GUIDANCE

Guidance on information requirements and chemical safety assessment

Appendix R7-1 for nanoforms applicable to Chapter R7a and R7c Endpoint specific guidance

Draft (Internal) Version 3.0

June 2021

1
2

NOTE

Please note that the present document is a proposed amendment to specific extracts **only** of the following guidance documents:

Appendix R7-1 to Chapter R.7a. (section 3 only)

Appendix R7-2 to Chapter R7c (section 2.1.3 only)

This document was prepared by the ECHA Secretariat for the purpose of this consultation and includes only the parts open for the current consultation, i.e. the above mentioned sections.

The full guidance documents (version before proposed amendments) are available on the ECHA website at:

https://echa.europa.eu/documents/10162/13632/appendix_r7a_nanomaterials_en.pdf (version 2.0 published May 2017).
https://echa.europa.eu/documents/10162/13632/appendix_r7c_nanomaterials_en.pdf (version 2.0 published May 2017).

The numbering and headings of the sub-sections that are displayed in the document for consultation correspond to those used in the currently published guidance document; this will enable the comparison of the draft revised sub-sections with the current text if necessary.

After conclusion of the consultation and before final publication the updated sub-sections will be implemented in the full documents.

LEGAL NOTICE

This document aims to assist users in complying with their obligations under the REACH Regulation. However, users are reminded that the text of the REACH Regulation is the only authentic legal reference and that the information in this document does not constitute legal advice. Usage of the information remains under the sole responsibility of the user. The European Chemicals Agency does not accept any liability with regard to the use that may be made of the information contained in this document.

Guidance on information requirements and chemical safety assessment

Extracts from:
Appendix R7-1 Recommendations for nanomaterials applicable to Chapter R7a - Endpoint specific guidance
Appendix R7-2 Recommendations for nanomaterials applicable to Chapter R7c - Endpoint specific guidance **Reference:** xxxx
Cat. Number: xxxxxx
ISBN: xxxxxxxx
DOI: xxxxxx
Publ.date: xxxxxx
Language: EN

If you have questions or comments in relation to this document please send them (indicating the document reference, issue date, chapter and/or page of the document to which your comment refers) using the Guidance feedback form. The feedback form can be accessed via the ECHA Guidance website or directly via the following link:
https://comments.echa.europa.eu/comments_cms/FeedbackGuidance.aspx

European Chemicals Agency

Mailing address: P.O. Box 400, FI-00121 Helsinki, Finland
Visiting address: Telakkakatu 6, Helsinki, Finland

1 DOCUMENT HISTORY

Version	Changes	Date
Version 1	First edition	April 2012
Version 2	• Update of section **Error! Reference source not found.** on sample preparation, to provide specific indications on the parameters for characterisation, pre-requisites and preparation for any nanomaterial. • Update of section 2.2.1 on water solubility, to include alternative guidelines that could be used for this endpoint and to flag the non-applicability of insolubility as a waiver for other endpoints • Update of section **Error! Reference source not found.** on partition coefficient n-octanol/water, to strengthen the message that guidelines recommended for this endpoint for non-nanomaterials are not applicable for nanomaterials and recommend other parameters that could be considered instead. • Update of section **Error! Reference source not found.** on adsorption/desorption, to clarify that the methods recommended in the parent guidance for this endpoint are not applicable for nanomaterials and recommend other parameters that could be considered instead. • New advisory note on testing and sampling strategy and sample preparation for human health endpoints (section 0); • Reorganisation of the (general) advice regarding non-testing methods in section 0 instead of under each specific endpoint to avoid repetition • Update of advisory notes on consideration on lung overload (section **Error! Reference source not found.**); • Update of the section on repeated dose toxicity (section 3.2.2); • Update of the section on mutagenicity (section 3.2.3)	May 2017
Version 3	• Update the advisory note on testing and sampling strategy and sample preparation for human health endpoints (section 0); • Update of the section on repeated dose toxicity (section 3.2.2.); • Update of the section on mutagenicity (section 3.2.3.)	May 2020

PREFACE

Three appendices concerning information requirements (appendices to IR&CSA Guidance Chapters R7a, R7b and R7c) have been developed in order to provide advice to registrants for use when preparing REACH registration dossiers that cover "nanoforms"[1].

The advice provided in this document focuses on specific recommendations for testing materials that are nanoforms of substances[2]. As most of the guidelines and publications are referring to nanomaterials or nanoparticles, also the terms 'nanomaterial' and'nanoparticle' are used. Annex VI defines the terms "nanoform"and "set of similar nanoforms"[3] and establishes the requirements for characterisation of the identified nanoforms/sets of similar nanoforms of the substance. A glossary is available to clarify the proper meaning of the terms used in the guidance.
Part of the advice provided is not strictly nanoform specific and may for instance also be applicable to other particulate forms of substances (e.g. relevance of dissolution rate). However, when such advice has been included, it is because it is considered especially relevant for nanoforms and should be part of the nanoform specific guidance.

In the absence of availability of any suitable specific provision (either because the endpoint is not relevant for nanoforms, because the guidance already provided is considered to be equally applicable to nanoforms as to non-nanoforms, or because more research or adaptation is needed before developing advice) no additional guidance for the information requirement has been included in this appendix.

This appendix intends to provide advice specific to nanoforms and does not preclude the applicability of the general principles given in Chapter R.7a (i.e. the parent guidance). Moreover, when no advice has been given in this appendix for a specific endpoint the advice provided in the parent Guidance should be followed.

Please note that this document (and its parent guidance) provides specific guidance on meeting the information requirements set out in Annexes VI to XI to the REACH Regulation.

General information for meeting the information requirements such as collection and evaluation of available information, and adaptation of information requirements is available in Chapter R.2 to R.5 of Guidance on IR&CSA).

Moreover, when considering the use of data already available, "Guidance on information requirements and chemical safety assessment – *Appendix R.6-1 for nanoforms applicable to the Guidance on QSARs and Grouping* of *Chemicals"* [1] may be useful as it provides an approach on how to read-across the hazard data between nanoforms (and the non-nanoform) of the same substance.

---

[1] *ECHA Guidance 'Appendix for nanoforms applicable to the Guidance on Registration and Substance Identification'* https://echa.europa.eu/documents/10162/13655/how_to_register_nano_en.pdf/

[2] See Annex VI of the REACH Regulation (EU) 1907/2006, as amended by Commission Regulation (EU) 2018/1881 to address nanoforms of substances.

[3] In this document often the term "set of nanoforms" is used instead of "set of similar nanoforms", but it should be always interpreted as "set of similar nanoforms", as defined in Annex VI.

Table of Contents

Glossary of terms 7
Appendix R7-1 to Chapter R.7a 10
3 RECOMMENDATIONS FOR TOXICOLOGICAL INFORMATION REQUIREMENTS for NANOFORMS 10
3.1. General advisory notes 10
3.1.1. General advisory note on testing with nanoforms and sampling strategy and sample preparation for human health endpoints 10
3.1.2. Advisory note on the consideration of assay interference 19
3.2. Specific advice for individual endpoints 21
3.2.1. Acute toxicity 21
3.2.2. Repeated dose toxicity 22
3.2.3. Mutagenicity and Carcinogenicity 31
Appendix R7-2 to Chapter R.7c 35
2.1.3 Guidance on Toxicokinetics 35
2.1.4 Recommended approach for gathering toxicokinetics information on nanomaterials according to REACH 37
REFERENCES 44

Table of Tables

Table 1: Potential sources of nanomaterials interference with commonly used assays 19

Table of Figures

Figure 1: Decision process for selecting the applicable guidance document 10
Figure 2: Schematic representation of the relationship between retained lung burden and duration of exposure leading to the phenomenon of lung overload 27

Glossary of terms[4]

Accumulation: the gradual build-up over time of nanoparticles in a tissue or organ.

ADME: absorption, distribution, metabolism, excretion

Agglomerate: a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components [2], [3], [4], [5]
Aggregate: a particle comprising of strongly bound or fused particles [2].

Bronchoalveolar lavage (BAL): The sample containing cells, particles, and secretions, obtained by flushing the small airways and alveoli of the lungs with saline while the animal is anesthetized.

BALF: bronchoalveolar lavage fluid

Bioavailability: The proportion of a substance in the systemic circulation compared with the total amount of substance that has been ingested or inhaled (modified from [6]).

Biodegradation: Degradation of a substance resulting from interaction with the biological environment [6].

Biodurability: The tendency to resist chemical and biochemical alteration through dissolution and enzymatic biodegradation or chemical disintegration within biological media (modified from [6]). Biodurability (dissolution and biodegradation) is measured using *in vitro* acellular and cellular tests.

Biopersistence: The ability of a material to persist in the body due to its biodurability and resistance to physiological clearance [6]. It is determined using *in vivo* methods.

Biotransformation: Alteration of a substance resulting from interaction with biological systems [6].

Clearance: (1) In general toxicology, volume of blood or plasma or mass of an organ effectively cleared of a substance by *elimination* (*metabolism* and *excretion*) divided by the time of elimination. Total clearance is the sum of the clearances of each *eliminating* organ or tissue for a given substance. (2) In pulmonary toxicology, the volume or mass of lung cleared divided by the time of *elimination* is used qualitatively to describe removal of any inhaled substance which deposits on the lining surface of the lung [7].

Dissolution half-life/half-time: A time interval that corresponds to a concentration decrease by a factor of 2 [6].

Dissolution:Dissolution as used herein is the process by which a soluble nanomaterial in an aqueous medium or biological environment is converted to the constituent ions or molecules [5].

Dissolution rate: The rate at which ions or molecules are released from the surface of a solid into the surrounding liquid medium [6].

[4] As most of the guidelines and publications are referring to nanomaterials or nanoparticles, also the terms 'nanomaterial' and 'nanoparticle' are used in addition to the "nanoform". Nanoform, is a recent REACH regulatory term which was not used in the scientific studies or authority reports mentioned in this guidance.

Dispersion: Microscopic multi-phase system in which discontinuities of any state (solid, liquid or gas: discontinuous phase) are dispersed in a continuous phase of a different composition or state [6]. Dispersion may also refer to the "act of" dispersion.

Impaired clearance: a continuously increasing prolongation of lung clearance of poorly soluble particles or fibres when the retained lung burden exceeds a certain threshold (modified from [7]). It can be caused by toxicity (impairment of alveolar macrophages function or cytotoxicity), or by overload of alveolar macrophages.

Lung burden: The amount of test chemical that can be analytically measured in the lung at a given time point (modified from [7]).

Lung overload: a phenomenon of impaired clearance in which the deposited dose of inhaled poorly soluble particles of low toxicity (PSLT) in the lung overwhelms clearance from the alveolar region leading to a reduction in the ability of the lung to remove particles. Lung particle overload results in an accumulation of particles greater than that expected under normal physiological clearance. This definition is relevant for all species (not just rat). This definition is independent of the underlying mechanism(s) (e.g. macrophage mobility impairment). A key issue is that increased particle retention due to large lung burdens needs to be differentiated from that due to high cytotoxicity particles (e.g. quartz) [8].

Nanofibre: fibre with a length-to-diameter ratio of > 3:1 (by partial analogy to the WHO fibre concept [9]) and with one or more external dimensions below 100 nm.

Nanoform: a form of a natural or manufactured substance containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm, including also by derogation fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm [3], [2].

Nanomaterial: a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %. By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials [3].

Nanoparticle: nano-object with all three external dimensions in the nanoscale where nanoscale is defined as the size range from approximately 1–100 nm [10]. This covers all particles with any external dimension on the nanoscale including "nanofibres" (two external dimensions in the nanoscale) and "nanoplates" (one external dimension in the nanoscale).

Particle: a minute piece of matter with defined physical boundaries [2].

Simulated body fluid: a solution with an ion concentration close to that of a physiological fluid.

PEO: post-exposure observation

Poorly soluble particle (PSP): solid aerosol particles deposited in the lung that do not undergo rapid dissolution and clearance [7]. The defintion is restricted to lung and to aerosols. A PSP is generally understood as having a solubility of less than 0.1 g dissolved in 100 ml dissolvent within 24 hours [7]. Examples of dissolvent are the simulated biofluids which

include artificial lung lining fluid that contains salts and proteins or in an acidic environment that mimics the lysosomal fluid of macrophages. Specific criteria determining a PSP were recently elaborated [8].

Poorly soluble particles of low toxicity (PSLT): a PSP which does "*not cause more than minimal and transient granulocytic inflammation up to a lung burden causing overload in the rat"* [8]*.***(Q)SAR**: quantitative structure–activity relationship

'Set of similar **nanoforms':** under REACH Regulation, it is a group of nanoforms character ised in accordance with section 2.4 of REACH where the clearly defined boundaries in the parameters in the points 2.4.2 to 2.4.5 of the individual nanoforms within the set still allow to conclude that the hazard assessment, exposure assessment and risk assessment of these nanoforms can be performed jointly[5] [2].

Solubility: the proportion of a solute in a solvent under equilibrium conditions (i.e. in a saturated state) [11].

[5] A justification shall be provided to demonstrate that a variation within these boundaries does not affect the hazard assessment, exposure assessment and risk assessment of the similar nanoforms in the set. A nanoform can only belong to one set of similar nanoforms.

Appendix R7-1 to Chapter R.7a

3 RECOMMENDATIONS FOR TOXICOLOGICAL INFORMATION REQUIREMENTS for NANOFORMS

3.1. General advisory notes

3.1.1. General advisory note on testing with nanoforms and sampling strategy and sample preparation for human health endpoints

These advisory notes do not describe any detailed protocol but, instead, aim to provide useful advice with regard to specific aspects that are particularly important for nanoforms testing, and references to relevant resources. For a test material fulfilling the definition of a nanoform, the testing strategy (Fig. 1) is dependent on its solubility and/or dissolution rate in water. Dissolution rate in relevant biological fluids is an important parameter in toxicokinetics evaluation, and may also be used for grouping and building of the read-across or to substantiate the weight of evidence in the cases where existing studies with nanoforms are available.

Figure 1 below shows a decision tree that can be used to determine whether nanospecific advice should be used, or, due to the conclusions on the nanoform's properties, the advice provided by the parent guidance can be followed instead.

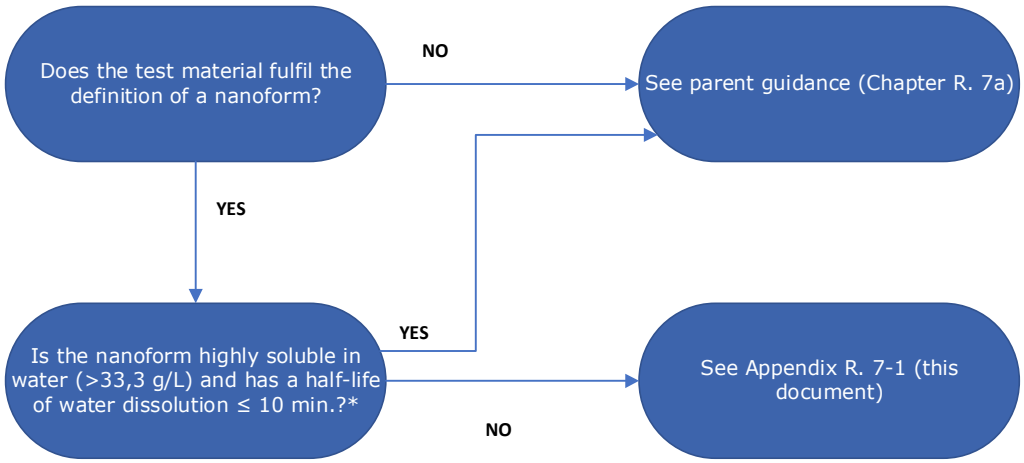

Figure 1: ***Decision process for selecting the applicable guidance document***

*The given values may be updated when more validated information becomes available with regard to testing in relevant biological media.

3.1.1.1. Considerations on solubility and dissolution rate

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) has stated that many nanomaterials have considerable solubility and that for "*these materials the interaction with living systems remains close enough to the bulk chemical agent to justify the use of well-established toxicological testing procedures and approaches*" [12]. Water solubility may give a first indication on the (non) biopersistence of a nanomaterial [14]. EFSA (draft) Guidance on Technical Requirements [11] considers, a solubility equal to or higher than 33.3 g/L as a high solubility in water, in line also with the SCCS Guidance (2019) [16] and with the categories for degrees of solubility proposed by JECFA [17] and the European and US Pharmacopoeias [11]. Based on a pragmatic and consistent approach among different

regulatory bodies, a similar value for water solubility is recommended also here to decide whether this guidance is applicable (see Fig. 1). In the context of risk assessment in occupational settings, BAuA [15] proposed that nanomaterials with a water solubility above 100 mg/L could be considered as soluble[7] and thus not biopersistent.

The latest approaches for the risk assessment of nanomaterials recommend a strategy in which the dissolution rate and equilibrium in water is a first key element [13], [11]. Dissolution rate is a kinetic process which informs not only whether a substance dissolves but also on how long it takes to dissolve. Dissolution rate depends on the size and surface properties of the particles and fibres, and also on the nature of media where the release of ions occurs, on the temperature, the pH and the presence of substances interacting with the nanoform's surface. The pH and temperature should be specified and relate to biologically relevant conditions. Another important factor in the dissolution of nanoparticles is aggregation [18]. Aggregation decreases the available external surface area thereby reducing the extent of dissolution [19]. It has been observed that the dissolution rate decreases as the aggregation state of nanoparticles increases.

To determine the dissolution rate in water, the protocol proposed by EFSA [11] is recommended. In the EFSA (draft) Guidance on Technical Requirements [11] a high dissolution rate in water is defined as a half-life of 10 min or less corresponding to a dissolved fraction equal to or higher than 88% in 30 min [11]. In this context EFSA (draft) Guidance on Technical Requirements refers to '*concentration corresponding to exposure at maximum use level*'. A similar threshold for dissolution rate in water is proposed in this guidance (see Fig. 1).

Annex VII, section 7.7. column 1, of the REACH Regulation specifies, that (beside the water solubility testing) "*for nanoforms, in addition the testing of dissolution rate in water as well as in relevant biological and environmental media shall be considered.*" (see section 2.2.1)[9].

The determination of the dissolution rate (*k*) as well as the dissolution half-life in biological fluids provides an insight on how a certain particle may interact with its biological environment [18] and allows an informed decision for the design of the repeated dose studies. The dissolution rates are important parameters in toxicokinetics evaluation, which can also be used for grouping and read-across. Recently a method and thresholds [20] have been proposed for grouping nanoparticles based on dissolution rates in phagolysosomal simulant fluid.

Until dissolution occurs, the kinetics of nanomaterials are governed by their particulate nature, whereas after dissolution the (dissolved) ions or molecules determine the toxicokinetics [21]. Thus, if the dissolution rate is not very high, uptake of particles may occur. A very high dissolution rate is considered close to instantly dissolved [13], and means that the material dissolves quickly at the site of entry, so that cellular uptake in the lung or gastrointestinal tract occurs almost exclusively as the dissolved species. When particles remain present for a longer duration, they can be taken up by macrophages or translocated across the epithelium as particles. Effects due to release of ions or molecules after distribution to cells/tissues (i.e. the so-called "Trojan-horse" mechanism, in which nanoparticles are internalized within cells, then release high levels of toxic ions), should be investigated, as the local dissolution in the macrophages and in tissues may lead to a different toxicity profile as compared to the solute itself. For example, although the toxicity of ZnO nanoforms is predominantly driven by the release of Zn^{2+} ions, *in vivo* studies showed that ZnO nanoparticles, but not the soluble ions, triggered the recruitment of eosinophils to the lungs [22]. In addition, the exposure to ZnO nanoparticles caused chronic effects that lasted up to four weeks. No aqueous extract caused

[7] Please note this value is only used as an indication for (non) biopersistence and should not be used as a threshold for solubility/insolubility in other contexts (such as triggering a waiver for insolubility for environmental endpoints)

[9] Please note that this draft is an extract document of the Appendix to Chapter R.7a for nanomaterials, and the section 2.2.1. referenced here is in that document and not in this extract.

such sustained inflammation, probably because soluble ions are rapidly cleared from the lungs [23]. Most probably the observed toxicity with ZnO nanoparticles is the result of the combined effects of the nanoparticles and of Zn^{2+} ions [24].

The dissolution of a nanomaterial is a time-dependent process (depending on the rate of solubilisation and the surface area) and is directly related to its *in vitro* or *in vivo* biopersistence and biodurability that decrease with increasing dissolution rate [14]. In contact with a biological environment, the nanoparticles may undergo changes involving dissolution, re-precipitation, protein coating and agglomeration. Such events are summarized as biotransformation. Biotransformation could impact clearance and can also contribute to the biokinetics of nanoparticles [25] and potentially to their toxicity [26]. Therefore, biotransformation is an important element to investigate in the context of toxicokinetics. A method to assess biotransformation was recently described [25]. However, as no standard method applicable to all nanoforms is currently available, the method used must be justified and thoroughly documented. The information on biotransformation is not a mandatory requirement but it is considered important for the implementation of grouping and read-across using dissolution rates [25].

Dissolution studies in relevant biological fluids can also be performed to substantiate the weight of evidence in cases were studies are already available and fulfill the criteria described in Section 3.2.2. OECD [6] lists as examples of biological media the artificial pulmonary interstitial fluid (Gamble's) balanced electrolyte solution (neutral) and alveolar lysosomal fluid (ALF) (acidic) as well as gastric (acidic) and intestinal (neutral) fluids. Different biological media may influence both the kinetics of dissolution and the saturation concentration depending in particular on pH and/or enzymatic action [27]. In addition, some water insoluble nanomaterials may be non-biodurable in biological fluids and this can be assessed from data on the dissolution rate. In general, the most appropriate route of administration, having regard to the likely route of human exposure, determines which biological media are relevant [13].

The OECD Guidance documents on inhalation toxicity studies [7] states that "*In order to decide whether your material is poorly soluble, the solubility of a solid material may be assessed by measuring solubility in a simulated biofluid. A poorly soluble material is generally understood as having a solubility of less than 0.1 g dissolved in 100 ml dissolvent within 24 hours*." Examples of dissolvent are the simulated biofluids. For the inhalation route of exposure, the simulated lung lining fluid and (macrophage) phagolysosomal simulant fluid are relevant for the assessment of dissolution via this route of exposure [7]. The data in the simulated lung lining fluid are important as this medium simulates the extracellular environment. Assessment of the stability in lysosomal conditions is important for the investigation of biopersistence and intracellular accumulation [18]). Within the cell, the nanomaterials generally distribute to lysosomes, where degradation can occur due to the acidic conditions and the presence of enzymes. Using an optimized abiotic flow-through method for 24 (nano)forms of 6 substances, Koltermann-Jülly et al. 2018 [20] proposed a grouping based on the dissolution rates in phagolysosomal fluid (pH 4.5). For example, a high dissolution rate ($k>100$ ng/cm^2/h) for a non-persitent nanoform corresponds to an abiotic dissolution ranging from 30% to 100% after 7 days. Indeed, a dissolution rate of 30-100% within 7 days is in line with a complete clearance during 21 days post-exposure.

It is important to assess the dissolution rate in both, simulated lung lining fluid and phagolysosomal fluid. For example, if dissolution only occurs in the lysosomal fluid but not in the simulated lung lining fluid, uptake of particles by lung macrophages as well as translocation across the lung epithelium can occur, as the lung lining fluid has been reported to stimulate the nanoparticle uptake by human alveolar epithelial cells [28]. Following translocation the nanomaterials may relocate at a different location than lung. In this case the read-across to the solute is not possible, since the dissolution of the nanoform (and release of the solute) takes place at a different location, which may lead to a different distribution profile. Comparisons of the distribution profiles for particles and solutes would help to assess whether

read-across to the soluble form is possible.

For the oral route, dissolution of nanomaterials in simulated gastric fluid and macrophage phagolysosomal fluid is relevant. The macrophage phagolysosomal fluid may provide insight on the persistence of the nanoform after cellular uptake. A nanomaterial is considered to dissolve quickly or to have a high dissolution rate if 12% or less of the material (mass based) remain as particles after 30 min of *in vitro* digestion in gastrointestinal fluid compared to the particulate concentration at the beginning of the dissolution test [5]. If the nanoform does not dissolve quickly in the gastrointestinal fluid then dissolution in lysosomal fluid should be performed. A half-life of ca. 24 h is considered indicative of high dissolution rate in lysosomal fluid. This would result in 12% or less of the material (mass based) remaining at 72 h compared to the particulate concentration at the beginning of the dissolution test [5].

For dermal conditions, the dissolution rate in artificial sweat could be used.

3.1.1.2. Available methods for solubility and dissolution testing

Different analytical techniques/methods and their suitability to measure solubility of nanomaterials have been reviewed [29]. The determination of the solubility of nanomaterials in water should be done according to OECD TG 105 with specific considerations for nanoforms [30]. The pH and temperature should be specified for solubility testing and relate to biologically relevant conditions [31].

In the solubility/dissolution kinetics experiments, the solid phase needs to be separated from the liquid phase in order to avoid artefactual high solubility values by also analysing solid materials. Presence of particles can be checked qualitatively by e.g. Tyndall analysis and solid nano particles can be removed by ultracentrifugation or ultrafiltration [29].

In water, so far, no specific OECD testing guideline (TG) is available for determination of solubility and dissolution rate for nanomaterials but some may be adapted e.g. OECD TG 105 [32], [27], [29] and ISO 19057 [33].

Dissolution studies on particle systems are often carried out by inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma atomic emission spectroscopy (ICP-AES) and atomic absorption spectroscopy (AAS), which are a few of the most employed methods for quantitative analysis. Real-time dissolution kinetics measurements can be performed using Dynamic Light Scattering (DLS), UV/vis spectrometry, fluorescence spectroscopy and confirmed using inductively coupled plasma mass spectrometry (ICP-MS) [34].

In a complex matrix, it is acknowledged that measuring dissolution is highly challenging. At present there is no one universal robust, rapid test method for regulatory testing that is applicable for all types of nanomaterials and all types of matrices [35]. During the NANoREG project , several methods have been identified for future use (e.g. ultrafiltration UF-ICP-MS/AES, ultracentrifugation UC-ICP-MS/AES, single particle sp-ICP-MS, and colorimetry). UF-ICP-MS/AES was considered a relatively easy and highly robust method that can be used for a rather broad range of nanomaterials if no nanomaterial-matrix interactions take place. If these interactions do take place, sp-ICP-MS is considered a good choice for measurement, given that the material is not below the size detection limits. Nevertheless, to select the best suitable method, knowledge of physical-chemical properties of the nanomaterial is crucial and it is recommended to use a combination of techniques [35].

Due to the fact that the assessment of dissolution in biological media is currently performed only by non-validated methods, the used protocols need to be well documented and justified.

Albeit not validated for nanomaterials, information from bioelution studies could be useful for read-across for metallic compounds. An ESAC Opinion on Scientific Validity of the Bioelution Test Method was finalised in July 2020 [36].

ENV/JM/MONO(2018)11 [37] compiled the available information on the determination of the biodurability (through dissolution) of different NMs included in the OECD Sponsorship Programme for the Testing of Manufactured Nanomaterials as indicator for their toxicity. With cellular and acellular *in vitro* tests, the dissolution of NMs is determined using static and dynamic (such as flow-through) protocols in the presence of different biological and environmental media with different pH values and chemical compositions . New studies found that dynamic abiotic dissolution systems adequately predicted the overall pulmonary biopersistence of several nanoforms [20] [25]. A recent OECD Guidance document [30] provides guidance for the methods addressing dissolution rate and dispersion stability for nanomaterials, in particular with the development of the dynamic testing or flow through system that could be applied to estimate the dissolution in biological media. There is also ongoing work on the WNT project 1.5 on "Determination of Solubility and Dissolution Rate of Nanomaterials in Water and Relevant Synthetic Biological Media" aiming to provide harmonised approaches for testing solubility and dissolution rate of nanomaterials. In addition, development of an OECD Guidance document on "Integrated *in vitro* approach for intestinal fate of orally ingested nanomaterials" is under developement. It is based on a bi-step approach in which the dissolution behavior of nanoform in simulated digestion environment is coupled with a biological evaluation of nanoparticles uptake/passage in a cellular model of intestinal barrier.

As long as no standard methods are available, detailed description and justification of the method including adequate controls (and reference materials, if possible) is required.

3.1.1.3. Test material characterization and reporting and sample preparation

The introductory text of Annexes VII-X of the REACH Regulation (EC) No 1907/2006 amended for nanomaterials, provides that: "*Without prejudice to the information submitted for other forms, any relevant physicochemical, toxicological and ecotoxicological information shall include characterisation of the nanoform tested and test conditions. A justification shall be provided where QSARs are used or evidence is obtained by means other than testing, as well as a description of the range of the characteristics/properties of the nanoforms to which the evidence can be applied*."

Prior to toxicological testing, the sample characterization and preparation including special considerations on dispersion and dosimetry, should be performed, as advised in the OECD Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials [38], and as specified in Section 2.1.1 of this Appendix. Additional useful information can be found in the report of the OECD expert meeting on the physical chemical properties of manufactured nanomaterials and test guidelines [39]. A harmonized preparation of the test sample will enable the comparison of the data and their further use. Information on the characterisation of test material serves multiple purposes:

a) enables linking to the identity of the nanoform/set of nanoforms being covered in the dossier and therefore supports data relevance.
b) provides general information on the material's properties 'as test sample' to support handling/storage and repeatability/reproducibility of results, and
c) facilitates the comparability and use of toxicological data for grouping of the nanoforms of a substance or justifying read-across between nanoforms, and between nanoforms and the non nanoforms (For further information see *Appendix R.6-1 for nanoforms applicable to the Guidance on QSARs and Grouping* [1]).

Section 2.1.1 and 2.2 of this Appendix explain in detail the importance of these physico

chemical parameters for toxicological testing and also gives information on how these parameters can be determined. A critical review of the methods used to characterize the manufactured nanomaterials to assess their health and safety risks, describing the limitations and accessibility of each method is also available [40].

The chemical composition, the physicochemical properties, and the interaction of the nanomaterials with biological systems may in some cases influence aspects of exposure, deposition (lung), systemic uptake and bioaccumulation, dissolution, toxicokinetics and toxicity [40]. The hazards posed by all possible forms of the substance covered by a registration, including nanoforms/set of nanoforms, must be addressed by the toxicological and ecotoxicological information provided in the registration dossier. In order to demonstrate that the results of a study are relevant for the hazard of a specific nanoform or set of nanorforms in the dossier, some information on the tested substance should be reported in the endpoint study record under the test material information field in IUCLID (please see more under "How to prepare registration dossiers for substances with nanoforms" (https://echa.europa.eu/documents/10162/22308542/howto_prepare_reg_dossiers_nano_en.pdf/) in https://echa.europa.eu/manuals). The following parameters must be provided:

- Chemical composition (as described in the ECHA *Guidance for identification and naming of substances under REACH and CLP*);
- Characterisation parameters of the test material (as described in the ECHA Guidance *Appendix for nanoforms applicable to the Guidance on Registration and Substance Identification*
    - Number based particle size distribution with indication of the number fraction of constituent particles in the size range within 1 nm – 100 nm;
    - Shape and aspect ratio;
    - Description of surface functionalisation or treatment;
    - Specific surface area.

Moreover, *Appendix R6-1 for nanoforms applicable to the guidance on QSARs and Grouping of Chemicals* [1] provides an approach on how to justify the use of hazard data between nanoforms (and the non-nanoform(s)) of the same substance. The Guidance details some (additional) parameters that may be required to be able to assess whether the available hazard data are applicable for different nanoforms of a substance. The registrant may wish to consider taking into account such parameters when characterising the test material, in order to be able to follow the above-mentioned guidance. For example, the dissolution rate, surface chemistry and dispersability have been reported as a founding basis for the grouping of the nanomaterials ([1], [41]).

Nanoforms characterization (e.g. size, dissolved ion fraction, shape) in the exposure system at least at the start and end of the *in vitro* experiments is important to confirm their presence in the test system and to observe potential changes that they may undergo. This characterisation should also include the assessment of the state of agglomeration. This is an important parameter to investigate also for in vivo studies, and characterisation of aerosols is recommended in OECD 412/413. These TGs also state that "*scanning and/or transmission electron microscopy should always be used periodically (e.g., monthly) for monitoring and qualitative confirmation of particle size and shape for all particulates, not just nanomaterials*."

Suitable suspension-based techniques include the Dynamic Light Scattering (DLS), analytical ultracentrifugation (AUC) or asymmetrical flow field-flow fractionation (AF4) coupled with inductively coupled plasma mass spectrometry (AF4/ICP-MS).

DLS has been used in many European projects (e.g. NANoREG, NanoReg2) in order to characterize nanomaterials in the medium. However, DLS is suitable to investigate the particle

size distribution and works well with monodisperse, non-agglomerated and spheroidal nanomaterials. DLS is therefore, more appropriate in studies with more simple environments such as the ones used in dissolution studies. DLS cannot detect the presence of small aggregates and it is not able to resolve small modifications of the particle size distributions (PSDs) happening under physiological conditions [42].

Analytical ultracentrifugation (AUC) is one of the most accurate techniques for the characterization of nanoparticles in the liquid phase because it can resolve PSDs with high resolution and detail also in the sub-nm range [43]. AUC combines separation, concentration and detection steps into one single measurement improving total analysis times and reducing experiment complexity [42].

AF4 is the most commercialized flow field-flow (FFF) fractionation technique and by far the most widely used FFF technique for nanomaterials. It shows good selectivity and size resolution. By showing differences in PSDs, AF4 is also very useful for monitoring particle modifications, such as the adsorption of macromolecules on the surface, or dissolution or aggregation processes [44]. The online coupling of AF4 with multiangle light scattering (MALS), UV, differential refractive index (dRI) or fluorescence detection, or offline combination with TEM or UV, allows for detailed characterization of the physicochemical properties of different populations within a polydisperse sample with respect to size, molar mass or concentration, shape, conformation, structure, and aggregation state [45].

The capability of multi-element analysis is an added benefit when coupling AF4 with mass spectrometry. AF4/ ICP-MS offer substantial advantages for detecting nanoparticles and assessing many parameters in complex matrices over traditional characterization methods such as microscopy, light scattering, and filtration [46]. AF4-ICP-MS has a good size resolution and can detect a very small nanoparticle size (up to 2 nm).

ICP-MS is a suitable method to quantify the dissolved fraction in the culture media, especially for metal nanomaterials. Single particle spICP-MS is a new promising method derived from using the inductively coupled plasma mass spectrometer (ICP-MS) in the time-resolved mode. The coupling of FFF to spICP-MS, allows nanoparticle dimensions to be determined at very low concentration levels, by choosing one or two specific chemical elements with the common instruments, or multi-elements with fast scanning quadrupoles of TOF (time-of-flight) mass analyzers [44]. Within the work alongside OECD projects to support and accelerate the development of Test Guidelines and Guidance Documents related to nanomaterial test methods, NanoHarmony project Task 1.4 (Determination of concentrations of ENMs in biological samples using spICP-MS) focuses on the optimisation and standardisation of spICP-MS but other techniques will also be addressed.

An integrated assessment of residual solids with respect to transformations of shape, size distribution, and crystallinity by protocols for preparation, analysis and statistical image analysis, using TEM, optionally supported by XPS and EDX is available [25]. However, XPS is currently not widely available.SEM and TEM are the “gold standard” techniques for nanoparticle size-, shape-, crystallinity- and composition- characterisation. While TEM is a highly valuable technique for the size analysis of nanomaterials, simple TEM sample preparation methods (by evaporation of the solution) do not distinguish between agglomerates formed during the drying of the sample on the support (grid) and aggregates already present in the NP suspension. Cryo-TEM might help to overcome this drawback by an ultra-fast conversion of the NP suspension into a vitrified film on the grid, allowing direct morphological visualization of nanomaterials at near native state, and thus, reducing the risk of aggregation and of the introduction of artefacts due to sample preparation [47]. However, the technique needs laborious sample preparation and particles or aggregates larger than 300 nm cannot be imaged.

The information on transformation is not a mandatory requirement but it is considered important for the implementation of grouping and read-across.

An important step in the sample preparation for *in vitro* or *in vivo* studies is dispersion. A generally applicable standard operation procedure (SOP) is currently not available for the dispersion of a dry powder in liquids. Since the use of a certain protocol or analytical technique depends on the type of nanomaterial and medium, the dispersion efficiency of the applied protocol and the stability of the dispersion should be tested and properly documented. A dispersion protocol can be considered effective if it yields samples which consist as much as possible of non-agglomerated/non-aggregated particles [11]. Reference materials or self-generated and properly characterised and documented test materials are essential for controlling and comparing the performance of analytical methods used for nanomaterial characterisation and in their validation [5]. Dispersion stability must be verified periodically. ISO/TR 13097:2013 (Guideline for the characterization of dispersion stability) provides technical guidance on the characterisation of dispersion stability [48].

The EU-project NanoDefine developed a specific optimised dispersion protocol for a number of priority nanomaterials laid down in the form of SOPs [57]. Whenever applicable, these SOPs are reccommended to be used. Several dispersion protocols have been developed and published [30], [49], [50], [51], [52], [53], [54], [55], [56]. More dispersion protocols are also available via the websites of international organisations (e.g. OECD; European Commission-JRC; and their respective research projects such as NanoGenoTox – http://www.nanogenotox.eu; Nanopartikel – http://www.nanopartikel.info; NanoDefine – http://www.nanodefine.eu; NANoREG – www.nanoreg.eu).

As long as no test guidelines are available, detailed description and justification of the method including adequate controls (and reference materials, if possible) is required.

Before *in vivo* administration of a nanoform, the possible interactions with the administration vehicle need also to be determined and reported. For example, the nanoforms may adsorb on the walls of the delivery system and may no longer be available, thus affecting the delivered dose [58]. The check of lab-ware retention capability is essential in order to make experiments reliable and improve the reproducibility of *in vivo* studies.

The "aging" of nanoforms during the storage may also influence the outcome of a toxicity study. For example, the biological action of freshly prepared and aged silver nanoparticles is strongly different due to the different amounts of released ions [59].

To help to determine the concentration of nanoforms in biological samples, data from the OECD WPMN project "Guidance on the determination of concentrations of NPs in biological samples for (eco)toxicity studies" is expected to become available by the end of 2021.

3.1.1.4. Biological Sampling

Two OECD test guidelines, OECD TG 412 (Subacute Inhalation Toxicity: 28-day Study) and OECD TG 413 (Subchronic Inhalation Toxicity: 90-day Study) have been updated to enable the testing and characterisation of effects of nanomaterials and are currently available. The OECD guidance document GD 39 on inhalation toxicity testing has also been updated and validated for nanomaterials testing and should be consulted in the design of the studies [7].

The biological samples (e.g.organs and tissues, blood cells, body fluids) to be collected in the *in vivo* toxicological studies are specified in the relevant test guidelines. However, if there is an indication that the nanomaterials would be distributed in other tissues not listed in the OECD TGs, then the collection of these additional tissues (including the sampling method) is recommended.

It is advised to handle and store the samples adequately to allow the performance of later

analysis (e.g. storage by chemical or physical tissue fixation for microscopy [60], freezing for burden analysis ([61], [62]. Caution should be exercised to use the most appropriate storage method for specific tissues as the available information is based on a limited number of nanomaterials and tissues.

3.1.1.5. Use of Non-Animal Testing Approaches [10]

Article 25 of the REACH regulation specifies that testing on vertebrate animals should be conducted only as a last resort, i.e. only when all other scientific available methods have been evaluated. Therefore, in order to fill any data gaps, there is a legal obligation to evaluate all the existing data and data from non-animal methods of hazard assessment before considering any new tests using vertebrates. Registrants are advised to stay informed on ongoing developments and validation efforts of the OECD and the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM), as well as on the regulatory acceptance of new methods by ECHA [63]. Implementation of non-animal approaches for nanomaterials requires the prior consideration of all available information, including nanomaterial characterisation, which is a critical requirement for grouping and read-across and quantitative structure–activity relationships (QSARs). In addition, relevant and reproducible *in vitro* systems may be used. Adverse Outcome Pathways (AOPs) relevant to nanomaterials are under development at the OECD and offer new approaches to integrated assessment (https://aopwiki.org and and OECD EAGMST https://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm).

In silico models and read-across

Regarding the use of non-testing data for nanomaterials, it is necessary to take into account that:

- The use of *in silico* models (e.g. QSARs) for nanomaterials still needs to be validated. Thus, the use of such models for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied on a case-by-case basis only. However, results from non-testing methods can be useful information in the context of weight of evidence or can provide essential information for the planning of an animal test. A range of *in silico* models, such as those used to determine nanomaterial kinetics, QSARs and physiologically based pharmacokinetic (PBPK) models have been developed for nanomaterials ([64], [65], [66] [67], [68], [69]. *In silico* modeling could be also useful to provide an initial prediction of deposited and retained dose in the lung (e.g. the Multi-Path Particle Dosimetry (MPPD) model). As with any *in silico* model, care must be employed with regard to the applicability domains of these models.
- The use of grouping and read-across approaches is another step to consider before performing animal testing. Annex VI of the REACH Regulation as amended stipulates that "*where technically and scientifically justified, the methodologies set out in Annex XI.1.5 shall be used within a registration dossier when two or more forms of a substance are "grouped" for the purposes of one, more or possibly all the information requirements*". In this respect, it is advised to consider the ECHA guidance *Appendix R.6-1 for nanoforms applicable to the Guidance on QSARs and Grouping of the Chemicals* [1] when data on other (nano)forms[11] of the same substance are available. Regarding read-across and/or grouping between (nano)forms of different substances the advice provided in the ECHA Guidance Chapter R.6 on QSARs and Grouping of the Chemicals [70] and its nanospecific appendix [1] may be considered. Lamon et al. 2018 reviewed the available approaches and case studies on the grouping of NMs to read-across hazard endpoints. The grouping frameworks identified hazard classes depending

[10] This advice is applicable for all endpoints relevant for human health, i.e. not only to those having a nanospecific entry in this document.

[11] The term (nano)form intends to cover nanoforms and non-nanoforms of the substance

on PC properties, hazard classification modules in control banding (CB) approaches, and computational methods, that can be used for grouping for read-across [71]. Grouping frameworks are currently being developed, e.g. by the EU project GRACIOUS, to substantiate nanospecific read-across and grouping also for regulatory purposes (https://www.h2020gracious.eu/).

***In vitro* studies**

This section discusses the possible contribution of *in vitro* data to the adaptation possibilities.

In accordance with Article 13(1) of the REACH regulation, "*Information on intrinsic properties of substances may be generated by means other than tests, provided that the conditions set out in Annex XI are met*". The information from *in vitro* tests should always be considered before performing an animal test.
It has been shown that *in vitro* assays (e.g. [72], [73], [74]) that are not yet formally validated but can be demonstrated to be scientifically valid, may be useful on a case-by-case-basis for the assessment of nanomaterials, and can be used as part of a weight of evidence approach that involves thorough physicochemical characterisation of NMs, *in vitro* screening tests including '-omics', the use of non-testing approaches (*in silico* models, read across) and the use of OECD and EURL ECVAM validated/ approved *in vitro* methods. [75], [16], [76] include provisions for the acceptance of data from *in vitro* studies.

According to OECD [77] for *in vitro* testing the "*Characterisation of the materials should be undertaken in the cell culture medium used both at the beginning of treatment and, where methodologies exist, after treatment. The intent when applying nanomaterials to a cell culture medium is to create conditions that are comparable, to the extent possible, with the biological and physiological conditions within the in vivo system*". Considerations for *in vitro* testing of NMs, which should lead to increased reliability and relevance are given in the scientific literature [78]. Drasler et al. 2017 [78], concluded that the most important criteria to produce reliable and robust data from *in vitro* nanotoxicological assays are: (i) detailed NM characterization, including physicochemical properties before, during and after the testing, (ii) use of comparable and realistic dose metrics and test conditions, and (iii) implementation of chemical positive and negative controls and reference NMs allowing for comparison between studies, both intra- and interlaboratory.

An analysis of the applicability of *in vitro* methods to NMs and recommendations for the adaptations needed is available [79].

3.1.2. Advisory note on the consideration of assay interference

Various nanomaterials have on occasion been found to interfere with several commonly used assays for determining their cellular or toxic effects. For example, some nanomaterials may contribute to the absorbance or fluorescence of colorimetric or fluorometric assays. In addition, due to their large surface area, nanomaterials may bind to assay components including the substrates (e.g. CNT with the reagent in MTT 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide assays; [80], Fe_3O_4 with WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium) [81] or the biomarker being measured, (e.g. lactate dehydrogenase (LDH) and cytokine proteins; see for example [82]). Please note that other factors such as coatings or impurities may also have an influence on the assay.

A summary list of potential sources of nanomaterial interference with commonly used *in vitro* assays has been developed by Kroll *et al.* [83] and is reproduced in the table below.

Table 1: Potential sources of nanomaterials interference with commonly used assays

Cytotoxicity assay	Detection principle	Nanomaterials interference	Altered readout	Nanomaterials type
Cell viability				
MTT/WST-1	Colorimetric detection of mitochondrial activity	Adsorption of substrate	Reduced indication of cell viability	Carbon nanomaterials
LDH	Colorimetric detection of LDH release	Inhibition of LDH	Reduced indication of necrosis	Trace metal-containing nanomaterials
Annexin V/ Propidium iodide	Fluorimetric detection of phosphatidylserine exposure (apoptosis marker) Propidium iodide staining of DNA (necrosis marker)	Ca^{2+}-depletion Dye adsorption	Reduced indication of apoptosis Reduced indication of necrosis	Carbon nanomaterials
Neutral red	Colorimetric detection of intact lysosomes	Dye adsorption	Reduced indication of cell viability	Carbon nanomaterials
Caspase	Fluorimetric detection of Caspase-3 activity (apoptosis marker)	Inhibition of Caspase-3	Reduced indication of oxidative stress	Carbon nanomaterials
Stress response				
Dichlorofluorescein (DCF)	Fluorimetric detection of ROS production	Fluorescence quenching	Reduced indication of oxidative stress	Carbon nanoparticles
Inflammatory response				

ELISA (enzyme-linked immunosorbent assay)	Colorimetric detection of cytokine secretion	Cytokine adsorption	Reduced indication of cytokine concentration	Carbon nanoparticles Metal oxide nanoparticles

It should be noted that the above list is not exhaustive and the potential for inhibition or enhancement of test results should always be investigated. The agglomeration, dispersion and/or dose may influence the outcome of the test.

Assay interference is not limited to colorimetric assays. Interference with medium components such as medium depletion or corona formation by adsorption of essential nutrients or interaction with buffer salts, resulting in increased disssolution of nanoparticles was reported [84], [85].

Within some standard methodologies, the method requires the use of spiked sample (addition of a known reference/control sample) to test for inhibition or enhancement of the spiked control. This is evaluated by assessing the measured value against the expected value, which should be a cumulative value of the spike and of the sample.

Assay interference should always be investigated wherever possible, irrespective of standard method requirement. In case it would not be possible, this should be justified. Furthermore, for many of the studies reported, it is not possible to ascertain whether the assays were adequately controlled to assess for interference. Thus, if other methods for assessing interference are not available, as a general precaution, it is reccommended to use more than one assay to assess the studied endpoint or effect, as for example advised by Landsiedel *et al*. [86] for genotoxicity studies. The potential for inhibition or enhancement of the test result may impact the validity of relevant *in vitro* test methods. For example carbon nanotubes are suspected to interfere with the MTT assay [87] or WST-1 assay [81] and this may cause issues with tests such as OECD TG 431/EU B.40 bis Human Skin Model tests (EPISKIN™, EpiDerm™) which use the MTT assay. However, knowledge on nanomaterial assay interference is incomplete and so precautions to ensure the validity of an assay, such as the above-mentioned use of control spikes could be used.

Due to the potential for interference resulting in misleading results in numerous assays, utmost care should be taken in testing for such interference and appropriate controls should be used for the assays.

3.2. Specific advice for individual endpoints

3.2.1. Acute toxicity

The parent guidance Section 7.4 provides the general testing strategy for acute toxicity. The advice provided in the parent guidance should be followed together with the recommendations given in this section.

According to the revised REACH Annex VII, Section 8.5.1 Column 2: *"For nanoforms, a study by the oral route shall be replaced by a study by the inhalation route (8.5.2), unless exposure of humans via inhalation is unlikely, taking into account the possibility of exposure to aerosols, particles or droplets of inhalable size"*.

Hence, when the dossier covers nanoforms, testing by a route other than inhalation needs to be justified. The inhalation route is appropriate if exposure of humans via inhalation is likely taking into account the possibility of exposure to aerosols, particles or droplets of an inhalable

size. Guidance on the interpretation of physico-chemical data regarding respiratory absorption can be found in Table R.7.12-2 in Chapter R.7c of the Guidance on IR&CSA. The information derived from in vitro models ranging from cell lines grown in monoculture to lung-on-a chip (LOC) microphysiological systems and in vitro airway models aiming to characterise the cellular and molecular mechanisms responsible for the effects of inhaled toxicant exposure [88] could be useful in a WoE approach and provide an initial insight in the toxicity of the substance.

In the event there is no exposure of humans via the inhalation route then the oral route (as for most substances) is the default first choice of administration at Annex VII.

The current acute inhalation test guidelines employ death as an endpoint (OECD TG 403 and TG 436) or replace lethality as an endpoint with evident toxicity as fixed concentration procedure (FCP) (OECD TG 433). It is however, acknowledged that none of these test guidelines contain provisions specific for nanomaterials. Nevertheless, the recently updated OECD GD 39 provides some advice applicable for the acute inhalation in case of testing nanomaterials [7]. As sometimes for low density materials it is not feasible to reach the top dose, the physico-chemical properties including tested material density should be taken into account when dosing for the acute studies. The OECD GD 39 emphasizes that "*Knowledge of dustiness and particle size for solid test chemicals will allow for selection of the ideal testing approach and starting concentration that will enhance respirability (e.g., through the use of micronization)*" and that "*The particle size distribution should be determined at least once during a single exposure study for each concentration level using an appropriate method of measurement.* ". OECD GD 39 also states that "*Aerosol testing at greater than 2 mg/L should only be attempted if a respirable particle size can be achieved*." For nanomaterials, the degree of agglomeration increases with dose. The degree of agglomeration affects the aerodynamic diameter and thereby the deposition in the lung. For oral exposure, a higher degree of agglomeration can reduce the absorption. The consequences of the dose on agglomeration should therefore be taken into account when designing and interpreting acute toxicity tests.

3.2.2. Repeated dose toxicity

Annexes VIII-X, Section 8.6.1. and Annexes IX-X, Section 8.6.2., Column 2 of REACH Regulation (EC) No 1907/2006 amended (underlined text) for nanoforms' testing, provide that:

"*For nanoforms toxicokinetics shall be considered including recovery period and, where relevant, lung clearance.*"

and under "*Further studies shall be proposed by the registrant or may be required by the Agency in accordance with Articles 40 or 41 in case of*:" third indent:

"*indications of an effect for which the available evidence is inadequate for toxicological and/or risk characterisation. In such cases it may also be more appropriate to perform specific toxicological studies that are designed to investigate these effects (e.g. immunotoxicity, neurotoxicity, and in particular for nanoforms indirect genotoxicity),*".

In contrast to acute toxicity testing, the information requirements for repeated dose testing of nanoforms do not prescribe inhalation as default route. However, Column 2 of Annex VIII, Section 8.6.1. and of Annex IX, Section 8.6.2. specify that "*Testing by the inhalation route is appropriate if exposure of humans via inhalation is likely taking into account the vapour pressure of the substance and/or the possibility of exposure to aerosols, particles or droplets of an inhalable size".* Therefore, inhalation can, in most situations, be seen as the most likely route of exposure to nanomaterials, and thus the following focuses on inhalation testing.

The testing protocols to be followed in repeated dose testing by inhalation are OECD TG 412 [89] and TG 413 [90] together with the guidance provided in OECD GD 39 [7]. When testing poorly soluble solid aerosols that are likely to be retained in the lungs, Option B in the Annex of OECD TG 412/413 should be used. OECD GD 39 defines the poorly soluble particles (PSPs) as "*Solid aerosol particles deposited in the lung that do not undergo rapid dissolution and clearance*" and states that "*A poorly soluble material is generally understood as having a solubility of less than 0.1 g dissolved in 100 ml dissolvent within 24 hours*." [7]. A recent expert group [8] on the hazards and risks of poorly soluble particles of low toxicity, convened that PSPs can be defined as particles for which their alveolar macrophage-mediated clearance rate is not shortened by their dissolution rate in the lung and also gives more specific criteria determining a PSP. For chemicals that accumulate in the lung or translocate into specific accumulating organs following repeated exposures, toxicokinetic shall be considered, as the accumulated dose is partly a function of clearance. Therefore, for these nanoforms the lung clearance investigation is considered relevant. This is also in agreement with OECD TG 412/413, which require the measurements of lung burden when a range-finding study or other information demonstrates that a poorly soluble aerosol is likely to be retained in the lung. For PSPs, the rat lung burden is an important issue to consider in the toxicological outcome and therefore a special chapter within this section is included.

For fibre-like particles, in addition to the overload of macrophages, frustrated phagocytosis has also been proposed as playing a role in their toxicity [91] and may determine impaired lung clearance, even below overload. The activated macrophages stimulate an amplified pro-inflammatory cytokine response from the adjacent pleural mesothelial cells [92]. This mechanism of toxicity is not addressed further in this guidance. The OECD GD 39 specifies that for chemicals with fibre geometry, additional specific observations, such as interstitial and pleural detection and possibly quantification, depending on fibre dimensions, may be needed when performing an OECD TG 412/413 study [7].

When considering the testing strategy for repeated dose toxicity (Section 7.5.6. of REACH Guidance R.7.a.) of nanoforms it should be noted that:

- Especially for workers (and in some cases for consumers, e.g. in case of sprayable products) inhalation is the most likely route of exposure to nano(particles), nano aerosols and dust. Hence, the repeated dose toxicity studies should be performed via inhalation, unless there is convincing information (e.g. uses, dissolution rate) that justifies another route. Any modification of the protocols described in OECD TG 412 and 413 ([89] and [90]) should be justified;

- When dose range finding studies or repeated dose studies are performed, for PSPs, it is recommended to collect additional toxicokinetic data as described in *Appendix R7-2 for nanomaterials applicable to Chapter R7c Endpoint specific guidance*). In addition, to make full use of the test, if there is a particular concern it is recommended to address it within the study design (e.g. accumulation, specific organ toxicity, etc.).

- When testing PSPs, it may be necessary for a range-finding study to be longer than 14 days to allow for a robust assessment of test chemical solubility and lung burden [7]. Dissolution data in simulated lung fluid and lysosomal fluid are important for an informed decision before starting the animal studies. Annex VII, section 7.7. column 1, of the REACH Regulation specifies, beside the water solubility data requirement, that "*for nanoforms, in addition the testing of dissolution rate in water as well as in relevant biological and environmental media shall be considered.*". Therefore the information on dissolution must in principle be available before the *in vivo* testing.

- Exposure concentrations should be selected to cover the entire range of lung burdens, i.e., concentrations ranging from those which do not delay clearance to those which do delay clearance. Similarly, post-exposure periods should not be markedly shorter than one elimination half-time of the particulate test article [7].

- The doses to be used in repeated dose inhalation studies should not exceed the maximum tolerated concentration. However, the doses in hazard tests needs to cover a range of doses to be able to detect any potential hazard. This means the inhalation studies with PSPs should include an exposure concentration high enough to produce a toxic effect or an overload of particle clearance inducing lung inflammation, as well as exposures not causing lung particle overload. A similar approach was proposed for the design of chronic inhalation studies [8]. OECD TG 412 [89] and TG 413 [90] provide advice on doses to be used. This includes the provision that the highest dose should elicit unequivocal toxicity without causing undue stress to animals or affecting their longevity.

- For nanomaterials, the degree of agglomeration increases with dose. The degree of agglomeration affects the aerodynamic diameter and thereby the deposition in the lung. For oral exposure, a higher degree of agglomeration can reduce the absorption. The consequences of the dose on agglomeration should therefore be taken into account for designing and interpreting repeated dose toxicity tests. *In silico* modeling could be useful to provide an initial prediction of deposited and retained dose in the lung. For instance, the Multi-Path Particle Dosimetry (MPPD) model can be used to assess the influence that the characteristics of the aerosols (MMAD, GSD, solubility, density, hygroscopicity) can have on the dose [93].

- Information from the dose–range finding studies and lung burden data are useful for the interpretation of a study outcome. Knowing the retained lung burden during exposure and at different time points post-exposure, the following can be determined: nanomaterials lung clearance and retention kinetics, critical dosimetric comparisons to clearance and retention data of well-established benchmark materials (allowing comparative Dose - Response relationships to be analysed) [94]. The selection of PSP benchmark materials should to be justified. Humans and rodents differ significantly in their biokinetic functions so the effects of nanmomaterials observed in rodents do not directly translate to humans. Nevertheless, the lung burden data cannot be used to exclude the relevance of toxicological findings in the experimental animal for human risk assessment [89], [90]. A detailed discussion on the concept of rat lung burden of PSPs and its impact in the toxicological assessment is provided in section 3.2.2.1.

- Although lung burden measurement is mandatory at only one post-exposure observation (PEO) period in Option B (at PEO-1) of OECD TGs 412/413, the TGs provide that a minimum of two lung burden measurements are necessary when investigating clearance kinetics. If the use of two post-exposure time points is considered sufficient, lung burden measurements may be performed at PEO-1 (main study) and at PEO-2 (recovery group) only, if timing for evaluation of recovery and lung clearance can be aligned. Lung burden should be measured for all concentrations [7]. Lung burden measurement at three time points allow curve fitting on post-exposure clearance kinetics. The need for additional post-exposure observations, the duration of the post-exposure interval and the timing of the post-exposure observations (PEOs) are determined by the study director based upon results from, among others, the range-finding study.

- The data on lung burden and clearance are also important in the context of read-across of the hazard data from studies using different nanoforms of the same substance. Incorporating measurement of retained lung burden in the study design allows a coupling of subchronic and subacute inhalation data with other appropriate test data (from shorter-term inhalation,and cellular and acellular assays) which can facilitate grouping and may result in saving animals [94]. For example for nanoforms with different particle sizes, the same external concentrations can result in differences in

retained dose [94]. Conversely, different external concentrations can result in the same retained dose for different particle sizes.

- Assessment of organ burden in potential target tissues other than lung should be considered to estimate the systemic availability. As a first indication of translocation, detection and burden measurements in local lymph nodes may be determined in addition to lung burden measurements. To monitor the fate and effects of PSPs in the body it is recommended to collect the samples at several time points and/or from different organs and tissues. Data from range-finding studies, if proven robust, could be used to determine the appropriate sampling times in order to investigate these toxicokinetic parameters (i.e. nanomaterials burden in secondary organs). If satellite animals are used for lung burden, the same animals should be used for any other organ burden assessment deemed necessary by the study director. In ISO TR 22019 [21] the liver, spleen, lung, brain, kidney, lymph nodes at the organ of entry, and bone marrow are considered relevant organs for the toxicokinetics of NPs. Other relevant tissues include reproductive organs (uterus and testis) and adipose tissue [53] [95]. The issue of bioaccummulation and organ burden is further discussed in section 2.1.3 of Appendix R7-2 to Chapter R.7c.

- Since the lower respiratory tract (i.e., the alveoli) is the primary site of deposition (depending on agglomerate size) and retention for inhaled nanoparticles, bronchoalveolar lavage (BAL) analysis is a useful complementary technique to histopathology predict and quantitatively estimate pulmonary inflammation and damage (for further information on BAL parameters please see OECD TGs 412 and 413 [52] [53]). BAL analysis allows dose-response and time-course changes of alveolar injury to be suitably investigated. Therefore, for nanomaterials testing, it is mandatory to include BAL analysis (further details are given in Section R.7.5 (repeated dose toxicity) of *Chapter R7.a of the Guidance on IR&CSA* (Endpoint specific guidance) [11]. BAL fluid should be analysed for all the concentrations [7].

- It is strongly advised to use more than one dose-describing metric and to include the mass metric. The choice of method(s) selected should be justified as described in Section 3.2.2.2.

OECD TG 412, TG 413, and GD 39 should be consulted when designing developmental and reproductive toxicity studies using TG 422, TG 421, TG 443 and TG 414 with exposure by the inhalation route.

With regard to the 'old' studies performed with nanoforms prior to the update of OECD TG 412/413, several conditions need to be fulfilled in order for these studies to be considered appropriate:

- The test material is well characterised (i.e. size distribution, shape and aspect ratio, surface treatment and specific surface area is known) and ensure the representativeness for the registered NF/set of NF, and
- The parameters from the testing guidelines valid at the time when the study was performed are covered. In case that only the respiratory system has been investigated and not all the organs listed for histopathology by the testing guidelines, the study cannot be seen as equivalent to an OECD 412 or 413.

If the study fulfils the conditions above, then it can be considered for the use under WoE. The updated testing guidelines specify for the substances likely to be retained in the lung that BAL and lung burden measurements at specified PEO should be performed. For the studies performed prior this update, for which this information is missing, the information must be substantiated using weight of evidence approach with further data:

- dissolution studies in artificial lung lining fluid and lysosomal fluid.
- in cases where no toxicity is seen in an old repeated dose study, the lack of toxicity seen in vivo can be substantiated with data from studies assesing cytotoxicity and inflammation markers with the registered nanoform in relevant cell lines. Overall, the cytotoxicity assays should always consider the cell type and nanomaterials used, therefore a generalized recommendation cannot be given [78]. Various factors could have contributed to the "no effects" outcome seen in the old in vivo study such as agglomeration, dispersion protocol, adherence of the tested substance to the delivery system etc.

For the oral studies (OEC 407, OECD 408, OECD 421/422) the same principles apply. If a proper characterisation of the test material (nanoform) is provided (i.e. size distribution, shape and aspect ratio, surface treatment and specific surface) and ensure the representativeness for the registered NF/set of NF, and if the study has been conducted according to the testing guidelines (including analysis of all the organs listed in the TG) then the study can be considered for the use of WoE. The information from such a study must be substantiated with further data such as dissolution studies in artificial gastrointestinal fluid and lysosomal fluid. In cases where no toxicity is seen in the repeated dose study, cytotoxicity studies in relevant mammalian cell lines could substantiate the lack of intrinsic toxicity seen *in vivo*. If an available oral study complies with all the criteria listed above, it can be used only for the relevant route of exposure, i.e. oral.

3.2.2.1. Advisory note on the consideration of lung burden within inhalation toxicity assessment

This section describes the concept of rat lung burden of PSPs and the associated effects, the differences between species and the extrapolation of the results to humans. Care should be taken when interpreting lung burden in the context of human risk assessment. Lung effects observed in animals exposed to PSPs by inhalation should be considered relevant for the hazard in humans unless it can be clearly substantiated otherwise.
Results from inhalation studies in rats have shown that the PSPs can induce serious adverse pulmonary effects if inhaled in high concentrations due to material accumulation, as lung clearance mechanisms are not able to remove materials at the same time or at a higher rate as the dose is delivered. This condition named "lung overload", occurs when the retained particle burden in the lung exceeds a certain threshold [96]. A recent expert group [8] on the hazards and risks of poorly soluble particles with low toxicity (PSLT) proposed the following expert consensus definition "*Lung Particle Overload is a phenomenon of impaired clearance in which the deposited dose of inhaled PSLT in the lung overwhelms clearance from the alveolar region leading to a reduction in the ability of the lung to remove particles. Lung Particle Overload results in an accumulation of particles greater than that expected under normal physiological clearance. This definition is relevant for all species (not just rat). This definition is independent of the underlying mechanism(s) (e.g. macrophage mobility impairment). A key issue is that increased particle retention due to large lung burdens needs to be differentiated from that due to high cytotoxicity particles (e.g. quartz).*"During prolonged exposure of rats to PSPs, the lung burden of particles increases until equilibrium is reached between deposition and clearance of particles [97] as shown by the curves A, B and C in Figure 2. This equilibrium can be reached very fast or may take up to many days. Below the lung overload threshold, particles are cleared via normal mechanisms at a constant clearance rate, in general generating little or only a minor or reversible response (exposure concentrations in curves A and B).

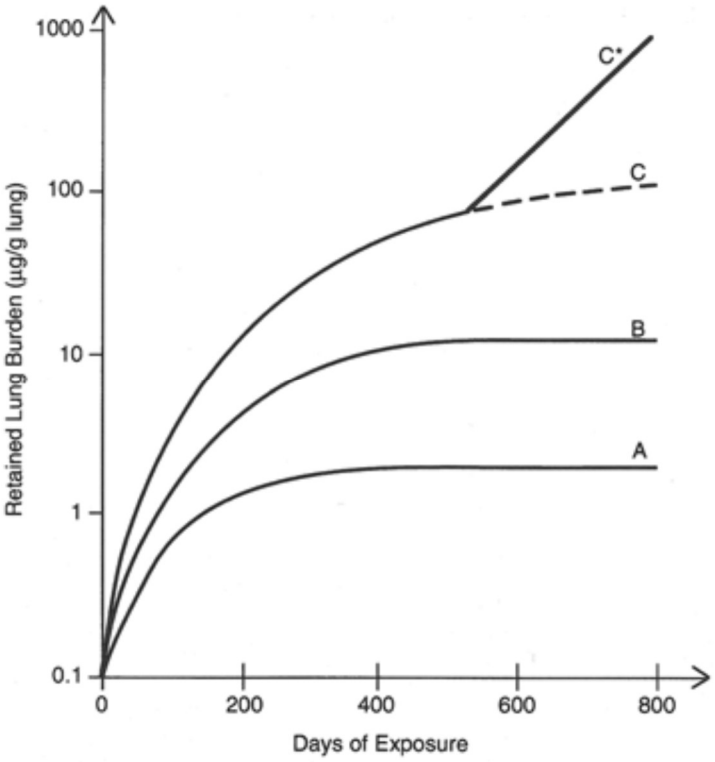

Figure 2: Schematic representation of the relationship between retained lung burden and duration of exposure leading to the phenomenon of lung overload.

Curves A, B, and C are associated with progressively increasing exposure doses. If the exposure level is sufficiently high and the duration of exposure sufficiently long, alveolar macrophage-mediated clearance of particles can be overwhelmed. When this occurs, the retained lung burden increases linearly with further exposure (curve C*). Reproduced from [97].

Once the threshold has been reached, the clearance mechanisms of the lung become overloaded. This is typified by a progressive impaired particle clearance from the deep lung, reflecting a breakdown in alveolar macrophage (AM)-mediated dust removal due to the loss of AM mobility [96]. This is shown in the C* curve of Figure 2 whereby at the point of threshold, particle retention increases rather than an equilibrium being established (as demonstrated by the dashed line).

The result of this net increase in particle accumulation is persisting lung inflammation, cessation of alveolar-mediated clearance and an increase in accumulation of particle laden macrophages and/or free (non-phagocyted) particles within the lung alveoli. The potential progression of inflammatory reactions toward a granulomatous type in rats was found to depend on the exposure duration and the level of the particle (surface) burden in the lung [98] as well as of the volumetric load of alveolar macrophages [99].

The situation of lung burden is most commonly associated with repeated inhalation exposure of rats to PSPs but it can also occur after single or repeated instillation of PSPs into the lung (due to a high deposition fraction as a result of direct instillation) or possibly as a result of a single massive inhalation exposure [100]. Since this phenomenon occurs at relatively high exposure levels of respirable PSPs it is often argued that the observed adverse effects are a product of the lung burden caused by experimental conditions and not always a true reflection on the intrinsic toxic potential of the particles to cause inflammation, fibrosis and cancer. Exposure to highly reactive or toxic particles may cause inflammation, fibrosis and cancer at lower exposure levels (non-overload conditions) due to intrinsic properties of the particles themselves.

Care should be taken when interpreting lung burden in the context of human risk assessment. Lung effects observed in animals exposed to PSPs by inhalation should be considered relevant for the hazard in humans unless it can be clearly substantiated otherwise. When designing a new study, the doses to be used in repeated dose inhalation studies should not exceed the maximum tolerated dose. However, the doses in hazard tests needs to cover both lower doses (seen as a valid approach to determine the lung overload together with the time-course measurement of actual lung particle burdens [8]) as well as higher doses which enable to detect any potential hazard so that the study can fulfill its regulatory purposes. OECD TGs 412 [89] and 413 [90] provide advice on dosages to be used. This includes the provision that the highest dose should elicit unequivocal toxicity without causing undue stress to animals or affecting their longevity.

In the studies performed with PSPs the measurement of changes in lung burden over post-exposure time(s) provides essential information on lung clearance and allows clarification of the deposited vs the exposed particle amount. Different imaging techniques may also be used for a semi-quantitative assessment of the PSPs in the tissue [101]. The assessment of the dissolution potential as an indicator for biopersistence can also be done using *in vitro* systems [18].

Information on clearance and biopersistance is important in the context of toxicokinetics, read-across and weight of evidence.

Rat is currently considered the most sensitive species for inhalation toxicity testing for nanoparticles [102]. However, as it can be difficult to interpret the findings of overload of alveolar macrophages in rat studies, a better understanding of the rat lung burden and its relevance to humans is needed. A recent study that analysed the issues of impaired clearance and the relevance for human health hazard and risk assessment [103] pointed out that while rats and humans have a clear difference in lung clearance kinetics of PSPs, the fact that the appropriate dose metric often is unknown and the level of clearance impairment is seldom assessed makes it difficult to relate the effects observed to an impaired clearance. Several studies have assessed the responses to lung overload in different species, and the relevance of this data for humans. For instance, in a comparative study assessing the long-term pulmonary response of rats, mice and hamsters to inhalation of ultrafine grade titanium dioxide [62], the same air concentrations caused overload effects in rats and mice but not in hamsters. Also, the inflammatory and pathological responses were less severe in mice than in rats and they diminished with time irrespective of the similar lung burdens ([62], [104]). However, in relation to the relevance of animal data for humans, other studies have pointed out that the lung responses to high lung burdens of PSP of low toxicity can be qualitatively similar in rats and humans [105]. Based on experience with exposure of coal miners, a primate-specific interstitial particle sequestration compartment is hypothesed [106]. Borm *et al* [99] discuss whether this mechanism could explain why humans, in contrast to rats, seem not to have an increased risk of lung cancer under lung overload conditions [99]. Nevertheless, there seems to be some conditional evidence for particle overload associated with impaired clearance in coal miners [99]. While lung inflammation after PSPs exposure does not necessarily lead to tumour formation in humans and carcinogencity [8], fibrosis is however, a response seen upon exposure to PSPs in both rats and humans, though not in mice [103]. Independent of the underlying mechanism(s) (e.g. macrophage mobility impairment), the concept of lung overload is relevant for all species (not just rat) and under overload conditions, the rat was considered "*not to be unique in its inflammatory, hyperplastic, and fibrotic responses to PSLT*" albeit the rat is more sensitive [8].

Therefore, the use of existing rodent data, obtained after exposure to high doses of PSPs, cannot automatically be dismissed as irrelevant for humans in the context of risk assessment and the interpretation of such data should be approached with caution. In the case of adverse effects observed in animals under overload conditions, the relevance for humans has to be assumed *a priori*; any claimed non-relevance for humans must be supported by data.

For further information, Bos et al. 2019 [103] and Driscol and Borm, 2020 [8] provide a detailed analysis on the pulmonary toxicity in rats following inhalation exposure to PSPs and

discuss the relevance of rat hazard studies for human health and risk assessment. There are also other several review articles covering the subject of lung overload such as Miller [97], who provided an in-depth discussion of particle deposition, clearance and lung overload. Borm *et al* [107] discussed the importance of overload in the context of risk assessment whereas in an editorial of Borm et al, [99] the state of the art concerning lung particle overload concepts is summarized. These reviews also present different views on how to assess lung overload and how to interpret the data and emphasize the fact that the topic is still under debate.

In conclusion, based on the current understanding, lung effects observed in experimental animals exposed to PSPs by inhalation should be considered relevant for humans. When designing a new study, the doses in hazard tests needs to cover both, lower doses as well as higher doses to enable to detect any potential hazard and to fulfill its regulatory purposes.

3.2.2.1.1. Metrics

The question of which dose metric best describes the association between deposited dose in the lung, and subsequent inflammation and impaired clearance function is particularly relevant. There have been several suggested metrics but volumetric load of AM and surface area appear to be the most relevant [99] in interpreting lung overload-related as well as other adverse effects and in establishing limit concentrations. Morrow *et al*. [96] hypothesised that overload begins when the particulate volume exceeds approximately 60 μm^3/AM (which produces a 6% increase in the average alveolar macrophage volume) and that total cessation of AM-mediated clearance occurs when the particulate volume exceeds 600 μm^3/AM (producing a 60% increase in the average alveolar macrophage volume). Extending the Morrow concept, Pauluhn ([108], [109]) modelled a generic particle displacement volume threshold for agglomerated PSPs.
Oberdoerster et al. [110] suggested that the particle surface area better correlates the overload with impaired clearance. Several studies suggest that, particle surface area correlates well with induced pathogenic events in lung ([107], [111], [112]). In a study by Tran *et al*. [112] data from a series of chronic inhalation experiments on rats with two poorly soluble dusts (titanium dioxide and barium sulphate) was analysed. The results indicated that when lung burden was expressed as particle surface area, there was a clear relationship with the level of inflammation and translocation to the lymph nodes. Most usefully, based on the shape of the statistical relationship for lung response to particles, the authors suggested the presence of a threshold at approximately 200–300 cm^2 of lung burden for "low-toxicity dusts" in rats.

Whilst some studies indicate mass as a less sensitive indicator of lung overload [113], the mass concentration is still important because there is already a large body of data and research on the exposure to and toxicity of particles using the mass-based metric. Therefore, for the sake of comparison(s), the mass concentration should always be reported.

Other studies ([114]) found that the particle number or the number of functional groups in the surface of nanoparticles ([115], [116]) was the best dose metric.

The most relevant dose metric seems to vary depending on the specific nanoparticle in question. Surface areas, mass, and the particle number should be reported in order to establish the dose metric that best describes the association between deposited dose in the lung, overload conditions and the subsequent pathogenic effects and in order to establish the dose metric most relevant for risk assessment.

It is therefore vital to fully characterise test materials, so that the measured response can be retrospectively correlated with multiple-dose metrics, without the need for repeat testing. In general, the more metrics are reported the better.
In conclusion, it is strongly advised to use more than one different dose-describing metric and to justify the choice of the selected methods. However, since all regulatory limits and effects values are based on mass concentration, this is still default and needs to be reported.

3.2.2.1.2. Main recommendations for lung burden

- Data from existing studies performed with high doses of PSPs showing adverse effects cannot automatically be dismissed as irrelevant for humans. When planning/performing, new studies, the use of excessively high doses should be avoided (in order not to exceed the maximum tolerated dose). However, the doses in hazard tests should include an exposure concentration high enough to produce a toxic effect or an overload of particle clearance inducing lung inflammation, as well as concentrations not causing lung particle overload.
- Lung burden data provide useful information on the pulmonary (retained) dose as well as on clearance behaviour and may support the read-across and weight of evidence approaches. Lung burden and BALF must be measured for all concentrations [7].
- The most relevant metric should be used and mass metric should always be included. It is strongly recommended to use more than one metric.

3.2.2.2. Indirect genotoxicity

The term 'indirect genotoxicity' is mentioned in the REACH section on repeated dose studies (i.e. Annex VIII section 8.6.1, and Annex IX section 8.6.2): "*Further studies shall be proposed by the registrant or may be required by the Agency in accordance with Articles 40 or 41 in case of […] - indications of an effect for which the available evidence is inadequate for toxicological and/or risk characterisation. In such cases it may also be more appropriate to perform specific toxicological studies that are designed to investigate these effects (e.g. immunotoxicity, neurotoxicity, and in particular for nanoforms indirect genotoxicity)*,".

Formally, primary genotoxicity and secondary genotoxicity can be distinguished, as described by several authors [117], [118], [119]. Primary genotoxicity is a genotoxic effect caused by a substance that interacts with or is internalised in the target cell. Primary genotoxicity can be either 1) direct genotoxicity, where the substance enters the cell nucleus of the target cell, interacts directly with DNA (or disturbs the mitotic apparatus) to produce DNA lesions (or chromosomal modifications); or 2) indirect genotoxicity, where the substance does not directly interact with DNA of the target cell, but generates reactive oxygen/nitrogen species (ROS/RNS) inducing oxidative DNA damage, or induces damages to the mitosis apparatus or to enzymes involved in e.g. DNA replication or DNA repair, or antioxidant activity. Secondary genotoxicity involves mechanisms in which the substance does not interact directly with the target cell but produces a particle-induced inflammatory response in neighbouring cells/tissues that generates ROS/RNS and induces a (secondary) genotoxic effect in the target cell.

Both primary and secondary mechanisms (as defined above) were reported to play a role in the genotoxic effects induced *in vivo* by nanomaterials [120], [121], [122], [123]).

In the scope of REACH column 2 of Annex VIII, 8.6.1 and of Annex IX, 8.6.2, the term "indirect genotoxicity" refers to oxidative DNA damage (which results from ROS/RNS generated either in the target cell or by inflammatory response in neighbouring cells). The observed indirect genotoxicity may thus be due to both primary and secondary genotoxicity mechanisms (as defined above).

The most frequently described mechanism to explain the genotoxicity of nanomaterials is an indirect mechanism, i.e. via generation of oxidative species or indirect consequences of inflammation [124], [86], [125], [126], [127]. The specific toxicological studies to investigate the indirect genotoxicity (i.e. DNA damage due to high levels of reactive oxygen/nitrogen species) could be triggered by available robust data showing that the nanoform induces a significant inflammation and/or the generation of high levels of oxidative species by the tested nanomaterial after repeated dose administration (e.g. 28-day study at Annex VIII). The

observed inflammation would be considered as relevant to trigger specific genotoxicity studies only in case it is persistent. In case an *in vivo* genotoxicity test required to address the data requirement under section 8.4 of REACH show positive results that are relevant for classification and labelling for mutagenicity hazard, such 'specific toxicological studies' would not be needed. The comet assay appears to be a suitable test to assess such genotoxic effects, because 1) the standard comet assay can detect some DNA damages induced by oxidative stress and 2) its sensitivity can be increased by using endonuclease (e.g. OGG1, FPG) to detect oxidised DNA bases that are not detected in the standard comet assay [127]. It is noted that the modified comet assay to detect oxidative DNA damages is not yet validated and the current OECD TG 489 (from 2016) for the standard comet assay does not provide recommendations for the modified comet assay. Moreover, the influence of inflammation on the comet assay results needs to be further investigated.

3.2.3. Mutagenicity and Carcinogenicity

The parent guidance[12] Section R.7.7 provides the general testing strategy for mutagenicity and carcinogenicity. The advice provided in the parent guidance should be followed together with the recommendations given in this section. The text below provides advice only on the mutagenicity endpoint.

The assessment of mutagenicity/genotoxicity generally relies on the investigation of *in vitro* (and possibly *in vivo*) effects for three major endpoints: i.e. gene mutation, clastogenicity and aneugenicity. It is now widely accepted, based on international collaborative studies and the large databases available, that no single assay can detect all genotoxic substances and that a battery of tests should be implemented.

Different groups have published several reviews on the genotoxic assessment of nanomaterials [128], [129], [126], [130] and the most recent comprehensive review [127] extended previous works with critical analysis of published data. All these reviews agreed on a number of recommendations that are described in this section (and also mentioned in recent guidance documents from EFSA [5] and SCCS [16]).

3.2.3.1. Bacterial (Ames) mutagenicity assays are not recommended

The bacterial reverse mutation (Ames) test (OECD TG 471 [131]/EU B.12/13: Bacterial reverse mutation test (*in vitro*)) detects point mutations in *Salmonella typhimurium* and *Escherichia coli* ([132], [133]; [134]). However, the strains of bacteria used in the standard assays do not appear to have the ability to take up/internalise nanoparticles, as they lack mammalian mechanisms of endocytosis, pinocytosis, and phagocytosis [135], [136], [86], [137], [127]. The updated text of Annex VII of REACH quoted in section 3.2.3.2 below reflects the agreement by the scientific community that the standard bacterial Ames assay test is usually not adequate to be part of the battery of mutagenicity tests for 'poorly soluble' particles ([86], [128], [135], [129], [126], [130], [127]). In 2014, OECD 43 [77] already stated that *'The use of the Ames test (TG 471) is not a recommended test method for the investigation of the genotoxicity of nanomaterials'*.

3.2.3.2. Recommended approach for gathering mutagenicity information on nanomaterials according to REACH

The revised Annex VII of REACH, for section '8.4.1 *In vitro* gene mutation study in bacteria', column 2 is now reflecting the agreement not to recommend the Ames test and, for the endpoint 8.4.1 *In vitro* gene mutation study in bacteria, column 2 reads "*The study does not need to be conducted for nanoforms where it is not appropriate. In this case other studies involving one or more in vitro mutagenicity study(ies) in mammalian cells (Annex VIII,*

[12] Endpoint specific guidance (Chapter R.7a, July 2017)

sections 8.4.2. and 8.4.3 or other internationally recognised in vitro methods) shall be provided".

In order to assess the *in vitro* mutagenicity of nanomaterials, the Ames test must not be used, unless there is evidence that the nanomaterial penetrates the cell wall of the bacteria or that indirect genotoxic effects due to generation of reactive oxygen species outside the bacteria. In most cases, the Ames test is not appropiate and one or more *in vitro* test(s) on mammalian cells must be performed at Annex VII instead. The registrant must provide at least one of the following tests:

1. one test detecting gene mutation: the *in vitro* mammalian cell gene mutation test using the thymidine kinase gene (according to OECD TG 490, latest updated in 2016) or the *in vitro* mammalian cell gene mutation test using the Hprt and xprt genes (according to OECD TG 476, latest updated in 2016),
2. one test detecting chromosomal aberration: the *in vitro* mammalian cell micronucleus test (according to OECD TG 487, latest updated in 2016), or the *in vitro* mammalian chromosomal aberration test (according to OECD TG 473, latest updated in 2016).

Given that the standard REACH data requirement at Annex VII (Ames test) investigates gene mutation, in case of a nanoform registered at Annex VII, ECHA considers appropriate that the registrant provides data from an *in vitro* test detecting also gene mutation, i.e. performed according to OECD TG 490 or OECD TG 476. At Annexes VIII to X, the registrant should provide data from two *in vitro tests*, one detecting gene mutation (OECD TG 490 or OECD TG 476) and one detecting chromosomal aberration (OECD TG 487 or OECD TG 473).

3.2.3.3. Recommendations for the *in vitro* tests

During the OECD/WPMN[13] expert meeting on the Genotoxicity of Manufactured Nanomaterials in Ottawa, Canada in November 2013 [77], several consensus statements were agreed and recommended to investigate the genetic toxicity of nanomaterials. These recommendations are further supported by more recent scientific literature (e.g. see reviews by Magdolenova *et al*. [126], Pfuhler *et al*. [129], Doak *et al*. [135]; Dekkers et al [138]; Elespuru et al. [127]).

3.2.3.3.1. Nanomaterials characterization in the test medium

When investigating nanomaterials, one must have or generate detailed data on the intrinsic properties of the studied nanomaterial as described in section 3.1.1.3 (e.g. size, shape, crystallinity, surface treatment …) as well as on its properties under the test condition. For *in vitro* genotoxicity test, the "characterization of the test material should be undertaken in the cell culture medium used during the test, both at the beginning of treatment and, where methodologies exist, after treatment" [127]. The recent JRC report describes relevant characterisation methods and apply them for gold, silica and silver nanoparticles [139]. Such characterisation helps to monitor the dispersion of the nanomaterial and to ensure that the cells are exposed to a sufficient effective dose under the *in vitro* conditions.

3.2.3.3.2. Verification of uptake into target cells

The verification of cellular uptake by appropriate methods is highly advised for *in vitro* genotoxicity tests. The importance of cell uptake was pointed out by the reviews mentioned above, the Nanogenotox report [140], the OECD report [77], and recent guidance documents from EFSA [5] and SCCS [16]. Several parameters (e.g. agglomeration and protein coating) can influence cell uptake.

In vitro tests should be performed on cell lines that have demonstrated the ability to take up

[13] WPMN, Working Party on Manufactured Nanomaterials

nanoparticles. A recent JRC report [141] showed that several cell lines (Caco-2, A549, CHO, V79 and TK6) demonstrated their ability to internalise the tested nanomaterials (gold, silica and silver nanoparticles). If a test is performed on a cell line for which the uptake capacity is unknown, the test data should demonstrate the uptake ability of the studied cell line. Such data can be generated using optical- and electron-based microscopy techniques (e.g. fluorescence microscopy, transmission electron microscopy) ([142], [143], [144], [145], [141]). It is noted that the fluorophore used for labelling may either influence the uptake or detach from the nanomaterial (which would complicate the interpretation of the analysis). The measurement of intracellular nanomaterial can be more or less challenging depending on the nature of the studied nanomaterial.

3.2.3.3.3. Recommendations to avoid interference with uptake or endpoint analysis

An *in vitro* mutagenicity test usually includes an experiment in the absence of metabolic activation system (S9mix) and another experiment in the presence of S9mix. The use of S9 mix in *in vitro* studies can affect the outcome of the tests: like for any other tested chemical, S9 can induce the formation of mutagenic metabolites (in case the nanomaterial can be metabolised); also, the addition in the culture medium of proteins (contained in S9) can modify the cellular uptake of nanomaterials ([126], [135] and [129]). It it thus recommended to perform *in vitro* tests in the presence of S9 only for the NMs composed of organic materials.

Cytotoxicity measurements described in relevant test guidelines (e.g*. relative population doubling, cloning efficiency or relative total growth) are appropriate to determine the top concentration that should be used for nanomaterials in vitro tests.* Moreover, the cytotoxicity should be assessed concurrently with the genotoxicity (not in separate experiments) [77] [127]. Since a variety of proliferaton or cytotoxicity assays employ colometry or fluorometry, dye interference should be checked beforehand (cf. 3.1.2).

"The intent when applying nanomaterials to a cell culture medium is to create conditions that are comparable, to the extent possible, with the biological and physiological conditions within the in vivo system" [64]].

According to Annex VIII 8.4.2 of REACH, a micronucleus test (OECD TG 487 [146]) or a chromosomal aberration test (OECD 473 [147]) is required. Nanogenotox concluded that the guideline for the testing of chemicals *in vitro* mammalian cell micronucleus test (OECD TG 487) is "applicable for nanomaterials but may need some adaptation in order to provide predictive results *in vivo*" [140].

A project on the adaptation of the *in vitro* mammalian cell micronucleus assay (TG 487 [146]) for nanomaterials testing is ongoing in the OECD WPMN rolling work plan (Project 4.95: Guidance Document on the Adaptation of *In Vitro* Mammalian Cell Based Genotoxicity TGs for Testing of Manufactured Nanomaterials). The project focuses on the physico-chemical characterisation of nanomaterials and protocol modifications (selection of cell type with respect to uptake mechanisms, use of cytochalasin B, timing of exposure to nanomaterials, specification of controls, dose ranges and dose metrics). Two intermediary reports studying gold, silica and silver nanoparticles were produced by this project and recently published: in 2018, on the physicochemical characterisation in water and in serum-containing cell culture media [139]; in 2020, on the *in vitro* cytotoxicity and cellular uptake in five different cell lines [141].

At this time, it is recommended to conduct genotoxicity tests for nanoforms according to the available OECD test guidelines, as for any other forms of a substance, with the exception of specific methods adaptations required for nanomaterials, as mentioned here and in the OECD guidance on Sample Preparation and Dosimetry [38]. For instance, if the *in vitro* micronucleus assay is performed using a mitosis blocking agent (cytochalasin B), it is recommended to add this agent post-treatment or to use a delayed co-treatment protocol, in order to ensure a period of exposure of the cell culture system to the nanomaterial in the absence of

cytochalasin B ([77], [5], [16], [127]). Moreover, cell lines derived from the expected target tissue(s) should be utilised, if available and possible.

3.2.3.4. *In vivo* test and exposure of target tissue

Prior to conducting an *in vivo* genotoxicity study, there is a need to have relevant toxicokinetic data or to conduct toxicokinetic investigations to assess whether the nanomaterial reaches the target tissue, where the target tissue is not the site of contact. An in vivo test is not applicable for detecting the genotoxicity of a nanomaterial if the nanomaterial does not reach the target tissue [77].

In the absence of toxicokinetic information demonstrating systemic availability and/or exposure of target tissue(s), it is recommended to investigate the genotoxic effects in the site of contact tissue(s). *The basis for selecting the route of administration for testing should be to consider the route most applicable to human exposure(s)* [77]. Currently inhalation is considered the most likely route for human exposure to nanoforms - at least for workers - (See R.7.a, Section R.7.5.6). The selected route of administration should be justified (and the issue of exposure of target tissues should be addressed).

Appendix R7-2 to Chapter R.7c

2.1.3 Guidance on Toxicokinetics

In the revised Annex VIII, Section 8.8.1. of REACH, a new requirement has been inserted in Column 2: *"For nanoforms without high dissolution rate in biological media a toxicokinetics study shall be proposed by the registrant or may be required by the Agency in accordance with Article 40 or 41 in case such an assessment cannot be performed on the basis of relevant available information, including from the study conducted in accordance with 8.6.1. The choice of the study will depend on the remaining information gaps and the results of the chemical safety assessment".*

The parent guidance R.7c (Section 7.12) [148] provides the general guidance on toxicokinetics and gives a general overview on the main principles of toxicokinetics for (dissolved molecular/ionic) substances. The advice provided in the parent guidance should be followed together with the recommendations given in this section when relevant. The advice in this section/appendix specifically applies to nanoforms without a high dissolution rate in biological media as described in Appendix R7-1 for nanomaterials applicable to Chapter R.7a Endpoint specific guidance, Version 3.0 –November2020, section 3.1.1.

It is acknowledged that the OECD TG 417 for toxicokinetics [149], generally intended for the oral route, does not contain specific provisions for nanomaterials. It does not either contain specific advice for administration of nanomaterials via the inhalation route. Furthermore, for dissolved chemicals the tissue distribution is concentration dependent, and an equilibrium is generally obtained between blood and organ concentration, whereas nanoparticles are rapidly removed from the circulation by cells of the mononuclear phagocytic system (MPS) [21]. Therefore, plasma is usually not a suitable sample to monitor NP exposure and plasma kinetic parameters such as plasma AUC are generally not relevant. Therefore, OECD TG 417 is not applicable to nanomaterials. Once a new test guideline applicable to nanomaterials is available, it should be used.

A standard project submission form (SPSF) for a new test guideline (TG) on toxicokinetics, specific to nanoforms, has been approved in April 2020. The TG is expected to be finalized by 2025. Until then, it is recommended to follow the advice given in this document and for example in the updated OECD GD 39 [7], OECD TGs 412 [89] and 413 [90], and in the ISO technical Report on toxicokinetics of nanomaterials [21].

The OECD TG 412 (Subacute Inhalation Toxicity: 28-day Study) [89] and the OECD TG 413 (Subchronic Inhalation Toxicity: 90-day Study) [90] have specific provisions for nanomaterials and are also suggesting additional investigations that may aid in the understanding of the toxicokinetics of the test substance. OECD TGs 412 and 413 require the measurements of lung burden when a range-finding study or other information demonstrates that poorly soluble particles (PSPs) are likely to be retained in the lung. For chemicals that accumulate in the lung or translocate/accumulate into specific organs following repeated exposures, a toxicokinetic investigation is recommended as the accumulated dose is partly a function of clearance. The updated OECD GD 39 [7] provides assistance on the conduct and interpretation of inhalation studies. In addition, it provides some advice on how to include toxicokinetic measurements in an inhalation toxicity study. The ISO technical Report on toxicokinetics of nanomaterials [21] provides useful considerations for performing toxicokinetic studies with nanomaterials. These include considerations on which factors may influence the toxicokinetics of nanomaterials, what are the analytical challenges regarding detection limits or quantification of nanomaterials in biological samples or what are the issues relevant for dosing conditions.

One particularity that differentiates the nanomaterials from the non-nanoform counterpart is the potential ability for some of them to translocate from the respiratory tract to secondary

target organs [150], [151]. Certain nanomaterials occur in the form of larger aggregates/agglomerates, and their behaviour in the body may not be too different from the bulk counterpart. However, other nanomaterials may become systemically available. Depending on size and surface modifications, the nanomaterials are prone to lymphatic transport mostly via the mononuclear phagocytic system [152] but they may also be directly translocated from the respiratory system into the blood [153], [150], [154], [155], [156], [157]. As lung burden, also secondary organ burden is dependent upon the transport of nanomaterials to, and clearance from, the respective organs. Subsequent to pulmonary deposition, translocation of nanomaterials was seen in secondary organs such as the liver, heart, spleen, or kidney [158]. In an acute inhalation study with gold nanoparticles in human volunteers [159], [160], gold was detected in the blood and urine within 15 min to 24 h after exposure, and was still present 3 months after exposure. Levels were greater following inhalation of 5 nm (primary diameter) particles compared to 30 nm particles. These authors also showed that the gold particles accumulated at sites of vascular inflammation. Since almost all types of nanoparticles, and especially those with small size, are very likely to be cleared through kidneys, they may therefore accumulate in the kidneys, causing some adverse effects [161], [162]. The nanoparticles deposited on the nasal mucosa of the upper respiratory tract (URT) may translocate to the olfactory bulb of the brain and also via the trigeminus (URT neuronal route) as has been shown in rats [163]. Nanoparticles deposited in the lower respiratory tract (LRT) may cross the air-blood-barrier into blood and enter the brain across the blood-brain-barrier or take a neuronal route from enervated tracheo-bronchial epithelia via the vagus nerve [163].

In ISO TR 22019 [21] the liver, spleen, lung, brain, kidney, lymph nodes at the organ of entry and bone marrow are considered relevant organs for the toxicokinetics of nanomaterials. The examples above imply that data obtained in the past for larger particles of these materials may no longer be valid for the nanoform [164]. It is acknowledged that nanoforms' properties may alter the ADME (absorption, distribution, metabolism, and excretion) behaviour in comparison to non-nano-sized forms.

The toxicokinetic profile of nanomaterials may depend on several physicochemical parameters, e.g. composition, size, shape, surface area, agglomeration/aggregation state, surface properties (including surface charge), hydrophobicity dissolution and biotransformation (see section 3.1.1.1. in *Appendix R.7-1 for nanomaterials applicable to the Chapter R.7a)*. Therefore, nanomaterials may be able to reach parts of the body that are otherwise protected from exposure to particulate materials by biological barriers [165]. Specifically, it is noted that nanomaterials can have high potential for accumulation. Hence, in case of accumulation, determination of kinetics becomes an important indicator for a potential health risk. In addition, toxicokinetic information provides insights into potential target organs and organ burden that ultimately may lead to toxicity.
It is noted that detecting and quantifying nanoparticles in biological tissue(s) is still analytically and technically challenging. Therefore, it is recommended that the methods used and their limitations are adequately documented.

Finally, toxicokinetic information may be used to evaluate if a nanomaterial behaves differently from a similar nanomaterial or a corresponding non nanoform.

Investigation of systemic availability is important information for the assessment of health effects of chemicals. In the case of PSPs, it is therefore relevant to determine whether or not they may cross biological barriers. Translocation may be further influenced by the properties listed in Section 3.1 of *Appendix R.7-1 for nanomaterials applicable to the Chapter R.7a*.

In vivo information on the possible behaviour of the nanomaterials can be supplemented with *in vitro* and *in silico* predictions based on physicochemical and other data. This information may for example be used for grouping nanomaterials and to justify the use of toxicological data between different forms of a substance (Appendix R.6-1 for nanomaterials applicable to

the Guidance on QSARs and Grouping) [1]. However, information on toxicokinetics alone cannot be used to waive any required toxicity study.

2.1.4 Recommended approach for gathering toxicokinetics information on nanomaterials according to REACH

A toxicokinetics study can be required under REACH under the conditions that a nanoform does not have a high dissolution rate in biological media, and that the available information is not sufficient to assess the toxicokinetic behaviour of the nanoform. As for all other forms of substances, the standard information requirements defined by the REACH regulation can give useful information to help making a judgement about the toxicokinetic properties of nanoforms (See Section R.7.12.2.1 in Chapter R.7c Guidance on Information Requirements and Chemical Safety Assessment) [148]. The revised Annex VIII, Section 8.8.1., Column 2 contains three elements to be considered:

- The dissolution rate of the nanoform in biological media,
- Toxicokinetic information that can be obtained in connection with a 28-day (or 90-day) inhalation study (Annex VIII, Section 8.6.1.),
- The choice of a toxicokinetics study depends on the information gaps and the results of the chemical safety assessment.

Firstly, data on solubility and dissolution rate in relevant biological fluids and testing media is an essential starting point in understanding a particle's behaviour and ADME properties and to set boundaries for considering a nanoform as "poorly soluble" (See Section 3.1.1 of *Appendix R.7-1 for nanomaterials applicable to the Chapter R.7a*.). Determination of the dissolution rate provides an insight into how a specific particle may interact with its biological environment [18]. Physico-chemical parameters like agglomeration may have an impact on the dissolution rate. Dissolution may be seen as a kinetic parameter as until dissolution occurs, the toxicokinetics of nanomaterials are governed by the particulate nature, whereas after dissolution, it is the (dissolved) ions of molecules that determine the toxicokinetics [21].

General advice regarding dissolution for nanoforms is given in Appendix R.7-1 for nanomaterials applicable to Chapter R7a, Section 3.1.1..

Secondly, toxicokinetic information can also be obtained from an adequate 28-day or 90-day repeated dose toxicity inhalation study (OECD TG 412 or 413) where the test material is well characterized.
In order to minimize animal use it is highly recommended to collect as much toxicokinetics data as possible from the experiments required under REACH. For example, when dose range finding studies or repeated dose, reproductive or genotoxicity studies are performed, for poorly soluble nanoparticles, several additional toxicokinetics investigations could be considered such as:

- Organ and tissue burden: in the current context, toxicokinetic information is limited to information on the potential for accumulation in tissues (which is related to persistency and elimination), rather than a full set of toxicokinetic parameters. Therefore, testing should focus on determining (possible increase in) concentrations in different organs. Lung burden is discussed in section 3.2.2. of Appendix R7-1 for nanomaterials applicable to Chapter R7a and in this section under distribution and accumulation in 2.1.4.2.2.
- Sampling at several time points in different organs to monitor the fate and accumulation of the particles in the body (data from range-finding studies could be used to determine the appropriate sampling times).
- For gaining insight in toxicokinetics, in line with the recommendations in OECD TG 412 and TG 413, a minimum of three time points post-exposure are recommended to estimate the post-exposure clearance kinetics and hence the potential for accumulation

in several relevant organs [7]. As a consequence, in case of a sub-acute or sub-chronic inhalation study (OECD TG 412 and TG 413), samples (e.g. organs and tissues) for toxicokinetic information can be collected from the animals already required for these studies when these PEOs are included. [110], [111]. The duration of a repeated dose toxicity study is considered sufficient if an equilibrium between deposition and clearance of particles [94] has been reached in tissue concentrations. In case there is indication that relevant tissues have not yet reached equilibrium between deposition and clearance of particles [94] at the last day of exposure, there may be a need for longer exposure durations. For some nanoforms, persistency and bioaccumulation may be such that longer exposure durations may not be sufficient for the development of any adverse effects that may occur in humans. If this is the case, an assessment based on internal concentrations could be an alternative. If this is the case, an assessment based on internal tissue concentrations could be an alternative.

- Urine sampling and nanoparticle content determination. If particles or ions are found in the urine, it is a proof that some level of systemic uptake has occurred.

Thirdly, the choice of a study and the study design depends on the information gaps and the results of the chemical safety assessment. This means that it depends on the one hand on the type of data available for the toxicokinetics assessment and on the other hand on how well the hazard, and exposure have been characterised. The quality of hazard characterization is directly linked to the quality of the data available for the toxicological endpoints. Exposure characterization is key in the context of determining the most appropriate route of exposure. As explained also in section 2.2.2. of Appendix R7-1 for nanomaterials applicable to Chapter R7a, for the repeated dose toxicity, especially for workers (and in some cases for consumers, e.g. in case of sprayable products), inhalation is the most likely route of exposure to (nano)particles present in nano aerosols and dust. Column 2 of sections 8.6.1. and 8.6.2. of REACH Annexes specify that "*Testing by the inhalation route is appropriate if exposure of humans via inhalation is likely taking into account the vapour pressure of the substance and/or the possibility of exposure to aerosols, particles or droplets of an inhalable size*" and there is possibility of exposure to particles of an inhalable size. However, there may be cases where there is convincing information (e.g. uses, dissolution rate, etc.) that justifies another route.

2.1.4.1. Detection methods of nanoforms in tissues and organs

Optical- or electron microscopic qualitative determination of the presence of nanoparticles in the relevant tissues when (technically) feasible. Alternatively, other methods such as multiplexed imaging by use of laser desorption/ionization mass spectrometry LDI-MS, Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) etc could be used [166], [167]. However, these are qualitative methods with limitations with regard to organ burden or organ tissue distribution. Specific labelling of nanomaterials to follow their fate *in vivo* can be done by using radioactive isotopes as radiolabels or fluorescent dyes. A disadvantage of specific labelling is that the label can detach from the nanomaterial. By using isotopic labeling, Raman spectroscopy, and fluorescence spectroscopy many carbon nanomaterials were subjected to pharmacokinetic and biodistribution evaluations both quantitatively and qualitatively [168].

Inductively coupled plasma mass spectrometry (ICP-MS) is a common technique for absolute quantification of the cellular uptake of metal or metal oxide nanoparticles [169]. However, this procedure reveals an average nanoparticle mass concentration only and it will not allow to differentiate between single particles and agglomerated or ionic species. Also, it will not give information about the sizes of nanoparticles. A more specific method for the quantitative analysis is single particle inductively coupled plasma mass spectrometry (spICP-MS) [170]. In spICP-MS each signal corresponds to a single particle, and the frequency of ICP-MS signals can be used to estimate the NP number concentration. However, the intensity of signals is related to the amount of the chemical element and thus to the sizes of the respective nanoparticles. Using laser ablation ICP-MS (LA-ICP-MS) it is possible to detect even smaller nanoparticles above background levels, which may not be possible with spICP-MS[169]. The current

analytical methodology and techniques developed for the quantification of the ADME processes of nanomaterials/nanoparticles *in vivo*, focusing on those used for quantification in different biomatrices, such as blood, tissues, organs, and biomedical processes have been reviewed in detail [171]. ICP-MS based techniques can be challenging in some cases, and still need improvement. For example spICP-MS, while a very promising method, is not yet widely used and is expensive.
To help to determine the concentration of nanoparticles in tissues and excreta, data from the OECD WPMN project "Guidance on the determination of concentrations of nanopartocles in biological samples for (eco)toxicity studies" is expected to become available by the end of 2021.

It is useful to keep the samples to allow later analysis (e.g. storage by freezing or tissue fixation for microscopy [60], freezing for burden analysis [61], [62]. Use of extra animals for the additional analyses should be avoided, where possible, and additional animals should only be included when scientifically justified. However, it is important to balance between performance of additional analyses and demonstration of toxicity. It is noted that according to OECD TG 412 and TG 413, option B, animals of satellite groups can be used for organ burden analysis. If satellite animals are used for lung burden, the same animals should be used for any other organ burden assessment deemed necessary by the study director.

The physical-chemical properties of nanoparticles might change in different environments, e.g. as pristine material, in dosing medium, body fluids, and in tissues. Therefore, physical-chemical characterization may need to be determined at various stages of the toxicokinetic testing [21].

2.1.4.2. Possible types of data and scenarios to be considered

2.1.4.2.1. Cases where there are existing data available on repeated dose toxicity or other high tier studies

In these cases repeated dose studies (RDT) via inhalation route (OECD TG 412 or 413) or RDT studies via the oral route (OECD TG 407 or 408) and/or a screening study (OECD TG 421, 422), a pre-natal developmental toxicity (PNDT) study (OECD TG 414) and/or Extended one generation reproductive toxicity study (EOGRTS) (OECD TG 443) performed via oral or inhalation route are available. It needs to be determined if the data generated by these studies contain information useful for toxicokinetics investigations as described above and if the substance on which the data are available is representative for the registered substance.

- In cases where the test material is not well characterized (e.g. no information on the particle size or surface area, no info on surface treatment) or when the test result is generated on the non nanoform, even if high tier or toxicokinetic data are available, these data cannot be considered relevant for nanoform/sets of nanoforms and new data is needed.

- In cases where the test material is well characterized but the studies do not include the toxicokinetics investigations described above, additional investigations can be performed to make a toxicokinetics assessment (specific investigations such as dissolution rate in relevant biological media, translocation studies, *in vitro* tests, physico-chemical properties and modelling). If it cannot be argued that the duration of the toxicity study is sufficient to address the potential hazard of nanoforms or potential organ burden, further studies are required along with the investigations described above. The determination of the dissolution rate in biological fluids provides an insight on how a certain particle may interact with its biological environment [18]. Therefore, this is an important parameter in toxicokinetics evaluation, which should also be used for grouping and read-across.

Further advise on the use of existing repeated dose studies is given in Appendix R7-1 to Chapter R.7a, section 3.2.2.

2.1.4.2.2. Cases where new repeated dose toxicity data via inhalation route needs to be generated (data gap in dossier)

Several parameters with relevance to the toxicokinetics should be investigated when performing a new repeated dose study (OECD TG 412, 413, 407 or 408, or similar e.g. OECD 421 or 422) in order to answer the following questions:

Absorption

- Do the nanomaterials enter into organs and tissues of the body?
- What can be considered as evidence for systemic absorption?

For the risk assessment of non nanoforms of substances, more detailed information on absorption is useful for refinement of the route-to-route extrapolation. Because route-to-route extrapolation for nanomaterials is unknown at present, knowledge on absorption can only be used in a qualitative manner to identify that a nanomaterial is absorbed. However, it is important to have insight if there is a potential for accumulation of a nanomaterial in different target organs. Information on accumulation would also provide insight in the extent of absorption. The level of absorption may change with the dose. At high doses the nanoform(s) may agglomerate resulting in absorption of a smaller fraction of the administered dose. The extent of absorption can be estimated based on the amount of nanoparticles present in key organs like lungs, liver, spleen and kidneys. Since especially small sized nanoparticles are likely to be cleared through kidneys, they may therefore accumulate in the kidneys [161].

The presence of nanoparticles in secondary organs (i.e. any organ beyond the portal of entry), in serum/blood or urine can be seen as evidence for systemic absorption. However, due to methodological limitations, a non-detection of nanoparticles in secondary organs especially by microscopic methods cannot be used as evidence to conclude that there is no systemic absorption. In cases where a validated or widely used methodology (see section 2.1.4.1.) is utilised to assess the presence of nanoparticles in secondary organs, the evidence of non-absorption may be considered acceptable. The presence of nanoparticles in serum/blood and urine in view of evidence of absorption is qualitative in nature.

Translocation studies via *ex vivo* tissues (skin, intestinal epithelium) and *in vitro* barrier systems have still limited precision to be predictive for systemic absorption. Therefore, these studies can currently not be used to conclude that there is no systemic absorption.

For the non nanoforms of substances, the physicochemical property log P/log K_{ow} provides an indication of the likelihood for accumulation. However, for nanoforms, this property has no predictive value. The dissolution rate in physiological media may however give a qualitative indication of the potential for accumulation. Modelling of absorption and accumulation based on physicochemical properties is currently not sufficiently advanced.

Determination of organ burdens, especially after limited exposure duration, can be hampered by the analytical detection limit. Hence, the methodology used needs to be thoroughly documented and the system validation must be explained.

Distribution and accumulation

- How to assess distribution of nanomaterials in the body?

The distribution and potential for accumulation in lung and body can be assessed with *in vivo* data based on multiple time points. The lung burden determination in OECD TGs 412 and 413 is mandatory at only one post-exposure observation (PEO) period in Option B (at PEO-1). However, these OECD test guidelines state that a minimum of two lung burden measurements are necessary when investigating clearance kinetics but recommend three post-exposure time points. Other organs can also be collected from the animals used in these tests to determine potential accumulation/burden. The assessments at these time points can be used to estimate the accumulation and/or half-life of the nanomaterials in specific organs. This information is to be used to assess the deposition and clearance of particles [94] in the repeated dose toxicity study. If studies of sufficient duration are not feasible, an assessment based on internal concentration could be an alternative. The three time-points post-exposure as described in the OECD TGs are an absolute minimum to obtain insight in the accumulation/elimination rate of nanoparticles.

The scheduling of the post-exposure time points depends on the expected clearance, and considerations as described in OECD TG 412 and OECD TG 413 are in place. A period of a few days is considered too limited to assess the potential for elimination or accumulation in tissues. More guidance and differentiation between nanomaterials that show differences in dissolution rate will be developed in the future OECD TG on toxicokinetics for nanomaterials. Alternatively or in addition, accumulation in organs could be evaluated by measuring organ burdens at different time-points during exposure.

- Which are the relevant organs for accumulation?

In ISO TR 22019 [21] the liver, spleen, lung, brain, kidney, lymph nodes at the organ of entry and bone marrow are considered relevant organs for the investigation of the toxicokinetics of nanoparticles. Subsequent to pulmonary uptake, translocation of nanoparticles to secondary organs such as the liver, heart, spleen, brain, kidney [158] or bone marrow and to local lymph nodes has been reported [172]. In an inhalation chronic low-dose study with CeO_2 nanoparticles, a significant cerium burden could be determined for all time points in all major non-pulmonary organs (liver, kidneys, spleen), with liver bearing the highest content followed by the skeleton [172].

Thereby, liver, spleen, lung, brain, kidney, heart, lymph nodes at the organ of entry and bone marrow, in addition to the organ(s) of entry, represent a relevant set of organs that should be investigated.

If additional toxicokinetics investigations are performed, the assessment of the bone marrow is also of importance for *in vivo* genotoxicity testing, in order to verify whether the test substance reached the target organ. Other organs that may be of relevance for triggering concern and possible further testing for immune, neurological, cardiovascular and reproductive effects are the lymph nodes at the port of entry, brain, thymus, heart, testis/ovaries. Knowledge on the distribution to specific organs can be used to prioritize on which organs (in addition to the standard requirements for the portal of entry and the liver) further genotoxicity studies could be performed.

With regard to the PNDT/screening study/EOGRTS the possible accumulation of nanoparticles in reproductive organs of the parental animals is of interest. In addition, it would be relevant to obtain information on the nanomaterials present in the placenta and their diaplacentar transfer. The potential for accumulation of nanoparticles in organs of the pups is also of interest.

Moreover different exposure routes/methods of administration can lead to different biodistribution of the nanomaterials. For example, radiolabelled gold nano particles in different sizes (1.4-200 nm) administered by intra-oesophageal instillation to healthy adult female rats resulted in detectable amounts of nanoparticles (ng/g organ) in the stomach, small intestine,

liver, spleen, kidney, heart, lung, blood and brain after 24 h as measured by gamma-spectroscopy, with the highest accumulation in secondary organs observed with the smallest particles [173]. When gold nanoparticles were delivered intra-tracheally to rats, the majority of nanoparticles remained in the lungs (> 95% of the initial dose, ID) with < 1% of the ID translocated to the kidneys, liver, blood and urine, and < 0.01 of the ID reaching the spleen, uterus and heart [155].
Existing information suggests that the half-life of nanoparticles can vary in different organs. For example in a study with CeO_2 nanoparticles, following chronic low-dose inhalation, it was concluded that the liver has a low accumulation rate, whereas kidneys, the skeleton and bone marrow seem to have a steady increase in nanoparticles burden over time [172].
Furthermore, translocation between organs, although very low, has been observed [153].
Early studies in rodents provided rough estimates that <1% mass of administered nanoparticles with a diameter of <50 nm will translocate [153]. The reported estimates are most frequently around 0.3% or less of the administered dose for a given tissue at 24 h post-exposure [160].
There is a complex distribution pattern that may change over time. However, the inclusion of three time points post-exposure investigations would give an indication of the potential for accumulation over time.

- How to follow the distribution and accumulation?

The ISO Technical Report on Toxicokinetics on nanomaterials [21] provides considerations for performing toxicokinetic studies with nanomaterials and considerations on the analytical challenges regarding the detection limits or the quantification of nanoparticles in biological samples or on the issues relevant for dosing conditions. The appropriate analytical method(s) depend(s) on the nanomaterials. Depending on the nanomaterial, it may be of relevance to identify whether the nanomaterial is present as constituent particles or as agglomerates/aggregate and whether there are degradation products as detached labelling, ions or transformed nanomaterials present. Inclusion of a control group is important to take potential background exposure into consideration.
Detection of secondary structures formed from the original nanomaterial (e.g. by salt precipation) may also be relevant to inform on the possible modification of the nanomaterial and the mechanism of its absorption and distribution.

Elimination/clearance

- Are the nanoparticles cleared from the body?
- How to determine the rate of elimination/accumulation

The elimination/clearance has a direct impact on the organ burden. Therefore, the measurement of organ burden over time also gives a quantitative estimation of elimination.
The detection of nanoparticles in urine and faeces provides no reliable information on accumulation and kinetics. However, the nanoparticles presence in urine may serve as an indication for systemic absorption and elimination.
These investigations may also be performed within a PNDT study (OECD TG 414), a screening study (OECD TG 421 or 422), or an EOGRTS study (OECD TG 443).
Similar considerations with regard to the toxicokinetics investigations as described for the studies performed via inhalation route apply in principle for the studies via the oral route.
Detailed advice on how to generate new toxicokinetic information within a repeated dose toxicity study via the oral route is provided in EFSA Guidance on nanotechnologies in the food and feed chain [5].

Dermal route of exposure

Regarding the dermal route, to date only very small nanoparticles (such as quantum dots) were found to penetrate the barrier compromised (UV radiated) skin of SKH-1 mice *in vivo*,

thereby reaching the lower epidermal layers and the dermis [116]. However, although the data on skin penetration of nanomaterials is inconsistent [174], the properties, surface modification and structuring of nanomaterials may influence the penetration of the dermal barrier. Furthermore, skin thickness, skin humidity, temperature, barrier integrity, mechanical flexion may increase their dermal uptake. Absorption through intact skin has been shown to occur for nanomaterials smaller than 4 nm, while penetration of nanomaterials larger than 45 nm may only take place in severely damaged skin [175].

REFERENCES

[1] ECHA, "Appendix R.6-1 for nanomaterials applicable to the Guidance on QSARs and Grouping," [Online]. Available: http://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment.

[2] E. P. a. t. Council, "REGULATION (EC) No 1907/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL," 2006.

[3] E. Commission, "Official Journal of the European Union COMMISSION RECOMMENDATIONof 18 October 2011on the definition of nanomaterial (2011/696/EU)," 2011. [Online]. Available: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011H0696&from=EN. [Accessed November 2020].

[4] International Organization for Standardization, "ISO/TS 80004-6:2013(en) Nanotechnologies — Vocabulary — Part 6: Nano-object characterization," ISO, 2013.

[5] C. EFSA, A. Hardy, B. D. T. Halldorsson, M. Jeger, H. Knutsen, S. More, H. Naegeli, H. Noteborn, C. Ockleford, A. Ricci, G. Rychen, J. Schlatter, V. Silano, R. Solecki, D. Turck, M. Younes, Q. Chaudhry, F. Cubadda, D. Gott, A. Oomen, W. S, M. Karamitrou, R. Schoonjans and A. Mortensen, "Guidance on risk assessment of the application of nanoscience nanotechnologies in the food and feed chai n: Part 1, human and animal health.," *EFSA Journal,* vol. 16, no. 7, 2018.

[6] OECD, "ENV/JM/MONO(2018)11 Assessment of Biodurability of Nanomaterials and their Surface ligands Series on the Safety of Manufactured Nanomaterials No. 86," 2018.

[7] OECD, "Guidance Document No. 39 on Acute Inhalation Toxicity Testing. Series on Testing and Assessment, Paris.," OECD, 2018.

[8] K. Driscoll and B. P.J.A., "Expert workshop on the hazards and risks of poorly soluble low toxicity particles," *Inhalation Toxicology,* vol. 32, no. 2, pp. 53-62, 2020.

[9] World Health Organisation, "Determination of airborne fibre number concentrations : a recommended method, by phasecontrast optical microscopy (membrane filter method).," WHO, Geneva, 1997.

[10] ISO, "ISO/TS 80004-2:2008 International Organization for Standardization. Technical Specification: Nanotechnologies—terminology and Definitions for Nano-objects—Nanoparticle, Nanofibre and Nanoplate," ISO, 2008.

[11] European Food Safety Authoritym SCIENTIFIC COMMITTEE AND EMERGING RISKS UNIT, "EFSA GUIDANCE ON TECHNICAL REQUIREMENTS FOR REGULATED FOOD AND FEED PRODUCT APPLICATIONS TO ESTABLISH THE PRESENCE OF SMALL PARTICLES INCLUDING NANOPARTICLES," European Food Safety Authority, 2020.

[12] (SCENIHR) Scientific Committee on Emerging and Newly-Identified Health Risks, "The appropriateness of existing methodologies to assess the potential risks associated with engineered and adventitious products of nanotechnologies," 2006. [Online]. Available: http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_003b.pdf.

[13] S. Dekkers, A. Oomen, E. Bleeker, R. Vandebriel, C. Micheletti, J. Cabellos, G. Janer, N. Fuentes, S. Vázquez-Campos, T. Borges, M. Silva, A. Prina-Mello, D. Movia, F. Nesslany, A. Ribeiro, P. Leite, M. Groenewold, F. Cassee, A. Sips and e. al, "Towards a nanospecific approach for risk assessment," *Regulatory Toxicology and Pharmacology,* vol. 80, p. 46–59, v 2016.

[14] J. Arts, M. Hadi, M. Irfan, A. Keene, R. Kreiling, D. Lyon, M. Maier, K. Michel, T. Petry, U. Sauer, D. Warheit, K. Wiench, W. Wohlleben and R. Landsiedel, "A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping)," *Regulatory Toxicology and Pharmacology,* vol. 71, no. 2, Supplement, pp. S1-S27, 2015.

[15] BAUA. Federal Institute for Occupational Safety and Health, "Manufactured Nanomaterials. Announcement on Hazardous Substances 527," [Online]. Available: http://www.baua.de/en/Topics-from-A-to-Z/Hazardous-Substances/TRGS/pdf/Announcement-527.pdf?__blob=publicationFile&v=3.

[16] SCCS (Scientific Committee on Consumer Safety), "Guidance on the Safety Assessment of Nanomaterials in Cosmetics. SCCS/1611/19," 2019.

[17] Joint FAO/WHO Expert Committee on Food Additives (JECFA), "COMBINED COMPENDIUMOF FOOD ADDITIVE SPECIFICATIONS. Volume 4 Analytical methods, test procedures and laboratory solutions used by and referenced in the food additive specifications," F A O J E C F A M o n o g r a p h s, Rome, 2006.

[18] W. Utembe, K. Potgieter, A. Stefaniak and M. Gulumian, "Dissolution and biodurability: Important parameters needed for risk assessment of nanomaterials," *Particle and fibre toxicology,* vol. 12, no. 11, 2015.

[19] P. Borm, F. Klaessig, T. Landry, B. Moudgil, J. Pauluhn and T. K., "Research strategies for safety evaluation of nanomaterials, part V: role of dissolution in biological fate and effects of nanoscale particles.," *Toxicol Sci. ,* vol. 90, p. 23, 2006.

[20] J. Koltermann-Jülly, J. Keller, A. Vennemann, K. Werle, P. Müller, L. Ma-Hock, R. Landsiedel, M. Wiemann and W. Wohlleben, "Abiotic dissolution rates of 24 (nano)forms of 6 substances compared to macrophage-assisted dissolution and in vivo pulmonary clearance: Grouping by biodissolution and transformation," *NanoImpact,* vol. 12, pp. 29-41, 2018.

[21] International Organization for Standardization, "ISO/TR 22019:2019 Nanotechnologies — Considerations for performing toxicokinetic studies with nanomaterials," ISO, 2019.

[22] W.-S. Cho, R. Duffin, C. Poland, A. Duschl, G. Oostingh and W. e. a. MacNee, "Differential pro-inflammatory effects of metal oxide nanoparticles and their soluble ions in vitro and in vivo; zinc and copper nanoparticles, but not their ions, recruit eosinophils to the lungs," *Nanotoxicology,* vol. 6, no. 1, pp. 22-35, 2012.

[23] H. Braakhuis, M. Park, I. Gosens and e. al., "Physicochemical characteristics of nanomaterials that affect pulmonary inflammation.," *Part Fibre Toxicol,* vol. 11, no. 18, 2014.

[24] S. Keerthana and A. Kumar, "Potential risks and benefits of zinc oxidenanoparticles: a systematic review," *Critical Reviews in Toxicology,* vol. 50, no. 1, pp. 47-71, 2020.

[25] J. G. Keller, U. M. Graham, J. Koltermann-Jülly, R. Gelein, L. Ma-Hock, R. Landsiedel, M. Wiemann, G. Oberdörster, A. Elder and W. Wohlleben, "Predicting dissolution and transformation of inhaled nanoparticles in the lung using abiotic flow cells: The case of barium sulfate.," *Sci Rep,* vol. 10, p. 458, 2020.

[26] Y. Qi, Y. Liu, T. Xia and e. al., "The biotransformation of graphene oxide in lung fluids significantly alters its inherent properties and bioactivities toward immune cells.," *NPG Asia Mater ,* vol. 10, p. 385–396 , 2018.

[27] S. Misra, A. Dybowska, D. Berhanu, S. Luoma and E. Valsami-Jones, "The complexity of nanoparticle dissolution and its importance in nanotoxicological studies," *Science of The Total Environment,* vol. 438, p. 225–232, 2012.

[28] A. Thorley, P. Ruenraroengsak, T. Potter and T. Tetley, "Critical Determinants of Uptake and Translocation of Nanoparticles by the Human Pulmonary Alveolar Epithelium," *ACS Nano,* vol. 8, no. 11, p. 11778–11789, 2014.

[29] R. Tantra, H. Bouwmeester, E. Bolea, C. Rey-Castro, C. A. David, D. J.-M. David, J. Jarman, F. Laborda, J. Laloy, K. N. Robinson, A. K. Undas and M. van der Zande, "Suitability of analytical methods to measure solubility for the purpose of nanoregulation," *Nanotoxicology,* vol. 10, no. 2, pp. 173-184, 2016.

[30] OECD, "GUIDANCE DOCUMENT FOR THE TESTING OF DISSOLUTION AND DISPERSION STABILITY OF NANOMATERIALS AND THE USE OF THE DATA FOR FURTHER ENVIRONMENTAL TESTING AND ASSESSMENT STRATEGIES No 318," OECD, 2020.

[31] M. Avramescu, P. Rasmussen, M. Chénier and e. al., "Influence of pH, particle size and crystal form on dissolution behaviour of engineered nanomaterials.," *Environ Sci Pollut Res,* vol. 24, p. 1553–1564, 2017.

[32] OECD, "TG 105 Water solubility. OECD Test Guideline for the testing of chemicals. Organisation for," OECD, 1995.

[33] International Organisation for Standardisation (ISO), "Nanotechnologies - Use and application of acellular in vitro tests and methodologies to assess nanomaterial biodurability.," ISO, 2017.

[34] J. E. Eixenberger, C. B. Anders, R. J. Hermann, R. J. Brown, K. M. Reddy, A. Punnoose and D. G. Wingett, "Rapid dissolution of ZnO nanoparticles induced by biological buffers significantly impacts cytotoxicity," *Chemical research in toxicology,* vol. 30, no. 8, p. 1641–1651, 2017.

[35] NANoREG Final Report Part 1, "NANoREG, a common European approach to the regulatory testing of nanomaterials," 2017.

[36] J. R. C. EU Commission, "Bioaccessibility testing (Bioelution) of metals, inorganic metals compounds and metals-containing materials: simulated gastric fluid," European Union Reference Laboratory for alternatives to animal testing - EURL ECVAM, 2020.

[37] OECD, "ASSESSMENT OF BIODURABILITY OF MANUFACTURED NANOMATERIALS AND THEIR SURFACE LIGANTS," *Series on the Safety of Manufactured Nanomaterials No 86,* no. 11, 2018.

[38] OECD, "Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterial. Series on the Safety of Manufactured Nanomaterials No. 36. ENV/JM/MONO(2012)40," 2012. [Online]. Available: http://www.oecd.org/science/nanosafety/publications-series-safety-manufactured-nanomaterials.htm.

[39] OECD, "REPORT OF THE OECD EXPERT MEETING ON THE PHYSICAL CHEMICAL PROPERTIES OF MANUFACTURED NANOMATERIALS AND TEST GUIDELINES , No. 41," 2014.

[40] X. Gao and G. Lowry, "Progress towards standardized and validated characterizations for measuring physicochemical properties of manufactured nanomaterials relevant to nano health and safety risks," *NanoImpact,* vol. 9, pp. 14-30, 2018.

[41] J. Arts, M. Irfan, A. Keene, R. Kreiling, D. Lyon, M. Maier, K. Michel, N. Neubauer, T. S. U. Petry, D. Warheit, K. Wiench, W. Wohlleben and R. Landsiedel, "Case studies putting the decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping) into practice.," *Regulatory Toxicology and Pharmacology,* vol. 76, pp. 234-261, 2016.

[42] S. Gioria, F. Caputo, P. Urban, C. Maguire, S. Bremer-Hoffmann, A. Prina-Mello, L. Calzolai and D. Mehn, "Are existing standard methods suitable for the evaluation of nanomedicines: some case studies," *Nanomedicine,* vol. 13, no. 5, 2018.

[43] J. Walter, G. Gorbet, T. Akdas, D. Segets, B. Demeler and W. Peukert, "2D analysis of polydisperse core–shell nanoparticles using analytical ultracentrifugation," *Analyst,* vol. 142, pp. 206-217, 2017.

[44] C. Contado, "Field flow fractionation techniques to explore the "nano-world"," *Analytical and Bioanalytical Chemistry,* vol. 409, p. 2501–2518, 2017.

[45] J. Runyon, M. Ulmius and L. Nilsson, "A perspective on the characterization of colloids and macromolecules using asymmetrical flow field-flow fractionation," *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* vol. 442, pp. 25-33, 2014.

[46] D. Mitrano, A. Barber, A. Bednar, P. Westerhoff, P. Higgins and J. Ranville, "Silver nanoparticle characterization using single particle ICP-MS(SP-ICP-MS) and asymmetrical flow field flow fractionation ICP-MS(AF4-ICP-MS)," *Journal of Analytical Atomic Spectrometry,* July 2012.

[47] D. Mehn, F. Caputo, M. Rösslein, L. Calzolai, F. Saint-Antonin, T. Courant, P. Wick and D. Gilliland, "Larger or more? Nanoparticle characterisation methods for recognition of dimers," *RSC Advances,* no. 44, 2017.

[48] ISO, "ISO/TR 13097:2013 Guidelines for the characterization of dispersion stability," ISO, 2013.

[49] P. Bihari, M. Vippola, S. Schultes, M. Praetner, A. Khandoga, C. Reichel, C. Coester, T. Tuomi, M. Rehberg and F. Krombach, "Optimized dispersion of nanoparticles for biological in vitro and in vivo studies.," *Particle and Fibre,* vol. 5, p. 14, 2008.

[50] N. Jacobsen, G. Pojan, H. Wallin and K. Jensen, "Nanomaterial dispersion protocol for toxicological studies in ENPRA. Internal ENPRA Project Report. Copenhagen, Denmark: National Research Centre for the Working Environm ent.," Copenhagen, Denmark: National Research Centre for the Working Environm ent., 2010.

[51] K. Jensen, Y. Kembouche, E. Christiansen, N. Jacobsen, H. Wallin, C. Guiot, O. Spalla and O. Witschger, "In: Jensen KA and Thieret N (eds.). Final protocol for producing suitable manufactured nanomaterial exposure media. Report. The generic NANOGENOTOX dispersion protocol. Standard operation procedure (SOP) and background documentation. Available online: ht".

[52] J. Taurozzi, V. Hackley and M. Wiesner, "NIST Special Publication 1200-1: reporting guidelines for the preparation of aqueous nanoparticle dispersions from dry materials. Version, 1, 1. https://doi.org/10.6028/NIST.SP.1200-1," 2012a.

[53] J. Taurozzi, V. Hackley and M. Wiesner, "NIST Special Publication 1200-2: preparation of nanoparticle dispersions from powdered material using ultrasonic disruption. Version, 2, 1. https://doi.org/10.6028/NIST.SP.1200-2," 2012b.

[54] J. Taurozzi, V. Hackley and M. Wiesner, "NIST Special Publication 1200-3: preparation of a nanoscale TiO2 aqueous dispersion for toxicological or environanomaterialsnanomaterialsental testing. Version, 1, 2. https://doi.org/10.6028/NIST.SP.1200-3," 2012c.

[55] J. Taurozzi, V. Hackley and M. Wiesner, "NIST Special Publication 1200-4: preparation of nanoscale TiO2 dispersion in biological test media for toxicological assessment. Version, 1, 1. https://doi.org/10.6028/NIST.SP.1200-4," 2012d.

[56] J. Mast and P.-J. De Temmerman, "Protocol(s) for size-distribution analysis of primary nanomaterials perticles in air, powders and liquids.," RIVM , 2016.

[57] A. Mech, H. Rauscher, K. Rasmussen, D. Gilliland, F. Babick, V. Hodoroaba, A. Ghanem, W. Wohlleben, H. Marvin, K. Löschner, R. Brüngel and C. Friedrich, "The NanoDefine Methods Manual. Part 3: Standard Operating Procedures (SOPs), EUR 29876 EN,," Publications Office of the European Union, ISBN 978-92-76-11955-5, Luxembourg, 2020.

[58] U. Holzwarth, U. Cossio, J. Llop and W. Kreyling, "Unpredictable Nanoparticle Retention in Commonly Used Plastic Syringes Introduces Dosage Uncertainties That May Compromise the Accuracy of Nanomedicine and Nanotoxicology Studies," *Front. Pharmacol,* 6 November 2019.

[59] S. Kittler, C. Greulich, J. Diendorf and M. a. E. M. Köller, "Toxicity of Silver Nanoparticles Increases during Storage Because of Slow Dissolution under Release of Silver Ions," *Chemistry of Materials,* vol. 22, no. 16, pp. 4548-4554, 2010.

[60] C. Mühlfeld, B. Rothen-Rutishauser, D. Vanhecke, F. Blank, P. Gehr and M. Ochs, "Visualization and quantitative analysis of nanoparticles in the respiratory tract by transmission electron microscopy," *Particle and Fibre Toxicology,* vol. 4, no. 1, 2007.

[61] K. E. Levine, R. A. Fernando, M. Lang, A. Essader and B. A. Wong, "Development and validation of a high-throughput method for the determination of titanium dioxide in rodent lung and lung-associated lymph node tissues," *Analytical Letters,* vol. 36, no. 3, pp. 563-576, 2003.

[62] E. Bermundez, J. Mangnum, B. Wong, B. Asgharian, P. Hext, D. Warheit and J. Everitt, "Pulmonary Responses of Mice, Rats, and Hamsters to Subchronic Inhalation of Ultrafine Titanium Dioxide Particles," *Toxicological Sciences,* vol. 77, no. 2, pp. 347-357, 2004.

[63] ECHA, "Practical guide: How to use alternatives to animal testing," [Online]. Available: https://echa.europa.eu/practical-guides.

[64] G. Bachler, N. von Goetz and K. Hungerbühler, "A physiologically based pharmacokinetic model for ionic silver and silver nanoparticles," *Int. J. Nanomedicine,* vol. 8, pp. 3365-3382, 2013.

[65] Z. Lin, N. Monteiro-Riviere and J. Riviere, "Pharmacokinetics of metallic nanoparticles," *Wiley Interdiscip Rev Nanomed Nanobiotechnol,* vol. 7, no. 2, pp. 189-217, 2015.

[66] Z. Lin, N. Monteiro-Riviere and J. Riviere, "A physiologically based pharmacokinetic model for polyethylene glycol-coated gold nanoparticles of different sizes in adult mice,"

Nanotoxicology, vol. 10, no. 2, pp. 162-172, 2016.

[67] P. Hinderliter, K. Minard, G. Orr, W. Chrisler, B. Thrall, J. Pounds and J. Teeguarden, "ISDD: A computational model of particle sedimentation, diffusion and target cell dosimetry for in vitro toxicity studies," *Particle and Fibre Toxicology,* vol. 7, no. 1, pp. 1-20, 2010.

[68] Y. Mu, F. Wu, Q. Zhao, R. Ji, Y. Qie, Y. Zhou, Y. Hu, C. Pang, D. Hristozov, J. Giesy and B. Xing, "Predicting toxic potencies of metal oxide nanoparticles by means of nano-QSARs," *Nanotoxicology,* vol. 10, no. 9, pp. 1207-1214, 2016.

[69] G. M. DeLoid, J. M. Cohen, G. Pyrgiotakis, S. V. Pirela, A. Pal, J. Liu, J. Srebric and P. Demokritou, "Advanced computational modeling for in vitro nanomaterial dosimetry," *Particle and Fibre Toxicology,* vol. 12, no. 32, 2015.

[70] ECHA, "Guidance on information requirements and chemical safety assessment Chapter R.6: QSARs and grouping of chemicals," [Online]. Available: http://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment.

[71] L. Lamon, K. Aschberger, D. Asturiol, A. Richarz and A. Worth, "Grouping of nanomaterials to read-across hazard endpoints: a review," *Nanotoxicology,* vol. 13, no. 1, pp. 100-118, 2019.

[72] M. Wiemann, A. Vennemann, U. G. Sauer, K. Wiench, L. Ma-Hock and R. Landsiedel, "An in vitro alveolar macrophage assay for predicting the short-term inhalation toxicity of nanomaterials," *Journal of Nanobiotechnology,* vol. 14, no. 16, 2016.

[73] M. Kingston, J. C. Pfau, J. Gilmer and R. Brey, "Selective inhibitory effects of 50-nm gold nanoparticles on mouse macrophage and spleen cells," *Journal of Immunotoxicology,* vol. 13, no. 2, 2016.

[74] S. Petrache Voicu, D. Dinu, C. Sima, A. Hermenean, A. Ardelean, E. Codrici, M. Stan, O. Zărnescu and A. Dinischiotu, "Silica Nanoparticles Induce Oxidative Stress and Autophagy but Not Apoptosis in the MRC-5 Cell Line," *Int. J. Mol. Sci,* vol. 16, pp. 29398-29416, 2015.

[75] Scientific Committee on Health, Environmental and Emerging Risks (SCHEER), "Memorandum on weight of evidence and uncertainties. Revision 2018," European Commission, 2018.

[76] ISO, "ISO/TR 16196:2016. Nanotechnologies - Compilation and description of sample preparation and dosing methods for engineered and manufactured nanomaterials," 2016.

[77] OECD, " Genotoxicity of Manufactured Nanomaterials : Report of the OECD Expert Meeting. Series on the Safety of Manufactured Nanomaterials No. 43. ENV/JM/MONO(2014)34," 2014. [Online]. Available: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2014)34&doclanguage=en.

[78] B. Drasler, P. Sayre, K. Steinhäuser, A. Petri-Fink and B. Rothen-Rutishauser, "In vitro approaches to assess the hazard of nanomaterials," *NanoImpact,* vol. 8, pp. 99-116, 2017.

[79] OECD, "Series on the safety of manufactured nanomaterials No. 85. In: Evaluation of in Vitro Methods for Human Hazard Assessment Applied in the OECD Testing Programme for the Safety of Manufactured Nanomaterials. ENV/JM/MONO(2018)4.," OECD, 2018.

[80] L. Belyanskaya, P. Manser, P. Spohn, A. Bruinink and P. Wick, "The reliability and limits of the MTT reduction assay for carbon nanotubes – cell interaction," *Carbon,* vol. 45, no. 13, p. 2643–2648, 2007.

[81] H. K. B. W. L. P. G. M. Z. B. D. S. M. J.-J. L. M. A. H. A. D. M. F. L. M. F. a. B. S. Guadagnini R., "Toxicity screenings of nanomaterials: challenges due to interference with assay processes and components of classic in vitro tests.," *Nanotoxicology,* vol. 9, no. S1, pp. 13-24, 2015.

[82] M. Davoren, E. Herzog, A. Casey, B. Cottineau, G. Chambers, H. Byrne and F. Lyng, "In vitro toxicity evaluation of single-walled carbon nanotubes on human A549 lung 56 cells," *Toxicology in Vitro,* vol. 21, no. 3, p. 438–448, 2007.

[83] A. Kroll, M. Pillukat, D. Hahn and J. Schnekenburger, "Current in vitro methods in nanoparticle risk assessment: Limitations and challenges," *European Journal of Pharmaceutics and Biopharmaceutics,* vol. 72, no. 2, p. 370–377, 2009.

[84] J. Eixenberger, C. Anders, R. Hermann, R. Brown, K. Reddy and A. a. W. D. Punnoose, "Rapid Dissolution of ZnO Nanoparticles Induced by Biological Buffers Significantly Impacts Cytotoxicity," *Chemical Research in Toxicology,* vol. 39, no. 8, pp. 1641-1651, 2017.

[85] E. Petersen, T. Henry, J. Zhao, R. MacCuspie, T. Kirschling, M. Dobrovolskaia, V. Hackley, B. Xing and J. and White, "Identification and Avoidance of Potential Artifacts and Misinterpretations in Nanomaterial Ecotoxicity Measurements," *Environmental Science & Technology,* vol. 48, no. 8, pp. 4226-4246, 2014.

[86] R. Landsiedel, M. Schulz, M. Kapp and F. Oesch, "Genotoxicity Investigations on Nanomaterials: Methods, Preparation and Characterization Test Material, Potential Artifacts and Limitations - Many Questions, Some Answers," *Mutation Research,* vol. 681, no. 2-3, p. 241–258, 2009.

[87] J. Wörle-Knirsch, K. Pulskamp and H. Krug, "Oops they did it again! Carbon nanotubes hoax scientists in viability assays," *Nano Letters,* vol. 6, no. 6, p. 1261–1268, 2006.

[88] S. Faber and S. McCullough, "Through the Looking Glass: In Vitro Models for Inhalation Toxicology and Interindividual Variability in the Airway," *Applied In Vitro Toxicology,* pp. 115-128, June 2018.

[89] OECD, "Test No. 412: Subacute Inhalation Toxicity: 28-Day Study, OECD Guidelines for the Testing of Chemicals, Section 4," 2009. [Online]. Available: http://www.oecd-ilibrary.org/environment/test-no-412-subacute-inhalation-toxicity-28-day-study_9789264070783-en.

[90] OECD, "Test No. 413: Subchronic Inhalation Toxicity: 90-day Study, OECD Guidelines for the Testing of Chemicals, Section 4," 2009. [Online]. Available: http://www.oecd-ilibrary.org/environment/test-no-413-subchronic-inhalation-toxicity-90-day-study_9789264070806-en.

[91] K. Donaldson, F. Murphy, R. Duffin and C. Poland, "Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma," *Particle and Fibre Toxicology,* vol. 7, no. 5, 2010.

[92] F. Muphy, A. Schinwald, C. Poland and K. Donaldson, "The mechanism of pleural inflammation by long carbon nanotubes: interaction of long fibres with macrophages stimulates them to amplify pro-inflammatory responses in mesothelial cells," *Particle and Fibre Toxicology,* vol. 9, no. 8, 2012.

[93] A. Oller and G. and Oberdörster, "Incorporation of dosimetry in the derivation of reference concentrations for ambient or workplace air: A conceptual approach," *Journal of Aerosol Science,* vol. 99, pp. 40-45, 2016.

[94] G. Oberdörster and T. Kuhlbusch, "In vivo effects: methodologies and biokinetics of inhaled nanomaterials.," *NanoImpact,* vol. 10, pp. 38-60, 2018.

[95] O. A. K. P. J. N. W. H. L. M. V. H. B. E. d. J. W. Geraets L, "Tissue distribution and elimination after oral and intravenous administration of different titanium dioxide nanoparticles in rats.," *Part Fibre Toxicol,* 2014.

[96] P. Morrow, "Possible mechanisms to explain dust overloading of the lungs," *Fundamental and Applied Toxicology ,* vol. 10, no. 3, pp. 369-384, 1988.

[97] F. Miller, "Dosimetry of particles in laboratory animals and humans in relationship to issues surrounding lung overload and human health risk assessment: A critical review," *Inhalation Toxicology,* vol. 12, pp. 19-57, 2000.

[98] J. Keller, W. Wohlleben, L. Ma-Hock, V. Strauss, S. Gröters, K. Küttler, K. Wiench, C. Herden, G. Oberdörster, B. van Ravenzwaay and R. Landsiedel, "Time course of lung retention and toxicity of inhaled particles: short-term exposure to nano-Ceria," *Archives of Toxicology,* vol. 88, no. 11, pp. 2033-2059, 2014.

[99] P. Borm, F. Cassee and G. Oberdörster, "Lung particle overload: old school – new

] insights?," *Particle and Fibre Toxicology ,* vol. 12, no. 10, 2015.

[10 J. Mauderly, "Usefulness of animal models for predicting human responses to long- term
0] inhalation of particles," *Chest,* vol. 109, no. 3, Supplement, pp. 65S-68S, 1996.

[10 A. Ostrowski, D. Nordmeyer, A. Boreham, C. Holzhausen, L. Mundhenk, C. Graf, M.
1] Meinke, A. Vogt, S. Hadam, J. Lademann, E. Rühl, U. Alexiev and A. Gruber, "Overview about the localization of nanoparticles in tissue and cellular context by different imaging techniques," *Beilstein Journal of Nanotechnology,* vol. 6, p. 263–280, 2015.

[10 J. Kratchman, B. Wang and G. Gray, "Which is most sensitive? Assessing responses of
2] mice and rats in toxicity bioassays.," *J Toxicol Environ Health,* vol. 81, pp. 173-183, 2018.

[10 P. Bos, I. Gosens, L. Geraets, C. Delmaar and F. Cassee, "Pulmonary toxicity in rats
3] following inhalation exposure to poorly soluble particles: The issue of impaired clearance and the relevance for human health hazard and risk assessment," *Regulatory Toxicology and Pharmacology,* vol. 109, 2019.

[10 E. Bermudez, J. Mangnum, B. Asgharian, B. Wong, E. Reverdy, P. Hext, D. Warheit and J.
4] Everitt, "Long-term pulmonary response of three laboratory rodents to subchronic inhalation of pigmentary titanium dioxide particles," *Toxicological Sciences,* vol. 77, no. 2, pp. 347-357, 2002.

[10 E. Kuempel, M. Attfield, L. Stayner and V. Castranova, "Human and Animal Evidence
5] Supports Lower Occupational Exposure Limits for Poorly-Soluble Respirable Particles: Letter to the Editor re: 'Low-Toxicity Dusts: Current Exposure Guidelines Are Not Sufficiently Protective' by Cherrie, Brosseau, Hay and Donalds," *The Annals of Occupational Hygiene,* vol. 58, no. 9, pp. 1205-1208, 2014.

[10 D. Gregoratto, M. Bailey and J. Marsh, "Modelling particle retention in the alveolar-
6] interstitial region of the human lungs," *Journal of Radiological Protection,* vol. 30, no. 3, pp. 491-512, 2010.

[10 P. Borm, R. Schins and C. Albrecht, "Inhaled particles and lung cancer, part B: Paradigms
7] and risk assessment," *International Journal of Cancer,* vol. 110, no. 1, pp. 3-14, 2004.

[10 J. Pauluhn, "Subchronic 13-week inhalation exposure of rats to multiwalled carbon
8] nanotubes: toxic effects are determined by density of agglomerate structures, not fibrillar structures," *Toxicological Science,* vol. 113, no. 1, pp. 226-242, 2010.

[10 J. Pauluhn, "Poorly soluble particulates: Searching for a unifying denominator of
9] nanoparticles and fine particles for DNEL estimation," *Toxicology,* vol. 279, no. 1-3, pp. 176-188, 2011.

[11 G. Oberdorster, J. Ferin and B. Lehnert, "Correlation between particle size, in vivo particle
0] persistence, and lung injury," *Environmental Health Perspectives,* vol. 102 (Supplement 5), pp. 173-179, 1994.

[11 A. Elder, R. Gelein, J. Finkelstein, K. Driscoll, J. Harkema and G. Oberdörster, "Effects of
1] subchronically inhalaed carbon black in three species. I. Retention kinetics, lung inflammation, and histopathology," *Toxicological Sciences,* vol. 88, no. 2, pp. 614-629, 2005.

[11 C. Tran, D. Buchanan, R. Cullen, A. Searl, A. Jones and K. Donaldson, "Inhalation of
2] poorly soluble particles. II. Influence of particle surface area on inflammation and clearance," *Inhalation Toxicology,* vol. 12, pp. 1113-1126, 2000.

[11 D. Warheit, J. Hansen, I. Yuen, D. Kelly, S. Snajdr and M. Hartsky, "Inhalation of high
3] concentrations of low toxicity dusts in rats results in impaired pulmonary clearance mechanisms and persistent inflammation," *Toxicology and Applied Pharmacology,* vol. 145, no. 1, pp. 10-22, 1997.

[11 K. Wittmaack, "Dose and Response Metrics in Nanotoxicology: Wittmaack Responds to
4] Oberdoerster et al. and Stoeger et al.," *Environmental Health Perspectives,* vol. 115, no. 6, p. A291–A292., 2007.

[11 D. Warheit, T. Webb, V. Colvin, K. Reed and C. Sayes, "Pulmonary bioassay studies with
5] nanoscale and fine-quartz particles in rats: Toxicity is not dependent upon particle size but on surface characteristics," *Toxicological Sciences,* vol. 95, no. 1, pp. 270-280, 2007.

[116] D. Warheit, T. Webb, K. Reed and S. S. C. Frerichs, "Pulmonary toxicity study in rats with three forms of ultrafine-TiO2 particles: Differential responses related to surface properties," *Toxicology,* vol. 230, no. 1, p. 90–104, 2007.

[117] R. P. Schins and A. M. Knaapen, "Genotoxicity of poorly soluble particles.," *Inhal. Toxicol.,* vol. 1, p. 189–198, 2007.

[118] P. Borm, L. Tran and K. Donaldson, "The carcinogenic action of crystalline silica: A review of the evidence supporting secondary inflammation-driven genotoxicity as a principal mechanism, Critical Reviews in Toxicology," *Critical Reviews in Toxicology,* vol. 41, no. 9, p. https://doi.org/10.3109/10408444.2011.576008, 2011.

[119] S. Evans, M. Clift, N. Singh, M. J. de Oliveira, M. Burgum, J. Wills, T. Wilkinson, G. Jenkins and S. Doak, "Critical review of the current and future challenges associated with advanced in vitro systems towards the study of nanoparticle (secondary) genotoxicity.," *Mutagenesis,* vol. 32, p. 233–241, 2017.

[120] J. Lovrić, S. J. Cho, F. M. Winnik and D. Maysinger, "Unmodified cadmium telluride quantum dots induce reactive oxygen species formation leading to multiple organelle damage and cell death.," *Chem. Biol.,* vol. 12, p. 1227–1234, 2005.

[121] J. Modrzynska, T. Berthing, G. Ravn-Haren, N. Jacobsen, W. I. Konow, K. Loeschner, A. Mortensen, S. A. Thoustrup and U. Vogel, "Primary genotoxicity in the liver following pulmonary exposure to carbon black nanoparticles in mice.," *Particle and Fibre Toxicology,* vol. 15, no. 2, pp. 1-12, 2018.

[122] H. Wallin, Z. Kyjovska, S. Poulsen, N. Jacobsen, A. Saber, S. Bengtson, P. Jackson and U. Vogel, "Surface modification does not influence the genotoxic and inflammatory effects of TiO2 nanoparticles after pulmonary exposure by instillation in mice.," *Mutagenesis,* vol. 32, pp. 47-57, 2017.

[123] S. Evans, M. Clift, N. Singh, J. Wills, N. Hondow, T. Wilkinson, M. Burgum, A. Brown, G. Jenkins and S. Doak, "In vitro detection of in vitro secondary mechanisms of genotoxicity induced by engineered nanomaterials.," *Particle and Fibre Toxicology,* vol. 16, no. 8, pp. 1-14, 2019.

[124] L. Gonzalez, D. Lison and M. Kirsch-Volders, Genotoxicity of engineered nanomaterials: a critical review, vol. 2, Nanotoxicology, 2008, p. 252–273.

[125] K. Donaldson, C. Poland and R. Schins, Possible genotoxic mechanisms of nanoparticles: criteria for improved test strategies, vol. 4, Nanotoxicology, 2010, p. 414–420.

[126] Z. Magdolenova, A. Collins, A. Kumar, A. Dhawan, V. Stone and M. Dusinska, "Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles," vol. 8, no. 3, pp. 233-278, 2014.

[127] R. S. Elespuru, M. Pfuhler, T. Aardema, S. Chen, A. Doak, C. Doherty, J. Farabaugh, M. Kenny, B. Manjanatha, M. Mahadevan, G. Moore, L. Ouédraogo, J. Stankowski and J. Tanir, "Genotoxicity Assessment of Nanomaterials: Recommendations on Best Practices, Assays, and Methods.," *Toxicological Sciences,* vol. 164, no. 2, p. 391–416, 2018.

[128] Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment, "Statement on genotoxicity assessment of nanomaterials and experimental considerations. Available at:," *http://webarchive.nationalarchives.gov.uk/20140506144244/http://www.iacom.org.uk/statements/documents/nanomaterialsfinal2012.pdf Accessed June 15, 2020..*

[129] S. Pfuhler, R. Elespuru, M. Aardema, S. Doak, E. Donner, M. Honma, M. Kirsch-Volders, R. Landsiedel, M. Manjanatha, T. Singer and J. Kim, "Genotoxicity of Nanomaterials: Refining Strategies and Tests for Hazard Identification," *Environmental and Molecular Mutagenesis,* vol. 54, no. 4, p. 229–239, 2013.

[130] KEMI, "Nanomaterials and Genotoxicity: A Literature Review (Report 13/16).," Swedish Chemicals Agency, 2016.

[131] OECD, "Test No. 471: Bacterial Reverse Mutation Test, OECD Guidelines for the Testing of Chemicals, Section 4," 1997. [Online]. Available: http://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en.

[132] B. Ames, J. McCann and E. Yamasaki, "Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome," *Mutation Research,* vol. 31, no. 6, pp. 347-363, 1975.

[133] D. Maron and B. Ames, "Revised Methods for the Salmonella Mutagenicity Test," *Mutation Research,* vol. 113, no. 3-4, pp. 173-215, 1983.

[134] D. Gatehouse, S. Haworth, T. G. E. Cebula, L. Kier, T. Matsushima, C. Melcion, T. Nohmi, S. Venitt and E. Zeiger, "Recommendations for the Performance of Bacterial Mutation Assays," *Mutation Research,* vol. 312, no. 3, pp. 217-233.

[135] S. Doak, B. Manshian, G. Jenkins and N. Singh, "In vitro genotoxicity testing strategy for nanomaterials and the adaptation of current OECD guidelines," *Mutation Research,* vol. 745, no. 1-2, p. 104–111, 2012.

[136] EFSA Scientific Committee, "Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment. (https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2011.2379)," *EFSA Journal ,* vol. 9, no. 9, p. 2379, 2011.

[137] D. B. Warheit and E. Donner, "Rationale of genotoxicity testing of nanomaterials: Regulatory requirements and appropriateness of available OECD test guidelines.," *Nanotoxicology,* vol. 4, pp. 409-413, 2010.

[138] S. Dekkers, A. Oomen, E. Bleeker, V. R. C. Micheletti, J. Cabellos, G. Janer, N. Fuentes, S. Vazquez-Campos, T. Borges and e. al., Towards a nanospecific approach for risk assessment., vol. 80, Regulatory Toxicology and Pharmacology,, 2016, pp. 46-59.

[139] Drewes C.C et al, Physicochemical characterisation of gold, silica and silver, EUR 29054 EN, Publications Office of the European Union, Luxembourg, ISBN 978-92-79-77705-9, doi 10.2760/818663, PUBSY No. JRC110379, 2018.

[140] Nanogenotox, Final Report of the NANOGENOTOX EU Joint Action, 2013.

[141] A. Bogni, J. Ponti, C. Drewes, A. Kinsner-Ovaskainen and S. Bremer-Hoffmann, In vitro cytotoxicity and cellular uptake evaluation of gold, silica and silver nanoparticles in five different cell lines: Caco-2, A549, CHO, V79 and TK6, European Commission, Ispra, 2020, JRC120791, 2020.

[142] N. Donahue, H. Acar and S. Wilhelm, "Concepts of nanoparticle cellular uptake, intracellular trafficking, and kinetics in nanomedicine.," *Advanced Drug Delivery Reviews,* vol. 143, pp. 68-96, 2019.

[143] D. Van Der Zwaag, N. Vanparijs, S. Wijnands, R. De Rycke, B. De Geest and L. Albertazzi, "Super resolution imaging of nanoparticles cellular uptake and trafficking.," *ACS Appl. Mater. Interfaces,* vol. 8, p. 6391–6399, 2016.

[144] C. Kim, X. Li, Y. Jiang, B. Yan, G. Tonga, M. Ray, D. Solfiell and V. Rotello, "Cellular imaging of endosome entrapped small gold nanoparticles.," *MethodsX,* vol. 2, pp. 306-315, 2015.

[145] D. Peckys and N. De Jonge, "Visualizing gold nanoparticle uptake in live cells with liquid scanning transmission electron microscopy," *Nano Lett.,* vol. 11, p. 1733–1738, 2011.

[146] OECD, "Test No. 487: In Vitro Mammalian Cell Micronucleus Test, OECD Guidelines for the Testing of Chemicals, Section 4," 2010. [Online]. Available: http://www.oecd-ilibrary.org/environment/test-no-487-in-vitro-mammalian-cell-micronucleus-test_9789264091016-en.

[147] OECD, "Test No. 473: In vitro Mammalian Chromosome Aberration Test, OECD Guidelines for the Testing of Chemicals, Section 4," 1997. [Online]. Available: http://www.oecd-ilibrary.org/environment/test-no-473-in-vitro-mammalian-chromosome-aberration-test_9789264071261-en.

[148] ECHA, "Guidance on Information Requirements and Chemical Safety AssessmentChapter R.7c: Endpoint specific guidance," 2017.

[149] OECD , "Test No. 417: Toxicokinetics," 2010. [Online]. Available: https://www.oecd-ilibrary.org/environment/test-no-417-toxicokinetics_9789264070882-en.

[150] G. Oberdörster, A. Elder and A. Rinderknecht, "Nanoparticles and the Brain: Cause for

0] Concern?," *J Nanosci Nanotechnol.,* vol. 9, no. 8, p. 4996–5007, 2009.

[15 1] Kreyling, WG, M. Semmler-Behnke, S. Takenaka and W. Möller, "Differences in the Biokinetics of Inhaled Nano- versus Micrometer-Sized Particles," *Accounts of Chemical Research,* vol. 46, no. 3, pp. 714-722, 2013.

[15 2] R. Landsiedel, E. Fabian, L. Ma-Hock and e. al., "Toxico-/biokinetics of nanomaterials.," *Arch Toxicol,* vol. 86, p. 1021–1060, 2012.

[15 3] W. Kreyling, M. Semmler, F. Erbe, P. Mayer, S. Takenaka, H. Schulz, G. Oberdorster and A. Ziesenis, "Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low," *J. Toxicol. Environ. Health, Part A,* vol. 65, p. 1513– 1530, 2002.

[15 4] T. Kato, T. Yashiro, Y. Murata, D. Herbert, K. Oshikawa, M. Bando, S. Ohno and Y. Sugiyama, "Evidence that exogenous substances can be phagocytized by alveolar epithelial cells and transported into blood capillaries.," *Cell Tissue Res.,* vol. 11, no. 1, p. 47051, 2003.

[15 5] G. Kreyling, S. Hirn, W. Möller, C. Schleh, A. Wenk, G. Celik, J. Lipka, M. Schäffler, N. Haberl, B. Johnston, R. Sperling, G. Schmid, U. Simon, W. Parak and M. Semmler-Behnke, "Air–Blood Barrier Translocation of Tracheally Instilled Gold Nanoparticles Inversely Depends on Particle Size.," *ACS Nano.,* vol. 8, no. 1, pp. 222-233, 2014.

[15 6] M. S.-B. J. S. W. S. A. W. P. M. S. T. &. G. O. Wolfgang G. Kreyling, "Size dependence of the translocation of inhaled iridium and carbon nanoparticle aggregates from the lung of rats to the blood and secondary target organs," *Inhalation Toxicology,* vol. 21:sup1, pp. 55-60, 2009.

[15 7] W. Kreyling, W. Möller, U. Holzwarth, S. Hirn, A. Wenk, C. Schleh, M. Schäffler, N. Haberl, N. Gibson and J. Schittny, "Age-Dependent Rat Lung Deposition Patterns of Inhaled 20 Nanometer Gold Nanoparticles and their Quantitative Biokinetics in Adult Rats," *ACS Nano,* vol. 12, no. 8, pp. 7771-7790, 2018.

[15 8] P. Laux, C. Riebeling, A. M. Booth, J. D. Brain, J. Brunner, C. Cerrillo, O. Creutzenberg, I. Estrela-Lopis, T. Gebel, G. Johanson, H. Jungnickel, H. Kock, J. Tentschert, A. Tlili, A. Tentschert, A. M. Sips, R. A. Yokel and A. Luch, "Biokinetics of nanomaterials: The role of biopersistence.," *NanoImpact,* vol. 6, pp. 69-80, 2017.

[15 9] M. R. Miller, J. B. Raftis, J. P. Langrish, S. G. McLean, P. Samutrtai, S. Connell, S. Wilson, A. Vesey, P. Fokkens, A. Boere, P. Krystek, C. Campbell, P. Hadoke, K. Donaldson, F. R. Cassee, D. E. Newby, R. Duffin and N. L. Mills, "Inhaled Nanoparticles Accumulate at Sites of Vascular Disease.," *ACS nano,* vol. 11, no. 5, p. 4542–4552, 2017a.

[16 0] M. R. Miller, J. B. Raftis, J. P. Langrish, S. G. McLean, P. Samutrtai, S. Connell, S. Wilson, A. Vesey, P. Fokkens, A. Boere, P. Krystek, C. Campbell, P. Hadoke, K. Donaldson, F. R. Cassee, D. E. Newby and R. Duffin, "Nanoparticles Accumulate at Sites of Vascular Disease.," *ACS nano,* vol. 11, no. 10, p. 10623-10624, 2017b.

[16 1] T. Wu and M. Tang, "Review of the effects of manufactured nanoparticles on mammalian target organs.," *J Appl Toxicol.,* vol. 38, pp. 25-40, 2018.

[16 2] I. Iavicoli, L. Fontana and G. Nordberg, "The effects of nanoparticles on the renal system," *Critical Reviews in Toxicology,* vol. 46, no. 6, pp. 490-560, 2016.

[16 3] W. Kreyling, "Discovery of unique and ENM— specific pathophysiologic pathways: Comparison of the translocation of inhaled iridium nanoparticles from nasal epithelium versus alveolar epithelium towards the brain of rats," *Toxicol Appl Pharmacol.,* vol. 299, pp. 41-46, 2016.

[16 4] M. Riediker, D. Zink, W. Kreyling, G. Oberdörster, A. Elder, U. Graham, I. Lynch, A. Duschl, G. Ichihara, S. Ichihara, T. Kobayashi, N. Hisanaga, M. Umezawa, T.-J. Cheng, R. Handy, M. Gulumian, S. Tinkle and F. Cassee, "Particle toxicology and health - where are we?," *Part Fibre Toxicol,* vol. 16, no. 19, 2019.

[16 5] Kemi, "Uptake and biodistribution of nanoparticles – a review," Kemi, Swedish Chemicals Agency, 2016.

[16 6] B. Yan, S. Kim, C. Kim, K. Saha, D. Moyano, Y. Xing, Y. Jiang, A. Roberts, F. Alfonso, V. Rotello and R. Vachet, "Multiplexed Imaging of Nanoparticles in Tissues Using Laser

Desorption/Ionization Mass Spectrometry," *Journal of the American Chemical Society,* vol. 135, no. 34, p. 12564–12567, 2013.

[167] I. Gunsolus and C. Haynes, "Analytical Aspects of Nanotoxicology," *Analytical Chemistry,* vol. 88, no. 1, p. 451–479, 2016.

[168] S.-T. Yang, X. Liu and J. Xie, "Biodistribution and Pharmacokinetics of Carbon Nanomaterials In Vivo," in *Biomedical Applications and Toxicology of Carbon Nanomaterials*, C. Chen and H. Wang, Eds., 2016.

[169] I. Hsiao, F. Bierkandt and P. e. a. Reichardt, "Quantification and visualization of cellular uptake of TiO2 and Ag nanoparticles: comparison of different ICP-MS techniques.," *Nanobiotechnol.,* vol. 14, no. 50, 2016.

[170] D. Mozhayeva and C. Engelhard, "A critical review of single particle inductively coupled plasma mass spectrometry – A step towards an ideal method for nanomaterial characterization," *J. Anal. At. Spectrom.,* vol. 35, pp. 1740-1783, 2020.

[171] L. Wang, L. Yan, ,. J. Liu, C. Chen and Y. and Zhao, "Quantification of Nanomaterial/Nanomedicine Trafficking in Vivo," *Analytical Chemistry,* vol. 90, pp. 589-614, 2018.

[172] J. Tentschert, P. Laux, H. Jungnickel, J. Brunner, I. Estrela-Lopis, C. Merker, J. Meijer, H. Ernst, L. Ma-Hock, J. Keller, R. Landsiedel and A. Luch, "Organ burden of inhaled nanoceria in a 2-year low-dose exposure study: dump or depot?," *Nanotoxicology,* vol. 14, no. 4, pp. 554-576, 2020.

[173] C. Schleh, M. Semmler-Behnke, J. Lipka, A. Wenk, S. Hirn, M. Schäffler, G. Schmid, U. Simon and W. Kreyling, "Size and surface charge of gold nanoparticles determine absorption across intestinal barriers and accumulation in secondary target organs after oral administration.," *Nanotoxicology.,* vol. 6, no. 1, pp. 36-46, 2012.

[174] K. Roach, S. AB and J. Roberts, "Metal nanomaterials: Immune effects and implications of physicochemical properties on sensitization, elicitation, and exacerbation of allergic disease," *Journal of Immunotoxicology,,* vol. 16:1, pp. 87-124,, 2019.

[175] F. Filon, M. Mauro, G. Adami, M. Bovenzi and M. Crosera, "Nanoparticles skin absorption: New aspects for a safety profile evaluation.," *Regul. Toxicol. Pharmacol.,* vol. 72, p. 310–322, 2015.

[176] ECHA, "Guidance on information requirements and chemical safety assessment Chapter R.7a: Endpoint specific guidance," [Online]. Available: http://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment.

[177] K. Wittmaack, "In search of the most relevant parameter for quantifying lung inflammatory response to nanoparticle exposure: Particle number, surface area, or what?," *Environmental Health Perspectives,* vol. 115, no. 2, pp. 187-194, 2007.

[178] D. Eastmond, A. Hartwig, D. Anderson, W. Anwar, M. Cimino, I. Dobrev, G. Douglas, T. Nohmi, D. Phillips and C. Vickers, "Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme," *Mutagenesis,* vol. 24, no. 4, pp. 341-9, 2009.

[179] ECHA, "How to prepare registration dossiers that cover nanoforms: best practices," [Online]. Available: http://echa.europa.eu/documents/10162/13655/how_to_register_nano_en.pdf/f8c046ec-f60b-4349-492b-e915fd9e3ca0.

[180] E. Blanco, H. Shen and M. Ferrari, "Principles of nanoparticle design for overcoming biological barriers to drug delivery.," *Nat Biotechnol.,* vol. 33, pp. 941-951, 2015.

[181] L. Mortensen, G. Oberdörster, A. Pentland and L. Delouise, "In vivo skin penetration of quatum dot nanopartciles in the murine model: the effects of UVR.," *Nano Lett.,* vol. 8, pp. 2779-2787, 2008.

[182] T. Xia, R. Hamilton, J. Bonner, E. Crandall, A. Elder, F. Fazlollahi, T. Girtsman, K. Kim, S. Mitra and S. e. a. Ntim, "Interlaboratory evaluation of in vitro cytotoxicity and inflammatory responses to engineered nanomaterials: The NIEHS Nano GO Consortium.," *Environ. Health Perspect.,* vol. 121, p. 683–690, 2013.

[18 J. G. U. K.-J. J. e. a. Keller, "Predicting dissolution and transformation of inhaled

3] nanoparticles in the lung using abiotic flow cells: The case of barium sulfate.," *Sci Rep,* vol. 10, p. 458, 2020.

[18 4] N. H., "Translocation of particles deposited in the respiratory system: a systematic review and statistical analysis.," *Environ Health Prev Med.,* vol. 17, no. 4, pp. 263-274, 2012.

[18 5] M. S.-B. S. T. a. W. M. Wolfgang G. Kreyling, "Differences in the Biokinetics of Inhaled Nano- versus Micrometer-Sized Particles," *Accounts of Chemical Research,* vol. 46, no. 3, pp. 714-722, 2013.

[18 6] J.-K. Chen, C.-C. Ho, H. Chang, J.-F. Lin, C. Yang, M.-H. Tsai and P. Tsai H-T & Lin, "Particulate nature of inhaled zinc oxide nanoparticles determines systemic effects and mechanisms of pulmonary inflammation in mice," *Nanotoxicolog,* vol. 9, no. 1, pp. 43-53, 2015.

[18 7] vjh, "bnm," *kl.*

[18 8] W. M, A. Vennemann, U. Sauer, K. Wiench, L. Ma-Hock and R. & Landsiedel, "An in vitro alveolar macrophage assay for predicting the short-term inhalation toxicity of nanomaterials," *Journal of Nanobiotechnology,* vol. 14, 2016.

[18 9] A. Kroll, C. Dierker, C. Rommel and e. al., "Cytotoxicity screening of 23 engineered nanomaterials using a test matrix of ten cell lines and three different assays.," *Part Fibre Toxicol,* vol. 8, no. 9, 2011.

[19 0] D. A. S. S. H. D. C. M. V. S. Chen H, "In vivo study of sperical gold nanoparticles: inflammatory effects and distribution in mice," *PLoS One,* 2013.

[19 1] L. C. F. U. G. B. d. C. J. R. d. G. J. B. D. C.-P. T. Silva AH, "Visceral fat increase and signals of inflammation in adipose tissue after administration of titanium dioxide nanoparticles in mice.," *Toxicol Ind Health,* pp. 147-158, 2017.

[19 2] Y. Y. S. H. N. N. N. K. A. Y. K. H. T. S. N. H. Y. T. T. Y. Morishita Y, "Distribution and histologic effects of intravenously administered amorphous nanosilica particles in the testes of mice.," *Biochem Biophys Res Commun,* pp. 297-301, 2012.

[19 3] L. H. X. K. Liu Y, "Distribution and Biological Effects of Nanoparticles in the reproductive system.," *Curr Drug Metab,* pp. 478-496, 2016.

[19 4] H. F. Z. X. L. L. Z. Y. Z. Y. Ye L, "Toxic effects of TiO2 nanoparticles in primary cultured rat sertoli cells are mediated via a dysregulated Ca2+/PKC/p38 MAPK/NF-kB cascade.," *J Biomed Mater Res A,* pp. 1374-1382, 2017.