
**Nanotechnologies — Lung burden
mass measurement of nanomaterials
for inhalation toxicity tests**

*Nanotechnologies — Mesure de la masse de la charge pulmonaire des
nanomatériaux pour les études de toxicité par inhalation*

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Foreword

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Introduction

Inhalation is a primary route of exposure to aerosolized nanomaterials and therefore appropriate inhalation toxicity tests are required to address risk assessment needs for these materials. For this reason, the Organisation for Economic Cooperation and Development (OECD) recently updated its inhalation toxicity test guidelines 412 (subacute) and 413 (subchronic) to make them applicable to nanomaterials.^{[1][2]} These revised test guidelines require post-exposure lung burden measurements to be undertaken when a range-finding study or other relevant information suggests that inhaled test nanomaterials are poorly soluble with low dissolution rate and likely to be retained in the lung. The measurements of lung burden inform on pulmonary deposition and retention of nanomaterials in the lung. At least three lung burden measurements are needed to evaluate clearance kinetics.

This document gives information on how to derive clearance kinetic parameter values using lung burden measurement data. This document complements OECD TG 412^[1] and OECD TG 413^[2]. As References [1], [2] and [3] only provide limited information on methods for lung burden measurement for nanomaterials or the derivation of lung clearance kinetics, this document provides useful supporting information for conducting inhalation studies based on OECD TG 412^[1] and OECD TG 413^[2].

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Nanotechnologies — Lung burden mass measurement of nanomaterials for inhalation toxicity tests

1 Scope

The document provides information on the measurement of nanomaterial mass in tissue after inhalation exposure, which can inform on lung clearance behaviour and translocation.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 80004 (all parts), *Nanotechnologies – Vocabulary*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in the ISO 80004 series and the following apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

3.1

aerodynamic diameter

diameter of a spherical particle with a density of 1 000 kg/m³ that has the same settling velocity as the particle under consideration

Note 1 to entry: Aerodynamic diameter is related to the inertial properties of *aerosol* (3.2) particles and is generally used to describe particles larger than approximately 100 nm.

[SOURCE: ISO/TR 27628:2007, 2.2^[4]]

3.2

aerosol

metastable suspension of solid or liquid particles in a gas

[SOURCE: ISO/TR 27628:2007, 2.3^[4]]

3.3

mass median aerodynamic diameter

MMAD

calculated *aerodynamic diameter* (3.1) which divides the particles of a measured *aerosol* (3.2) distribution in half based on the mass of the particles where fifty percent of the particles by mass will be larger than the median diameter and fifty per cent of the particles will be smaller than the median

[SOURCE: EPA IRIS Glossary^[11]]

3.4

manufactured nanomaterial

nanomaterial (3.8) intentionally produced for commercial purposes to have selected properties or composition

[SOURCE: ISO 80004-1:2023, 3.1.9, modified — "for commercial purposes" has been added to the definition.]

3.5

mixture

mixture composed of two or more substances in which they do not react

Note 1 to entry: A solution is a mixture as well.

[SOURCE: GHS, 2011^[8]]

3.6

mobility

propensity for an *aerosol* (3.2) particle to move in response to an external influence, such as an electrostatic field, thermal field or by diffusion

[SOURCE: ISO/TR 27628:2007, 2.9^[4], modified — the domain "<aerosols>" has been removed.]

3.7

nanofibre

nano-object with two similar external dimensions in the nanoscale and the third dimension significantly larger

Note 1 to entry: A nanofibre can be flexible or rigid.

Note 2 to entry: The two similar external dimensions are considered to differ in size by less than three times and the significantly larger external dimension is considered to differ from the other two by more than three times.

Note 3 to entry: The largest external dimension is not necessarily in the nanoscale.

[SOURCE: ISO 80004-1:2023, 3.3.5, modified — Notes 1 and 2 to entry have been added.]

3.8

nanomaterial

material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale

Note 1 to entry: This generic term is inclusive of *nano-object* and nanostructured material.

Note 2 to entry: See also engineered nanomaterial, manufactured nanomaterial and incidental nanomaterial.

[SOURCE: ISO 80004-1:2023, 3.1.4, modified — Note 1 to entry has been replaced.]

3.9

nanoparticle

nano-object with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly

Note 1 to entry: If the dimensions differ significantly (typically by more than 3 times), terms such as *nanofibre* (3.7) or *nanoplate* may be preferred to the term nanoparticle.

Note 2 to entry: Ultrafine particles may be nanoparticles.

[SOURCE: ISO 80004-1:2023, 3.3.4, modified — "where the lengths of the longest and the shortest axes of the nano-object do not differ significantly" has been added to the definition and Note 2 to entry has been added.]

3.10**nanotube**

hollow *nanofibre* ([3.7](#))

[SOURCE: ISO 80004-1:2015, 3.3.8]

3.11**single-walled carbon nanotube****SWCNT**

SWCNT single-walled carbon nanotube consisting of a single cylindrical graphene layer

Note 1 to entry: The structure can be visualized as a graphene sheet rolled into a cylindrical honeycomb structure.

3.12**multi-wall carbon nanotube****MWCNT**

MWCNT multi-walled carbon nanotube composed of nested, concentric or near-concentric graphene sheets with interlayer distances similar to those of graphite

Note 1 to entry: The structure is normally considered to be many *single-walled carbon nanotubes* ([3.11](#)) nesting each other, and would be cylindrical for small diameters but tends to have a polygonal cross-section as the diameter increases.

3.13**particle**

minute piece of matter with defined physical boundaries

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general definition applies to particle *nano-objects*.

[SOURCE: ISO 26824:2013, 3.1.1^[5]]

3.14**poorly soluble particle**

inhaled test particles that are likely to be retained in the lung

[SOURCE: OECD TG 412, paragraph 2^[1]]

3.15**primary particle**

original source particle of *agglomerates* or *aggregates* or mixtures of the two

Note 1 to entry: Constituent particles of agglomerates or aggregates at a certain actual state may be primary particles, but often the constituents are aggregates.

Note 2 to entry: Agglomerates and aggregates are also termed secondary particles.

[SOURCE: ISO 26824:2022, 3.1.4^[5]]

3.16**secondary particle**

particle formed through chemical reactions in the gas phase (gas to particle conversion)

[SOURCE: ISO/TR 27628:2007, 2.17^[4]]

4 Abbreviated terms

AAS	Atomic absorption spectrometry
AgNP	Silver nanoparticles
AuNP	Gold nanoparticles
BALF	Bronchoalveolar lavage fluid
CoO	Cobalt oxide
CuO	Copper oxide
DEMC	Differential electrical mobility classifier
DEMS	Differential electrical mobility spectrometer
ECA	Elemental carbon analysis
GD	Guidance document
GHS	Globally harmonized system
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
LALN	Lung-associated lymph node
MMAD	Mass median aerodynamic diameter
NDIR	Non-dispersive infrared
OECD	Organisation for Economic Cooperation and Development
PEO	Post-exposure observation
sp-ICP-MS	Single particle ICP-MS
TG	Test guideline
TiO ₂	Titanium dioxide
UV-Vis	Ultraviolet-visible spectrometry
WPMN	Working Party on Manufactured Nanomaterials
ZnO	Zinc oxide

5 Use of lung burden measurements for risk assessment of nanomaterials

The concept of lung overload hypothesis was first proposed in Reference [13]. The determination of lung burden of inhaled nanomaterials is therefore of great relevance to assess a possible lung overload.[14] [15] Morrow[13] has also proposed that a continuously increasing prolongation of particle lung clearance occurs when the retained lung burden exceeds a certain threshold. Decreased clearance capacity of alveolar macrophages will lead to inflammatory reactions and to an increase in the translocation of the inhaled particles to interstitium and lung-associated lymph nodes[16].

Lung burden data can be used for the risk assessment of poorly soluble with low dissolution rate particles (e.g. as obtained from tests according to References [1] and [2]). When pulmonary effects are

driving the human health risk assessment, risk assessors need to evaluate whether the occurrence of the pulmonary effects is better characterized by administered exposure concentration or by retained dose in the lungs. The human equivalent dose and lifetime human exposure may be calculated for risk estimation. Applications of such principles are available in literature, e.g. References [17] and [18]. Another value of lung burden data is the possibility of reading across hazard data from studies using the same material with different primary particle sizes.^[19] The same external concentrations can result in differences in retained dose. Conversely, different external concentrations can result in the same retained dose for different particle sizes^[20].

6 Inhalation exposure and tissue sampling to determine lung burden

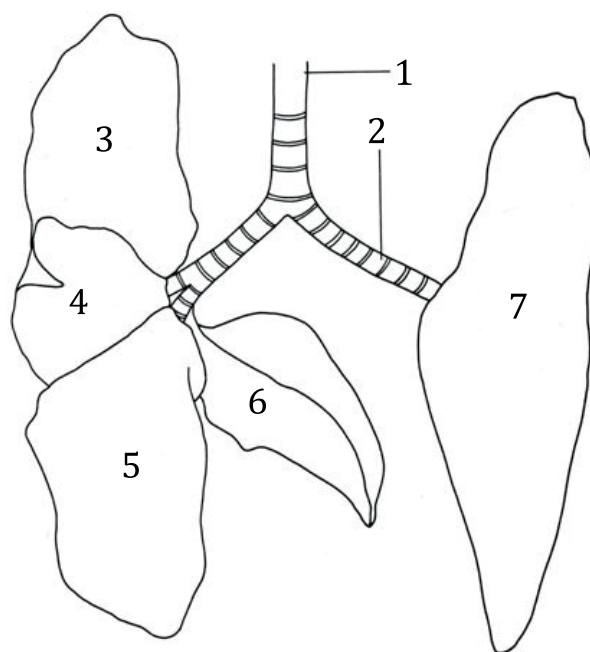
6.1 Inhalation exposure

For inhalation exposure of nanomaterials, nanomaterials are frequently generated in situ or powdered forms of nanomaterials are dispersed and generated and delivered into the inhalation chamber. The generation of nanomaterials aerosol for inhalation toxicity testing is described in ISO/TR 19601^[21] and ISO 10801^[22], and monitoring of such aerosols in the inhalation chamber is described in ISO 10808^[10]. References [10], [21] and [22] also provide methods of aerosol concentration monitoring and physicochemical characterization as well as OECD test guidelines.

6.2 Lung burden evaluation in single or multiple lobes

For inhalation toxicity testing of nanomaterials, please refer to References [1] and [2]. Depending on the type of nanomaterial, the study director can use data from a range-finding study to determine the appropriate post-exposure duration as well as the optimal number and timing of sampling intervals for a repeated exposure inhalation study. Although the TGs require using one lung (right lung) for lung burden measurement and the other lung (left lung) for histopathological evaluation, recent studies with AgNPs and AuNPs demonstrated that nanoparticles deposit in the rat lung lobes evenly, thus, any lobe can be used for lung burden measurement.^{[23][24]} As shown in Figure 1, the right lung lobe consisted of four lobes. Soluble nanoparticles with high dissolution rate such as silver nanoparticles^{[23][25]} as well as poorly soluble with low dissolution rate particles such as gold nanoparticles^[24] were evenly deposited in rat lungs after subacute (5 d) inhalation exposure. Using any lobe for lung burden measurements opens the opportunity to use the remaining lobes for other measurements, such as histopathological tissue preparation and BALF assay, in the same rat. Such an approach can maximize the number of endpoints measured and has the potential to reduce the number of animals used in testing. Although fibrous or plate forms of nanomaterials such as carbon nanotubes and graphenes were not tested and proven for even deposition throughout the lung lobes, some lung burden and thereafter lung clearance kinetic study has been conducted for carbon nanotubes.

A recent study given in Reference [26] on the lung deposition and retention of multi-walled carbon nanotubes (MWCNTs) [where the mass median aerodynamic diameter (MMAD) is 1,015 µm] after 28 d of inhalation and for 28 d post-exposure showed that the lung clearance kinetics of MWCNTs can be effectively evaluated using one lobe from the right lung.^[26] The BAL fluid was collected from the right lung after occluding the post-caval lobe and left lung. The left lung was then used to evaluate the histopathology and the post-caval lobe to evaluate the lung burden.^[24] In another recent study, quantitative analyses of lung burdens on various shapes of carbon nanomaterials including printex-90 carbon black (50 mg/m³), nanomaterial NM-401 (0,5 mg/m³ and 1,5 mg/m³), NM-403 (1,5 mg/m³), and MWCNT-7 (1,5 mg/m³) nanotubes were conducted after 28-d inhalation exposure. Their MMAD was 940 nm for printex-90 carbon black, 790 nm for NM-401, 1 940 nm for NM-403 and 1 780 nm for MWCNT. The middle right lobe was separated and used for lung burden analysis successfully^[27].

**Key**

- 1 trachea
- 2 left bronchus
- 3 superior lobe
- 4 middle lobe
- 5 inferior lobe
- 6 post-caval lobe
- 7 left lung

SOURCE: Reference [3]. Reproduced with the permission of the authors.

Figure 1 — Rodent trachea and lungs

6.3 Post-exposure observation points

Although References [1] and [2] prescribe only one mandatory sampling point [post-exposure observation (PEO)-1], it is recommended to conduct two additional sampling points (PEO-2 and PEO-3) right after the termination of exposure (PEO-1, post-exposure observation) to conduct toxicokinetic or particokinetics studies. The concept of “particokinetics” is introduced to address the dynamic biological behaviour of ENMs at the molecular level (including gravitational sedimentation, dispersion, aggregation and interaction with biomolecules in suspending media), cellular level (including cellular uptake, transport, biotransformation and elimination) and whole-organism level (including absorption, distribution, metabolism and excretion *in vivo*).^{[28]-[32]} In addition, lung burden measurement at exposure d-1 (6-h exposure) can provide information about the solubility of test nanomaterials and the retention trend after the designed exposure period, because lung retention time and biopersistence increases as particles are poorly soluble.

Additional satellite groups can be added to the main study to evaluate recovery, persistence, delayed occurrence of toxicity or lung burden for a post-treatment period of an appropriate length. Designs of main studies with satellite groups are shown in Annexes A and B. The study director should modify the design of a study based on the physicomaterial characteristics and kinetics of a test chemical to achieve the most robust data.

All satellite groups are exposed concurrently with the experimental animals in the main study and at the same concentration levels and there should be concurrent air or vehicle controls as needed. The scheduling and design of satellite groups depend on whether the test chemical is a solid aerosol and is

likely to result in lung retention following [Annexes A](#) and [B](#). If the test chemical is likely to result in lung retention, the main study is conducted as described in option B in [Annex B](#); otherwise, the main study is conducted as described in option A in [Annex A](#) (used for test chemicals as gas, vapour, aerosol or a mixture thereof). Satellite groups can be included to evaluate recovery in option A in [Annex A](#); option B in [Annex B](#) (used when testing chemicals that are likely to be retained in the lungs) provides for satellite groups for the evaluation of recovery and/or for lung burden measurements. Satellite recovery groups at PEO-2 consist of five males and five females per concentration in option A and option B in [Annexes A](#) and [B](#), respectively.

These recovery groups are exposed concurrently with the experimental animals in the main study and at the same concentration levels, and there should be concurrent air or vehicle controls as needed. When testing poorly soluble with low dissolution rate solid aerosols that are likely to be retained in the lungs, one or two additional satellite groups of five males per concentration may be added to measure lung burden at different post-exposure time points (see option B in [Annex B](#)). These additional lung burden measurements (i.e. PEO-2 and/or PEO-3) may be added to the design when the study director would like to understand the post-exposure clearance kinetics of the test substance. Since three-time points are generally required to provide information on clearance kinetics, lung burden measurements are performed within 24 h after exposure termination (PEO-1) and at two additional PEOs (PEO-2 and PEO-3). However, the use of two-time points may provide sufficient information under some circumstances, such as when the main objective is to identify whether clearance is very slow. Lung burden measurements are preferably performed in males, which have a higher minute volume than females and may thus have greater lung burdens. OECD guidelines provide following options.

- The study director can choose to schedule PEO-3 before the recovery group (PEO-2) (if included), if considered more appropriate.
- If the use of two post-exposure time points is considered sufficient, lung burden measurements can be performed at PEO-1 (main study) and at PEO-2 (recovery group) only, if the timing for evaluation of recovery and lung clearance can be aligned to one another. The satellite group at PEO-3 can then be omitted from the study.
- The study director can choose to perform lung burden measurements at PEO-1 (main study) and at PEO-3 (satellite group) and to use both sexes of the recovery groups (PEO-2) for BALF analysis.

Sometimes, for the lung clearance kinetic study, three PEOs can be insufficient to derive the toxicokinetic parameters.

7 Available methods for lung burden measurements

7.1 General

The lung burden of nanomaterials can be evaluated by various methods. In simple terms, these can be divided into:

- a) measurement without digestion of lung tissue, such as radioisotope labelling and direct imaging of particles in intact tissue samples, and
- b) measurement after digestion of lung tissues.

[Annex C](#) summarises the literature on lung burden analysis of nanomaterials. For measuring lung burden after digestion of lung tissue, acids, alkali or protease enzymes are generally used to digest lung tissue but digesting agents should be selected based on the physicochemical properties of nanomaterials. Then, the extracted nanomaterials can be quantified by specific methods or instruments to determine their elemental composition. In this document, the available methods for lung burden measurement are separately described by the types of nanomaterials such as carbon nanomaterials, metal-based nanomaterials, polymeric nanomaterials and others.

7.2 Carbon nanomaterials

As carbon nanomaterials such as carbon black, nanodiamond, graphene, carbon nanotube, and carbon nanofibre are not dissolved in buffers that are used for the lysis of lung tissues, these materials can be extracted from lung tissue by the acids, alkalis, and protease enzymes. However, acids (e.g. hydrochloric acid, nitric acid and sulfuric acid) and alkalis can change the physicochemical properties by inducing defects and oxidation/reduction.^{[33][34]} The modified physicochemical properties of carbon nanomaterials by the treatment of acids and alkalis can induce inaccuracy in instrumental analyses. Recently, the digestion of lung tissue using the proteinase K enzyme has been proposed as an alternative method to digest lung tissues without damaging the structure of carbon nanomaterials.^{[33][35]} The collected carbon nanomaterials can be quantified by the elemental carbon analysis (ECA) with high performance liquid chromatography (HPLC),^{[36][37]} non-dispersive infrared (NDIR) analysis,^[38] near infrared fluorescence imaging,^[39] ultraviolet-visible (UV-Vis) spectrophotometer^{[26][35]} and standard morphometric point counting methods of histology slices.^[40] The ECA is performed using an organic carbon (OC)/ elemental carbon (EC) analyzer based on NIOSH Manual of Analytical Methods (NMAM) 5040.^[41] The UV-Vis spectrophotometer analysis uses a wavelength of the near-infrared region such as 750 nm because biological materials such as haemoglobin and proteins show minimal absorbance in this region.^[35] Notably, the concentration of carbon nanomaterials measured by an organic carbon (OC/EC analysis is an absolute value, while that of UV-Vis spectrophotometer and NDIR analysis is a relative value calculated from the standard curve fit). In addition, the labelling of carbon nanomaterials such as Technetium-99 m (^{99m}Tc) and yttrium-86 (^{86}Y) radioisotopes can be a method for lung burden analysis.^{[42][43]} However, it should be noted that the labelled particles have different physicochemical properties from the pristine particles and the labelling materials can be OC, carbonate (CC) and EC.

7.3 Metal-based nanomaterials

When metal-based nanomaterials are labelled with radioisotopes or have imageable properties such as superparamagnetic iron oxide nanoparticles (SPION), the lung burden can be directly measured from the excised lung without digestion processes. Nanomaterials labelled with radioisotopes such as ^{105}Ag , ^{195}Au , $^{59}\text{Fe}_2\text{O}_3$, and ^{48}V -radiolabelled titanium dioxide (TiO_2) particles were successfully tested for lung burden analysis.^{[28][44][45][46]} In addition, nanomaterials having imageable properties such as SPION particles were quantified by magnetic particle imaging.^[47] However, the labelling method using radioisotopes has the same limitations as described in 7.2. The imaging method also has limitations in that only relative quantification is possible.

Lung burden measurement of metal-based nanomaterials can be divided into two steps: sample preparation and quantification. If a sample preparation procedure is sufficient to dissolve both lung tissue and the nanomaterial of interest (e.g. use of hydrochloric acid, nitric acid and sulfuric acid), the digestate can be filtered or centrifuged then analyzed using an appropriate spectrometry technique such as atomic absorption spectrometry (AAS) or inductively coupled plasma mass spectrometry (ICP-MS). The quantification of nanomaterials by these methods was implemented for cerium oxide, titanium dioxide and aluminum oxide.^{[48]–[54]} The selection of acids and their combinations should depend on whether the digestion buffer can dissolve nanomaterials. The use of alkalis such as solvable® can be applied to digest lung tissue. While some nanomaterials such as copper oxide (CuO), cobalt oxide (CoO), and zinc oxide (ZnO) are dissolved during acid-assisted sample digestion procedures, other nanomaterials such as Au and TiO_2 are not dissolved. Thus, samples with these nanomaterials require pre-treatment to dissolve any tissue followed by digestion to fully dissolve the nanomaterial. Digestates can be filtered or centrifuged followed by analysis using AAS or ICP techniques. AAS was used for aluminum oxide^[55], silver^[56] and gold nanoparticles^[57].

When the concentration of nanomaterials is measured by an ICP instrument from samples of acid digestion or metal-dissolved supernatant after digesting fluid treatment, it is difficult to differentiate whether the measured concentration of metals is from the particulate form of nanomaterials, dissolved ions of nanomaterials or elements from lung tissue. To overcome this limitation, the extraction of metal-based nanomaterials as a particulate form is needed. In recent studies, the use of protease enzymes showed a good efficacy to lysis lung tissue. Although poorly-soluble with low dissolution rate nanomaterials do not dissolve in contact with the protease enzymes, the potential for dissolution of

nanomaterials during the tissue digestion process should be evaluated before lysing the lung tissue. If nanomaterials dissolve in alkalis or by protease enzymes, the use of acids is highly recommended.

The dissolved metal ions can be quantified by an ICP instrument and the measured concentrations can be converted to the concentration of nanomaterials based on the chemical composition. For nanomaterials composing elements that are abundant in the tissue, the subtraction of measured concentration with vehicle control is needed and perfusion is highly recommended to exclude the effect of components in blood. As some metals such as silica are evaporated during the acid digestion process, the availability of metals to this application should be tested before the experiment.^[58] The collection of intact nanomaterials from the lung tissue is thus a good way to measure lung burden because this method can distinguish the origin of the detected elements. When particulate forms of nanomaterials are collected, the quantification can be performed by measuring metal elements using an instrument such as ICP-MS or measuring particulate form nanomaterials using instruments such as single-particle (sp)-ICP-MS, UV-Vis spectrophotometer and fluorimeter^[59].

7.4 Polymeric nanomaterials and others

Polymeric nanomaterials such as polystyrene and polypropylene are resistant to acids, alkalis and protease enzymes.^[60] Therefore, any tissue digestion methods excluding organic solvents can be used to collect polymeric nanomaterials. Then, the measurement method for the collected nanomaterials should be decided based on the nanomaterial-specific properties. For example, when polymeric nanomaterials are labelled with dyes or fluorophores, the standard curve fit with absorbance or fluorescence can be used for quantification. However, the current levels of technology for the quantification of non-labelled polymeric nanomaterials should be improved. Any other nanomaterials that were not discussed above can follow the same procedures such as

- a) collection of nanomaterials, and
- b) measurement of concentration.

When nanomaterials are dissolved or destructed during the process of collecting nanomaterials from the lung, the measurement method for the dissolved molecules should be selected based on the physicochemical properties of nanomaterials.

8 Application of lung burden data to toxicokinetics of nanomaterials

8.1 General

Lung burden measurements performed during repeated exposure studies in rats provide a metric of retained dose and can be helpful in understanding the toxicity of poorly soluble particles with low dissolution rates. However, each retained lung burden can have a different kinetic history due to burden-specific changes in clearance. Lung burden data can be used for the risk assessment of poorly soluble with low dissolution rate particles (e.g. as obtained from tests according to References ^[1] and ^[2]). When pulmonary effects are driving the human health risk assessment, risk assessors need to evaluate whether the occurrence of the pulmonary effects are better characterized by exposure concentration or by retained dose in the lungs. The human equivalent dose and lifetime human exposure may be calculated for risk estimation^{[17][18]}.

8.2 Sampling points

Although lung burden measurement is mandatory at only one post-exposure observation period in option B (at PEO-1), more lung burden measurements may be needed to provide information on clearance kinetics and persistence/progression response, especially for poorly soluble with low dissolution rate particles or some soluble particles with high dissolution rate. It is also advantageous to have some more sampling points such as exposure d-1 (6-h exposure) to estimate daily retention and additional sampling points during PEOs to fit better for the retention curve. The daily retention obtained from 6-h exposure will help to estimate the clearance tendency of deposited nanomaterials and the additional

sampling points would be helpful to find better inflection points for some nanomaterials having two-phase clearance kinetics.

8.3 Particle lung clearance and retention kinetics

8.3.1 General

The clearance of particles from the alveolar or pulmonary region of the lungs has been usually regarded as a first-order process, which implies a constant proportion of particle is eliminated per unit time. [13][61] This model has been used mainly because it provides a kinetically suitable description of lung clearance and a relatively simple dosimetric approach. [62] The fraction of organ concentration per initial organ concentration at PEO-1 was used for estimating retention and clearance kinetics, applying an appropriate first-order clearance model.

8.3.2 One-compartment first-order clearance model

The first-order model is defined by [Formula \(1\)](#). The retention half-time ($T_{1/2}$) is derived using λ and natural log (2) as shown in [Formula \(2\)](#).

$$M(t) = P \exp(-\lambda t) \quad (1)$$

where

$$M(t) = M(0) \exp(-\lambda t);$$

$M(0)$ as the lung burden at $t = 0$ d;

$M(t)/M(0)$ as the retention fraction, i.e. the lung burden at the time of a fraction of the initial lung burden;

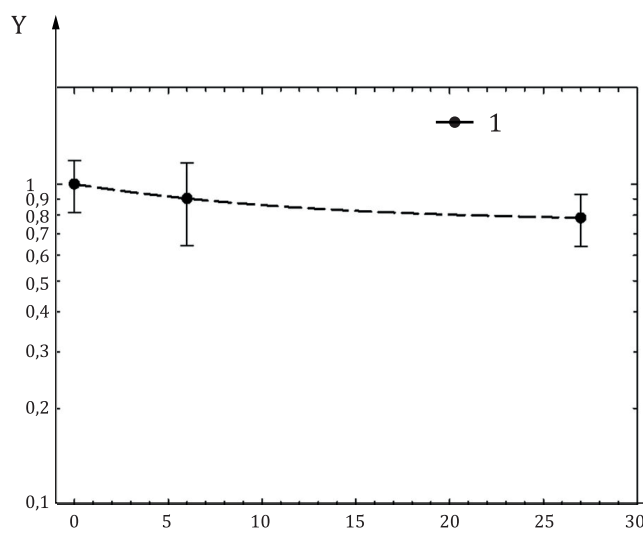
P is the fraction of lung burden cleared (1,0 for one-compartment model);

λ is the clearance rate per day for one-compartment model;

t is the time, in d.

$$T_{1/2} = \frac{\ln(2)}{\lambda} \approx \frac{0,693}{\lambda} \quad (2)$$

The example of first-order model of clearance is poorly soluble with low dissolution rate gold nanoparticles (AuNP) as shown in [Figure 2](#).



Key

X retention fraction for AuNP

Y time, in d

1 AuNP

Single AuNP in the Y axis signifies that the group treated with AuNP only, not combined with AgNP[32].

Figure 2 — Lung retention fraction of AuNP at 1-d, 7-d, and 28 p 28-d inhalation exposure to rats

Table 1 — Retention kinetics of AuNP

First order model AuNPs	
$T_{1/2}$ d	Elimination rate λ d ⁻¹
81,5	0,008 5
SOURCE: Reference [32]. Reproduced with the permission of the authors.	

Rats were exposed subacutely (28 d) to an aerosol of gold nanoparticles (10,8 nm) at a mass concentration of $17,7 \mu\text{g}/\text{m}^3 \pm 1,7 \mu\text{g}/\text{m}^3$ and lung burdens were determined at 1-d, 7-d and 28 d post-exposure. The data are presented in Figure 1 and the estimated $T_{1/2}$ and elimination rate in Table 1. Most poorly soluble particles with low dissolution rate follow the first-order model.

8.3.3 Two-compartment first-order model

Some nanoparticles which are soluble with high dissolution rate may form poorly soluble with low dissolution rate secondary nanoparticles after reacting soluble ions with biomolecules.[25][46][32] The two-phase model or two-exponential time-decay function used computer programming based on Formula (3), prior to which the retention fractions were converted to logarithmic variables. The retention half-time ($T_{1/2}$) was derived using λ_1 , λ_2 , and natural log (2) as shown in Formula (4).

$$M(t) = P_1 \exp(-\lambda_1 t) + P_2 \exp(-\lambda_2 t) \quad (3)$$

where

$$M(t)=M(0)\exp(-\lambda t);$$

P_1 is the fraction of lung burden cleared by fast phase;

λ_1 is the fast clearance rate per day for two-compartment model;

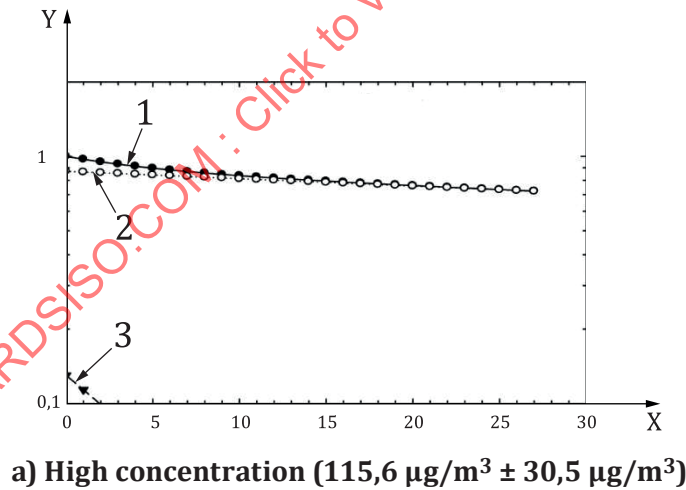
P_2 is the fraction of lung burden cleared by slow phase;

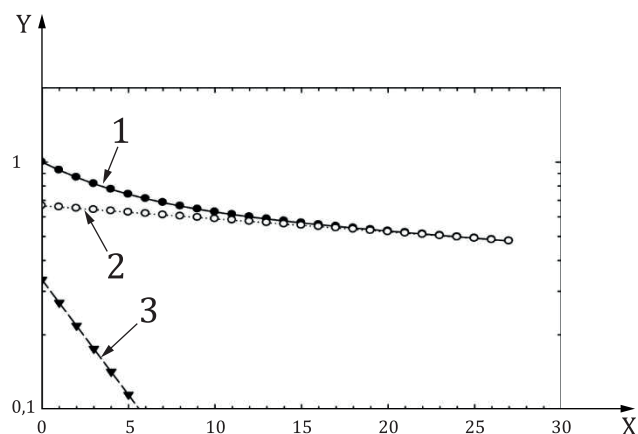
λ_2 is the slow clearance rate per day for two-compartment model;

t is the time, in d.

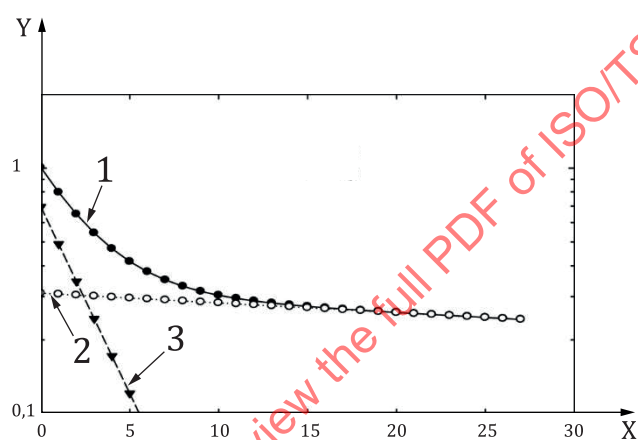
$$T_{1/2} = \frac{\ln(2)}{\lambda} \approx \frac{0,693}{\lambda} \quad (4)$$

An example of the two-compartment first-order model is silver nanoparticles (AgNP). The lung burdens of Ag from AgNPs were measured on PEOs of 1-d, 7-d, and 28-d to obtain quantitative mass concentrations per lung. Lung burden measurement suggested that Ag from AgNPs was cleared through two different modes: fast and slow clearance. The fast clearance component was concentration-dependent with half-times ranging from two days to four days and clearance rates of 0,35 d⁻¹ to 0,17 d⁻¹ from low to high concentrations. The slow clearance had half-times of 100-d, 57-d, and 76-d and clearance rates of 0,009 d⁻¹, 0,012 d⁻¹, and 0,007 d⁻¹ for the high, moderate, and low concentration exposure (see [Figure 3](#) and [Table 2](#)). The fast clearance component which was concentration-dependent can be dependent on the dissolution of AgNPs and the slow clearance would be due to slow clearance of the low dissolution AgNPs secondary particles originating from silver ions reacting with biogenic anions. These secondary AgNPs can be cleared by mechanisms other than dissolution such as mucociliary escalation, translocation to the lymphatic system or other organs[25][46][32].





b) Moderate concentration ($81,5 \mu\text{g}/\text{m}^3 \pm 11,4 \mu\text{g}/\text{m}^3$)



c) Low concentration ($31,2 \mu\text{g}/\text{m}^3 \pm 8,5 \mu\text{g}/\text{m}^3$)

Key

X time, in d

Y retained fraction, in log

1 $y = P_1 \exp(-d_1 t) + P_2 \exp(-d_2 t)$

2 $y = P_2 \exp(-d_2 t)$; slow clearance

3 $y = P_1 \exp(-d_1 t)$; fast clearance

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Figure 3 — Clearance of kinetics of Ag after 28-d of AgNP exposure and post-exposure

Table 2 — Clearance kinetics of Ag

Concentration	Fast clearance		Slow clearance	
	$T_{1/2}$ d	Rate d^{-1}	$T_{1/2}$ d	Rate d^{-1}
High	4,04	0,171	100,46	0,007
Moderate	3,23	0,21	56,82	0,012
Low	1,98	0,35	76,17	0,009

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The clearance $T_{1/2}$ of soluble nanomaterials with high dissolution rate is influenced by the exposure concentration in both slow and fast clearance. Lower concentration cleared faster than higher concentration. Compared to AgNPs, the $T_{1/2}$ of lung clearance of poorly soluble with low dissolution

rate nanomaterials ranged 60-d to 90-d, as seen in TiO_2 and gold nanoparticles. If $T_{1/2}$ is much longer than these $T_{1/2}$, it can be lung overload effects.^{[63][32]} Overload lung burdens by poorly soluble with low dissolution rate nanomaterials have been shown to induce chronic lung pathology such as fibrosis and lung cancer.^[64] The determination of change in lung burden over time with relevant $T_{1/2}$ are important parameters for the risk assessment to ascertain if chronic pulmonary effects are due to the physicochemical properties of the nanomaterials studied or due to overload of a low toxicity nanoparticles.

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Annex A (informative)

Option A for test scheme for 28-d and 90-d studies — Gases, vapours, liquid aerosols, and fast dissolution solid aerosols

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Examinations in the main study at PEO-1 and in the satellite groups at PEO-2		Exposure group	Main study: PEO-1 ^a	Satellite group: PEO-2 ^b	Total animals
—	Clinical observations	0	HP (LL) + BAL (RL) 5 f/5 m (28-d)	HP (LL) + BAL (RL) 5 f/5 m (28-d and 90-d)	
—	Body weight measurements	C ₁	HP (LL) + BAL (RL) 5 f/5 m (28-d)	HP (LL) + BAL (RL) 5 f/5 m (28-d and 90-d)	
—	Food/water consumption				
—	Clinical pathology	C ₂	HP (LL) + BAL (RL) 5 f/5 m (28-d)	HP (LL) + BAL (RL) 5 f/5 m (28-d and 90-d)	
—	Gross pathology/organ weights	C ₃	HP (LL) + BAL (RL) 5 f/5 m (28-d)	HP (LL) + BAL (RL) 5 f/5 m (28-d and 90-d)	
—	Lung weight-left lung				
—	Histopathology-left lung		Σ = 40 (28-d) or 80 (90-d)	Σ = 40	Σ = 80 (28-d) or 120 (90-d)
—	BALF-right lung				
Key 0 : control group Cx : exposure concentration BAL : bronchoalveolar lavage HP : histopathology LB : lung burden LL : left lung RL : right lung f : female m : male PEO : post-exposure observations PEO-1 : within one day after the last exposure day PEO-2 : within x weeks after the last exposure day PEO-3 : within y weeks after the last exposure day TBD : to be determined ^a Mandatory ^b Optional. NOTE All test chemicals use option A except solid aerosols, which use option B in Annex B which describes how a study director can customize these two options to optimize the hazard assessment of a test chemical.					

Annex B (informative)

Option B for test schemes for 28-d and 90-d studies — Poorly soluble aerosols

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Table B.1 — Scheme B.1: Test scheme of the examinations in the main study

Examinations in the main study	Exposure group	Main study: PEO-1 ^a		Total animals
PEO-1 (f and m):	0	HP (LL) + BAL (RL) 5 f/5 m (28-d)	LB (RL) (+TBD) 5 m (28-d and 90-d)	
— Clinical observations	C ₁	HP (LL) + BAL (RL) 5 f/5 m (28-d)	LB (RL) (+TBD) 5 m (28-d and 90-d)	
— Body weight measurements	C ₂	HP (LL) + BAL (RL) 5 f/5 m (28-d)	LB (RL) (+TBD) 5 m (28-d and 90-d)	
— Food/water consumption	C ₃	HP (LL) + BAL (RL) 5 f/5 m (28-d)	LB (RL) (+TBD) 5 m (28-d and 90-d)	
— Clinical pathology				
— Gross pathology/organ weights				
— Lung weight- left lung (f and m)				
— Histopathology- left lung (f and m)				
— BALF- right lung (f and m).				
PEO-1 (satellite groups, m only):				
— Lung burden- right lung				
— Other parameters to be determined by the study director.				
		$\Sigma = 40$ (28-d) or 80 (90-d)	$\Sigma = 20$	$\Sigma = 60$ (28-d) or 100 (90-d)

^a Mandatory.

Table B.2 — Scheme B.2: Test scheme of the examinations in the satellite groups at PEO-2 and PEO-3

Examinations in the satellite groups at PEO-2 and/or PEO-3	Exposure group	Satellite groups		Total animals
		PEO-2 ^a	PEO-3 ^a	
PEO-2 (f and m):	0	HP (LL) + BAL (RL) 5 f	HP (LL) + LB (RL) 5 f	LB (RL) (TBD) 5 m
— Lung weight- left lung (f and m)	C ₁	HP (LL) + BAL (RL) 5 f	HP (LL) + BAL (RL) 5 f	LB (RL) (TBD) 5 m
— Histopathology- left lung (f and m)	C ₂	HP (LL) + BAL (RL) 5 f	HP (LL) + BAL (RL) 5 f	LB (RL) (TBD) 5 m
— BALF- right lung (f only)	C ₃	HP (LL) + BAL (RL) 5 f	HP (LL) + BAL (RL) 5 f	LB (RL) (TBD) 5 m
— Lung burden- right lung (m only)				
PEO-3 (m only):				
— Lung burden- right lung				
— Other parameters to be determined by the study director				
		$\Sigma = 40$	$\Sigma = 20$	$\Sigma = 60$

^a Optional.

NOTE The number of animals of PEO-2 and PEO-3 in Reference [1] is identical to that in Reference [2]. The need for additional satellite groups at PEO-2 and/or PEO-3, the duration of the post-exposure interval, and the timing of the PEOs are determined by the study director based upon the purpose of the study and the results of a range-finding study and/or other relevant information. For example, PEO-1, PEO-2 and/or PEO-3 are used when multiple lung burden measurements are needed for evaluating clearance kinetics (see Reference [3]).

Annex C (informative)

Lung burden measurement methods

[Table C.1](#) lists different lung burden measurement methods.

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Table C.1 — Lung burden measurement methods

Nanomaterial	Characteristics of nanomaterials	Study design	PEO	Lung burden method	Observations	Reference
Fullerene	55 nm and 0,93 µm	Male rats, 3 h/d, 10 d, nose-only, 2,22 mg/m ³ for 55 nm; 2,35 mg/m ³ for 0,93 µm	D 0, 1, 5 and 7	Tissue lysis: magnesium perchlorate and toluene	$T_{1/2}$: 26 d (55 nm), 29 d (0,93 µm)	[36]
	50 nm and 1 µm	Male mice and male rats, 3 h/d, 5 d/week for 13 weeks, nose-only; 0,5 mg/m ³ and 2 mg/m ³ for 50 nm; 2 mg/m ³ , 15 mg/m ³ and 30 mg/m ³ for 1 µm	D 0, 14, 28 and 56	Tissue lysis: magnesium perchlorate and toluene Measurement: HPLC	Mice $T_{1/2}$: 15 d to 16 d for both particles Rats $T_{1/2}$: 61 d (50 nm), 27 d (1 µm)	[37]
SWCNT	0,78 nm diameter, >500 nm length	Mice, intratracheal instillation at 10 µg/mouse, 18 µg/mouse and 26 µg/mouse	D 0, 3, 7, 14 and 21	Tissue lysis: proteinase K Measurement: spectrophotometer at 638 nm, 691 nm, and 782 nm wavelengths Near-infrared fluorescence imaging	A slow decreasing trend was observed.	[39]
	44 nm diameter, 2,5 µm length	Male rats, intratracheal instillation at 0,20 mg/rat and 0,55 mg/rat	D 1, 3, 7, 28, 91, 175 and 364	Measurement: NDIR analysis	Rarely cleared from the lung within 1 year	[38]
MWCNT	Tangled, MMAD 381 nm to 1 015 nm, 2,73 µm diameter, 37,4 µm length.	Male rats, 6 h/d, 5 d/week for 28-d, nose-only, 0,257 mg/m ³ , 1,439 mg/m ³ and 4,253 mg/m ³	D 1, 7 and 28	Right caudal lobe Tissue lysis: digestion followed by measurement: OC/Reference [41]	$T_{1/2}$: 35,55 d	[26]
	16,7 nm diameter, 3,55 µm length	Female mice, pharyngeal aspiration	D 1	Tissue lysis: proteinase K Measurement: UV-Vis	Retention rate about 99,5 %	[35]
	CEN NM-401, MMAD 79 nm; NM-403, MMAD 1940 nm; carbon black, MMAD 940 MWCNT-7, MMAD 1780	Female SD rat, 28-d; CEN-401 0,5 mg/m ³ and 0,15 mg/m ³ ; CEN 403 1,5 mg/m ³ ; MWCNT-7 1,5 mg/m ³	3-d; 30-d; 180-d	TGA	—	[27]
	Thermally weak CNT and MWNT-7	—	Whole lung	Digestion followed by programmed thermal analysis	—	[33]
Graphene	8 nm to 25 nm thickness, 20 µm lateral, 5 µm lateral and <2 µm lateral	Male mice, pharyngeal aspiration to 4 µg/mouse or 40 µg/mouse	4 h and D 1, 7, 28, and 56	Standard morphometric point counting methods of histology slices	About 50 % of particles cleared at 2 months after treatment	[40]

Table C.1 (continued)

Nanomaterial	Characteristics of nanomaterials	Study design	PEO	Lung burden method	Observations	Reference
AgNPs	18,1 nm to 19,6 nm	Male rats, 6 h/d, 5 d/week for 28-d, nose-only, 31,2 µg/m ³ , 81,8 µg/m ³ and 115,6 µg/m ³	Whole lung D 1, 7, and 28	Tissue lysis: nitric acid Measurement: AAS	Early phase: $T_{1/2}$: 1,98 d to 4,04 d Late phase: $T_{1/2}$: 56,82 to 100,46 d	[25]
	10,86 nm	Male rats 10,12 µg/m ³ 28 d	Whole lung; D 1, 7 and 28	ICP-MS after acid digestion	Fast clearance 3,1 d	[32]
	20 nm radiolabelled ¹⁰⁵ Ag	Female rats, intratracheal inhalation for 1,5 h, 13,5 µg ± 3,6 µg (deposited mass)	Whole lung 0,75 h, 4 h, and Day 1, 7 and 28	Gamma isotope counter for radiolabelled ¹⁰⁵ Ag	After 28 d post exposure, the cleared fraction rises marginally to 0,94 while 2/3 of the remaining [¹⁰⁵ Ag]AgNP are retained in the lungs	[46]
	Evaporation and condensation generated 14 nm to 15 nm	84 d; 49 µg/m ³ , 117 µg/m ³ , 381 µg/m ³ , male and female SD rats	Whole lung; D 1, 28 and 84	Analyzed by a Zeeman graphite furnace atomic absorption spectrophotometer	—	[53]
AuNPs	20 nm ¹⁹⁵ Au-radiolabelled AuNP	Female rats, intratracheal inhalation for 2 h 1 µg/l	Whole lung, 1 h, 1-d, 7-d and 28-d	Gamma counter for radiolabelled ¹⁹⁵ Au	$T_{1/2}$: 23 d	[44]
	10,82 nm	28-d; 17,68 µg/m ³ ; male SD rat	Whole lung, PEO-1, 7-d and 28-d	ICP-MS after acid digestion	$T_{1/2}$: 81,5 d	[32]
	13 nm and 105 nm	Male rats, 6 h/d, 5 c/d at 12,8 µg/m ³ for 13 nm particles and 13,7 µg/m ³ for 105 nm particles	D1, 3 and 28	Tissue lysis: nitric acid Measurement: atomic absorption spectrophotometer	$T_{1/2}$: 44,5 d (13 nm) and 179,5 d (105 nm)	[20]
Iron oxide	130 nm (DLS)	Female rats, endotracheal intubation and aerosol exposure at 0,5 mg/kg bw	Up to 13 d	Magnetic particle imaging technique	$T_{1/2}$: 2,6 d, only relative quantification is possible	[47]
	22 nm radiolabelled ⁵⁹ Fe ₂ O ₃	Male rats, intratracheal instillation at 4 mg/rat	D 1, 7, 21, 50	Radioactivity detector	Lung clearance rate was 3,06 µg/d	[28]
CuO	15 nm to 22 nm	Male rats, 11,6 mg/m ³ for five consecutive days, nose-only	D 1, 22	Tissue lysis: nitric acid (HNO ₃ :H ₂ O ₂ :H ₂ O – 2:1:3) Measurement: ICP-MS	Negligible levels were detected at D 22	[50]
TiO ₂	⁴⁸ V-radiolabelled, 20 nm TiO ₂	Female rats, intratracheal inhalation for 2 h, 0,15 µg/l Deposited mass: 1,4 µg	Whole lung, 1 h, 4 h, D 1, 7 and 28	Radioactivity detector by isotope counter	$T_{1/2}$: 25 d	[45]
	20 nm	Male rats, 6 h inhalation at 15 mg/m ³	0 h, 3 h, 6 h, 12 h, D 1, 2, 3, 7 and 14	Tissue lysis: nitric acid (HNO ₃) and hydrogen peroxide (H ₂ O ₂) Measurement: ICP-MS	Progressive decrease over 14 d	[52]