Towards a nanospecific approach for risk assessment

Susan Dekkers [a], [*], Agnes G. Oomen [a], Eric A.J. Bleeker [a], Rob J. Vandebriel [a], Christian Micheletti [b], Joan Cabellos [c], Gemma Janer [c], Natalia Fuentes [c], Socorro Vázquez-Campos [c], Teresa Borges [d], Maria João Silva [e], Adriele Prina-Mello [f], Dania Movia [f], Fabrice Nesslany [g], Ana R. Ribeiro [h], Paulo Emílio Leite [h], Monique Groenewold [a], Flemming R. Cassee [a], Adrienne J.A.M. Sips [a], Aart Dijkzeul [i], Tom van Teunenbroek [i], Susan W.P. Wijnhoven [a]

[a] National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
[b] ECAMRICERT, Vicenza, Italy
[c] LEITAT, Technological Center, Barcelona, Spain
[d] Directorate-General of Health (DGS) and PToNANO, Lisbon, Portugal
[e] National Institute of Health Doutor Ricardo Jorge (INSA) and PToNANO, Lisbon, Portugal
[f] Trinity College Dublin (TCD), Dublin, Ireland
[g] Institute Pasteur de Lille (IPL), Lille Cedex, France
[h] National Institute of Metrology Quality and Technology (INMETRO), Rio de Janeiro, Brazil
[i] Ministry of Infrastructure and the Environment (Min I&M), The Hague, the Netherlands

ARTICLE INFO

ABSTRACT

In the current paper, a new strategy for risk assessment of nanomaterials is described, which builds upon previous project outcomes and is developed within the FP7 NANoREG project. NANoREG has the aim to develop, for the long term, new testing strategies adapted to a high number of nanomaterials where many factors can affect their environmental and health impact. In the proposed risk assessment strategy, approaches for (Quantitative) Structure Activity Relationships ((Q)SARs), grouping and read-across are integrated and expanded to guide the user how to prioritise those nanomaterial applications that may lead to high risks for human health. Furthermore, those aspects of exposure, kinetics and hazard assessment that are most likely to be influenced by the nanospecific properties of the material under assessment are identified. These aspects are summarised in six elements, which play a key role in the strategy: exposure potential, dissolution, nanomaterial transformation, accumulation, genotoxicity and immunotoxicity.

With the current approach it is possible to identify those situations where the use of nanospecific grouping, read-across and (Q)SAR tools is likely to become feasible in the future, and to point towards the generation of the type of data that is needed for scientific justification, which may lead to regulatory acceptance of nanospecific applications of these tools.

1. Introduction

It is generally accepted that the recent and upcoming large variety in nanomaterials provides a challenge for assessing their risk. Because nanomaterials of the same chemical composition can have many different physicochemical properties (e.g. size, shape, charge, etc.), the variation of different nanoforms is much larger compared to non-nanomaterials (Maynard et al., 2006). Whereas it has been indicated that — for now — the risks of nanomaterials should be assessed on a case-by-case basis for each individual nanoform with its specific size, shape, surface chemistry, etc. (e.g. SCENIHR, 2009; EFSA Scientific Committee, 2011), it is also recognized that it will require a lot of experimental animals as well as time, effort, and money to obtain for each case the necessary physicochemical, exposure and hazard data for all relevant exposure scenarios and endpoints. For a

more sustainable situation, many initiatives have been taken to explore ways that enable a risk assessment of nanomaterials without the need to subject each individual nanoform to a full battery of experimental tests. Important aspects of these new approaches include amending tools like (Q)SARs, grouping, read-across and high-throughput screening/testing for nanomaterials. For successful applicability of such new approaches it is crucial that sufficient good quality nanospecific information becomes available (OECD, 2014a,b; Tantra et al., 2015).

In this paper, we describe a new strategy for risk assessment of nanomaterials in which we integrate and expand aforementioned approaches to guide the user how to prioritise those nanomaterial applications that may lead to high exposure or high toxic potential and ultimately high risks for human health. Additionally, we identify those aspects of the exposure, kinetics or hazard assessment that are most likely to be influenced by the specific properties of the nanomaterial(s) under assessment. It is to be noted that the focus is on human health; the potential risks for environment are also of importance, though beyond the scope of this paper and therefore remain to be further investigated in a future dedicated document. Further, the scientific knowledge on nanomaterials is not sufficient yet for defining benchmarks, cut-off values, validation and subsequent regulatory acceptance of nanospecific applications of (Q)SARs, grouping and read-across tools. In this paper the current knowledge will be integrated to identify those situations where the use of such nanospecific tools is likely to become feasible and regulatory acceptable in the near future, and to point towards the generation of the type of data that is needed for regulatory acceptance.

Currently, there is no indication that nanomaterials will lead to other toxicological endpoints than those known for non-nanomaterials (Nel et al., 2014; Donaldson and Poland, 2013; Gebel et al., 2014). For this reason, current regulatory frameworks on the safe use of chemicals, such as the regulatory framework for chemicals REACH (Registration, Evaluation, Authorisation and restriction of Chemicals; EU2006), are generally considered suitable to address the risks of nanomaterials (EC, 2012a,b; OECD, 2013). Within Europe REACH Guidance is being modified or developed to explain this (ECHA, 2012), while there is also a call to adapt the legal text, especially with regard to the information requirements on physicochemical properties (DG Growth, 2016; Roberts, 2016). Some (European) legislation has recently been adapted to set rules for the identification of nano-enabled applications (e.g. Cosmetics Regulation EC No 1223/2009 (EU, 2009) and Biocidal Product Regulation 528/2012 (EU, 2012)). The approach proposed in this paper is developed within the NANoREG project, which mainly focusses on REACH. However, in a later stage, it can be made applicable within other regulatory frameworks as well.

In parallel to the regulatory discussion, there is a scientific challenge to provide further insights in the specific properties that are crucial in the behaviour and toxicity of nanomaterials. These insights can aid in performing a proper and efficient risk assessment for nanomaterials in the future, preferably in a way that accelerates the rate at which the information needed for risk assessment can be generated. The proposed approach described below, facilitates further development of such insights by identifying:

a) those applications of nanomaterials that have the highest potential to cause adverse human health effects (due to high exposure and/or toxicity)
b) those aspects of exposure, kinetics or hazard that are most important to address in the human health risk assessment of nanomaterials,

c) those situations where the use of nanospecific grouping, read-across and (Q)SARS is likely to become feasible and potentially regulatory acceptable in the near future, and
d) the type of information needed for this regulatory acceptance.

The proposed approach is developed to be applicable to nanomaterials that are already on the market. However, elements of this approach, such as use of grouping and read-across methods and aspects most important to address the nanospecific issues within the risk assessment, will also be applicable to safe innovation approaches during the development of new nanomaterials in the research and development phase (Sips et al., 2015).

Nanomaterials are prone to many possible changes during their life cycle, like (partial) dissolution or degradation, complexation, aggregation, agglomeration, etc. Because these changes may differ from the changes of non-nanomaterials, the influence of these changes on the exposure and hazard of the nanomaterial should be assessed throughout its whole life cycle, from the manufacturing of the nanomaterial, through the different stages of the life cycle, including various uses, disposal and waste treatment.

The proposed approach is built on the extensive knowledge already developed in other European research projects or by other international organisations and committees. The most important sources of knowledge used are given in Table 1. The existing knowledge is subdivided in knowledge on newly developed risk assessment strategies (column 2), read-across and grouping approaches (column 3) and other supporting information (column 4). The most recent and relevant publications used in this paper, are mentioned.

1.1. Current knowledge on the nanospecific behaviour and toxicity

There is still a lot of debate on the terms nanospecific behaviour and toxicity, because differences in the behaviour and toxicity between nanomaterials and non-nanomaterials are not related to a nanospecific threshold below 100 nm, but more likely to be a gradual magnification of the intrinsic hazard by decreasing size (Donaldson and Poland, 2013). Nevertheless, in this paper the terms nanospecific behaviour and toxicity are still used to indicate changes in the response, interaction, behaviour and toxicity associated with the decreasing geometrical size of the (nano)materials.

The most distinctive feature of our approach is its focus on nanospecific issues in not only the hazard, but also the exposure assessment and kinetic behaviour. In other words, it makes use of the specific physicochemical properties that determine the nanospecific behaviour that influences to what extent and in which way nanomaterials come into contact and interact with the human body. Examples of such properties are dissolution rate and reactivity. These properties may change during the life cycle of a nanomaterial and are partly depending on interactions with the surrounding environment, which may lead to a different behaviour of nanomaterials in different situations.

The nanospecific behaviour is especially relevant for: a) exposure (deposition and agglomeration), b) absorption and distribution (transport across biological barriers like gut epithelium, blood-brain barrier, or skin), c) accumulation, and d) toxic potency (dose-response relationships).

Based on epidemiological and experimental research on the effects of (ultra)fine particles, it is known that small particles can cause inflammation, fibrosis, lung cancer, cardiovascular effects, neurodegenerative effects and teratogenic effects (Chen et al., 2016; Oberdorster et al., 2009). These health effects can also be caused by non-nanomaterials and are therefore not only restrictive or specific for nanomaterials. However, the nanospecific behaviour can lead to

Table 1
Summary of most important sources of information for the proposed approach.

Sources	Types of information used for the current approach		
	Risk assessment strategy	Read-across and grouping approaches	Other supporting information
MARINA	Bos et al., 2015; Oomen et al, 2014	Oomen et al., 2015	
GUIDEnano	S.W.P. Wijnhoven and P. Van Kesteren, personal communication	M. Park and G. Janer, personal communication	
ITSnano	Stone et al., 2014	Stone et al., 2014	Stone et al., 2014 Intelligent testing strategy for nanomaterials
SUN	Malsch et al., 2015		
ECETOC		Arts et al., 2014, 2015, 2016	Arts et al., 2015 Testing strategy
ECHA/RIVM/ JRC		ECHA, 2013 ECHA, 2016	Regulation (EC) No 1907/2006 (EU, 2006); Hankin et al., 2011 (RIP-oN2 on information requirements); Aitken et al., 2011 (RIP-oN3 on chemical safety assessment); ECHA, 2011 (updated chapters of the guidance on IR & CSA)
OECD	OECD, 2013	OECD, 2014a,b	OECD, 2007—2016 Testing programme of manufactured nanomaterials
SCENIHR	SCENIHR, 2009		SCENIHR, 2007 Test guidelines
RIVM/ ARCADIS		Sellers et al., 2015	

differences in the critical dose levels or location of such health effects, e.g. due to the crossing of particular barriers leading to a (higher) dose at a target site (Donaldson and Poland, 2013). Here, it should also be realised that where mass is generally used as a dose descriptor for non-nanomaterials (e.g. mg/m^3), other metrics may be more suitable to compare the toxicity of non-nanomaterials with nanomaterials (e.g., the surface area of nanomaterials per m^3) (Delmaar et al., 2015). For instance, when effects are caused by interactions with the surface of a toxicant, nanomaterials may appear much more toxic than non-nanomaterials using mass as a dose descriptor due to their relatively high surface area per unit mass. The dose-response relationship may be similar when surface area is used as a dose descriptor.

2. Proposed approach

2.1. Elements

Although the strategies summarised in Table 1 have different aims and anticipated users, there is a considerable overlap in the various aspects used in the different approaches. This overlap includes aspects related to the toxicokinetics (such as solubility, absorption and kinetics), toxicodynamics (such as genotoxicity, and immunotoxicity), exposure (such as the most relevant routes of exposure) and aspects that need to be considered when using grouping or read-across approaches. Using the strategies of Table 1 and the current knowledge on the nanospecific behaviour described in the previous paragraphs, six overlapping elements have been identified by a small group of NANoREG experts as most important nanospecific determinants within the risk assessment of nanomaterials. The selected elements were subsequently reviewed, amended and prioritised by a larger group of partners within the NANoREG project. Below, the six elements are shortly explained and a short argumentation for the selection of the element is given. More details on how these elements are incorporated within the proposed approach can be found in the description of the different phases.

2.1.1. Exposure potential

Exposure potential is included early in the present approach because exposure assessment is, in addition to hazard assessment, essential for performing risk assessment. Although some of the determinants for exposure (e.g. transformation) are also addressed in the other elements, these other elements mainly focus on the

toxicokinetics and toxicodynamics of the nanomaterials in relation to human health effects. The element exposure potential also includes the other determinants (e.g. routes of exposure or the amount of nanomaterials used) that are important in identifying the "hotspots" for exposure throughout the entire lifecycle of the nanomaterial under assessment.

2.1.2. Dissolution

Dissolution is the key element to identify whether a nanomaterial is stable enough to exert nanospecific behaviour. It is very important to know if a nanomaterial dissolves into its molecular or ionic form and how fast, where and under which circumstances this takes place. If a nanomaterial immediately falls apart into its molecular or ionic form before it reaches its potential target, it exerts no nanospecific behaviour and it is suggested to perform the classical (non-nanomaterial) risk assessment approach. If not, the nanospecific behaviour and effects should be further evaluated. How dissolution occurs can have a huge impact on the exposure potential, behaviour and effects of a nanomaterial in humans (including absorption, translocation to secondary organs and accumulation in tissues).

2.1.3. Transformation

This element is important since nanomaterials may be transformed during their life cycle. The stability of their original appearance during manufacturing and the subsequent transformations (including the coating, corona, agglomeration, aggregation and disintegration to smaller units, dissolution, precipitation, adsorption and desorption, combustion, abrasion, oxidation and reduction) is very important for their behaviour and effects in humans and the environment.

2.1.4. Accumulation

The ability of nanomaterials to accumulate in the human body may increase the likelihood for effects after long-term exposure. Some nanomaterials have been shown to accumulate in the body. Although it is not always known if this accumulation results in toxic effects or not, accumulation is a serious reason for concern in risk assessment and therefore needs to be included as one of the elements in our approach.

2.1.5. Genotoxicity

This element is an important mechanism of toxicity, also for nanomaterials, since genotoxicity is one of the potential

mechanisms that may lead to cancer and, if germ cells are affected, also to developmental and reproductive effects. It is known that nanomaterials can induce genotoxicity by directly or indirectly damaging or interacting with a DNA molecule (Chen et al., 2005; Donaldson et al., 2010a,b; Singh et al., 2009; Louro et al., 2015).

2.1.6. Immunotoxicity

Another important mechanism of toxicity of nanomaterials is the onset or triggering of an immune response, causing for example inflammation, immune stimulation or immunosuppression. In its chronic form, inflammation may lead to several health effects such as fibrosis, cirrhosis, lung cancer, cardiovascular diseases, neurological diseases, etc. There are different pathways by which nanomaterials can trigger an immune response, but not all cellular immune responses will lead to notable inflammation.

2.2. Main objectives and users of the different phases

The proposed approach consists of different phases, in which critical aspects of the six elements are addressed. The main objectives of the approach are:

a) to prioritise those applications of nanomaterials that have the highest potential to cause human health effects and
b) to identify the most important information needed to address the nanospecific issues within the risk assessment.

The first objective (prioritisation of applications) is addressed in the first phase, while the second objective (identification of information) is mainly addressed in the second and further phases.

The objective of the first phase is screening and prioritisation. From this phase, one should be able to get a rough idea on the potential of a specific nanomaterial to cause adverse health effects, by identifying:

a) materials that have the highest potential to be hazardous (flagged red),
b) materials for which the classical (non-nanomaterial) risk assessment approach can be performed (flagged green) and
c) materials that need further evaluation (flagged orange).

It is expected that only a few of the nanomaterials that are currently on the market will fall into the 'red' or 'green' category, because manufacturers will probably try to avoid the use of nanomaterials which may be hazardous or quickly lose their functionality by falling apart into their ionic or molecular form. Therefore, the orange group will probably be the largest group, for which further ranking is needed to indicate a relatively 'high', 'medium' or ' low' potential to cause harmful effects. Furthermore, the information obtained in this phase should give direction to further steps within the information gathering process for risk assessment by identifying the most relevant exposure scenarios and possibilities to use read-across and grouping primarily based on the hazard classification of non-nanomaterials or similar nanomaterials.

The objective of the second and further phases is to identify the most important information needed to address the nanospecific issues within the risk assessment. These phases should give direction to the most important information needed depending on the specific application, life cycle stage and exposure situation. Furthermore, these phases will identify possibilities for grouping and read-across primarily based on physicochemical properties and *in vitro* data.

The proposed approach should be suitable for different uses by policy makers, regulators and industry. Policy makers and regulators can predominantly benefit from using the first phase of the approach to prioritise those applications that need to be addressed most urgently. Industry can use the first phase to get an initial impression on the suitability of the application of the nanomaterial in a specific product based on the potential of a specific nanomaterial to cause adverse health effects during the different life stages of that product. The second and further phases can be used by regulators and industry to identify the most important information needs to address the nanospecific issues and/or investigate the possibilities for grouping or read-across.[1]

In Fig. 1, an overview is given of the different phases with the various relevant elements depicted for each phase. Each of the six elements has its own colour. Next to the elements, the different aspects are depicted in the same colour(s) as the elements they relate to. More details on the various phases of the flow chart and elements within that phase are described below.

3. More detailed description of phase I

3.1. Input phase I

In the following section, the reader will be guided through the first phase of the proposed approach (see Fig. 1). Going through the flow chart, suitable information should be gathered or generated within each of the boxes. The flow chart starts in phase I on the upper left side of the figure, where the dashed black arrow 'Start' points to the grey box 'Nano?' To determine whether the investigated material is a nanomaterial, information is needed on physicochemical characteristics size and/or surface area. Other information needed in other boxes of the first phase is the aspect ratio (shape and size), rigidity, biopersistence, dissolution and reactivity of the nanomaterial. For exposure, possible applications, production volumes and production process as well as operational conditions are important. A more detailed description can be found in the paragraphs underneath. The information needed in the first phase is often available from manufacturers or can be obtained through analytical or acellular assays. An overview of the relevant information for going through the entire flow chart is given in Table SI-1 of the Supplementary information. The way to precede further in phase I of the flow chart is described for each of the boxes below.

3.2. Physicochemical characteristics (phase I)

3.2.1. Nano?

The first phase starts with determining if the material indeed is a nanomaterial (see in Fig. 1, the dotted line from the left). There has been a lot of discussion on the definition of a nanomaterial and multiple definitions are used in various international organisations, committees and jurisdictions all over the world. Here we use the EC recommendation (European Parliament and Council, 2011) on the definition:

'Nanomaterial' means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm—100 nm.

This definition is not a risk based definition, but is considered the most appropriate one to be used for regulatory purposes. The use of this definition might be updated depending on new scientific

---

[1] Irrespective of the most important information needs identified within the proposed approach, information requirements set by regulatory frameworks, e.g. REACH regulation, should be met.
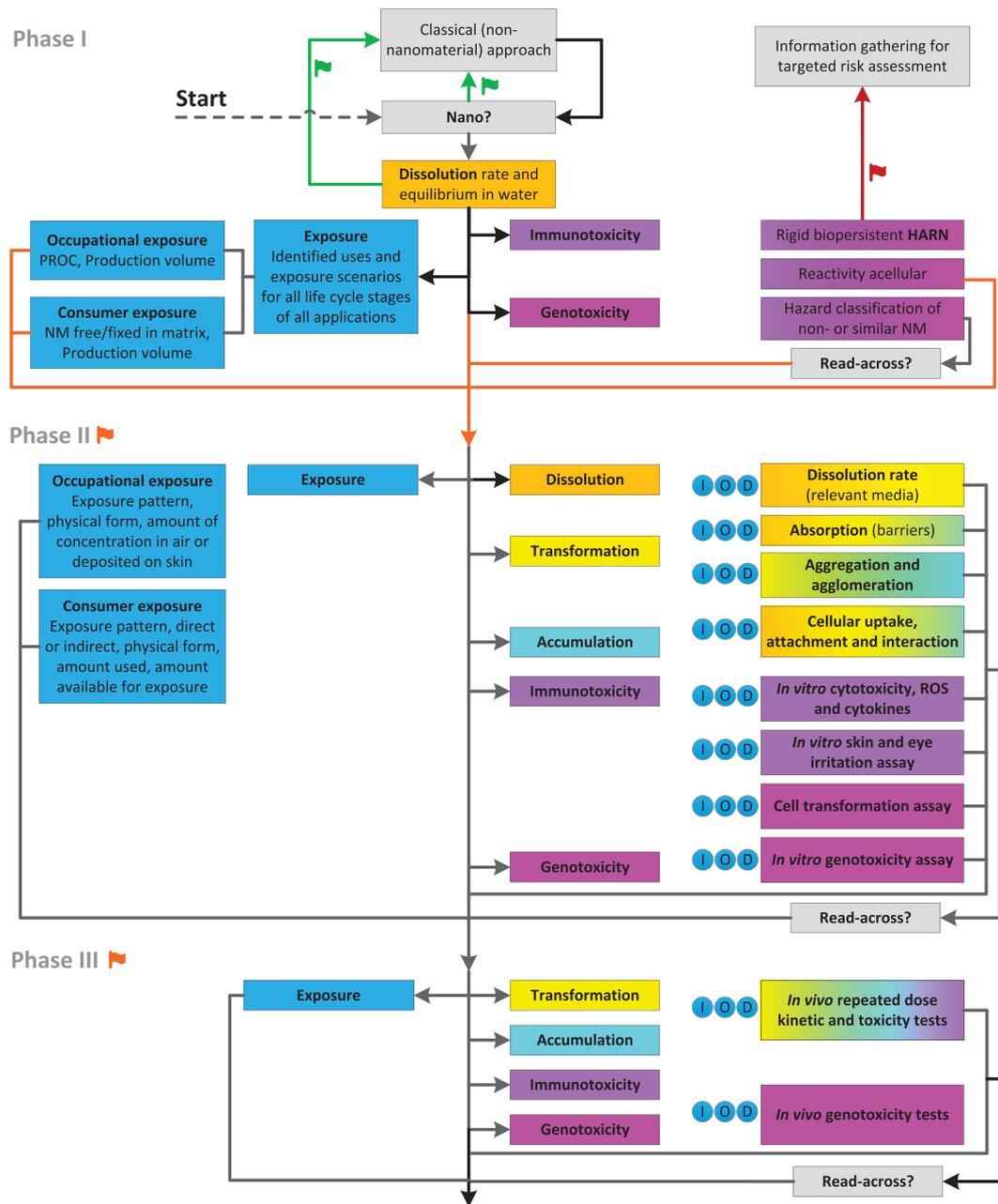
Fig. 1. Overview of the different phases of the flow chart **Green arrows**: the material is no nanomaterial or has such a high dissolution rate in water that it falls apart into its molecular or ionic form before it reaches its target => the classical (non-nanomaterial) risk assessment approach can be performed. **Red arrow**: the material is a "rigid and biopersistent High Aspect Ratio Nanomaterial (HARN)" => substitution or information gathering for targeted risk assessment to evaluated the potential to cause mesothelioma is needed. **Orange arrows**: the material does not meet the criteria for classical (non-nanomaterial) risk assessment or targeted risk assessment to evaluate the potential to cause mesothelioma => use information of phase I for prioritisation and/or further evaluation following the proposed elements related to the kinetics, toxicity and exposure in phase II, III and further. **Black arrows**: evaluation of the nanomaterial following the proposed elements related to the kinetics, toxicity and exposure in phase I, II, III and further. PROC = process and operational conditions. I: inhalation route of exposure. O: oral route of exposure. D: dermal route of exposure.

evidence and regulatory developments towards a more risk based definition. The analytical methods to determine whether a material meets the criteria of the EC recommendation have been evaluated in different reports by Joint Research Centre (JRC) and within work package 2 of the NANoREG project (JRC, 2012, 2014a, 2014b; 2015; De Temmerman et al., 2014). If the material does not meet the criteria of a nanomaterial as described in the EC recommendation, it can be evaluated using the information on the chemical composition(s) of the non-nanomaterial (follow the green arrow in Fig. 1), effectively leaving the current approach. If the material does meet the criteria of a nanomaterial, the black arrow in Fig. 1 should

be followed and dissolution in water should be evaluated.

3.2.2. Dissolution rate and equilibrium in water

The water solubility is conventionally measured using the OECD Test Guideline (TG) 105, which defines the water solubility of a substance as the saturation mass concentration of the substance in water at a given temperature and proposes two methods to measure it for conventional substances (the column elution method and the flask method). This OECD TG is already used for aggregated and agglomerated nanomaterials but it needs to be revised and refined especially for nanomaterials that disperse into small

primary nanoparticles (OECD, 2014a,b). Several approaches for the risk assessment of nanomaterials propose to use the outcomes of these types of tests to distinguish soluble from non-soluble nano-materials (BAuA, 2013; Arts et al., 2015, 2016). However, as no equilibrium will be reached in many situations relevant for human health risk assessment, the water solubility does not provide sufficient insight in the possibility of uptake of nanomaterials as physiologically relevant time frames are not considered (Oomen et al., 2015). It might be more informative to use the dissolution rate, because the information whether a nanomaterial will fall apart into its molecular or ionic form and at what rate before (or after) it reaches its potential target is far more relevant. OECD GD 29 describes how the dissolution rate of metals and metal compounds in aqueous media can be measured. However, there are no nano-specific guidelines for such tests and also no proposed cut-off values to distinguish soluble from non-soluble nanomaterials are proposed (Tantra et al., 2015). Therefore, a comparison of the dissolution rate of the nanomaterial to that of the chemical components of which it is composed, might give an indication on the possibility to use the data of the non-nanomaterial (read-across). If a nanomaterial has a very fast dissolution rate (i.e. close to instantly dissolved), the nanomaterial can be evaluated using the information on the chemical composition(s) of the non-nanomaterial (follow the green arrow in Fig. 1 towards the box "classical (non-nanomaterial) approach"). If a nanomaterial does not have a very fast dissolution rate or a slower dissolution rate than its non-nano counterpart, the black arrow down in Fig. 1 should be followed and the nanospecific behaviour and effects should be further evaluated, both for the kinetics and hazard (right side of the figure) and for the exposure (on the left).

3.3. Exposure (phase I)

3.3.1. Exposure

The first indication of exposure for workers and consumers is based on a similar (qualitative) approach for identifying priorities in exposure scenarios as described earlier in the NANoREG project within work package 3 (NANoREG, 2015). In this report, exposure scenarios of the highest potential occupational (and environmental) exposure along the life cycle of currently marketed nanomaterials have been prioritised. The approach starts with the identification of the main applications in which the nanomaterial is used. After this step, a life cycle for the nanomaterial and each of its main applications is mapped, followed by the identification of identified uses (IU)[2] and exposure scenarios (ES)[3] for each life cycle stage of each application (NANoREG, 2015).

3.3.2. Occupational exposure

In order to get a more specific understanding of the occupational exposure, the information gathered on identified uses (IU) is coupled to Contributing Exposure Scenarios (CES) for each life cycle stage of each application. CES are a set of specific conditions that are corresponding to one worker's or consumer's activity. CES can be directly linked to Process and Operational Conditions (PROCs) for occupational exposure, for which ranking values have been determined within ECETOC Targeted Risk Assessment (TRA) (ECETOC,

Table 2
Ranking of the occupational exposure potential in phase I based on production volume and PROCs of the most important occupational scenarios within the life cycle of each application of the nanomaterial.

PROC[a]	Production volume		
	high	medium	low
high	high	high	high
medium	high	medium	medium
low	medium	low	low

[a] Process and Operational Conditions (PROCs) for occupational exposure.

2012). The ranking of the PROCs within ECETOC TRA is mainly based on dustiness, energy in the process, enclosure level of the process, concentration in the preparation, duration of the activity, ventilation and the use of personal protection (ECETOC, 2012; Technical report 114). A first ranking of the occupational exposure can be obtained by combining the ranking values of the PROCs with the estimated production volume of the nanomaterial of the application (see Fig. 1 and Table 2). Also the most important route(s) of exposure are important for further determination of the strategy in the next phase of the approach.

3.3.3. Consumer exposure

For consumer exposure, no ranking of the exposure scenarios was performed earlier in the NANoREG project (NANoREG, 2015), because of the absence of information on the main determinant of consumer exposure (the transfer factor[4]). However, when the main applications are known in which the nanomaterial is used, the most important exposure aspects for phase I of the proposed approach can be selected based on information from NANoREG (2015), RIVM (2015) and Wijnhoven et al. (2009). For consumer exposure, the first ranking is based on the production volume of the nanomaterial in the application in combination with the way the nanomaterial is incorporated in the consumer product (fixed within a matrix or freely available) (see Table 3). Products containing freely available nanoparticles suspended in liquids or airborne aerosols (e.g. spray applications) are expected to cause a higher consumer exposure than products in which the nanomaterials are fixed or incorporated into a solid matrix (e.g. a bicycle frame). However, it is not always clear if and how the nanoparticles are fixed in the matrix of the product and if they will stay fixed or migrate, evaporate, wash out, wear off, etc. during the use of the product. In addition, the most important route(s) of exposure are important for further determination of the strategy in the next phase of the approach.

3.4. Kinetic and hazard aspects (phase I)

3.4.1. Rigid biopersistent HARN

One of the established mechanisms of toxicity of nanomaterials is the potential of rigid and biopersistent high aspect ratio (fibre-like) nanomaterial (HARN) to cause "frustrated phagocytosis" by macrophages after inhalation. This may lead to mesothelioma, a specific form of cancer also known from exposure to asbestos (Donaldson et al., 2010). Information needed for determining whether a nanomaterial is a potential "rigid biopersistent HARN", includes the aspect ratio, rigidity as well as the biopersistence of the nanomaterial under investigation. Rigid biopersistent HARN materials with a length (L) of ≥ 5 μm, a diameter (D) of < 3 μm and a L/D ratio of > 3, should either be substituted by an alternative

---

[2] REACH definition of Identified Use (IU): a use of a substance on its own or in a mixture, that is intended by an actor in the supply chain, including his own use, or a use that is made known to him in writing by an immediate downstream user.

[3] Exposure Scenarios (ES) should address the manufacture and identified uses. According to REACH Annex I, registrants who are required to carry out a Chemical Safety Assessment (CSA) with exposure assessment have to address all stages of the life cycle of the substance including those resulting from the manufacture and identified uses if they happen in the EU (e.g. the use of substances in articles).

[4] Transfer factor is the fraction (0—1) of the substance transferred from the product to the air, mouth or skin and represents a realistic worst-case dose available for exposure.

Table 3
Ranking of the consumer exposure potential in phase I based on production volume and way of incorporation in the exposure matrix (free/fixed) of the most important consumer exposure scenarios within the life cycle of each application of the nanomaterial.

Fixed in matrix/free	Production volume		
	high	medium	low
free	high	high	high
fixed in matrix	medium	low	low

substance or evaluated for their potential to cause this specific type of health effect. This would mean avoiding most elements in phase II and III of our approach and instead target information gathering on the potential to cause mesothelioma (follow red arrow to the box "Information gathering for targeted risk assessment").

3.4.2. Reactivity acellular

One of the most important hypotheses of nanospecific toxicity is the increased surface reactivity of nanomaterials due to their relatively large surface-to-volume ratio and sometimes also surface modification. Due to the relatively large surface-to-volume ratio and specific functionalisation of nanomaterials, the reactivity of nanomaterials can be enhanced compared to non-nanomaterials. This reactivity may trigger the generation of reactive oxygen species (ROS), leading to oxidative stress and subsequent inflammation in biological tissues.

For metal and metal oxide nanoparticles, the surface reactivity can for example be predicted using the conduction band energy levels in combination with the solubility (Zhang et al., 2013). Based on this publication, metal and metal oxide particles can be ranked for their potential to cause oxidative stress *in vitro* as well as acute inflammation after inhalation *in vivo*. For other nanomaterials and other exposure routes, a first indication on the reactivity of nanomaterials can be obtained by using acellular assays, for example by measuring ROS (reactive oxygen species) formation or FRAS (Ferric Reducing Ability of Serum) (Arts et al., 2015; Hsieh et al., 2013; Nel et al., 2014). It should be noted that these assays only provide a first indication of the oxidative properties, since the local environment, i.e. cell culture media *in vitro* or body fluids *in vivo*, can influence the reactivity of the nanomaterials in various ways, for example by containing antioxidants or by altering the nanoparticles surface due to biomolecule corona formation (Riebeling et al., 2016). The results in the band gap analysis or acellular reactivity assays are used to define further hazard ranking or subgroups (see Table 4). In addition, the results can give direction to further investigation of the reactivity in cellular environments in phase II.

3.4.3. Hazard classification of non- or similar NM

Another important indication on the toxicodynamics of a nanomaterial can be obtained by looking at the hazard classification of the chemical components of the nanomaterial (the non-nanomaterial) or a similar nanomaterial. It can be expected that nanomaterials made of chemical components that are classified to be for example genotoxic or sensitising will also have genotoxic and/or sensitising properties. Although there may be differences with respect to the critical dose levels and target organs, the possibilities to use read-across and grouping for these specific end-points based on the hazard classification of the non-nanomaterial or similar nanomaterials might be considered (follow the arrow in Fig. 1 towards the box "Read-across?").

Guidance on categorisation and read-across for chemicals has recently been published by ECHA and OECD (ECHA, 2013; OECD, 2014a,b; ECHA, 2016). The use of read-across and grouping

primarily based on the hazard classification of non-nanomaterials or similar nanomaterials is probably only feasible and regulatory acceptable if this would lead to a classification of the nanomaterial into the highest category which requires risk reducing measurements that would prevent or minimise human exposure in all life cycle stages of the nanomaterial. If the chemical components of the nanomaterial (the non-nanomaterial) or a similar nanomaterial are not classified or if no (non-nano) counterpart can be identified, this does not mean that the nanomaterials will not cause these specific health effects. Further evaluation of the other elements related to the kinetics, toxicity and exposure in phase II is then recommended by following the orange arrow down in Fig. 1.

3.5. Output phase I: prioritisation and ranking

Based on the information on size, shape and dissolution rate in water obtained in the first boxes of phase I, a first 'red', 'green' or 'orange' flag can be assigned to a nanomaterial as follows:

a) materials that have the highest potential to be hazardous (flagged red),
b) materials for which the classical (non-nanomaterial) risk assessment approach can be performed (flagged green) and
c) materials that need further evaluation (flagged orange).

Within the orange group further ranking for prioritisation can be obtained by combining further exposure and hazard ranking to indicate a high, medium or low potential to cause harmful effects within the life cycle of each application of the nanomaterial. The kinetic behaviour is not explicitly included in this further ranking, but is already implicitly taken into account by only including nanomaterials that do not have a fast dissolution in water in the orange group.

Further exposure ranking for occupational exposure is based on the production volume of the nano-enabled application and the PROCs of the exposure scenarios within the life cycle of each application of the nanomaterial. Further exposure ranking for consumer exposure is based on the production volume of the nano-enabled application and the incorporation of the nanomaterial into the matrix within the life cycle of each application of the nano-material.

Further hazard ranking is based on classification and reactivity:

- If one of the chemical components of a nanomaterial or a similar nanoform is classified as carcinogenic, mutagenic or reprotoxic (CMR, all categories), this nanomaterial is ranked 'high' with respect to its hazard. The medium classification category describes nanomaterials that are not classified as CMR but as (respiratory) sensitisers or irritating substances, whereas a material with only acute toxicity or no classification for toxicity is ranked low.
- If a nanomaterial has a high reactivity as predicted by the band gap analysis for metal and metal oxide nanoparticles or acellular ROS or FRAS assays for non-metal nanomaterials, it is also ranked 'high' with respect to its hazard. At the moment, the

Table 4
Further hazard ranking in phase I based on classification and reactivity.

Reactivity	Classification		
	high	medium	low
high	high	high	medium
low	high	medium	low

criteria for high reactivity are not precisely defined for each of the assays.

- If the chemical components or similar nanoforms are not classified and the nanomaterial does not have a high reactivity, the nanomaterial is ranked 'low' with respect to its hazard. The total hazard ranking results in the medium category when the classification is medium with a low reactivity, or when the classification is low with a high reactivity (Table 4). If no information on the hazard classification or reactivity is available, the material will be ranked 'high' with respect to its hazard.

Combining the exposure ranking with the hazard ranking of the most important occupational and consumer exposure scenarios within the life cycle of each application of the nanomaterial gives a further ranking in three subgroups to indicate a high, medium or low potential to cause harmful effects.

Please note that 'low' in Table 5 does not mean that further action for risk assessment is not needed. Information in line with regulatory requirements needs to be complete. However, this grouping (high, medium, low) can be used for prioritisation as indicated in the introduction.

Table 5
Combined ranking of potential exposure and hazard in phase I of the most important occupational and consumer exposure scenarios within the life cycle of each application of the nanomaterial.

Exposure	Hazard		
	high	medium	low
high	high	high	medium
medium	high	medium	medium
low	medium	low	low

3.6. Output phase I: information used in phase II

The materials that are flagged 'green' or 'red' will not enter phase II of this flow chart. The materials that are flagged 'green' will need to be evaluated according to the classical non-nanomaterial risk assessment approach. The materials that are flagged 'red' will skip most elements in phase I, II and maybe also III, to enable targeted information gathering on the potential to cause mesothelioma.

The group of materials that are flagged 'orange' will enter phase II. The rankings as described in the previous paragraphs are not used in phase II as such. However, the information on which these subgroups are based is used to give direction to those elements that should be addressed in phase II by preference. The information indicating which elements are most important is different for each exposure situation and should be evaluated on a case-by-case basis. Although it is not possible to describe this for each situation, an attempt to a general description of which information of phase I indicates which type of information is most relevant to obtain in each of the boxes in phase II is given in the paragraphs underneath. In addition, a few specific examples are described to give some more insight into this process.

4. Description phase II

As depicted with the blue circles in Fig. 1, the most important route(s) of exposure is (are) key in determining which type of information is relevant to obtain in each of the boxes in phase II. The route of exposure also indicates the relevant media for testing the dissolution rate and also the specific *in vitro* models (cell types and endpoints) to be investigated. In addition, information on the

hazard classification of the chemical components of the nanomaterials may also point towards relevant cell types and endpoints to be investigated.

Relevant cell types for *in vitro* assays on the cytotoxicity, immunotoxicity and genotoxicity can be selected based on the information on the dissolution rate in relevant media and *in vitro* absorption together with the limited amount of knowledge available on the absorption, distribution and translocation of nanomaterials (in general).

Below a more detailed description of the type of information and possible methods to generate this information is given for each of the boxes in Fig. 1. Most information needed in the second phase can be obtained through analytical and *in vitro* assays. An overview of the relevant information for going through the entire flow chart is given in Table SI-1 of the Supplementary information.

4.1. Exposure (phase II)

4.1.1. Occupational exposure

In the second phase, it is proposed to extend the information obtained on the occupational exposure in the first phase with information on the exposure pattern (frequency and duration), physical form and concentration (in air) or amount (deposited on skin). These determinants were selected because they have the largest influence on the final ranking score in the NANoREG report (NANoREG, 2015) and information on these determinants is generally available for most exposure scenarios.

Using the additional information further ranking of the most important occupational exposure scenarios is possible.

4.1.2. Consumer exposure

For consumer exposure, it is proposed to obtain additional information on the exposure pattern (including direct or indirect exposure, frequency and duration), physical form, amount used and/or amount available for exposure in the second phase. This selection was based on ECETOC TRA (ECETOC, 2012), ConsExpo Nano (RIVM, 2015) and Wijnhoven et al. (2009). The amount available for exposure is based on the release out of the matrix of a product, which is often very difficult to measure. However, based on the product description, an indication of the potential release of the nanomaterial out of the matrix of the product can be obtained. In general, the potential release from solid consumer products is expected to be less than the release from liquid or powdered products. In addition, incorporation of the nanomaterial into the solid matrix of the consumer product itself (e.g. incorporation of silver nanomaterials into textile fibres) will probably lead to less release of the nanomaterial than applying a coating to the surface of a solid consumer product (e.g. spraying a coating containing silver nanoparticles onto the textile product). If no information is available, the assumption that all material is released may be considered as worst-case assumption.

Using the additional information, further ranking of the most important consumer exposure scenarios is possible.

4.2. Kinetic and hazard aspects (phase II)

4.2.1. Dissolution rate (relevant media)

Recently the different analytical methods available to measure solubility of nanomaterials have been described (Tantra et al., 2015). Although a wide variety of techniques are available with the capability to measure total dissolved species or free ions, but not both, only a limited number of them is suitable for measurement in biological media. Electrochemical and colorimetric based detection schemes are able to measure the latter, whilst atomic spectrometry based techniques are able to measure the former if

combined with separation techniques such as ultrafiltration or ultracentrifugation.

In general, the exposure route is determining what a relevant medium is. For the inhalation route of exposure, dissolution in lung airway epithelial lining fluid and (macrophage) phagolysosomal simulant fluid is relevant. The oral route can be covered by measuring dissolution of nanomaterials in food matrices, gastrointestinal tract simulation fluid and macrophage phagolysosomal fluid. For dermal conditions, the dissolution rate in artificial sweat could be used.

In general, the dissolution rate in relevant media can provide information on the forms or speciation (coated or uncoated nanoparticle, agglomerate, aggregate, ionic and molecular form) of the nanomaterial when it comes into contact with the relevant areas in the human body, when it is absorbed and when it is distributed and translocated into specific organs and/or cellular compartments. This information is very important, because the extent and rate in which the nanomaterial transforms into these different forms of the material (including the extent to which it is dissolved) greatly influences its kinetic behaviour and toxicity. For some nanomaterials, the toxicity is mainly determined by the extent and rate in which it releases ions, while the toxicity of other nanomaterials is mainly determined by the particulate properties that induce an inflammatory response (Cho et al., 2012). It should be noted that more complex nanomaterials cannot be seen as a homogeneous objects when evaluating the solubility rate.

4.2.2. Absorption (barriers)

In vitro test methods simulating pulmonary (MucilAir™) or gastrointestinal barriers (Caco2) have been developed within the NANoREG project based on existing protocols (ECVAM, 2013), but these still need to be validated. Other physiological barrier models based on cell cultures and *ex vivo* tissues have also been used within the NANoREG project to simulate the blood brain barrier (Domínguez et al., 2014) and the oral mucosa barrier. 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 TG 428) but it still needs to be validated for nanomaterials.

For the inhalation route, generally only a very small percentage of insoluble nanomaterials is translocated or accumulated in extra-pulmonary organs. Studies with partially soluble nanomaterials typically show a larger percentage of particle translocation to extra-pulmonary organs as compared to the insoluble particles. However, it should be noted that with the current analytical tools it is difficult to determine whether either the particles themselves or another form of the material (e.g. molecular or ionic) are translocated.

For the oral route, it is known that the vast majority of ingested nanomaterials are rapidly passing through the gastrointestinal tract (GT) and are excreted via faeces. Nanomaterial absorption in the GT decreases with increasing material size (Schleh et al., 2012; Powell et al., 2010). Therefore, aggregation and agglomeration state of the nanomaterial influences its bioavailability. The rate of nanomaterial agglomeration in different vehicles is affected by the pH level. The different pH conditions in the GT and the presence of digestion enzymes might influence the behaviour (i.e. ion release, dissolution) of some nanomaterials. It has been suggested that positively charged materials exhibit poor bioavailability due to electrostatic repulsion and mucus entrapment (Hoet et al., 2004; Kermanizadeh et al., 2015). For nanomaterials dissolution rates in physiologically relevant media like gastrointestinal simulated fluid has been suggested to be the decisive factor determining oral uptake.

Nanomaterial size appears to be highly significant for dermal penetration. Materials larger than 100 nm in one or more dimensions do not seem to penetrate through the stratum corneum.

Aggregation and agglomeration state is crucial in the degree of penetration and potential translocation (Kermanizadeh et al., 2015).

Information on absorption into the body provides information on the need to consider only local or also systemic effects. However, it is often difficult to distinguish between complete absence and little transport in *in vitro* barrier systems. For nanomaterials even very little uptake may result in relevant internal levels due to low elimination and accumulation in time. This should be considered when data from *in vitro* barrier models is used. Currently, the scientific knowledge on the behaviour of nanomaterials within the human body is not sufficiently developed to predict the distribution and translocation of nanomaterials throughout the human body after inhalation, dermal or oral exposure. Without specific modifications, most poorly soluble nanomaterials that reach the systemic circulation are mainly distributed to tissues that are rich in reticuloendothelial cells, such as liver and spleen. However, the nanospecific physiologically based pharmacokinetic (PBPK) models developed to date mostly concern PBPK models in rats and mice for a specific type of nanomaterial (Bachler et al., 2013; Lin et al., 2015, 2016). Development of more general PBPK models and extrapolation of these models to humans should, in the near future, make it possible to predict the distribution and translocation of several types of nanomaterials in the human body. In the meantime, one may use absorption rates in combination with intravenous kinetic models developed for specific nanomaterials to estimate internal dose levels, taking into account the physicochemical properties of the nanomaterial and the nanomaterial on which the kinetic model is based (e.g. Van Kesteren et al., 2015). Based on these estimated internal dose levels relevant internal barrier models and relevant cell types for *in vitro* assays can be selected. When, for example, a nanomaterial is likely to reach the systemic circulation, *in vitro* blood-brain or placental barrier models might be relevant, though it should be noticed that such *in vitro* models cannot distinguish between low and no translocation. For nanomaterials that are likely to be distributed to the liver, hepatic cell lines should be considered for *in vitro* genotoxicity testing.

4.2.3. Aggregation and agglomeration

Some of the analytical methods used to determine if a material meets the criteria of the EC recommendation (JRC, 2012; 2014a; b; 2015; De Temmerman et al, 2014) can also be used to determine the aggregation and agglomeration. The most suitable methods should be selected taking the environment or matrix surrounding the nanomaterial into account. If inhalation is one of the most important routes of exposure, information on the aggregation and agglomeration as estimated by the size distribution of the aerodynamic diameter of the aerosol is very important to determine the deposition in the respiratory tract and subsequent translocation from the lungs to the blood stream, which are largely dependent on the diameter of the aggregated or agglomerated nanomaterials.

The largest level of deposition is at the smaller sub-micron size range (<0.1 μm), with particles able to penetrate the trachea-bronchial and alveolar regions. Deposition of particles in the range >0.5 μm is related to their aerodynamic diameter whilst for particles <0.5 μm deposition is related to their diffusion equivalent diameter (Schulz et al., 2000).

The average agglomeration number (AAN) has been proposed to assess the dispersibility of nanomaterials (Arts et al., 2015, 2016). Nanomaterials that remain dispersed as constituent particles (with AAN <3) are defined as 'mobile', since they may potentially move between body compartments.

Information on the aggregation and agglomeration of a nanomaterial can be used to predict the ability of absorption,

translocation and distribution in the body, which can be used in the selection of relevant internal barrier models and relevant cell types for *in vitro* assays.

4.2.4. Cellular uptake, attachment and interaction

Information on the cellular uptake, attachment and interaction of nanomaterials can be studied using flow-cytometry, microscopy and inductively coupled plasma mass spectrometry (ICP-MS). Flow-cytometry and ICP-MS can measure quantitatively, but cannot distinguish between externally attached and fully internalised nanomaterials. Furthermore, ICP-MS cannot distinguish between dissolved ions and nanoparticles and can only be used for electron-dense material and not for detecting liposomes, polymers, or dendrimers. Confocal microscopy gives qualitative insight into the subcellular localisation and three-dimensional structure of particles. Transmission electron microscopy (TEM) can be used to confirm subcellular particle localisation and three-dimensional structure with high resolution. This method allows semi-quantitative assessments, but the procedure is time-consuming. Combining TEM with energy-dispersive X-ray spectroscopy (EDX) makes it possible to confirm the elemental composition of the nanoparticles (Kettiger et al., 2013).

Several *in vitro* assays have been tested and further developed within the NanoREG project based on standard toxicity protocols developed for pharmaceutical products, but these still need to be validated for nanomaterials. One may also consider studying the cellular uptake, attachment and interaction in the same *in vitro* assay(s) used to investigate the cytotoxicity and cytokine induction.

Information on cellular uptake, attachment and interaction gives a first indication on the possible mechanisms of toxicity, such as damaging different cellular targets through the release of ions, the generation of ROS or the binding and interaction with intracellular proteins (Fröhlich, 2013; Nel et al., 2009). For example, direct interaction of a nanomaterial with DNA can only occur if the nanomaterial is taken up by the cell and is able to reach the DNA within the nucleus.

4.2.5. In vitro cytotoxicity, ROS and cytokines

There are many *in vitro* assays based on a range of cell types and endpoints to investigate cytotoxicity, ROS generation and cytokine induction. Several *in vitro* assays have been tested and further developed within the NANoREG project. These include standard protocols for MTS assay, the neutral red assay (adapted from OECD 432), micronucleus assay (OECD 487), mammalian gene mutation test (OECD490), colony forming efficacy (OECD 476), the comet assay (Collins, 2004), ROS detection by dichlorofluorescin diacetate (DCFDA) and interleukin expression upon exposure of NM to cells, but these still need to be validated for nanomaterials since in several cases the nature of the nanomaterials may interfere with detection methodologies (OECD, 2013).

Some of these assays allow studying the cellular uptake, attachment, interaction, cytotoxicity, ROS generation and/or cytokine induction in the same *in vitro* system. The appropriate assay should be selected taking into account that nanomaterials often show major interference with the *in vitro* assay or read-out system. Furthermore, it is essential to test for endotoxin contamination before studying the immunotoxicity of nanomaterials *in vitro* (Dobrovolskaia et al., 2009, 2016).

As for non-nanomaterials, the results of these *in vitro* assays cannot be used to predict Human Limit Values. However, they will give insight in the potential mechanisms of toxicity. Measuring the levels of pro-inflammatory cytokines and other inflammatory mediators may give insight into the mechanisms of the immunomodulating effects of a nanomaterial *in vitro*, such as inflammasome activation or dendritic cell maturation (Elsabahy and Wooley,

2013). In addition, they may give a first indication on the ability of the nanomaterials to cause immunotoxic effects *in vivo*. Cellular ROS assays provide information on the ability of nanomaterials to generate ROS within a cellular environment. Measuring the cytotoxicity is important for a good interpretation of the results of the *in vitro* cytokine and genotoxicity assays. In addition, *in vitro* cytotoxicity assays may give insight into the mechanisms of cytotoxicity, including damaging the plasma membrane, mitochondria, lysosomes or DNA through the release of ions, the generation of ROS or the binding and interaction with intracellular proteins (Fröhlich, 2013; Nel et al., 2009).

4.2.6. In vitro skin and eye irritation tests

Several *in vitro* skin and eye irritation tests are available, including the rat skin transcutaneous electrical resistance (TER) test (OECD TG 431), the reconstructed human epidermis (RHE) skin irritation test (OECD TG 439), the Bovine Cornea Opacity Permeability (BCOP) test (OECD TG 437), the isolated chicken eye (ICE) test (OECD TG 438), and an *in vitro* cell assay (OECD TG 460). These assays were developed for the evaluation of skin and eye irritation of chemical substances, but not all of them have been validated for chemical substances yet and none of them have been validated for nanomaterials (SCENIHR, 2015).

Information on *in vitro* skin and eye irritation gives an indication on the ability of the nanomaterials to cause these effects *in vivo*.

4.2.7. Cell transformation assay

Several *in vitro* cell transformation assays (CTAs) are available to assess initiation and tumour promotion potentials, but none of them have been validated for chemical substances or nanomaterials. CTAs measure induction of phenotypic alterations characteristic of tumourigenic cells. CTAs mimic some key stages of *in vivo* multistep carcinogenesis and have been shown to have a good concordance with rodent bioassay results, detecting both genotoxic and non-genotoxic carcinogens (Creton et al., 2012).

Information on *in vitro* cell transformation ability gives an indication on the possible mechanisms of carcinogenicity and an indication on the ability of the nanomaterials to cause these types of effects *in vivo*.

4.2.8. In vitro genotoxicity

The strategy for *in vitro* genotoxicity testing of nanomaterials needs to include the detection of the most relevant events for the multistep process of malignancy (gene mutations, clastogenicity and aneugenicity). At each stage of the testing strategy, expert judgment is necessary to decide on the relevance of a result considering the existing weight of evidence.

Tests for gene mutation in mammalian cells can be used, e.g., the mouse lymphoma TK gene mutation assay (MLA) OECD TG 476 which uses the autosomal thymidine kinase (Tk) gene as a reporter of mutations in the L5178Y/Tk $\pm$ mouse lymphoma cell line or the hypoxanthine guanine phosphoribosyltransferase (Hprt) gene forward mutation assay in Chinese hamster ovary (CHO) cells (Silva et al., 2005). In addition, the chromosomal aberration and the cytokinesis-blocked micronucleus (CBMN) tests are sensitive and reliable assays for the analysis of chromosome damage in mammalian cells. The former is used for detection of structural chromosome aberrations, i.e., chromatid- and chromosome-type breaks and rearrangements in cultured mammalian cells (OECD, 1997). The CBMN test allows the detection of micronuclei in the cytoplasm of interphase cells (Fenech, 2000) containing whole chromosomes (aneugenic events) or chromosome fragments (clastogenic events) during cell division (OECD, 2010). On the other hand, a genotoxicity assay that has been strongly recommended for regulatory purposes is the alkaline single cell electrophoresis, or

comet assay (EFSA, 2012; Tice et al., 2000). The comet assay is a sensitive and cost-effective method for the identification of DNA strand breaks and oxidative DNA lesions (formamido-pyrimidine-DNA-glycosylase modified comet assay) having the added advantage of requiring a very low quantity of substance or material for analysis compared to other *in vitro* genotoxicity assays. Several *in vitro* genotoxicity assays have been tested and further developed within the NANoREG project (adapted from OECD 487, OECD 476 and OECD 490), but these still need to be validated for nanomaterials.

Information on *in vitro* genotoxic mechanisms gives an indication on the possible genotoxicity and the ability of the nanomaterials to cause cancer. Positive results indicate that these genotoxic endpoints might need to be investigated *in vivo* (or read-across to *in vivo* studies with similar materials should be considered). Before performing *in vivo* tests kinetic information is needed to assess which target tissues might be reached (including germ cells for potential reproductive effects). Negative results might in the future be sufficient to rule out these genotoxic effects, provided the most relevant test methods, cell types, and dose levels have been tested according to high quality standards to gather enough weight of evidence. In the future, when more scientific knowledge becomes available, it might also be possible to use *in silico* methods to build stronger predictions (e.g. on validated nano-QSARs) and support the weight of evidence.

4.3. Output phase II

In contrast to the output of phase I, the information obtained in phase II does not lead to a ranking of nanomaterial applications. However, the output gives direction to the information that needs to be obtained in phase III.

5. Description phase III and further

In phase III, additional information on other determinants or exposure measurements may be obtained to give further insight into the risks associated to critical exposure scenarios. Guided by information obtained on the kinetics and hazard in phase II, *in vivo* studies to confirm the potential absorption, irritation, immunotoxicity and genotoxicity indicated by the *in vitro* studies might be needed. Which information from phase II may trigger the type of information to be gathered in phase III (and further) is different for each nanomaterial application and exposure situation. Although it is not possible to describe this for each situation, a general description of which information of phase II may trigger the need to generate which type of information in phase III is given in the paragraphs underneath. An overview of the relevant information for going through the entire flow chart is given in Table SI-1 of the Supplementary Information.

Positive results of *in vitro* absorption assays may trigger further investigation of the ability of a nanomaterial to become systemically available (and possibly cause systemic effects) in an *in vivo* repeated dose kinetic and toxicity test. Negative results of *in vitro* absorption assays should be interpreted with care, because it is often difficult to distinguish between complete absence and little transport in *in vitro* barrier systems. Therefore, negative results may indicate the need for more information on the dissolution, transformation and systemic toxicity of the nanomaterials under investigation. Together with information on the size, aggregation, agglomeration as well as information on the lack of absorption, systemic distribution and toxicity of similar nanomaterials or non-nanomaterials, the possibility of read-across might be considered.

The results of *in vitro* assays investigating cellular uptake, attachment, interaction, cytotoxicity, ROS generation and/or

cytokine induction will give insight in the possible mechanisms of toxicity, which may trigger the measurement of specific parameters (cytokines, oxidative stress markers) in *in vivo* studies. Eventually this may also highlight the relevance of specific endpoints to be considered.

Positive results of *in vitro* genotoxic assays may trigger further investigation of genotoxicity by *in vivo* genotoxicity testing (or read-across to *in vivo* studies with similar materials should be considered). Before *in vivo* genotoxicity tests are performed, information on the kinetics of the nanomaterial is needed, to enable the selection of the relevant tissues.

Positive results of *in vitro* cell transformation and *in vivo* genotoxicity studies together with observed systemic availability, expected accumulation and toxicity (e.g. inflammatory effects) from *in vivo* repeated dose toxicity tests may trigger long-term repeated dose kinetic and toxicity testing to rule out accumulation and long term effects, including carcinogenic, cardiovascular and adverse reproductive effects.

6. Discussion and conclusions

Performing risk assessment for each individual nanoform on a case-by-case basis would require a lot of experimental animals as well as time, effort, and money. The proposed approach, based on six elements, provides alternative ways to address the risk assessment of nanomaterials, by prioritising those applications with the highest potential health risks, identifying the most important information to address the nanospecific issues or perform risk assessment across different nanoforms (e.g. using (Q)SARs, grouping or read-across).

The prioritisation is just a first indication on the potential health risk of a nano-enabled application. Because it should only be used for prioritisation, applications within the 'low' risk category should not be disregarded for further evaluation. Potential health risks of all categories ('low', 'medium' and 'high') still need to be verified and refined. Possibly, in the first phase of the approach, not all exposure situations have been identified or unexpected toxicokinetic or toxicodynamic effects have not been identified.

The proposal suggests specific steps to gather certain pieces of key information. It should be noted that these selected pieces of information might not always be easy to obtain or generate. Within REACH, industry is responsible to provide sufficient information to ensure safe use of the application of the nanomaterial. These information requirements can be met in different ways, including the use of read-across and grouping. The methods proposed to obtain the selected pieces of information should be seen as suggestions. In case similar information can be obtained with other methods or tests, which might for example appear (scientifically) more suitable for specific cases, these can also be used. Clearly, the completeness, quality and uncertainty of the information are of utmost importance, but this is not always possible to verify. Without good quality data, and the ability to assess the quality of the data, the information obtained or generated might be inadequate for risk assessment.

It is also widely accepted that the scientific knowledge on nanomaterials is not yet sufficient for defining all benchmarks or cut-off values needed within this approach or for broad application of nanospecific (Q)SARs, grouping and read-across tools. With the current approach it is possible to identify those situations where defining such benchmarks or cut-off values is likely to become feasible in the near future, and which type of data needs to be generated for scientific justification. Some of the benchmarks and cut-off values are rather general and applicable to many different situations, while others are more specific for the nanomaterial application and exposure situation. To give an indication of the type

of data that is needed for the scientific justification of these cut-off values, a more general and a more specific example of a cut-off value are described. Furthermore, an outlook on the feasibility to obtain this type of data in the near future is given. Only the type of data, not the amount and level of confidence needed for regulatory acceptability is described.

Within the first phase of the approach, a cut-off value on dissolution rate in water is needed to distinguish those materials that fall apart into their molecular or ionic form before they reach the human body from those materials that have a slower dissolution rate enabling at least some of the material to reach the human body as a nanomaterial. Although no exact cut-off value has been proposed, the dissolution rate needs to be very fast (i.e. close to instantly dissolved) to justify the use of the "classical (non-nanomaterial) approach". The type of data needed to define this cut-off value is data on the dissolution rate of a large systematic set of different types and variations of nanomaterials in water together with information on the forms or speciation (coated or uncoated nanoparticle, agglomerate, aggregate, ionic and molecular form) of these nanomaterials when they come into contact with the human body. The latter is difficult to obtain, as it requires a lot of knowledge on the behaviour of different types of nanomaterials in different types of environments, including air, water and different product matrices. However, it might be possible to define a conservative cut-off value for dissolution rate in water based on worst case assumptions in the near future, taking relevant time-frames for hazard assessment into account.

A more specific example are the cut-off values to predict the acute inflammatory effects of metals and metal oxides nanoparticles after inhalation as proposed by Zhang et al. (2013). These cut-off values are based on analytical data (on conductivity band gap and dissolution in bronchial epithelial growth medium), *in vitro* data (on the potential to cause oxidative stress) and *in vivo* data (on the acute inflammation after inhalation) of a limited set of metal and metal oxide nanoparticles. The data needed for a more thorough scientific justification are similar data of a larger systematic set of other (combinations of) metal and metal oxide nanoparticles. It seems feasible to obtain this type of data in the near future.

To make the proposed approach work, multiple benchmark and cut-off values need to be defined for basically each of the aspects described throughout all three phases. Therefore, the approach still needs to be further developed and tested by guiding several case studies through the different phases. These case studies will also make it possible to obtain a more complete overview of those situations where defining benchmarks and cut-off values is likely to become feasible in the near future. In general, systematic sets of high quality data are needed to identify, verify and validate which nanomaterial characteristics influence which aspect of the exposure, kinetics or toxicity.

The proposed approach, including the type of information linked to the various elements and endpoints, is based on the current state of knowledge and is flexible enough to accommodate future insights and knowledge of nanomaterials. Further elaboration and refinement of especially phase III (and further) is needed based on experience with case studies. Although the current approach focusses only on the human risk assessment of nanomaterials, the approach can be expanded to environmental risk assessment in the future.

To conclude, the proposed risk assessment strategy, which is based on six elements, can be used to prioritise those nanomaterial applications that may lead to high risks for human health. The different phases of the flow chart guide the user to the most important information needed to address the nanospecific issues within the risk assessment, depending on the specific nanomaterial application, life cycle stage and exposure situation. Furthermore, the approach can also be used to identify those situations where the use of nanospecific grouping, read-across and (Q)SAR tools is likely to become feasible in the future, and to point towards the generation of the type of data that is needed for scientific justification, which may lead to regulatory acceptance of nanospecific applications of these tools.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.yrtph.2016.05.037.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.yrtph.2016.05.037.

References

Aitken, R.A., Bassan, A., Friedrichs, S., Hankin, S.M., Hansen, S.F., Holmqvist, J., Peters, S.A.K., Poland, C.A., Tran, C.L., 2011. Specific Advice on Exposure Assessment and Hazard/Risk Characterisation for Nanomaterials under REACH (RIP-oN 3) — Final Project ReportRNC/RIP-oN3/FPR/1/FINAL.

Arts, J.H., Hadi, M., Keene, A.M., Kreiling, R., Lyon, D., Maier, M., Michel, K., Petry, T., Sauer, U.G., Warheit, D., Wiench, K., Landsiedel, R., 2014. A critical appraisal of existing concepts for the grouping of nanomaterials. Regul. Toxicol. Pharmacol. 70 (2), 492—506. http://dx.doi.org/10.1016/j.yrtph.2014.07.025, 2014 Nov.

Arts, J.H., Hadi, M., Irfan, M.A., Keene, A.M., Kreiling, R., Lyon, D., Maier, M., Michel, K., Petry, T., Sauer, U.G., Warheit, D., Wiench, K., Wohlleben, W., Landsiedel, R., 2015a. A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping). Regul. Toxicol. Pharmacol. 71 (2 Suppl. I), S1—S27. http://dx.doi.org/10.1016/j.yrtph.2015.03.007, 2015 Mar 15.

Arts, J.H., Irfan, M.A., Keene, A.M., Kreiling, R., Lyon, D., Maier, M., Michel, K., Neubauer, N., Petry, T., Sauer, U.G., Warheit, D., Wiench, K., Wohlleben, W., Landsiedel, R., 2016 Apr. Case studies putting the decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping) into practice. Regul. Toxicol. Pharmacol. 76, 234—261. http://dx.doi.org/10.1016/j.yrtph.2015.11.020. Epub 2015 Dec 10.

Bachler, G., von Goetz, N., Hungerbühler, K., 2013. A physiologically based pharmacokinetic model for ionic silver and silver nanoparticles. Int. J. Nanomed. 2013 (8), 3365—3382. http://dx.doi.org/10.2147/IJN.S46624.

BAuA, 2013. Announcements on Hazardous Substances- Manufactured Nanomaterials Announcement 527. Joint Ministerial Gazette (GMBl), May 2013. No25, pp. 498—511. http://www.baua.de/en/Topics-from-A-to-Z/Hazardous-Substances/TRGS/pdf/Announcement-527.pdf?__blob=publicationFile&v=3.

Bos, P.M., Gottardo, S., Scott-Fordsmand, J.J., van Tongeren, M., Semenzin, E., Fernandes, T.F., Hristozov, D., Hund-Rinke, K., Hunt, N., Irfan, M.A., Landsiedel, R., Peijnenburg, W.J., Sánchez Jiménez, A., van Kesteren, P.C., Oomen, A.G., 2015. The MARINA risk assessment strategy: a flexible strategy for efficient information collection and risk assessment of nanomaterials. Int. J. Environ. Res. Public Health 12 (12), 15007—15021. http://dx.doi.org/10.3390/

ijerph121214961, 2015 Nov 27.

Chen, M., von Mikecz, A., 2005. Formation of nucleoplasmic protein aggregates impairs nuclear function in response to SiO2 nanoparticles. Exp. Cell Res. 305 (1), 51–62, 2005 Apr 15.

Chen, R., Hu, B., Liu, Y., Xu, J., Yang, G., Xu, D., Chen, C., 2016. Beyond PM2.5: the role of ultrafine particles on adverse health effects of air pollution. Biochim. Biophys. Acta 30074–30075, 2016 Mar 16. pii: S0304-4165.

Cho, W.S., Duffin, R., Thielbeer, F., Bradley, M., Megson, I.L., Macnee, W., Poland, C.A., Tran, C.L., Donaldson, K., 2012. Zeta potential and solubility to toxic ions as mechanisms of lung inflammation caused by metal/metal oxide nanoparticles. Toxicol. Sci. 126 (2), 469–477, 2012 Apr.

Collins, A.R., 2004. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol. Biotechnol. 26 (3), 249–261, 2004 Mar Review.

Creton, S., Aardema, M.J., Carmichael, P.L., Harvey, J.S., Martin, F.L., Newbold, R.F., O'Donovan, M.R., Pant, K., Poth, A., Sakai, A., Sasaki, K., Scott, A.D., Schechtman, L.M., Shen, R.R., Tanaka, N., Yasaei, H., 2012. Cell transformation assays for prediction of carcinogenic potential: state of the science and future research needs. Mutagenesis 27 (1), 93–101. http://dx.doi.org/10.1093/mutage/ger053, 2012 Jan.

Delmaar, C.J., Peijnenburg, W.J., Oomen, A.G., Chen, J., de Jong, W.H., Sips, A.J., Wang, Z., Park, M.V., 2015. A practical approach to determine dose metrics for nanomaterials. Environ. Toxicol. Chem. 34 (5), 1015–1022. http://dx.doi.org/10.1002/etc.2878, 2015 May.

De Temmerman, P., Verleysen, E., Lammertyn, J., Mast, J., 2014. Semi-automatic size measurement of primary particles in aggregated nanomaterials by transmission electron microscopy. Powder Technol. 261 (2014), 191–200.

DG Growth, 2016. Nanomaterials. Available at: http://ec.europa.eu/growth/sectors/chemicals/reach/nanomaterials/index_en.htm.

Dobrovolskaia, M.A., Germolec, D.R., Weaver, J.L., 2009. Evaluation of nanoparticle immunotoxicity. Nat. Nanotechnol. 4, 411–414.

Dobrovolskaia, M.A., Shurin, M., Shvedova, A.A., 2016. Current understanding of interactions between nanoparticles and the immune system. Toxicol. Appl. Pharmacol. 15 (299), 78–89. http://dx.doi.org/10.1016/j.taap.2015.12.022, 2016 May.

Domínguez, A., Suárez-Merino, B., Goñi-de-Cerio, F., 2014. Nanoparticles and blood-brain barrier: the key to central nervous system diseases. J. Nanosci. Nanotechnol. 14, 766–779.

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

Donaldson, K., Poland, C.A., Schins, R.P., 2010a. Possible genotoxic mechanisms of nanoparticles: criteria for improved test strategies. Nanotoxicology 2010 (4), 414–420.

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

EC, 2012a. Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee — Second Regulatory Review on Nanomaterials {SWD(2012) 288 Final}. COM(2012) 572 final. Commission of the European Communities (EC), Brussels, Belgium. http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52012DC0572&from=EN.

ECHA, 2011. New Appendixes. Chapters R.7a, R.7b and R.7c of the Guidance on Information Requirements and Chemical Safety Assessment (IR & CSA) with Recommendations for Registering nanomaterials.).

ECHA, 2012. Guidance on Information Requirements and Chemical Safety Assessment — Appendix R7-1 Recommendations for Nanomaterials Applicable to Chapter R7a Endpoint Specific Guidance. Guidance for the Implementation of REACH. European Chemicals Agency (ECHA), Helsinki, Finland. Available at: https://echa.europa.eu/documents/10162/22334053/draft_appendix_r7a_v2_peg_en.pdf.

ECHA, JRC, RIVM, 2016. Usage of (Eco)toxicological Data for Bridging Data Gaps between and Grouping of Nanoforms of the Same Substance. Elements to Consider. http://echa.europa.eu/documents/10162/13630/eco_toxicological_for_bridging_grouping_nanoforms_en.pdf.

EC, 2012b. Second Regulatory Review on Nanomaterials. COM(2012) 572 Final, EC, Brussel 2-10-2012. Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52012DC0572.

ECETOC, 2012. ECETOC TRA Version 3: Background and Rationale for the Improvements. Technical Report No. 114. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium.

ECHA, 2013. Grouping of Substances and Read-across Approach Part 1: Introductory Note and Part 2: Read-across Illustrative Example (Analogue Approach: Similarity Based on Breakdown Products). Accessible via. https://echa.europa.eu/support/grouping-of-substances-and-read-across.

ECVAM, 2013. Permeability Assay on Caco-2 Cells. Protocol no 142. ECVAM, 2013. Available at: http://ecvam-dbalm.jrc.ec.europa.eu/.

EFSA Scientific Committee, 2011. Scientific opinion on guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. EFSA J. 9, 2140.

EFSA, 2012. Minimum Criteria for the Acceptance of in vivo Alkaline Comet Assay Reports. European Food Safety Authority. European Food Safety Authority (EFSA), Parma, Italy.

Elsabahy, M., Wooley, K.L., 2013. Cytokines as biomarkers of nanoparticle immunotoxicity. Chem Soc Rev. 42 (12), 5552–5576. http://dx.doi.org/10.1039/c3cs60064e, 2013 June 21.

EU (2006) Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. O. J. L 396: 1–849.

EU, 2009. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products (recast). O. J. L 342, 59–209.

EU, 2012. Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. Off. J. Eur. Union L 167, 1–123.

Fenech, M., 2000 Nov 20. The in vitro micronucleus technique. Mutat Res. 455 (1–2), 81–95. Review.

Fröhlich, E., 2013. Cellular targets and mechanisms in the cytotoxic action of non-biodegradable engineered nanoparticles. Curr. Drug Metab. 14 (9), 976–988, 2013 Nov.

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

Hankin, S.M., Peters, S.A.K., Poland, C.A., Foss Hansen, S., Holmqvist, J., Ross, B.L., Varet, J., Aitken, R.J., 2011. Specific Advice on Fulfilling Information Requirements for Nanomaterials under REACH (RIP-oN 2) — Final Project. ReportRNC/RIP-oN2/FPR/1/FINAL.

Hoet, P.H., Brüske-Hohlfeld, Salata, OV, 2004. Nanoparticles - known and unknown health risks. J. Nanobiotechnol. 2 (1), 12, 2004 Dec 8.

Hsieh, S.F., Bello, D., Schmidt, D.F., Pal, A.K., Stella, A., Isaacs, J.A., Rogers, E.J., 2013. Mapping the biological oxidative damage of engineered nanomaterials. Small 9 (9–10), 1853–1865. http://dx.doi.org/10.1002/smll.201201995, 2013 May 27.

JRC, 2012. Linsinger T et al, Requirements on measurements for the implementation of the European Commission definition of the term nanomaterial. JRC, Ispra, Italy.

JRC, 2014a. Rauscher H. And Roebben G. Towards a Review of the EC Recommendation for a Definition of the Term "nanomaterial". Part 1: Compilation of Information Concerning the Experience with the Definition. JRC, Ispra, Italy.

JRC, 2014b. Rauscher H. And Roebben G. Towards a Review of the EC Recommendation for a Definition of the Term "nanomaterial". Part 2: Assessment of Collected Information Concerning the Experience with the Definition. JRC, Ispra, Italy.

JRC, 2015. Rauscher H. And Roebben G. Towards a Review of the EC Recommendation for a Definition of the Term "nanomaterial". Part 3: Scientific-technical Evaluation of Options to Clarify the Definition and to Facilitate. JRC, Ispra, Italy.

Kermanizadeh, A., Balharry, D., Wallin, H., Loft, S., Møller, P., 2015. Nanomaterial translocation–the biokinetics, tissue accumulation, toxicity and fate of materials in secondary organs–a review. Crit. Rev. Toxicol. 45 (10), 837–872. http://dx.doi.org/10.3109/10408444.2015.1058747.

Kettiger, H., Schipanski, A., Wick, P., Huwyler, J., 2013. Engineered nanomaterial uptake and tissue distribution: from cell to organism. Int. J. Nanomed. 8, 3255–3269.

Lin, Z., Monteiro-Riviere, N.A., Riviere, J.E., 2015. Pharmacokinetics of metallic nanoparticles. Wiley Interdiscip. Rev. Nanomed Nanobiotechnol 7 (2), 189–217. http://dx.doi.org/10.1002/wnan.1304, 2015 Mar-Apr.

Lin, Z., Monteiro-Riviere, N.A., Riviere, J.E., 2016. A physiologically based pharmacokinetic model for polyethylene glycol-coated gold nanoparticles of different sizes in adult mice. Nanotoxicology 10 (2), 162–172. http://dx.doi.org/10.3109/17435390.2015.1027314, 2016 Mar.

Louro, H., Bettencourt, A., Gonçalves, L.M., Almeida, A.J., Silva, M.J., 2015. Role of nanogenotoxicology studies in safety evaluation of nanomaterials. In: Thomas, Sabu, Grohens, Yves, Ninan, Neethu (Eds.), Nanotechnology Applications in Tissue Engineering. Elsevier Inc., Oxford, UK, pp. 263–288.

Malsch, I., Subramanian, V., Semenzin, E., Hristozov, D., Marcomini, A., 2015. Supporting decision-making Sustain. Nanotechnol. Environ Syst Decis 35, 54–75. http://dx.doi.org/10.1007/s10669-015-9539-4.

Maynard, A.D., Aitken, R.J., Butz, T., Colvin, V., Donaldson, K., Oberdörster, G., Philbert, M.A., Ryan, J., Seaton, A., Stone, V., Tinkle, S.S., Tran, L., Walker, N.J., Warheit, D.B., 2006. Safe handling of nanotechnology. Nature 444 (7117), 267–269, 2006 Nov 16.

NANoREG, 2015. Critical Exposure Scenarios - Deliverable 3.1. Available at: http://www.nanoreg.eu/images/2015_05_20_NANoREG_Factsheet_D3.1.pdf.

Nel, A.E., Mädler, L., Velegol, D., Xia, T., Hoek, E.M., Somasundaran, P., Klaessig, F., Castranova, V., Thompson, M., 2009. Understanding biophysicochemical interactions at the nano-bio interface. Nat. Mater. 8 (7), 543–557. http://dx.doi.org/10.1038/nmat2442, 2009 Jul.

Nel, A., Xia, T., Meng, H., Wang, X., Lin, S., Ji, Z., Zhang, H., 2014. Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. Accounts Chem. Res. 2013 (46), 607–621.

Oberdorster, G., Oberdorster, E., Oberdorster, J., 2009. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ. Health Perspect. 113 (7), 823–839. http://dx.doi.org/10.1289/ehp.7339.

OECD, 1997. OECD guidelines for the testing of chemicals: in vitro mammalian chromosome aberration test. In: OECD (Ed.), Test No. 473: 1997.

OECD, 2010. OECD guidelines for the testing of chemicals: in vitro mammalian cell micronucleus test. In: OECD (Ed.), Test No. 487: 2010.

OECD, 2014a. Report of the OECD expert meeting on the physical chemical properties of manufactured nanomaterials and test guidelines. OECD Ser. Saf. Manuf. Nanomater. 15. No. 41, ENV/JM/MONO.

OECD, 2014b. Environment, Health and Safety Publications. Guidance on Grouping of Chemicals, second ed. Series on Testing & Assessment No. 194, ENV/JM/MONO(2014)4.

OECD, 2013. Recommendation of the Council on the Safety Testing and Assessment of Manufactured Nanomaterials, 19 September 2013-C(2013)107. Available at: http://webnet.oecd.org/OECDACTS/Instruments/ListByTypeView.aspx.

OECD, 2007-2016. Testing Programme of Manufactured Nanomaterials. Available at: http://www.oecd.org/chemicalsafety/nanosafety/overview-testing-programme-manufactured-nanomaterials.htm.

Oomen, A.G., Bleeker, E.A., Bos, P.M., van Broekhuizen, F., Gottardo, S., Groenewold, M., Hristozov, D., Hund-Rinke, K., Irfan, M.A., Marcomini, A., Peijnenburg, W.J., Rasmussen, K., Jiménez, A.S., Scott-Fordsmand, J.J., van Tongeren, M., Wiench, K., Wohlleben, W., Landsiedel, R., 2015. Grouping and read-across approaches for risk assessment of nanomaterials. Int. J. Environ. Res. Public Health. 12 (10), 13415—13434. http://dx.doi.org/10.3390/ijerph121013415, 2015 Oct 26.

Oomen, A.G., Bos, P.M., Fernandes, T.F., Hund-Rinke, K., Boraschi, D., Byrne, H.J., Aschberger, K., Gottardo, S., von der Kammer, F., Kühnel, D., Hristozov, D., Marcomini, A., Migliore, L., Scott-Fordsmand, J., Wick, P., Landsiedel, R., 2014. Concern-driven integrated approaches to nanomaterial testing and assessment—report of the NanoSafety Cluster Working Group 10. Nanotoxicology 8 (3), 334—348. http://dx.doi.org/10.3109/17435390.2013.802387, 2014 May.

Powell, J.J., Faria, N., Thomas-McKay, E., Pele, L.C., 2010. Origin and fate of dietary nanoparticles and microparticles in the gastrointestinal tract. J. Autoimmun. 34 (3), J226—J233. http://dx.doi.org/10.1016/j.jaut.2009.11.006, 2010 May Review.

Riebeling, C., Wiemann, M., Schnekenburger, J., Kuhlbusch, T.A., Wohlleben, W., Luch, A., Haase, A., 2016. A redox proteomics approach to investigate the mode of action of nanomaterials. Toxicol. Appl. Pharmacol. 15 (299), 24—29. http://dx.doi.org/10.1016/j.taap.2016.01.019, 2016 May.

RIVM, 2015. ConsExpo Nano. https://www.consexponano.nl/.

Roberts, G., 2016. Commission Rejects Idea of EU Nano Register: Officials Also Confirm Changes to REACH Annexes Won't Apply before 2018 Deadline. Chemical Watch: Global risk and regulation news, 16 March 2016/Europe, Nanomaterials. Available at: https://chemicalwatch.com/45776/commission-rejects-idea-of-eu-nano-register.

Schleh, C., Semmler-Behnke, M., Lipka, J., Wenk, A., Hirn, S., Schäffler, M., Schmid, G., Simon, U., Kreyling, W.G., 2012. Size and surface charge of gold nanoparticles determine absorption across intestinal barriers and accumulation in secondary target organs after oral administration. Nanotoxicology 6 (1).

Schulz, H., Brand, P., Heyder, J., 2000. In: Gehr, Heyder (Eds.), Particle-lung Interactions: Chapter 5: Particle Deposition in the Respiratory Tract. Marcel Dekker Inc., New York — Basel. Available at: ftp://ftp.cdc.gov/pub/Documents/OEL/02.%20Kuempel/References/Schulz_2000-Particle-lung%20interactions.pdf.

SCENIHR, 2007. The Appropriateness of the Risk Assessment Methodology in Accordance with the Technical Guidance Documents for New and Existing Substances for Assessing the Risks of Nanomaterials, pp. 21—22. June 2007.

SCENIHR, 2009. Risk Assessment of Products of Nanotechnologies, 19 January 2009.

SCENIHR, 2015. Guidance on the Determination of Potential Health Effects of Nanomaterials Used in Medical Devices, 6 January 2015.

Sellers, K., Deleebeeck, N.M.E., Messiaen, M., Jackson, M., Bleeker, E.A.J., Sijm, D.T.H.M., Van Broekhuizen, F.A., 2015. Grouping Nanomaterials a Strategy towards Grouping and Read-across RIVM Report 2015—0061.

Singh, N., Manshian, B., Jenkins, G.J., Griffiths, S.M., Williams, P.M., Maffeis, T.G., Wright, C.J., Doak, S.H., 2009. NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials 30 (23—24), 3891—3914.

Silva, M.J., Costa, P., Dias, A., Valente, M., Louro, H., Boavida, M.G., 2005. Comparative analysis of the mutagenic activity of oxaliplatin and cisplatin in the Hprt gene of CHO cells. Environ. Mol. Mutagen 46 (2), 104—115, 2005 Aug.

Sips, A.J.A.M., Noorlander, C.W., Lehman, H.C., Hoehener, C., 2015. NANoREG Safe-by-design (SbD) Concept. Available at: http://www.nanoreg.eu/images/20150530_SbD_Working_Draft_EU_US_and_ProSafe.pdf.

Stone, V., Pozzi-Mucelli, S., Tran, L., Aschberger, K., Sabella, S., Vogel, U., Poland, C., Balharry, D., Fernandes, T., Gottardo, S., Hankin, S., Hartl, M.G., Hartmann, N., Hristozov, D., Hund-Rinke, K., Johnston, H., Marcomini, A., Panzer, O., Roncato, D., Saber, A.T., Wallin, H., Scott-Fordsmand, J.J., 2014. ITS-NANO-—prioritising nanosafety research to develop a stakeholder driven intelligent testing strategy. Part Fibre Toxicol. 11 (9) http://dx.doi.org/10.1186/1743-8977-11-9, 2014 Feb 13.

Tantra, R., Bouwmeester, H., Bolea, E., Rey-Castro, C., David, C.A., Dogné, J.M., Jarman, J., Laborda, F., Laloy, J., Robinson, K.N., Undas, A.K., van der Zande, M., 2015. Suitability of analytical methods to measure solubility for the purpose of nanoregulation. Nanotoxicology 22, 1—12, 2015 May.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen 35 (3), 206—221.

Van Kesteren, P.C., Cubadda, F., Bouwmeester, H., van Eijkeren, J.C., Dekkers, S., de Jong, W.H., Oomen, A.G., 2015. Novel insights into the risk assessment of the nanomaterial synthetic amorphous silica, additive E551, in food. Nanotoxicology 9 (4), 442—452. http://dx.doi.org/10.3109/17435390.2014.940408, 2015 May.

Wijnhoven, S.W.P., Dekkers, S., Hagens, W.I., De Jong, W.H., 2009. Exposure to Nanomaterials in Consumer Products. RIVM Letter Report 340370001/2009. http://www.rivm.nl/dsresource?objectid=rivmp:13485&type=org&disposition=inline.

Zhang, H., Ji, Z., Xia, T., Meng, H., Low-Kam, C., Liu, R., Pokhrel, S., Lin, S., Wang, X., Liao, Y.P., Wang, M., Li, L., Rallo, R., Damoiseaux, R., Telesca, D., Mädler, L., Cohen, Y., Zink, J.I., Nel, A.E., 2013. Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. ACS Nano 6 (5), 4349—4368. http://dx.doi.org/10.1021/nn3010087, 2012 May 22, Epub 2012 Apr 24.