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


a AkzoNobel, Technology and Engineering, Arnhem, Netherlands
b Shell Health, Shell International B.V., The Hague, Netherlands
c BASF SE, Ludwigshafen, Germany
d Afcon 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

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 decision-making framework for the grouping and testing of nanomaterials.
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 as 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 on 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 (BÄuA, 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 BÄuA 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 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 nanomaterial’s 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 TiO₂ 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, Grieber 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 interactions).
Overview and interdependence of the criteria used for the grouping of nanomaterials and corresponding tiers of the DF4nanoGrouping.

Supplementary criteria that may relate to the nanomaterial’s MoA

Key criteria with direct relevance for nanomaterial grouping

Intrinsic material properties (3.1)

- Water solubility (3.1.1)
- Composition (3.1.2)
- PSS (3.1.2)
- Primary particle size (3.1.1)
- Surface area (3.1.3)
- Composition and rigidity (3.1.4)
- Aspect ratio (3.1.2)
- Rigidity (3.1.2)

System-dependent properties (3.3)

- Biopersistence (3.4.1)
- Dissolution rate in BSF (3.3.1)
- Release of toxic ions (3.3.2)
- Surfactant reactivity (3.3.2)
- Corona formation (3.3.4)
- Surface area in situ (3.3.5)

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. 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. 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.
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 GRPs, which do not have surface reactivity and do not elicit a specific cellular effect and do not prevail in

---

**Fig. 2.** The decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping).
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 particular 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 MnO reactivity in FRAS or cytchrome c assays; dispersibility: AAN ≥ 3; no cellular effects at ≤10 μg/cm² (Kroll et al., 2011). Confirmatory threshold value in respect to low toxic potency, i.e. NOAEC in short-term inhalation study (STIS) >10 mg/m³.

**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 ≤10 mg/m³ 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-nanomaterials. 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/m³ 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/m³ (considering an average agglomerate density of 2.0 g/cm³ 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/m³ (BAuA, 2013). By comparison, for respirable non-nanosized dust, an OEL of 1.25 mg/m³ (based on an average particle density at the workplace of 2.5 g/cm³) 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.

<table>
<thead>
<tr>
<th>Grouping criteria or qualifiers</th>
<th>Relevance for HA or RA</th>
<th>Threshold values for grouping</th>
<th>Benchmark materials</th>
<th>Assignment to main group or sub-grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td>Use</td>
<td>Nanomaterial exposure likely? If so: In which form?</td>
<td>cf. Chapter 3.2 and Supplementary Information Table S3</td>
<td>Benchmark materials for release of free nanoparticles are unavailable Dustiness of powders Low: pellet-like, non friable solids Medium: crystalline, granular solids High: fine, light powders Droplet size affecting inhalability Respirable: &lt;10 μm Inhalable: &lt;100 μm Not inhalable: &gt;100 μm Nanomaterial migration from food matrix Low: &lt;10 mg of substances/dm² of food contact surface (overall migration limit) High: &gt;10 mg of substances/dm² of food contact surface (overall migration limit)</td>
<td>‘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</td>
</tr>
<tr>
<td>Release</td>
<td>Release (i.e. exposure) likely? If so: In which form? Do the released fragments contain nanoparticles?</td>
<td>cf.</td>
<td>Benchmark materials for release of free nanoparticles are unavailable Dustiness of powders Low: pellet-like, non friable solids Medium: crystalline, granular solids High: fine, light powders Droplet size affecting inhalability Respirable: &lt;10 μm Inhalable: &lt;100 μm Not inhalable: &gt;100 μm Nanomaterial migration from food matrix Low: &lt;10 mg of substances/dm² of food contact surface (overall migration limit) High: &gt;10 mg of substances/dm² of food contact surface (overall migration limit)</td>
<td>‘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</td>
</tr>
<tr>
<td>Exposure route</td>
<td>Determination of most relevant biological pathways and corresponding endpoints</td>
<td>cf. corresponding grouping criteria relevant for the respective route of exposure</td>
<td>Inhalation exposure</td>
<td>Dermal exposure</td>
</tr>
<tr>
<td>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</td>
<td>Water solubility</td>
<td>Screening for loss of nanomaterial structure: 1st estimation of bioreactivity</td>
<td>Assignment to main group 1 (soluble nanomaterials): &gt;100 mg/L (BAuA, 2013)</td>
<td>ZincCl₂ (high)</td>
</tr>
<tr>
<td></td>
<td>Shape (and aspect ratio)</td>
<td>Screening for fiber effects (and for appropriate dose metrics)</td>
<td>Indication for main group 2 (biopersistent HAR NMs): Size, shape, aspect ratio fulfill WHO definition of ‘respirable fibers’: Aspect ratio &gt;1:3 (i.e. HAR NMs), length &gt; 5 μm, diameter &lt; 3 μm ‘Not main group 2’: Granular nanomaterials</td>
<td>Certified reference materials with 1 and 3 nanoscale dimensions, all with diameters &lt; 100 nm, available from: NIST (USA); BAM (DE); IRMM (JRC-BE)</td>
</tr>
<tr>
<td></td>
<td>Composition</td>
<td>Screening for toxic ion release (inorganic nanomaterials) Nanoized and bulk materials may share similar properties</td>
<td>Indication for main group 3 (passive nanomaterials): &lt;0.1% of toxic component (element or molecule; EP and Council of the EU, 2008)</td>
<td>Dependent upon characterization method</td>
</tr>
</tbody>
</table>


* 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://libc.jrc.ec.europa.eu/our_activities/nanotechnology/nanomaterials-repository).

* 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 nanof orm.

* Relative amount of nanomaterial of entire amount of released fragment.


* (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.

<table>
<thead>
<tr>
<th>Grouping criteria or qualifiers</th>
<th>Relevance for HA or RA</th>
<th>Threshold values for grouping</th>
<th>Benchmark materials</th>
<th>Assignment to main group or sub-grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>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</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>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</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dissolution rate in biological fluids</td>
<td>After ‘water solubility’ (cf. Tier 1; Table 2), the dissolution rate provides further estimations of a nanomaterial’s in vivo ‘biopersistence’</td>
<td>Nanomaterials with a dissolution rate &gt;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):</td>
<td>Cricodolite asbestos (biopersistent, PC; non-fibrous benchmark material not yet determined) Chrysotile asbestos (dissolves in biological fluids, NC) cf. Donaldson et al. (2010)</td>
<td>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)</td>
</tr>
<tr>
<td>Surface reactivity</td>
<td>Cellular and apical toxicity</td>
<td>Indication for assignment to main group 3 (passive nanomaterials): Passive: &lt;10% of MnO2 reactivity in FRAS or cytochrome c assays Assignment to main group 4 (active nanomaterials): Active: &gt;10% of MnO2 reactivity in FRAS or cytochrome c assays</td>
<td>Passive: BaSO4, NM-220 Active: MnO2</td>
<td>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)</td>
</tr>
<tr>
<td>Dispersibility</td>
<td>Mobility</td>
<td>Assignment to main groups 2 (biopersistent HAR NMs) and 4 (active nanomaterials): Mobile*: AAN &lt; 3 Indication for main group 3 (passive nanomaterials): Large: AAN &gt; 3 or diameter above 100 nm (may be grouped together with micron-scale materials, if appropriate)</td>
<td>Ag NM-300 (AAN &lt; 3) SiO2 NM-203 (small agglomerates) SiO2 NM-200 (large agglomerates)</td>
<td>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)</td>
</tr>
<tr>
<td>Cellular effects</td>
<td>Preliminary prediction of apical toxicity</td>
<td>Indication for nanomaterial assignment to main group 3 (passive nanomaterials): No effect at ≤10 μg/cm² (krolli et al., 2011)</td>
<td>Passive: BaSO4, NM-220 Active: ZnO NM-110 and NM-111 (ion effects); CeO2 NM-211 and NM-212 (surface-related effects)</td>
<td>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)</td>
</tr>
<tr>
<td><strong>In vitro genotoxicity</strong></td>
<td>Preliminary prediction of apical toxicity</td>
<td>Not yet determined</td>
<td>Not yet determined</td>
<td></td>
</tr>
</tbody>
</table>

**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’)**

<table>
<thead>
<tr>
<th>Grouping criteria</th>
<th>Threshold values for sub-grouping</th>
<th>Benchmark materials</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apical toxic effects</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Inflammation in STIS at an aerosol concentration of ≤10 mg/m³</td>
<td>CeO2 NM-211 and NM-212</td>
<td></td>
</tr>
<tr>
<td>2. Necrosis in STIS at an aerosol concentration of ≤10 mg/m³</td>
<td>ZnO NM-110 and NM-111</td>
<td></td>
</tr>
<tr>
<td><strong>Toxic potency in STIS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. NOAEC: ≤0.1 mg/m³</td>
<td>MWCNT NM-400, quartz DQ12</td>
<td></td>
</tr>
<tr>
<td>2. NOAEC: ≤1 mg/m³</td>
<td>CeO2 NM-211 and NM-212</td>
<td></td>
</tr>
<tr>
<td>3. NOAEC: ≤10 mg/m³</td>
<td>SiO2 naked and TiO2 NM-105</td>
<td></td>
</tr>
<tr>
<td>4. NOAEC: &gt;10 mg/m³ (confirms Group 3)</td>
<td>BaSO4 NM-220</td>
<td></td>
</tr>
<tr>
<td>Grouping criteria</td>
<td>Relevance for HA or RA</td>
<td>Threshold values for grouping</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>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</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary particle size</td>
<td>Size is indispensable to identify a nanomaterial</td>
<td>Size is not a primary grouping criterion, but it influences dose metrics depending on MoA via mass, surface (or number)</td>
</tr>
<tr>
<td>Surface area</td>
<td>Many of the most relevant MoAs are surface-induced</td>
<td>Surface area is not a primary grouping criterion, but it is essential to convert dose metrics</td>
</tr>
<tr>
<td>Surface chemistry</td>
<td>Many of the most relevant MoAs are surface-induced</td>
<td>For grouping, composition is more important than surface chemistry. Subgrouping relies on the system-dependent consequences of surface chemistry: surface reactivity and dispersibility</td>
</tr>
<tr>
<td>Crystallinity</td>
<td>Addressed in the context of 'composition'</td>
<td></td>
</tr>
<tr>
<td>Hydrophobicity</td>
<td>Altered mobility: Hydrophobic materials have stronger corona formation and are cleared differently than hydrophilic materials</td>
<td>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</td>
</tr>
<tr>
<td>Hydrosolubility</td>
<td>Altered mobility: Hydrophobic materials have stronger corona formation and are cleared differently than hydrophilic materials</td>
<td>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</td>
</tr>
<tr>
<td>Corona formation</td>
<td>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</td>
<td></td>
</tr>
</tbody>
</table>


<sup>a</sup> 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).

<sup>b</sup> Further benchmark materials required for all relevant scenarios; data from OECD is emerging.

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

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 indispensable 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 and 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 whole 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.

<table>
<thead>
<tr>
<th>Relevant life cycle stage for grouping</th>
<th>Production of NM</th>
<th>Formulation / industrial use</th>
<th>Uses</th>
<th>Disposal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Form</td>
<td>Release</td>
<td>Exposure</td>
<td>Form</td>
</tr>
<tr>
<td>Oral</td>
<td>Powder</td>
<td>No (industrial hygiene)</td>
<td>No</td>
<td>Powder</td>
</tr>
<tr>
<td>Liquid</td>
<td>No (industrial hygiene)</td>
<td>No</td>
<td>Liquid</td>
<td>No (industrial hygiene)</td>
</tr>
<tr>
<td>Bound in matrix</td>
<td>No (industrial hygiene)</td>
<td>No</td>
<td>Bound in matrix</td>
<td>No (industrial hygiene)</td>
</tr>
<tr>
<td></td>
<td>Powder</td>
<td>No (industrial hygiene)</td>
<td>No</td>
<td>Powder</td>
</tr>
<tr>
<td>Radiation</td>
<td>Liquid</td>
<td>No (industrial hygiene)</td>
<td>No</td>
<td>Liquid</td>
</tr>
<tr>
<td>Bound in matrix</td>
<td>No (industrial hygiene)</td>
<td>No</td>
<td>Bound in matrix</td>
<td>No (industrial hygiene)</td>
</tr>
<tr>
<td></td>
<td>Powder</td>
<td>Dust</td>
<td>High dustiness</td>
<td>Powder</td>
</tr>
<tr>
<td></td>
<td>Low dustiness</td>
<td>No dust</td>
<td>No</td>
<td>No dust</td>
</tr>
<tr>
<td></td>
<td>Medium dustiness</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Inhalation</td>
<td>Liquid</td>
<td>No (industrial hygiene)</td>
<td>No</td>
<td>Liquid</td>
</tr>
<tr>
<td></td>
<td>No aerosol</td>
<td>No</td>
<td>No aerosol</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Bound in matrix</td>
<td>No (industrial hygiene)</td>
<td>No</td>
<td>Bound in matrix</td>
</tr>
<tr>
<td></td>
<td>No abrasion</td>
<td>No</td>
<td>No abrasion</td>
<td>No</td>
</tr>
</tbody>
</table>

Groups defined by legal provisions; specifically designed exposure scenarios:

- Foods
- 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 (Neil 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 TiO₂, 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 CeO₂ 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 (Frijjiter-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 CeO₂ 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-nanomaterial substances or nanomaterials (Wagner et al., 2014). While the traditional OECD method of K<sub>ow</sub> 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 DG4nanoGrouping, 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-nanized 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 aequous 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; Pujalvé 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-nanized 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 modulations 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 (Cr2O3, Co3O4, CoO, MnO2, MnO, 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 micronized 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 (AAI) 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 AAI <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 affect nanomaterial translocation rate and extent from the blood into organs and tissues (biodistribution). Particular attention should be paid to the ‘internal exposure’ or ‘intrinsic 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 CeO2 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 NCI-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 MMVs with lengths >20 μm had disappeared from the lung compared to only 17% of the highly biopersistent crocidolite. The longer 22 and 27 μm MMVs disappeared more rapidly than the shorter 15 and 17 μm MMVs, 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 (GBP)). Moreno-Horn and Gebel (2014) define GBP 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 GBP, 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 (BÄuA, 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.,...
During inhalation exposure to CeO\textsubscript{2} NM-211, the dose rate of CeO\textsubscript{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 alveolar 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 nanosized 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; Kreiling 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; Lankelid 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.
findings, also in an in vivo study using mini-pigs, different uncoated or coated TiO2 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, PSS: <20 nm, TEM; 59 nm, DLS; or polyvinylpyrrolidone-coated, PSS: <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 SiO2 (PSS: 7 nm) or to 1000 mg/kg SiO2 NM-202 (PSS: 10–25 nm) for 28 days did not result in distinctly elevated SiO2 levels in the body tissues. After 84 days of exposure, however, SiO2 accumulated in the spleen of the rats treated with the commercially available amorphous SiO2, and a significant increase in the occurrence of liver fibrosis was observed in the rats treated with SiO2 NM-202 (Van der Zande et al., 2014). Treating rats orally with up to 1042 mg/kg bw TiO2 (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 TiO2 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) inflammation 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-1β. 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). Inflammamosome 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; Golbambaki 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 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 (Teguuarden 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., 2013). 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 CeO2 nanoparticles with negative zeta potential as compared to those with positive zeta potential (Pati et al., 2007). Negative zeta potentials have been observed to correlate with better nanomaterial dispersion in water (Buesen et al., 2014; Wohleben 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 TiO2 and carbon black, oxidative stress reactions have been suggested as predominant mechanisms of toxicity, whereas SiO2 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 SiO2 nanomaterials, Tarantini et al. (2014b) exposed rats orally byavage 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 SiO2 with or without surface functionalization, two surface-functionalized ZrO2 nanomaterials, or BaSO4 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 SiO2. By contrast, for TiO2 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 TiO2 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 TiO2 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 TiO2 nanoparticles show evidence of carcinogenicity in humans (IARC, 2010). Alternatively, 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 toxicity 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); cf. 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 (ECCOTO) 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 nanof orm. 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-nanof orm 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 ECTOC 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.


Toxicol. Sci. 37, 37–53.
W凤凰网. 2015年12月23日.”

and quartz. Toxicol. Lett. 186, 152–159


