Regulatory Toxicology and Pharmacology 71 (2015) S1–S27

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping)

Josje H.E. Arts [a], Mackenzie Hadi [b], Muhammad-Adeel Irfan [c], Athena M. Keene [d], Reinhard Kreiling [e], Delina Lyon [f], Monika Maier [g], Karin Michel [h], Thomas Petry [i], Ursula G. Sauer [j], David Warheit [k], Karin Wiench [c], Wendel Wohlleben [c], Robert Landsiedel [c,*]

[a] AkzoNobel, Technology and Engineering, Arnhem, Netherlands
[b] Shell Health, Shell International B.V., The Hague, Netherlands
[c] BASF SE, Ludwigshafen, Germany
[d] Afton Chemical, Richmond, VA, USA
[e] Clariant Produkte GmbH, Sulzbach, Germany
[f] Shell Health, Shell Oil Company, Houston, TX, USA
[g] Evonik Degussa GmbH, Hanau, Germany
[h] Henkel AG & Co. KGaA, Düsseldorf, Germany
[i] Toxminds BVBA, Brussels, Belgium
[j] Scientific Consultancy – Animal Welfare, Neubiberg, Germany
[k] DuPont Haskell Global Centers for HES, Newark, DE, USA

ARTICLE INFO

Article history:
Received 11 March 2015
Available online 26 March 2015

Keywords:
Nanomaterials
Grouping
Read-across
Intrinsic material properties
System-dependent properties
Biopersistence
Biodistribution
Cellular effects
Apical toxic effects
Risk assessment

ABSTRACT

The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) 'Nano Task Force' proposes a *Decision-making framework for the grouping and testing of nanomaterials* (DF4nanoGrouping) that consists of 3 tiers to assign nanomaterials to 4 main groups, to perform sub-grouping within the main groups and to determine and refine specific information needs. The DF4nanoGrouping covers all relevant aspects of a nanomaterial's life cycle and biological pathways, i.e. intrinsic material and system-dependent properties, biopersistence, uptake and biodistribution, cellular and apical toxic effects. Use (including manufacture), release and route of exposure are applied as 'qualifiers' within the DF4nanoGrouping to determine if, e.g. nanomaterials cannot be released from a product matrix, which may justify the waiving of testing. The four main groups encompass (1) soluble nanomaterials, (2) biopersistent high aspect ratio nanomaterials, (3) passive nanomaterials, and (4) active nanomaterials. The DF4nanoGrouping aims to group nanomaterials by their specific mode-of-action that results in an apical toxic effect. This is eventually directed by a nanomaterial's intrinsic properties. However, since the exact correlation of intrinsic material properties and apical toxic effect is not yet established, the DF4nanoGrouping uses the 'functionality' of nanomaterials for grouping rather than relying on intrinsic material properties alone. Such functionalities include system-dependent material properties (such as dissolution rate in biologically relevant media), bio-physical interactions, *in vitro* effects and release and exposure. The DF4nanoGrouping is a

Abbreviations: AOP, adverse outcome pathway; APPIE, Association of Powder Process Industry; ASTM, American Society for Testing and Materials; ATP, adenosine triphosphate; BAM, *German Federal Institute for Materials Research and Testing*; BAuA, *German Federal Institute for Occupational Safety and Health*; BET, (Method of) Brunauer, Emmett and Teller; BSAI, biological surface adsorption index; BSF, biological simulation fluid; BSI, British Standards Institute; bw, body weight; CEN, *European Standardization Organization*; CNT, carbon nanotube; DLS, dynamic light scattering; DNEL, derived-no-effect-level; DMEL, derived-minimal-effect-level; ECETOC, European Centre for the Ecotoxicology and Toxicology of Chemicals; ECHA, European Chemicals Authority; EFSA, European Food Safety Authority; EPA, Environmental Protection Agency; DF4nanoGrouping, Decision-making framework for the grouping and testing of nanomaterials; FP7, 7th Research Framework Programme; FRAS, ferric reducing ability of serum; GBP, respirable granular biodurable particles; HA, hazard assessment; HAR NM (alternatively HARN), high aspect ratio nanomaterial; IRMM, Institute for Reference Materials and Measurements; ISO (TC), International Standardization Organization (Technical Committee); JRC, Joint Research Centre; LDH, lactate dehydrogenase; MPS, mononuclear phagocyte system; MoA, mode-of-action; MNT, micronucleus test; MWCNT, multi-walled carbon nanotube; NC, negative control; NIOSH, National Institute for Occupational Safety and Health; NIST, National Institute of Standards and Technology; NOAEC, no observed adverse effect concentration; OECD, Organization for Economic Co-operation and Development; OEL, occupational exposure limit; PC, positive control; PPS, primary particle size; PSF, phagolysosomal simulation fluid; (Q)SAR, (quantitative) structure activity relationship; RA, risk assessment; REACH, Registration, Evaluation, Authorisation (and Restriction) of Chemicals; ROS, reactive oxygen species; SCCS, Scientific Committee on Consumer Safety; SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks; SEM, scanning electron microscope; SSA, specific surface area; STIS, short-term inhalation study; STOS, short-term oral study; TEM, transmission electron microscope; TG, test guideline; TNF-α, tumor necrosis factor alpha; UBA, *German Federal Environmental Agency*; VSSA, volume-specific surface area; WHO, World Health Organization.

* Corresponding author at: Experimental Toxicology and Ecology, BASF SE, 67056 Ludwigshafen, Germany.
E-mail address: robert.landsiedel@basf.com (R. Landsiedel).

http://dx.doi.org/10.1016/j.yrtph.2015.03.007
0273-2300/

hazard and risk assessment tool that applies modern toxicology and contributes to the sustainable development of nanotechnological products. It ensures that no studies are performed that do not provide crucial data and therefore saves animals and resources.

Information box: definitions of terms used in the present article

Benchmark material: A (nano-)material, which has been tested and evaluated according to standard criteria and to which new materials may reliably be compared for grouping purposes (Kuempel et al., 2012).

(Certified) reference material: A material that has undergone a process for validation or round robin assessment as 'reference material', thereby having fulfilled specific predefined requirements for, e.g., its homogeneity and stability (Stefaniak et al., 2013).

Intrinsic material properties: Characteristics of the material that are determined independently of the biological environment or test system. Accordingly, intrinsic material properties include chemical composition and impurities, primary particle size (PPS), surface area, water solubility and shape or aspect ratio.

Mode-of-action (MoA): Mechanisms by which substances may elicit cellular or apical toxic effects. To date, only a limited number of such mechanisms have been discerned for nanomaterials (cf. Chapters 3.5 and 3.6 'Grouping of nanomaterials by cellular and apical toxic effects' for further information on different MoAs).

Nanoform: As defined by the EU Commission's NANO SUPPORT Project (2012), the term 'nanoform' is used for REACH registration dossiers that (seem to) also address other forms (e.g. bulk). Thus, a nanoform registered 'alone' (not along with non-nanoforms) would be a nanomaterial.

Nanomaterial: In line with the EU definition (EU Commission, 2011), 'nanomaterial' is an overarching term to describe materials containing particles with external dimensions in the size range 1–100 nm.

Nanoparticle: A specific nanosized 'pieces of matter' (EU Commission, 2011).

Substance: The EU Regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH; EP and Council of the EU, 2006) defines a substance a chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurity deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition. Accordingly, in the present article, 'substance' is used as an overarching term encompassing nanosized and non-nanosized substances in all forms regardless of their state of dissolution.

System-dependent properties: Characteristics that are linked to the material's functionality in its environment, such as surface reactivity, dissolution rate, and dispersibility. The outcome of measurements of system-dependent properties is affected by the given surroundings, i.e. the choice of the test system (culture media, supplements, dispersing agents, etc.) or of the product application. System-dependent properties constitute bio-physical interactions of the particles with their environment. Accordingly, 'systems' may be, e.g., matrices in which a nanomaterial is embedded in a product, exposure media (aerosols, suspensions, etc.), or biological systems that the nanomaterial comes into contact with.

"*form ever follows function*" (Louis Sullivan, 1896)

1. Introduction

Given the vast number of nanotechnological products entering the market and the multitude of different nanomaterials already available, hazard and risk assessments of each and every single variant of nanomaterial are impracticable and undesirable for economic reasons and stand in contradiction to the legal requirement to reduce animal testing (EP and Council of the EU, 2006, 2010). The 'grouping' concept aims at making substance hazard assessment more efficient. In its guidance documents, the European Chemicals Agency (ECHA, 2013) describes grouping as the process of uniting substances into a common group *if they are structurally similar with physico-chemical, toxicological, ecotoxicological and/or environmental fate properties that are likely to be similar or to follow a regular pattern*. Such similarities may be due to common functional groups, common precursors, or likely common breakdown products. Within a group, each individual substance may not need to be tested. Instead, endpoint-specific effects of an unknown substance may be derived from the endpoint-specific effects of further substances within the group. 'Read-across' is the application of the grouping concept to fill a data gap within a group of substances by using data from the same endpoint from another substance or other substances (ECHA, 2013; *cf.* Information box – for the definitions of terms as they are used in the present article).

For substances in general, technical guidance documents on grouping are available, e.g. from the Organization for Economic Cooperation and Development (OECD) or the ECHA (ECHA, 2008, 2012a,b, 2013, 2014; OECD, 2014a). By contrast, to date there are no specific regulatory frameworks for the grouping of nanomaterials. However, this topic is addressed in different publications, and preliminary guidance is provided in the context of substance-related legislation or the occupational setting. In an extensive review, the *European Centre for Ecotoxicology and Toxicology of Chemicals Task Force on Nanomaterials* (ECETOC Nano TF) assessed such available concepts for the grouping of nanomaterials for human health risk assessment (Arts et al., 2014). Based upon this review, in the present article, the ECETOC Nano TF proposes a functionality-driven *Decision-making framework for the grouping and testing of nanomaterials* (DF4nanoGrouping) that aims to group nanomaterials by their specific mode-of-action (MoA; *cf.* Information box) that results in an apical toxic effect.

In its review (Arts et al., 2014), the ECETOC Nano TF came to the conclusion that nearly all of the currently available approaches involve some form of grouping by intrinsic (material) properties or system-dependent properties that constitute bio-physical interactions. Of note, whereas the term 'physico-chemical characterization' is widely used in the literature, for the purpose of grouping, the ECETOC Nano TF distinguishes between 'intrinsic material properties' ('material properties') on the one hand and 'system-dependent properties' constituting bio-physical interactions on the other hand (Wiesner, 2014; *cf.* Information box for the definitions of these terms).

The grouping of non-nanosized substances is often based on (quantitative) structure–activity relationships ((Q)SARs) alone, and also the above-mentioned ECHA guidance only allows for grouping based upon structural similarities (ECHA, 2013). By contrast, all existing approaches for the grouping of nanomaterials already go beyond the determination of mere (Q)SARs. For instance, the United States National Institute for Occupational Safety and Health (NIOSH) and the British Standards Institute (BSI) distinguish between (1) soluble, (2) biopersistent and low toxicity, (3) biopersistent and high toxicity, and (4) fibrous particles (or high aspect ratio nanomaterials, HAR NMs) (Kuempel et al., 2012; BSI, 2007; reviewed in Arts et al. (2014)).

Similarly, the German Federal Institute for Occupational Safety and Health (BAuA, 2013; Gebel et al., 2014) proposes three categories for nanomaterials based upon their predominant toxicological MoA, while noting that some nanomaterials might be assignable to more than one category or to none of these categories. The BAuA distinguishes between (1) nanomaterials whose toxicity is mediated by their chemical composition; (2) rigid biopersistent respirable fibrous nanomaterials; and (3) respirable granular biodurable particles (GBPs). The *Guidance on the protection of the health and safety of workers from the potential risks related to nanomaterials at work* (RPA and IVAM, 2014), produced for the EU Commission, lists shape (in respect to HAR NMs), persistence, water solubility, dustiness and flammability as essential material properties to categorize levels of concern, further taking into account nanomaterial exposure assessment.

Overall, the existing concepts for the grouping of nanomaterials are founded on different aspects of the nanomaterial's life cycle throughout its biological pathway from production to disposal (Fig. 1, see also Arts et al., 2014). These aspects include the nanomaterial's intrinsic material properties and system-dependent properties, specific types of use and release, exposure route, biopersistence, uptake, and biodistribution and cellular and apical toxic effects. However, while none of the currently available concepts consistently addresses all of these aspects, the ECETOC Nano TF considers this necessary for a meaningful grouping of nanomaterials: Apical toxic effects caused by nanomaterials are not solely influenced by intrinsic material properties (let alone, a single intrinsic material property) or by system-dependent properties (Arts et al., 2014; Oomen et al., 2014a,b). Instead, nanomaterials readily undergo pronounced interactions with their respective surroundings. These interactions may change at the different stages of the nanomaterials' life cycles, and this specific feature of nanomaterials underlines the need for a functionality-driven and exposure-based grouping concept (*cf.* Supplementary Information (SI) text and SI Table S1 for two examples addressing the hazard assessment of carbon allotropes and TiO_2 that provide further scientific evidence for this need).

Against this background, the functionality-driven *Decision-making framework for the grouping and testing of nanomaterials* (DF4nanoGrouping) proposed by the ECETOC Nano TF uses and combines all of the different tools for grouping which are already at hand. It addresses the complexity of all aspects of possible nanomaterial interactions with its environment by taking into account all of the above-mentioned aspects of the different stages of the nanomaterials' biological pathways throughout their life cycles. Thereby, it is functionality-driven, and the components of the DF4nanoGrouping correlate with the different steps of the adverse outcome pathway (AOP) concept (Ankley et al., 2010), even though definite AOPs have not yet been established for nanomaterials. The starting point of the general AOP concept implies addressing the 'chemical properties' of a substance, which correspond to the 'intrinsic material properties' of the DF4nanoGrouping. In a sequential series, this starting point of the AOP is succeeded by 'molecular initiating events' or 'macro-molecular interactions' (corresponding to 'system-dependent properties' that constitute bio-physical interactions), cellular responses (corresponding to 'cellular effects'), and organ, organism and population responses (corresponding to 'apical toxic effects') (Ankley et al., 2010).

Consistent with the DF4nanoGrouping, Pastoor et al. (2014) suggest a comprehensive framework for bringing together knowledge to enable effective decision-making. The so-called RISK21 framework is presented as a *problem formulation-based, exposure-driven, tiered data acquisition approach* that incorporates exposure and toxicity estimates and their respective uncertainties to guide informed human health safety decisions as soon as sufficient evidence is acquired to address the specific problem formulation (Pastoor et al., 2014). Similarly, Grieger et al. (2014) call for structured decision support tools such as risk ranking approaches for decision-making regarding the use of nanomaterials in products and applications.

For the time being, the DF4nanoGrouping is restricted to the purpose of human health hazard assessment (and not environmental

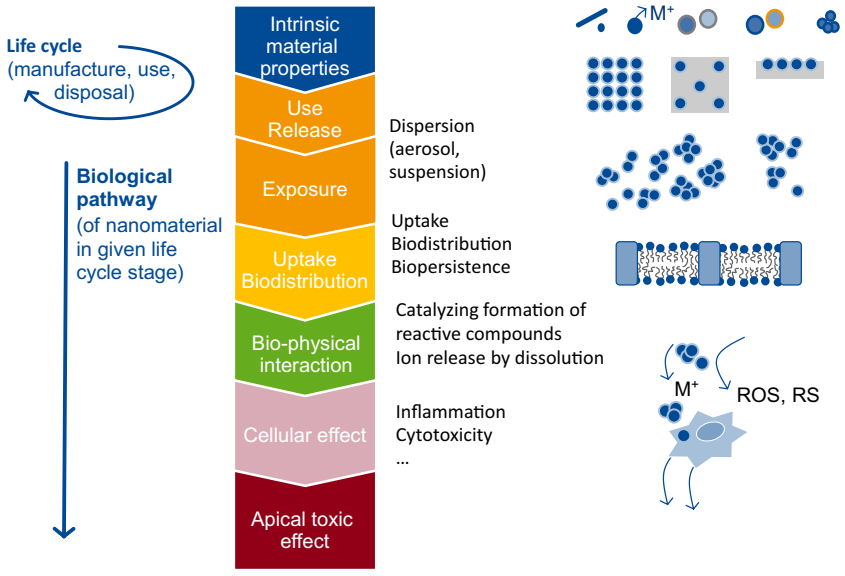

Fig. 1. Life cycle and biological pathway of nanomaterials (Adapted from: Landsiedel et al. (2010) and Oomen et al. (2014b); M+: metal ion; ROS: reactive oxygen species; RS: reactive species).

Table 1
Overview and interdependence of the criteria used for the grouping of nanomaterials and corresponding tiers of the DF4nanoGrouping.

	Intrinsic material properties (3.1)[a]			System-dependent properties (3.3)		Biopersistence, uptake, and biodistribution (3.4) Cellular effects (3.5) Apical toxic effects (3.6)
	Definition of nanomaterial[b]	Key criteria with direct relevance for nanomaterial grouping	Supplementary criteria that may relate to the nanomaterial's MoA	Key criteria with direct relevance for nanomaterial grouping	Supplementary criteria that may relate to the nanomaterial's MoA	
[c]	Tier 0	Tier 1		Tier 2		Tier 2 (*in vitro*)/Tier 3 (*in vivo*)
Properties assigned to identical Table rows are interdependent[d]	Water solubility (3.1.1) PPS (3.1.2) Surface area (3.1.3) Composition and crystallinity (3.1.4) Surface chemistry (3.1.5)	Water solubility (3.1.1) Composition (3.1.4)		Dissolution rate in BSF, release of toxic ions (3.3.1)		Biopersistence (3.4.1) Release of toxic ions (3.5)
		PPS (3.1.2) Aspect ratio (3.1.2) Rigidity (3.1.2)		Dissolution rate in BSF (3.3.1)		If biopersistent: potential for apical toxic effects (3.6)
				Surface reactivity (3.3.2)	Corona formation (3.3.4) Surface area *in situ* (3.3.5)	Oxidative stress Membrane damage (3.5) Macrophage activation Inflammation (3.6)
		Surface charge (3.1.5) Hydrophobicity (3.1.5)		Size in relevant media, dispersibility (3.3.3)		Uptake, biodistribution and clearance (3.4.2 and 3.4.3)

Abbreviations: BSF: biological simulation fluid; DF4nanoGrouping: decision-making framework for the grouping and testing of nanomaterials; MoA: mode-of-action; PPS: primary particle size.

[a] The numberings refer to the sub-chapter that the respective criterion is presented in further detail.

[b] These intrinsic material properties may also be used within the tiers of the DF4nanoGrouping to sub-group the nanomaterials assigned to a given main group (*cf.* Chapter 2.2).

[c] Tier of the DF4nanoGrouping during which the respective criteria are (predominantly) applied (*cf.* Chapter 2.2).

[d] Interdependence of properties, e.g.: Water solubility correctly estimates or underestimates solubility in BSF, which again correctly estimates or underestimates solubility *in vivo*, i.e. biopersistence (but not vice versa).

hazard assessment) of nanomaterials. Physical hazards elicited by, e.g. substance flammability, are not taken into consideration, and the DF4nanoGrouping excludes nanomaterials intended for medical application routes (such as intravenous or transdermal application) or that are specifically designed for therapeutic effects. The DF4nanoGrouping focuses on potential effects upon inhalation, the predominant route of exposure for nanomaterials, whereas the dermal and oral routes of exposure are only briefly addressed.

2. The decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping)

2.1. Delineation of the DF4nanoGrouping

The DF4nanoGrouping consists of three tiers to assign nanomaterials to one of four main groups, to perform subgrouping within the main groups, and to determine and refine further specific information needs. An overview of the grouping criteria used in the course of the three tiers of the DF4nanoGrouping is provided in Table 1 and Fig. 2. These criteria cover all relevant aspects of a nanomaterial's life cycle and biological pathways, i.e. intrinsic material properties, system-dependent properties, biopersistence, uptake, and biodistribution, and cellular and apical toxic effects. The intrinsic and system-dependent properties selected for the DF4nanoGrouping are similar to the ones indicated in the recent OECD report on the *physico-chemical properties of manufactured nanomaterials and test guidelines* (OECD, 2014b). A comprehensive description of the four main groups of nanomaterials and the three tiers of the DF4nanoGrouping are presented in the following Chapter 2.2. Additionally, the aspects 'use, release and route of exposure' are addressed as 'qualifiers' during all tiers of the grouping process. The rationale for assigning these aspects a special role is explained in Chapter 2.3. Chapter 3 provides detailed information on the criteria that have been selected for the DF4nanoGrouping discussing the specific role each criterion may play for the grouping and hazard and risk assessment of nanomaterials.

As Table 1 and Fig. 2 reveal, a Tier 0 may precede the DF4nanoGrouping. During this pre-tier, commonly used intrinsic material properties may be used to identify a nanomaterial (*cf.* Information box for definition). During Tier 1 of the DF4nanoGrouping, intrinsic material properties are used as key criteria to assign nanomaterials to main group 1 'soluble nanomaterials' and for a preliminary assignment to main groups 2–4, i.e. biopersistent HAR NMs, passive nanomaterials, and active nanomaterials (*cf.* Chapter 2.2). These four main groups have been adapted from the grouping schemes proposed by BSI (2007), Kuempel et al. (2012), BAuA (2013), Gebel et al. (2014). During Tier 2, system-dependent properties are used as key criteria to corroborate the assignment of nanomaterials to main group 2 (biopersistent HAR NMs) and to distinguish between passive and active nanomaterials (main groups 3 and 4). A nanomaterial, which does not show any relevant activity in Tier 2, is assigned to main group 3 (passive nanomaterials), whereas a nanomaterial that shows relevant activity in regard to any single Tier 2 grouping criterion is assigned to main group 4 (active nanomaterials). Data from Tier 2 may also be used to assign those nanomaterials to main group 1 which are not water-soluble, but soluble in biological media. Additional system-dependent properties may be used as supplementary criteria if they relate to the nanomaterial's given MoA. Tier 3 allows confirming the distinction between passive and active nanomaterials, and data from Tier 3 may be used to assign those nanomaterials to main group 1 that are not biopersistent *in vivo*. Likewise, Tier 3 allows for sub-grouping of active nanomaterials based on the results of short-term *in vivo* studies. This information may also be used to determine specific further information needs.

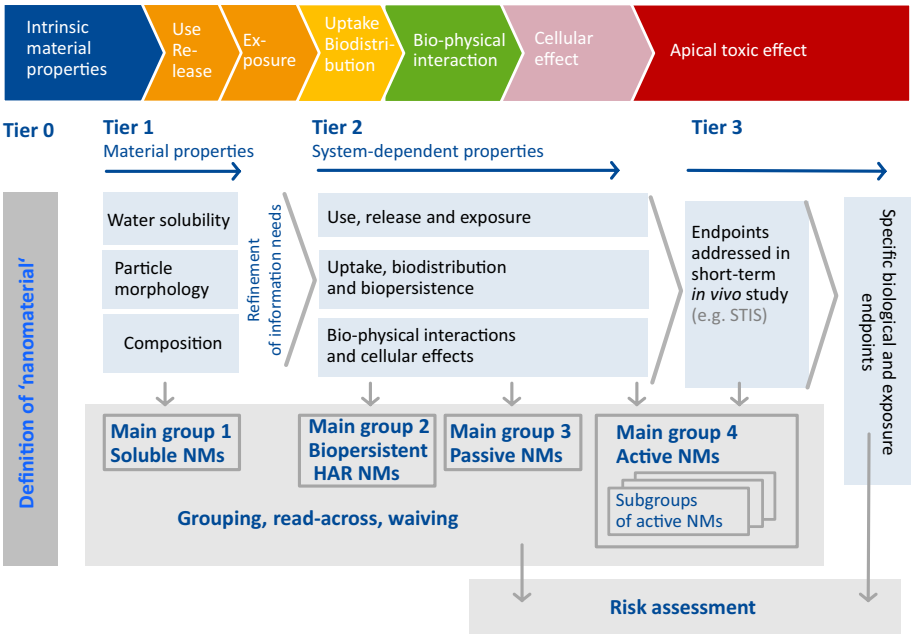

Fig. 2. The decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping).

The DF4nanoGrouping aims to group nanomaterials according to their specific MoA that may result in an apical toxic effect. Clearly, this is a function of a nanomaterial's intrinsic material properties. However, the exact correlation of intrinsic material properties and apical toxic effect is not yet established, and different intrinsic material properties may interact in complex ways, which are not yet fully understood. Intrinsic material properties of nanomaterials are highly relevant for grouping, but such grouping approaches may not be effective at the moment. Therefore, the tiered approach of the DF4nanoGrouping utilizes 'functionalities' of nanomaterials in addition to intrinsic material properties (Table 1). Functionalities include system-dependent material properties, bio-physical interactions as well as *in vitro* effects and release and exposure. Tier-by-tier, 'related' higher-tier criteria (presented as 'interdependencies' in the same rows in Table 1) provide information that increasingly reflects the biological complexity and biological circumstances of the corresponding *in vivo* property or apical toxic effect. For instance, the (Tier 1) intrinsic material property 'water solubility' is related to the more complex (Tier 2) system-dependent property 'dissolution rate/solubility in biological simulation fluids (BSF)', which in return is related to the more complex criterion (Tier 3) '*in vivo* solubility/biopersistence'.

2.2. The four main groups and three tiers of the DF4nanoGrouping

The three tiers of the DF4nanoGrouping allow a stepwise assignment of nanomaterials to the following four main groups, sub-grouping within the main groups, and the determination of further, specific information needs.

Main group 1 (soluble nanomaterials)

The main group 1 encompasses non-biopersistent nanomaterials, for which the chemical composition is more important for risk assessment than the as-produced nanostructure.

- *Key threshold value:* In accordance with BAuA (2013), in the DF4nanoGrouping, nanomaterials whose water solubility exceeds 100 mg/L are defined as soluble.
- *Nanomaterial assignment to main group 1 within the DF4nanoGrouping:* In Tier 1, nanomaterials are assigned to main group 1 or 'not main group 1'. Data from Tier 2 may be used to additionally assign those nanomaterials to main group 1 which are not water-soluble, but soluble in biological media. Likewise, in Tier 3, nanomaterials may be assigned to main group 1 if their pulmonary half-life is less than 40 days (*cf.* the threshold value set for biopersistent fibers in BAuA (2014a)).
- *Consequence of nanomaterial assignment to main group 1:* No further nano-specific sub-grouping, and no nano-specific hazard assessment. Instead, read-across of the properties of the dissolved materials to the corresponding bulk materials will be applied.

Main group 2 (biopersistent HAR NMs)

The main group 2 encompasses HAR NMs that are rigid and fulfill the WHO criteria for respirable fibers and the criterion for biopersistence (BAuA, 2014a). Biopersistent HAR NMs may elicit toxic effects due to their morphology and prolonged half-life in the organism.

- *Key threshold values:* Aspect ratio: <3:1, length: >5 μm; diameter: <3 μm; (WHO, 2005); biopersistence, i.e. dissolution rate > 100 mg/L (taken over from BAuA, 2013) or pulmonary half-life upon intratracheal instillation: ⩾40 days (BAuA, 2014a). Fiber diameter may be used as a proxy for rigidity.
- *Nanomaterial assignment to main group 2 within the DF4nanoGrouping:* Indication for assignment to main group 2 based upon size and aspect ratio in Tier 1. Final assignment based upon dissolution rate in BSF (Tier 2) or *in vivo* biopersistence (Tier 3).
- *Consequence of nanomaterial assignment to main group 2:* Different biopersistent HAR NMs may be assigned to further sub-groups based upon their degrees of water solubility or biopersistence (e.g. if they range in the same 25% intercept between the negative control (NC) and the positive control (PC)) and additionally taking into account release-related qualifiers.

Main group 3 (passive nanomaterials)

The main group 3 encompasses biopersistent, non-fibrous nanomaterials, such as GBPs, which do not have surface reactivity and do not elicit a specific cellular effect and do not prevail in

biological fluids in a well-dispersed form. *In vivo*, the 'passive state' of nanomaterials is confirmed in that they do not elicit apical toxic effects and in that they are not biodistributed from the site of contact or outside the mononuclear phagocyte system (MPS). Regardless of their 'passive state', high doses of these nanomaterials – like other particles – may elicit effects on account of their particulate nature, especially by dust inhalation. Additionally, nanomaterials that are not released from their matrix in products – in any way – are also assigned to the main group 3 (passive nanomaterials).

- *Key threshold values:* Toxic component (element or molecule) <0.1% (EP and Council of the EU, 2008); surface reactivity: <10% of Mn_2O_3 reactivity in FRAS or cytochrome c assays; dispersibility: AAN $\geqslant$ 3; no cellular effects at $\leqslant$10 μg/cm^2 (Kroll et al., 2011). Confirmatory threshold value in respect to low toxic potency, i.e. NOAEC in short-term inhalation study (STIS) >10 mg/m^3.
- *Nanomaterial assignment to main group 3 within the DF4nanoGrouping:* Based on data obtained in Tier 2. The Tier 2 assignment may be confirmed or revised by Tier 3 data from *in vivo* studies (STIS).
- *Consequence of nanomaterial assignment to main group 3:* Passive nanomaterials are considered to possess no or only very low hazard potential.

Main group 4 (active nanomaterials)

The main group 4 encompasses biopersistent, non-fibrous nanomaterials with a hazard potential that is determined based upon chemical composition, dissolution rate, surface reactivity, dispersibility, or cellular effects. *In vivo*, 'active' nanomaterials are expected to elicit apical toxic effects at lower doses. Results of *in vivo* studies may be used to sub-group and rank 'active nanomaterials'.

- *Key threshold values:* Nanomaterials are assigned to main group 4 'active nanomaterials' if they are not assigned to the main groups 1, 2, or 3, i.e. if any single decisive property (or combinations of properties) listed for main groups 1–3 is (or are) not met. For confirmation of nanomaterial assignment to main group 4, a NOAEC in STIS $\leqslant$10 mg/m^3 may be used. Effects and toxic potency determined in the STIS may also be used for sub-grouping.
- *Nanomaterial assignment to main group 4 within the DF4nanoGrouping:* Based on data obtained in Tier 2. The Tier 2 assignment may be confirmed or revised by Tier 3 data from *in vivo* studies (STIS).
- *Consequence of nanomaterial assignment to main group 4:* Further sub-grouping by the degree of mobility in air (dustiness) and in physiological fluids (dispersibility), as well as by *in vitro* and *in vivo* (STIS) effects and uptake, biopersistence, and biodistribution.

Grouping is an instrument to aid risk assessment. One element of risk assessment is the estimation of 'no effect levels'. According to Annex 1 of the EU Regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH; EP and Council of the EU, 2006), a derived-no-effect-level (DNEL) is required as part of the Chemical Safety Assessment. Based upon the risk assessment, risk management measures are taken, including the setting of occupational exposure limits (OELs). Differing exposure limits are being enforced for specific types of nanomaterials that reflect the main groups of the DF4nanoGrouping. In 2007, the British Standards Institute (BSI) was one of the first institutions to suggest OEL values for different groups of nanomaterials, and it related them to the existing OEL values of the corresponding non-nanosized materials. BSI (2007) proposed 0.5 of the existing OEL for soluble nanomaterials, 0.01 fibers/mL for fibrous nanomaterials, 0.066 of the existing OEL for insoluble nanomaterials without specific toxicity, and 0.1 of the existing OEL for nanomaterials with a specific toxicity.

More recently, the German Federal Institute for Occupational Safety and Health recommended seeking air concentrations <10,000 fibers/m^3 for fibers (BAuA, 2013). Since legally binding, health-based occupational limit values for manufactured nanomaterials are currently not available, BAuA (2013) suggested an 'assessment criterion' of <0.5 mg/m^3 (considering an average agglomerate density of 2.0 g/cm^3 at the workplace) for biopersistent, non-fibrous nanomaterials without specific toxicity, and for nanomaterials with specific (chemical composition-related) toxicity, an 'assessment criterion' of <0.1 mg/m^3 (BAuA, 2013). By comparison, for respirable non-nanosized dust, an OEL of 1.25 mg/m^3 (based on an average particle density at the workplace of 2.5 g/cm^3) has been laid down (BAuA, 2014b). An extensive meta-analysis of chronic rat inhalation studies with GBP materials (Gebel, 2012) found the difference in carcinogenic potency between GBP nanomaterials and GBP micromaterials to be low (i.e. a factor of 2.0–2.5 referring to the dose metrics mass concentration). Of note, however, the human health relevance of lung tumors observed in the rat after particle inhalation is at least questionable, as reviewed in an ECETOC report on pulmonary overload (ECETOC, 2013).

Taking into account these considerations, for main group 1 of the DF4nanoGrouping (soluble nanomaterials), the DNEL of the respective dissolved material may be applied, for biopersistent HAR NMs (main group 2) – derived-minimal-effect-levels (DMELs) for fibers, and for passive nanomaterials (main group 3) – general DNELs derived from the no-observed-adverse-effect-concentrations (NOAECs) obtained in long-term studies (*cf.* 3.6.4 'Toxic potency').

2.2.1. Tier 1: nanomaterial assignment to main groups based on intrinsic material properties

Taking into account the exposure-related qualifiers as appropriate (*cf.* Chapter 2.3), 3 essential intrinsic material properties are addressed as key criteria for nanomaterial grouping (Table 2; in the following, the figure assigned to each grouping criterion refers to the chapter in which its specific role for grouping is discussed in further detail):

- Water solubility (3.1.1).
- Particle morphology (primary particle size (PPS) and shape, including aspect ratio and surface area (3.1.2)).
- Chemical composition (3.1.4).

These key criteria are used to assign soluble nanomaterials to the main group 1 and to provide an indication for the assignment of non-soluble nanomaterials' to the main groups 2, 3, or 4.

2.2.2. Tier 2: nanomaterial assignment to main groups based on functionality

Tier 2 serves to assign non-soluble nanomaterials to the main groups 2–4 and to sub-group the nanomaterials within the main groups 2 (biopersistent HAR NMs) and 4 (active nanomaterials). During Tier 2, the following key system-dependent properties (assessed in relevant media) and *in vitro* toxicological criteria are applied (Table 3):

- Dissolution rate (including loss of nanostructure and release of toxic ions; 3.3.1).
- Surface reactivity (3.3.2).
- Dispersibility (3.3.3).

Table 2
DF4nanoGrouping: application of qualifiers, e.g. for exposure-based waiving of testing, and performance of Tier 1. Grouping criteria, threshold values and benchmark materials for nanomaterial assignment to main group 1 (with resulting read-across of dissolved material) and indications for assignment to main groups 2–4.

Grouping criteria or qualifiers	Relevance for HA or RA	Threshold values for grouping	Benchmark materials[a]	Assignment to main group or sub-grouping
Application of the qualifiers 'use, release, and route of exposure', e.g. for exposure-based waiving of testing (To be taken into consideration during each tier)				
Use	Nanomaterial exposure likely? If so: In which form?	*cf.* Chapter 3.2 and Supplementary Information Table S3		
Release	Release (i.e. exposure) likely? If so: In which form? Do the released fragments contain nanoparticles?	Dustiness of powders[b] Low: pellet-like, non friable solids Medium: crystalline, granular solids High: fine, light powders Droplet size affecting inhalability Respirable: ⩽10 μm Inhalable: ⩽100 μm Not inhalable: >100 μm Nanomaterial migration from food matrix Low: <10 mg of substances/dm^2 of food contact surface (overall migration limit) High: >10 mg of substances/dm^2 of food contact surface (overall migration limit)	Benchmark materials for release of free nanoparticles are unavailable Dustiness of powders[b] Low: polyvinyl chloride pellets, waxes Medium: soap powder, sugar High: cement, TiO_2 NM-100 – NM-105 (RPA and IVAM, 2014) Release by machining Soft plastics (low[c]) Brittle epoxy (high[c]) Release by weathering or aging UV-protected polyethylene (low[c]) Epoxy or polyamide (high[c])	'No release' of free nanomaterials, e.g. due to reactivity within the matrix (example: X-Seed®, a concrete hardening accelerator), can be used to assign a nanomaterial to main group 3 (passive nanomaterials). Further (non-nanospecific sub-grouping) may then be performed by product matrix
Exposure route	Determination of most relevant biological pathways and corresponding endpoints	Not applicable	*cf.* corresponding grouping criteria relevant for the respective route of exposure	Inhalation exposure Dermal exposure Oral exposure
Tier 1: Nanomaterial assignment to main group 1 and indications for main groups 2–4 making use of key criteria, i.e. essential intrinsic material properties indicating predominant MoAs				
Water solubility	Screening for loss of nanomaterial structure: 1st estimation of biopersistence	Assignment to main group 1 (soluble nanomaterials): >100 mg/L (BAuA, 2013)	$ZnCl_2$ (high) ZnO NM-110, NM-111 (limited) TiO_2 NM-100 – NM-105 (low)	Read-across of the dissolved materials (that have been assigned to main group 1) by composition
Shape (and aspect ratio)	Screening for fiber effects (and for appropriate dose metrics)	Indication for main group 2 (biopersistent HAR NMs): Shape, size, aspect ratio fulfill WHO definition of 'respirable fibers': Aspect ratio > 3:1 (i.e. HAR NMs), length > 5 μm, diameter < 3 μm 'Not main group 2': Granular nanomaterials	Certified reference materials with 1 and 3 nanoscale dimensions, all with diameters < 100 nm, available from: NIST (USA); BAM (DE); IRMM (JRC-BE)[d,e] Rigid HAR NMs: Asbestos and Mitsui MWCNT-7 (Grosse et al., 2014)	Tier 2 assignment to main group 2 (biopersistent HAR NMs): Dissolution rate Rigidity (or diameter as a proxy) Surface reactivity (*cf.* Table 3) AND: Use and release: The actually released material may have no 'high aspect ratio' properties, even though the pristine nanomaterial was a HAR NM
Composition	Screening for toxic ion release (inorganic nanomaterials) Nanosized and bulk materials may share similar properties	Indication for main group 3 (passive nanomaterials): <0.1% of toxic component (element or molecule; EP and Council of the EU, 2008) Indication for main group 4 (active nanomaterials): ⩾0.1% of toxic component (element or molecule)	Dependent upon characterization method	Tier 2 assignment to main group 3 (passive nanomaterials): No significant release OR No toxic element AND Low surface reactivity AND Low dispersibility AND Low *in vitro* effects (*cf.* Table 3) Tier 2 assignment to main group 4 (active nanomaterials): All nanomaterials that either have a toxic element or high surface reactivity or high dispersibility or high *in vitro* effects 'Composition' may be applied to sub-group nanomaterials (assigned to main groups 2–4 in Tier 2) by most potent element (e.g. zinc for ZnO)

Abbreviations: BAM: *German Federal Institute for Materials Research and Testing*; BAuA: *German Federal Institute for Occupational Safety and Health;* CNT: carbon nanotubes; HA: hazard assessment; HAR NMs: high aspect ratio nanomaterials; IRMM: Institute for Reference Materials and Measurements; JRC: Joint Research Centre; NRCWE: National Research Centre for the Working Environment; NIST: National Institute of Standards and Technology; RA: Risk assessment; WHO: World Health Organization.

[a] NM-x numberings of benchmark materials (e.g. 'ZnO NM-110') refer to the respective numberings of the 'OECD reference nanomaterials' as they have been coded in the list of the OECD Sponsorship Program for the Testing of Manufactured Nanomaterials (http://www.oecd.org/science/nanosafety/ and http://ihcp.jrc.ec.europa.eu/our_activities/nanotechnology/nanomaterials-repository).

[b] Reflecting the kinds of dustiness that may occur with nanomaterials and indicating benchmark materials that are used as examples for dustiness, even though not all of them might be present in the nanoform.

[c] Relative amount of nanomaterial of entire amount of released fragment.

[d] *cf*: NIST: http://www.nist.gov/mml/bbd/rm-8027-092414.cfm; BAM: http://www.nano-refmat.bam.de/en/; IRMM: https://ec.europa.eu/jrc/en/reference-materials.

[e] (Certified) reference materials both <100 nm and ⩾100 nm diameters, with 1, 2, or 3 nanoscale dimensions, incl. determination of size distribution and surface area, in preparation within the FP7 project NanoDefine (see: http://www.nanodefine.eu/index.php/links/2-uncategorised/1-welcome).

Table 3
DF4nanoGrouping: application of qualifiers, e.g. for exposure-based waiving of testing, and performance of Tiers 2 and 3. Grouping criteria, threshold values and benchmark materials for nanomaterial assignment to main group 2–4 and for sub-grouping.

Grouping criteria or qualifiers	Relevance for HA or RA	Threshold values for grouping	Benchmark materials[a]	Assignment to main group or sub-grouping
Application of the qualifiers 'use, release, and route of exposure', e.g. for exposure-based waiving (To be taken into consideration during each tier) cf. Table 2				
Tier 2: Nanomaterial assignment to main groups 2–4, corroboration and sub-grouping making use of key criteria, i.e. essential system-dependent material properties and in vitro effects				
Dissolution rate in biological fluids	After 'water solubility' (*cf.* Tier 1; Table 2), the dissolution rate provides further estimations of a nanomaterial's *in vivo* 'biopersistence'	Nanomaterials with a dissolution rate >100 mg/L: may be moved to main group 1 For fibers: Low dissolution rate is an indication for main group 2 (biopersistent HAR NMs):	Crocidolite asbestos (biopersistent, PC; non-fibrous benchmark material not yet determined) Chrysotile asbestos (dissolves in biological fluids, NC) *cf.* Donaldson et al. (2010)[b]	Assignment to main group 2 (biopersistent HAR NMs): Release AND Shape, size, aspect ratio, rigidity (*cf.* Table 2) AND Low dissolution rate Sub-grouping of nanomaterials assigned to main groups 2 or 4, e.g. by similar dissolution rates (e.g. the same 25% range between NC and PC)
Surface reactivity	Cellular and apical toxicity	Indication for assignment to main group 3 (passive nanomaterials): Passive: <10% of Mn_2O_3 reactivity in FRAS or cytochrome c assays Assignment to main group 4 (active nanomaterials): Active: $\geqslant$10% of Mn_2O_3 reactivity in FRAS or cytochrome c assays	Passive: $BaSO_4$ NM-220 Active: Mn_2O_3	Assignment to main group 3 (passive nanomaterials): No release OR Low dispersibility AND No toxic element AND Low surface reactivity AND Low *in vitro* effects Sub-grouping of nanomaterials assigned to main groups 2 or 4, e.g. by similar surface reactivity (e.g. the same 25% range between NC and PC)
Dispersibility	Mobility[c]	Assignment to main groups 2 (biopersistent HAR NMs) and 4 (active nanomaterials): Mobile[c]: AAN < 3 Indication for main group 3 (passive nanomaterials): Large: AAN $\geqslant$ 3 or diameter above 100 nm (may be grouped together with micron-scale materials, if appropriate)	Ag NM-300 (AAN < 3)[b] SiO_2 NM-203 (small agglomerates) SiO_2 NM-200 (large agglomerates)	Assignment to main group 3 (passive nanomaterials): No release OR Low dispersibility AND No toxic element AND Low surface reactivity AND Low *in vitro* effects Sub-grouping of nanomaterials assigned to main groups 2 or 4, e.g. by similar degrees of dispersibility (e.g. the same 25% range between NC and PC)
Cellular effects	Preliminary prediction of apical toxicity	Indication for nanomaterial assignment to main group 3 (passive nanomaterials): No effect at $\leqslant$10 μg/cm^2 (Kroll et al., 2011) Nanomaterial assignment to main group 4 (active nanomaterials): Effect at $\leqslant$10 μg/cm^2	Passive: $BaSO_4$ NM-220 Active: ZnO NM-110 and NM-111 (ion effects); CeO_2 NM-211 and NM-212 (surface-related effects)	Assignment to main group 3 (passive nanomaterials): No release OR Low dispersibility AND No toxic element AND Low surface reactivity AND Low *in vitro* effects Sub-grouping of nanomaterials assigned to main groups 2 or 4, e.g. by similar degrees of cellular toxicity (e.g. the same 25% range between NC and PC)
In vitro genotoxicity	Preliminary prediction of apical toxicity	Not yet determined	Not yet determined	

Grouping criteria	Threshold values for sub-grouping	Benchmark materials
Tier 3: Confirmation of nanomaterial assignment to main group, sub-grouping by apical toxic effects, and refinement of information needs (examples relate to inhalation exposure and numberings of group assignments indicate increasing degrees of 'activeness')		
Apical toxic effects	1. Inflammation in STIS at an aerosol concentration of $\leqslant$10 mg/m^3 2. Necrosis in STIS at an aerosol concentration of $\leqslant$10 mg/m^3	1. CeO_2 NM-211 and NM-212 2. ZnO NM-110 and NM-111
Toxic potency in STIS	1. NOAEC: $\leqslant$0.1 mg/m^3 2. NOAEC: $\leqslant$1 mg/m^3 3. NOAEC: $\leqslant$10 mg/m^3 4. NOAEC: >10 mg/m^3 (confirms Group 3)	1. MWCNT NM-400, quartz DQ12 2. CeO_2 NM-211 and NM-212 3. SiO_2.naked and TiO_2 NM-105 4. $BaSO_4$ NM-220

In vivo genotoxicity	Not yet determined			Not yet determined
Recovery/Progression	1. Recovery (full or partial) of effects within half-life of lung clearance 2. No recovery/progression			1. TiO_2 NM-100 – NM-105 2. MWCNT NM-400
Organ burden and clearance(Biopersistence)	1. Physiological clearance in STIS at an aerosol concentration of 1 mg/m^3 2. Decelerated clearance in STIS at an aerosol concentration of 1 mg/m^3 3. Accelerated clearance Biopersistent nanomaterials are defined as having a pulmonary half-life upon intratracheal instillation equal to or exceeding 40 days (BAuA, 2014a)			1. TiO_2 NM-100 – NM-105 2. CeO_2 NM-211 and NM-212 3. $BaSO_4$ NM-220
Biodistribution	1. Local availability 2. Availability in MPS >1 mass% of dose 3. Systemic availability outside MPS >1 mass% of dose			1. CeO_2 NM-211 and NM-212 2. Not yet determined 3. Not yet determined
Grouping criteria	Relevance for HA or RA	Threshold values for grouping	Benchmark materials[a]	
Supplementary criteria, i.e. properties that are not used independently, but that are covered by properties of Tier 1 or Tier 2 of the DF4nanoGrouping or that may be applied to support the grouping and sub-grouping within the DF4nanoGrouping				
Primary particle size	Size is indispensible to identify a nanomaterial	Size is not a primary grouping criterion, but it influences dose metrics depending on MoA via mass, surface (or number)	Certified reference materials with 1 and 3 nanoscale dimensions, all with diameters <100 nm, available from: NIST (USA); BAM (DE); IRMM (JRC-BE)[d]	
Surface area	Many of the most relevant MoAs are surface-induced	Surface area is not a primary grouping criterion, but it is essential to convert dose metrics	Certified reference materials with BET values from 0.102–550 m^2/g, covering nano-sized and bulk materials, available from: NIST (USA); BAM (DE); IRMM (JRC, BE); APPIE (JP)[d]	
Surface chemistry	Many of the most relevant MoAs are surface-induced	For grouping, composition is more important than surface chemistry. Subgrouping relies on the system-dependent consequences of surface chemistry: surface reactivity and dispersibility	ZnO NM-110 vs. NM-111 TiO_2 NM-103 vs. NM-104	
Crystallinity		Addressed in the context of 'composition'		
Surface charge	Interaction with lipids: Negatively charged (and hydrophilic) materials evade corona formation longer; positively charged materials may damage membranes	Secondary evidence for nanomaterial assignment to main groups 3 (passive) or 4 (active nanomaterials) – joint evaluation with 'dispersibility': If materials within (sub-)group are very heterogeneous in hydrophobicity and surface charge, data of the extreme cases should verify that uptake and biodistribution do not counter-indicate the grouping Positive: $\zeta > 10$ mV; neutral: $\zeta = -10$–10 mV; negative: $\zeta < -10$ mV	Positive: CeO_2 NM-212 Negative: SiO_2 NM-203	
Hydrophobicity	Altered mobility: Hydrophobic materials have stronger corona formation and are cleared differently than hydrophilic materials	Secondary evidence for nanomaterial assignment to main groups 3 (passive) or 4 (active nanomaterials) – joint evaluation with 'dispersibility': If materials within (sub-)group are very heterogeneous in hydrophobicity and surface charge, data of the extreme cases should verify that uptake and biodistribution do not counter-indicate the grouping	Not yet determined	
Corona formation		The predictive value of this system-dependent property is sufficiently addressed by the intrinsic properties of hydrophobicity and surface charge, supplemented by the system-dependent dispersibility		

Abbreviations: AAN: average agglomeration number; APPIE: Association of Powder Process Industry; BAM: *German Federal Institute for Materials Research and Testing*; DF4nanoGrouping: decision-making framework for the grouping and testing of nanomaterials; FRAS: ferric reducing ability of serum; HA: hazard assessment; IRMM: Institute for Reference Materials and Measurements; JRC: Joint Research Centre; MoA: mode-of-action; MPS: mononuclear phagocyte system; MWCNT: multi-walled carbon nanotube; NC: negative control; NIST: National Institute of Standards and Technology; NOAEC: no-observed-adverse-effect concentration; PC: positive control; RA: risk assessment.

[a] The numberings in the column 'benchmark materials' correspond to the numberings in the column 'group assignment (and threshold values, if applicable)'. AND: NM-x numberings of benchmark materials (e.g. 'ZnO NM-110') refer to the respective numberings of the 'OECD reference nanomaterials' as they have been coded in the list of the OECD Sponsorship Program for the Testing of Manufactured Nanomaterials (http://www.oecd.org/science/-nanosafety/ and http://ihcp.jrc.ec.europa.eu/our_activities/nanotechnology/nanomaterials-repository).

[b] Further benchmark materials required for all relevant scenarios; data from OECD is emerging.

[c] Mobile nanomaterials: nanomaterials that remain dispersed as constituent particles and therefore may potentially move between body compartments; surface charge, hydrophobicity, dispersibility determine mobility.

[d] *cf:* NIST: http://www.nist.gov/mml/bbd/rm-8027-092414.cfm; BAM: http://www.nano-refmat.bam.de/en/; IRMM: https://ec.europa.eu/jrc/en/reference-materials; APPIE: https://www.nmij.jp/english/info/lab/mtrl-charct/.

- Effects observed in the *in vitro* macrophage assay, *in vitro* genotoxicity assays, and *in vitro* barrier penetration studies using relevant test systems reflecting the primary target organ (3.4 and 3.5).

During Tier 1 and Tier 2, no further properties, apart from the mentioned key criteria, are necessary for decision-making, i.e. to assign nanomaterials to the corresponding main groups. Additional intrinsic material and system-dependent properties may however be relevant for sub-grouping, i.e. PPS (3.1.2), surface area (3.1.3), crystallinity (3.1.4), surface chemistry (3.1.5), surface charge (3.1.5), hydrophobicity (3.1.5), and corona formation (3.3.4), which also relates to surface area *in situ* (3.3.5). These properties are listed as supplementary criteria in the lower part of Table 3. Sub-grouping is especially relevant for the main groups 2 (biopersistent HAR NMs) and 4 (active nanomaterials) since nanomaterials assigned to these groups may possess specific hazards. Additionally, size and surface area are indispensible to establish the appropriate dose metrics that is relevant to investigate specific MoAs (Landsiedel et al., 2014a).

2.2.3. Tier 3: confirmation of nanomaterial assignment to main groups und sub-grouping based on short-term in vivo studies

Tier 3 allows confirming or revising the assignment of nanomaterials to the main groups. Further, sub-grouping may be performed by information obtained in short-term *in vivo* studies, such as the STIS and to define and refine additional information needs. Toxicological criteria, exemplified for the inhalation route of exposure, include (Table 3):

- Lung burden, systemic uptake, *in vivo* biopersistence, biodistribution, assessed by STIS (3.6).
- Apical toxic effects and toxic potency, assessed by STIS (3.6).
- *Ex vivo* genotoxicity screening combined with STIS (3.6).

2.3. Application of the qualifiers 'use, release and route of exposure' for exposure-based waiving

Within the DF4nanoGrouping, the aspects of nanomaterial use, release and route of exposure play a special role. (Of note, 'use' is defined as handling during all life cycle stages of the nanomaterial and explicitly includes nanomaterial manufacture, production and consumer use.) Even one single variant of a specific type of nanomaterial may be intended for a broad spectrum of different *uses.* Vice versa, different types of nanomaterials may be intended for one specific type of use. Likewise, depending on the final product for which the given nanomaterial is foreseen, a broad variety of different *release scenarios* are foreseeable, including no release at all and different degrees of release by machining, weathering or aging. Again, these properties may be very similar for a variety of different types of nanomaterials, or they may differ considerably within a group of nanomaterials with very similar intrinsic material properties. These examples further highlight that the determination of intended use and realistic release scenarios (throughout the life cycle of the given nanomaterial, starting from production, use within industry (e.g. formulation) to consumer use and disposal) directly affect the nanomaterial's most likely *route of exposure* and exposure potential.

Presently, the DF4nanoGrouping focuses on intended use conditions covering the most realistic exposure scenarios for workers or consumers. Accidental exposure to nanomaterials may occur at all life cycle stages and could form a separate exposure group. For the time being, however, acute overexposure by accidental uptake is not further considered in the DF4nanoGrouping. Likewise, humans may potentially be exposed to nanomaterials if they are present in the environment, e.g. when a nanomaterial is discharged or contained in the sludge. Nevertheless, since such 'indirect' human exposure is considered to be much lower than 'direct' exposure at the workplace or during use, it is also not further addressed in the grouping concept.

Grouping by use, release and exposure may be debatable since most scenarios are not mutually exclusive and further uses and release scenarios may exist – even if they may only be minor. However, nanomaterials that fuse or react with their matrix in the final product (e.g. concrete seeding crystals; Bräu et al., 2012) are examples for passive nanomaterials forming a group of 'no-release'. For such nanomaterials, grouping by no-release is effectively an exposure-based waiving.

Due to the special role that the aspects 'use, release and route of exposure' play, within the DF4nanoGrouping, these aspects are used as qualifiers for grouping instead of direct grouping criteria. For a given nanomaterial, the grouping criteria on intrinsic and system-dependent properties, nanomaterial biopersistence, uptake, and biodistribution or cellular and apical toxic effects are affected by the use and release scenarios and the resulting relevant route(s) of exposure. Therefore, these qualifiers may serve to support grouping decisions in order to adapt data requirements, i.e. to tailor subsequent testing to specific needs or to justify waiving of testing. Furthermore, the qualifiers may serve to determine appropriate forms of nanomaterial preparation for testing (e.g. aerosols for inhalation exposure).

The qualifiers 'use, release, and route of exposure' may be integrated into any given tier of the grouping process. Table 4 illustrates how the qualifiers are combined and applied to determine hotspots, i.e. groups of nanomaterials for which human exposure is likely. The table addresses possible uses of nanomaterials (listed in the accessory table at the bottom of Table 4) and distinguishes relevant life cycle stages for grouping, i.e. production, formulation and industrial use, consumer use, and, finally, disposal (first level column scaling). Further, the table distinguishes between the most relevant routes of exposure (first level row scaling) and finally differentiates by the form in which the given nanomaterial may prevail, i.e. as powder, liquid or bound in matrix (second level row scaling). Jointly addressing these aspects, Table 4 indicates the likelihood of nanoparticle release and exposure, highlighting in red the hotspots for which exposure is likely. The most relevant exposure scenarios for nanomaterials known to date (e.g. dustiness in production) are addressed.

The following examples demonstrate how Table 4 may be applied for decision-making during nanomaterial grouping:

- *Intended use:* Nanomaterial in sunscreen (tube); *route of exposure under investigation:* dermal; *nanomaterial form:* liquid; *potential release:* yes, by direct skin contact; *exposure potential:* yes (hotspot). Resulting qualifier: Risk assessment required for dermal route.
- *Intended use:* Nanomaterial in sunscreen (tube); *route of exposure under investigation:* inhalation; *nanomaterial form:* liquid; *potential release:* no, since no aerosol is formed; *exposure potential:* no inhalation exposure. Resulting qualifier: No risk assessment required for this route of exposure.
- *Intended use:* Nanomaterial in sunscreen (spray); *route of exposure under investigation:* inhalation; *nanomaterial form:* liquid; *potential release:* yes, since aerosol is formed; *exposure potential:* yes, due to respirable droplet size (hotspot). Resulting qualifier: Risk assessment required for inhalation route.

In the final life cycle stage 'disposal', no human exposure or environmental emission of nanomaterials is expected from controlled combustion (Walser et al., 2012). Although many inorganic nanomaterials are presumably persistent, they mostly end up in the glassy matrix of the slag, and are partially present in the

Table 4
Application of the qualifiers 'use, release and route of exposure' to determine 'hotspots' with likely occupational or consumer exposure.

Relevant life cycle stage for grouping		Production of NM			Formulation / industrial use			Use			Disposal		
		Form	Release	Exposure	Form	Release	Exposure	Form	Release	Exposure	Form	Release	Exposure
Route of exposure	Oral	Powder	No (industrial hygiene)	No	Powder	No (industrial hygiene)	No	Powder	Dependent on the use	Specific exposure groups (see below)	Powder	No release from ignition or disposal	No
		Liquid	No (industrial hygiene)	No	Liquid	No (industrial hygiene)	No	Liquid	Dependent on the use	Specific exposure groups (see below)	Liquid	No release from ignition or disposal	No
		Bound in matrix	No (industrial hygiene)	No	Bound in matrix	No (industrial hygiene)	No	Bound in matrix	Migration	High	Bound in matrix	No release from ignition or disposal	No
										Low			
									No migration	No			
	Dermal	Powder	No (industrial hygiene)	No	Powder	No (industrial hygiene)	No	Powder	Direct skin contact	Yes	Powder	No release from ignition or disposal	No
									No contact	No			
		Liquid	No (industrial hygiene)	No	Liquid	No (industrial hygiene)	No	Liquid	Direct skin contact	Yes	Liquid	No release from ignition or disposal	No
									No contact	No			
		Bound in matrix	No (industrial hygiene)	No	Bound in matrix	No (industrial hygiene)	No	Bound in matrix	Migration	High	Bound in matrix	No release from ignition or disposal	No
										Low			
									No migration	No			
	Inhalation	Powder	Dust	High dustiness	Powder	Dust	High dustiness	Powder	Dust	High dustiness	Powder	No release from ignition or disposal	No
				Medium dustiness			Medium dustiness			Medium dustiness			
				Low dustiness			Low dustiness			Low dustiness			
			No dust	No		No dust	No		No dust	No			
		Liquid	No (industrial hygiene)	No	Liquid	No (industrial hygiene)	No	Liquid	Aerosol	Respirable droplets	Liquid	No release from ignition or disposal	No
										Inhalable droplets			
										Not inhalable			
									No aerosol	No			
		Bound in matrix	No (industrial hygiene)	No	Bound in matrix	Abrasion/ aging	Respirable particles	Bound in matrix	Abrasion/ weathering	Respirable particles	Bound in matrix	No release from ignition or disposal	No
							Inhalable particles			Inhalable particles			
							Not inhalable			Not inhalable			
						No abrasion	No		No abrasion/ no weathering	No			

Groups defined by legal provisions; specifically designed exposure scenarios

Food
Medicinal products
Cosmetics
Biocides
Agrochemicals

Footnote to Table 4:
Red: Hotspot, i.e. concern due to potential exposure

filtered fly ash (Walser et al., 2012). As a result, there is no potential uptake via any of the exposure routes during the life cycle step 'disposal', and no hotspot was identified in Table 4.

During all tiers, the release and exposure potential of a nanomaterial may be investigated addressing relevant endpoints as appropriate (e.g. release of substance from articles (dustiness) determined by methods allowing to assess release by weathering or machining) and taking into account the intended uses and most likely routes of exposure. Based upon the outcome of such assessments, the qualifiers 'use, release and route of exposure' may be applied to justify waiving of testing in case of low toxicological potency or the selection of specific higher tier assays.

3. Criteria for the grouping of nanomaterials

3.1. Grouping of nanomaterials by intrinsic material properties

Due to nanomaterial complexity, their structure will involve features generally not measured for bulk substances, including size, surface area and shape or aspect ratio, as well as traditional structural measurements, such as chemical composition. It is vital to understand the specific intrinsic material properties of a nanomaterial prior to beginning toxicological testing (Warheit, 2008; Keene et al., 2014). Accordingly, the assessment of intrinsic material properties is the starting point for nanomaterial grouping in the preceding Tier 0 and in Tier 1 of the DF4nanoGrouping. In addition, correctly identifying the nanomaterial's material properties may allow the determination of some of the risks factors inherent with occupational exposure of nanomaterials, particularly for inhalation exposure (RPA and IVAM, 2014).

For a given nanomaterial, its intrinsic material properties will be useful for grouping if they pertain to certain adverse health effects regardless of the nanomaterial's interactions with the environment. Examples include the chemical composition that may be a predictor of the cellular effects elicited by nanomaterials that shed toxic ions or the shape, i.e. high aspect ratio and rigidity, which may indicate particles acting according to the fiber paradigm. Other material properties, however, will change once the given nanomaterial is placed in a different environment. For instance, a nanomaterial may agglomerate (affected by the system-dependent property 'dispersibility') so that it is no longer present in its PPS. In such cases, the respective intrinsic material property will be less relevant for grouping by mechanisms that rely on size.

Defining their intrinsic material properties will allow some nanomaterials to be initially placed into commonly accepted groups, such as the ones suggested by the NIOSH, the BSI, the BAuA, or RPA and IVAM. By determining water solubility, aspect ratio, and chemical composition (together with biopersistence and apical toxic effects), many nanomaterials may be assigned as being soluble, biopersistent and low or high toxicity, or HAR NMs (BSI, 2007; Kuempel et al., 2012; Gebel et al., 2014; RPA and IVAM, 2014).

In the following chapters, water solubility, PPS, shape, and aspect ratio, surface area, chemical composition, and surface chemistry are presented as relevant intrinsic material properties for nanomaterial characterization and grouping. The rationale for each property is discussed. Additionally, the Supplementary Information Table S2 provides specific information on currently preferred analytical techniques and the dynamic ranges of the respective properties that are relevant for grouping. If possible, standardized methodologies are listed, since the standardization of analytical techniques is vital to proper grouping. Many measurement methods have standards accepted by the International Standardization Organization (ISO), although these methods have not necessarily been designed for nano-sized objects (Izak-Nau and Voetz, 2014). Further, Table S2 indicates technical limitations or caveats for the characterization methods and lists relevant benchmark materials (*cf.* Information box for definition), if available. These benchmark materials would ideally have a similar MoA as other substances that are placed into the particular group. Benchmark materials allow some ranking of toxicity among members of a group, allowing for exposure control bands (Kuempel et al., 2012).

Of note, NM-x numberings of benchmark materials (or of specific nanomaterials mentioned in the text, e.g. 'ZnO NM-110') refer to the respective numberings of the 'OECD reference nanomaterials' as they have been coded in the list of the OECD Sponsorship Program for the Testing of Manufactured Nanomaterials (http://ihcp.jrc.ec.europa.eu/our_activities/nanotechnology/nanomaterials-repository and http://www.oecd.org/science/nanosafety/).

3.1.1. Water solubility

In the DF4nanoGrouping, 'water solubility' is used as Tier 1 key criterion to assign nanomaterials to main group 1 ('soluble nanomaterials'). 'Water solubility' is interdependent with the system-dependent property/Tier 2 key criterion 'dissolution rate in BSF, and the Tier 3 criteria '*in vitro*/*in vivo* biopersistence/release of toxic ions'.

Solubility is conventionally described as the maximum mass of a (nano-)material that is found in molecularly dissolved state in a given volume of solvent containing a particulate material under specific conditions. Dissolved species may either be ions or single molecules that have dissolved from organic nanomaterials. Within the DF4nanoGrouping, water solubility is addressed as intrinsic material property (and oftentimes, it is tabulated), whereas the time-dependent dissolution rate of a nanomaterial in different biological media is addressed as system-dependent property (*cf.* Chapter 3.3).

The water solubility of a material provides a first indication of its (non-)biopersistence. However particle dissolution may be reversed by recrystallization (Kuempel et al., 2012; Wohlleben et al., 2013; BAuA, 2013; Li et al., 2014). For ionic compounds, dissolved metal ions are one of the primary mechanisms of cytotoxicity (Nel et al., 2013). The German Federal Institute for Occupational Safety and Health (BAuA, 2013) has set a threshold value of 100 mg/L to distinguish between soluble and non-soluble nanomaterials.

3.1.2. Particle morphology: primary particle size, shape, and aspect ratio

'*PPS, shape and aspect ratio*' may be used in Tier 0 to identify a nanomaterial. In the DF4nanoGrouping, these intrinsic material properties are used as Tier 1 key criteria to indicate that a nanomaterial might belong to main group 2 'biopersistent HAR NMs'. 'PPS, shape and aspect ratio' are interdependent with the system-dependent properties/Tier 2 key criteria 'size in relevant media/dispersibility' and the Tier 2 and 3 criteria '*in vitro*/*in vivo* uptake, biodistribution, and clearance'.

The PPS, the shape and resulting aspect ratio of nanomaterials may influence biological interactions such as adsorption sites, cellular deposition and cellular or systemic uptake, clearance, and biological effects, such as inflammation-related responses (Oberdörster et al., 2005; Teeguarden et al., 2007; Kettler et al., 2014). Therefore, assessing size and shape (and aspect ratio) is imperative for nanomaterial grouping. It may further serve to prioritize inhalation testing (since the size of the nanomaterial greatly affects lung deposition) and to understand how to minimize potential hazards caused by nanomaterials.

In accordance with the guidance from the German Federal Institute for Occupational Safety and Health (BAuA, 2013, 2014a),

fibers that fulfill the criteria of the World Health Organization (WHO, 2005; i.e. that have an aspect ratio of 3:1 or higher, a length >5 μm, and a diameter <3 μm) may be expected to elicit asbestos-like effects – acting by a mechanism also described as the 'fiber pathogenicity paradigm' (Poland et al., 2009). Additionally, the rigidity and biopersistence of such HAR NMs contribute to their increased hazard potential by inhalation exposures (Donaldson et al., 2006, 2010, 2011). Accordingly, the above-mentioned BAuA, NIOSH, and BSI grouping approaches have defined a separate group for (biopersistent) nanomaterials that may elicit 'fiber toxicity'.

Importantly, size and shape may change depending on the nanoparticles' surroundings. For instance, the primary particles of many types of nanomaterials, including TiO_2, form aggregates or agglomerates when dispersed in air or in liquid vehicles (Yi et al., 2013), and saline causes gold nanoparticles to agglomerate (Keene and Tyner, 2011). Therefore, the state of the particles (powder, suspension, etc.), both as manufactured and as intended for use, should be understood before testing takes place. Since *in situ* nanomaterial size and shape depends on prior dispersion, it is treated as a system-dependent property designated 'dispersibility' (ECHA, 2012c; *cf.* Chapter 3.3).

3.1.3. Surface area

'Surface area' may be used in Tier 0 to identify a nanomaterial. In the DF4nanoGrouping, it may also be used as supplementary criterion. 'Surface area' is interdependent with the system-dependent property/Tier 2 key criterion 'surface reactivity', with the Tier 2 supplementary criteria 'corona formation' and 'surface area *in situ*' and with the Tier 2 and 3 criteria '*in vitro*/*in vivo* oxidative stress, membrane damage, macrophage activation, and inflammation'.

Surface area is considered an important factor for nanomaterial toxicology because all interactions between a nanoparticle and its environment occur at the particle surface. With decreasing particle size, down to the nanoscale, the surface area increases exponentially. Thereby, also the fraction of the particle's atoms present on its surface increases. Surface area greatly depends on the PPS, shape and porosity.

Frequently, the increase in catalytic surface is the desired feature of a nanomaterial since it enables the formation of more reaction products per time and mass of the catalyzing particles. With increasing surface area, such nanomaterials may increasingly catalyze the formation of reactive species, such as reactive oxygen species (ROS), in biological systems. Another example of the high surface activity of nanomaterials is the rapid formation of protein coronas when dispersed in biological fluids (Bello et al., 2009). Thereby, the intrinsic material property 'surface area' is closely related to the system-dependent properties 'surface charge' and 'corona formation' (*cf.* Chapter 3.3).

Brown et al. (2001), Duffin et al. (2007) described that large surface area nanoparticles made from low-toxicity materials exerted higher levels of inflammatory response in lung cells, indicating that surface area is an important factor in triggering inflammatory responses. Also in a STIS investigating CeO_2 NM-211 and NM-212, the surface area of the particles provided the dose metrics with the best correlation of the two substances' inflammatory responses and better correlated than mass or volume in the lung (Keller et al., 2014). Many studies recognized surface metrics as the best-suited parameters for comparative toxicological testing of nanomaterials (Oberdörster, 2009; Rushton et al., 2010; Wang and Fan, 2014). Since different properties of nanomaterials affect different stages of the events leading to apical toxic effects, a unifying dose metric for all types of nanomaterials is unlikely (Braakhuis et al., 2014; Landsiedel et al., 2014b).

3.1.4. Chemical composition (including impurities and crystallinity)

'Chemical composition' and 'crystallinity' may be used in Tier 0 to identify a nanomaterial. In the DF4nanoGrouping, 'composition' is used as Tier 1 key criterion to indicate that a nanomaterial might belong to the main groups 3 or 4 ('passive' and 'active nanomaterials', respectively). 'Composition' is interdependent with the system-dependent property/Tier 2 key criterion 'dissolution rate' and with the Tier 2 and 3 criteria '*in vitro*/*in vivo* biopersistence and release of toxic ions'.

For many nanomaterials, the chemical composition, i.e. the nanomaterial's chemical identity on the molecular level without consideration of nanostructures, is an important determinant of toxic effects. This has been discussed in detail in different reviews (*e.g.* Donaldson and Poland, 2013; Landsiedel et al., 2014a; Wang and Fan, 2014), as well as in publications related to the EU-funded project Particle_Risk (Johnston et al., 2013), the German project nanoGEM (Buesen et al., 2014; Landsiedel et al., 2014b), or the United States Environmental Protection Agency (EPA) ToxCast™ program (Wang et al., 2013). The chemical composition's potential to drive toxicity requires special attention if the nanomaterial is produced from a substance that is known to induce a specific toxic effect, i.e. carcinogenicity (Pietruska et al., 2011) or is likely to dissolve releasing toxic ions.

As for non-nanosized materials, impurities and stabilizers may drive nanomaterial toxicity (Nel et al., 2006; Keene et al., 2014). Similarly, the crystal phase of a nanomaterial that may differ even for substances of the same chemical composition, may affect its toxicity. For instance, amorphous silica is innocuous, while crystalline silica is carcinogenic (Fruijtier-Polloth, 2012). Due to the influence of chemical composition on toxicity, several classification schemes include a group for nanomaterials that bases their biopersistence and toxicity on this intrinsic material property (BSI, 2007; BAuA, 2013).

3.1.5. Surface chemistry, surface charge and surface hydrophobicity

'Surface chemistry, surface charge and hydrophobicity' may be used in Tier 0 to identify a nanomaterial. In the DF4nanoGrouping, these intrinsic material properties may be used as supplementary criteria. They are interdependent with the system-dependent property/Tier 2 key criterion 'size in relevant media/dispersibility' and with the Tier 2 and 3 criteria '*in vitro*/*in vivo* uptake, biodistribution and clearance'.

Surface chemistry encompasses the chemical composition of the material's surface and intentional or inadvertent organic surface modifications (Hellack et al., 2013; Wohlleben et al., 2013). In accordance with the definitions for 'intrinsic material properties' and 'system-dependent properties', intentional surface modifications are listed as 'intrinsic material properties' whereas inadvertent surface modifications (specifically, corona formation) are assigned as system-dependent properties (*cf.* Chapter 3.3). Chemical surface functional groups, such as amines on dendrimers, may affect nanomaterial toxicity (Kim et al., 2008), and many laboratories use such modifications to lessen nanoparticle interactions with proteins and to mitigate the toxicity of organic and inorganic nanomaterials (Aillon et al., 2009; Kong et al., 2011). Small changes in functionalization groups that change a nanoparticle's surface charge have been shown to significantly affect cellular uptake, protein absorption, and cytotoxicity (Patil et al., 2007; Moghadam et al., 2012; Mattix et al., 2013). By contrast, the biological effects of other nanomaterials of the same composition but with different surface functionalization were not greatly influenced by surface functionalization (Hellack et al., 2013; Wohlleben et al., 2014a).

Surface charge is measured from the zeta potential or the electrophoretic mobility of the particle at a given pH and temperature (Table S2). In addition to the zeta potential, it is important to note

the isoelectric point, i.e. the pH where the zeta potential is equal to zero (Izak-Nau and Voetz, 2014). In colloidal science, particles in solution with zeta potentials below −20 mV or above +20 mV are considered as electrostatically stabilized against agglomeration with each other (Evans and Wennerström, 1999).

Surface charge is an intrinsic material property, since it is independent of the system in which the material prevails (Brunner et al., 2006; Schulze et al., 2008; Wang et al., 2014). Kim et al. (2008) found positively charged polyamidoamine dendrimers to be more cytotoxic in Chinese hamster ovary cells than neutral or negatively charged particles and concluded that the charge of a nanoparticle might affect its biodistribution. Wang et al. (2014) found the nanoparticles' positive zeta potential to be neutralized by corona formation immediately after their deposition in lung lining fluid. However, once taken up into cellular lysosomes, the acidic environment in combination with protease and lipase activity was able to remove the corona. Thereby, the charged surface was again revealed, and the naked particle was free to interact with the lysosomal membrane (Wang et al., 2014).

While surface charge is not of primary relevance for grouping, monitoring the surface chemistry of organic and inorganic nanomaterials (Suzuki, 2003; Baer et al., 2010) provides additional structural information relevant for grouping. The surface chemistry of nanomaterials has a fundamental influence on system-dependent properties, such as aggregation, agglomeration, and biomolecule interaction, and it may vary depending on the synthesis method used (Kim et al., 2013). This variation may also have an effect on the nanomaterial's toxicity, since it affects surface reactivity, which in return influences oxidative reactions, i.e. one of the primary mechanisms of cytotoxicity (Nel et al., 2013). Similarly, differences in ATP-ase activity (ATP: adenosine triphosphate) and in the cellular uptake of particles were observed in variants of CeO_2 nanomaterials created during assorted synthesis techniques (Dowding et al., 2013).

Hydrophobicity has been addressed as key property to determine the uptake, fate and transport of non-nanosized substances or nanomaterials (Wagner et al., 2014). While the traditional OECD method of K_{ow} partition coefficient is not generally considered to be applicable to determine the hydrophobicity of nanomaterials (OECD, 2012), other characterization methods for this property have not yet gained wide acceptance. For powder materials, inverse gas chromatography is relatively simple and is increasingly being applied. Nevertheless, the even simpler method of measuring the water contact angle on a pressed powder substrate may still be advantageous for hydrophobicity assessment.

The biological surface adsorption index (BSAI) has been suggested as a more complete approach to assess a nanomaterial's surface composition. The BSAI results in five quantitative descriptors, i.e. hydrophobicity, hydrogen bond, polarity/polarization, and lone-pair electrons (Xia et al., 2011; Chen et al., 2014). Up to now, however, a simple correlation of all indices with *in vivo* effects is unavailable. Therefore, within the DF4nanoGrouping, the basic properties of charge and hydrophobicity have been selected as preferred grouping criteria, in the expectation that methods for the determination of nanomaterial hydrophobicity will shortly become accepted.

Knowledge on how altered surface chemistries affect system-dependent properties, cellular or apical toxic effects due may be used to design and produce safer nanomaterials, since manipulation of the surface chemistry may minimize its toxic potential. Nevertheless, also the OECD guidance confirms that the surface charge of a nanomaterial is not of primary relevance for grouping. Instead, it is a proxy to estimate agglomeration tendency (by colloidal theories), which in return contributes significantly to mobility (OECD, 2012), i.e. the nanomaterial's potential to move between body compartments (*cf.* Chapter 3.3.3).

3.1.6. Grouping of nanomaterials by intrinsic material properties – conclusion

Grouping of nanomaterials by specific intrinsic material properties is based on the assumption that the characteristic in question is one of the primary influences of toxicity. Overall, some intrinsic material properties, such as high aspect ratios, may be direct predictors of biological effects. Other intrinsic material properties, such as chemical composition and water solubility, may provide a first estimate of the nanomaterial's likely toxic potential, whereas further properties are not by themselves predictive, but need to be combined with the outcome of *in vitro* or *in vivo* studies to allow nanomaterial grouping. Depending on the respective type of nanomaterial and the given intrinsic material property, grouping may lead to an understanding on how the unique characteristics of nanomaterials contribute to potential toxicological risks. If a reliable grouping may be established, it allows for a more efficient production, use, and disposal of nanomaterials, affecting the whole life cycle of the material. This may provide the basis for the safer use and development of nanomaterials.

However, most nanomaterials will not be accurately grouped based solely on their intrinsic material properties. While high aspect ratio is an intrinsic material property allowing the assignment of the corresponding nanomaterials into a 'group with higher hazard potential' and water solubility into a group of 'soluble particles', other nanomaterials may need to be grouped using additional aspects of grouping discussed below depending on the toxicity endpoint. In addition, it is likely false to assume that nano-size alone dictates toxicity (Gebel et al., 2014).

3.2. The qualifiers use, release, and route of exposure

3.2.1. Use

With the increasing application of nanomaterials in industry and consumer products (Chen et al., 2014), the list of specific uses of nanomaterials is continuously increasing. The German Federal Environmental Agency suggests a classification framework to group products containing nanomaterials into the following categories: substances, cosmetics, health care, food and feed, coatings and inks, cleaning and disinfection, rubber products, building and construction, textiles, paper products, and complex objects and other products (UBA, 2014). Specifically for carbon nanotubes (CNTs), Nowack et al. (2013) describe nine relevant use and release scenarios, i.e. injection molding, manufacturing, sports equipment, electronics, windmill blades, fuel system components, tires, textiles, incineration, and landfills, with each having specific exposure routes and patterns. The properties of fragments released from nanocomposite materials and the actual probability of release were assessed as being primarily linked to the matrix in which the nanomaterial is incorporated (i.e. the 'system' is referred to in the term 'system-dependent properties'), with only secondary modulation by the embedded nanomaterial (Kingston et al., 2014). Since investigations using fragments released during the use of plastics, cements and coatings revealed that toxicological effects are predominantly elicited by the material system that incorporates the nanomaterial (Wohlleben et al., 2011; Saber et al., 2012; Kaiser et al., 2013; Kuhlbusch and Canady, 2014; Saber and Jensen, 2014; Smulders et al., 2014), within the DG4nanoGrouping, 'use' has been included as a relevant qualifier for nanomaterial grouping.

3.2.2. Release

In the Supplementary Information, Table S3 presents parameters for determining the impact of relevant release scenarios sorted by the corresponding most relevant route of exposure. These include the dustiness or droplet size of aerosolized nanomaterials or particle sizes upon machining or weathering. Hence,

nanomaterial volatility and dustiness, two intrinsic material properties, are addressed as indicators of nanomaterial release. Substances, which are inherently 'dusty', are likely to have increased toxicity due to increased pulmonary deposition and possible absorption after inhalation exposure.

Intensive investigations have assessed nanomaterial release from matrices focusing on the product's use phase under conditions like weathering or aging. Overall, the potential release, e.g., of MWCNTs or silica from different polymer systems, is likely to be low (Kingston et al., 2014; Wohlleben et al., 2014a,b). Release by machining or mechanical treatment of an article with embedded nanomaterial should be investigated if elevated exposures may occur locally and temporally during the mechanical treatment. This is especially relevant if, e.g., high-shear professional handling may lead to aerosol generation. In outdoor uses, hydrolysis and UV radiation degrade polymer matrices, and fractions of embedded nanomaterials may be released (Ging et al., 2014). Even though the corresponding rates of release and released masses will be low, they may nevertheless be relevant. Accordingly, nanomaterial release by weathering or aging of an article with embedded nanomaterial substance should be assessed, if appropriate.

3.2.3. Route of exposure

Table S3 distinguishes between inhalation, oral and dermal exposure (while additionally mentioning special exposure scenarios for nanomaterials that are intended for use in food, pharmaceuticals, or cosmetics). Handling of nanomaterial powders is the most critical occupational exposure scenario, however, inhalation exposure may also be relevant for consumers. Ingestion mostly reflects both incidental and intentional exposure to nanomaterials in food and consumer products. The potential of dermal exposure is especially high for consumers using e.g. cosmetic products and for workers in occupational settings.

Of note, the DF4nanoGrouping implies that general methods for industrial hygiene are fully implemented. Along with exposure-based hazard assessment, it will be possible to advise on further specific risk management measures, such as, in the occupational setting, respiratory protection, wearing of specific gloves, etc.

3.2.4. The qualifiers use, release, and route of exposure – conclusion

The importance of exposure estimations that are based on relevant use and release scenarios for hazard assessment is highlighted in various research projects (Stone et al., 2014; Pastoor et al., 2014; Duncan and Pillai, 2015). Most importantly, it is also reflected in the regulatory environment. Sensitive uses, e.g. with direct exposure of humans or the environment, such as in food, medical devices, agrochemicals, biocides and cosmetics, are treated by separate, specific regulations. The European Food Safety Authority (EFSA, 2011) distinguishes 6 different exposure scenarios for 'engineered' nanomaterials in food and feed products: (1) no persistence of the nanomaterial in preparations and formulations as marketed; (2) no nanomaterial migration from food contact materials (i.e. no exposure); (3) complete nanomaterial transformation in the food or feed matrix before ingestion; (4) nanomaterial transformation during digestion. Finally, in scenarios (5) and (6) it is assumed that some or all of the nanomaterial persists in the food or feed matrix and in gastrointestinal fluids and information on a non-nanosized substance of the same chemical composition is (or is not) available. Hence, the EFSA guidance allows for reduced information requirements when there is no exposure to the nanomaterial either because it does not migrate from food contact materials or if it completely degrades or dissolves so that no nanomaterial will be absorbed (EFSA, 2011).

Specifically for the determination of potential health effects of nanomaterials used in medical devices, the EU Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2015) recommends a phased approach based on potential release and characteristics of the nanomaterials to avoid unnecessary testing, i.e. (1) particle release, (2) particle distribution and persistence, (3) hazard assessment (toxicological evaluations), (4) risk characterization and assessment: *In phase 1, an evaluation of the potential for the device to release nanoparticles either directly or due to wear of the device during use should be carried out. In phase 2, the aim is to determine the distribution of the particles released and also their persistence potential. In phase 3, the hazard is assessed using appropriate toxicity tests taking account of the exposure characteristics and potential for persistence in specific organs. This will provide input for the final risk characterization (phase 4). The estimated risk needs to be compared to the risk from the use of comparable devices not incorporating nanomaterials in judging the acceptability of the risk.*

Within the REACH regulation, hazard and exposure assessment form the two fundamental pillars of risk assessment. Nevertheless, in Annex XI, Paragraph 3, 'substance-tailored exposure-driven testing' is discussed as a possible waiving argument for testing. Higher tier testing (in accordance with the information requirements from Annex VIII–X of the REACH regulation) may be omitted if exposure assessment demonstrates that exposure is either absent or non-significant throughout the life cycle of a substance (EP and Council of the EU, 2006).

The combination of intrinsic material properties and realistic release and exposure scenarios during the various stages of a nanomaterial's life cycle reveals to which forms of a nanomaterial humans may potentially be exposed and which therefore should be submitted to further evaluation. On the other hand, exposure-based waiving with regard to hazard testing should be possible, if no or negligible exposure exists and information on intrinsic material properties and system-dependent properties is available. For instance, nanomaterials solely used in non-spray cosmetic sunscreen lotions would have to be assessed for dermal effects and dermal penetration, but may not require extensive data on inhalation toxicity.

3.3. Grouping of nanomaterials by system-dependent properties

System-dependent properties provide essential information on potential hazards that may arise from nanomaterial exposure. The system-dependent properties of a nanomaterial are dependent upon, and influenced by, the environment in which it is located during use. A substance that has low toxicity as a powder may become more hazardous in a particular solvent if it begins to break down into toxic ions. Alternatively, wetting of nanomaterials or incorporation into solid matrices are established means of exposure control by reducing dustiness. Numerous types of nanomaterials will aggregate or agglomerate in biological matrices, such as blood or culture media that are supplemented with serum. This may change their size and shape as well as surface characteristics. Many of these parameters are dependent upon the specific matrix used during the measurement. Therefore, it may be necessary to characterize the system-dependent properties of a nanomaterial using multiple assay conditions or using one standardized technique that utilizes a particular matrix.

The following chapters present and discuss surface reactivity, dispersibility, and dissolution in relevant media as key criteria for nanomaterial grouping in Tier 2 of the DF4nanoGrouping, highlighting their influence on potential cellular or apical toxic effects (i.e. their interdependence with higher tier criteria within the DF4nanoGrouping). For these parameters, the Supplementary Information Table S4 lists preferred characterization methods, dynamic ranges, and benchmark materials. Some of the system-dependent properties require functional assays that extend

standardized characterization methods to investigate intrinsic material properties. Additionally, 'corona formation' and 'surface area *in situ*' are presented. These system-dependent properties are closely related to the key criterion 'surface reactivity' and therefore are used as supplementary criteria in the DF4nanoGrouping.

3.3.1. Dissolution rate

In the DF4nanoGrouping, 'dissolution rate' is used as Tier 2 key criterion to assign nanomaterials to main group 2 'biopersistent HAR NMs'. It is interdependent with the lower tier intrinsic material properties 'composition', 'crystallinity', 'water solubility', 'PPS and aspect ratio' and the Tier 2 and 3 criteria '*in vitro*/*in vivo* biopersistence and release of toxic ions'.

Whereas the intrinsic material property 'water solubility' describes equilibrium in a saturated aqueous solution, a nanomaterial's dissolution describes a time-dependent process (depending on the rate of solubilization and the surface area). Further, dissolution may be determined in more physiologically relevant media, such as phagolysosomal simulation fluids (PSF) or phosphate buffered saline (Stefaniak et al., 2005; Gamble, 2011; Misra et al., 2012). As kinetic property, the dissolution rate is directly related to a nanomaterial's *in vitro* or *in vivo* biopersistence that decreases with increasing dissolution rate. Therefore, the dissolution rate is expected to be a better reflection of the biopersistence of a nanomaterial than the intrinsic material property 'water solubility'.

The dissolution rate is an essential factor for the grouping and risk assessment of nanomaterials. Some nanomaterials, such as silver or zinc oxide nanomaterials or cadmium-based quantum dots, may release toxic ions, particularly in biological samples (Sharma et al., 2012; Nel et al., 2013; Gebel et al., 2014; Landsiedel et al., 2014a; Pujalté et al., 2014). Alternatively, completely (bio)soluble nanomaterials with very high dissolution rates will lose their nanospecific features so that only ions will become systemically available. For such nanomaterials, hazard identification and risk assessment may rely on data from the non-nanosized bulk material or the dissolved ions. Also the particulate fraction remaining after incubation may be relevant for grouping and risk assessment due to recrystallization phenomena that may directly link to toxicological effects (Li et al., 2014; Molina et al., 2014).

3.3.2. Surface reactivity (incl. electronic resonance (band gap), abiotic ROS generation, redox and photocatalytic activities)

In the DF4nanoGrouping, 'surface reactivity' is used as Tier 2 key criterion to distinguish 'passive nanomaterials' (main group 3) from 'active nanomaterials' (main group 4). 'Surface reactivity' is interdependent with the lower tier intrinsic material properties 'surface area', 'surface chemistry', 'surface charge' and 'hydrophobicity' as well as the Tier 2 supplementary criteria 'corona formation' and ''surface area *in situ*' and the Tier 2 and 3 criteria '*in vitro*/*in vivo* oxidative stress, membrane damage, macrophage activation, and inflammation'.

Materials may absorb energy from their surrounding system to promote bound electrons into a state of higher excitation energy (i.e., for semiconductor metal oxides, the conduction band). When the electrons return to the ground state (i.e. valence band), energy is restored to the surrounding system by emission of heat or light or by the redox cycle. The energetic gap between these two bands (i.e. the band gap) is measurable by UV–Vis spectroscopy. Such processes constitute the technical functionality of e.g. semiconducting metal oxides used as (photo)catalysts or UV absorbers. The potential of metal oxide nanomaterials to initiate oxidative stress via ROS generation is believed to be one of their primary MoAs of toxic effects. ROS reactivity may even be calculated via the band gap and redox state energies (Burello and Worth, 2011; Zhang et al., 2012), even though this cannot be performed universally on all groups of materials and hence is not recommended. Instead, abiotic ROS generation should be determined in the Ferric Reducing Ability of Serum (FRAS) assay.

Based upon the outcome of the FRAS assay, Hsieh et al. (2013) grouped a broad variety of 138 different nanomaterials by their MoAs related to the inorganic particle surface and slight modulation by additional surface charges. Although it is potentially of even higher physiological relevance than the FRAS assay, fewer data are available on using cytochrome c as the protein that carries the redox-cyclable iron-complex to determine abiotic ROS generation. Nevertheless, the available cytochrome c assay-based data confirms the suggested ranking of materials using data from the FRAS assay (Zhang et al., 2012). Regardless of the ROS screening method applied, due to the composition of the most reactive materials (Cr_2O_3, Co_3O_4, CoO, MnO_2, Mn_2O_3, NiO, Cu, CuO, Ag, and ZnO), synergistic effects from nanomaterial metal ion dissolution should be considered in parallel (Chusuei et al., 2013).

If nanomaterials are intended for use under exposure to light that is resonant with the electronic absorbance spectrum (especially UV filters or pigments in cosmetics), photocatalytic activity is accepted as critical property. Several standardized methods based on the discoloration of dyes have been adapted to nanomaterials from the OECD sponsorship program (OECD, 2014b).

3.3.3. Dispersibility

In the DF4nanoGrouping, 'dispersibility' is used as Tier 2 key criterion to distinguish 'passive nanomaterials' (main group 3) from 'active nanomaterials' (main group 4). It is interdependent with the lower tier intrinsic material properties 'PPS, surface chemistry, surface charge and hydrophobicity' as well as the Tier 2 and 3 criteria '*in vitro*/*in vivo* uptake, biodistribution and clearance'.

Dispersibility is the measure of the distribution of a solid material in a suspending medium, and the 'smallest dispersible unit' of a material is the asymptote of particle size with increasing shear force (e.g. by ball milling or probe sonication) (ASTM, undated). Although its determination frequently relies on estimations and procedural conventions (Taurozzi et al., 2010), assessing dispersibility may provide indications on how a material will perform in a biological fluid, which in turn may be an indication of its *in vivo* uptake and biodistribution and provide relevant information for *in vitro* dose metrics (DeLoid et al., 2014). Only particles with surface functionalizations that are designed to minimize corona formation (*cf.* Chapter 3.3.4) remain in the systemic circulation for longer periods of time. This may be achieved most effectively by steric stabilization with long flexible polymer chains, such as polyethylene glycol, and further be aided by zwitterionic or strong negative charges (Hühn et al., 2013; Kim et al., 2013).

Dispersibility is an important characteristic in understanding the behavior of nanoparticles, yet it is one of the more difficult ones to quantify due to the high polydispersity of agglomerates (Powers et al., 2006). Consequently, measurement techniques need to tolerate a polydisperse distribution of free proteins, lipids, natural matter, and nanoparticles, on the one hand, and their micron-sized homo- or hetero-agglomerates, on the other hand (Landsiedel et al., 2010). The simplest property to screen for is full dispersibility with average agglomeration numbers (AAN) of 1 (ECHA, 2012c; Choi et al., 2009, 2010; Hühn et al., 2013; Wohlleben et al., 2013). Nanomaterials that remain dispersed as constituent particles (with AAN <3) are defined as 'mobile', since they may potentially move between body compartments. Nanomaterial mobility is jointly determined by dispersibility, surface charge and hydrophobicity.

3.3.4. 'Corona formation': medium-related alterations in surface chemistry

In the DF4nanoGrouping, 'corona formation' and 'surface area *in situ*' may be used as supplementary criteria to the Tier 2 key

criterion 'surface reactivity' that serves to distinguish 'passive nanomaterials' (main group 3) from 'active nanomaterials' (main group 4). 'Surface reactivity', 'corona formation', and ''surface area *in situ*' are interdependent with the lower tier intrinsic material properties 'surface area', 'surface chemistry', 'surface charge' and 'hydrophobicity' and the Tier 2 and 3 criteria '*in vitro*/*in vivo* oxidative stress, membrane damage, macrophage activation, and inflammation'.

As soon as nanomaterials come into contact with biological fluids, they readily adsorb proteins and/or phospholipids. In *in vitro* studies, addition of fetal calf serum or other protein-rich supplements to the culture media with resulting protein adsorption onto the nanoparticle surfaces was found to mitigate cellular toxicity (Docter et al., 2014; Gebel et al., 2014). Due to this process of 'corona formation' (Monopoli et al., 2011, 2012), previously designated as 'opsonization', 'naked' nanoparticles will never reach the cells of the organism (Landsiedel et al., 2014a). Protein binding to nanoparticles is a factor distinctly influencing their systemic availability and translocation, e.g. if immunoglobulin G or fibrinogen bound to their surface target particles for immune responses, such as phagocytosis. As reviewed by Landsiedel et al. (2012), the binding of proteins often causes a high loss of nanoparticles from the circulation and fast transfer to organs of the mononuclear phagocyte system (MPS), such as the liver and spleen. While the composition of adsorbed proteins may be very specific and also depends upon the composition of the surroundings (Monopoli et al., 2012), the overall adsorption affinity is mostly determined by the intrinsic properties of hydrophobicity and positive charge (Xia et al., 2011; Chen et al., 2014), which also correlate directly with fast removal of nanoparticles from the blood and uptake into the MPS (Landsiedel et al., 2012; Johnston et al., 2013).

System-dependent alterations in surface chemistry affect the net charge of the particle, and protein coronas may lead to a net negative charge and zeta potential of the nanomaterials (Sayes et al., 2007; Alkilany and Murphy, 2010). Nevertheless, different nanomaterials (with very different toxicological effects) tend to all have the same net charge in serum-containing medium (Landsiedel et al., 2010). A deviation from that rule may be an indication of weak corona formation, and may correlate with low agglomeration (but not necessarily so).

3.3.5. Surface area in situ

Cf. Chapter 3.3.4, first paragraph, for the status of 'surface area *in situ*' within the DF4nanoGrouping.

In situ, the surface area of the nanoparticle that is accessible to biomolecules of a given size and diffusion constant is likely to change due to the reduced interstitial pore sizes inside agglomerates, and due to binding of other biomolecules to the surface (Monopoli et al., 2011, 2012). The techniques for measuring surface area in situ are limited to simulations and calculations from assessments of size and shape including dynamic light scattering (DLS) or atomic force microscopy (Izak-Nau and Voetz, 2014). For nanomaterials in dispersion, the surface area may be measured using the nuclear magnetic resonance relaxation of the liquid containing the nanomaterial (Fairhurst and Prescott, 2011). However, none of these techniques have gained wide acceptance yet, and this property is only being used as supplementary criterion in the DF4nanoGrouping.

3.3.6. Grouping of nanomaterials by system-dependent properties – conclusion

The determination of system-dependent properties is vital for nanomaterial grouping. In fact, it is a key feature of nanomaterials that they elicit pronounced interferences with their surroundings, and this very feature is the reason why nanomaterial grouping must be performed differently than the grouping of conventional substances. Due to the pronounced influence of system-dependent properties of nanomaterials on their cellular and apical toxic effects, grouping of nanomaterials cannot rely on structural similarities alone, but must be functionality-driven. As such, system-dependent properties correspond to the AOP step of 'molecular initiating effects', which also directly relates to subsequent cellular and organ effects, while taking into account the biopersistence, uptake, and biodistribution properties of a material.

Of note, even though 'corona formation' plays a role in a nanomaterial's mobility and cellular effects, the predictive value of this system-dependent property is sufficiently addressed by the intrinsic properties hydrophobicity and surface charge, supplemented by the system-dependent property dispersibility. Therefore, these three properties have been selected key criteria within the DF4nanoGrouping, whereas 'corona formation' and 'surface area *in situ*' are addressed as supplementary criteria.

3.4. Grouping of nanomaterials by biopersistence, uptake, and biodistribution

After nanomaterial grouping by intrinsic material properties taking into account the qualifiers use, release and route of exposure, the next step of the DF4nanoGrouping implies determining whether the respective nanomaterial under investigation is likely to persist *in vitro* or *in vivo*, whether it may be taken up into the organism and, if so, how it is distributed within the body. The unique intrinsic material properties of nanomaterials relative to the corresponding bulk materials and especially their variability and modification diversity may either facilitate or hamper the biopersistence of nanomaterials in the organism as well as their translocation across respiratory, gastrointestinal or dermal barriers (depending on the route of exposure). The intrinsic material properties may further influence nanomaterial translocation rate and extent from the blood into organs and tissues (biodistribution). Particular attention should be paid to the 'internal exposure' or 'internal dose', i.e. the nanomaterial's concentration in different tissues and organs, as a driver of the biologically effective dose. Notwithstanding, Gebel et al. (2014) caution that biodistribution is much more strongly affected by the route of exposure than by intrinsic or system-dependent properties. Similarly, Yokel et al. (2014) reported that biodistribution and retention of CeO_2 nanomaterials upon intravenous injection into rats was very similar regardless of particle size or shape.

Relevant information to group nanomaterials by biopersistence, uptake, and biodistribution may be obtained in *in vitro* studies. To investigate uptake through the skin, an accepted *in vitro* test method is available, i.e. the *in vitro* skin absorption method in accordance to OECD Test Guideline (TG) 428 (Supplementary Information Table S5). By contrast, to date *in vitro* test methods simulating the pulmonary or gastrointestinal barriers have not yet passed the stage of test method development, and on short notice validated or accepted *in vitro* models are not to be expected (Hittinger et al., 2014; Murgia et al., 2014). The human lung adenocarcinoma cell line NCl-H441 has been suggested as a useful *in vitro* model to study particle transport across the distal lung epithelial barrier (Salomon et al., 2014). *In vitro* investigations addressing nanomaterial intestinal uptake are frequently conducted using the human intestinal Caco-2 cells (Tarantini et al., 2014a). Nevertheless, Tarantini and co-workers cautioned that the undifferentiated Caco-2 cells are not fully polarized and only display some phenotypic characteristics of human enterocytes and, further, that M cells or a mucus barrier are lacking in Caco-2 cell-based test systems. Gantzsch et al. (2014) proposed a modified phospholipid vesicle-based permeation assay to jointly assess the gastrointestinal dissolution and permeation of nanomaterials, since this model appears more robust and easier to handle than

Caco-2 cell monolayers, especially under physiological dynamic flow conditions.

Importantly, data may also be collected in the course of *in vivo* toxicity assays, such as the STIS (Table S5). The 5-day study protocol of the STIS, first described by Arts et al. (2007) and further elaborated by Ma-Hock et al. (2009a) is designed to assess lung burden, i.e. the amount of nanomaterial retained in the primary target organ, just as organ burden in lung-draining mediastinal lymph nodes, in the blood and in secondary organs, such as liver, kidneys, spleen and brain. Thereby, the STIS reveals if and how a nanomaterial becomes systemically available (uptake and biodistribution), and this information is valuable for the grouping of nanomaterials by their potential to induce systemic effects. Additionally, the STIS incorporates a time-course experimental design to investigate effects immediately after the 5-day exposure period and after a 3-week post-exposure period. Therefore, it also provides information on nanomaterial biopersistence and the reversibility or progression of effects. These criteria are relevant for the grouping of nanomaterials by their potential to elicit chronic effects (Ma-Hock et al., 2009a, 2013, 2014; Landsiedel et al., 2014b).

3.4.1. Biopersistence

Biopersistence is defined as the property of a material to persist in a cell, tissue, organ or organism. The biopersistence of a nanomaterial may affect its pulmonary retention and clearance (and hence also lung burden) as well as the nanomaterial's systemic uptake and biodistribution, which are presented in the following chapters. Nanomaterial biopersistence may be reduced if the material is degraded (e.g. by phagolysosomal dissolution). *In vivo*, nanomaterials may be poorly soluble or of moderate or high solubility. Nanoparticle toxicity (other than indirect toxic effects elicited by dissolved ions) is only likely for non-soluble or poorly soluble (i.e. biopersistent) particles. Biopersistence also determines the toxic potential of HAR NMs (Donaldson et al., 2010). This was shown in a rat short-term inhalation study applying 30 mg/m^3 of four different man-made vitreous fibers (diameters approx. 1 μm; lengths 15, 17, 22, and 27 μm, respectively) or 10 mg/m^3 crocidolite asbestos (diameter 0.3 μm; length 5.7 μm) (Hesterberg et al., 1996). One year post-exposure, >95% of the MMVFs with lengths >20 μm had disappeared from the lung compared to only 17% of the highly biopersistent crocidolite. The longer 22 and 27 μm MMVFs disappeared more rapidly than the shorter 15 and 17 μm MMVFs, suggesting that long fibers were dissolving or breaking (Hesterberg et al., 1996).

The relevance of biopersistence for hazard assessment is underlined by the fact that it is already taken into account in available concepts for the grouping of nanomaterials. For instance, in the scheme from Gebel et al. (2014) categories two and three address the nanomaterials' biopersistence ((1) nanomaterials whose toxicity is mediated by their chemical composition; (2) rigid biopersistent respirable fibrous nanomaterials; and (3) respirable granular biodurable particles (GBPs)). Moreno-Horn and Gebel (2014) define GBPs as being persistent in biological systems without possessing relevant toxicity that is either mediated by specific, released substances or by relevant functional surface properties. Other authors have suggested a similar group of 'poorly soluble particles', 'poorly soluble low toxicity particles', or 'low-toxicity dusts' (*cf.* e.g. Kuempel et al., 2012, 2014; Donaldson and Poland, 2013). In spite of their low inherent toxicity, inhaled GBPs, due to their biopersistence, may cause pulmonary inflammation and secondary mutagenicity that may ultimately lead to lung cancer (Gebel et al., 2014). The German Federal Institute for Occupational Safety and Health (BAuA, 2014a) prescribes categorizing fibers meeting the WHO criteria (*cf.* Chapter 3.1.2) as carcinogenic, if 4 × 0.5 mg intratracheally instilled, suspended fibers are retained in the rat lung with a half-life exceeding 40 days.

As a rule, biopersistence is inversely related to the dissolution rate of the respective material in relevant biological media. However, it cannot always be derived from this system-dependent property. For instance, $BaSO_4$ was found to be hardly soluble in water, poorly soluble in simulation fluid, but nevertheless of very low biopersistence *in vivo* (Konduru et al., 2014). Therefore, the initial assessment of a nanomaterial's biopersistence by its water solubility should be revisited when data on the dissolution rate in BSFs and *in vivo* organ burden over time are available. Water-soluble nanomaterials are generally not biopersistent – whereas some insoluble nanomaterials may nevertheless be non-biopersistent in BSFs or *in vivo*. Hence water solubility is a conservative predictor of non-biopersistence and does not result in wrongful exclusion of biopersistent nanomaterials.

3.4.2. Deposition in the lung, pulmonary clearance, and lung burden

Nanomaterials may enter the lung as airborne dust or dispersed or nebulized particles, with fractions below 3 μm (for the rat) reaching the non-ciliated alveolar region. The proportion of an inhaled amount of a nanomaterial that reaches the lung and that is not exhaled may be deposited on the surface of the lung. The deposited fraction carried within macrophages may initially be small. Over time, particles may progressively be removed from the alveoli by macrophages, which internalize the particles and move, as particle-laden cells, to the mucociliary escalator. During this process, dissolution and/or degradation of the materials by phagocytic cells may take place as well. To a limited extent, nanoparticles may also translocate across the air-blood-barrier as free particles (ICRP, 1994; Semmler-Behnke et al., 2007; Landsiedel et al., 2012). Accordingly, at a given point in time, the lung burden of a nanomaterial is dependent upon the amount of nanomaterial that has been deposited in the lung and its pulmonary clearance.

Inhaled particles may damage lung cells, especially if the macrophages' clearance capacity is overwhelmed. The clearance capacity is impaired once 6% of the macrophage volume has been filled, and macrophage stasis occurs at 60% filling of the macrophage volume (Morrow, 1988, 1994; Morrow et al., 1996). Therefore, determination of the lung burden and clearance rates of inhaled nanoparticles is especially relevant to determine whether pulmonary particle overload is likely, which may further lead to particle uptake and hence biodistribution (Kuempel et al., 2014). Apart from the degree of biopersistence, cumulative lung burden appears dependent upon the size of agglomerated particles and the likelihood of their disintegration. Nanoparticle agglomerates that form in aerosols and do not disintegrate in the lung are unlikely to become systemically available (Landsiedel et al., 2012, 2014a; Konduru et al., 2014; Molina et al., 2014), but might nevertheless remain in the lung or be cleared from there.

Overall, lung burden is low e.g. ranging from approximately 1% for surface-functionalized ZrO_2 to approximately 15% for ZnO and CeO_2 nanomaterials immediately after a 5-day exposure period (Landsiedel et al., 2014b). Lung burden of 4-week inhalation exposure to 50 mg/m^3 $BaSO_4$ NM-220 was also low (0.84 mg/lung; corresponding to 1.3% deposition) and decreased by 95% over 34 days (Konduru et al., 2014). Konduru et al. conclude that particle dissolution most likely explains the lower pulmonary biopersistence (and toxicity) of $BaSO_4$ nanoparticles compared to poorly soluble nanoparticles, such as CeO_2 and TiO_2.

Test substance concentration and exposure duration, and different intrinsic material and system-dependent properties, such as chemical composition including surface characteristics, size, water solubility, and dissolution in relevant media, may affect nanomaterial pulmonary deposition, clearance, and retention and the resulting lung burden at a given point in time, which in return directly influences nanomaterial biodistribution (Van Ravenzwaay et al.,

2009; Geraets et al., 2012; Ma-Hock et al., 2013; Keller et al., 2014; Konduru et al., 2014; Landsiedel et al., 2014b; Silva et al., 2014; *cf.* Supplementary Information for a summary of the results of these studies).

During inhalation exposure to CeO_2 NM-211, the dose rate of CeO_2 deposition drove an initial neutrophil-dominated inflammatory reaction (Keller et al., 2014). During 4 weeks of exposure, cell counts shifted to a macrophage-dominated inflammation that progressed toward a granulomatous reaction depending on the duration and amount of particles retained in the lung (Keller et al., 2014; Pauluhn, 2014). Hence, particle deposition and retention in the lung may affect apical toxic effects.

3.4.3. Systemic uptake and biodistribution upon inhalation

Typically, systemic uptake of nanomaterials lies in ranges below 1% of the retained dose, and it may occur especially under high-dose conditions. Systemic availability may be a result of the particles' ability to cross the air-blood barrier. In this case, they are present in the blood as free particles. If the nanoparticles dissolve in the lung, released ions may become systemically available. Alternatively, alveolar macrophages may take up the particles in the lung and then enter the blood stream in which case the particles are not present in the blood outside the cells. These differences in particle uptake mechanisms may affect tissue distribution.

Specifically for GBPs, low rates of absorption and biodistribution have been recorded (generally below 0.1–0.2% of the applied dose) without relevant differences in the translocation of nano-sized or micron-sized GBPs. If taken up systemically, nanoparticles are prone to lymphatic transport, but they may also be translocated with the circulatory system. By contrast, absorption via the olfactory system, if it occurs, does not seem to be specific for the nanosized variants of a material. Also nanoparticles that enter the blood stream are mostly taken up by the MPS that acts as a depot for nanoparticles. This explains observed extra-pulmonary accumulations in the liver and spleen, which are the first organs that particles circulating in the blood encounter. Surface charge affects nanoparticle uptake by the MPS. Neutral nanoparticles are taken up to a lesser extent, and therefore may have prolonged half-lives in the blood becoming available for increased uptake by other organ systems. However, organ uptake is also dependent on additional factors, such as the partition coefficient, and smaller-sized nanoparticles were observed to have a much greater biodistribution than larger-sized nanoparticles. Also within tissues, nanoparticles are preferentially located in phagocytically active cells, where they may have half-lives in the range of weeks or even up to years. Just as determined for lung burden, also secondary organ burden is dependent upon nanomaterial transport to and clearance from this organ. Repeated exposure to nanoparticles may lead to tissue accumulation if exposure levels and systemic availability are high enough, as is also known for bulk substances (Landsiedel et al., 2012; Kreyling et al., 2013; Gebel et al., 2014; Moreno-Horn and Gebel, 2014; Oomen et al., 2014).

Since secondary organ burden is very low, a number of studies have investigated nanomaterial organ distribution upon intravenous or intraperitoneal application (De Jong et al., 2008; Fabian et al., 2008; Van Ravenzwaay et al., 2009; Lankveld et al., 2010; Yang et al., 2013; Semmler-Behnke et al., 2014). These routes of application provide information for assessing systemic particle translocation and putative adverse effects based upon maximized bioavailability. However, intravenous or intraperitoneal administration do not represent realistic exposure scenarios for nanomaterials intended for non-medical uses, and biodistribution patterns and organ ratios may differ considerably depending on the administration route (Semmler-Behnke et al., 2008; Moreno-Horn and Gebel, 2014). Therefore, these routes of application are not further addressed in the present decision-making framework.

In different rat STIS investigating a broad spectrum of different TiO_2, CeO_2, SiO_2, ZrO_2, $BaSO_4$ and ZnO nanomaterials, most nanoparticles were found only in the animals' lungs and lung-draining lymph nodes. Only polyacrylate-coated SiO_2 was also identified in the spleen. None of the other nanoparticles were recorded in organs other than the respiratory tract, indicating that their translocation rate into secondary organs was negligibly low (Van Ravenzwaay et al., 2009; Morfeld et al., 2012; Landsiedel et al., 2014b). Keller et al. (2014) reported a very low content of cerium in the liver of rats exposed to 25 mg/m^3 CeO_2 NM-212 for 4 weeks by inhalation. Also 28 days post-exposure following a single intratracheal instillation of CeO_2 nanoparticles in rats, less than 1% of the administered dose was retained in extra-pulmonary tissues (Molina et al., 2014), and 7 days after intratracheal instillation of $BaSO_4$ nanoparticles in rats, only 0.15% of the dose was detected in the organs, with predominant accumulation in the bone compartment (29%). Overall, instilled and inhaled $BaSO_4$ nanoparticles were cleared from the organism quickly, but resulted in higher tissue retention rates compared to oral administration (Konduru et al., 2014). With decreasing size (down to very small PPS of 1.4 nm) and resulting increasing SSA, gold nanoparticles were found to be more likely to cross the air-blood barrier upon intratracheal instillation. However, relative to the amount of nanoparticles that had been taken up systemically, their retention in the secondary organs appeared unrelated to the SSA (Semmler-Behnke et al., 2008; Kreyling et al., 2014).

In an extensive review of GBP tissue distribution and systemic effects determined in *in vivo* rat and mouse acute, sub-acute and sub-chronic studies with at least 14-day post-exposure observation periods, Moreno-Horn and Gebel (2014) reported overall low systemic TiO_2 or CeO_2 nanoparticle translocation regardless of the route of application. If particles were found in extra-pulmonary tissues at all, they were mainly restricted to the liver (but also occurred to minimal extents in other organs, including the brain). For CeO_2 nanoparticles, a 28-day inhalation study using male mice provided evidence of nephrotoxicity (Aalapati et al., 2014), a finding that, however, was not observed in any studies using rats (Moreno-Horn and Gebel, 2014). Also for silver nanoparticles, systemic translocation was low, as assessed in a sub-chronic inhalation study with rats, in spite of its higher bioavailability than the poorly soluble TiO_2 or CeO_2 nanoparticles (Moreno-Horn and Gebel, 2014).

In summary, whereas most nanomaterials do not accumulate in secondary organs, determination of tissue distribution during initial screening tests, such as the STIS, allows grouping nanomaterials by potential secondary organ burden and clearance rates within 3-week post-exposure. Information on tissue distribution and biopersistence is relevant to rank nanomaterials by their potential to cause systemic apical toxic effects (and the likelihood of progression toward chronic effects) and also to select relevant follow-up studies.

3.4.4. Dermal and gastrointestinal absorption

The most important criterion to group nanomaterials with expected dermal or gastrointestinal exposure routes is to address whether these substances are likely to become systemically available. Generally, the available *in vitro* and *in vivo* studies do not report unintentional permeability or systemic availability of dermally applied nanomaterials, e.g. for nanomaterials used in sunscreen lotions (Landsiedel et al., 2012). In an *in vitro* porcine skin absorption model, neither micron-sized zinc oxide, nor different micron-sized titanium oxide penetrated the stratum corneum (Gamer et al., 2006). Also UVB-damaging of the skin only slightly enhanced the dermal penetration of TiO_2 or ZnO nanoparticles in sunscreen formulations, whereas transdermal absorption was not detected (Monteiro-Riviere et al., 2011). Consistent with these

findings, also in an *in vivo* study using mini-pigs, different uncoated or coated TiO_2 nanoparticles did not penetrate significantly through the intact normal epidermis (Sadrieh et al., 2010).

As a rule, nanomaterials reaching the gastrointestinal tract are excreted with the feces. Nevertheless, for some nanomaterials very low levels are absorbed and become systemically available (Landsiedel et al., 2012). Immediately after 28-day oral administration of 90 mg/kg body weight (bw) silver nanoparticles (uncoated, PPS: <20 nm, TEM; 59 nm, DLS; or polyvinylpyrrolidone-coated, PPS: <15 nm, TEM; 49 nm, DLS) to rats, silver was present in all examined organs, with the highest levels recorded in the liver and spleen. Silver concentrations in the organs highly correlated with the amount of Ag ions in the nanoparticle suspension, indicating that mainly Ag ions passed through the intestines. Eight weeks after dosing, silver was cleared from most organs, but not from the brain or testis (Van der Zande et al., 2012). Exposing rats orally to 2500 mg/kg bw commercially available amorphous SiO_2 (PPS: 7 nm) or to 1000 mg/kg SiO_2 NM-202 (PPS: 10–25 nm) for 28 days did not result in distinctly elevated SiO_2 levels in the body tissues. After 84 days of exposure, however, SiO_2 accumulated in the spleen of the rats treated with the commercially available amorphous SiO_2, and a significant increase in the occurrence of liver fibrosis was observed in the rats treated with SiO_2 NM-202 (Van der Zande et al., 2014). Treating rats orally with up to 1042 mg/kg bw TiO_2 (80% anatase, 20% rutile; 26 nm, SEM; 38 nm, DLS) or up to 536 mg/kg bw ZnO nanoparticles (90 nm, SEM; 202 nm, DLS) for 13 weeks did not result in significantly increased TiO_2 organ levels. Zn concentrations in the liver and kidney were significantly increased, and they were minimally increased in the spleen and brain (Cho et al., 2013).

3.4.5. Grouping of nanomaterials by biopersistence, uptake, and biodistribution – conclusion

Grouping of nanomaterials by biopersistence, uptake, and biodistribution, taking into account the qualifiers use, release, and exposure route, may provide first indications of expected local or systemic apical toxic effects. Furthermore, the same key intrinsic material properties and system-dependent properties that affect nanomaterial uptake and biodistribution (i.e. composition, solubility, size, surface area and charge) are also considered to be main drivers for toxicity. Most nanomaterials do not penetrate the stratum corneum of the skin, and only minimal amounts enter the systemic circulation from the lung and gastrointestinal tract. If nanomaterial exposure does not lead to systemic uptake, demonstrated in the STIS (or short-term oral studies or relevant *in vitro* test methods, such as OECD TG 428 if the dermal route of exposure is relevant) by a lack of secondary organ burden, potential health risks are limited to local effects. Notwithstanding, especially for the respiratory route of exposure, local effects at the primary site of contact have to be taken into consideration and evaluated for nanomaterial grouping.

3.5. Grouping of nanomaterials by cellular effects

Nanomaterials may induce cellular effects by a number of different mechanisms of toxicity, i.e. (1) membrane damage including cationic phagolysosome damage, (2) generation of ROS, oxidative stress, redox activities, and photo-catalytic effects, (3) inflammasome activation and cytokine and chemokine production, (4) the cytotoxic effects of toxic ions, (5) fiber effects, and (6) DNA damage (Meng et al., 2009; Nel et al., 2006, 2013; Landsiedel et al., 2014a; Visalli et al., 2015).

The release of cellular lactate dehydrogenase (LDH) allows assessing membrane damaging potential. Additionally, the human red blood cell lysis assay has been suggested as a relevant model to investigate phagolysosome damage, leading to inflammasome activation and release of the pro-inflammatory cytokine IL-1ß. Even though erythrocytes are not involved in pulmonary inflammatory processes, the same mechanisms appear involved in red blood cell lysis or phagolysosomal damage (Pavan et al., 2014). Inflammasome activation, in return, is determined by measuring the induction or release of relevant cytokines and chemokines, such as interleukins and tumor necrosis factor alpha (TNF-α). Different endpoint detection methods are available to investigate cytotoxicity and fiber-induced toxicity, including measurements of impaired mitochondrial activity (reduction of tetrazolium salts, such as MTT and WST-1) and colony formation ability, or apoptotic reactions (caspase-3/-7 or Annexin V/propidium iodide assays) (Landsiedel et al., 2014a; Sauer et al., 2014).

Oxidative stress reactions have been described as hierarchically evolving responses. At low levels of oxidative stress, antioxidant enzymes are induced that neutralize the ROS. Inflammatory reactions may occur if the cells' antioxidant capacities are overwhelmed. Finally, at prolonged, high oxidative stress levels, the cells may become apoptotic (Nel et al., 2006; Meng et al., 2009; Colognato et al., 2012). Therefore, the potential of a nanomaterial to generate ROS has been addressed as a key element affecting its cell and tissue damaging potential (Nel et al., 2006; Colognato et al., 2012). Generation of ROS and release of toxic ions have been linked to indirect genotoxic effects of the respective nanomaterials. Generally, there is conflicting and inconsistent evidence regarding the genotoxicity of nanomaterials *in vitro*. Few nanomaterials of specific particle morphology (distinct single-walled CNT and small gold nanoparticles) have been shown to interact with the DNA and the spindle apparatus *in vitro*, respectively (Singh et al., 2009; Colognato et al., 2012). There is, however, no consistent evidence of nanomaterials having direct genotoxic effects *in vitro* and *in vivo* (Landsiedel et al., 2009; Tarantini et al., 2014a; Golbamaki et al., 2015).

In vitro assays to determine DNA damaging potential include the *in vitro* micronucleus test (MNT) or the mammalian cell hypoxanthine phosphoribosyl transferase (HPRT) gene mutation assay (Landsiedel et al., 2009, 2014a; Supplementary Information Table S6).

As a rule, cellular effects are assessed in *in vitro* test methods that use cells and tissues of the corresponding organs of primary contact related to the expected human exposure route (i.e. pulmonary, dermal or intestinal cells) or cells and tissues of relevant target organs, such as hepatocytes or neuronal cells. The need to select appropriate dispersing agents, to consider and avoid nanomaterial interferences with assay detergents and to calculate *in vitro* effective doses, i.e. the amount of applied nanomaterial that reaches the *in vitro* system within the given exposure time, and to relate *in vitro* doses to realistic *in vivo* doses (avoid unrealistically high dosages), which all affect the relevance of the outcome of *in vitro* studies, have been discussed extensively elsewhere (Teeguarden et al., 2007; Stone et al., 2009; Guadagnini et al., 2013; Cohen et al., 2014; Landsiedel et al., 2014a).

In vitro-in vivo comparisons oftentimes yield unsatisfactory results, especially in regard to predicting *in vivo* hazard potency (Sayes et al., 2007; Landsiedel et al., 2014a). By contrast, the *in vitro* alveolar macrophage assay was found to be promising in regard to predicting the outcome of *in vivo* rat intratracheal instillation studies or short-term inhalation studies ranked according to no-observed-adverse-effect-levels (NOAEL; Wiemann and Bruch, 2009; Wiemann et al., 2015). This *in vitro* alveolar macrophage assay investigates LDH release, glucuronidase (as a sign of macrophage activation or incomplete phagocytosis), tumor necrosis factor alpha (TNF-α) and ROS. Thereby, it allows assessing many known mechanisms of cellular toxicity in a dose-dependent manner (Wiemann and Bruch, 2009; Wiemann et al., 2015, Table S6). The relevance of alveolar macrophages as suitable *in vitro* test

systems is explainable by the unique role these phagocytically active cells play in the biopersistence, uptake, and biodistribution of nanomaterials (*cf.* Chapter 3.4).

A number of intrinsic material properties and system-dependent properties of nanomaterials (and of testing conditions that influence these characteristics) crucially affect their uptake into cells and the resulting cellular effects they may cause (Nel et al., 2006, 2013; Zhu et al., 2013; Wang and Fan, 2014). An extensive review by Kettler et al. (2014) concludes that nanoparticle uptake into non-phagocytic cells depends strongly on particle size, with an uptake optimum at approximately 50 nm. Further, increasingly positive or negative surface charges have been shown to increase particle uptake (Kettler et al., 2014). Cellular uptake studies using A549 cells cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum showed preferential uptake of CeO_2 nanoparticles with negative zeta potential as compared to those with positive zeta potential (Patil et al., 2007). Negative zeta potentials have been observed to correlate with better nanomaterial dispersion in water (Buesen et al., 2014; Wohlleben et al., 2013.

Taking into account their intrinsic material and system-dependent properties, nanomaterials may be grouped by one or more mechanisms of cellular toxicity. For instance, for GBPs, such as TiO_2 and carbon black, oxidative stress reactions have been suggested as predominant mechanisms of toxicity, whereas SiO_2 nanomaterials were reported to cause membrane damage, ZnO and Ag nanomaterials cytotoxicity by the dissolution of toxic ions, and HAR NMs fiber effects (Nel et al., 2013; Landsiedel et al., 2014a). Within a group of common mechanism of cellular toxicity, different nanomaterials may be sub-grouped by the severity of the respective *in vitro* effect. To date, findings regarding *in vitro* genotoxic effects of nanomaterials are often inconsistent (and much less pronounced than effects caused by corresponding non-nanosized positive controls) and further difficult to relate to *in vivo* genotoxic effects. Overall, a nanomaterial's potential to induce genotoxicity appears mainly dependent upon its chemical composition and surface properties (Landsiedel et al., 2009; Haase et al., 2015).

3.5.1. Grouping of nanomaterials by cellular effects – conclusion

Grouping nanomaterials by cellular effects determined in relevant *in vitro* assays, such as the *in vitro* alveolar macrophage assay, while taking into account important intrinsic and system-dependent properties and realistic exposure scenarios for relevant stages of the nanomaterial's life cycle, is a further functionality-driven aspect of the DF4nanoGrouping that supports nanomaterial hazard and risk assessment. The experimental design of these studies will be important and should include fundamental parameters such as dose/response and time course characteristics, as well as appropriate benchmark control materials to better interpret the results of the *in vitro* assays (Hristozov et al., 2012; National Research Council, 2013; Krug, 2014; Landsiedel et al., 2014a).

3.6. Grouping of nanomaterials by apical toxic effects

3.6.1. Standard (screening) test methods for grouping by apical toxic effects

The STIS is suggested as standard (screening) test method for inhalation as route of exposure during Tier 3 of the DF4nanoGrouping. It allows the investigation of local and systemic toxicity, provides excellent correlation to 90-day inhalation studies and further allows the determination of local deposition and systemic distribution. Further, the STIS may be combined with *ex vivo* genotoxicity studies (Supplementary Information Table S6; Ma-Hock et al., 2009a, 2009b, 2013, 2014; Landsiedel et al., 2010, 2014b).

Frequently, nanomaterial respiratory tract effects are investigated in intratracheal instillation studies. Even though the limitations of these studies as compared to inhalation studies have been described (Driscoll et al., 2000), by bridging data obtained in instillation studies to a well-studied control material for which long-term inhalation data are available, these limitations may be at least partially mitigated (Gordon et al., 2014). According to Gordon et al., intratracheal instillation studies of nanomaterials should include dose–response data, time-course assessments (e.g. 1 day, 1 week, 1 month, and 3 months post-exposure), and doses should be relevant to potential worker exposures and not so high as to cause lung overload. Furthermore, dose bridging between instillation and inhalation should be done in terms of deposited lung doses, derived either via direct measurements or model estimates (Gordon et al., 2014).

For the oral route of exposure, the short-term oral study (STOS) does not have a defined exposure protocol yet. Currently, oral studies applying different exposure durations and exposure schemes up to 28-day exposure are addressed as 'short-term' studies. For instance, to investigate the genotoxic effects of amorphous SiO_2 nanomaterials, Tarantini et al. (2014b) exposed rats orally by gavage to 5–20 mg/kg bw of the nanomaterials for three consecutive days. Buesen et al. (2014) administered 1000 mg/kg bw/day of four amorphous SiO_2 with or without surface functionalization, two surface-functionalized ZrO_2 nanomaterials, or $BaSO_4$ NM-220 orally by gavage for 28 days.

3.6.2. Local effects in the respiratory tract (for the inhalation exposure route)

Many nanomaterials do not cause adverse effects upon inhalation. If effects are observed, they are mostly refined to the lung and recorded as different forms and degrees of inflammatory reactions. ZnO nanomaterials have additionally been reported to elicit necrotic reactions in the upper respiratory tract (Van Ravenzwaay et al., 2009; Klein et al., 2012; Landsiedel et al., 2010, 2014b; Ma-Hock et al., 2013, 2014). Accordingly, nanomaterials may be grouped by their potential to cause local reactions in the different parts of the respiratory tract. Arts et al. (2007) showed similarities in apical toxic effects between three different forms of amorphous SiO_2. By contrast, for TiO_2 and carbon-based nanomaterials, differences in apical toxic effects induced in the respiratory tract upon short-term and sub-chronic exposure allowed differentiating between distinct forms of TiO_2 nanoparticles (Warheit et al., 2007) or carbon allotropes (Ma-Hock et al., 2009a, 2009b, 2013; DeLorme et al., 2012).

3.6.3. Progressiveness of local effects

If inflammatory reactions are observed in the lung (for the inhalation exposure route), further grouping should address whether effects are progressive. If so, the corresponding nanomaterials ultimately may have the potential to initiate tumor development. Lung carcinogenicity was shown in the rat for nanosized carbon black and TiO_2 nanoparticles, and Gebel et al. (2014) just as the EU Commission's Scientific Committee on Consumer Safety (SCCS, 2014) considered these effects to be relevant for extrapolation to humans. Further, the potency of nanosized GBPs to induce inflammatory reactions in the lung was assessed as being higher than for micron-sized GBPs when comparing mass concentration exposure, the standard measured variable at the workplace (Gebel et al., 2014). On the other hand, none of the epidemiological studies examining workers exposed to carbon black or TiO_2 nanoparticles show evidence of carcinogenicity in humans (IARC, 2010). Additionally, Nikula et al. (1997, 2001) observed the biokinetics and lung responses elicited in rats versus non-human primates or humans upon inhalation of particulate materials to differ considerably, indicating that, for that effect, the rat might not be a good model for extrapolation to humans.

3.6.4. Toxic potency in the respiratory tract

The toxic potency of a nanomaterial in the respiratory tract may be assessed using no-observed-adverse-effect-concentration (NOAEC) values that correspond to the highest test substance concentration that does not elicit local toxic effects in the STIS. Within a given main group, nanomaterials may be sub-grouped by toxic potency, i.e. by similar NOAEC values. NOAEC values may also be used to derive OEL values (calculated, e.g., as suggested by Gordon et al. (2014)), such as DNELs or DMELs (for substances, such as fibers, that may be carcinogenic); *c.f.* 2.2 for further details on OELs for nanomaterials assigned to the 4 main groups of the DF4nanoGrouping.

3.6.5. Toxic potency in secondary organs

Whereas data from long-term studies are too sparse to provide evidence whether nanoparticles may accumulate to an extent high enough to cause adverse chronic systemic effects, to date, no convincing evidence for a relevant specific systemic toxicity of nano-sized GBPs could be identified (Gebel et al., 2014; Moreno-Horn and Gebel, 2014). Therefore, within the DF4nanoGrouping, toxic potency in secondary organs is considered to be of minor relevance for grouping. Notwithstanding, as discussed in further detail in Chapter 3.4 (Grouping of nanomaterials by biopersistence, uptake, and biodistribution), in principle, nanomaterial grouping should also address the nanomaterials' potential to cause chronic local or systemic effects.

3.6.6. Use of bulk material toxicity data for nanomaterial grouping

In grouping nanomaterials by their potential to cause apical toxic effects, it is further relevant to compare their hazard profiles to those of the corresponding bulk materials of identical chemical composition. Extensive toxicological data on a variety of bulk materials, by various routes of exposure, are available. Such comparative assessments allow applying nanomaterial grouping to determine whether, and if so, which hazard information on bulk materials provides important clues for nanomaterials of identical chemistry.

3.6.7. Grouping of nanomaterials by apical toxic effects – conclusion

Comparative investigations of broad spectra of nanomaterials *in vivo* in rat short-term inhalation studies allow grouping nanomaterials. Generally, grouping by apical toxic effects is the final step of the grouping concept. It may allow the selection of relevant further (*in vivo*) tests, but it may also link back to earlier steps of the grouping concept if effects were observed that had not been anticipated. Accordingly, *in vivo* testing is performed to confirm or disapprove grouping performed during earlier steps.

4. Conclusion and outlook

The *Decision-making framework for the grouping and testing of nanomaterials* (DF4nanoGrouping) presented by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) Task Force on Nanomaterials consists of three tiers to assign nanomaterials to four main groups, to perform sub-grouping within the main groups, and to identify and refine specific subsequent information needs for those nanomaterials for which the information needs could not be fulfilled by grouping and read-across.

Currently, intrinsic material properties may be used to define if a substance is in fact a nanomaterial, e.g. in accordance to the EU recommendation (EU Commission, 2011). Intrinsic material properties may also be relevant for grouping even though this is not yet possible for most nanomaterials. For many nanomaterials, it is not yet understood how intrinsic material properties relate to apical toxic effects, i.e. the (the degree and type of) interdependence of intrinsic material properties and bio-physical interactions leading to apical toxic effects. Therefore, while grouping by intrinsic material properties appears highly relevant, it is not yet useable. Hence, the DF4nanoGrouping follows a functionality-driven approach instead of being predominantly based upon intrinsic materials properties. Of note, the focus on intrinsic material properties such as composition, size, surface area or coatings to identify a nanomaterial and different nanoforms of a substance does not compromise a subsequent grouping of these nanoforms for the purpose of hazard and risk assessment by functionality-driven properties.

Within the DF4nanoGrouping, relevant and useful intrinsic material and function-related properties have been selected as 'key criteria' (while others were considered 'supplementary') and were sorted into three tiers by increasing complexity thereby enabling a step-by-step approach to apical toxic effects. By making use of the different perspectives of nanomaterials, i.e. intrinsic material and system-dependent properties, bio-physical interactions, biopersistence, uptake and biodistribution as well as *in vitro* cellular and, finally, apical toxic effects, the DF4nanoGrouping is a multiple perspective grouping concept. It makes use of grouping criteria that are as simple as possible and as complex as necessary to enable a relevant and justifiable grouping. Within the DF4nanoGrouping, pragmatic methods, many of which standardized, and specific benchmark materials, predominantly from the OECD sponsorship program, are suggested for each grouping criterion.

In practice, functionality-driven grouping is closely linked to (or even undistinguishable from) integrated approaches for the testing and assessment of nanomaterials, and both of these processes take into account the life cycle of a nanomaterial and its biological pathways (Fig. 1, Oomen et al., 2014a,b; Tollefsen et al., 2014). Similar to AOPs, biological pathways may encompass a multitude of interlinked steps that are not necessarily already fully understood for each and every type of nanomaterial. Nevertheless, application of the grouping concept (while potentially making use of all of the steps of the life cycle and biological pathway of a nanomaterial) does not require that all pieces of knowledge concerning the respective steps are already available. Based upon the available knowledge, grouping may begin at any step of the biological pathway of a nanomaterial, i.e. not only with intrinsic material properties, but also with any aspect closer to the apical toxic effect.

Thereby, the DF4nanoGrouping may be applied and further developed at the same time making use of further knowledge on the relationship between intrinsic and system-dependent properties as it becomes available. Specifically, future research should address the following knowledge gaps: *In vitro* models that allow the prediction of *in vivo* effects and toxic potency of nanomaterials, especially *in vitro* barrier models, remain to be developed. Likewise, promising approaches, such as the *in vitro* macrophage assay require standardization and validation. Regarding *in vivo* databases as a basis for nanomaterial grouping, there is a need for long-term exposure data.

Importantly, case studies covering a broad spectrum of different types of nanomaterials should be conducted to provide proof-of-evidence of the relevance of the DF4nanoGrouping and to further develop and refine it as necessary.

Based upon the outcome of such case studies, future research should aim at making available a decision tree und REACH guidance on how the DF4nanoGrouping may be integrated into the REACH registration process for substances that have to be registered in the nanoform. Thereby, very early on in the assessment process, supporting information is provided concerning the application of read-across and grouping, e.g. with the non-nanoform of the substance or with groups of nanomaterials (Patlewicz et al., 2013; Ball et al., 2014). Therefore, one option would be to

add the DF4nanoGrouping to Annex VI of the REACH regulation or to provide guidance on its use in a separate document.

The DF4nanoGrouping is a hazard and risk assessment tool that applies modern toxicology and contributes to the sustainable development of nanotechnological products. The grouping approach presented in the DF4nanoGrouping is effective, efficient, and safe and moves away from a more traditional check-box approach to regulatory toxicology by ensuring that no studies are performed that are not needed, that do not provide crucial data, and that therefore would lead to a waste of animals and resources.

Conflict of Interest

This manuscript was prepared by members of the ECTOC Task Force on nanomaterials. TP represents and JA, MH, MI, RK, DL, MM, KM, DW, KW, WW, RL are employees of companies producing and/or marketing nanomaterials. UGS was hired by ECETOC to support this Task Force. The authors alone are responsible for the content and writing of the paper.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

The authors thank the members of the ECETOC Scientific Committee for their feedback. Special thanks go to Dr. Alan Poole, Secretary General of ECETOC, and Ms. Christine Yannakas of ECETOC for their extensive administrative support. RL and MAI received funding from the EU FP7 project MARINA.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.yrtph.2015.03.007.

References

Note: All websites were accessed in January and February 2015.

Aalapati, S., Ganapathy, S., Manapuram, S., Anumolu, G., Prakya, B.M., 2014. Toxicity and bio-accumulation of inhaled cerium oxide nanoparticles in CD1 mice. Nanotoxicology 8, 786–798.

Aillon, K.L., Xie, Y., El-Gendy, N., Berkland, C.J., Forrest, M.L., 2009. Effects of nanomaterial physico-chemical properties on in vivo toxicity. Adv. Drug Deliv. Rev. 61, 457–466.

Alkilany, A.M., Murphy, C.J., 2010. Toxicity and cellular uptake of gold nanoparticles: what we have learned so far? J. Nanopart. Res. 12, 2313–2333.

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrrano, J.A., Tietge, J.E., Villeneuve, D.L., 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ. Toxicol. Chem. 29, 730–741.

Arts, J.H.E., Muijser, H., Duistermaat, E., Junker, K., Kuper, C.F., 2007. Repeated (5-day) inhalation toxicity of three types of synthetic amorphous silicas in Wistar rats: evaluation of their toxicity up to 3 months after exposure. Food Chem. Toxicol. 45, 1856–1867.

Arts, J.H.E., Hadi, M., Keene, A.M., Kreiling, R., Lyon, D., Maier, M., Michel, K., Petry, T., Sauer, U.G., Warheit, D., Wiench, K., Landsiedel, R., 2014. A critical appraisal of existing concepts for the grouping of nanomaterials. Regul. Toxicol. Pharmcol. 70, 492–506.

ASTM, undated. American Society for Testing and Materials Standard D3053 on Terminology Relating to Carbon Black. see: doi: 10.1520/D3053.

Baer, D.R., Gaspar, D.J., Nachimuthu, P., Techane, S.D., Castner, D.G., 2010. Application of surface chemistry analysis tools for characterization of nanoparticles. Anal. Bioanal. Chem. 396 (3), 963–1002.

Ball, N., Bartels, M., Budinsky, R., Klapacz, J., Hays, S., Kirman, C., Patlewicz, G., 2014. The challenge of using read-across within the EU REACH regulatory framework; how much uncertainty is too much? Dipropylene glycol methyl ether acetate, an exemplary case study. Regul. Toxicol. Pharmacol. 68 (2), 212–221.

BAuA, 2013. Bundesanstalt für Arbeitsschutz und Arbeitsmedizin. Bekanntmachung zu Gefahrstoffen. Hergestellte Nanomaterialien. – German Federal Institute for Occupational Safety and Health. Announcement regarding hazardous substances. Manufactured nanomaterials. BekGS 527. BMBl (Nr. 25). pp. 498–511. see: <http://www.baua.de/de/Themen-von-A-Z/Gefahrstoffe/TRGS/Bekanntmachung-527.html>.

BAuA, 2014a. Bundesanstalt für Arbeitsschutz und Arbeitsmedizin. Technische Regel für Gefahrstoffe 905. Verzeichnis krebserzeugender, erbgutverändernder oder fortpflanzungsgefährdender Stoffe. – German Federal Institute for Occupational Safety and Health. Technical rule regarding hazardous substances. Register of carcinogenic, mutagenic or toxic to reproduction substances. GMBl 2013, 510-522. see: <http://www.baua.de/de/Themen-von-A-Z/Gefahrstoffe/TRGS/TRGS-905.html>.

BAuA, 2014b. Bundesanstalt für Arbeitsschutz und Arbeitsmedizin. Begründung zum Allgemeinen Staubgrenzwert (2014/2001) in TRGS 900, Arbeitsplatzgrenzwerte, Nr. 12 GMBl 2014, 271, Bek. d. BMAS v. 14.2.2014. IIIb 3, 35125 – 5. In: Bekanntmachung von Technischen Regeln – Justification of the general dust limit in the technical rule on hazardous substances 900 (occupational exposure limits Nr. 12). see: <http://www.baua.de/cae/servlet/contentblob/664342/publicationFile/47939/900-allgemeiner- staubgrenzwert.pdf>.

Bello, D., Hsieh, S.-F., Schmidt, D., Rogers, E., 2009. Nanomaterials properties versus biological oxidative damage: implications for toxicity screening and exposure assessment. Nanotoxicology 3, 249–261.

Braakhuis, H.M., Park, M.V., Gosens, I., De Jong, W.H., Cassee, F.R., 2014. Physicochemical characteristics of nanomaterials that affect pulmonary inflammation. Part. Fibre Toxicol. 11 (1), 18.

Bräu, M., Ma-Hock, L., Hesse, C., Nicoleau, L., Strauss, V., Treumann, S., Wiench, K., Landsiedel, R., Wohlleben, W., 2012. Nanostructured calcium silicate hydrate seeds accelerate concrete hardening: a combined assessment of benefits and risks. Arch. Toxicol. 86, 1077–1087.

Brown, D.M., Wilson, M.R., MacNee, W., Stone, V., Donaldson, K., 2001. Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines. Toxicol. Appl. Pharmacol. 175, 191–199.

Brunner, T.J., Wick, P., Manser, P., Spohn, P., Grass, R.N., Limbach, L.K., Bruinink, A., Stark, W.J., 2006. In vitro cytotoxicity of oxide nanoparticles: comparison to asbestos, silica, and the effect of particle solubility. Environ. Sci. Technol. 40 (14), 4374–4381.

BSI, 2007. British Standards Institute. Nanotechnologies – Part 2: Guide to safe handling and disposal of manufactured nanomaterials. PD 6699-2:2007. ISBN 978 0 580 60832 2.

Buesen, R., Landsiedel, R., Sauer, U.G., Wohlleben, W., Strauss, V., Groeters, S., Kamp, H.G., van Ravenzwaay, B., 2014. Effects of SiO_2, ZrO_2, and $BaSO_4$ nanomaterials with or without surface functionalization upon 28-day oral exposure to rats. Arch. Toxicol. 88, 1881–1906.

Burello, E., Worth, A.P., 2011. A theoretical framework for predicting the oxidative stress potential of oxide nanoparticles. Nanotoxicology 5 (2), 228–235.

Chen, R., Zhang, Y., Darabi Sahneh, F., Scoglio, C.M., Wohlleben, W., Haase, A., Monteiro-Riviere, N.A., Riviere, J.E., 2014. Nanoparticle surface characterization and clustering through concentration-dependent surface adsorption modeling. ACS Nano 8 (9), 9446–9456.

Cho, W.S., Kang, B.C., Lee, J.K., Jeong, J., Che, J.H., Seok, S.H., 2013. Comparative absorption, distribution, and excretion of titanium dioxide and zinc oxide nanoparticles after repeated oral administration. Part. Fibre Toxicol. 10, 9.

Choi, H.S., Ipe, B.I., Misra, P., Lee, J.H., Bawendi, M.G., Frangioni, J.V., 2009. Tissue- and organ-selective biodistribution of NIR fluorescent quantum dots. Nano Lett. 9 (6), 2354–2359.

Choi, H.S., Ashitate, Y., Lee, J.H., Kim, S.H., Matsui, A., Insin, N., Bawendi, M.G., Semmler-Behnke, M., Frangioni, J.V., Tsuda, A., 2010. Rapid translocation of nanoparticles from the lung airspaces to the body. Nat. Biotechnol. 28 (12), 1300–1303.

Chusuei, C.C., Wu, C.H., Mallavarapu, S., Hou, F.Y., Hsu, C.M., Winiarz, J.G., Aronstam, R.S., Huang, Y.W., 2013. Cytotoxicity in the age of nano: the role of fourth period transition metal oxide nanoparticle physicochemical properties. Chem. Biol. Interact. 206 (2), 319–326.

Cohen, J.M., Teeguarden, J.G., Demokritou, P., 2014. An integrated approach for the in vitro dosimetry of engineered nanomaterials. Part. Fibre Toxicol. 11, 20.

Colognato, R., Park, M.V.D.Z., Wick, P., De Jong, W.H., 2012. Interactions with the human body. In: Fadeel, B., Pietroiusti, A., Shvedova, A.A. (Eds.), Adverse Effects of Engineered Nanomaterials: Exposure, Toxicology and Impact on Human Health. Elsevier, Amsterdam.

De Jong, W.H., Hagens, W.I., Krystek, P., Burger, M.C., Sips, A.J.A.M., Geertsma, R.E., 2008. Particle size dependent organ distribution of gold nanoparticles after intravenous administration. Biomaterials 29, 1912–1919.

DeLoid, G., Cohen, J.M., Darrah, T., Derk, R., Rojanasakul, L., Pyrgiotakis, G., Wohlleben, W., Demokritou, P., 2014. Estimating the effective density of engineered nanomaterials for in vitro dosimetry. Nat. Commun. 5, 3514.

Delorme, M.P., Muro, Y., Arai, T., Banas, D.A., Frame, S.R., Reed, K.L., Warheit, D.B., 2012. Ninety-day inhalation toxicity study with a vapor grown carbon nanofiber in rats. Toxicol. Sci. 128 (2), 449–460.

Docter, D., Bantz, C., Westmeier, D., Galla, H.J., Wang, Q., Kirkpatrick, J.C., Nielsen, P., Maskos, M., Stauber, R.H., 2014. The protein corona protects against size- and dose-dependent toxicity of amorphous silica nanoparticles. Beilstein J. Nanotechnol. 5, 1380–1392.

Donaldson, K., Aitken, R., Tran, L., Stone, V., Duffin, R., Forrest, G., Alexander, A., 2006. Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety. Toxicol. Sci. 92, 5–22.

Donaldson, K., Murphy, F.A., Duffin, R., Poland, C.A., 2010. Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. Part. Fibre Toxicol. 7, 5.

Donaldson, K., Murphy, F., Schinwald, A., Duffin, R., Poland, C.A., 2011. Identifying the pulmonary hazard of high aspect ratio nanoparticles to enable their safety-by-design. Nanomedicine 6, 143–156.

Donaldson, K., Poland, C.A., 2013. Nanotoxicity: challenging the myth of nano-specific toxicity. Curr. Opin. Biotechnol. 24, 724–734.

Dowding, J.M., Das, S., Kumar, A., Dosani, T., McCormack, R., Gupta, A., Sayle, T.X.T., Sayle, D.C., von Kalm, L., Seal, S., Self, W.T., 2013. Cellular interaction and toxicity depend on physico-chemical properties and surface modification of redox-active nanomaterials. ACS Nano 7 (6), 4855–4868.

Driscoll, K.E., Costa, D.L., Hatch, G., Henderson, R., Oberdörster, G., Salem, H., Schlesinger, R.B., 2000. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. Toxicol. Sci. 55 (1), 24–35.

Duffin, R., Tran, L., Brown, D., Stone, V., Donaldson, K., 2007. Proinflammogenic effects of low-toxicity and metal nanoparticles *in vivo* and *in vitro*: Highlighting the role of particle surface area and surface reactivity. Inhal. Toxicol. 19 (10), 849–856.

Duncan, T.V., Pillai, K., 2015. Release of engineered nanomaterials from polymer nanocomposites: diffusion, dissolution and desorption. ACS Appl. Mater. Interfaces 7 (1), 2–19.

ECETOC. 2013. TR 122. Poorly soluble particles. Lung overload. December 2013.

ECHA, 2008. Guidance on information requirements and chemical safety assessment. Chapter R.6. QSARs and grouping of chemicals. European Chemicals Agency. p. 134. see: <http://echa.europa.eu/documents/10162/13632/information_requirements_r6_en.pdf>.

ECHA, 2012a. Assessment of read-across in REACH. European Chemicals Agency, p. 58.

ECHA, 2012b. Background Paper. An introduction to the assessment of read-across. Experts workshop on read-across assessment with the active support of Cefic LRI. 2-3 Oct 2012. European Chemicals Agency. p. 8. see: <http://echa.europa.eu/documents/10162/5649897/ws_raa_20121003_background_paper_an_introduction_to_the_assessment_of_read-across_in_echa_en.pdf>.

ECHA, 2012c. Guidance on information requirements and chemical safety assessment. Appendix R7-1 Recommendations for nanomaterials applicable to Chapter R7a Endpoint specific guidance. European Chemicals Agency ECHA-12-G-03-EN, Apr 2012. p. 60. see: <http://echa.europa.eu/documents/10162/13632/appendix_r7a_nanomaterials_en.pdf>.

ECHA, 2013. Grouping of substances and read-across approach. Part I. Introductory note. European Chemicals Agency ECHA-13-R-02-EN, Apr 2013. p. 11.

ECHA, 2014. Human health and environmental exposure assessment and risk characterization of nanomaterials. Best practice for REACH registrants. Third GAARN meeting – Helsinki, 30 Sep 2013. ECHA-14-R-10-EN. p. 12.

EFSA, 2011. European Food Safety Authority. EFSA guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. EFSA J. 9 (5), 2140.

EP and Council of the EU, 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. OJ L 396/1, 30 Dec 2006.

EP and Council of the EU 2008. Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. OJ L 353/1, 31 Dec 2008.

EP and Council of the EU, 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. OJ L 276/33, 20 Oct 2010.

EU Commission, 2011. Commission recommendation on the definition of nanomaterial. OJ L 275/38, 18 October 2011.

Evans, D.F., Wennerström, H., 1999. The Colloidal Domain: Where Physics, Chemistry, Biology, and Technology Meet, 2nd ed. John Wiley and Sons Inc., Hoboken NJ, USA, p. 672 (ISBN: 978-0-471-24247-5).

Fabian, E., Landsiedel, R., Ma-Hock, L., Wiench, K., Wohlleben, W., van Ravenzwaay, B., 2008. Tissue distribution and toxicity of intravenously administered titanium dioxide nanoparticles in rats. Arch. Toxicol. 82 (3), 151–157.

Fairhurst, D., Prescott, S., 2011. The use of nuclear magnetic resonance as an analytical tool in the characterization of dispersion behavior. Spectrosc. Eur. 23 (4), 13–16.

Fruijtier-Polloth, C., 2012. The toxicological mode of action and the safety of synthetic amorphous silica—a nanostructured material. Toxicology 294, 61–79.

Gamble, J.F., 2011. Crystalline silica and Lung cancer: a critical review of the occupational epidemiology literature of exposure-response studies testing this hypothesis. Crit. Rev. Toxicol. 41, 404–465.

Gamer, A.O., Leibold, E., van Ravenzwaay, B., 2006. The *in vitro* absorption of microfine zinc oxide and titanium dioxide through porcine skin. Toxicol. In Vitro 20, 301–307.

Gantzsch, S.P., Kann, B., Ofer-Glaessgen, M., Loos, P., Berchtold, H., Balbach, S., Eichinger, T., Lehr, C.-M., Schaefer, U.F., Windbergs, M., 2014. Characterization and evaluation of a modified PVPA barrier in comparison to Caco-2 cell monolayers for combined dissolution and permeation testing. J. Control. Release 175, 79–86.

Gebel, T., 2012. Small difference in carcinogenic potency between GBP nanomaterials and GBP micromaterials. Arch. Toxicol. 86 (7), 995–1007.

Gebel, T., Foth, H., Damm, G., Freyberger, A., Kramer, P.-J., Lilienblum, W., Röhl, C., Schupp, T., Weiss, C., Wollin, K.-M., Hengstler, J.G., 2014. Manufactured nanomaterials: categorization and approaches to hazard assessment. Arch. Toxicol. 88, 2191–2211.

Geraets, L., Oomen, A.G., Schroeter, J.D., Coleman, V.A., Cassee, F.R., 2012. Tissue distribution of inhaled micro- and nano-sized cerium oxide particles in rats: results from a 28-day exposure study. Toxicol. Sci. 127, 463–473.

Ging, J., Tejerina-Anton, R., Ramakrishnan, G., Nielsen, M., Murphy, K., Gorham, J.M., Nguyen, T., Orlov, A., 2014. Development of a conceptual framework for evaluation of nanomaterials release from nanocomposites: environmental and toxicological implications. Sci. Total Environ. 473–474, 9–19.

Golbamaki, N., Rasulev, B., Cassano, A., Robinson, R.L.M., Benfenati, E., Leszczynski, J., Cronin, M.T., 2015. Genotoxicity of metal oxide nanomaterials: review of recent data and discussion of possible mechanisms. Nanoscale 7 (6), 2154–2198.

Gordon, S.C., Butala, J.H., Carter, J.M., Elder, A., Gordon, T., Gray, G., Sayre, P.G., Schulte, P.A., Tsai, C.S., West, J., 2014. Workshop report: strategies for setting occupational exposure limits for engineered nanomaterials. Regul. Toxicol. Pharmacol. 68 (3), 305–311.

Grieger, K.D., Redmon, J.H., Money, E.S., Widder, M.W., van der Schalie, W.H., Beaulieu, S.M., Womack, D., 2014. A relative ranking approach for nano-enabled applications to improve risk-based decision making: a case study of Army materiel. Environ. Syst. Decis. (epub ahead of print 24 Dec 2014, doi: 101007/s10669-014-9531-4).

Grosse, Y., Loomis, D., Guyton, K.Z., Lauby-Secretan, B., El Ghissassi, F., Bouvard, V., Benbrahim-Tallaa, L., Guha, N., Scoccianti, C., Mattock, H., Straif, K., 2014. International agency for research on cancer monograph working group. Carcinogenicity of fluoro-edenite, silicon carbide fibres and whiskers, and carbon nanotubes. Lancet Oncol. 15, 1427–1428.

Guadagnini, R., Halamoda Kenzaoui, B., Cartwright, L., Pojana, G., Magdolenova, Z., Bilanicova, D., Saunders, M., Juillerat, L., Marcomini, A., Huk, A., Dusinska, M., Fjellsbø, L.M., Marano, F., Boland, S., 2013. Toxicity screenings of nanomaterials: challenges due to interference with assay processes and components of classic *in vitro* tests. Nanotoxicology (epub ahead of print 27Jul 2013, doi:10.3109/17435390.2013.829590. (09 Mar 2015: still only online)).

Haase, A., Dommershausen, N., Schulz, M., Landsiedel, R., Luch, A., 2015. Genotoxicity testing of different surface-functionalized SiO_2, ZrO_2 and silver nanomaterials. Mutat. Res. (submitted for publication)

Hellack, B., Hulser, T., Izak, E., Kuhlbusch, T., Meyer, F., Spree, M., Voetz, M., Wiggers, H., Wohlleben, W., 2013. Characterization of all nanoGEM materials. see: www.nanogem.de/cms/nanogem/upload/Veroeffentlichungen/nano-GEM_Del1.3.1_Characterization_Materials_2013_04_24.pdf.

Hesterberg, T.W., Miller, W.C., Musselman, R.P., Kamstrup, O., Hamilton, R.D., Thevenaz, P., 1996. Biopersistence of man-made vitreous fibers and crocidolite asbestos in the rat lung following inhalation. Fundam. Appl. Toxicol. 29, 267–279.

Hittinger, M., Juntke, J., Kletting, S., Schneider-Daum, N., de Souza Carvalho, C., Lehr, C.-M., 2014. Preclinical safety and efficacy models for pulmonary drug delivery of antimicrobials with focus on in vitro models. Adv. Drug Deliv. Rev. http://dx.doi.org/10.1016/j.addr.2014.10.011.

Hristozov, D.R., Gottardo, S., Critto, A., Marcomini, A., 2012. Risk assessment of engineered nanomaterials: a review of available data and approaches from a regulatory perspective. Nanotoxicology 6, 880–898.

Hsieh, S.F., Bello, D., Schmidt, D.F., Pal, A.K., Stella, A., Isaacs, J.A., Rogers, E.J., 2013. Mapping the biological oxidative damage of engineered nanomaterials. Small 9 (9–10), 1853–1865.

Hühn, D., Kantner, K., Geidel, C., Brandholt, S., De Cock, I., Soenen, S.J., Rivera Gil, P., Montenegro, J.M., Braeckmans, K., Müllen, K., Nienhaus, G.U., Klapper, M., Parak, W.J., 2013. Polymer-coated nanoparticles interacting with proteins and cells: focusing on the sign of the net charge. ACS Nano 7 (4), 3253–3263.

IARC, 2010. International Agency for Research on Cancer Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 93. IARC, Lyon, France, pp. 166–171.

ICRP (International Commission on Radiological Protection), 1994. ICRP Publication No. 66: Human respiratory tract model for radiological protection. Ann. ICRP 24, 1–3.

Izak-Nau, E., Voetz, M., 2014. As-produced: intrinsic physico-chemical properties and appropriate characterization tools. In: Wohlleben, W., Kuhlbusch, T.A.J., Lehr, C.-M., Schnekenburger, J. (Eds.), Safety of Nanomaterials Along their Lifecycle: Release, Exposure, and Human Hazards. CRC Press, London, UK, pp. 3–23, ISBN 978-1-46-656786-3.

Johnston, H.J., Pojana, G., Zuin, S., Jacobsen, N.R., Møller, P., Loft, S., Semmler-Behnke, M., McGuiness, C., Balharry, D., Marcomini, A., Wallin, H., Kreyling, W.,

Donaldson, Tran, L., Stone, V., 2013. Engineered nanomaterial risk. Lessons learnt from completed nanotoxicology studies: potential solutions to current and future challenges. Crit. Rev. Toxicol. 43, 1–20.
Kaiser, J.P., Roesslein, M., Diener, L., Wick, P., 2013. Human health risk of ingested nanoparticles that are added as multifunctional agents to paints: an in vitro study. PLoS One 8 (12), e83215.
Keene, A.M., Tyner, K.M., 2011. Analytical characterization of gold nanoparticle primary particles, aggregates, agglomerates, and agglomerated aggregates. J. Nanopart. Res. 13, 3465–3481.
Keene, A.M., Bancos, S., Tyner, K.M., 2014. Considerations for In Vitro Nanotoxicity Testing. Handbook of Nanotoxicology, Nanomedicine and Stem Cell Use in Toxicology. John Wiley & Sons Ltd, Chichester, UK, ISBN 978 1 118 43926 5.
Keller, J., Wohlleben, W., Ma-Hock, L., Groeters, S., Küttler, K., Strauss, V., Wiench, K.E., Herden, C., Oberdörster, G., van Ravenzwaay, B., Landsiedel, R., 2014. Time-course of lung retention and toxicity of inhaled particles: short-term exposure to nano-Ceria. Arch. Toxicol. 88, 2033–2059.
Kettler, K., Veltman, K., van de Meent, D., van Wezel, A., Hendriks, A.J., 2014. Cellular uptake of nanoparticles as determined by particle properties, experimental conditions, and cell type. Environ. Toxicol. Chem. 33 (3), 481–492.
Kim, Y., Klutz, A.M., Jacobson, K.A., 2008. Systematic investigation of polyamidoamine dendrimers surface-modified with poly(ethylene glycol) for drug delivery applications: synthesis, characterization, and evaluation of cytotoxicity. Bioconjug. Chem. 19 (8), 1660–1672.
Kim, S.T., Saha, K., Kim, C., Rotello, V.M., 2013. The role of surface functionality in determining nanoparticle cytotoxicity. Acc. Chem. Res. 46 (3), 681–691.
Kingston, C., Zepp, R., Andrady, A., Boverhof, D., Fehir, R., Hawkins, D., Wohlleben, W., 2014. Release characteristics of selected carbon nanotube polymer composites. Carbon 68, 33–57.
Klein, C.L., Wiench, K., Wiemann, M., Ma-Hock, L., van Ravenzwaay, B., Landsiedel, R., 2012. Hazard identification of inhaled nanomaterials: making use of short-term inhalation studies. Arch. Toxicol. 86 (7), 1137–1151.
Konduru, N., Keller, J., Ma-Hock, L., Gröters, S., Landsiedel, R., Donaghey, T.C., Brain, J.D., Wohlleben, W., Molina, R.M., 2014. Biokinetics and effects of barium sulfate nanoparticles. Part. Fibre Toxicol. 11, 55.
Kong, B., Seog, J.H., Graham, L.M., Lee, S.B., 2011. Experimental considerations on the cytotoxicity of nanoparticles. Nanomedicine 6 (5), 929–941.
Kreyling, W.G., Semmler-Behnke, M., Takenaka, S., Möller, W., 2013. Differences in the biokinetics of inhaled nano- versus micrometer-sized particles. Acc. Chem. Res. 46 (3), 714–722.
Kreyling, W.G., Hirn, S., Möller, W., Schleh, C., Wenk, A., Celik, G., Lipka, J., Schäffler, M., Haberl, N., Johnston, B.D., Sperling, R., Schmid, G., Simon, U., Parak, W.J., Semmler-Behnke, M., 2014. Air-blood barrier translocation of tracheally instilled gold nanoparticles inversely depends on particle size. ACS Nano 8 (1), 222–233.
Kroll, A., Dierker, C., Rommel, C., Hahn, D., Wohlleben, W., Schulze-Isfort, C., Göbbert, C., Voetz, M., Hardinghaus, F., Schnekenburger, J., 2011. Cytotoxicity screening of 23 engineered nanomaterials using a test matrix of ten cell lines and three different assays. Part. Fibre Toxicol. 8 (1), 1–19.
Krug, H.F., 2014. Nanosafety research – are we on the right track? Angew. Chem. Int. Ed. 53 (46), 12304–12319.
Kuempel, E.D., Castranova, V., Geraci, C.L., Schulte, P.A., 2012. Development of risk-based nanomaterial groups for occupational exposure control. J. Nanopart. Res. 14, 1029.
Kuempel, E.D., Attfield, M.D., Stayner, L.T., Castranova, V., 2014. Human and animal evidence supports lower occupational exposure limits for poorly-soluble respirable particles. Letter to the editor in respect to: low-toxicity dusts: current exposure guidelines are not sufficiently protective, by Cherrie, Brosseau, Hay and Donaldson. Ann. Occup. Hyg. 58 (9), 1205–1208.
Kuhlbusch, T.A.J., Canady, R., 2014. Case study: the life cycle of conductive plastics based on MWCNTs. In: Wohlleben, W., Kuhlbusch, T.A.J., Lehr, C.-M., Schnekenburger, J. (Eds.), Safety of Nanomaterials along their Lifecycle: Release, Exposure, and Human Hazards. CRC Press, London, UK, ISBN 978-1-46-656786-3, Ch. 18.
Landsiedel, R., Kapp, M.D., Schulz, M., Wiench, K., Oesch, F., 2009. Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations – many questions, some answers. Mutat. Res. 681 (2–3), 241–258.
Landsiedel, R., Ma-Hock, L., Kroll, A., Hahn, D., Schnekenburger, J., Wiench, K., Wohlleben, W., 2010. Testing metal-oxide nanomaterials for human safety. Adv. Mater. 22, 2601–2627.
Landsiedel, R., Fabian, E., Ma-Hock, L., Wohlleben, W., Wiench, K., Oesch, F., van Ravenzwaay, B., 2012. Toxico-/biokinetics of nanomaterials. Arch. Toxicol. 86, 1021–1060.
Landsiedel, R., Sauer, U.G., Ma-Hock, L., Schnekenburger, J., Wiemann, M., 2014a. Pulmonary toxicity of nanomaterials: a critical comparison of published *in vitro* assays and *in vivo* inhalation or instillation studies. Nanomedicine 9 (16), 2557–2585.
Landsiedel, R., Ma-Hock, L., Hofmann, T., Wiemann, M., Strauss, V., Treumann, S., Wohlleben, W., Gröters, S., Wiench, K., van Ravenzwaay, B., 2014b. Application of short-term inhalation studies to assess the inhalation toxicity of nanomaterials. Part. Fibre Toxicol. 11, 16.
Lankveld, D.P.K., Oomen, A., Krystek, P., Troost-De Jong, A., Noorlander, C.W., Van Eijkeren, J.C.H., Geertsma, R.E., De Jong, W.H., 2010. The kinetics of the tissue distribution of silver nanoparticles of differents sizes. Biomaterials 31, 8350–8361.
Li, R., Ji, Z., Chang, C.H., Dunphy, D.R., Cai, X., Meng, H., Zhang, H., Sun, B., Wang, X., Dong, J., Lin, S., Wang, M., Liao, Y.P., Brinker, C.J., Nel, A., Xia, T., 2014. Surface interactions with compartmentalized cellular phosphates explain rare earth oxide nanoparticle hazard and provide opportunities for safer design. ACS Nano 8, 1771–1783.
Ma-Hock, L., Burkhardt, S., Strauss, V., Gamer, A.O., Wiench, K., van Ravenzwaay, B., Landsiedel, R., 2009a. Development of a short-term inhalation test in the rat using nano-titanium dioxide as a model substance. Inhal. Toxicol. 21, 102–118.
Ma-Hock, L., Treumann, S., Strauss, V., Brill, S., Luizi, F., Mertler, M., Wiench, K., Gamer, A.O., van Ravenzwaay, B., Landsiedel, R., 2009b. Inhalation toxicity of multiwall carbon nanotubes in rats exposed for 3 months. Toxicol. Sci. 112, 468–481.
Ma-Hock, L., Strauss, V., Treumann, S., Küttler, K., Wohlleben, W., Hofmann, T., Gröters, S., Wiench, K., van Ravenzwaay, B., Landsiedel, R., 2013. Comparative inhalation toxicity of multi-wall carbon nanotubes, graphene, graphite nanoplatelets and low surface carbon black. Part. Fibre Toxicol. 10, 23.
Ma-Hock, L., Hofmann, T., Landsiedel, R., van Ravenzwaay, B., 2014. A short-term inhalation study protocol: designed for testing of toxicity and fate of nanomaterials. Methods Mol. Biol. 1199, 207–212.
Mattix, B., Moore, T., Uvarov, O., Pollard, S., O'Donnell, L., Park, K., Horne, D., Dhulekar, J., Reese, L., Nguyen, D., Kraveka, J., Burg, K., Alexis, F., 2013. Effects of polymeric nanoparticle surface properties on interaction with brain tumor environment. Nano Life 3 (4), 1343003.
Meng, H., Xia, T., George, S., Nel, A.E., 2009. A predictive toxicological paradigm for the safety assessment of nanomaterials. ACS Nano 3, 1620–1627.
Misra, S.K., Dybowska, A., Berhanu, D., Luoma, S.N., Valsami-Jones, E., 2012. The complexity of nanoparticle dissolution and its importance in nanotoxicological studies. Sci. Total Environ. 438, 225–232.
Moghadam, B.Y., Hou, W.C., Corredor, C., Westerhoff, P., Posner, J.D., 2012. The role of nanoparticle surface functionality in the disruption of model cell membranes. Langmuir 28 (47), 16318–16326.
Molina, R.M., Konduru, N.V., Jimenez, R.J., Wohlleben, W., Brain, J.D., 2014. Bioavailability, distribution and clearance of tracheally instilled, gavaged or injected cerium dioxide nanoparticles and ionic cerium. Environ. Sci. Nano 1, 561.
Monopoli, M.P., Walczyk, D., Campbell, A., Elia, G., Lynch, I., Bombelli, F.B., Dawson, K.A., 2011. Physical-chemical aspects of protein corona: relevance to *in vitro* and *in vivo* biological impacts of nanoparticles. J. Am. Chem. Soc. 133 (8), 2525–2534.
Monopoli, M.P., Åberg, C., Salvati, A., Dawson, K.A., 2012. Biomolecular coronas provide the biological identity of nanosized materials. Nat. Nanotechnol. 7, 779–786.
Monteiro-Riviere, N.A., Wiench, K., Landsiedel, R., Schulte, S., Inman, A.O., Riviere, J.E., 2011. Safety evaluation of sunscreen formulations containing titanium dioxide and zinc oxide nanoparticles in UVB sunburned skin: an *in vitro* and *in vivo* study. Toxicol. Sci. 123 (1), 264–280.
Moreno-Horn, M., Gebel, T., 2014. Granular biodurable nanomaterials: no convincing evidence for systemic toxicity. Crit. Rev. Toxicol. 44 (10), 849–875.
Morfeld, P., Treumann, S., Ma-Hock, L., Bruch, J., Landsiedel, R., 2012. Deposition behavior of inhaled nanostructured TiO2 in rats: fractions of particle diameter below 100 nm (nanoscale) and the slicing bias of transmission electron microscopy. Inhal. Toxicol. 24 (14), 939–951 (Erratum in: Inhal. Toxicol. 25(5), 307 (2013)).
Morrow, P.E., 1988. Possible mechanisms to explain dust overloading of the lungs. Fundam. Appl. Toxicol. 10 (3), 369–384.
Morrow, P.E., 1994. Mechanisms and significance of "particle overload". In: Toxic and Carcinogenic Effects of Solid Particles in the Respiratory Tract: Proceedings of the 4th International Inhalation Symposium, March 1993 in Hannover, Germany. International Life Sciences Institute Press, Washington DC, USA, pp. 17–25.
Morrow, P.E., Haseman, J.K., Hobbs, C.H., Driscoll, K.E., Vera, V., Oberdörster, G., 1996. The maximum tolerated dose for inhalation bioassays: toxicity vs. overload. Fundam. Appl. Toxicol. 29 (2), 155–167.
Murgia, X., de Souza Carvalho, C., Lehr, C.-M., 2014. Overcoming the pulmonary barrier: new insights to improve the efficiency of inhaled therapeutics. Eur. J. Nanomed. 6 (3), 157–169.
NANO SUPPORT Project, 2012. Scientific technical support on assessment of nanomaterials in REACH registration dossiers and adequacy of available information. AA No. 07.0307/2010/581080/AA/D3 between DG Environment (DG ENV) and the Joint Research Centre (JRC). Final Report on analysis and assessment (Task I, step 3&4&5) and options for adapting REACH (Task II, step 1), Frans M. Christensen (JRC Technical Responsible) Nanosafety & Regulatory Methods Competence Group I.04 Nanobiosciences Unit JRC-IHCP, addressed to DG ENV D.3 Attn.: Mr. H. Laursen (ENV D.3 Technical Responsible), Ref. Ares(2012)314828, 16 Mar 2012, see: <http://ec.europa.eu/environment/chemicals/nanotech/pdf/jrc_report.pdf>.
National Research Council, 2013. Research Progress on Environmental, Health, and Safety Aspects of Engineered Nanomaterials. National Academies Press, Washington, D.C..
Nel, A.E., Xia, T., Madler, L., Li, N., 2006. Toxic potential of materials at the nanolevel. Science 311 (5761), 622–627.
Nel, A.E., Xia, T., Meng, H., Wang, X., Lin, S., Ji, Z., Zhang, H., 2013. Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. Acc. Chem. Res. 46 (3), 607–621.

Nikula, K.J., Avila, K.J., Griffith, W.C., Mauderly, J.L., 1997. Lung tissue responses and sites of particle retention differ between rats and cynomolgus monkeys exposed chronically to diesel exhaust and coal dust. Fundam. Appl. Toxicol. 37, 37–53.

Nikula, K.J., Vallyathan, V., Green, F.H., Hahn, F.F., 2001. Influence of exposure concentrations or dose on the distribution of particulate material in rat and human lungs. Environ. Health Perspect. 109, 311–318.

Nowack, B., David, R.M., Fissan, H., Morris, H., Shatkin, J.A., Stintz, M., Zepp, R., Brouwer, D., 2013. Potential release scenarios for carbon nanotubes used in composites. Environ. Int. 59, 1–11.

Oberdörster, G., Maynard, A., Donaldson, K., Castranova, V., Fitzpatrick, J., Ausman, K., Carter, J., Karn, B., Kreyling, W., Lai, D., Olin, S., Monteiro-Riviere, N., Warheit, D., Yang, H.ILSI Research Foundation, 2005. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part. Fibre Toxicol. 2, 8.

Oberdörster, G., 2009. Safety assessment for nanotechnology and nanomedicine: concepts of nanotoxicology. J. Int. Med. 267 (1), 89–105.

OECD, 2012. Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials. Series on the safety of manufactured nanomaterials. No. 36. ENV/JM/MONO(2012) 40, OECD, Paris, France, 18 Dec 2012.

OECD, 2014a. Guidance on grouping of chemicals, second edition. Series on testing and assessment No. 194. ENV/JM/MONO(2014) 4, OECD, Paris, France, 14 Apr 2014.

OECD, 2014b. Report of the OECD expert meeting on the physical chemical properties of manufactured nanomaterials and test guidelines. OECD Environment, Health, and Safety Publications. Series on the safety of manufactured nanomaterials. No. 41. ENV/JM/MONO(2014) 15, OECD, Paris, France, 4 Jul 2014.

Oomen, A.G., Bos, P.M.J., Fernandes, T.F., Hund-Rinke, K., Boraschi, D., Byrne, H.J., Aschberger, K., Gottardo, S., van der Kammer, F., Kühnel, D., Hristozov, D., Marcomini, A., Migliore, L., Scott-Fordsmand, J., Wick, P., Landsiedel, R., 2014a. Concern-driven integrated approaches to nanomaterial testing and assessment –Report of the NanoSafety Cluster Working Group 10. Nanotoxicology 8, 334–348.

Oomen, A., Bos, P.M.J., Landsiedel, R., 2014b. Concern-driven safety assessment of nanomaterials: an integrated approach using material properties, hazard, biokinetic and exposure data and considerations on grouping and read-across. In: Wohlleben, W., Kuhlbusch, T.A.J., Lehr, C.-M., Schnekenburger, J. (Eds.), Safety of Nanomaterials along their Lifecycle: Release, Exposure, and Human Hazards. CRC Press, London, UK, ISBN 978-1-46-656786-3 (Ch. 16).

Pastoor, T.P., Bachman, A.N., Bell, D.R., Cohen, S.M., Dellarco, M., Dewhurst, I.C., Doe, J.E., Doerrer, N.G., Embry, M.R., Hines, R.N., Moretto, A., Phillips, R.D., Rowlands, J.C., Tanir, J.Y., Wolf, D.C., Boobis, A.R., 2014. A 21st century roadmap for human health risk assessment. Crit. Rev. Toxicol. 44 (Suppl. 3), 1–5.

Patil, S., Sandberg, A., Heckert, E., Self, W., Seal, S., 2007. Protein absorption and cellular uptake of cerium oxide nanoparticles as a function of zeta potential. Biomaterials 28 (31), 4600–4607.

Patlewicz, G., Ball, N., Booth, E.D., Hulzebos, E., Zvinavashe, E., Hennes, C., 2013. Use of category approaches, read-across and (Q)SAR: general considerations. Regul. Toxicol. Pharmacol. 67, 1–12.

Pauluhn, J., 2014. Derivation of occupational exposure levels (OELs) of low-toxicity isometric biopersistent particles: how can the kinetic lung overload paradigm be used for improved inhalation toxicity study design and OEL-derivation? Part. Fibre Toxicol. 11 (1), 72.

Pavan, C., Rabolli, V., Tomatis, M., Fubini, B., Lison, D., 2014. Why does the hemolytic activity of silica predict its pro-inflammatory activity? Part. Fibre Toxicol. 11 (1), 76.

Pietruska, J.R., Liu, X., Smith, A., McNeil, K., Weston, P., Zhitkovich, A., Hurt, R., Kane, A.B., 2011. Bioavailability, intracellular mobilization of nickel, and HIF-1a activation in human lung epithelial cells exposed to metallic nickel and nickel oxide nanoparticles. Toxicol. Sci. 124 (1), 138–148.

Poland, C.A., Duffin, R., Donaldson, K., 2009. High aspect nanoparticles and the fibre pathogenicity paradigm. In: Sahu, S.C., Casciano, D.A. (Eds.), Nanotoxcity: From In Vivo and In Vitro Models to Health Risks. Wiley-VCH Verlag GmbH, Weinheim, Germany, ISBN 978-0-470-74137-5, Chapter 4.

Powers, K.W., Brown, S.C., Krishna, V.B., Wasdo, S.C., Moudgil, B.M., Roberts, S.M., 2006. Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. Toxicol. Sci. 90 (2), 296–303.

Pujalté, I., Passagne, I., Dalculsi, R., de Portal, C., Ohayon-Courtès, C., L'Azou, B., 2014. Cytotoxic effectsand cellular oxidative mechanisms of metallic nanoparticles on renal tubular cells: impact of particle solubility. Toxicol. Res. (epub ahead of print 23 Dec 2014, doi: 101039/C4TX00184B).

RPA, IVAM, 2014. RPA – Risk and Policy Analysts Ltd., UK; IVAM - Research and Consultancy on Sustainability, University of Amsterdam, NL. Guidance on the protection of the health and safety of workers from the potential risks related to nanomaterials at work. COM 2.4, June 2014. Publication commissioned by the European Union Programme for Employment and Social Solidarity – PROGRESS (2007–2013).

Rushton, E.K., Jiang, J., Leonard, S.S., Eberly, S., Castranova, V., Biswas, P., Elder, A., Han, X., Gelein, R., Finkelstein, J., Oberdörster, G., 2010. Concept of assessing nanoparticle hazards considering nanoparticle dosemetric and chemical/biological response metrics. J. Toxicol. Environ. Health A 73 (5), 445–461.

Saber, A.T., Jacobsen, N.R., Mortensen, A., Szarek, J., Jackson, P., Madsen, A.M., Jensen, K.A., Koponen, I.K., Brunborg, G., Gützkow, K.B., Vogel, U., Wallin, H., 2012. Nanotitanium dioxide toxicity in mouse lung is reduced in sanding dust from paint. Part. Fibre Toxicol. 9, 4.

Saber, A.T., Jensen, K.A., 2014. Case study: paints and lacquers with silica nanoparticles. In: Wohlleben, W., Kuhlbusch, T.A.J., Lehr, C.-M., Schnekenburger, J. (Eds.), Safety of Nanomaterials along their Lifecycle: Release, Exposure, and Human Hazards. CRC Press, London, UK, ISBN 978-1-46-656786-3, Ch. 17.

Sadrieh, N., Wokovich, A.M., Gopee, N.V., Zheng, J., Haines, D., Parmiter, D., Siitonen, P.H., Cozart, C.R., Patri, A.K., McNeil, S.E., Howard, P.C., Doub, W.H., Buhse, L., 2010. Lack of significant dermal penetration of titanium dioxide from sunscreen formulations containing nano- and submicron-size TiO_2 particles. Toxicol. Sci. 115 (1), 156–166.

Salomon, J.J., Muchitsch, V.E., Gausterer, J.C., Schwagerus, E., Huwer, H., Daum, N., Lehr, C.-M., Ehrhardt, C., 2014. The cell line NCI-H441 is a useful in vitro model for transport studies of human distal lung epithelial barrier. Mol. Pharm. 11 (3), 995–1006.

Sauer, U.G., Vogel, S., Aumann, A., Hess, A., Kolle, S.N., Ma-Hock, L., Wohlleben, W., Dammann, M., Strauss, V., Treumann, S., Gröters, S., Wiench, K., van Ravenzwaay, B., Landsiedel, R., 2014. Applicability of rat precision-cut lung slices in evaluating nanomaterial cytotoxicity, apoptosis, oxidative stress, and inflammation. Toxicol. Appl. Pharmacol. 276, 1–20.

Sayes, C.M., Reed, K.L., Warheit, D.B., 2007. Assessing toxicity of fine and nanoparticles: comparing in vitro measurements to in vivo pulmonary toxicity profiles. Toxicol. Sci. 97 (1), 163–180.

SCCS, 2014. Scientific Committee on Consumer Safety. Opinion for clarification of the meaning of the term "sprayable applications/products" for the nano forms of Carbon Black CI 77266, Titanium Oxide and Zinc Oxide. Adopted at the 7th SCCS plenary meeting 23 Sept 2014. SCCS/1539/14, rev. of 16 Dec 2014, p. 13.

SCENIHR, 2015. The Scientific Committee on Emerging and Newly Identified Health Risks. Opinion on the guidance on the determination of potential health effects of nanomaterials used in medical devices. 6 Jan 2015.

Semmler-Behnke, M., Takenaka, S., Fertsch, S., Wenk, A., Seitz, J., Mayer, P., Oberdörster, G., Kreyling, W.G., 2007. Efficient elimination of inhaled nanoparticles from the alveolar region: evidence for interstitial uptake and subsequent reentrainment onto airways epithelium. Environ. Health Perspect. 115 (5), 728–733.

Semmler-Behnke, M., Kreyling, W.G., Lipka, J., Fertsch, S., Wenk, A., Takenaka, S., Schmid, G., Brandau, W., 2008. Biodistribution of 1.4- and 18-nm gold particles in rats. Small 4 (12), 2108–2111.

Semmler-Behnke, M., Lipka, J., Wenk, A., Hirn, S., Schäffler, M., Tian, F., Schmid, G., Oberdörster, G., Kreyling, W.G., 2014. Size dependent translocation and fetal accumulation of gold nanoparticles from maternal blood in the rat. Part. Fibre Toxicol. 11, 33.

Sharma, A., Madhunapantula, S.V., Roberson, G.P., 2012. Toxicological considerations when creating nanoparticle based drugs and drug delivery systems? Expert Opin. Drug Metab. Toxicol. 8 (1), 47–69.

Silva, R.M., Doudrick, K., Franzi, L.M., TeeSy, C., Anderson, D.S., Wu, Z., Mitra, S., Vu, V., Dutrow, G., Evans, J.E., Westerhoff, P., van Winkle, L.S., Raabe, O.G., Pinkerton, K.E., 2014. Instillation versus inhalation of multiwalled carbon nanotubes: exposure-related health effects, clearance, and the role of particle characteristics. ACS Nano 8 (9), 8911–8931.

Singh, N.B., Manshian, B., Jenkins, G.J., Griffiths, S.M., Williams, P.M., Maffeis, T.G., Wright, C.J., 2009. Nanogenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials 30, 3891–3914.

Smulders, S., Luyts, K., Brabants, G., Landuyt, K.V., Kirschhock, C., Smolders, E., Golanski, L., Vanoirbeek, J., Hoet, P.H., 2014. Toxicity of nanoparticles embedded in paints compared with pristine nanoparticles in mice. Toxicol. Sci. 141 (1), 132–140.

Stefaniak, A.B., Guilmette, R.A., Day, G.A., Hoover, M.D., Breysse, P.N., Scripsick, R.C., 2005. Characterization of phagolysosomal simulant fluid for study of beryllium aerosol particle dissolution. Toxicol. In Vitro 19, 123–134.

Stefaniak, A.B., Hackley, V.A., Roebben, G., Ehara, K., Hankin, S., Postek, M.T., Lynch, I., Fu, W.E., Linsinger, T.P., Thünemann, A.F., 2013. Nanoscale reference materials for environmental, health and safety measurements: needs, gaps and opportunities. Nanotoxicology 7 (8), 1325–1337.

Stone, V., Johnston, H., Schins, R.P.F., 2009. Development of in vitro systems for nanotoxicology: methodological considerations. Crit. Rev. Toxicol. 39, 613–626.

Stone, V., Pozzi-Mucelli, S., Tran, L., Aschberger, K., Sabella, S., Vogel, U.B., Poland, C., Balharry, D., Fernandes, T., Gottardo, S., Hankin, S., Hartl, M.G., Hartmann, N., Hristozov, D., Hund-Rinke, K., Johnston, H., Marcomini, A., Panzer, O., Roncato, D., Saber, A.T., Wallin, H., Scott-Fordsmand, J.J., 2014. ITS-NANO – prioritising nanosafety research to develop a stakeholder driven intelligent testing strategy. Part. Fibre Toxicol. 11, 9.

Sullivan, L.H., 1896. The Tall Office Building Artistically Considered. Lippincott's Magazine, pp. 403–409.

Suzuki, N., 2003. X-ray photoelectron spectroscopy and its application to carbon. Carbon Alloys: Novel Concepts Develop Carbon Sci. Technol. 211–222.

Tarantini, A., Lanceleur, R., Mourot, A., Lavault, M.T., Casterou, G., Jarry, G., Hogeveen, K., Fessard, V., 2014a. Toxicity, genotoxicity and proinflammatory effects of amorphous nanosilica in the human intestinal Caco-2 cell line. Toxicol. In Vitro (epub ahead of print 13 Nov 2014, doi: 10.1016/j.tiv.2014.10.023).

Tarantini, A., Huet, S., Jarry, G., Lanceleur, R., Poul, M., Tavares, A., Vital, N., Louro, H., João Silva, M., Fessard, V., 2014b. Genotoxicity of synthetic amorphous silica nanoparticles in rats following short-term exposure. Part 1: oral route. Environ. Mol. Mutagen. (epub ahead of print 15 Dec 2014, doi: 101002/em.21935).

Taurozzi, J.S., Hackley, V.A., Wiesner, M.R., 2010. Ultrasonic dispersion of nanoparticles for environmental, health and safety assessment – issues and recommendations. Nanotoxicology 5, 711–729.

Teeguarden, J.G., Hinderliter, P.M., Orr, G., Thrall, B.D., Pounds, J.G., 2007. Particokinetics in vitro: dosimetry considerations for in vitro nanoparticle toxicity assessments. Toxicol. Sci. 95, 212–300 (Erratum in: Toxicol. Sci. 97, 614).

Tollefsen, K.E., Scholz, S., Cronin, M.T., Edwards, S.W., de Knecht, J., Crofton, K., Garcia-Reyero, N., Hartung, T., Worth, A., Patlewicz, G., 2014. Applying adverse outcome pathways (AOPs) to support integrated approaches to testing and assessment (IATA). Regul. Toxicol. Pharmacol. 70 (3), 629–640.

UBA, 2014. Umweltbundesamt – Federal Environmental Agency. – Assessment of impacts of a European register of products containing nanomaterials. Texte 23/2014. In: Herman, A., Diesner, M.O., Abel, J., Hawthorne, C., Greßmann, A. (Eds.), Project No. (FKZ) 360 01 072, Report No. (UBA-FB) 001907/E, 142 p.

Van der Zande, M., Vandebriel, R.J., van Doren, E., Kramer, E., Herrera Rivera, Z., Serrano-Rojero, C.S., Gremmer, E.R., Mast, J., Peters, R.J.B., Hollman, P.C.H., Hendriksen, P.J.M., Marvin, H.J.P., Peijnenburg, A.A.C.M., Bouwmeester, H., 2012. Distribution, elimination, and toxicity of silver nanoparticles and silver ions in rats after 28-day oral exposure. ACS Nano 6 (8), 7427–7442.

Van der Zande, M., Vandebriel, R.J., Groot, M.J., Kramer, E., Rivera, Z.H., Rasmussen, K., Ossenkoppele, J.S., Tromp, P., Gremmer, E.R., Peters, R.J.B., Hendriksen, P.J., Marvin, H.J.P., Peijnenburg, A.A.C.M., Bouwmeester, H., 2014. Sub-chronic toxicity study in rats orally exposed to nanostructured silica. Part. Fibre Toxicol. 11, 8.

Van Ravenzwaay, B., Landsiedel, R., Fabian, E., Burkhardt, S., Strauss, V., Ma-Hock, L., 2009. Comparing fate and effects of three particles of different surface properties: nano-TiO_2, pigmentary TiO_2 and quartz. Toxicol. Lett. 186, 152–159.

Visalli, G., Bertuccio, M.P., Iannazzo, D., Piperno, A., Pistone, A., Di Pietro, A., 2015. Toxicological assessment of multi-walled carbon nanotubes on A549 human lung epithelial cells. Toxicol. In Vitro 29 (2), 352–362.

Wagner, S., Gondikas, A., Neubauer, E., Hofmann, T., von der Kammer, F., 2014. Spot the difference: Engineered and natural nanoparticles in the environment—release, behavior, and fate – Finde den Unterschied: synthetische und natürliche Nanopartikel in der Umwelt – Freisetzung, Verhalten und Verbleib. Angew. Chem. 126, 12604–12626.

Walser, T., Limbach, L.K., Brogioli, R., Erismann, E., Flamigni, L., Hattendorf, B., Juchli, M., Krumeich, F., Ludwig, C., Prikopsky, K., Rossier, M., Saner, D., Sigg, A., Hellweg, S., Günther, D., Stark, W.J., 2012. Persistence of engineered nanoparticles in a municipal solid-waste incineration plant. Nat. Nanotechnol. 7, 520–524.

Wang, A., Berg, E.L., Polokoff, M., Yang, J., El-Badawy, A., Reif, D., Kleinstreuer, N., Marinakos, S., Badireddy, A.R., Gavett, S., Rotroff, D., Gangwal, S., Rabinowitz, J., Matson, C., Tolavma, T., Wiesner, M., Houck, K., 2013. Ranking and profiling nanomaterial (NM) bioactivity by ToxCast high-throughput screening (HTS). In: Poster at the 52nd Annual Meeting of the Society of Toxicology (SOT), 10–14 Mar 2013.

Wang, J., Fan, Y., 2014. Lung injury induced by TiO_2 nanoparticles depends on their structural features: size, shape, crystal phases, and surface coating. Int. J. Mol. Sci. 15 (12), 22258–22278.

Wang, X.Z., Yang, Y., Li, R., McGuinnes, C., Adamson, J., Megson, I.L., Donaldson, K., 2014. Principal component and causal analysis of structural and acute in vitro toxicity data for nanoparticles. Nanotoxicology 8 (5), 465–476.

Warheit, D.B., Webb, T.R., Reed, K.L., Frerichs, S., Sayes, C.M., 2007. Pulmonary toxicity study in rats with three forms of ultrafine-TiO_2 particles: differential responses related to surface properties. Toxicology 230, 90–104.

Warheit, D.B., 2008. How meaningful are the results of nanotoxicity studies in the absence of adequate material characterization? Toxicol. Sci. 101 (2), 183–185.

WHO, 2005. World Health Organization. Air quality guidelines for Europe, 2nd edition, see: <http://www.euro.who.int/__data/assets/pdf_file/0005/74732/E71922.pdf>.

Wiemann, M., Bruch, J., 2009. Comparison of *in vitro* and *in vivo* findings. In: Kuhlbusch, T.K.J., Nau, K., Krug, H. (Eds.), NanoCare. Health Related Aspects of Nanomaterials. Final Scientific Report. Dechema, Frankfurt, Germany, pp. 68–73.

Wiemann, M., Vennemann, A., Bruch, J., Wiench, K., Ma-Hock, L., Landsiedel, R., 2015. Evaluation of a macrophage assay for predicting nanomaterial-induced inhalation toxicity. Manuscript in preparation.

Wiesner, M.R., 2014. Some thoughts on exposure. In: Presentation at the 4th International Conference on Safe Production and Use of Nanomaterials. 18–20 Nov 2014, Grenoble, France.

Wohlleben, W., Brill, S., Meier, M.W., Mertler, M., Cox, G., Hirth, S., von Vacano, B., Strauss, V., Treumann, S., Wiench, K., Ma-Hock, L., Landsiedel, R., 2011. On the lifecycle of nanocomposites: comparing released fragments and their in-vivo hazards from three release mechanisms and four nanocomposites. Small 7 (16), 2384–2395.

Wohlleben, W., Ma-Hock, L., Boyko, V., Cox, G., Egenolf, H., Freiberger, H., Hinrichsen, B., Hirth, S., Landsiedel, R., 2013. Nanospecific guidance in REACH: a comparative physico-chemical characterization of 15 materials with methodical correlations. J. Ceram. Sci. Technol. 4, 93–104.

Wohlleben, W., Kuhlbusch, T., Lehr, C.-M., Schnekenburger, J., 2014a. Safety of Nanomaterials along their Lifecycle: Release, Exposure and Human Hazards. CRC Press, London, UK, ISBN 978-1-46-656786-3.

Wohlleben, W., Vilar, G., Fernández-Rosas, E., González-Gálvez, D., Gabriel, C., Hirth, S., Vazquez-Campos, S., 2014b. A pilot interlaboratory comparison of protocols that simulate aging of nanocomposites and detect released fragments. Environ. Chem. 11 (4), 402–418.

Xia, X.R., Monteiro-Riviere, N.A., Mathur, S., Song, X., Xiao, L., Oldenberg, S.J., Fadeel, B., Riviere, J.E., 2011. Mapping the surface adsorption forces of nanomaterials in biological systems. ACS Nano 5 (11), 9074–9081.

Yang, K., Gong, H., Shi, X., Wan, J., Zhang, Y., Liu, Z., 2013. *In vivo* biodistribution and toxicology of functionalized nano-graphene oxide in mice after oral and intraperitoneal administration. Biomaterials 34 (11), 2787–2795.

Yi, J., Chen, B.T., Schwegler-Berry, D., Frazer, D., Castranova, V., McBride, C., Knuckles, T.L., Stapleton, P.A., Minarchick, V.C., Nurkiewicz, T.R., 2013. Whole-body nanoparticle aerosol inhalation exposures. J. Vis. Exp. 75, e50263.

Yokel, R.A., Unrine, J.M., Wu, P., Wang, B., Grulke, E.A., 2014. Nanoceria biodistribution and retention in the rat after its intravenous administration are not greatly influenced by dosing schedule, dose, or particle shape. Environ. Sci. Nanotechnol. 1, 549.

Zhang, H., Ji, Z., Xia, T., Meng, H., Low-Kam, C., Liu, R., Pokhrel, S., Lin, S., Wang, X., Liao, Y.-P., Wang, M., Li, L., Rallo, R., Damoiseaux, R., Telesca, D., Mädler, L., Cohen, Y., Zink, J.I., Nel, A.E., 2012. Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. ACS Nano 6 (5), 4349–4368.

Zhu, M., Nie, G., Meng, H., Xia, T., Nel, A., Zhao, Y., 2013. Physicochemical properties determine nanomaterial cellular uptake, transport and fate. Acc. Chem. Res. 46 (3), 622–631.