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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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

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

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2 # DOCUMENT HISTORY

Version	Changes	Date
Version 1	First edition	April 2012
Version 2	• New Advisory note on testing strategy and toxicokinetics (section 3.1); • Update of advisory notes on consideration on lung overload (section 3.1.1); • Update of the section on repeated dose toxicity (section 3.2.4); • Update of the section on mutagenicity (section 3.2.6)	Xxxx 2017

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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 for nanomaterials[1].

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.

[1] See Recommendation on the definition of nanomaterial adopted by the European Commission

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Appendix R7-1 to Chapter R.7a

3. RECOMMENDATIONS FOR TOXICOLOGICAL INFORMATION REQUIREMENTS for NANOMATERIALS

3.1 General advisory notes

These advisory notes do not propose a protocol but aim to provide useful advice and references to relevant resources.

The sample characterization and preparation, including special considerations on dispersion, and dosimetry for toxicological testing of nanomaterials should be performed as advised in the OECD Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials [1]and the report of the OECD expert meeting on the physical chemical properties of manufactured nanomaterials and test guidelines [2].

For a comprehensive understanding of the toxicological data, the robust study summaries should report in IUCLID under the relevant test materials fields (e.g. "*Test material information, Specific details on test material used for the study*" and the "Test material record", "*Details on test material*" or "*Confidential details on test material*", as considered appropriate, the following parameters of the tested particle(s):

- Chemical composition (including crystalline structure)
- Impurities
- Surface chemistry
- Size
- Shape
- Surface area
- Solubility (as dissolution behaviour in relevant biological fluids and testing media)
- Dispersibility (refers to the relative number or mass of particles in a suspending medium, and relates to stability [3], aggregation and agglomeration in relevant media, and is dependent on e.g. van der Waals energy, Hamaker constant, zeta potential.)
- Dustiness
- Biological reactivity (e.g. ROS production)
- Photoreactivity
- Stability in storage
- Rigidity for fibres

Having sufficient knowledge of the test material may facilitate the usage of toxicological data for grouping of the nanoforms of a substance (Further information at *Appendix R.6-1: Recommendations for nanomaterials applicable to the Guidance on QSARs and Grouping* [4]).

The biological samples to be collected in the *in vivo* toxicologogical studies should be relevant i.e. based on the expected/known ADME/toxicokinetic characteristics of the nanomaterial. Liver, spleen, brain (including the olfactory bulb and the hippocampus), kidney, bone marrow, lung and lymph nodes and pleural tissue (for fibres) are typical examples of tissues to which nanomaterials are known to distribute. Lung is strongly advised, e.g. in order to set external exposure – lung dose and enable an insight on the 'internal dose'.

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 [5]freezing for burden analysis ([6], [7]

Please note that information about toxicokinetics and nanomaterials can be found in the *Appendix R7-2 Recommendations for nanomaterials applicable to Chapter R7c Endpoint specific guidance* [8].

3.1.1 Consideration of rat lung overload within inhalation toxicity assessment

The term 'lung overload' or 'particle overload' as it is also known, is a phenomenon associated with exposure to poorly soluble particles (PSP), with generally low toxicity and occurs when a threshold dose of particles is achieved within the lung. During sub-chronic and chronic exposure to PSP, the lung burden of particles increases until a steady state or equilibrium is achieved between deposition and clearance of particles [9]as shown by the A, B and C traces in Figure 1. Below the lung overload threshold, particles are cleared via normal mechanisms at a normal clearance rate, generating little or no appreciable response.

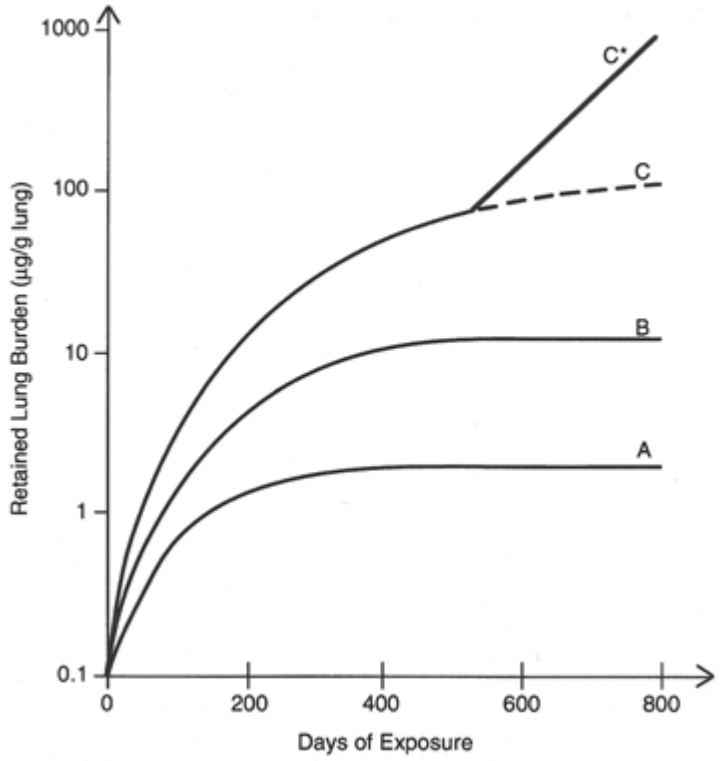

Figure 1: Schematic representation of the relationship between retained lung burden and length of exposure leading to the phenomenon of lung overload. Curves A, B, and C are associated with progressively increasing exposure concentrations. If the exposure level is sufficiently high and the length of exposure sufficiently long, alveolar macrophage-mediated clearance of particles can be overwhelmed. When this occurs, retained lung burden increases linearly with further exposure (curve C*). Reproduced from [9].

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. This is shown in the C* trace of Figure 1 whereby at the point of threshold, particle retention occurs exponentially rather than an equilibrium being established (as demonstrated by the dashed line).

The result of this rapid net increase in particle accumulation is lung inflammation, cessation of alveolar-mediated clearance and an increase in accumulation of particle laden macrophages within the lung alveoli. The continued build-up of particles leads to a higher rate of transfer to lymph nodes and accumulation of particles in the lung interstitia. Persistent inhalatory exposure leads to chronic inflammation which in turn is likely to lead to fibrosis, alveolar cell

proliferation (hyperplasia), the conversion of cells to cell types not normally associated with the specific lung location (metaplasia). The final result may be local tumour formation (neoplasia) as shown in Figure 2 ([10]; [9]; [11]). The progression of the inflammatory reactions toward a granulomatous type was found to depend on the exposure duration and the amount of the particle (surface) burden in the lung [12].

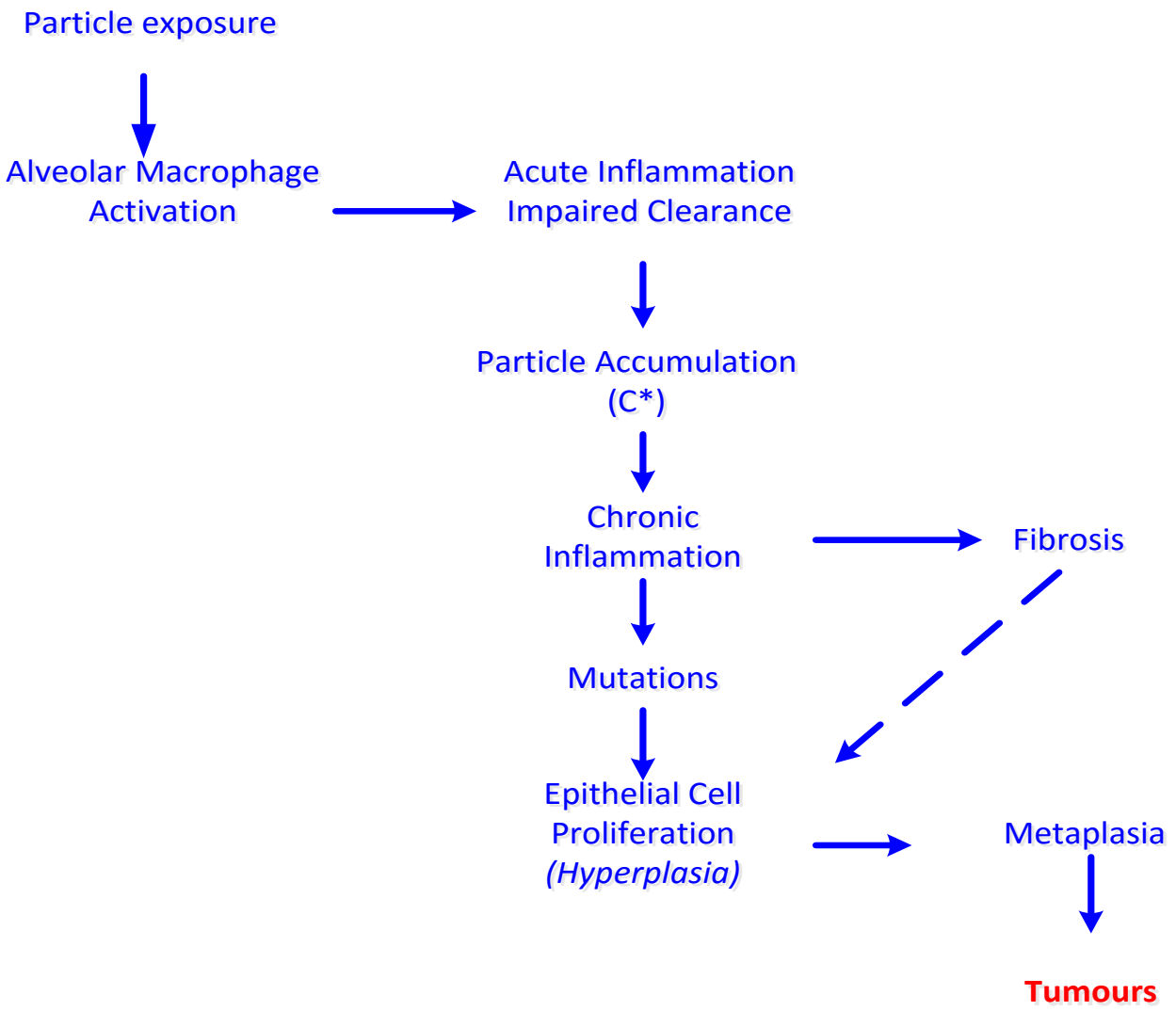

Figure 2: Suggested pathogenic sequence of effects of chronically inhaled particles in rats. Adapted from [11].

The driving force behind this cascade of effects is thought to be the particle load rather than an intrinsic property of the particles themselves and this phenomenon that occurs at high particle inhalatory exposure is known as lung overload. The situation of overload is most commonly associated with repeated inhalation exposure to particles but it can also occur after single or repeated instillation of particles 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 [10]. Since this phenomenon occurs at high level of inhalatory exposure, it is often argued that the observed effects are a product of the particle overload caused by experimental conditions and not necessarily a true reflection on the intrinsic toxic potential of the particles to cause inflammation, fibrosis and cancer.

The generation of overload conditions may be seen as a point of weakness within a study design and hinder accurate hazard and risk assessment, the suggested differences in species

susceptibility introducing further uncertainty. In a retrospective analysis by [13] they analysed studies considering the lifetime tumour occurrence in rats after repeated dose short term intratracheal instillation of 19 different PSP with low toxicity. Among other drawbacks within the studies (such as the lack of low-dose studies) the authors pointed towards significant issues with study design that resulted in lung overload in the test subjects. [13]concluded that, when the lung-overload threshold is exceeded, rats develop lung tumours from ongoing inflammation as opposed to particle-specific toxicity, whilst humans do not. Furthermore, the authors suggested that the reported results for PSP in rats were not a reliable basis for predicting human lung cancer risk. They argued that the response of rats to PSP lung overload is stereotyped and unique to that species and pointed towards human exposure to justify this. Specifically, they noted that workers historically exposed to potentially lung-overloading burdens of inhaled dust (e.g., coal workers, underground miners using diesel equipment) do not exhibit an established lung-cancer excess despite the potential for lung overload. However, a recent epidemiological study evaluating the underlying cause of death for 9033 underground coal miners from 31 US mines after 37 years of follow-up (Graber et al. 2014), found a significant relationship between coal mine dust exposure and lung cancer mortality. Hence, the data obtained from rats may still be useful to predict the effects in humans.

Regarding the particular sensitivity to lung overload between different species, in a comparative study assessing the long-term pulmonary response of rats, mice and hamsters to inhalation of pigmentary grade titanium dioxide, the authors found species differences ([7]). Lung burden was shown to be lower in hamsters at concentrations which caused overload in rats and mice. 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 [14]. 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 [15]and that studies in mice and hamsters were also negative for some particles that have been classified as known human carcinogens ([16]; [17]).

The interpretation of data obtained after high doses of PSP particles should be approached with caution and appropriate consideration should be given to the mechanistic driver behind any pathogenic effects detected. The reason for this is to establish the relevance of the results to humans and whether alteration of the default assessment factors is warranted or appropriate in the derivation of exposure limits.

For further information, review articles covering this subject include: [9], which provides an excellent in-depth discussion of particle deposition, clearance and lung overload; [18], which discuss the importance of overload in the context of risk assessment.

Due to the issues highlighted above, in the absence of the relevant data on particles clearance, the studies performed with PSP administered at concentrations that could induce lung overload would be difficult to interpret and of an ambiguous regulatory value.

Therefore, to prevent the repetition of a study, for the PSP particles it is highly recommended to perform lung burden determinations. The measurements of changes in lung burden over time post-exposure allow to distinguish between a highly soluble, semi-soluble, and non-soluble particle, to clarify the deposited vs the exposed particle amount and bring essential information on the clearance. In addition, this data could also be important in the context of read-across and weight of evidence.

The question of which dose metric best describes the association between deposited dose in the lung, overload conditions and subsequent pathogenic effects is particularly pertinent. There have been several suggested metrics with the first being **particle volume** as suggested by [19]. These authors hypothesised that overload begins when the particulate volume exceeds approximately 60 μm^3/AM (which produces a 6% increase in the average AM 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 AM volume). Such a driver of lung overload has also been more recently suggested for carbon nanotubes ([20], [21]).

Other metrics have also been found to be important in driving the lung overload. Oberdoerster et al. [22]suggested the retained **surface area** as an appropriate metric for correlating overload with retarded clearance, particularly if nanoparticles are involved. There are several studies which suggest that, as metric, particle surface area correlates well with induced pathogenic events ([23]; [18], [24]). In a study by Tran et al. (2000), 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. In relation to surface area as a driving metric, due to their known high level of surface area, the potential for overload effects may be increased with those nanomaterials which exhibit a high biologically accessible surface area.

Another important metric is **mass**. Whilst some studies indicate mass as a less sensitive indicator of lung overload [25], the mass concentration is important because there is already a large body of research on exposure to and toxicity of particles using the mass-based metric.

Other studies [26], [27]found that the **particle number** was the best dose metric while others (Warheit *et al.* 2007a, b)) found that the **number of functional groups** in the surface of nanoparticles influenced their toxicity.

3.1.2 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, nanoparticles may bind to assay components including the substrates (e.g. CNT with the reagent in MTT assays; [28]) or the biomarker being measured, (e.g. LDH and cytokine proteins, see for example [29]).

A summarised list of potential sources of interferences with commonly used assays has been developed by Kroll et al. [30]and is reproduced in the table below.

Table 1: Potential sources of interferences with commonly used assays

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)	Ca2+-depletion Dye adsorption	Reduced indication of apoptosis Reduced indication of necrosis	Carbon nanoparticles
Neutral red Caspase	Colorimetric detection of intact lysosomes Fluorimetric detection of Caspase-3 activity (apoptosis marker)	Dye adsorption Inhibition of Caspase-3	Reduced indication of cell viability Reduced indication of oxidative stress	Carbon nanoparticles Carbon nanoparticles
Stress response				
DCF	Fluorimetric detection of ROS production	Fluorescence quenching	Reduced indication of oxidative stress	Carbon nanoparticles
Inflammatory response				
ELISA	Colorimetric detection of cytokine secretion	Cytokine adsorption	Reduced indication of cytokine concentration	Carbon nanoparticles Metal oxide nanoparticles

It should be noted that this list is not exhaustive and the potential for inhibition or enhancement of test results should always be investigated.

Within some standard methodologies such as ISO 29701 (Nanotechnologies - endotoxin tests on nanomaterial samples for in vitro systems-- Limulus amebocyte lysate (LAL) test [31]), 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.

Any alteration to the test outcome may indicate inhibition (measurement of a value lower than expected) or enhancement (measurement of a value higher than expected) of the assay. The use of spiked samples is encouraged as it allows a simple yet effective method of investigating potential assay interference and would give greater confidence in derived results. This is especially important due to the uncertainty that surrounds the effect of nanomaterials on the performance of routinely used assays.

The use of such methods to investigate possible inhibition or enhancement of results should be carried out wherever possible irrespective of standard method requirement; however, this may not always be possible. In many of the studies reported it is not possible to ascertain whether the assays were adequately controlled to assess for interference. Thus, as a general precaution, it is advisable to use more than one assay to assess the studied endpoint or effect,

as advised by Landsiedel et al. [32]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 [33] and as such may cause issues with tests such as OECD TG 431/EU B.40 Human Skin Model tests (EPISKIN™, EpiDerm™) due to their use of 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 Skin and eye irritation/corrosion and respiratory irritation

The test method(s) described in the guidance are considered applicable to nanomaterials. However, regarding the use of non-testing data, i.e. Sections 7.2.3.1, 7.2.4.1 (on non-human data), Appendixes R.7.2-2 and R.7.2-3 (on QSARs and expert systems) and Figures R.7.2-2 and R.7.2-3 (on integrated testing strategy) it is necessary to take into account that the use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this. the use of *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by-case basis only.

3.2.2 Skin and respiratory sensitisation

The test method(s) described in the guidance are considered as applicable to nanomaterials as they are to other substances. However, regarding the use of non-testing data, i.e. Sections R7.3.3.1, R7.3.4.1 and R7.3.5.1 (on non-human data), and Figure R.7.3-1 (on integrated testing strategy) of the parent guidance it is necessary to take into account that the use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this, the use of *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by-case basis only.

3.2.3 Acute Toxicity

When considering the testing strategy for acute toxicity (Section R.7.4.6.3), with respect to new data generation it should be noted that the route of exposure to be used for acute toxicity evaluation depends on the nature of the substance (e.g. gas or not, molecular weight, log Kow, solid with inhalable particle size (e.g. nanomaterials)) and should reflect the most likely route of human exposure. Consequently the ITS for acute toxicity endpoint (Figure R.7.4-1 of the parent guidance) has not only to consider if the substance is gaseous or not, but also if the substance is inhalable.

Regarding the use of non-testing data, i.e. Sections R7.4.3.1, R7.4.4.1 (on non-human data), and R7.4.5.1(on classification and labelling) it is necessary to take into account that the use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis only.

3.2.4 Repeated dose toxicity

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

- As, especially for workers, inhalation may be the most likely route for nano(particles) exposure, the repeated dose toxicity studies are recommended to be performed via inhalation, unless there is compelling information (e.g. uses, dissolution rate, etc.) that justifies another route. In this respect further modification of the protocols described in the OECD TG 412 and 413 ([34] and [35])may be required with full justification;
- When dose ranging studies or repeated dose studies are performed, for poorly-soluble nanomaterials, several basic toxicokinetics parameters could be considered (further details in Appendix R7-2 Recommendations for nanomaterials applicable to Chapter R7c Endpoint specific guidance [8]);
- When performing an inhalation test for PSP of low toxicity the lung overload should be addressed. Due to the issues highlighted in section 3.1.1(Consideration of rat lung overload within inhalation toxicity assessment) it is highly recommended to perform lung burden determination;
- To monitor the fate of the particles in the body it is recommended to sample at several time points in different organs/compartments (data from range-finding studies could be used to determine the appropriate sampling times);
- Since the lower respiratory tract (i.e., the alveoli) is the primary site of deposition 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, 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) [36]

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 the use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this, the use of *in silico* models for nanomaterials has also yet to be established or accepted. Therefore, the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by-case basis only.

3.2.5 Reproductive and development toxicity

Regarding the use of non-testing data, i.e. Section R7.6.4.1 (on non-human data), and R7.6.6.2 (on integrated testing strategy) it is necessary to take into account that the use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by-case basis only.

3.2.6 Mutagenicity and Carcinogenicity

3.2.6.1 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.

One such test, the bacterial reverse mutation (Ames) test (OECD TG 471/EU B.12/13: Bacterial reverse mutation test (in vitro)), uses amino acid requiring strains of *Salmonella*

typhimurium and *Escherichia coli* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs ([37]; [38]; [39]). The principle of this bacterial reverse mutation test is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid (histidine or tryptophan). The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain (OECD TG471, [40]). A positive test indicates that the test substance can act as a mutagen, and may hold carcinogenic potential (as cancer is often linked to DNA damage).

Generally, the major drawback of the Ames test is that it is difficult to translate prokaryotic data into eukaryotic genotoxicity testing, and the test may generate false positive results [41]. Indeed, 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 [42]. In relation to nanomaterials, a recent review of the applicability of genotoxicity tests to NM questioned whether the Ames test was accurately representative of NM genotoxicity [32]. The Landsiedel study [32] reported that of those studies reviewed, results were predominantly negative (5/6 studies). The group speculated that it is likely that some NMs are not able to cross the bacterial wall, whilst others kill the test organism as they are bactericidal.

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.

3.2.6.2 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 3.2.6.1Therefore, the bacterial mutation assay should not be used as a single 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.

During the OECD/WPMN workshop on the Genotoxicity of Manufactured Nanomaterials in Ottawa, Canada in November 2013 [43], seven general recommendations 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. [44], Pfuhler et al. [45], Doak et al. [46]):

1. "*The use of the Ames test (TG 471* [40]*) 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 [47] or 490 [48]) 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 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. "*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*".

4. "*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*".

5. "*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 [49]) or a chromosomal aberration test (OECD 473 [50]) is required. A project on the adaptation of the *in vitro* mammalian cell micronucleus assay (TG 487 [49]) for NMs testing was approved in OECD WPMN rolling work plan. The study focuses on physico-chemical characterisation of NMs and protocol modifications (selection of cell type with respect to uptake mechanisms, use of cytochalasin B, timing of exposure to NMs, specification of controls, dose ranges and dose metrics). In the study, five selected cell types (primary human lymphocytes, TK6 cells, Caco-2 cells, A549 cells, V79 cells) and 3 nanomaterials (gold NPs, silver NPs, silica NPs) are tested. The study is expected to take place in 2015 and 2016. (Project 4.95: Guidance Document on the Adaptation of In Vitro Mammalian Cell Based Genotoxicity TGs for Testing of Manufactured Nanomaterials.

6. *"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 issue 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."*

7. "*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 NMs - at least for workers - (See R.7.a, Section R.7.5.6).

Regarding the use of non-testing data, i.e. Sections R7.7.3.1, R7.7.4.1 R7.7.10.1 R7.7.11.1 (on non-human data), R7.7.6.2 (on ITS on mutagenicity) and R.7.7.13 and Figure R.7.7-2of the parent guidance (on ITS on carcinogenicity) it is necessary to take into account that the use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by-case basis only.

Appendix R7-2 to Chapter R.7c

2.1.3 Guidance on Toxicokinetics

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 toxicokinetic properties of nanomaterials (See Section R.7.12.2.1). For a nanomaterial the toxicokinetic profile may depend on several physicochemical parameters, e.g. composition, size, shape, agglomeration/aggregation state, surface properties (including surface charge), hydrophobicity, and dissolution.

Data on solubility and dissolution in relevant biological fluids and testing media is an essential starting point in understanding a particle's behaviour and ADME properties.

In the case of poorly soluble particles 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* [4]). Therefore, 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 ranging studies or repeated dose/reproductive studies are performed, for poorly-soluble nanomaterials, several additional analysis 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 feasible. Alternatively, other methods such as multiplexed imaging by use of laser desorption/ionization mass spectrometry LDI-MS can be used ([51]; [52]).
- Sampling at several time points in different organs/compartment to monitor the fate of the particles in the body (data from range-finding studies could be used to determine the appropriate sampling times)

It could be useful to keep the samples to allow the performance of later analysis. (e.g. freezing of tissue for burden analysis, storage by freezing or tissue fixation for microscopy ([5]), freezing for burden analysis ([6], [7]))

Information on the possible behaviour of the nanomaterials can be supplemented also with *in vitro* and *in silico* predictions based on the physicochemical data.

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