In vitro approaches to assess the hazard of nanomaterials

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ABSTRACT

The rapid development of engineered nanomaterials demands for a fast and reliable assessment of their health hazard potential. A plethora of experimental approaches have been developed and are widely employed in conventional toxicological approaches. However, the specific properties of nanomaterials such as smaller size but larger surface area, and high catalytic reactivity and distinctive optical properties compared to their respective bulk entities, often disable a straightforward use of established *in vitro* approaches. Herein, we provide an overview of the current state-of the art nanomaterial hazard assessment strategies using *in vitro* approaches. This perspective has been developed based on a thorough review of over 200 studies employing such methods to assess the biological response upon exposure to a diverse array of nanomaterials. The majority of the studies under review has been, but not limited to, engaged in the European 7th Framework Programme for Research and Technological Development and published in the last five years. Based on the most widely used methods and/or the most relevant biological endpoints, we have provided some general recommendations on the use of the selected approaches which would the most closely mimic realistic exposure scenarios as well as enabling to yield fast, reliable and reproducible data on the nanomaterial-cell response *in vitro*. In addition, the applicability of the approaches to translate *in vitro* outcomes to leverage those of *in vivo* studies has been proposed. It is finally suggested that an improved comprehension of the approaches with its limitations used for nanomaterials' hazard assessment *in vitro* will improve the interpretation of the existing nanotoxicological data as well as underline the basic principles in understanding interactions of engineered nanomaterials at a cellular level; this all is imperative for their safe-by-design strategies, and should also enable subsequent regulatory approvals.

1. Introduction

Nanotechnology enables the engineering of nanomaterials *i.e.* materials with any external dimension or internal/structural dimension in the nanoscale, with remarkable new physical and chemical properties that differ from their bulk equivalents. This huge potential has led to an increasing growth of research and development activities and created an entire new class of materials which are used in a broad field of applications such as in optics, electronics (*e.g.* for efficient and cost-effective energy storage or their use as semiconductors) (Jariwala et al., 2013), and in the medical field as potential carriers for drug and gene delivery or as diagnostic tools and contrast agents (De Jong and Borm, 2008). However, these new properties and the increasing industrial production have raised concerns about potential adverse effects for human health; thus, a better understanding of cellular consequences upon the direct exposure of (human) cells to these engineered nanomaterials (NMs) is prerequisite for their safe and successful use in any applications.

The number of newly developed NMs with different core materials, sizes, shapes, and coatings is huge (McWilliams, 2016) and expectations from society, consumer and regulatory bodies about their safety are increasing. The characteristics of NMs can be influenced by various physico-chemical parameters, in addition, a proper safety assessment of every nanoform would be extremely cost-intensive and time-consuming. Moreover, the outcomes of animal testing regarding its predictive power for human beings exhibit certain limitations, mainly due to physiological and biochemical species dissimilarities (Shanks et al., 2009). In addition to that, the principle of the 3Rs – Replacement, Reduction and Refinement – has become an increasing public and legal demand which ethically supports the replacement of animal use with

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more human-relevant alternatives that do not rely on *in vivo* testing (Tornqvist et al., 2014). New concepts for efficient, cheaper and evidence-based testing strategies were proposed, based on the use of human primary cells and cell lines (Council NR, 2007). In addition, endpoints for health effects and *in vitro* tests of regulatory interest for conventional chemicals are contained in the Organisation for Economic Co-operation and Development (OECD) and its test guidelines documents (TG) (OECD, 2013a). These *in vitro* tests are rather narrow in their coverage of endpoints: they address genetic toxicity (*e.g.* (OECD, 2015a; OECD, 2014b; OECD, 2014c)), dermal absorption (OECD, 2004) and skin and eye irritation (OECD, 2013b; OECD, 2015b; OECD, 2015c), endocrine disruption (*e.g.* (OECD, 2015d)), and few other selected endpoints. But skin penetration has not been a major concern for NMs while endocrine disruptor effects for NMs are also not currently a focus of research or regulatory concern. Rather, the most relevant *in vitro* protocols for NMs align with the current major routes of NM exposures. Besides dermal (NMs in cosmetic products) and oral (NMs in food products) exposures the effects due to NM inhalation are currently considered to be the most relevant.

Cellular responses have been observed upon exposure to NMs and currently several hypotheses regarding how NMs induce adverse cellular effects exist: (i) *via* oxidative means (the oxidative stress paradigm) which then leads to pro-inflammatory effects (Donaldson et al., 2003), (ii) *via* the fibre paradigm (Dorger et al., 2001; Donaldson and Tran, 2004) (iii) through genotoxicity (Schins and Knaapen, 2007), and (iv) *via* NM dissolution, *i.e.* release of potentially toxic ions and/or other constituents (Bergin and Witzmann, 2013; Braakhuis et al., 2014). The fibre paradigm was highlighted in the paper by Poland et al. (Poland et al., 2008) in which it was shown that multi-walled carbon nanotubes caused granulomas in the peritoneal cavity. This paradigm however, can only be attributed to nanofibres in particular to those with the specific characteristics of high rigidity and high aspect ratio NMs (HARN) (Donaldson et al., 2010).

Other endpoints for NMs which can be examined *in vitro* include those which test for the biological fate of NMs at the cellular or multicellular levels such as size exclusion criteria for given key cell types (Zhu et al., 2013), and adverse effects such as fibrogenicity at these levels of organization (Azad et al., 2013).

The goal of some ongoing research is to unravel modes of action (MOA) of NMs using a plethora of functional assays which are designed to indicate certain MOA relevant to the toxicity and/or fate of NMs and to elucidate biokinetics of NMs, *e.g.* transport through interfaces like air-liquid interface (ALI). It is anticipated that the obtained information on MOA and biokinetics can later be used in weight of evidence analyses or tiered testing schemes in combination with other *in vivo* data, leading to reduction and eventual replacement of *in vivo* tests.

These approaches may help to reduce and/or replace and reduce animal testing according to the 3R strategy. With all these goals, it is critical to use environmentally or occupationally relevant NM concentrations, and to be able to relate these *in vitro* test concentrations to *in vivo* test exposures so that results can be correlated and used in a regulatory context.

A comprehensive review about the relevance of *in vitro* nanotoxicological studies in hazard assessment of NMs has been provided by Park and colleagues in 2009 (Park et al., 2009) where comparison of different cell types and exposure duration was discussed, along with, among others, dose response analysis and potential artefacts in the most commonly used nanotoxicological assays. Since then many new studies have been reported and this review has been developed based on an in-depth summary of over 200 literature reports on the assessment of biological hazard of NMs *in vitro*. The basis for the selection of the selected and relevant results, protocols, and guidance documents, where chosen from the project “A common European approach to the regulatory testing of Manufactured Nanomaterials” (NANoREG) (data deliverables from WP2, WP3, WP4 and WP5; www.nanoreg.eu; (Joint Research Centre, JRC, 2016)), together with the OECD working party of manufactured NMs (WPMN) activities. Peer reviewed publications from other the 7th framework programme for research and technological development (FP 7) projects and US research programmes have been included. However, as some aspects were not comprehensively covered in the above-mentioned literature pool, the authors have searched for other relevant studies employing the publicly available search tools (Web of Science, Pubmed, Google scholar). The aim of this overview is to provide general recommendations for researchers employing *in vitro* assays for assessment of NM hazard based on an extensive literature study. All the recommendations proposed in this manuscript have been developed based on the authors' perspective on the existing literature data on this matter, as well on their experiences with *in vitro* studies for regulatory submissions. Therefore, this overview of the most widely used methodological approaches can serve as a basis for future research directions including thoughts about reported pitfalls for some of the methods and/or approaches.

2. General considerations for *in vitro* test methods

2.1. Nanomaterials

The description of the source of NMs and characterization data has to be given with sufficient detail, including a thorough characterization of both the pristine materials as well as *in situ* (before, during and after the experiments). Additionally, the details of any dispersions methods used for the experiments need to be reported (*discussed in more details in the subchapter 2.3 Dose metrics*).The majority of the reviewed *in vitro* studies report the primary sizes of NMs (transmission electron microscopy (TEM), the hydrodynamic diameter (dynamic light scattering (DLS)) and the zeta potential in water, and/or phosphate-buffered saline (PBS) and/or cell culture medium (Stoehr et al., 2011; Anguissola et al., 2014; Huo et al., 2015a; Shannahan et al., 2015), and specific surface area (Huk et al., 2014; Armand et al., 2016a). In addition to DLS, in some cases, nanoparticle tracking analysis (Di Cristo et al., 2016), differential centrifugal sedimentation (Monopoli et al., 2011; Wan et al., 2015) and photon cross correlation spectroscopy (Gliga et al., 2014) are being used. Regarding light scattering techniques, careful data interpretation is required as agglomeration and sedimentation can occur simultaneously, particularly in the cell culture medium. Particle agglomeration in the cell medium can be, depending on the NM type, investigated by *e.g.* ultraviolet–visible spectroscopy (UV–Vis) (Gliga et al., 2014). Specific surface area can be determined by nitrogen adsorption (Huk et al., 2014); however this approach requires relatively high material masses and is carried out on a powder sample, which is not always feasible in nanotoxicological studies. Depending on the type, inductively coupled plasma (ICP) techniques including ICP-optical emission spectroscopy (-OES) and ICP-mass spectrometry (MS) can be applied for mass concentration of the metal, and UV–Vis for size determination of plasmonic NMs such as silver or gold nanoparticles (NPs) (Stoehr et al., 2011; Gliga et al., 2014; Pang et al., 2016). For soluble NMs, *e.g.* silver NPs or zinc oxide, kinetics of dissolution in cell culture media needs to be assessed over time (Huk et al., 2014; Mu et al., 2014). Noteworthy, transformations of NMs in time (particularly when stored in suspensions) has been suggested as one of the most significant contributors to the contradictory *in vitro* toxicity results observed in the literature for identical NMs; hence, aging needs to be addressed in parallel with the assessment of effects (Izak-Nau et al., 2015). Surface reactivity of NMs in a cell free environment can be measured by the electron spin resonance (ESR) technique; briefly, presence of free radicals can be detected by using so called spin traps, reagents that form adducts to stabilize the radicals, which then exhibit a paramagnetic resonance detectable by spectroscopy (Monopoli et al., 2011). Also, there are other techniques for detection of ROS/free radicals, for instance *via* cytochrome *c* reduction (Dikalov and Harrison, 2014), ferric-reducing ability of serum (FRAS) assay and dichlorofluorescein assay (Pal et al., 2014).

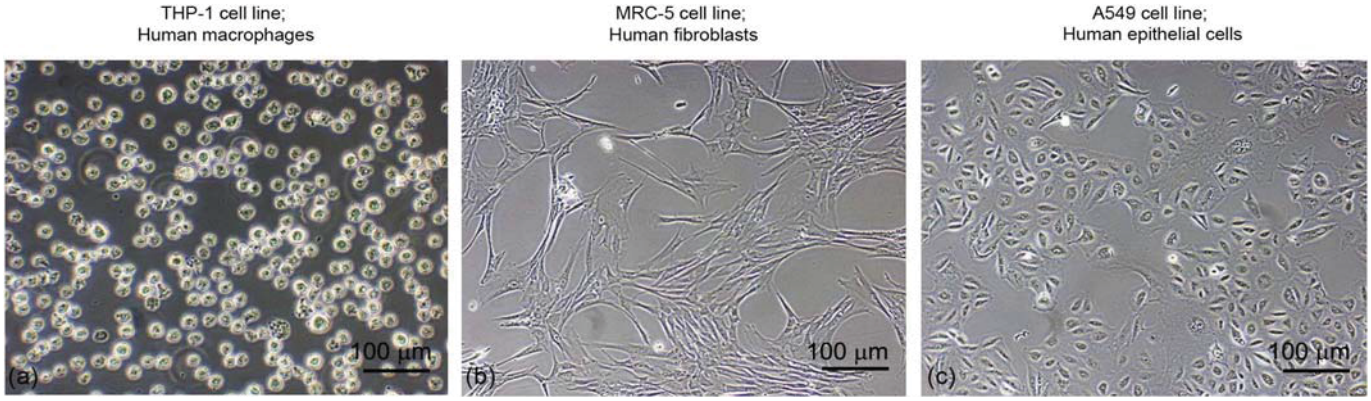

Fig. 1. Phase contrast light microscopy images of human cell lines: macrophages (THP-1, 1a), fibroblasts (MRC-5, 1b) and alveolar epithelial cells (A549, 1c).

The importance of the individual properties also differs with respect to the exposure scenarios. For inhalation – which is accepted as one of the most likely routes for NMs entering the body – the physico-chemical properties of NMs affecting material deposition and clearance from the lungs are of particular importance. It is important to understand that there is no individual particle property dictating this, but rather a combination of them, predominantly: (i) size of individual particles or agglomerates present in aerosols which influences the region of deposition in the respiratory system, (ii) surface reactivity as a predictor for induction of oxidative stress and pulmonary inflammation, and (iii) shape which has gained attention especially regarding the fibrous NMs which are prone to cause asbestos-like effects due to the diminished clearance/increased biopersistence[2] in the lungs (Utembe et al., 2015). With submerged *in vitro* studies, biodurability of NMs *e.g.* dissolution rate is also important, particularly for fast dissolving particles (as the effect is driven by their chemical composition), *e.g.* the released ions may present an important source of NM toxicity. When such dissolving NMs are phagocytized by alveolar macrophages the dissolution rate might even be accelerated compared to that in the epithelial lining fluid which can lead to enhanced inflammation in the lungs (reviewed in: (OECD, 2015c)).

Until now, no optimal set of techniques has been proposed for characterizing the physicochemical properties either for a specific type of NMs or for a general assessment of a variety NMs. However, elsewhere in this journal, Lowry et al. (2017 - in this issue) propose a minimal set of such properties broadly applicable to NM regulatory assessments (Lowry et al., 2017 - in this issue). In addition, the practical aspect of the techniques available at the researcher's organization needs to be taken into account. However, it is recommended that not only one single technique, but a combination of the appropriate ones should be employed for an accurate description of NMs. Such a strategy has also been proposed in a review from the FP 7 NanoTEST project (Dusinska et al., 2015) and has already been indicated previously by the European food and safety authority (EFSA (EFSA, 2011)), the Scientific Committee on Consumer Safety (SCCS (SCCS, 2012)) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR (SCENIHR, 2015)) guidance on use of NMs in food/feed, cosmetics and medical devices. The properties considered to be the most relevant for the toxicology of NMs include size, size distribution, shape, agglomeration state, density, surface area, surface charge, surface reactivity, solubility and crystalline phase (Gordon et al., 2014). In addition, the effective density which is an indicator of possible agglomerate formation (DeLoid et al., 2014); hydrophobicity (Farcal et al., 2015) and the extent of impurities/contaminants (Pulskamp et al., 2007) are important factors which can determine the hazard of the NMs.

[2] For definition of biopersistence and biodurability see Utembe et al., 2015: Biopersistence of is defined as the extent to which they are able to resist chemical, physical, and other physiological clearance mechanisms in the body. Biodurability, defined as the ability to resist chemical/biochemical alteration, is a significant contributor to biopersistence.

2.2. Cell cultures

In order to repeat previously published experiments, and for inter-laboratory comparisons, it is important to report the cell source, passage number, and precise cell culture method (Gstraunthaler and Hartung, 2002). This also includes *e.g.* the brand of the laboratory plastics used for culturing and in the assays, as well as a thorough description of the cell growth, morphology and differentiation before and during the test performance. Such a detailed description and characterization of the cells used is unfortunately missing in most of the reviewed publications, which makes replication or comparison very difficult. In some of the publications the cell proliferation rate, *i.e.* cell growth, was assessed (Armand et al., 2016a; Izak-Nau et al., 2015; Huk et al., 2015a) and the morphology has been observed by bright field microscopy (Huk et al., 2014).

The use of cell culture medium (foetal calf or bovine serum (FCS/FBS)), and the choice of the cultivating material (cell culture flasks in which the cells are grown) are not standardized. Also, the time of the cells in culture, meaning the passage number of cells in the experiment and the exposure duration, significantly differs among the publications.

It is important to justify the selection of the cells used. Fibroblasts (usually cell lines) (Fig. 1b) are commonly used to assess pro-fibrotic signals (Azad et al., 2013; Wang et al., 2015), whereas the rationale behind the selection of macrophages (Fig. 1a) is based on the fact that they are primary cells and that they act as the first responders upon exposure to NMs (Herd et al., 2015; Palomaki et al., 2015). In addition, many studies use intestine or lung epithelial (Fig. 1c) cells because they represent the primary organs of entry for NMs (Rothen-Rutishauser et al., 2012).

In most of the studies screened, cell lines are preferred over primary cells due to their homogeneity and greater stability; this yields better data reproducibility as it is impossible to exactly reproduce the isolates batches of primary cells. Primary cultures also face limitations such as the lack of tissue availability, limited number of cells that can be yielded from each isolation and donor-specific variations. Yet the advantage of primary cells is that they can (depending on the cell type) survive for prolonged periods of time thus enabling it more possible to analyse subacute or sub-chronic exposure duration effects *in vitro* with respect to the exposure duration. However, there are also other limitations of *in vitro* sub- and chronic exposure studies, such as the fast cell division of most of the cell lines and possible de-differentiation during prolonged time in culture. Also certain cell lines can be used for long-term experiments (Thurnherr et al., 2011), however they have to be subcultured routinely because of their continuous growth, and thus differentiation might not be well reflected. With initial testing of a new NM, the use of primary cells might be preferred over cell lines since in

certain cases cell lines might not be responsive for certain cell reactions because of their de-differentiation in culture. In addition, the use of human cell lines is supported since they more closely mimic human responses in comparison to rodent cell lines; rodent cell lines might, however, be important to include for comparison of the results with animal data.

Recently, the importance of specific disease-related endpoints and receptive relevant experimental conditions has been highlighted. One example is the human pulmonary edema model on a chip which is a co-culture of human alveolar epithelial and pulmonary microvascular endothelial cells stimulated with interleukin-2 (Huh et al., 2012). It is also emphasized that more advanced human *in vitro* models should be used to assess interactions and possible effects (Wick et al., 2014). However, so far no standardized and validated three-dimensional (3D) model, neither healthy nor diseased, has yet been accepted or approved for any NM type and application.

It is well-known that traditional *in vitro* cell monocultures can lack phenotypic details, physiological functions or only partially depict the complex cross-talks between cells. 3D culture models have been described to have the potential to be more predictive in toxicology testing thus filling a gap between two-dimensional (2D) system and animal experiments (Alepee et al., 2014). There are multitudes of new co-culture or 3D cell culture systems for different tissues developed during the last years, combining several relevant cell types for the same organ into one model-system which can be used to assess the interaction with NMs (Wick et al., 2014). However, only a few were carefully validated to whole organ or *in vivo* responses (Astashkina and Grainger, 2014). For instance, co-cultures of skin tissue composed of human epidermal keratinocytes and dermal fibroblasts which have been described in the 90s (Osborne and Perkins, 1991). As an alternative *in vitro* method for hazard identification of irritant substances reconstructed 3D human epidermis-based human models (OECD TG 439) (OECD, 2015c) have been designed which closely mimic the biochemical and physiological properties of the human epidermis. This assay has already been applied to test the irritation potential of NMs (Kim et al., 2016). In addition, since 2013 the OECD TG 431 (OECD, 2014a) is approved to use this *in vitro* system allowing the identification of corrosive chemical substances and mixtures; more efforts are required to develop and validate such advanced *in vitro* models for other tissue types. As of 2009, the OECD TG 437 Bovine Corneal Opacity and Permeability (BCOP) (OECD, 2013b) test method has been introduced to determine severely irritating substances. In the recent study by Kolle and colleagues (Kolle et al., 2016) a broad panel of NMs was tested using the combination of the EpiOcular™ Eye Irritation Test (OECD TG 492 (OECD, 2015b)) and the BCOP (OECD TG 437 (OECD, 2013b)). It has been proposed that this two-tier non-animal testing strategy could replace more traditional animal tests for these endpoints.

The use of cell lines in monoculture systems is recommended for the first stage NM hazard evaluation as they allow a better reproducibility and comparability for interlaboratory comparison of either the same NM type or among different NMs. Selection of cell lines strongly depends on the research question and the investigated endpoints. For a higher tier evaluation, the use of primary human cells and/or co-culture models is preferred to better understand NM mechanistic behaviour in more complex systems; however, such systems might be difficult to apply in high-throughput assays due to costs and reproducibility.

2.3. Dose metrics

2.3.1. Dispersion of NMs and physico-chemical NM characterization in suspensions

Harmonized protocols for NM suspension preparation are particularly important for the *in vitro* tests as both dispersion protocol and the cell culture media significantly alter their characteristics. For example, the standard operating procedure (SOP) developed under the Nanogenotox project aims to produce a highly dispersed state of NMs by ethanol pre-wetting of hydrophobic powders, followed by dispersion bovine serum albumin in water (NanoGenotox Joint Action report, addressed in (Farcal et al., 2015)). Besides powders, NMs can be obtained in pure or buffered water suspensions. Regardless, sonication might be required prior to addition to cell culture medium, but, importantly, this must be performed in water and subsequently diluted in culture media to the desired concentration for application to cells (DeLoid et al., 2017). Since probe sonication can induce production of reactive oxidative species (ROS) in suspension (and protein denaturation), ROS content must be checked using the cell free assays based on florigenic dyes which measure activity of ROS activity (Zhao and Riediker, 2014). In order to avoid interference of serum proteins with NMs, *in vitro* cell culture experiments can also be performed in serum-free medium, *i.e.* without the addition of FBS/FCS, or with reduced FBS/FCS content. Importantly, the amount of serum proteins, their pre-treatment (*e.g.* heat-inactivation) and the source can strongly effect NMs interactions with cells (Lesniak et al., 2010; Lesniak et al., 2012). It is important to mention that the cell behaviour in serum-free conditions has to be thoroughly observed. The use of stabilizers with mutagenic or toxic potency in the synthesis and dispersion preparations of NMs should be avoided where possible. For example, the detergent Tween 80 (already at a concentration of 0.008% *w/v*) caused a strong mutagenic effect whereas anionic surfactants (*e.g.* sodium dodecyl sulphate (SDS; 0.05%) and a polysaccharide chitosan (0.05% in 0.1% acetic acid) were found to be only weakly mutagenic (Huk et al., 2015a). Pluronic F127 has been described to effectively disperse carbon nanotubes (Kastrisianaki-Guyton et al., 2015); however, it also has been reported that the surfactant alone (*e.g.* Pluronic dispersants) can induce adverse effects, particularly upon sonication (Wang et al., 2013). It is difficult to recommend an optimal dispersant for all NMs since they differ in physico-chemical properties and it is, therefore, mandatory to include appropriate controls with the dispersant alone. Also, NM suspensions need to be tested for endotoxin presence such as lipopolysaccharide (LPS) using commercially available assays, for instance the limulus amebocyte lysate (LAL) endochrome test. At the moment, three different approaches of the *in vitro* LAL assay formats are available: gel-clot (semi-quantitative), turbidimetric, and chromogenic (quantitative) (Farcal et al., 2015; Dobrovolskaia et al., 2009; Giannakou et al., 2016a). As NMs interferences with the LAL assays are expected (due to the instance high adsorption capacity, optical properties, hydrophobicity, surface charge, and catalytic activities), the selection of the appropriate format is specific to the NM types and at least two assay formats should be used as to avoid either over- or underestimation of the endotoxin levels (Dobrovolskaia et al., 2009; Giannakou et al., 2016a). In all the assays, it is important to include: (i) “NM-only” controls in order to screen for the potential positive responses caused by NM interferences with the optical reading (absorbance, luminescence or fluorescence), (ii) “NM-plus-assay-reagents” controls to eliminate possible false positive responses arising from NM catalytic properties, *i.e.* the assay is run in the presence of NMs and all the assay reagents but in the absence of the tested biological sample, and (iii) the “inhibition/enhancement controls” for identifying false-positive or false-negative signals resulting from the interaction of NMs with the inflammatory signalling mediators, such as endotoxins and/or cytokines (Dobrovolskaia et al., 2009). Several approaches have been used traditionally to resolve interference of test samples with the LAL assays, *e.g.* diluting the test sample. However, if the interference cannot be overcome, additional methods may be required, such as: (i) extraction of endotoxin from NM formulation, *e.g.* by depyrogenation (dry heating in high temperature; yet this might have severe consequences on NM properties) or filtration (cannot be used for all the NM types), autoclaving and irradiation, as well as formaldehyde, ethylene oxide and gas plasma treatments. Furthermore, (ii) the endotoxin presence analysis *per se* can be done on particle free supernatants obtained by either dialysis of centrifugation. Overall, the optimal way is to avoid NM contamination by NM preparation using sterile, endotoxin-free

environment and reagents (Dobrovolskaia et al., 2009; Giannakou et al., 2016a).

Most of the *in vitro* experiments require a dilution of the NM stock solution in complex media, *i.e.* serum supplemented cell culture medium. A standard complete cell culture medium can be described as a buffered solution containing proteins such as serum albumin, globulins, other biomolecules such as vitamins and amino acids, and ionic salts (*e.g.* minimum essential medium (MEM), Dulbecco's modified Eagle medium (DMEM) or Roswell Park Memorial Institute (RPMI1640), and supplemented with 10% FBS or human-derived serum, as well as antibiotics (usually penicillin-streptomycin). Thus, the behaviour of NMs in such a complex environment will be dictated by the interaction with these components and can lead to dissolution, agglomeration, protein adsorption, *i.e.* the formation of a protein corona, or detachment of ligands (reviewed in: (Urban et al., 2016; Moore et al., 2015). It is therefore absolutely mandatory to characterize the NMs in the respective cell culture media because this will strongly affect the NM-cell interactions. It is recommended that primary NM size, hydrodynamic size and zeta potential values in water (or the relevant dispersant) are evaluated; for experiments under submerged conditions, the latter two need to be assessed also in cell culture medium. The state of agglomeration needs to be considered in all the media used (or compared water *vs.* cell culture medium). However, the characterization requirements strongly depend on the experimental setup therefore this might require experiment specific adjustments.

Besides the exposure conditions, *e.g.* particle concentrations, height of the medium on top of the cell layer, and perfusion with medium, cell culture medium composition is the most important factor influencing NM uptake and cellular responses (Meindl et al., 2017). The most important medium components which influence NM behaviour in cell culture media are the proteins (type and content, usually deriving from the serum source) which are affecting their state of agglomeration and sedimentation as well as their overall biological identity. The latter is well-known as the “protein corona paradigm” (Monopoli et al., 2011; Wan et al., 2015; Lundqvist et al., 2008; Monopoli et al., 2012; Cedervall et al., 2007; Walczyk et al., 2010). It has been noted that the type of NM protein corona, generated as a consequence of a specific test medium, can strongly influence the uptake and toxicity of individual NMs such that the *in vitro* results may be test system-specific (Gliga et al., 2014; Kim et al., 2014; Docter et al., 2015). It should be noted that adsorption of proteins on NM surface may function as steric protection colloids causing deagglomeration of NMs in protein-rich media (Schulze et al., 2008). Nonetheless, the use of proteins in cell culture media needs to be adapted with respect to the research question, *i.e.* with the estimated exposure route. For example, in experiments using lung cells *in vitro*, the use of pulmonary surfactant is recommended since inhaled NMs will come in contact with the surfactant covering the aqueous lining layer leading to surfactant covering of the materials (Schleh et al., 2013). In contrast, when investigating other exposure routes, in particular when mimicking NMs in the systemic circulation, the addition of blood serum proteins is prerequisite for a reliable cell exposure to NMs.

Protein adsorption on the NM surface can be, for instance, analysed with SDS-polyacrylamide gel electrophoresis gel based approaches which can be additionally coupled with *e.g.* electrospray liquid chromatography mass spectrometry (LC-MS/MS) (Wan et al., 2015), or the ability or a potential of NMs to adsorb proteins can be simply evaluated using the bicinchoninic acid assay (Pang et al., 2016). Depending on the NM type, hyperspectral imaging can be used to follow the extra- and intracellular alterations such as biomolecule adsorption and alteration in surface chemistry (Shannahan et al., 2015).

2.3.2. Realistic NM concentrations and dose delivered onto the cell surface, i.e. dosimetry

In order that the outcomes of *in vitro* studies can be useful in a regulatory context the results must be transformed to concentrations that would be meaningful in an *in vivo* setting. Concentrations of NMs and their respective doses should be realistic, *i.e.* relevant to human exposure scenarios such as occupational exposure limits for NMs (Gordon et al., 2014), *e.g.* using a model for calculation of the deposited NM concentrations based on the concentration in suspension (Hinderliter et al., 2010). For the *in vivo* dosimetry the evaluation can be done, for instance, applying the *In vitro* sedimentation, diffusion and dosimetry model (ISDD) model (Hinderliter et al., 2010) or the multiple-path particle dosimetry (MPPD) model (described below) (Cassee et al., 2002). Only in few studies, hydrodynamic size is compared in a time course before and after the exposure in cell culture media (Di Cristo et al., 2016; Gliga et al., 2014).

It should be noted that in mechanistic studies, unrealistically high NM concentrations (used both in *in vivo* rodent and – even to a higher extent – in *in vitro* assays) are sometimes required for determination of both the effect and no-effect levels of NMs. However, for assessment of potential NMs hazard to humans, NM concentrations should be selected based on realistic human exposure measurements, *i.e.* field studies. Nevertheless, such data are often scarce or unavailable for the majority of NM types. Therefore, the dose estimations are often calculated based on worst case assumptions (Dekkers et al., 2016). Overall, for trustworthy nanotoxicological *in vitro* data, first of all, there is a need for measurement data or at least estimations of realistic human exposure to NMs *via* the dermal, oral or respiratory route. In addition, it is recommended that the concentrations used in the submerged settings do not greatly exceed the level at which agglomeration is enhanced in order to avoid side effects of agglomeration and sedimentation affecting data interpretation (Huk et al., 2015b; Hirsch et al., 2011). For suspension experiments, use of 1–100 μg NMs/mL has been recommended, conditionally with the lower and higher limits at 0.125 and 200 μg/mL, respectively (Farcal et al., 2015). This has to be considered regardless of the fact that the maximum effect (100% cell death) cannot always be achieved as the administration of too high doses of NMs could cause interferences with the assays (Krug and Wick, 2011; Stone et al., 2009). The effect of NMs can be categorized depending on the calculated half maximal inhibitory concentration (IC_{50}) and the concentration that gives half-maximal response (EC_{50}) values (Farcal et al., 2015). A review article by Landsiedel and colleagues (Landsiedel et al., 2014) noted that high concentrations *in vitro* do not correlate with *in vivo* test concentrations. Most studies test acute exposure with high NM concentrations, and there are only a few describing long-term exposures over weeks or months to sub-chronic exposures (Armand et al., 2016a; Chen et al., 2015; Armand et al., 2016b). Some *in vitro* methods, such as ALI systems and other systems which examine how NMs cross other barriers, can largely avoid reverse dosimetry models as they offer determination of deposited NMs dose, and at the same time more closely have recently emerged as an optimal solution to overcome the issue with suspension cultures by a direct deposition of the NM onto the lung cell surface (Huh et al., 2012; Chortarea et al., 2015). The advantage of such systems is that the material mass can be fully controlled with monitoring the material deposition on the lung cell surface on-line, allowing the determination of a dose-effect correlation.

It is difficult to provide the justification for a defined concentration of NMs in *in vitro* assays; however, there are some approaches to understand the relation between real-world exposure scenarios and *in vitro* test concentrations. Since inhalation is assumed to be the major route for NMs entering the human body, the majority of dosing methods are found for the respiratory tract (Oberdörster et al., 2007; Oberdorster et al., 2015). One approach describes the measurement of NM concentration in the air in manufacturing laboratories to identify input levels for estimating NM mass retained in the human lung using the MPPD model. Based on a review of published NM concentration in the air at the manufacturing sites and using the MPPD model, Gangwal and colleagues (Gangwal et al., 2011) provided an estimation of the alveolar mass for selected NMs (*i.e.* titanium dioxide NPs, silver NPs and carbon nanotubes). The calculations propose that the estimated

alveolar retention of the NMs is likely to be proportional to a working-lifetime (45 years) exposure duration which can be expected in the range of 10 to 50 μg/cm^2 (alveolar mass retention). Based on the model, this corresponds to rather high concentrations of NM solutions used in *in vitro* studies (30 to 400 μg/mL) typically reported in the literature (Gangwal et al., 2011). Nevertheless, it is important to note that mechanisms of biological response to a high single dose compared to a repeated low dose exposure are expected to differ substantially. Therefore, in general, the *in vitro* assays cannot be directly correlated to life-time exposure scenarios, but rather to a daily exposure. A closer approximation to a subchronic exposure scenario can be achieved by repeated exposures to a substance (*e.g.* NMs) *in vitro*, even over a several week period. However, there the limitations with *in vitro* model systems since they might not be sustainable for subchronic durations. Therefore, experiments with such high concentrations and shorter durations have to be carefully interpreted since one has to consider that the cells receive a high concentration within a brief 24 h period and it might not be appropriate refer to as a life-time exposure when cell cultures are used. Besides the dose itself, the post-exposure time may significantly affect the NM dose rate. Even with similar lung burden observed after a short *vs.* long-term exposure, the dose rates might be significantly different in the *in vivo* situation due to the contribution of NM clearance. Lower dose-rates have been observed to lead to a longer-lasting and more persistent inflammation (Keller et al., 2014). Only a few publications have been found that consider such calculations. Occupationally relevant *in vivo* exposures (10 to 80 μg/mouse) and pulmonary fibroblast *in vitro* exposure studies, using *in vivo* dose equivalents (0.02 to 0.2 μg/cm^2), resulted in a dose-dependent transient pulmonary inflammation followed by fibroblast cell proliferation, alveolar wall thickening, and collagen I production culminating in persistent pulmonary fibrosis (Shvedova et al., 2008; Wang et al., 2010).

In addition to the aforementioned extracellular biological transformation of NMs in cell culture medium, also intracellular NM alterations such as biomolecule adsorption, alterations in surface chemistry and dissolution inside cellular compartments may influence cellular responses to NMs. For instance, using the hyperspectral dark field microscopy, such alterations of NMs in different intracellular compartments can be observed (Shannahan et al., 2015). The spectra of internalized NMs can be compared to that of NMs alone, or in conditions mimicking cellular compartments, *e.g.* artificial lysosomal fluid, or in other relevant biological environment such as lung or intestinal fluids.

When interpreting nanotoxicological data, attention should be paid to the administered and applied doses as well as with the information about human realistic exposure data. Discrepancy between the administered and deposited doses might be substantial. The deposited dose can be evaluated with dosimetry models or by experimentally assessing NM concentration on the cells or intracellularly, for instance with ICP-based techniques. However, as the human exposure concentrations and final doses are not available for many NM types, the comparison might be difficult. Nevertheless, the existing nanotoxicological data can provide a first idea on NM hazard and can serve as a basis for comparison of the potential hazard different NM types.

Both issues have been extensively discussed in the past and improvements are possible. The interpretation of NM-related cellular effects requires thorough understanding about the dose and number of NMs deposited on the cell surface. It has been reported that when comparing NMs with different properties – such as with the same chemical composition but different size, shapes or surfaces – reverse trends for cytotoxicity have been reported for the results presented as mass *vs.* surface area NM or number (Huk et al., 2014; Huk et al., 2015a). Dose metrics can be expressed in mass (μg/mL), surface area (m^2/mL) or particle number (n/mL); in the majority of studies the concentrations are expressed in mass units, *i.e.* as NM dispersion concentration (μg/ml), as NM mass per surface of exposure cells (μg/cm^2) or NM mass per individual cell (μg/cell), although it has been claimed that the number or volume of particles determines cytotoxicity of NMs (also reviewed in: (Park et al., 2009)). However, in inhalation toxicology as well as generally for *in vitro* toxicity, a correlation with surface area has been proposed as a recommended dose expression (Park et al., 2009; Oberdorster et al., 2005; Braakhuis et al., 2016) as it can be compared to *in vivo* exposure results (yet attention to differences in the method of exposures should be considered) (Elliott et al., 2017). The use of mass or number concentration per cell seems to be particularly appropriate for NM testing; however, it is recommended to express concentrations in all the three dose metrics (mass, surface area and number). This allows conversion from one dose to another when needed for determination of the best fitting dose metric for a toxic effect. If the particles are thoroughly characterised, this should be anyhow feasible.

Depending on the size (and agglomerate density), NMs might sediment slower compared to bulk materials, and can be delivered to the cell surface *via* diffusion (Limbach et al., 2005). Thus, deposition of NMs concentration/dose onto the per cell surface is the dominant factor determining the rate of uptake and not particle number NM concentration per volume or total surface area of the material (Limbach et al., 2005; Teeguarden et al., 2007). Hinderliter and colleagues have developed the ISDD to estimate the deposited NMs dose by a computational approach (Hinderliter et al., 2010) if experimental methods are not available. Applying a simulation approach, the ISDD model calculates the delivered dose and rate of transport of NMs using readily available parameters such temperature, media height, particle size in solution, agglomeration state and particle density. The model has been developed for spherical particles, though for fibres such a modelling approach does not yet exist. For instance, the ISDD model has been used to calculate the deposited concentration of silver NPs on lung cells. The results showed that a rather high concentration of 30 μg/mL applied for 24 h under submerged conditions reflect a working lifetime rather than an acute exposure scenario and result in both increased cytotoxicity and inflammatory responses (Herzog et al., 2014). However, it is of course questionable as to whether the effects observed within 24 h really reflect a long-term response and such results have to be interpreted carefully. Some other studies have recently been published on the improvement of the prediction of *in vitro* dosimetry, for instance by measuring the effective density of nano-agglomerates in suspension (DeLoid et al., 2014) or to improve the detection of particle size and agglomeration in complex biological media by using depolarized DLS (Balog et al., 2015). Although all these efforts to describe and measure the deposited NM concentration have increased and proved to be relevant within hazard and/or risk assessment, not many publications have employed such approaches thus far.

3. Interaction of NMs at the cellular level and their modes of actions

3.1. Biopersistence of NMs in relevant environments

The behaviour of NMs in physiological fluids, such as in mucus resembling the environment in the gastro-intestinal and respiratory tracts, in aqueous lining layer covered by surfactant (lung parenchyma), in blood or lymphatic fluid, is important to study because this might change their physico-chemical properties, such as size, agglomeration state and zeta potential, *i.e.* stability (Urban et al., 2016). The evaluation of NMs' biodegradability and biodurability is also recommended in mimicked intracellular lysosomal compartments, *e.g.* using either artificial lysosomal fluid or cell lysates (Shannahan et al., 2015; Gliga et al., 2014).

Regarding the inhalation route of exposure *in vivo*, lung lining fluid which is covered with pulmonary surfactant is the first biological structure with which inhaled and deposited NMs interact in the alveolar compartment. Pulmonary surfactants consist of lipids (90% w/w; mainly phospholipids) and proteins (10% w/w; mostly specific

surfactant proteins but also common proteins such as albumin or immunoglobulins are present (Wohlleben et al., 2016). Thus the interaction between NMs and surfactants is required for a complete NM characterization in relevant biological fluids. It has been proposed that, besides NM aerodynamic size, lipid affinity of NMs may strongly influence their deposition *in vivo*, meaning lipophilic NMs with high affinity towards pulmonary surfactants exhibit higher lung burden compared to hydrophilic NMs (Wohlleben et al., 2016). This is an important consideration also for the NM impact at the alveolar ALI (Marchetti et al., 2015).

It is difficult to compare the existing experimental data as different surfactant models have been used, including both the semi-synthetic or natural surfactant preparations. For example, evaluation of the surfactant protein D adsorbed to NMs with SDS-page and immunoblotting is a recommended approach (Marchetti et al., 2015) as it is applicable to a variety of NMs regardless of their composition, shape and size.

Among the studies under review, we have not found one applying the mineral fibre biopersistence protocol (Bernstein et al., 1994) to NMs directly. However, since length, thickness and biopersistence of fibre shaped MNs (*i.e.* carbon nanotubes), is an important factor for the prediction of the pathogenicity of fibres (Donaldson et al., 2013), biodurability of the NMs in relevant biological fluids needs to be addressed not only in NM inhalation studies, but in any outcomes related to their intracellular fate and distribution also in other cell types. In particular regarding the pulmonary exposure studies, as the agglomeration state and thus biopersistence of not only fibrous NMs but also other types of NMs is especially important for uptake and particle processing in alveolar macrophages (Goode et al., 2015).

3.2. Key physico-chemical parameters of NMs determining cellular uptake and fate

As recommended by the OECD the physico-chemical properties of NMs, including primary particle size, size distribution, composition, surface charge, shape, specific surface area, zeta potential, crystallinity, crystalline size, dissolution and solubility and redox potential should be considered in hazard assessment studies (OECD, 2009) since these parameters strongly influence the behaviour in physiological fluids and thus the uptake. In addition, agglomeration and aggregation, biopersistence, protein bio-corona, and dosimetry of the tested NMs are also recommended for consideration. Since most of the publications do not provide a detailed characterization of the NMs in complex cell culture media, the correlation of NM properties with cell uptake and intracellular fate is difficult. In addition to NM size and hydrophobicity, surface charge has been proposed to be one of the most factors influencing uptake of NMs in cells and their cellular fate (Fröhlich, 2012).

3.3. Uptake mechanisms

The most important biological parameters determining NM cellular uptake *in vitro* are cell size, proliferation rate and growth characteristics and expression of surface receptors involved in specific uptake-routes (Meindl et al., 2017). The uptake of NMs occurs *via* endocytotic pathways, *i.e.* phagocytosis which involves the ingestion of large particles or NM agglomerates (> 0.25 μm in diameter) and pinocytosis including macropinocytosis, clathrin-, and caveolin-mediated endocytosis and clathrin- and caveolin-independent endocytosis involving the ingestion of smaller particles (< 0.15 μm in diameter) (Conner and Schmid, 2003). NMs have been shown to be internalized in the cells mainly by pinocytosis, however the specific mechanisms differ with respect to the NM characteristics and cell types (Mahmoudi et al., 2012; Unfried et al., 2007). Other pathways have been reported since NMs of different composition have been found not to be membrane-bound, thus indicating alternative entering pathways (Geiser et al., 2005; Mu et al., 2012; Lesniak et al., 2005; Rothen-Rutishauser et al., 2006). For example, passive diffusion through membrane pores and passive uptake by van der Waals or steric interactions (subsumed as adhesive interactions) have been described (Rimai et al., 2000). Noteworthy, the composition of cell culture medium strongly affects uptake mechanism, most importantly by the protein content, and by exposure conditions, such as the NM concentration and factors related to the height of the cell culture medium covering exposed cells as well as, if applicable, medium perfusion (Meindl et al., 2017). Regarding NM characteristics, NMs surface charge is currently considered as one of the most important parameters. It has been suggested that cationic NM penetrate more easily through mammalian cell membranes in comparison to positive charged ones (Harush-Frenkel et al., 2008). Importantly, the well-studied and accepted protein corona paradigm (Lundqvist et al., 2008; Cedervall et al., 2007) in the nanotoxicology also has to be considered (Wan et al., 2015; Monopoli et al., 2012; Walczyk et al., 2010), since the protein corona formed on NM surface may significantly alter the NMs surface charge as seen by the cells. This means that even on NMs with different primary surface charges (even of opposite charges), a protein corona with a similar or the same charge may be formed in cell culture medium where exposure to cells occurs [82, 119 and reviewed in: {Corbo et al., 2016 #4276]. The latter needs to be taken into account when interpreting the surface charge of NMs with cellular uptake.

Noteworthy, the uptake mechanisms for the same NMs into different cell types can also vary (Tedja et al., 2012). All of the previously presented endocytotic pathways do have at least one aspect in common, that the internalized particle is ultimately located in an intracellular vesicle. Endocytosis of NMs results in the localisation of the particles first in early endosomes, then late endosomes, and those finally fuse with lysosomes (Li et al., 2013). For instance, the clathrin-mediated pathway has been reported to be involved in the uptake of chitosan NPs in A549 cells (within 1 h to 4 h) (Huang et al., 2002).

3.4. Intracellular localization of NMs

The intracellular distribution pattern of NMs is an important factor in investigating their biological responses; however different NM types require distinctive analytical methods. The method of choice for the intracellular detection of NMs depends on their characteristics such as chemical composition, fluorescence, size, and structure as well as on the cellular structure of interest (for a review see: (Elsaesser et al., 2010; Vanhecke et al., 2014)).

The information on intracellular localization of NMs can greatly help to elucidate mechanisms of NM-cell interactions. Confocal laser scanning microscopy and flow cytometry have been frequently applied for studying NM uptake, intracellular trafficking and semi-quantitative estimation of the uptake (Meindl et al., 2017). Some NMs, such as metal NMs, can be detected in cells *via* flow cytometry without additional modification by using the side scattering intensity signals (Zucker et al., 2016). However, often NMs require fluorescent labels. This can be a potential drawback of these techniques as such fluorescent modifications of NMs can substantially alter their biological behaviour compared to the non-labelled equivalents. In addition, especially with flow cytometry, the results can be misleading as it is difficult to distinguish the association of NMs with cells from the actual cellular uptake. Alternatively, “imaging flow cytometry” is an integrative approach combining flow cytometry analyses with confocal microscopy (Vranic et al., 2013).

Furthermore, ICP-based techniques coupled with MS (Armand et al., 2016a; Pang et al., 2016; Huo et al., 2015b) or atomic emission spectroscopy (Albanese et al., 2013) cannot distinguish between internalized NMs, extracellularly associated and/or just located between cells or within the extracellular fluid (reviewed in: (Collins et al., 2017)). However, this can be overcome by comparing NM uptake at 37 °C and at 4 °C: at the lower temperature, cells enter an energy-depleted state as well as the membrane rigidity is increased which disables both the energy dependent NM uptake as well as passive diffusion

through cell membranes (Vranic et al., 2013)]. Hence, at 4 °C no NM uptake is expected hence the entire signal from NMs is located extracellularly whereas at 37 °C both uptake of NMs and adherence onto cells occur. NM uptake can be estimated by subtracting measurement at 4 °C from that at 37 °C. On the other hand imaging techniques, such as hyperspectral dark field microscopy, enable intracellular detection of NMs based on the scattered light from the sample (Shannahan et al., 2015). Similarly, ion beam microscopy techniques, such as micro-proton-induced X-ray emission and micro-Rutherford backscattering, are powerful tools for spatially resolved elemental imaging and quantitative analysis at the single cell level (Armand et al., 2016a).

Frequently, TEM (Huk et al., 2014; Gliga et al., 2014; Mu et al., 2014; Huk et al., 2015a; Goode et al., 2015) or confocal Raman microspectroscopy (CRM) (Romero et al., 2011; Brautigam et al., 2014) are employed for a semi-quantitative space-resolved imaging approaches for simultaneous visualization of NMs and biological environment at a sub-cellular level (reviewed in: (Collins et al., 2017)). Namely, TEM with a resolution range from Angstrom to nanometer is the method of choice for resolving electron dense NMs and, when coupled to X-ray energy-dispersive spectrometry, it enables chemical identification of NMs present inside or around the cells. However, TEM is relatively cost-intensive and time-consuming, and is also not applicable for non-electron dense NMs; therefore it is not recommended for the first step evaluation of NM hazard assessment. CRM can overcome restrictions of optical microscopy (low resolution) and electron microscopy (cell destructive approach), it is economical and relatively fast (involving minimal sample preparation) and thus it can be recommended for the intracellular detection of NMs. Even though spatial resolution is limited compared to electron microscopy, the detection of unlabelled NMs and agglomerates in cells and tissue with CRM is sensitive and specific enough for the regulatory purposes (Brautigam et al., 2014). It is applicable also for time course imaging of individual cells and tracking cell metabolism of most cell types upon exposure to most NMs.

3.5. Translocation of NMs across tissue barriers

Numerous reports have shown that NM may overcome existing barriers such as lung, skin and gut and enter the blood circulation (Muhlfeld et al., 2008; Lundquist and Artursson, 2016; Lipka et al., 2010; Kreyling et al., 2014; Jatana and Delouise, 2014).

Most of monoculture or multicellular *in vitro* systems mimicking an epithelial barrier are cultivated on Transwell® membrane systems consisting of permeable membranes separating the apical and basolateral compartment. The pitfall of these systems is that the membranes are relatively thick, *i.e.* up to 10 µm, and adherence of NMs to the membranes can occur. Hence, depending on the composition and pore size, the membranes *per se* might, hamper translocation of NMs (Dekali et al., 2014; Braakhuis et al., 2015). Therefore, more realistic *in vitro* translocation models are required to reduce the membrane thickness and/or the material (Jud et al., 2015).

Several *in vitro* models are available to test the translocation of NM which can help to estimate the *in vivo* internal exposure (Braakhuis et al., 2015). NM penetration through tissue barriers can be evaluated with the dermal penetration assay *in vitro* (OECD TG 428 (OECD, 2004)). With this approach also assessment of skin absorption rates is feasible, yet this has been shown for chemicals (not NMs) (Fabian et al., 2017). However, validation of these systems using animal data is still lacking. Another approach is to use experimental data combined with *in silico* models allowing to predict the human bioavailability of NMs *in vivo* based on their *in vitro* translocation rate (Bachler et al., 2015) but again, *in vivo* data is required to test the power of such systems. The *in vitro* biokinetics research reviewed herein did not yet show a clear relationship between localisation and MOA.

However, reports on very low, or almost no barrier penetration by NMs has been observed in several *in vitro* studies. For example, some titanium dioxide and zinc oxide formulations did not penetrate the healthy skin barrier (reviewed in: (Monteiro-Riviere et al., 2011; Landsiedel et al., 2012)), and only minor translocation across the lung and intestinal barriers (reviewed in: (Braakhuis et al., 2015)). This again depends on size and material as reported by Kreyling and co-workers who found a size-dependent translocation of gold NPs across the air-blood barrier in rats (Kreyling et al., 2014).

3.6. Omics approaches for identification of MOA

Omics tools such as transcriptomics, proteomics, genomics/epigenomics, and metabolomics have mainly been used in effects-oriented nanosafety research for the purpose of hazard identification, and less for biomonitoring, or identification of specific biomarkers. Such approaches can present a supporting tool in elucidating the prevailing mechanisms of NM toxicity; however improvements in the correlation of such omics data sets with *in vitro* studies are required (Riebeling et al., 2017). Some promising outcomes have been reported, yet it is important to keep in mind that the approaches can present biases by the researcher and not so much of the methods themselves (Costa and Fadeel, 2016). Also, it has often been suggested that some biological effects of NMs can be manifested only after a normal cellular function has been challenged (*e.g.* after pathogen recognition) (Costa and Fadeel, 2016; Kodali et al., 2013).

When coupled with an appropriate bioinformatics evaluation, some omics approaches have resulted in the indication of novel and/or low-dose effects that had not been captured by conventional cellular assays (reviewed in: (Costa and Fadeel, 2016)). For example, a secretomic approach which is a proteomic analysis of proteins released into the supernatant has revealed some key events in the NM effect; some major differences were observed in the inflammation-related proteins and apoptosis induced in macrophages upon exposure to carbon nanotubes with respect to asbestos materials (Palomaki et al., 2015). Also, based on a microarray-based approach combined with secretomics, a so-called “no observed transcriptomic adverse effect level” was introduced. This suggests transcriptomics could be applied to benchmark potentially any type of toxicant, alone or in a mixture, in a predictive risk assessment framework (Pisani et al., 2015).

Overall, generation of this type of omics data presents the first step in identifying adverse outcome pathways describing toxicological key events starting from the first molecular interaction to the ultimate adverse outcome that can be quantitatively modelled using bioinformatics approaches. The combination of omics methods will support the knowledge of MOA that can then give evidence for grouping approaches for NMs (Riebeling et al., 2017).

Complementary to the majority of the endpoints evaluated in *in vitro* studies, use of omics approaches is recommended. They can be used for comparison of different *in vitro* settings, or compared to *in vivo* scenarios: after exposure to NMs, gene expression profiling approaches have confirmed closer resemblance of co-cultures *in vitro* to the *in vivo* situation, compared to monocultures *in vitro*. This suggests that co-cultures of relevant cell types can provide an improved system for high-throughput *in vitro* testing, and may reduce the need for animal testing of NMs (Snyder-Talkington et al., 2015). The bottlenecks so far are that only limited numbers of NMs have been tested, and costs and protocols for nanomaterial omics analyses are emerging. Therefore, different cell types and animal experiments should be included and more data is needed to validate individual co-culture systems before clear conclusion and recommendations about the applicability of these methods can be made.

3.7. Cellular reactions

3.7.1. Cytotoxicity

Regardless of the method, both primary cells and cell cultures are sensitive to alterations in their environments (*e.g.* temperature and pH

fluctuations, nutrient and waste concentration) prior to the addition of a tested substance, *e.g.* NMs. Therefore, it is important to control cell culture conditions in order to ensure that the measured cytotoxicity observed is due to NM effects rather than unfavorable culture conditions (Landsiedel et al., 2010).

Cytotoxicity assays can be classified based on the experimental approach for detection of cell death:

(i) *via* visualization of cellular morphological alterations using *e.g.* phase-contrast microscopy or (ii) *via* colorimetric or fluorimetric analysis. These methods (ii) can be further categorized into assays that assess plasma membrane integrity and the ones based on the cellular metabolism, *e.g.* mitochondrial activity (Lewinski et al., 2008) and were used in the majority of the reviewed studies. (iii) Depending on the research question, flow cytometry is frequently used for determination of specific mechanisms of cell death, *i.e.* for necrosis and apoptosis evaluation. (iv) As oppose to the cell death evaluation, proliferation can be used as a measure of cell viability/cytotoxicity of a substance. (v) Finally, cytotoxic action of NMs can be accessed *via* epithelial cell barrier damage.

(i) Cellular morphology visualization.

Solely the use of visualization techniques for determination of NM cytotoxicity might not be sufficient. It is recommended to use those techniques as a complementary tool to other cytotoxicity assays (described under (ii)).

(ii) Colorimetric or fluorimetric analysis.

(a) Compromised membrane integrity assessment.

To start with, exposure to NMs might results in compromised cell membrane integrity which allows leaking of cellular contents. Such cell viability assessment includes the neutral red uptake (NRU), trypan blue (TB) and lactate dehydrogenase (LDH; cytosolic enzyme) release assays (Stoehr et al., 2011; Gliga et al., 2014; Farcal et al., 2015; Goode et al., 2015). The principle of the NRU assay is based on the accumulation of the cationic dye in lysosomes upon crossing plasma membranes by diffusion. Spectrophotometric measurements of the cells exposure to neutral red allow distinguishing between live and dead cells as the uptake is decreased or the dye can be leaked out in the case of ruptured plasma membranes. Differently, TB dye is only permeable to cells with compromised membranes: dead cells are stained blue while live cells remain uncoloured. The amount of dead cells is usually determined *via* light microscopy. Similarly, the cytosolic enzyme LDH is released in the supernatants only from cells with compromised plasma membranes. Cell death is calculated based on the spectrophotometrically upon addition of reagents (tetrazolium based salts) enabling colorimetric reaction with the enzyme.

(b) Assessment of altered cellular metabolism (metabolic activity).

In order to determine the mechanisms behind the induced cell death, other colorimetric/fluorimetric cytotoxicity assays are being used in order to determine the mechanism behind the induced cell death, for example MTT (Armand et al., 2016a; Mu et al., 2014), MTS (Anguissola et al., 2014; Shannahan et al., 2015; Goode et al., 2015), WST-1 or resazurin (Farcal et al., 2015) assays. Tetrazolium salts can as well be used for evaluation of mitochondrial activity as mitochondrial dehydrogenase enzymes cleave the tetrazolium ring; these enzymes are functional only in active mitochondria (in viable cells). All those assays are based on the detection of dehydrogenase activity in viable cells. Therefore, all the testing conditions (*e.g.* presence of NMs or chemicals) can affect dehydrogenase activity or react with the reducing agents to generate formazan. This can cause discrepancy between the actual viable cell number and the cell number determined.

As high potential for NM interference with different cytotoxicological assays has been proven (Stone et al., 2009), each *in vitro* test system has to be evaluated for each individual type of NM to accurately assess for possible interferences (Kroll et al., 2012). The light absorption due to the presence of NMs can be corrected for the NM dispersion alone in the absence of cells (background subtraction) (Mu et al., 2014), however this is not always accurate as agglomeration and sedimentation of NMs can be different in the presence or absence of cells (Farcal et al., 2015; Kroll et al., 2012; Guadagnini et al., 2015). In order to elucidate the potential interference of NMs with any assay, one can compare the signals from the following: (i) the NMs dispersed in cell culture media alone, (ii) the cells exposed to the respective NM-containing medium, and (iii) untreated cells with NMs added later; the added value of the test under (iii) is to compare if the interference of NMs alone in cell culture medium differs from that in cell culture medium with conversed formazan (in the case of tetrazolium salt based assays, such as MTT and LDH), and (iv) the last measurement is done on (ultra/high speed) centrifuged supernatants from the NM-exposed cells so that potentially formed NM agglomerates can be removed. Based on these outcomes, depending on the severity and type of interference, some conditions (*e.g.* the highest NM concentrations) can be excluded from the following experiments.

(iii) Cell death evaluation: apoptosis and necrosis assays.

The evaluation of apoptosis and necrosis, *i.e.* programmed *vs.* accidental cell death, is often used as the first stage of NM toxicity evaluation. The apoptotic/necrotic cell proportion is usually estimated by flow cytometry: Annexin-V for loss of membrane asymmetry (apoptosis) is coupled with propidium iodide to detect necrotic cells. Since both the forms of cell death have been reported for cells after exposure to NMs (Kumar et al., 2015), testing for both is recommended.

(iv) Cell proliferation evaluation.

At the moment, the colony forming efficiency (CFE; platting efficiency) assay, is considered one of the most promising tests for general, first step NM toxicity evaluation (Dusinska et al., 2015). The method has been proven reliable, transferable and has been suggested useful as an early/first stage screening method (Kinsner-Ovaskainen et al., 2014). CFE is an example of a cell viability method that has been implemented in the genotoxicity testing (*e.g.* in the OECD TG 476 (OECD, 2015a)) as the first step in the genotoxicity assessment of chemicals. The CFE assay has been recommended as preferred over a test where interference with NMs cannot be excluded, however its applicability for multi-cellular systems has not yet been proven.

(v) Estimation of epithelial cell barrier damage: impedance-based measurements.

Another approach which enables the overcoming of interferences of NMs with cytotoxicity assays are impedance-based devices. They enable label-free alternatives to be used for evaluation of adherent cells (when adhered = viable) and integrity of (epithelial/endothelial) cell barrier. There are several commercially-available impedance devices that allow for sensitive real-time observations of cell changes (cell viability, motility, adhesion) upon exposure to NM throughout an experiment without the need for destructive cell sampling. It is applicable for a range of NMs and cell types. Reliability and efficiency of impedance-based methods have been compared with conventional cytotoxicity assays used for assessment of NM induced cellular damage (reviewed in: (Collins et al., 2017). Such approaches have been recently recommended for initial, first step, evaluation of material toxicity particularly for barrier systems (Collins et al., 2017).

3.7.2. Oxidative stress evaluation

Oxidative stress is the prevailing paradigm in the NM induced effects *in vitro* as it is linked to various adverse outcomes such as (pro-) inflammation, DNA damage, and general cytotoxicity. Cells generate ROS to maintain normal metabolism/homeostasis, but its overproduction can interfere with a variety of signal transduction pathways, even induce cell apoptosis. ROS detection in cells often uses fluorescent or non-fluorescent probes such as dihydroethidium or dichlorofluorescein (DCF) (Gliga et al., 2014; Goode et al., 2015; Driessen et al., 2015). On one hand, this approach allows for investigation of early intracellular ROS indicators at much lower NP concentrations than those needed for standard cytotoxicity assays, but on the other hand interference with NMs has been observed *e.g.* for multi-walled carbon nanotubes (Roesslein et al., 2013), graphene-based materials

(Creighton et al., 2013), cadmium selenide, titanium dioxide (Ong et al., 2014), iron oxide (Doak et al., 2009) and gold (Pfaller et al., 2010) NPs and thus can limit the range of tested particle concentrations. Therefore, an alternative method to report on cellular ROS generation induced by NMs is highly needed, such as a proteomic-based analysis of protein carbonylation as an oxidative stress measure (Driessen et al., 2015). As a complementary approach to this or prior the cellular experiments, acellular measurement methods are recommended for the evaluation of the potential of NMs to generate ROS already in the suspension medium such as the ESR approach for indirect assessment of surface reactivity (Driessen et al., 2015; He et al., 2014). As addressed in the Section 2 (General considerations for *in vitro* test methods), ROS can be detected also *via e.g.* cytochrome *c* reduction (Dikalov and Harrison, 2014; Delaval et al., 2017) or FRAS assay (Braakhuis et al., 2014). In addition, measurements of the photosensitized oxidation of dyes or ROS quenchers such a furfuryl alcohol or *tert*-butanol from solution in batch experiments can also be used to measure the ROS production rates for NMs' suspensions (Pickering and Wiesner, 2005).

3.7.3. (Pro-)inflammatory reactions

The analysis of the secreted soluble factors *i.e.* proteins such as cytokines, chemokines and growth factors are usually determined by the enzyme-linked immunosorbent assay (ELISA) (Huk et al., 2014; Wan et al., 2015; Farcal et al., 2015; Goode et al., 2015). It is based on the enzymatic reaction analysed with flow cytometry, or using a plate reader (absorbance, chemoluminescence or fluorescence measurements). However, with all these approaches, interference with NMs in cell culture supernatants or tissue/cell lysates is possible. The outcomes must be interpreted with respect to the cell viability data as the loss of cell viability at higher doses of NMs could prevent the cytokine secretion and thus lead to false negative results. In addition, due to the high protein adsorption capacity of some of the NM types, the released proteins (*e.g.* cytokines) have the possibility to adhere to the NMs and thus the protein/cytokine concentration may be underestimated as demonstrated for titanium dioxide, silicon dioxide and iron oxide NPs (Guadagnini et al., 2015). Therefore, instead of, or in parallel with, the cytokine release assessed with ELISA, the gene expression can be evaluated using the reverse transcription polymerase chain reaction such as for the selected pro-inflammatory genes' expression (Shannahan et al., 2015), or *i.e.* reporter cell lines can be used for that purpose which has been *e.g.* proven for transfected A549 cell line (Stoehr et al., 2011).

Overall, the cytotoxicity assays should always consider the cell type and MNs used, so therefore a generalized recommendation cannot be given. A battery of tests should be employed, along with an appropriate medium, time of exposure, appropriate controls and reference standards used, multiple *in vitro* cytotoxicity assays (including dye-free approaches), at least three representative cell lines (including different cell lines originating from the same organic system) (Kroll et al., 2011), five concentrations of MNs, and must include corresponding negative and positive assay controls; both the assay-specific ones and the NM-based controls are advised (*discussed in the section 3.7.7: Positive and negative controls*). For suspension experiments in which a dye is required, interference tests are mandatory. Conventional toxicological (and genotoxicological) assays have been developed to test soluble chemicals and not NMs. For that reason, individual cytotoxicity methods alone are currently not sufficient for evaluation of toxic potential of NMs. Prior evaluation and adaptation of the assays is recommended; further, multiple assays need to be employed for a reliable nanotoxicological assessment (Landsiedel et al., 2010; Kroll et al., 2011). These assays are commonly based on detection of light or fluorescence of a marker or chemical reaction products, therefore possible interference of NMs with the assay reagents or optical pathway needs to be carefully considered (Landsiedel et al., 2010). NM-specific adaptations of the conventional cytotoxicity assays (*e.g.* MTT and DCF assays) might eliminate NM interference with the method, thus still limited with high NM concentration (Kroll et al., 2012). In addition, *in vitro* assays are often pH-dependent hence the outcomes may be affected by acidic or basic NMs. Therefore, particularly in the case of NM concentrations exceeded the buffer capacities of biological media, acidity/alkalinity testing is recommended (Landsiedel et al., 2010).

3.7.4. Genotoxicity

Genotoxicity describes the potential damage of NMs to the genetic material in a cellular nucleus, *i.e.* DNA. In general, NM-induced genotoxicity can be classified as primary or secondary genotoxicity. Primary genotoxicity is defined as genetic damage induced by NMs themselves and not by an inflammatory response, whereas secondary genotoxicity implies involvement of inflammatory cells resulting in the oxidative damage of DNA by ROS (Van Berlo et al., 2012; KEMI, 2016; Nabiev et al., 2007). Conceptually, primary genotoxicity can be induced *via* direct or indirect mechanisms. The direct genotoxicity results from physical interactions of the materials with the genomic DNA which can occur for smaller NMs capable of reaching the nucleus through the nuclear pore complexes (Nabiev et al., 2007). Indirectly caused genetic damage can occur *via* various pathways; for instance *via* formation of ROS by metals and organic constituents (but not inducing inflammation) (Schins and Knaapen, 2007).

The OECD workshop findings (OECD, 2014d) provide very useful recommendations on genotoxicity testing *in vitro*. We agree on the inappropriateness of the bacterial based tests (such as Ames test (OECD TG 471 (OECD, 1997)) for genotoxicity of NMs due to the reasons stated in the OECD document (OECD, 2014d). Namely, uptake of NMs in bacterial cells can be limited due to the lack of endocytotic ability of bacteria used in the assays and diffusion of NMs across the bacterial cell wall may be limited the pores of the Ames Salmonella assay bacterium are < 30 nm. As reviewed by Landsiedel and colleagues in 2009, the outcomes of the Ames assay for all the NMs tested (*e.g.* zinc oxide, titanium dioxide, magnetic NPs) were negative (Landsiedel et al., 2009). In addition, some NMs have antibacterial properties which can lead to misinterpretations of the outcomes. In some cases, the size of bacteria can be even smaller than some types of NMs (Huk et al., 2014; Doak et al., 2012). As an alternative, *in vitro* mammalian cell gene mutation assay OECD TG 476 (OECD, 2015a) has been suggested (OECD, 2014d). For that assay, the use of an appropriate cell type is recommended *e.g.* the ones with a shorter cell cycle (12 h). However, to date, there are only few studies using this test (Huk et al., 2014; Huk et al., 2015a) and there has been no evidence in interference of NMs; therefore the approach is recommended, but further testing is required.

Another recommendation from the workshop was to consider specific modifications of the *in vitro* mammalian cell micronucleus assay (OECD TG 487 (OECD, 2014b)) which is used for evaluation of chromosome breakage leading to the formation of an additional nucleus (micronucleus) during cellular division. To prevent cytokinesis in the test, treatment with Cytochalasin-B (Cyt−B) is usually used; however this has been proven to strongly affect NM uptake (KEMI, 2016), therefore, post-treatment or delayed co-treatment with Cyt-B has been suggested (Gonzalez et al., 2011; ANSES, 2013). As of now, it can be reported that the OECD started to develop a guidance document on the NM-specific adaption of the *in vitro* mammalian cell micronucleus assay (OECD, 2015e).

Additionally, comet assay has been reported as a successfully applied approach for genotoxicity evaluation for NMs (Huk et al., 2014; Dusinska et al., 2015; Magdolenova et al., 2014); however the method is limited due to possibly interference with induced cytotoxicity upon incubation with the tested NMs – this can significantly impair reliable genotoxicity evaluation (Huk et al., 2015b). Also, false positive results have been reported due to presence of NMs in the 'comet head' which has raised concerns of the possible interference of the previously uptaken NMs with the assay components (Ferraro et al., 2016). Hence, the comet assay is an appropriate method for NMs' genotoxicity testing, but

only in combination with cytotoxicity evaluations so that genotoxicity can be related to concentrations above 80% viability. Other genotoxicity assays include *e.g.* the γ-H2AX foci formation as a marker for DNA double strand breaks which might provide higher sensitivity of the comet assay but with limited mechanistic insight for investigations of more precise mechanisms of genotoxicity (Gliga et al., 2014). Also the *in vitro* mammalian chromosome aberration OECD TG 473 (OECD, 2014c) and *in vivo* mammalian bone marrow chromosomal aberration OECD TG 475 (OECD, 2014e) tests might be suitable solutions also for NMs, yet only upon further testing for NMs.

Similar to the cytotoxicological methods, use of chemical and NM positive and negative controls is required for verification of individual genotoxicity assays *in vitro*: negative controls should elicit the background level of DNA damage, whereas positive controls should show highly significant damage, corresponding to the type of the damage which is measured with the individual assays used (Huk et al., 2015b). (*In more details discussed in the section 3.7.7. Positive and negative controls*).

Nevertheless, a recommended approach is to compare the outcomes of *in vitro* genotoxicological assays with that of the equivalent *in vivo* assay using the same NM dispersion and comparable exposure duration. However, some discrepancies can occur between both the settings. For instance, absence of genotoxic effects of gold NPs have been observed in comet/micronucleus *in vivo* and *in vitro* settings, whereas DNA damage induced by crystalline silica (quartz, DQ 12) NMs was measured in the *in vivo* Comet and micronucleus assays, but not *in vitro* (Downs et al., 2012). Oppositely, comparable concentrations of NMs can induce effect *in vitro* with absence of observed damage *in vivo* (even with pronounced inflammation reaction) which can be explained with the fact that considerably lower doses may reach the target cells *in vivo* than *in vitro*.

From a regulatory perspective, genotoxicity testing requires a battery of tests addressing different genotoxic and mutagenic endpoints, since no single method is capable of detecting all the different forms of genome damage which includes DNA lesions, chromosome aberration and mutations (Huk et al., 2015a; Doak et al., 2012). The current recommendations on genotoxicity of NMs are: (i) the use of mammalian tests over bacterial cells (as the later have limited ability to engulf MNs), such as the modified *in vitro* mammalian cell micronucleus assay OECD TG 487 (OECD, 2014b) or the *in vitro* mammalian cell gene mutation assay OECD TG 476 (OECD, 2015a) (ii) the use of non-cytotoxic concentrations; appropriate cytotoxicity tests should be part of the genotoxicity testing strategy, (iii) prolonged time (at least 24 h) of the treatment is recommended to ensure MN uptake by cells and access to DNA when the nuclear membrane is dissolved. Outcomes of the genotoxicological assays vary significantly at intra- and inter-laboratory comparisons. The reasons are mostly attributed to diversity of NM preparation and handling, therefore parallel studies with other endpoints are important (Huk et al., 2015b; OECD, 2015e; Pfuhler et al., 2013).

3.7.4.1. Carcinogenicity in vitro: Colony Transformation Assay (CTA). Besides the abovementioned approaches for evaluation of genotoxicity of NMs *in vitro*, there has been promising new approaches proposed. As an example of a complementary approach to *in vitro* genotoxicity test batteries is the *i.e.* Cell Transformation Assays (CTA) which measure potential cell transformation (that is one step in the multistep cancer process), and can detect both genotoxic and non-genotoxic carcinogens (Steinberg, 2017). In 2015, European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM) issued a Guidance document (protocol) proposing to use the "*In vitro* syrian hamster embryo (SHE) cell transformation assay" as part of a weight of evidence approach in the testing of substances for carcinogenic potential. Until now, only few results are available for NMs (Ponti et al., 2009; JRC, 2016) and further testing is required before issuing a final recommendation.

3.7.5. Other relevant endpoints

3.7.5.1. Immunotoxicity testing. Regardless of the route of exposure, NM will eventually come in contact with immune cells and reports have shown this may trigger adverse immune effect (Farrera and Fadeel, 2015) (and reviewed in: (Giannakou et al., 2016b; Fytianos et al., 2016). Effects of NM on the immune system have been described and recommendations from the 2008 National Cancer Institute workshop on immunology and nanotechnology have been proposed in terms of the immune cell models, mono- as well as co-culture systems, being suitable for a first evaluation of NM immunomodulatory effects (Dobrovolskaia et al., 2009). However, at the moment, there are no specific regulatory documents available for general immunotoxicity assessment of NMs (apart from some specific NM formulations). The adverse effects are not always readily detected using the conventional immunotoxicity methods; a battery of such NM-specific assays is required to assess NM effect on the immune system (Giannakou et al., 2016b).

3.7.5.2. High aspect ratio nanomaterials (HARN). In addition, HARN, such as carbon nanotubes, have been shown to induce (pro-)fibrotic responses both *in vivo* and *in vitro*, whereas the severity of the response is dependent on their physico-chemical characteristics (Vietti et al., 2016). Therefore this endpoint is recommended to be tested for fibre shaped NMs.

3.7.6. Multiple endpoint approaches

The combination of multiple endpoint-approaches such as the high throughput screening and the high content analysis has been proposed as promising approaches for fast cytotoxicity screening of a large panel of NMs (Anguissola et al., 2014) within hazard and risk assessment frameworks. However, similar possible NM interferences with assays reagents and optical pathways need to be considered as well as the appropriate positive and negative controls (discussed below).

3.7.7. Positive and negative controls

It is very important to mention that for all the assays described above at least chemical positive and negative controls have to be systematically included in experiments for quality control, to demonstrate correct performance of the assays and to ensure reproducibility (Huk et al., 2015b). Besides chemical controls, also NM-based controls are recommended in order to confirm the sensitivity of the techniques for NMs, to benchmark the NM biological effects, and in some cases also to assess potential NM interferences with the assay (Dusinska et al., 2015; Collins et al., 2017). Usually, untreated cells are used for negative controls and the assay data are presented normalised to these values (*e.g* (Huk et al., 2014).), whereas in some cases, the respective bulk materials are used (Wiemann et al., 2016). The majority of the studies under review compare the NM-induced effects with those caused by chemicals, *e.g.* H_2O_2 for cell viability, oxidative stress and genotoxicity (Armand et al., 2016a), LPS (Chortarea et al., 2015) or TNF-α (Huk et al., 2014) for the onset of inflammation, or respective salts (Stoehr et al., 2011; Mu et al., 2014). In most of the respiratory exposure-related studies, the same positive controls (chemicals (Clift et al., 2014) or DQ 12 (Chortarea et al., 2015; Wiemann et al., 2016)) are applied for all the endpoints. Only in some studies has NM-based reference material been reported, *e.g.* amine modified polystyrene NPs (Anguissola et al., 2014; Paget et al., 2014) as positive controls for cytotoxicity, oxidative stress, genotoxicity or apoptosis and activation of the inflammasome pathway and reviewed in: (Collins et al., 2017). In some studies, material with the same chemical composition but different morphology is used for positive control (*e.g.* long silver nanowires for cytotoxicity and cell death (Stoehr et al., 2011)) whereas poly-lactic-*co*-glycolic acid–-polyethylene oxide copolymer coated NPs or carboxylated nanodiamonds are examples of particle negative controls for cyto-and genotoxicity (Dusinska et al., 2015; Paget et al., 2014).

However, there is a considerable challenge in nanotoxicology to advise suitable nano-specific positive as well as negative controls

(Dusinska et al., 2015; Huk et al., 2015b). Up to date, there has not yet been a generally accepted recommendation given for nano-specific reference controls and standards since this does not only depend on the endpoint but also on the exposure route. As one example for cytotoxicity and apoptosis it is recommended to use the amine modified polystyrene NPs as a positive control NM, and their neutral equivalents (plain polystyrene NPs) or carboxylated polystyrene NPs for negative controls (Anguissola et al., 2014); for inflammation DQ 12 has been shown to be the most promising positive control, especially in respect to the inhalation route of exposure (Oberdörster et al., 2005). However, for genotoxicity, we agree with the proposal by Pfuhler and colleagues that NM-specific positive controls were not considered to be necessary for genotoxicity assays, because positive controls in this case relate to assay performance (Pfuhler et al., 2013).

Overall, a combination of system control measurements and interlaboratory comparison data is recommended to be performed for each individual cytotoxicity method to get an insight about the relative sources of assay variability, which can be more distinctive for NMs (compared to chemicals). The interlaboratory comparison of the MTS assay *in vitro* using A549 cells, recently provided by Elliot and the colleagues (Elliott et al., 2017), proposes that even using the same NM and cell type as well as the same assay, researcher's experimental performance may strongly influence the cytotoxicity assay outcomes. For example, the cell line source, media exchange, cell handling, and NM dispersion preparation and application to cells are critical to ensure protocol robustness and comparability of results. These general concepts should be applicable to most nanotoxicological tests.

4. Regulatory relevance: testing strategy

4.1. Categorization

The "grouping" concept of NMs aims to increase the hazard assessment of any material in a more efficient manner and with the possibility of cross-reading of toxicological properties. Such effects of an unknown substance might be predicted from endpoint-specific effects of the other substances classified in the same group. However, so far, no regulatory framework for such grouping approaches of NMs exists, but various efforts on this are described in the literature (Dekkers et al., 2016; Arts et al., 2016; Arts et al., 2015; Bos et al., 2015; Oosterwijk et al., 2016; Walser and Studer, 2015; Hendren et al., 2015). A systematic approach which is called "The Read-Across Assessment Framework" has been proposed by the European Chemicals Agency (ECHA) (ECHA, 2017). This framework uses relevant information from analogous substances to predict the properties for other, similar substances. However, this has been proposed for chemicals and it is questionable if such an approach also can be applied to NMs.

For example, the National Institute for Occupational Safety and Health (NIOSH) and the British Standardization Institute (BSI) distinguish between: (i) soluble, (ii) biopersistent and low toxicity, (iii) biopersistent and high toxicity, and (iv) fibrous NMs, *i.e.* HARN (Kuempel et al., 2012). Other grouping efforts reported by German Federal Institutes BAuA and BAM or the EU Commission have been reviewed by Arts et al. (Arts et al., 2014).

The European Centre for Ecotoxicology and Toxicology of Chemicals Task Force on Nanomaterials (ECETOC Nano TF) (Arts et al., 2016; Arts et al., 2015) has proposed a functionality-driven Decision-making framework for the grouping and testing of NMs (DF4nanoGrouping) with the aim to group them by their specific MOA that results in an apical toxic effect. The following three tiers to assign NMs into four main groups have been proposed, based on the data including 24 tested materials in specific case studies:

- DF4nanoGrouping Tier 1 – intrinsic material properties: water solubility, particle size and shape (aspect ratio) and composition, including surface functionalization, and noting the presence of material components or impurities;
- DF4nanoGrouping Tier 2 – system-dependent properties and *in vitro* effects: dissolution in biological media, surface reactivity, dispersibility, cellular effects and *in vitro* genotoxicity;
- DF4nanoGrouping Tier 3 – *in vivo* effects: apical toxic effects, toxic potency, *in vivo* genotoxicity, reversibility of effects, (primary and secondary) organ burden and clearance, biodistribution and biopersistence;
- DF4nanoGrouping – qualifiers: release (dustiness) and supplementary criteria, such as surface area, surface chemistry, surface charge, and hydrophobicity.

In addition, surface reactivity of NMs in a cell free setting can provide a useful tool for prediction of material cytotoxicity. For example, it has been shown that the redox potential of NMs measured by ESR correlated well with the outcomes of the proteomic-based analysis of protein carbonylation as an oxidative stress measure. Hence, such a redox profiling approach may be used for NM classification (grouping) according to their MOA using *e.g.* the ESR approach (Driessen et al., 2015) or the FRAS assay (Riebeling et al., 2016; Hsieh et al., 2013). Another example of an *in vitro* assay suitable for regulatory purposes predicting short-term inhalation toxicity has recently be proposed by the group of Landsiedel and co-workers (Wiemann et al., 2016) who tested an *in vitro* assay using rat derived alveolar macrophages.

A useful approach for ranking NMs has recently (2016) been proposed by the NANoREG project (JRC, 2016) which is in context with the European Chemicals Agency (ECHA) in the report called "Usage of (eco)toxicological data for bridging data gaps between and grouping nanoforms of the same substance – Elements to consider" (ECHA, 2016) aiming at clarifying rules for read-across and grouping of NMs. To start with, it is expected that NMs are classified to have genotoxic and/or sensitizing properties according to the classification of their chemical core, and the remaining evaluation process to be followed is presented in Fig. 2 below.

The authors conclude that specific case studies covering a broad spectrum of different types of NMs should be conducted to provide further proof-of-evidence for the suggested grouping concept with the perspective to integrate this into the REACH registration process for substances that have to be registered in the nanoform.

It is accepted that a single dose parameter is not sufficient to describe the toxic effects of NMs. Therefore other parameters such as size, surface area, surface reactivity, concentration and exposure time need to be included as well as different endpoints.

In the first phase of evaluation, NMs are classified in 3 categories: red (the highest potential to be hazardous), green (NMs for which the classical *i.e.* the non-NM, risk assessment approach can be performed), and orange (further evaluation needed). The *in vitro* part of the process includes genotoxicity and immunotoxicity evaluation in the phase I and is continued in the phase II ranking based on occupational and consumer exposure scenarios In phase III, guided by information obtained on the kinetics and hazard in phase II, additional information on other endpoints may be obtained by *in vivo* studies. In the case of observed positive effects of *in vitro* cell experiments, transformation of the NMs itself, and *in vitro* genotoxicity along with expected accumulation and immunotoxicity, this may trigger long-term repeated dose kinetic and toxicity testing *in vivo* to rule out accumulation and long-term effects, including carcinogenic, cardiovascular and adverse reproductive effects (presented as the final arrow in the Fig. 2).

5. Final conclusions and recommendations

The authors fully agree with Gordon and the colleagues (Gordon et al., 2014) who concluded that most *in vitro* test results we have available so far for NMs are not sufficient in and of themselves for final regulatory decisions on the possible toxicity of a given NM. However, based on the current literature research we conclude that the existing *in*

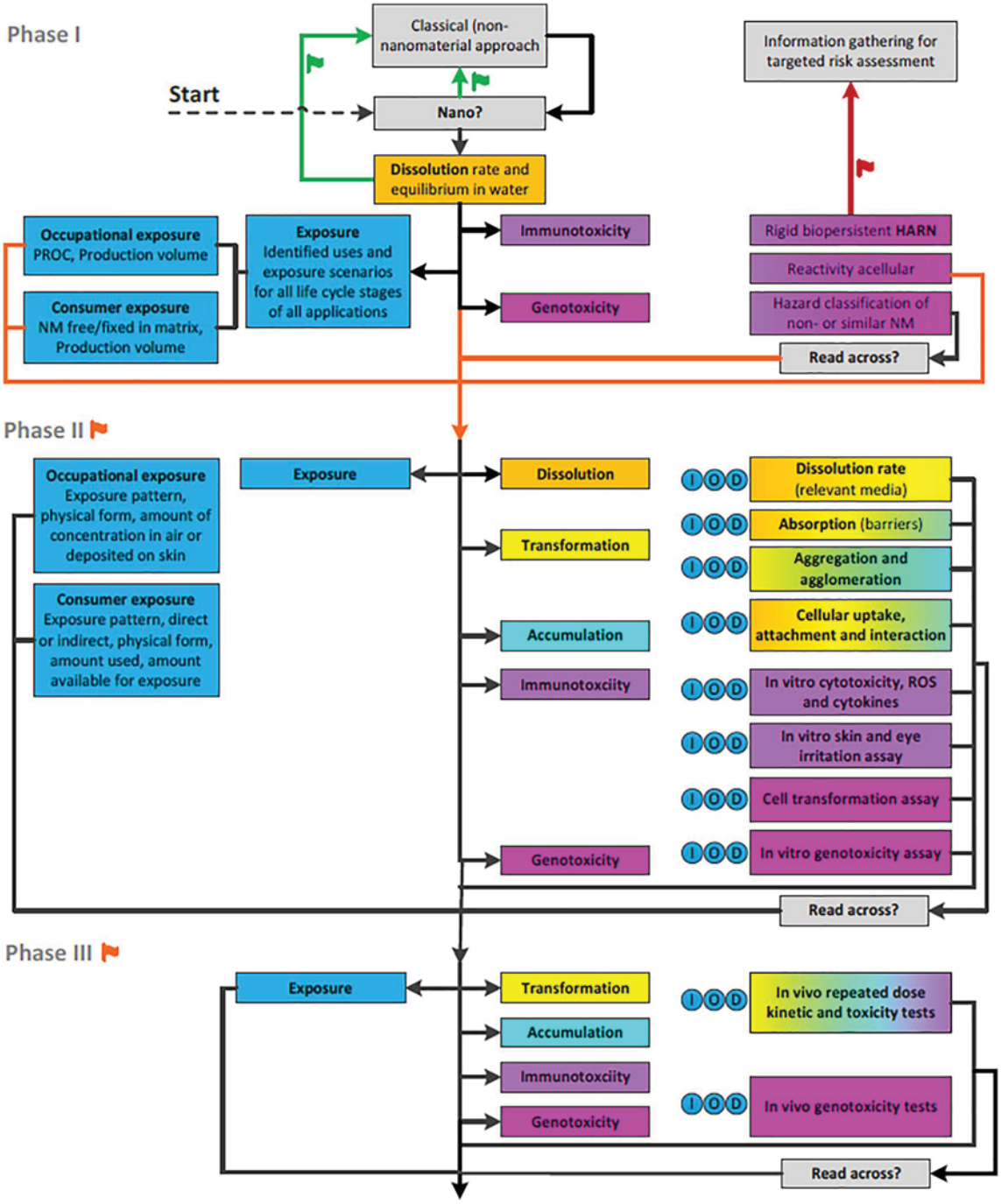

Fig. 2. Stepwise approach to categorize NMs according to Deliverable 1.1 of the NANoREG project (Adopted with permission from: NANoREG DR, D. 1.11, 2016 (JRC, 2016)). **Green arrows**: the material is no nanomaterial or has such a high dissolution rate in water that it falls apart into its molecular 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vitro assays are suitable for identifying the capacity of NM to induce potential human hazard effects. Hence, *in vitro* results may be useful in ranking NMs either as mechanistic studies enabling a deeper insight into mechanisms of NM-induced (potentially even nano-specific) effects, or for follow-on *in vivo* testing in certain circumstances (Wiemann et al., 2016), and can also contribute to the interpretation of *in vivo* results by identifying their MOA. In addition, the *in vitro* outcomes can serve as guidelines for design of *in vivo* experiments. It is recommended that several time points are included in the assessment of NMs' internalization and subsequent cellular response. This might importantly be affected by the naturally present biomolecules which can adhere on NM surface. The intracellular distribution pattern of MNs is an important factor in investigating nanotoxicological responses; however analytical method of choice depends on the type of the investigated NMs base on their physico-chemical characteristics.

The behaviour of MNs in physiological fluids is relevant, as is the

Table 1
Considerations for *in vitro* testing of NMs, which should lead to increased reliability and relevance.

Topics	Recommendations
NM characterization	1. More than one appropriate technique for physico-chemical (PC) characterization (before, during and after the cell experiments). 2. Use of the identified methods for measurement of the following PC endpoints: primary particle size distribution; hydrodynamic diameter; volume specific surface area; agglomeration behaviour; surface reactivity; dissolution kinetics, as applicable; aging of NMs (particularly in suspension). 3. The importance of individual properties differs with respect to the exposure scenarios. o Of particular importance: the identified PC properties of NMs affecting material deposition and clearance from the lungs.
Cell culture characterization	1. Report the cell source, passage number, and precise cell culture method. o Detailed description of the source and providers (cell culture medium, laboratory cell culturing material), a thorough description of the cell growth, morphology and differentiation before and during the test performance.
Cell culture choice	1. A justification of the selected cell culture. 2. Cell lines (homogenous and more stable and hence more reproducible; easily accessible) are preferred over the primary cells. 3. Human cell lines are supported (more closely mimic human responses); however, rodent cell line results are more comparable to *in vivo* animal data. 4. *e.g.* Fibroblasts for pro-fibrotic signals, macrophages as primary responders, intestine or lung epithelial cells due to their barrier functions.
Dose metrics/Dosimetry	1. Careful consideration of the dispersion protocol effects for NMs: *e.g.* avoidance of ROS generation and other effects on NM; avoidance of stabilizers with toxic or mutagenic potential. Tested for presence of endotoxins. 2. The effects of the protein corona formation should be considered, if possible. 3. Higher importance of the chronic (*e.g.* repeated) exposures for regulatory risk assessment. 4. High doses are required in mechanistic studies, however it needs to be considered if the concentration of MNs in tests would exceed the level at which agglomeration is enhanced. 5. Units: mass or number concentration per cell; however it is recommended that all the three mass metrics are reported (mass, surface area and number). In the likelihood of intracellular NM alterations or dissolution, detection of intracellular NM concentration is recommended. 6. Submerged test conditions: 1–100 μg MNs/mL, with the lower and higher limits at 0.125 and 200 μg/mL. It is recommended that the concentrations used in the submerged settings do not exceed greatly the level at which agglomeration is enhanced. Deposited dose on the cell surface needs to be calculated (specified methods or models). 7. Doses selected should be anchored by known human exposures; conversions from human exposures < - > *in vitro* exposures are suggested (*via e.g.* reverse dosimetry models). 8. Air-liquid interface (ALI) protocols for lung cell experiments may overcome the issue with suspension cultures by a direct deposition of a NM onto the lung cell surface. Use of ALI (and barrier protocols) avoids the need for many dosimetric conversions since most of the aerosolisation systems can measure the deposited NM concentration on-line thus allowing to obtain a dose-related effect (Braakhuis et al., 2016)
Cellular responses	1. Multiple assays for individual endpoints should be employed. 2. NM response need to be assessed in multiple (at least three) different cell types and/or co-cultures. 3. Possible NM interferences with assay reagents, products or optical pathways need to be considered. 4. Chemical positive and negative controls need to be included in each assay and the use of NM-based negative and positive controls is recommended.

evaluation of NMs' biodegradability and biopersistence in mimicked intracellular lysosomal compartments. Many of the more general considerations useful in assessing the reliability of an *in vitro* test are summarized in Table 1:

(i) From the publications reviewed we cannot recommend one cell model which can cover everything, since clearly the models or endpoints might differ for different (disease) stages. As a first stage hazard assessment, cell lines are be preferred over primary cells. For initial screening methods, relevant mono-cultures (*i.e.* oral, lung, and intestine) and CFE might be sufficient but then more complex models and more endpoints should be included, particularly the long-term effects mimicking sub-chronic exposures.

(ii) Dose metrics: it is not sufficient to provide information about NM concentration (*i.e.* μg/mL) or the surface exposed to cells. It is recommended that all the three mass metrics are reported (mass, surface area and number). The deposited dose and/or cell burden has to be given. Estimation methods are available to provide deposited doses for submerged cultures, and more realistic dosing methods are available to more directly measure deposited doses (such as ALI exposure systems). More studies should be performed using the concentrations expected with human exposure, namely those concentrations which are occupationally relevant or daily relevant. Companies should make *in vivo* data available that would support efforts to optimize and validate cell culture approaches.

(iii) Cellular uptake of NMs is an important aspect to consider in NM hazard assessment *in vitro*. However, attention should be given to comparison with *in vivo* situation. A characteristic of *in vitro* settings is that most of the cell types (or nearly all) internalize NM that are and deposited on the cell surface are engulfed. Therefore, the most important question *in vitro* as well as *in vivo* is how many NMs reach the surface of any specific cell type. These aspects are covered in the dosimetry paper within this special issue (Oberdörster et al., 2017 - in this issue) and are not further explored herein. (iv) Finally, the following tiered testing strategy for cell culture assays is recommended before *in vivo* tests are performed:

1. In addition to the physico-chemical characterization of NMs before, during and after the test performance, protein corona kinetics during and after the test, analysis of NMs stability/biodurability in relevant physiological fluids is required.
2. The choice of relevant cell models including thorough characterization of cell growth and differentiation, inclusion of endpoints mimicking the *in situ* cell response. Comparison between acute and long-term effects.
3. Assessment of cellular uptake, biokinetics, inclusion of *i.e.* screening methods (omics) to determine MOA.

To summarize, the most important criteria to produce reliable and robust data from *in vitro* nanotoxicological assays is: (i) detailed NM characterization, including physicochemical properties before, during and after the testing, (ii) use of comparable and realistic dose metrics and test conditions, (iii) implementation of chemical positive and negative controls and reference NMs allowing for comparison between studies, both intra- and interlaboratory. The use of NM-based controls is recommended. In order to avoid false negative or false positive results, at least two independent methods per individual tested endpoint need to be performed and with multiple relevant cell types or co-culture models and standardized cytotoxicological assays (also reviewed in: (Farcal et al., 2015)). The choice of relevant cell models includes a thorough characterization of cell growth and differentiation along with the endpoints mimicking the *in situ* cell response. With respect to the comparison between acute and long-term effects: for first initial

screening, mono-cultures seem to be appropriate. However, for more in-depth mechanistic studies 3D cultures should be preferred. If the hazard of inhaled NMs is investigated, then the use of ALI lung cell cultures in combination with dose-controlled NM aerosolisation systems is recommended.

Overall, it is foreseen that *in vitro* methods for NM hazard assessment have the potential to contribute to reduction of animal studies by setting priorities for further *in vivo* testing (Braakhuis et al., 2015). However, as currently the correlation between the *in vitro* and *in vivo* assays is not clarified yet, at the moment *in vitro* tests *per se* are not suitable for NM hazard and further risk assessment. The future direction should be driven towards standardization and validation of specific *in vitro* assays mimicking defined *in vivo* endpoints.

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