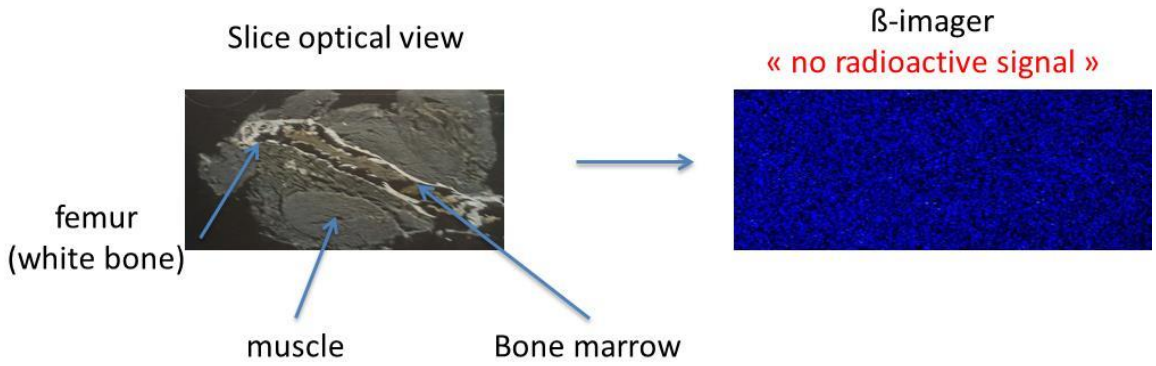


Figure 6-2. Lack of ^{14}C radiolabel detection of NM-401 in bone marrow 24 h after IV administration in a female rat.

Although detection of the ^{14}C isotope used to label the MWCNTs, is indicative of the presence of the CNTs in organs, there is the possibility that the label detected is not associated with the CNTs. In order to prove the presence of the label on the CNTs, concentrated extracts of the organs have been prepared that can be analysed by high resolution TEM. As shown in Figure 6-3, it was unambiguously demonstrated that the radioactive signal indeed corresponds to the presence of carbon nanotubes, with a direct visualization of the CNT walls, the walls inside, and finally measurement of the CNT diameter.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.
www.nanogenotox.eu
Co-funded by the Health Programme of the European Union

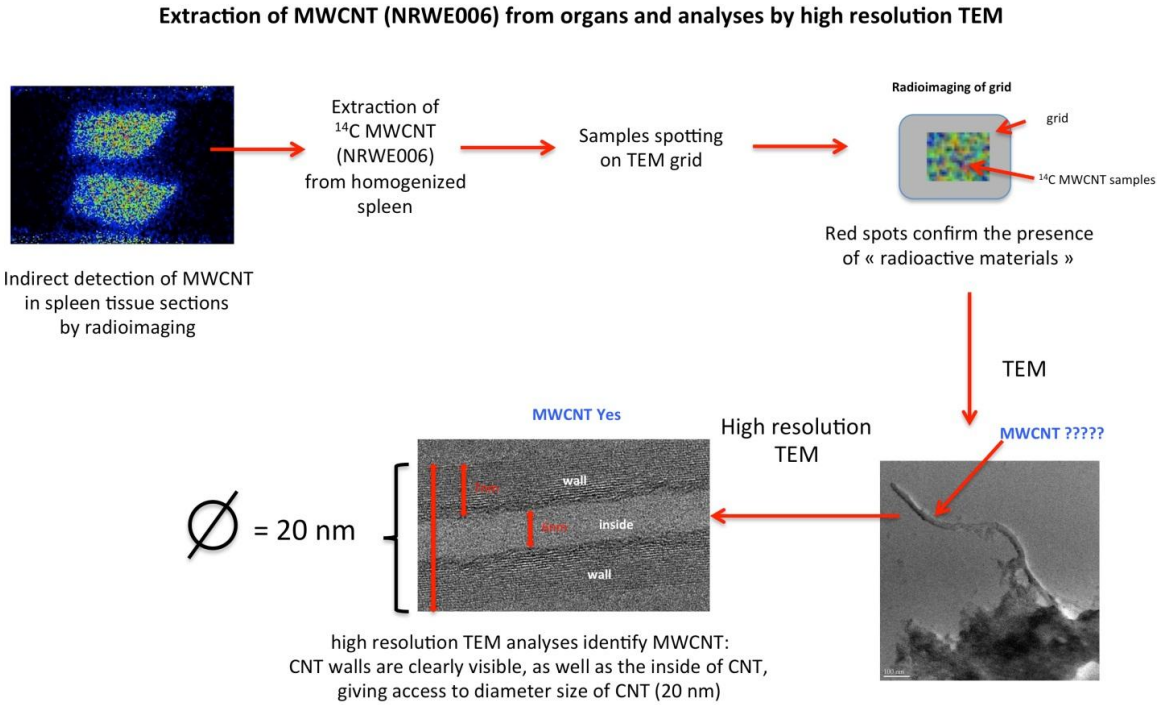


Figure 6-3. Demonstration of ^{14}C radiolabel associated with NRCWE-006 MWCNT.

6.3 Conclusions

Each type of MWCNT was found to display particular bioaccumulation and biopersistence properties in the various organs evaluated. NM-400 and NRCWE-006 showed much higher bioaccumulation than NM-401 and NM-402. In liver and spleen, at day 90 a marked reduction in the MWCNT level expressed as percentage of injected dose, was only observed for NM-400; for NM-401, NM--402 and NRCWE-006 no significant decrease was observed between day 6 and day 90. In lung, a significant reduction in the MWCNT level expressed as percentage of injected dose was only observed for NM-401. For NM-401, NM-402 and NRCWE-006, the data suggest a significant biopersistence of these MWCNTs in most organs beyond 3 months after administration. A comparison of the four investigated MWCNT nanomaterials for liver, spleen and lung is presented in Figures 6-3, 6-4 and 6-5.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

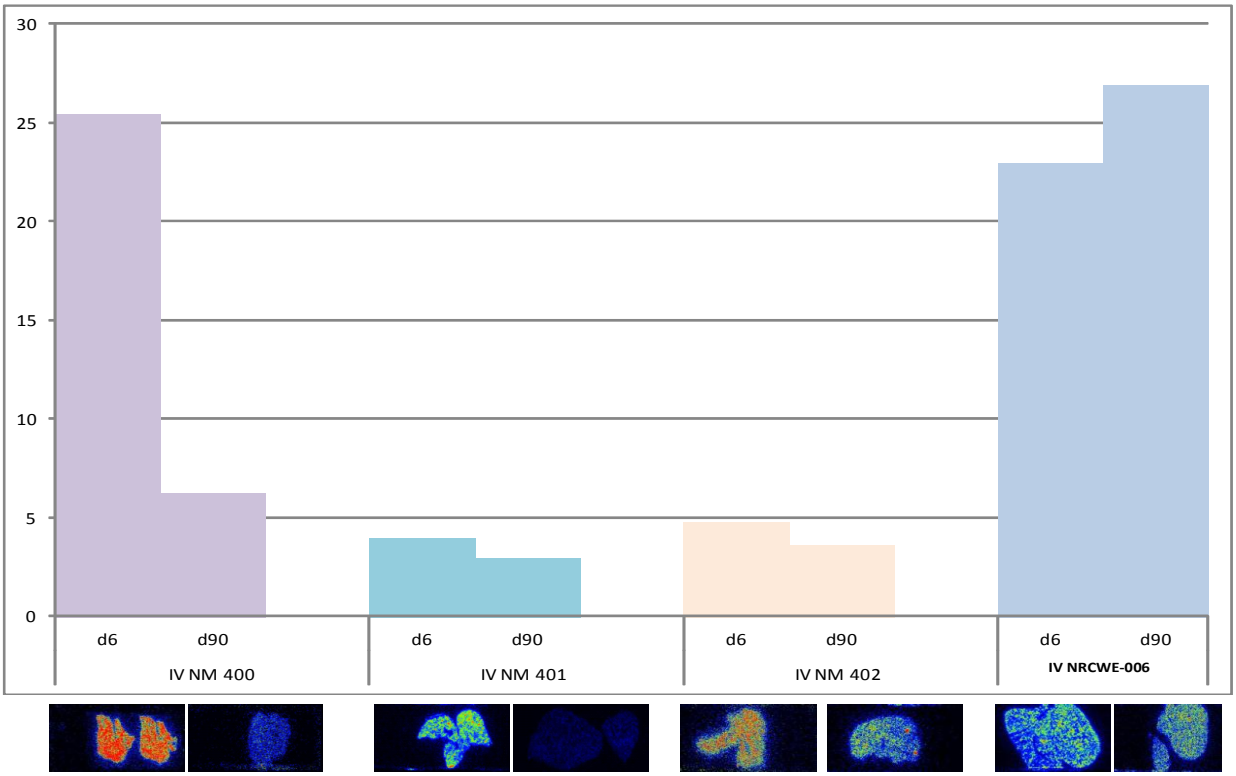


Figure 6-3. ^{14}C levels in tissues as percentage of the injected dose as indicator for MWCNT presence in the liver after repeated MWCNT administration.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

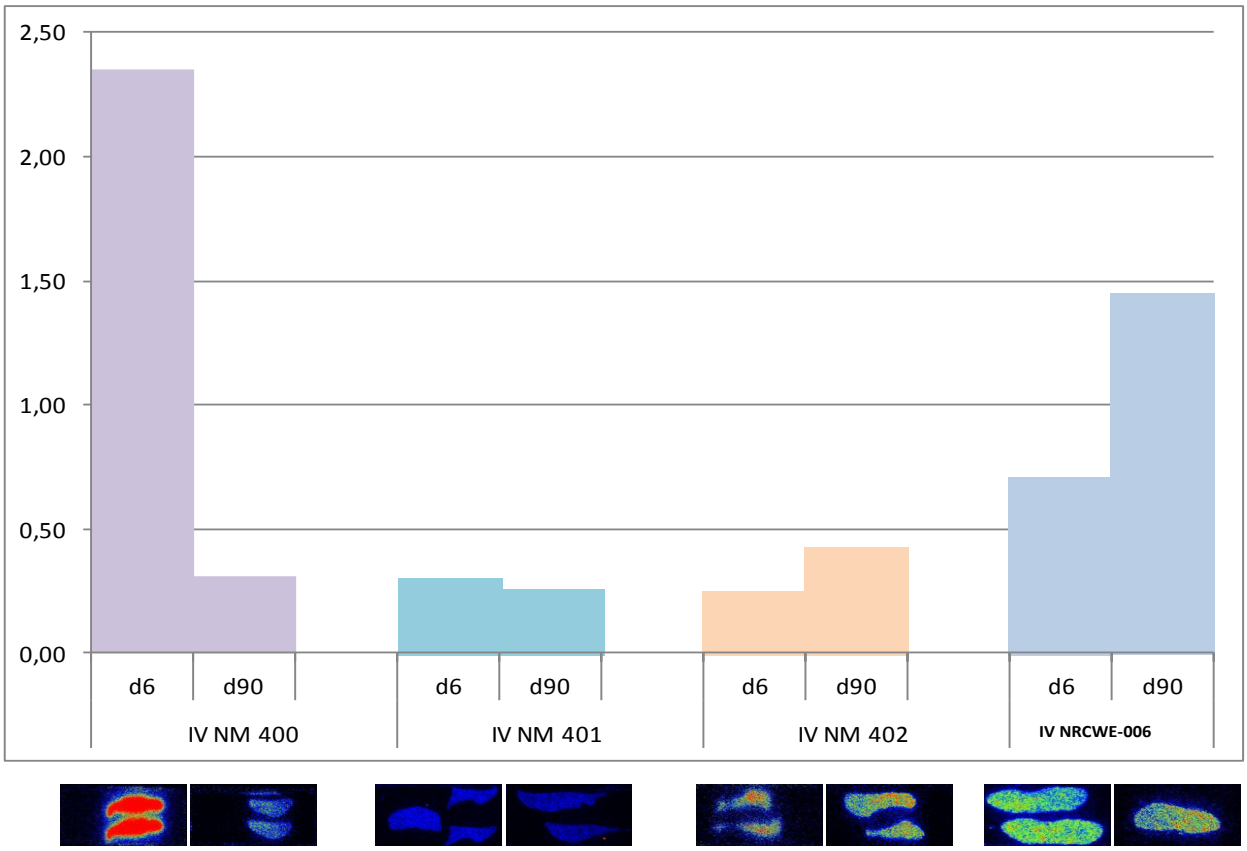


Figure 6-4. ^{14}C levels in tissues as percentage of the injected dose as indicator for MWCNT presence in the spleen after repeated MWCNT administration.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

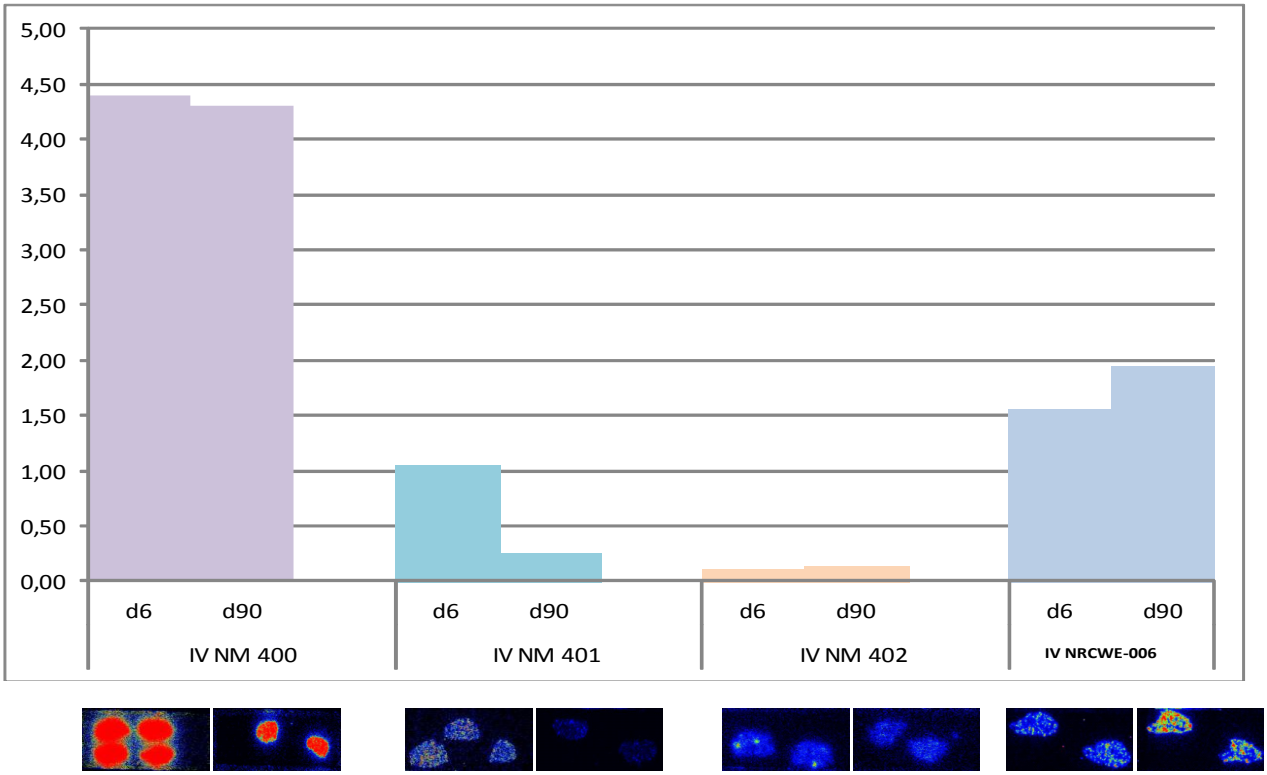


Figure 6-5. ^{14}C levels in tissues as percentage of the injected dose as indicator for MWCNT presence in the lung after repeated MWCNT administration.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

7. Conclusions and discussion

For all manufactured nanomaterials (MNs) investigated the oral administration resulted in a rather low uptake via the gastro intestinal tract even after repeated oral administration. The infrequent and incidental demonstration of some Ti in tissues beyond the GI-tract may indicate that for some individuals, uptake may be possible. However, it was also demonstrated that the faeces of control rats already contained an amount of Ti well above the Limit of Detection. As this level was for all animals investigated in the same order of magnitude it could be concluded that after the intravenous administration of TiO_2 nanomaterials there is no excretion into the GI-tract. For SAS the levels determined in liver and spleen as indicator organs for systemic uptake were similar to control levels or below the Limit of Detection indicating a very low absorption from the GI-tract. The organ levels after repeated oral administration suggest minor differences in either between male and female animals and the MN material (NM-200 and NM-203) investigated. For MWCNTs, translocation of the MWCNTs from the GI-tract into the systemic circulation or any of the organs investigated was not demonstrated.

For the single and repeated intravenous administrations, the results indicate that the TiO_2 investigated nanomaterials can remain in the body for a prolonged period of time, the exception being NM-105 anatase-rutile mixture with a size between 15-25 nm. For TiO_2 redistribution was noted for the spleen with at day 90 higher spleen levels (in µg/g organ) compared to the liver. However, the liver remains the organ with the highest uptake in view of its size. It should be considered that not only the levels expressed as percentage of the dose are important in the tissue distribution of nanomaterials. The levels expressed as µg/g organ can give a direct indication of the possible exposure of the cells within the organ and might be indicative for the induction of toxicity.

For the SAS, and most MWCNT nanomaterials a decrease in recovery was observed between day 2/day6 and day 90 after the repeated and single dosing indicating a clearance from the body. Major organs for the distribution of the nanomaterials are liver, spleen and lung and to a lesser extent the kidney. For SAS, following single dose IV administration, measurable concentrations slightly above the Limit of Quantification of Si were detected in the liver of male and female rats up until 90 days after administration. After repeated IV dose administration, a considerable concentration of Si is present in liver and spleen at day 6, with some particle- and gender-related differences regarding distributions to spleen and liver, while detectable concentrations are found in other organs as well. Si concentrations in liver and spleen of males remain high at day 14 and 30. At day 90, Si concentrations in spleen

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

and liver were still distinctly higher than in controls, both for male and female rats, and for both NM-200 and NM-203 SAS nanomaterials, suggesting that a longer time period is required for complete elimination of administered MNs from the body.

The four investigated MWCNTs showed a clear difference in their tissue distribution and tissue clearance. NM-401 and NM-402 showed much less distribution to the organs when compared with NM-400 and NRCWE-006. Whether this has an impact on possible toxicity is yet unknown. Each type of MWCNT was found to display particular bioaccumulation and biopersistence properties in the various organs evaluated. The data suggest for all four MWCNT investigated (NM-400, NM-401, NM-402, and NRCWE-006) some biopersistence of these MWCNT in the main organs beyond 3 months after administration.

After the intravenous administration most nanomaterials showed a rapid clearance (< 30 minutes) from the blood indicating a quick distribution to and uptake by the various organs.

In conclusion, for all TiO_2 and SAS manufactured nanomaterials investigated, the oral administration resulted in a rather low uptake via the GI-tract after repeated oral administration, whereas for MWCNTs no uptake from the GI-tract could be demonstrated. For the single and repeated IV administrations the results indicate that especially some TiO_2 and MWCNT nanomaterials are still present in the organs at day 90 after the last administration. For SAS in general a decrease in Si level was noted between day2/day 6 and day 90, although at day 90 Si could still be detected. Major organs for the biodistribution of the nanomaterials are liver, spleen and lung and to a limited extent the kidney. Although the IV administration can be considered an artificial route of exposure the results obtained clearly demonstrate that some nanomaterials can persist in organs for a prolonged period of time until at least 90 days, the last time point investigated in these studies.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

8. References

Aureli F, D'Amato M, De Berardis B, Raggi A, Turco AC, Cubadda F. Investigating agglomeration and dissolution of silica nanoparticles in aqueous suspensions by dynamic reaction cell inductively coupled plasma-mass spectrometry in time resolved mode. Journal of Analytical Atomic Spectrometry 2012;27(9):1540-1548.

Baek M, Chung H-E, Yu J, Lee J-A, Kim T-H, Oh J-M, Lee W-J, Paek S-M, Lee JK, Jeong J, Choy J-H, Choi S-J. Pharmacokinetics, tissue distribution, and excretion of zinc oxide nanoparticles. Int J Nanomedicine 7, 3081-3097, 2012.

Brandon EFA, Baars AJ, Te Biesebeek JD, Oomen AG, Bakker MI, De Heer C. Risk assessment of patulin intake from apple containing products by young children. World Mycotoxin Journal, 5, 391-403, 2012.

Enck P, Merlin V, Erckenbrecht JF, Wienbeck M. Stress effects on the gastrointestinal transit in the rat. Gut 30,455-459, 1989.

Eurachem. Eurachem Guide. The fitness for purpose of analytical methods. A laboratory guide to method validation and related topics. Eurachem, LGC, Teddington, UK, 1998. http://www.eurachem.org/index.php/publications/guides/mv

Flemming SE, Lee B. Growth performance and intestinal transit time of rats fed purified and natural dietary fibers. J Nutrition 113, 592-601, 1983.

Georgin D, Czarny B, Botquin M, Mayne-L'Hermite M, Pinault M, Bouchet-Fabre B, Carriere M, Poncy J-L, Chau Q, Maximilien R, Dive V, Taran F. Preparation of 14C-labeled multiwalled carbon nanotubes for biodistribution investigations J. Am. Chem. Soc., 131, 14658-14659, 2009.

Wang J, Zhou G, Chen C, Yu H, Wang T, Ma Y, Jia G, Gao Y, Li B, Sun J, Li Y, Jiao F, Zhao Y, Chai Z. Acute toxicity and biodistribution of different seized titanium dioxide particles in mice after oral administration. Toxicol Lett 168, 176-185, 2007.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

9. ANNEX 1. Quality control of dispersion used in the toxicokinetic studies

A full characterization of these nanomaterials is presented in the reports of WP4. For the in vivo studies to the tissue distribution of the nanomaterials the nanomaterials were only evaluated for their size distribution as an indication for the quality of the used dispersions.

1. Methods

1.1 TiO_2 dispersions

1.1.1 TiO_2 dispersion used for oral exposure

The hydrodynamic particle number/size distribution of the particles in the exposure liquids were analysed by photon correlation spectroscopy using a Dynamic laser scattering DLS Zetasizer nano ZS (Malvern Inc., UK.). The number distributions were calculated by the DTS software using the viscosity for H_2O (0.6864), temperature of 25°C and material refractive (R_i) and absorption indices (R_s) for TiO2 (Anatase: R_i 2.49; R_s 0.10 and rutile: R_i 2.90; R_s 0.10). NM-105 contains 85% anatase and was analysed as anatase. Vehicle controls were analysed using both settings. Scans were performed on all samples to determine optimal measure parameters. A laser attenuation factor of 2 and measurement position of 0.45 mm was optimal for all TiO2 containing samples. It was not possible to find optimal parameters for samples without TiO_2 particles (Vehicle control). These were analysed using a laser attenuation factor of 7 and a measurement position of 4.65 mm. All samples were analysed within 30 min after sonication, i.e. within the same time frame as the oral exposures occurred. Data quality was analysed by evaluating the intensity correllelogram, cumulants fit and the distribution fit of the laser scattering intensity data.

1.1.2. TiO_2 dispersion used for IV exposure

The size distribution of the TiO_2 particles (NM-100, NM-102, NM-103and NM-104) were determined directly after preparing the suspensions for IV administration in at least 3 separate measurements using tracking analysis of Brownian motion with a laser illuminated microscopical technique (LM20, NanoSight Ltd, UK).

For NM-105 DLS was used for evaluation of the dispersion quality. The particle size distribution in the NM-105 dispersions was determined at 633 nm and 90° with a Dynamic Laser Scatter Bi-90plus equipped with a correlator and avalanche photo detector (APD) (Brookhaven Instruments Corporation, US). Data were analyzed using the BIC Particle sizing Software, vs. 5.23. Samples were measured at 23°C in 4 mL disposable polystyrene cuvettes. For calculations of hydrodynamic size, we used the refractive index (Ri) of 2.20, for TiO2 and standard properties for H2O and solvents. The measurements were performed in triplicate (one run consists of three cycles of 2 min).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

1.2 SAS (SiO_2) dispersion

The quality of the NM-200 and NM-203 dispersions was evaluated using asymmetric flow field flow fractionation with optical (MALS and UV) and ICP-MS detection. Furthermore, an entirely novel method based on dynamic reaction cell inductively coupled plasma-mass spectrometry (DRC-ICP-MS) in time resolved mode has been developed for studying the agglomeration behaviour of the dispersed nanomaterials (Aureli et al. 2012). Since this method is more rapid than AF4-MALS-ICP-MS, it was used on a daily basis for a quick check of the quality of the dispersion after initial in-depth characterization of the two SAS NMs by AF4-MALS-ICP-MS. In all these determinations, the reference material ERM-FD 100 ‘‘colloidal silica in water’’ (IRMM, Geel, Belgium) was used as size calibrant.

1.3 MWCNT dispersion

The MWCNT dispersion used in the tissue distribution studies were evaluated by visual inspection and transmission electron microscopy.

2. Results

2.1 Quality of TiO_2 dispersions used for oral administration

The number/size distribution of the suspended TiO_2 particle preparations used for oral gavage was characterized by dynamic light scattering (DLS). Analysis of the vehicle solution was challenging, and the DTS software could not establish optimal measuring parameters. The count rate and the results (Z-average, intensity) varied highly between each of the 12 analysis, leading to a high Polydisperty Index and unreliable DLS data. Analysing the vehicle for rutile or anatase TiO_2 did not influence the results.

All TiO_2 containing suspensions yielded reliable results according to the DTS software (Table 1). The majority of particles occurred as agglomerates between 80–150 nm (peak number distributions, Figure 1). Suspensions of NM-103, NM-104 and NM-105 contained the smallest agglomerates (80-90 nm) whereas the largest agglomerates were observed with NM-101 and NM-102 (140-150 nm). NM-101 and NM-102 additionally showed a bi-modal size-distribution with another much less frequent mode of ~1 um. No agglomerates were observed between 3 µm and 10 µm (the upper limit of the Zetasizer nano ZS) for any of the materials.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 1. Z-average (intensity distribution peak) and polydispersity index (PdI) of the TiO2 suspensions used for oral gavage. Results are the mean of 12 analyses conducted on two separate days.

Material	Conc.	Vehicle	Z-Average (intensity)	PdI
NM-101	2.56	EtOH/RSA/PBS	278 nm	0.323
NM-102	2.56	EtOH/RSA/PBS	367 nm	0.290
NM-103	2.56	EtOH/RSA/PBS	127 nm	0.384
NM-104	2.56	EtOH/RSA/PBS	108 nm	0.274
NM-105	2.56	EtOH/RSA/PBS	93 nm	0.151
Control	0.00	EtOH/RSA/PBS	477 nm	0.817

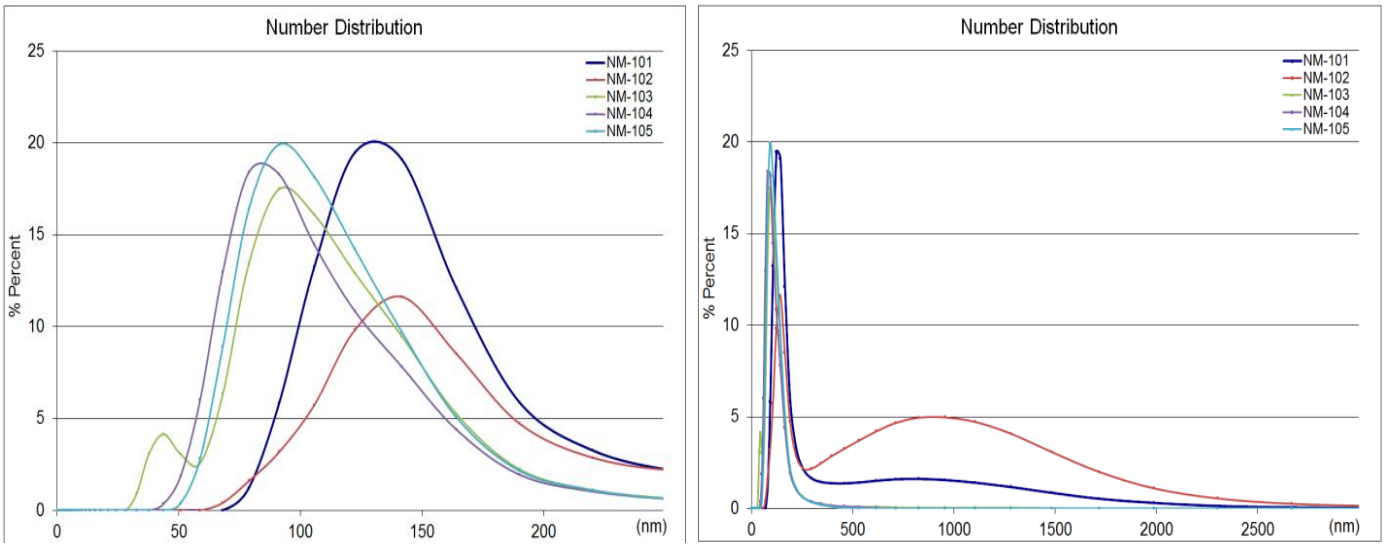


Figure 1. Hydrodynamic number/size distribution of NM-101-NM-105 (left and right). The majority of particles were observed as agglomerates between 50-200 nm. Suspensions of NM-101 and NM-102 contained an additional mode between 250 and 2500 nm. No agglomerates were observed above 3 µm. Results are the mean of 12 analyses conducted on two separate days.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Co-funded by the Health Programme of the European Union

www.nanogenotox.eu

2.2 Quality of TiO_2 dispersions used for IV administration

2.2.1 NanoSight evaluation of NM-100, NM-102, NM-103 and NM-104

Particle tracking with the NanoSight equipment was performed for NM-100, NM-102, NM-103 and NM-104. The evaluation was performed just after the dispersions were prepared. Representative figures are presented for NM-100 and NM-102 (Figures 2 and 3, respectively). For NM-100 one single peak was observed at the nominal size of the particle diameter (200-220 nm). For NM-102 different peaks could be identified indicating the occurrence of agglomeration/aggregation of the individual nanoparticles into larger clusters of nanoparticles. Aggregates of nanoparticles with a size of approximately 60 nm, 100 nm, 160 nm, and 250 nm could be identified by the observed peaks (Figure 3).

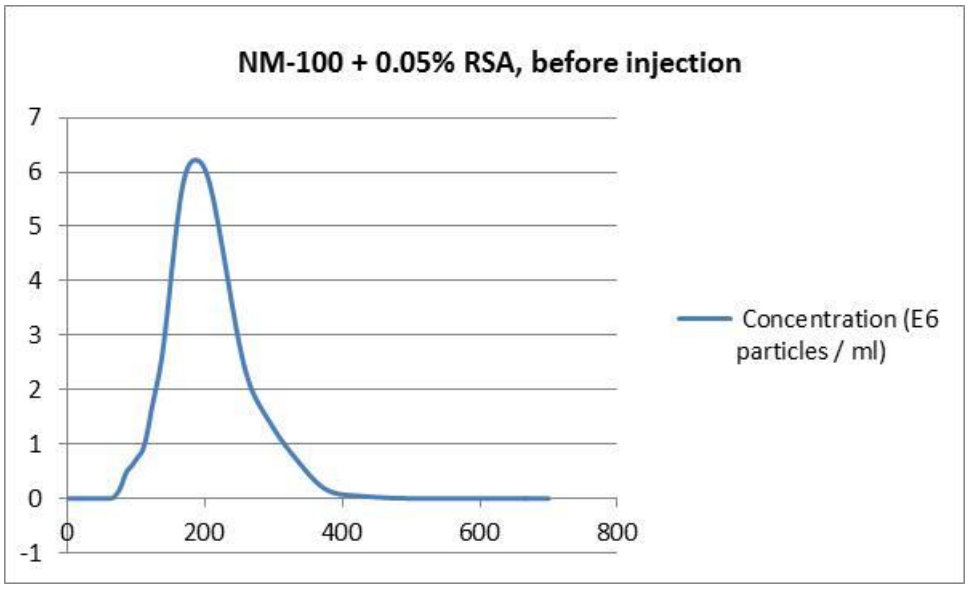


Figure 2. Particle size distribution of NM-100 as determined with the NanoSight particle tracking equipment. Mean of 3 independent measurements.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

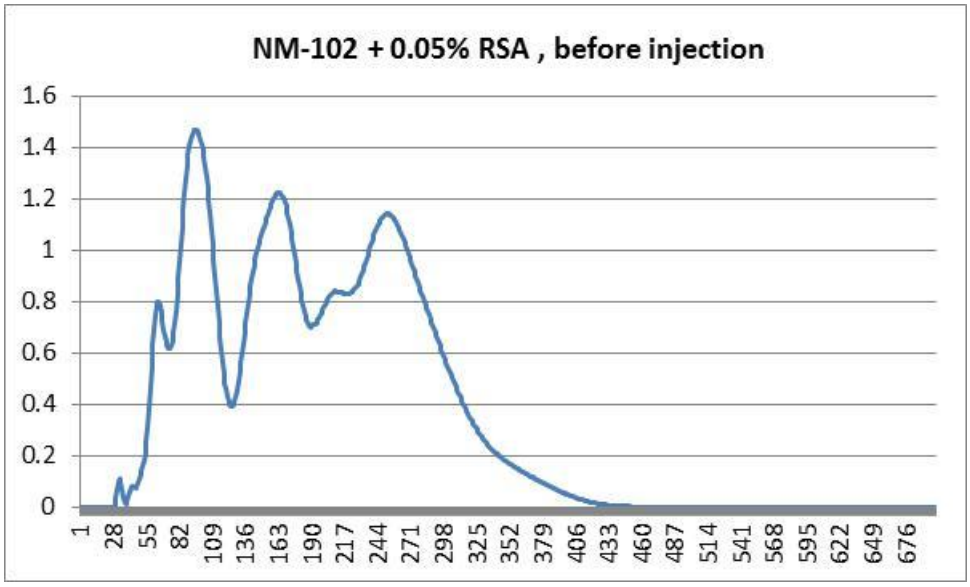


Figure 3. Particle size distribution of NM-102 as determined with the NanoSight particle tracking equipment. Mean of 3 independent measurements.

2.2.2 DLS evaluation of NM-105

DLS evaluation of NM-105 revealed a good dispersion quality of the stock solution used for preparing the final solutions (Figure 4). When the solution was diluted with 10x concentrated phosphate buffer used for IV administration clearly two peaks were observed indicating the presence of agglomerated particles. Based on number distribution the majority of the agglomerates had a mean diameter of approximately 60 nm (Figure 5). When determined after 20 minutes of preparation of the dilution the mean diameter of aggregates increased four times (Figure 6). The data of the measurements are presented in Table 2.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

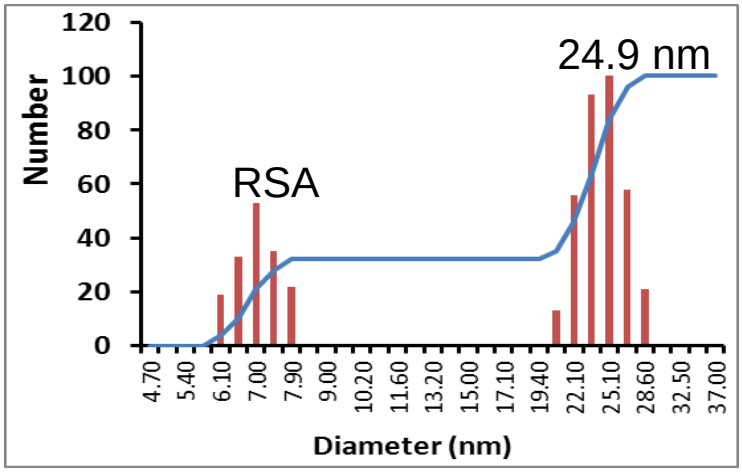


Figure 4 Multimodal size distribution results of NM-105 stock solution (2.56 mg/ml).

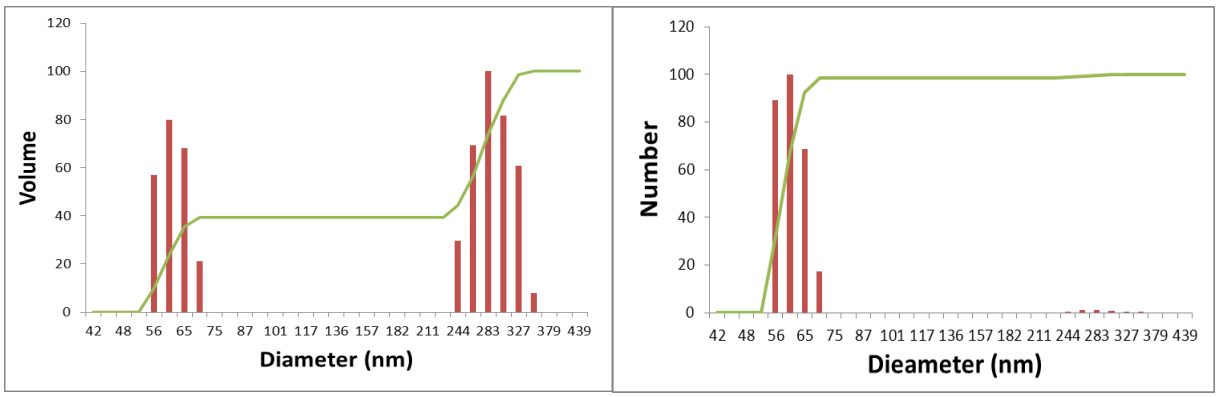


Figure 5 DLS results of NM-105 solution after dilution with 10x concentrated phosphate buffer.

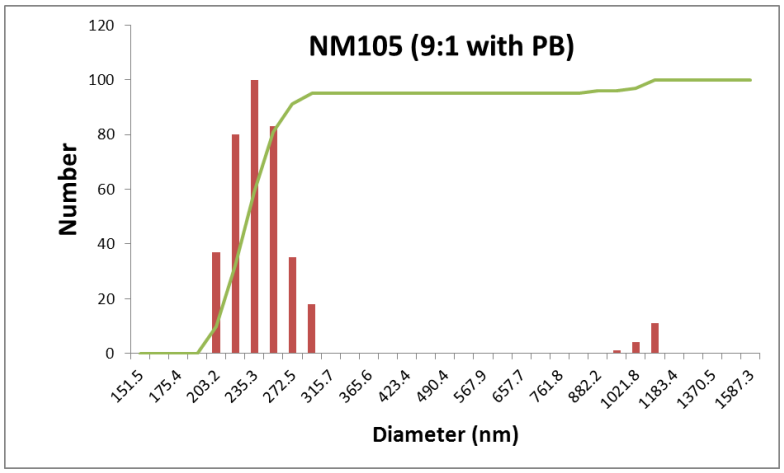


Figure 6 DLS results of NM-105 20 minutes after preparation of solution with phosphate buffer.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Table 2. Results of DLS measurements of NM-105, 20 minutes after preparation of the solution with phosphate buffer.

Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	234.8	85.1	0.131	9.9/ 98.89%
2	249.5	102.3	0.168	6.8/ 98.23%
3	263.5	116.7	0.196	9.0/ 95.57%
Mean	249.3	101.4	0.165	8.5/ 97.56%
Std.Error	8.3	9.1	0.019	0.9/ 1.02
Combined	248.9	102.1	0.168	9.3/ 97.56%

2.3 Quality of SAS (SiO_2) dispersions

A representative example of the results of the evaluation of the SAS dispersions is presented in Figure 7. A bimodal size distribution was observed with a first peak indicating the presence of the primary nanoparticles, and a second peak indicating the presence of agglomerates.

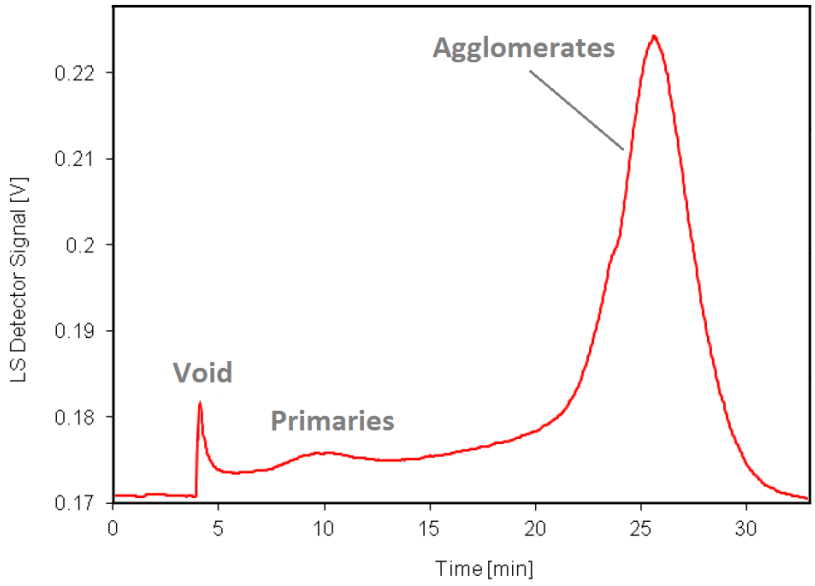


Figure 7. AF4-MALS fractogram for NM-203. The first peak (RT~10 min) corresponds to primaries (~20 nm diameter) whereas the second peak (RT~25 min) corresponds to agglomerates (~200-300 nm diameter).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

2.4 Quality of MWCNT dispersions

Optical microscopy of dispersed MWCNT solutions was performed just before iv injection to animal to check for the absence of bundles (> 20-50 µm). Figure 8 shows a representative example of such evaluation. Additional characterization by electronic microscopy (TEM) was also performed in order to get information on the diameter size of the MWCNT (Figure 9).

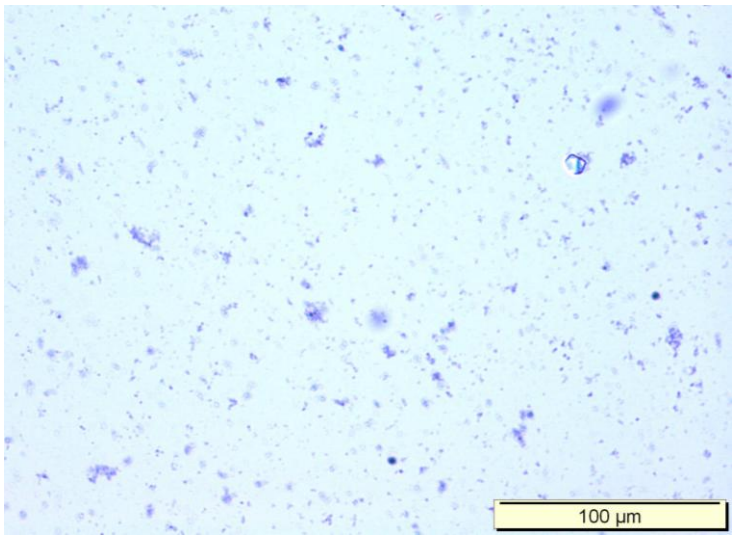


Figure 8. Example of light microscopy of MWCNT preparation before injection.

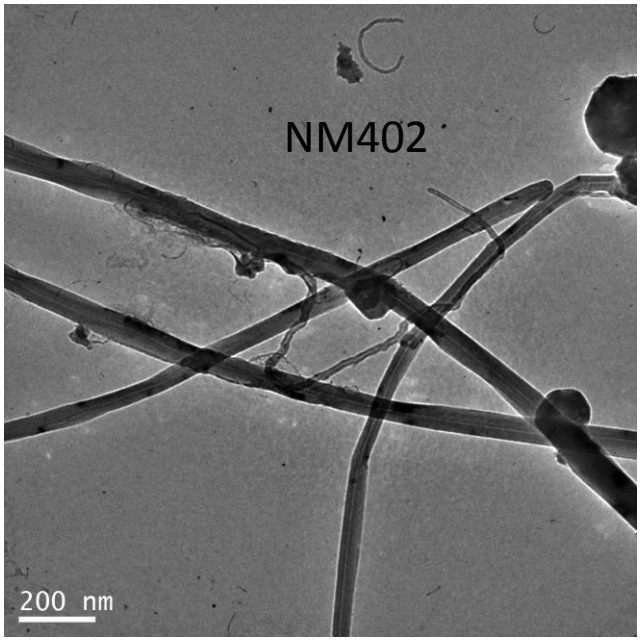


Figure 9 Representative photograph of TEM of NM-402.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.
www.nanogenotox.eu
Co-funded by the Health Programme of the European Union

10. ANNEX 2. Additional methods evaluated for Ti determination

At IMB-BAS four spectrometric techniques were tested for accurate and reliable measurement of Ti in sample after digestion: electrothermal atomic absorption spectrometry (ETAAS) for low Ti levels and flame AAS for high Ti levels, ICP-OES and ICP-MS. Instrumental parameters for all studied methods were optimized according to the manufacturers' recommendations.

Atomic Absorption Spectrometry (AAS)

ETAAS could be used for the determination of Ti in narrow concentration range using pyrolitically coated graphite tubes (pretreatment temperature 1200 °C, atomization temperature 2650 °C). Unfortunately even for highest atomization temperature signal tailing was observed combined with strong memory effect. Sensitive flame AAS measurement could be achieved only by using N_2O/acetylene flame, however, only for Ti concentrations above 10 mg/L. Overall it was concluded that AAS is not useful technique for Ti determination in biological samples.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

ICP-MS (Thermo Scientific, XSeries 2) was used for the determination of titanium concentration in sample solutions (optimal instrumental parameters are presented in Table 1).

Table 1. Optimal instrumental parameters for ICP-MS measurements.

Spectrometer:	**Thermo Scientific, XSeries 2**
Parameters:	
Power	1.4 kW
Argon flow:	
Cooling	17 l/min
Auxiliary	0.81 l/min
Carrier	0.73 l/min
Nebulizer	concentric
Sample flow rate	0.1 ml/min
Integration time	0.3 s
Replicates	3
Isotopes Ti	^{47}Ti, ^{48}Ti, ^{49}Ti, ^{50}Ti

Studies were performed for interfering elements (Ca, Cr, V and multielement mixture) and for the influence of biological matrices on Ti determination. Liver samples were spiked with known amounts of TiO_2 and measured by using all four Ti istopes as indicated in Table 1 Results obtained were also compared with measurements by ICP-OES. As can be seen from results presented in Table 2, the best Ti isotopes that could be used for liver samples are isotopes ^{49}Ti and ^{50}Ti. It is worth to mention that such experiments should be performed also

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

for other biological samples e.g. blood, spleen and other organs. Parallel measurements by ICP-OES and ICP-MS showed in some cases and some biological matrices statistically different results. The Ti measurement might be improved by using either an ICP-MS measurement in combination with a collision cell or a HR-ICP-MS. For use in the toxicokinetic study the ICP-OES was adopted as instrumental method.

Table 2. Recoveries from model experiments using ICP-MS for Ti determination.

	Added Ti (TiO2), µg/L	**Measured Ti / Added Ti (%)**			
		^{47}Ti	**^{48}Ti**	**^{49}Ti**	**^{50}Ti**
Microwave digestion, TiO_2 (HNO_3 +HF)	20	105%	130%	**102%**	103%
	110	102%	121%	**101%**	102%
	1100	104%	106%	**101%**	102%
Microwave digestion, TiO_2, liver, (HNO_3 +HF)*	20	1500%	280%	**108%**	106%
	110	1300%	120%	**106%**	103%
	1100	179%	108%	**102%**	102%

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by the Health Programme of the European Union

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Co-funded by
the Health Programme
of the European Union

• Contact •

Website:
www.nanogenotox.eu

E-mail:
nanogenotox@anses.fr

Coordinator: French Agency for Food, Environmental and Occupational Health & Safety (ANSES)

27-31, avenue du Général Leclerc
94701 Maisons-Alfort Cedex
France

anses
French agency for food, environmental and occupational health & safety

Partners

French Agency for Food, Environmental and Occupational Health Safety (France)	ANSES
Federal Institute of Risk Assessment (Germany)	BfR
French Atomic Energy Commission (France)	CEA
Institute of Mineralogy and Crystallography (Bulgaria)	IMC-BAS
Veterinary and Agrochemical Research Centre (Belgium)	CODA-CERVA
Finnish Institute of Occupational Health (Finland)	FIOH
Roumen Tsanev Institute of Molecular Biology Academy of Sciences (Bulgaria)	IMB-BAS
Institut national de recherche et de sécurité (France)	INRS
National Health Institute Doutor Ricardo Jorge (Portugal)	INSA
Scientific Institute of Public Health (Belgium)	IPH
Institut Pasteur of Lille (France)	IPL
Istituto superiore di sanità (Italy)	ISS
The Nofer institute of Occupational Medicine (Poland)	NIOM
National Research Centre for the Working Environment (Denmark)	NRCWE
National Institute for Public Health and the Environment (The Netherlands)	RIVM
Universitat Autònoma de Barcelona (Spain)	UAB

This document arises from the NANOGENOTOX Joint Action which has received funding from the European Union, in the framework of the Health Programme under Grant Agreement n°2009 21.
This publication reflects only the author's views and the Community is not liable for any use that may be made of the information contained therein.

Co-funded by the Health Programme of the European Union

NANOGENOTOX **Grant Agreement n° 2009 21 01**