ECHA
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GUIDANCE

Guidance on information requirements and Chemical Safety Assessment

Appendix R7-1 Recommendations for nanomaterials applicable to Chapter R7a Endpoint specific guidance and Appendix R7-2 Recommendations for nanomaterials applicable to Chapter R7c Endpoint specific guidance

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December 2016

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:

http://echa.europa.eu/documents/10162/13632/appendix_r7a_nanomaterials_en.pdf (version 1.0 published in April 2012).

http://echa.europa.eu/documents/10162/13632/appendix_r7c_nanomaterials_en.pdf (version 1.0 published in April 2012).

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.

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Guidance on information requirements and chemical safety assessment

Extracts from:

Appendix R7-1 Recommendations for nanomaterials applicable to Chapter R7a - Endpoint specific guidance

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Version 1	First edition	April 2012
Version 2	• New advisory note on testing and sampling strategy and sample preparation for human health endpoints (section 3.1.1); • Reorganisation of the (general) advice regarding non-testing methods in section 3.1.1 instead of under each specific endpoint to avoid repetition • Update of advisory notes on consideration on lung overload (section 3.2.1.1); • Update of the section on repeated dose toxicity (section 3.2.1); • Update of the section on mutagenicity (section 3.2.2)	Xxxx 2017

PREFACE

The three appendices concerning information requirements (appendices to R7a, R7b and R7c) have been developed in order to provide advice to registrants for use when preparing registration dossiers that cover "nanoforms" [1]

The advice provided in this document, focuses on specific recommendations for testing materials that are nanomaterials[2]. Part of the advice provided is not strictly nanospecific (e.g. may for instance be also applicable to other particulate materials). However, when included, it has been considered that the issue is especially relevant for nanomaterials and should be part of the nanospecific guidance. In the absence of any specific recommendation, either because the endpoint is not relevant for nanomaterials (e.g. flash point or surface tension), or the guidance already provided is considered to be equally applicable to nanomaterials or because more research is needed before developing advice, no additional guidance for the endpoint has been included in this appendix.

This appendix intends to provide advice specific to nanomaterials and does not preclude the applicability of the general principles given in Chapter R.7a (i.e. the parent guidance). The parent guidance applies when no specific information for nanomaterials has been given in this appendix.

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 *Appendix R.6-1: Recommendations for nanomaterials applicable to the Guidance on QSARs and Grouping of Chemicals* [1] may be useful as it provides an approach on how to justify the use of hazard data between nanoforms (and the non-nanoform) of the same substance.

[1] See Appendix 4 to the Guidance on Registration [9]
[2] See Recommendation on the definition of nanomaterial adopted by the European Commission

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3. RECOMMENDATIONS FOR TOXICOLOGICAL INFORMATION REQUIREMENTS for NANOMATERIALS

3.1 General advisory notes

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

These advisory notes do not propose a protocol but aim to provide useful advice with regard to specific aspects that are particularly important for nanomaterials testing, and references to relevant resources. For a testing material identified by the physico-chemical characterization as being a nanomaterial, the testing strategy is dependent on its solubility and dissolution potential in relevant biological fluids and testing media. Figure 1 below shows a decision tree to determine whether nanospecific advice should be used, or, due to the nanomaterial properties, the parent guidance can be used instead

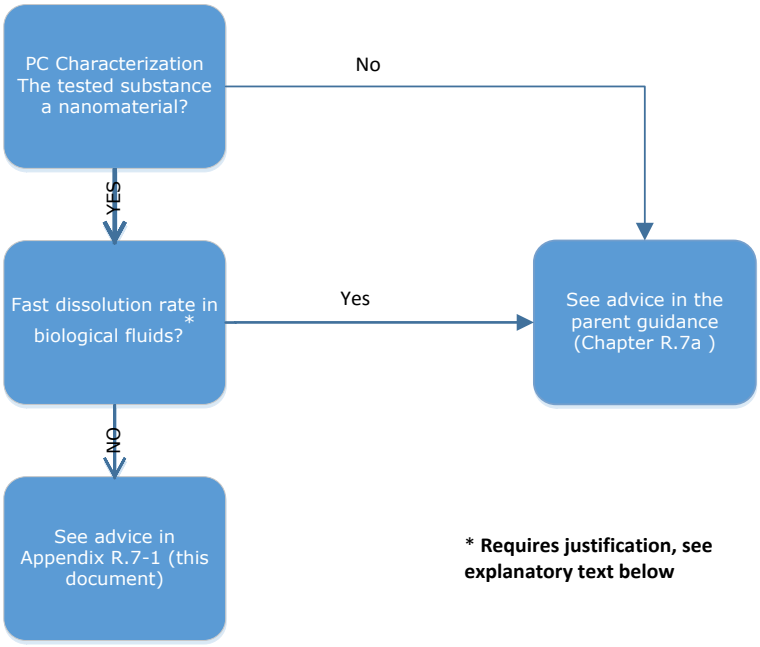

Figure 1: Decision tree for nanomaterials testing for human health endpoints

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) states that many nanomaterials will 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*" [2]. The latest approaches for the risk assessment of nanomaterials recommend a similar strategy in which the dissolution rate and equilibrium in water is a primary key element [3]. Water solubility may give a first indication on a nanomaterial (non)biopersistence [4]. For example, as an initial pragmatic approach to assess the biopersistence of nanomaterials in the context of risk assessment in occupational settings, BAuA [5] proposed that the nanomaterials with a water solubility above 100 mg/l could be considered as soluble[3] (and thus not biopersistent). The water-soluble nanomaterials are generally not biopersistent. Nevertheless, different biological

[3] 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)

media may influence both the kinetics of dissolution and the saturation concentration [6]. In addition, some water insoluble nanomaterials may be non-biopersistent in biological fluids and this can be assessed from the data on the dissolution rate. A nanomaterial's dissolution describes a time-dependent process (depending on the rate of solubilisation and the surface area) and it is directly related to a nanomaterial's in vitro or in vivo biopersistence that decreases with increasing dissolution rate [4]. Although no exact cut-off value has been proposed, the dissolution rate needs to be very fast [3].The determination of the dissolution rate provides an insight on how a certain particle may interact with its biological and environmental surroundings [7].

Consequently, for the nanomaterials for which there is evidence of fast dissolution in relevant biological fluids and testing media the advice provided in the parent guidance applies [8].

For the nanomaterials that do not have fast dissolution in relevant biological fluids and testing media, further guidance is given in this document.

3.1.1.1 Test material characterization and reporting

For the purpose of the toxicological testing, the sample characterization and preparation including special considerations on dispersion and dosimetry, should be performed, as much as possible, as advised in the OECD Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials (ENV/JM/MONO(2012)40), 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 (ENV/JM/MONO(2014)15). A harmonized preparation of the tested sample will enable the comparison of the data and their further use. Information on the characterisation of test material serves multiple purposes:

a) enables link to the identity (in this case also of the nanoform being covered in the dossier) and therefore supports data relevance,
b) facilitates interpretation of test results and
c) provides general info on material properties 'as test sample' to support handling/storage and repeatability/reproducibility of results.
d) May facilitate the usage of toxicological data for grouping of the nanoforms of a substance or justifying read-across between nanoforms, and between nanoforms and bulk form (Further information at *Appendix R.6-1: Recommendations for nanomaterials 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.

The chemical composition, the physicochemical properties as well as the interaction of the nanomaterials with the biological systems influences its potential hazard. In order to show that the test material(s) chosen are appropriate, some information should be reported at the endpoint study record under the test material information field in IUCLID. The parameters required for the identification nanoforms should be reported (see [9] for further advice on the type of information required):

- Chemical composition (as described in ECHA Guidance for identification and naming of substances under REACH and CLP)
- Size
- Shape and aspect ratio
- Surface chemistry

Moreover, the appendix R6-1: Recommendations for nanomaterials 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) 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 want to consider characterising the test material taking into account such parameters, 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 base for the grouping of the nanomaterials ([1], [10]).

3.1.1.2 Sampling

Currently there are no OECD test guidelines specifically adapted for nanomaterials testing for human health endpoints[4]. However, this document aims to give supplementary recommendations on specific aspects that, although not entirely nanomaterial specific (e.g. lung overload), are particularly important for nanomaterial testing.

The biological samples to be collected in the in vivo toxicological studies are specified in the relevant test guidelines. However, if there is indication that the nanomaterials would be distributed in other tissues not listed in the OECD TGs, then the collection of these additional tissues is recommended.

It could be useful to keep the samples to allow the performance of later analysis (e.g. storage by chemical or physical tissue fixation for microscopy [11], freezing for burden analysis ([12], [13]

3.1.1.3 Use of Non-Animal Testing Approaches [5]

Article 25 of the REACH regulation specifies that testing on vertebrate animals should be conducted only as a last resort, *i.e.* when all other avenues have been exhausted. Therefore, there is an obligation to look at existing data and non-animal methods of hazard assessment before considering any tests using vertebrates. Registrants are advised to stay informed of ongoing developments and validation efforts of the OECD and the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM), as well as the regulatory acceptance of new methods by ECHA [14]. Implementation of non-animal approaches for nanomaterials requires the prior consideration of all available information, including context-specific 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) specific to nanomaterials are under development at OECD and offer new approaches to integrated assessment.

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 has also yet to be established or accepted. Thus, the use these 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, in any case 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

[4] The update of OECD TG 412 and TG 413 to cover nanomaterials testing is currently on-going. The drafts (when publicly available) may already provide some guidelines for testing nanomaterials.
[5] This advice is applicable for all endpoints relevant for human health, not only to the ones having a nanospecific entry in this document.

to determine nanomaterial kinetics, QSARs and physiologically based pharmacokinetic (PBPK) models have been developed for nanomaterials ([15], [16], [17] [18], [19] [20] .

- The use of grouping and read-across approaches is another step to consider before performing animal testing. In this respect it is advised to consider the ECHA guidance: *Appendix R.6-1: Recommendations for nanomaterials applicable to the Guidance on QSARs and Grouping of the Chemicals* [1] when data on other (nano)forms[6] of the same substance are available. When considering the 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* [21] together with the advice provided in its nanospecific appendix [1] could be considered.

3.1.1.4 In vitro studies

In accordance with Article 13(1) of the REACH regulation, "*information on intrinsic properties of substances may be generated by means other than tests providing 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 many *in vitro* assays are applicable to nanomaterials when the nano-specific parameters are considered, and can be effectively used as part of a weight of evidence approach [22], [23], [24]. REACH Annex XI includes provisions for the acceptance of data from *in vitro* studies.

Valuable data can be derived from in vitro assays. In vitro tests have recently been described to measure nanoparticle effects on Rat NR8383 alveolar macrophages [25], mouse macrophage and spleen cells [26], MRC-5 human lung fibroblast cells [27].

Moreover, these studies can provide indications of likely mode of action, for instance whether toxicity is driven by Reactive Oxygen Species (ROS) formation or other mechanisms.

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" [28].

3.1.2 Advisory note on the consideration of assay inhibition/ enhancement (interference)

Various nanomaterials have on occasion been found to interfere with several commonly used assays utilised to determine 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; [29]) or the biomarker being measured, (e.g. lactate dehydrogenase (LDH) and cytokine proteins, see for example [30]).

A summarised list of potential sources of interferences with commonly used assays has been developed by Kroll et al. [31] and is reproduced in the table below.

Table 1: Potential sources of interferences with commonly used assays

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

Cytotoxicity assay	Detection principle	Nanoparticle interference	Altered readout	Particle type
Cell viability				
MTT	Colorimetric detection of mitochondrial activity	Adsorption of substrate	Reduced indication of cell viability	Carbon nanoparticles
LDH	Colorimetric detection of LDH release	Inhibition of LDH	Reduced indication of necrosis	Trace metal-containing nanoparticles
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 nanoparticles
Neutral red	Colorimetric detection of intact lysosomes	Dye adsorption	Reduced indication of cell viability	Carbon nanoparticles
Caspase	Fluorimetric detection of Caspase-3 activity (apoptosis marker)	Inhibition of Caspase-3	Reduced indication of oxidative stress	Carbon nanoparticles
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

1

It should be noted that this 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.

Within some standard methodologies such as ISO 29701:2010 (Nanotechnologies - endotoxin test on nanomaterial samples for in vitro systems-- Limulus amebocyte lysate (LAL) test [32]), 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.

The possible inhibition or enhancement of results should be investigated wherever possible irrespective of standard method requirement; however, this may not always be possible. 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 advisable to use more than one assay to assess the studied endpoint or effect, as for example advised by Landsiedel et al. [33] for the genotoxicity endpoint. The potential for inhibition or enhancement of the test result may impact numerous test methods. In certain cases, the potential for assay interference has been identified for some nanomaterials, for example carbon nanotubes are suggested to interfere with the MTT assay [34] 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 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 to validate obtained results.

3.2 Specific advice for endpoints

3.2.1 Repeated dose toxicity

As highlighted in the general testing strategy for the nanomaterials in Figure 1, for the nanomaterials that do not have a fast dissolution rate in relevant and soluble biological fluids and testing media, further guidance for testing is needed. The poorly soluble particles (PSP) are part of this category.

For the PSP, the rat lung burden is an important issue to consider in the toxicological outcome and therefore a special chapter within this section 3.2.1.1is 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 [35].

When considering the nanomaterials testing strategy for repeated dose toxicity (Section 7.5.6) it should be noted that:

- As, especially for workers (and for in some cases for consumers) inhalation may be the most likely route for nano(particles), nano aerosols and dust exposure. Hence, the repeated dose toxicity studies are recommended to be performed via inhalation, unless there is convincing information (e.g. uses, dissolution rate, etc.) that justifies another route. Any modification of the protocols described in the OECD TG 412 and 413 ([36] and [37]) should be justified;

- When dose range finding studies or repeated dose studies are performed, for PSP, it is recommended to collect additional toxicokinetic data as described in *Appendix R7-2 Recommendations 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 during the study.

- When performing an inhalation test for PSP of low toxicity the possibility for lung overload should be considered. The data on lung burden and clearance are essential arguments in the context of read-across.

- To monitor the fate and effects of PSP in the body it is recommended to collect the samples at several time points and/or in different organs. Data from range-finding studies, if proven robust, could be used to determine the appropriate sampling times). However, it is important to balance between performing additional analyses and indication of toxicity. It is not intended here to advice on use of extra animals for the additional analyses.

- Since the lower respiratory tract (i.e., the alveoli) is the primary site of deposition (depending on agglomerate size) and retention for inhaled nanoparticles, the bronchoalveolar lavage (BAL) analysis may be the technique of choice to quantitatively analyse hypothesis-based dose-effect parameters focusing on alveolitis and pulmonary inflammation and phospholipidosis. This allows for dose-response and time-course changes of alveolar injury to be suitably investigated. Therefore, for nanomaterials testing, it is highly recommended to include BAL analysis (further details in Section R.7.5 (repeated dose toxicity) of *Chapter R7.a of the Guidance on IR&CSA* (Endpoint specific guidance) [8].

- It is strongly advised to use more than one different dose-describing metrics and include the mass metric. The choice for the methods selected should be justified as described in Section 3.2.1.1.1.

Regarding the use of non-testing data, i.e. Sections R7.5.3.1, R7.5.4.1 (on non-human data), and R7.5.6.2 (on integrated testing strategy) it is necessary to take into account that:

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

This chapter describes the concept of rat lung burden of poorly soluble particles (PSP) and the associated effects, the differences to other species and the extrapolation of the results to humans, relevant dose metrics and suggested thresholds. Care should be taken when interpreting lung burden in the context of human risk assessment. Lung effects observed in animals exposed to PSP by inhalation should be considered relevant for humans unless it can be clearly substantiated otherwise. When designing a new study, the doses to be used in long-term inhalation studies should not exceed the maximum tolerated dose. The OECD TG 413 [37] provides advice on dosage 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.

Results from inhalation studies in rats have shown that the PSP, even if otherwise of low toxicity, can induce serious adverse pulmonary effects if inhaled in high doses and which will result in material accumulation as lung clearance mechanisms are not able to remove materials at the same time or higher rate as the dose is delivered. This condition named lung "particle overload", occurs when the retained lung burden exceeds a certain threshold [38].

As it can be difficult to interpret the findings of overload of alveolar macrophages in the rat studies, a better understanding of the rat lung burden and its relevance to human is needed.

The term 'lung overload', is a phenomenon associated with exposure to PSP, with generally low toxicity and occurs when a threshold level of particles is reached within the lung. During prolonged exposure of rats to PSP, the lung burden of particles increases until a steady state or equilibrium is reached between deposition and clearance of particles [39] as shown by the A, B and C curves in Figure 2. This can be reached very fast or take many days. Below the lung overload threshold (see section 3.2.1.1.1), particles are cleared via normal mechanisms at a normal clearance rate, in general generating little or no appreciable response.

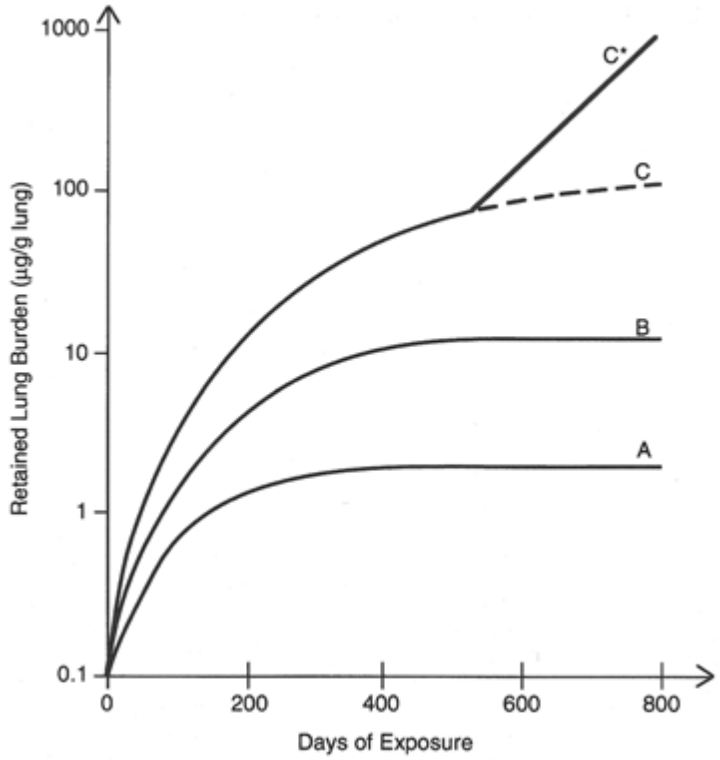

Figure 2: Schematic representation of the relationship between retained lung burden and duration of exposure leading to the phenomenon of lung overload. Curves A, B, and C are associated with progressively increasing exposure doses. If the exposure level is sufficiently high and the duration of exposure 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 [39].

Once the threshold has been reached, the clearance mechanisms of the lung become overloaded. This is typified by a progressive reduction of particle clearance from the deep lung, reflecting a breakdown in alveolar macrophage (AM)-mediated dust removal due to the loss of AM mobility [38][7]. This is shown in the C* curve of Figure 2 whereby at the point of threshold, particle retention occurs linearly rather than an equilibrium being established (as demonstrated by the dashed line).

The result of this net increase in particle accumulation is 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 the 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 [40]as well as of the volumetric load [41].

The situation of lung burden is most commonly associated with repeated inhalation exposure of rats to PSP but it can also occur after single or repeated instillation of PSP into the lung (due to high deposition fraction as a result of direct instillation) or possibly as a result of a single massive inhalation exposure [42]. Since this phenomenon occurs at high level of inhalatory exposure, 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

[7] Please note that the impact of particle characteristics (e.g. shape, size) resulting in non-recognition of particles by macrophages, and in a decreased phagocytic activity, cannot be excluded

(non-overload conditions) due to intrinsic properties of the particles themselves.

In the studies performed with PSP the measurement of changes in lung burden over time post-exposure provide essential information on the clearance and allows to clarify the deposited vs the exposed particle amount. Different imaging techniques may also be used for a semi-quantitative assessment of the nanomaterials in the tissue [43]. The assessment of biodurability in relation to the dissolution potential can also be done using *in vitro* systems [7].

The information on clearance and biosolubility is important in the context of read-across and weight of evidence.

Several studies assessed the responses to lung overload in different species, and the relevance of the 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 [13], 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 ([13], [44]). However, in relation to the relevance of animal data for humans, other studies pointed out that the lung responses to high lung burdens of PSP of low toxicity can be qualitatively similar in rats and humans [45]. Based on experience with exposure of coal miners a specific interstitial particle sequestration compartment is hypothesed [46], which may explain why humans, in contrast to rats, seem not to have an increased risk of lung cancer under lung overload conditions [41]. Nevertheless, there seems to be some conditional evidence for particle overload associated with impaired clearance in the coal miners [41].

Therefore, the use of existing data, which obtained after exposure to high doses of PSP, cannot automatically be dismissed as irrelevant in the context of risk assessment and the interpretation of such data should be approached with caution. In 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 has to be supported by data.

For further information, there are several review articles covering the lung overload subject such as Miller [39], which provides an excellent in-depth discussion of particle deposition, clearance and lung overload, [47] , which discuss the importance of overload in the context of risk assessment and [41] which review the state of art of the lung particle overload concepts. 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 debated.

In conclusion, care should be taken when interpreting lung burden measurements in the context of human risk assessment. Lung effects observed in animals exposed to PSP by inhalation should be considered relevant for humans unless it can be clearly substantiated otherwise.

3.2.1.1.1 Metrics

The question of which dose metric best describes the association between deposited dose in the lung, and subsequent pathogenic effects is particularly pertinent. There have been several suggested metrics but volumetric load of AM and surface area seem to be considered the most relevant [41]. Morrow et al. [38] 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). Such a driver of lung overload has also been more recently suggested for carbon nanotubes ([48], [49]).

Oberdoerster et al. [50] suggested that the particle surface area better correlates the overload with retarded clearance. Several studies suggest that, particle surface area correlates well with induced pathogenic events in lung ([47], [51], [52]). In a study by [52] 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".
Whilst some studies indicate mass as a less sensitive indicator of lung overload [53], the mass concentration is still important because there is already a large body of data and research on the exposure and toxicity of particles using the mass-based metric. Therefore, the mass dose should always be reported.

Other studies ([54], [55]) found that the particle number was the best dose metric while others ([56], [57]) found that the number of functional groups in the surface of nanoparticles influenced their toxicity.

The most relevant dose metric seems to vary depending on the specific nanoparticle in question. Particle volume, surface areas, mass, particle number as well as number of functional groups 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 conclusion, it is strongly advised to use more than one different dose-describing metrics and the choice for the methods selected should be justified.

3.2.1.1.2 Overview of the 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
- For new studies, the use of too high doses should be avoided (not to exceed the maximum tolerated dose)
- Lung burden data may bring useful information on clearance and may be supportive information in the context of read-across and weight of evidence
- 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 Mutagenicity and Carcinogenicity

3.2.2.1 Advisory note on the consideration of bacterial assay interference

Assessment of substances with regard to genotoxicity is generally based on a combination of tests to assess effects on three major endpoints of genetic damage associated with human disease: gene mutation, clastogenicity and aneuploidy. The bacterial reverse mutation (Ames) test (OECD TG 471 [58]/EU B.12/13: Bacterial reverse mutation test (in vitro)) detects point mutations in *Salmonella typhimurium* and *Escherichia coli* ([59], [60]; [61]).

It is now clear from the results of international collaborative studies and the large databases that are currently available for the assays evaluated, that no single assay can detect all genotoxic substances [62]. In relation to nanomaterials, a review of the applicability of genotoxicity tests to nanomaterial questioned whether the Ames test was accurately representative of nanomaterial genotoxicity [33]. The Landsiedel study [33] reported that of those studies reviewed, results were predominantly negative (5/6 studies). The group speculated that it is likely that some nanomaterials are not able to cross the bacterial wall, whilst others kill the test organism as they are bactericidal. The OECD Working Party on Manufactured Nanomaterials held an expert meeting, 'Genotoxicity of Manufactured nanomaterials', in 2013, where it was agreed that 'The use of the Ames test (TG 471) is not a

recommended test method for the investigation of the genotoxicity of nanomaterials'. This observation was based, among others, on the work by Doak et al. [63]) that suggested that "although the Ames test is a reliable genotoxicity screen for the analysis of chemicals, it does not appear to be suitable for the assessment of nanomaterials".

Based on this evidence, it is advisable that any data harvested from such bacterial mutation tests should be followed up with other assays after the initial screening, perhaps via implementation of a battery of standardised genotoxicity testing methods covering an as wide as possible variety of potential genotoxic mechanisms. In addition to the use of other assays, determination of cellular uptake by appropriate methods will help in the interpretation of in vitro genotoxicity assays.

3.2.2.2 General considerations for Mutagenicity and Carcinogenicity

The guidance gives a list of methods for *in vitro* testing for mutagenicity in Table R.7.7-2, and the list includes the *in vitro* gene mutation study, as specified in Annex VII of REACH (See Section 7.7.6.3). In this respect, it should be noted that solid particles, including some nanomaterials, may not penetrate the cell wall of bacteria and as such this assay may not allow a robust evaluation of (nano) material mutagenicity as discussed in the bacterial mutagenicity advisory note (See Section 3.2.2.1). Therefore, the bacterial mutation assay should not be used as the only test for (nano)particle mutagenicity, but instead be used in conjunction with a range of mammalian cell gene mutation tests to reduce the potential for confounded results due to interference with a test method. Measurement of cellular uptake by appropriate methods is highly advised for bacterial as well as for mammalian cell genotoxicity/mutagenicity tests. Moreover, the use of metabolic activation system (S9) 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 of proteins (contained in S9) can modify the cellular uptake of nanomaterials ([64], [63] and [65])

During the OECD/WPMN expert meeting on the Genotoxicity of Manufactured Nanomaterials in Ottawa, Canada in November 2013 [28], seven consensus statements were agreed and found useful to investigate the genetic toxicity testing of nanomaterials. Several of these recommendations are also supported in other scientific literature (e.g. see reviews by Magdolenova et al. [64], Pfuhler et al. [65], Doak et al. [63]):

1. "*The use of the Ames test (TG 471* [58]*) is not a recommended test method for the investigation of the genotoxicity of nanomaterials"*

   According to the recent discussions, it is advised to perform another in vitro mutagenicity study in mammalian cells, such as the gene mutation test on mammalian cell (OECD TG 476 [66] or 490 [67]) that is required according to 8.4.3. However, an in vitro gene mutation study in bacteria is a data requirement for Annex VII 8.4.1 with potentially important regulatory consequences (e.g. follow-up in vivo testing). Therefore, a negative outcome in the Ames test should be considered valid only if there is proof of bacterial wall penetration and on absence of bactericidal activity by the nanomaterial.

2. "*Measures of cytotoxicity based on cell proliferation that are described in the test guidelines are appropriate for determining the top concentration to be applied for in vitro tests of nanomaterials. It is appropriate in some cases to consider wider concentration spacing than the standard √10 in order to ensure that any potential concentration-response relationship is well characterized, and at concentrations not associated with cytotoxicity*."

3. "*The extent of cellular uptake is a critical factor to consider when interpreting test results. In some circumstances, a lack of uptake in a mammalian cell may indicate a low intrinsic hazard from a direct genotoxicity perspective*".

The importance of cell uptake was also pointed out by the EU Nanogenotox project (http://www.nanogenotox.eu/files/PDF/nanogenotox_web.pdf)

4. "*The test guidelines program should consider modification of the in vitro micronucleus assay to recommend, where cytochalasin B is used, its addition using a post-treatment or 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*".

According to Annex VIII 8.4.2, a micronucleus test (OECD TG 487 [68]) or a chromosomal aberration test (OECD 473 [69]) is required. The EU Nanogenotox project showed 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" (http://www.nanogenotox.eu/files/PDF/nanogenotox_web.pdf). A project on the adaptation of the *in vitro* mammalian cell micronucleus assay (TG 487 [68]) for nanomaterials testing was approved in 2015 in 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 study focuses on 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).

5. *"Prior to conducting an in vivo genotoxicity study, there is a need to conduct some toxicokinetic investigations to determine if the nanomaterial reaches the target tissue, where the target tissue is not the site of contact. In the absence of data to the contrary, the test is not applicable for detecting primary genotoxicity if the nanomaterial does not reach the target tissue."*

In 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).

6. "*There are insufficient data to recommend one route of administration over another. The basis for selecting the route of administration for testing should be to consider the route most applicable to human exposure(s).*"

Currently inhalation is considered the most likely route of human exposure for nanomaterials - at least for workers - (See R.7.a, Section R.7.5.6).

Appendix R7-2 to Chapter R.7c

2.1.3 Guidance on Toxicokinetics

A toxicokinetics study is not an information requirement under REACH. However, as for all the other substances, the standard information requirements defined by the REACH regulation can give useful information to help make a judgement about the possible toxicokinetics of nanomaterials (See Section R.7.12.2.1).

Information on the possible behaviour of the nanomaterials can be supplemented with *in vitro* and *in silico* predictions based on the physicochemical and other data. This information may be used in the grouping of nanomaterials to assist in the read-across of exposure and hazard characteristics, reducing the total number of tests required.

It is acknowledged that nanomaterials properties may alter the ADME behaviour in comparison to their non-nano-sized counterparts. 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, and dissolution. Therefore, nanomaterials may be able to reach unintended parts of the body that are otherwise protected from exposure to particulate materials by biological membrane barriers. Nevertheless, it might be a technical and analytical challenge to properly and adequately address toxicokinetics of nanomaterials.

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 "poorly soluble" vs. "readily soluble" particles in order to fulfil the REACH data requirements. The determination of the dissolution rate provides an insight on how a certain particle may interact with its biological and environmental surroundings [7].

In the case of PSP, it is paramount to determine whether or not they could translocate. The translocation behaviour across biological membranes may be further influenced by the properties listed in Section 3.1 of Appendix R.7-1*: Recommendations for nanomaterials applicable to the Chapter R.7a*. In addition to its intrinsic value for hazard assessment, the information on toxicokinetics is valuable to justify the use of toxicological data between different forms of a substance (*Appendix R.6-1: Recommendations for nanomaterials applicable to the Guidance on QSARs and Grouping* [1]). Therefore, in order to optimise animal use it is highly recommended to collect as much toxicokinetics data as possible from the experiments that are performed under the REACH requirements. For example, when dose range finding studies or main repeated dose/reproductive studies are performed, for poorly soluble nanomaterials, several additional analyses could be considered such as:

- Urine and faeces sampling
- Microscopic or electron microscopic qualitative determination of the presence of nanomaterials in the relevant tissues when (technically) feasible. Alternatively, other methods such as multiplexed imaging by use of laser desorption/ionization mass spectrometry LDI-MS can be used ([70], [71]).
- 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)
- Lung and tissue burden

It could be useful to keep the samples to allow the performance of later analysis. (e.g. storage by freezing or tissue fixation for microscopy ([11]), freezing for burden analysis ([12], [13])). However, it is important to balance between performing additional analyses and indication of toxicity. It is not intended here to advice on use of extra animals for the additional analyses unless scientifically justified.

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