GUIDANCE

Guidance on information requirements and chemical safety assessment

Appendix R7-2 for nanomaterials

applicable to Chapter R7c Endpoint specific guidance

Version 3.0

October 2021

LEGAL NOTICE

Guidance on information requirements and chemical safety assessment
Appendix R7-2 for nanomaterials applicable to Chapter R7c - Endpoint specific guidance

Reference: ECHA-21-H-03-EN
Cat. Number: ED-08-21-272-EN-N
ISBN: 978-92-9481-959-8
DOI: 10.2823/783979
Publ.date: October 2021
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 information request form. The information request form can be accessed via the Contact ECHA page at: *http://echa.europa.eu/contact*

European Chemicals Agency

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

Visiting address: Telakkakatu 6, 00150 Helsinki, Finland

DOCUMENT HISTORY

Version	Changes	Date
Version 1	First edition	April 2012
Version 2.0	• Update of section 1.1.1. on aquatic bioaccumulation, to explain the general limitations of K_{ow} as a basis for a waiver for nanomaterials and to provide advice on the applicability of the available OECD guidelines; • Update of section 1.1.2 on Effects on terrestrial organisms to provide advice on spiking methods and on use of different metrics. Please note that the numbering of the sections and sub-sections has changed from that in Version 1, the section numbers above refer to the updated numbering of the guidance as used in Version 2.0 (onwards).	May 2017
Version 3.0	The chapter was updated and restructured under: • 2.1.3. Guidance on Toxicokinetics • 2.1.4. Recommended approach for gathering toxicokinetics information on nanomaterials according to REACH ○ 2.1.4.1. Detection methods of nanoforms in tissues and organs ○ 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 ▪ 2.1.4.2.2. Cases where new repeated dose toxicity data via inhalation route needs to be generated (data gap in dossier)	October 2021

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.

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.7c [1] (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" [2] 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/regulations/nanomaterials

[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

1 RECOMMENDATIONS FOR ECOTOXICOLOGICAL ENDPOINTS for NANOMATERIALS:

1.1 Specific advice for endpoints

When following the endpoint specific advice provided by this guidance, please take into account that the advice regarding sample preparation provided in section 2.1.1 of *Appendix R7-1 to ECHA Guidance R.7.a* and the general advice on ecotoxicity and fate testing provided in section 1.1 of *Appendix R7-1 to ECHA Guidance R.7.b* are also applicable for this guidance.

1.1.1 Aquatic bioaccumulation

In the parent guidance, section R.7.10.2 describes the REACH Annex IX information requirements for aquatic bioaccumulation and the use of alternative information when measured data are not available. However, the prediction techniques described in the parent guidance and the use of surrogate information (e.g., the octanol-water partition coefficient K_{ow}), applicable for many classes of organic substances, may not be applicable to predict bioaccumulation potential of nanoparticles. In the case of nanomaterials, it is not normally possible to make log K_{ow} or solubility estimations since nanomaterials are dispersed and not in solution. However, measurement of n-octanol/water partition coefficient may still be of value for organic nanomaterials that are water soluble and have a high dissolution rate.

1.1.1.1 Non-testing data

Section R.7.10.3.2 of the parent guidance concerns non-testing data, e.g. quantitative structure-activity relationships (QSARs), bioconcentration factor (BCF) models based on log K_{ow} and grouping approaches for assessing aquatic bioaccumulation. The use of *in silico* models for nanomaterials has yet to be established or accepted and therefore, when used, needs to be thoroughly reported and justified. With regard to nanoparticles, it is often not possible to make bioaccumulation estimations based on log K_{ow} or solubility, as explained above and in Appendix R7-1 to ECHA Guidance R.7.a [3] Sections 2.2.1, 2.2.2 and 2.2.4. Nevertheless, non-testing methods and parameters such as those listed in *Appendix R7-1* to the ECHA IR&CSA Guidance chapter R.7.a, could be useful for this endpoint when considered as part of a weight of evidence approach.

Section R.7.10.3.4 of the parent Guidance describes other indicators for bioaccumulation potential. This includes a screening approach where potential bioaccumulation can be estimated from the value of the n-octanol/water partition coefficient (K_{ow}). Furthermore, REACH Annex IX 9.3.2 column 2 states that, for instance, a value for log $K_{ow} \leq 3$ could be used as a waiving argument to justify omitting the testing of bioaccumulation in aquatic species. This approach is not necessarily appropriate for nanoparticles, as prediction techniques based on equilibrium partitioning do not strictly apply to undissolved nanoparticles - as explained in Appendix R7-1 to chapter R.7a of the ECHA IR&CSA guidance Sections 2.2.1, 2.2.2 and 2.2.4. As outlined in OECD 40 [4], the K_{ow} value is often not suitable for predicting bioaccumulation for nanomaterials.

Taking into account the above, waiving the information requirement for bioaccumulation in aquatic species based on log K_{ow}, log K_{oc} or other screening methods is in most cases not appropriate for nanomaterials.

1.1.1.2 *In vivo* tests for aquatic bioaccumulation

The parent guidance section R.7.10.3.1 describes OECD TG 305 "Bioaccumulation in Fish: Aqueous and Dietary Exposure" [5] as an appropriate *in vivo* test method to fulfil the information requirement set for bioaccumulation in aquatic species in Annex IX 9.3.2. Further information on bioaccumulation testing strategies can be found in *Chapter R.11 of the Guidance on IR&CSA*, concerning PBT assessment.

OECD TG 305 is partially applicable for nanomaterials. It is applicable when the dietary exposure route is followed; the aqueous exposure route resulting in a *bioconcentration factor (BCF)* is not applicable for most nanomaterials if they remain as nanoparticles. For organic nanomaterials that are water soluble and/or would have a high dissolution rate, a BCF study is applicable via the aqueous route. However, there may be a need for additional considerations and testing for bioaccumulation of the particular form of such nanomaterials. The BCF is the ratio of the concentration of a substance in an organism to its concentration in water, once a steady state has been achieved. For nanoparticles, a BCF cannot be calculated as no thermodynamic equilibrium will be reached between the organism and the water phase [6] and a stable aqueous concentration cannot be maintained. Nevertheless, uptake and depuration rate as kinetic data can be assessed instead for nanomaterials and particles. Therefore provided these kinetic parameters are used and estimated, the flow-through method can still be applied for estimation of the nanomaterial's bioaccumulation potential ([4], [7], [8] and [9]).

A new OECD Guidance for assessing the apparent accumulation potential for nanomaterials is under development. This guidance, when available, will provide information on how to test nanomaterials via the dietary exposure and on how to measure and quantify the accumulation potential in fish. In the meantime, the existing draft GD on dietary exposure can give information on that exposure method[4] .

Other *In vivo* tests for bioaccumulation could be also used, apart from the testing in aquatic media, such as bioaccumulation in sediment and soil. OECD TG 315 Bioaccumulation in Sediment dwelling Benthic Oligochaetes [10] and OECD TG 317 Bioaccumulation in Terrestrial Oligochaetes [11] are in principle applicable for nanomaterials, but expert judgement will be required for performing the bioaccumulation tests and interpreting the results ([9], [12]). The results of applying these TGs (OECD TG 315 and OECD TG 317), taking into account the current challenge in testing bioaccumulation of nanomaterials in fish, may be used as weight of evidence in bioaccumulation assessment. Soil and sediment compartments are considered potential sinks for nanomaterials and therefore they are also relevant when considering nanomaterial fate in the environment.

In order for them to be considered reliable, whenever tests for bioaccumulation in aquatic or sediment and soil organisms are performed, the recommendations on sample preparation and ecotoxicity and fate testing given in Appendix R7-1 to chapter R7a, section 2.1.1. (Sample preparation) and Appendix R7-1 to R7b, section 2.1 (General advice on how to perform nanomaterials ecotoxicity and fate testing) should be followed. In addition, test concentrations should be monitored throughout the whole test duration to account for concentration-specific changes in dispersion and agglomeration/ aggregation characteristics, using a mass metric and nano-specific metrics such as surface area, particle number, when relevant ([9], [11]).

1.1.2 Effects on terrestrial organisms

1.1.2.1 Non-testing data

In the parent guidance (Chapter R7c), Section R.7.11.3.1, the possibility of using non-testing approaches e.g. QSAR, grouping and the equilibrium partitioning method (EPM) to estimate soil and terrestrial toxicity is explained.

With respect to nanomaterials, estimates based on "partitioning" are limited to distribution of a substance in molecular form (excluding ionic forms as explained in parent guidance). In the case of nanoparticles, the partitioning method may underestimate exposure in soil and sediment environments and overestimate the exposure in water. If the particle size is small,

[4] Available at: http://www.oecd.org/env/ehs/testing/draft-guidance-review-documents-monographs.htm

distribution via air may also occur. There are no estimation methods available for particle distribution, so this has to be dealt with on a case-by-case basis.

1.1.2.2 Testing data

Regarding testing for effects on terrestrial organisms, the methods described in the parent guidance Section R.7.11 are, in principle, also applicable for testing nanomaterials.
The application technique in e.g. sample preparation and spiking has been shown to have an effect on the availability of the nanomaterial and its level of ecotoxicity in soil [7]. Therefore it is essential that the sample preparation and spiking method applied are well justified and reported in detail, and that the recommendations set out in the OECD Guidance manual for the testing of manufactured nanomaterials: OECD's Sponsorship Programme; first revision [13] (OECD, 2009), Guidance Notes on Sample Preparation and Dosimetry for nanomaterials [14] and OECD 40 [4] are followed.

When performing the test, the test material needs to be homogenously dispersed in the soil. OECD 40 [4] describes different spiking methods; particles can be dispersed as aquatic dispersion into soil (wet spiking) or directly into test media (dry spiking), or put onto a carrier e.g. silica sand or spiked food. The optimal spiking method depends on both the test material and the test method. It will depend on the physicochemical properties of the nanomaterial, the target concentration, the medium, and the bioassay method selected, and preliminary data gathered prior to the test. For example, ZnO nanoparticles can be introduced to soil as aqueous dispersions prepared in the soil extracts to achieve homogenous distribution [15] and satisfactory spiking homogeneity can be achieved with Ag nanoparticles using soil as a solid carrier [7].

Unless the use of the mass metric only can be justified, nano-specific metrics such as particle number and surface area should in principle be used whenever relevant. Using multiple metrics allows retrospective correlation of the measured response with different dose metrics, (see Section 2.1.1 of Appendix to Chapter R7.b). If e.g. only the mass metric is recorded during the test, conversion between metrics increases the uncertainty in interpretation of the test results and therefore measurement of multiple metrics during testing is recommended (as highlighted in section 2.1.1 of *Appendix R7-1 to ECHA Guidance R.7.a)*.

In addition to these recommendations, it should be considered that measurements of the nanomaterial's concentration (using different metrics, e.g. particle number, surface area, or mass concentration) should be monitored throughout the test at all test concentrations to account for concentration-specific changes in dispersion and agglomeration/aggregation characteristics if possible ([10], [12].

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) [1] 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, section 2.1.1.

It is acknowledged that the OECD TG 417 for toxicokinetics [16], generally intended for the oral route, does not contain specific provisions for nanomaterials. Neither does it 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) [17]. 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 [18], OECD TGs 412 [19] and 413 [20], and in the ISO technical Report on toxicokinetics of nanomaterials [17].

The OECD TG 412 (Subacute Inhalation Toxicity: 28-day Study) [19] and the OECD TG 413 (Subchronic Inhalation Toxicity: 90-day Study) [20] 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 [18] 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 [17] 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 [21], [22]. 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 [23] but they may also be directly translocated from the respiratory system into the blood [24], [21], [25], [26], [27], [28]. 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 [29]. In an acute inhalation study with gold nanoparticles in human volunteers [30], [31], 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 [32], [33]. 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 [34]. 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 [34].

In ISO TR 22019 [17] 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 [35]. 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 2.1.1.1. 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 [36]. 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 2.1 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) [2]. 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 make 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) [1]. 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 2.1.1 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 [37]. 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 [17].

General advice regarding dissolution for nanoforms is given in Appendix R.7-1 for nanomaterials applicable to Chapter R7a, Section 2.1.1.

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 2.2.2. 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 [18]. Consequently, 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. The duration of a repeated dose toxicity study is considered sufficient if an equilibrium between deposition and clearance of particles [38] 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 [38] 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 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*". 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), laser-ablation-ICP-MS etc. could be used [39], [40]. It should be noted, however, that as with all imaging techniques, these are qualitative or semi-quantitative methods that have limitations in terms of 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 [41].

Inductively coupled plasma mass spectrometry (ICP-MS) is a common technique for absolute quantification of the cellular uptake of metal or metal oxide nanoparticles [42]. 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) [43]. 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 [42]. 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 [44]. 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 [45], freezing for burden analysis [46], [47]). 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 [17].

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 toxicity (RDT) studies 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, physic-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 [37]. 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 2.2.2.

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 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 [32].

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 [38] 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 [17] 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 [29] or bone marrow and to local lymph nodes has been reported [48]. 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 [48].

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 [49]. 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 [26].
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 [48]. Furthermore, translocation between organs, although very low, has been observed [24]. Early studies in rodents provided rough estimates that <1% mass of administered nanoparticles with a diameter of <50 nm will translocate [24]. The reported estimates are most frequently around 0.3% or less of the administered dose for a given tissue at 24 h post-exposure [31].
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 [17] 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 [50].

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 [51]. However, although the data on skin penetration of nanomaterials is inconsistent [52], 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 [53].

REFERENCES

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

[2] 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.

[3] ECHA, "Appendix R7-1 for nanomaterials applicable to Chapter R7a Endpoint specific guidance," [Online]. Available: http://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment.

[4] OECD, "Series on the Safety of Manufactured Nanomaterials- No. 40. Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines. Expert Meeting Report ENV/JM/MONO(2014)1," 2014. [Online]. Available: http://www.oecd.org/science/nanosafety/publications-series-safety-manufactured-nanomaterials.htm.

[5] OECD, "Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure, OECD Guidelines for the Testing of Chemicals, Section 3," 2012. [Online]. Available: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-3-degradation-and-accumulation_2074577x.

[6] G. Cornelis, "Fate descriptors for engineered nanoparticles: the good, the bad, and the ugly," *Environmental Science: Nano,* vol. 2, pp. 19-26, 2015.

[7] K. Hund-Rinke, K. Schlich and T. Klawonn, "Influence of application techniques on the ecotoxicological effects of nanomaterials in soil," *Environmental Sciences Europe,* vol. 24, no. 1, pp. 1-12, 2012.

[8] L. M. Skjolding, *Bioaccumulation and trophic transfer of engineered nanoparticles in aquatic organisms-PhD thesis DTU, Denmark,* 2015.

[9] K. Rasmussen, M. Gonzalez, P. Kearns, J. Riego Sintes, F. Rossi and P. Sayre, "Review of achievements of the OECD Working Party on Manufactured Nanomaterials' Testing and Assessment Programme. From exploratory testing to test guidelines," *Regulatory Toxicology and Pharmacology,* vol. 74, p. 147–160, 2016.

[10] OECD, "Test No. 315: Bioaccumulation in Sediment-dwelling Benthic Oligochaetes, OECD Guidelines for the Testing of Chemicals, Section 3," 2008. [Online]. Available: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-3-degradation-and-accumulation_2074577x.

[11] OECD, "Test No. 317: Bioaccumulation in Terrestrial Oligochaetes, OECD Guidelines for the Testing of Chemicals, Section 3," 2010. [Online]. Available: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-3-degradation-and-accumulation_2074577x.

[12] K. Hund-Rinke, M. Herrchen and K. Schlich, "Integrative test strategy for the environmental assessment of nanomaterials. UBA-FB 00," 2014. [Online]. Available: http://www.bmub.bund.de/fileadmin/Daten_BMU/Pools/Forschungsdatenbank/fkz_3712_65_409_nanomaterialien_teststrategie_bf.pdf.

[13] OECD, "Guidance manual for the testing of manufactured nanomaterials: OECD's sponsorship programme. First revision", Series on the safety of manufactured nanomaterials, ENV/JM/MONO(2009)20/REV," 2009. [Online]. Available: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2009)20/rev&doclanguage=en.

[14] 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.

[15] P. Kool, M. Ortiz and C. van Gestel, "Chronic toxicity of ZnO nanoparticles, non-nano ZnO and ZnCl2 to Folsomia candida (Collembola) in relation to bioavailability in soil," *Environ Pollut.,* vol. 159, no. 10, pp. 2713-2719, 2011.

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

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

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

[19] 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.

[20] 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.

[21] G. Oberdörster, A. Elder and A. Rinderknecht, "Nanoparticles and the Brain: Cause for Concern?," *J Nanosci Nanotechnol.,* vol. 9, no. 8, p. 4996–5007, 2009.

[22] 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.

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

[24] 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.

[25] 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.

[26] 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.

[27] W. Kreyling, M. Semmler-Behnke, J. Seitz, W. Scymczak, A. Wenk, P. Mayer, T. S and G. Oberdörster, "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.

[28] 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.

[29] 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.

[30] 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.

[31] 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.

[32] 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.

[33] 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.

[34] 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.

[35] 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.

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

[37] 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.

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

[39] 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.

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

[41] 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.

[42] 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.

[43] 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.

[44] 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.

[45] 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.

[46] 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.

[47] 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.

[48] 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.

[49] 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.

[50] EFSA, "Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health," *EFSA Journal,* vol. 19, no. 8, 2021.

[51] 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.

[52] 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.

[53] 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.

EUROPEAN CHEMICALS AGENCY
ANNANKATU 18, P.O. BOX 400,
FI-00121 HELSINKI, FINLAND
ECHA.EUROPA.EU