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Immunotoxicity, genotoxicity and epigenetic toxicity of nanomaterials: New strategies for toxicity testing?

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ABSTRACT

The unique properties of nanomaterials (NMs) are beneficial in numerous industrial and medical applications. However, they could also induce unintended effects. Thus, a proper strategy for toxicity testing is essential in human hazard and risk assessment. Toxicity can be tested *in vivo* and *in vitro*; in compliance with the 3Rs, alternative strategies for *in vitro* testing should be further developed for NMs. Robust, standardized methods are of great importance in nanotoxicology, with comprehensive material characterization and uptake as an integral part of the testing strategy. Oxidative stress has been shown to be an underlying mechanism of possible toxicity of NMs, causing both immunotoxicity and genotoxicity. For testing NMs *in vitro*, a battery of tests should be performed on cells of human origin, either cell lines or primary cells, in conditions as close as possible to an *in vivo* situation. Novel toxicity pathways, particularly epigenetic modification, should be assessed along with conventional toxicity testing methods. However, to initiate epigenetic toxicity screens for NM exposure, there is a need to better understand their adverse effects on the epigenome, to identify robust and reproducible causal links between exposure, epigenetic changes and adverse phenotypic endpoints, and to develop improved assays to monitor epigenetic toxicity.

1. Introduction

Innovative nanotechnology research aims to develop nanomaterials (NMs) that are small, smart and safe (3S), and thus can improve our everyday life without affecting negatively our health. In general, the safety evaluation of NMs is based on principles of risk assessment applied to bulk chemical substances. However, more information is needed especially on physicochemical properties of NMs, their behavior in different environments, and interactions with biological system. To understand which physicochemical properties of NMs are coupled with adverse effects is thus critical for designing 3S NMs following 'safe by design' as a paradigm that requires implementation of safety evaluation in designing NMs.

For proper hazard and risk assessment of NMs, both external and internal exposure needs to be defined by including reference to uptake of NMs by cells. Specific and relevant toxicity tests are needed to address all possible toxicity pathways. A relevant test battery includes *in vivo* and *in vitro* assays. As with other chemical testing, the 3Rs policy should be preferably followed (reduce, refine and replace the use of animals in research). Alternative *in vitro* tests are recommended for initial screening of cytotoxicity and genotoxicity of NMs, and also for further identification of underlying cellular mechanisms of toxicity. *In vitro* tests are fast, cost-effective and can be performed as high-throughput screening (HTS) assays on relevant cells from humans and other mammals. *In vitro* assays adapted for testing of NMs can be performed in a controlled manner taking physicochemical characterization and cellular uptake into account.

Recent research has raised concern about possible epigenetic toxicity and health effects induced by NMs (Jennifer and Maciej, 2013; Shyamasundar et al., 2015; Smolkova et al., 2015, 2017). Epigenetic toxicology is a novel area of research, that examines

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epigenetic alterations induced by environmental exposures and their implications for public health. An increasing number of chemicals referred to as epimutagens, among them ions such as chromium, arsenic, nickel, lead, copper, mercury, cadmium and organic tin, have been shown to have drastic impact on the epigenome by inducing changes in DNA methylation, modifications of histone proteins, affecting chromatin structure and miRNA expression (Arita and Costa, 2009; Cheng et al., 2012). It was shown that many non-genotoxic toxicants in subtoxic concentrations can affect epigenetic processes (Stefanska et al., 2012). Some of these substances in nano-size scale are used in industry, including nanomedicine (Klostergaard and Seeney, 2012; Guo et al., 2014). The epigenetic alterations driven by NMs, especially soft particles, have rarely been studied. A growing body of evidence indicates that environmentally-induced epigenetic alterations play a role in the onset of several human diseases, including cancer, mental disorders, obesity, and other severe conditions (reviewed in (Marczylo et al., 2016). At this point, a well-standardized animal-free approach to study epimutagens is not yet available. Here we discuss new approach in hazard assessment of NMs that combines characterization of NMs, cellular uptake, standard toxicity endpoints of cytotoxicity, oxidative stress, immunotoxicity and genotoxicity, as well as novel endpoints, particularly epigenetic toxicity.

2. Characterization of NMs

An important aspect of toxicity testing is characterization of the NMs in relevant media. The physicochemical parameters of NMs change, depending on the surrounding environment, and NMs should therefore be characterized both as manufactured (in pristine state) and as applied.

A major challenge in nanotoxicology today is the huge discrepancy in reported toxicity studies. This is partly due to different intrinsic properties of NMs, but more importantly, to distinct secondary characteristics related to cell culture medium and dispersion methods, which may have a huge impact on the results of the studies. In addition, inconsistent testing conditions, with NMs in different physiological media (biological fluids and tissues), could affect kinetics, distribution and interactions of the NMs with biological components (Kato et al., 2009; Magdolenova et al., 2012; Guadagnini et al., 2015). It is also important to identify and study degradation of the NMs in the biological environment, as the release of molecular debris could induce cytotoxic effects (Treuel and Nienhaus, 2012).

A recommended list of physicochemical properties, to be explored when testing NMs in relation to human health and environmental safety, includes particle size distribution (in solid and in liquid media), shape, agglomeration/aggregation, water solubility/dispersability, surface charge and surface properties, redox potential, and potential for radical formation (Bouwmeester et al., 2011). The characterization of NMs generally but especially of airborne NMs is complicated because of the dynamic behavior of NMs as an aerosol as well as the structural complexity of the individual particles and their functionalization.

2.1. Testing methods

In 2006, the OECD launched a Working Party on Manufactured Nanomaterials (WPMN) that set up an exploratory test programme to examine the information needs and testing methods for NMs. A guidance Manual was drafted and a list of reference NMs, as well as physicochemical properties relevant for the assessment of a NM was published (OECD, 2010). The OECD WPMN evaluated the methods used for physicochemical characterization (OECD, 2016a,b) showing that not all existing analytical methods for chemical characterization are suitable also for NMs (Rasmussen et al., 2016). The OECD WPMN proposed 26 physicochemical properties. Furthermore, NMs should be characterized at different stages of their life cycle, in their pristine form, as well as under actual test conditions (Hunt et al., 2013). However, only a few methods are available for characterization of NM properties after administration, and physicochemical characterization for toxicological testing has to meet this additional challenge.

The most frequently employed microscopy techniques for characterization of NMs are scanning and transmission electron microscopy (SEM and TEM), high-resolution transmission electron microscopy (HRTEM), TEM with energy-dispersive X-ray spectroscopy (EDS), atomic force microscopy (AFM), scanning tunnelling microscopy (STM) and others. Brunauer-Emmett-Teller (BET), dynamic or static light scattering (DLS and SLS), quasi-elastic light scattering (QELS), photon correlation spectroscopy (PCS), multi-angle light scattering (MALS), nanoparticle tracking analysis (NTA), and small angle neutron scattering (SANS), are commonly used nondestructive techniques that measure particle size in liquid dispersions. Other techniques that employ light scattering and can be used for size distribution analysis are small-angle X-ray scattering (SAXS) or wide-angle X-ray scattering (WAXS). Scanning mobility particle size (SMPS) spectrometry provides information on the size of particles, agglomerates, and aggregates (Rasmussen et al., 2017).

Recently, fractionation techniques have been combined with detection techniques to determine the size distribution of particles (NanoDefine, 2016). Field flow fractionation (FFF) techniques are very powerful chromatographic methods. The techniques most used are AF4 (asymmetric flow FFF) and FIFF (crossflow FFF). The nondestructive FFF techniques can be coupled with different detectors, such as DLS, MALS, PCS or inductively coupled plasma mass spectrometry (ICP-MS) (Rasmussen et al., 2017). Another widely used chromatographic technique is capillary hydrodynamic fractionation (CHDF). Also, single particle (sp) ICP-MS can provide information on size and size distribution of nanoparticles (Linsinger et al., 2014; Rasmussen et al., 2017). Raman spectroscopy, UV-VIS, nuclear magnetic resonance (NMR) and X-ray fluorescence (XRF) spectroscopy can be applied for chemical composition analysis and can identify a wide range of elements. Several methods exist for inducing X-ray fluorescence, such as irradiation of the sample by electrons or X-rays. A modification of this technique, total reflection X-ray fluorescence (TRXF) spectroscopy, is currently widely used in the electronic industry for quality control and ISO standards are also available (ISO, 2015). Some of the techniques used for chemical composition analysis, such as UV-VIS or FTIR can also be applied to characterize the surface chemistry of NMs.

There is no consensus yet on the optimal set of techniques and procedures to be applied, mainly because of the rapidly increasing variety of NMs and the limited comparative evaluations carried out on the advantages and constraints of each analytical method and technique applied in toxicological testing (Dusinska et al., 2015).

3. Cellular uptake, transport, tissue distribution and excretion of NMs

NMs have the potential to enter cells actively or passively, and to cross cellular barriers within the body, including the blood–brain barrier (Bhaskar et al., 2010). The uptake mechanism depends on intrinsic physicochemical characteristics of the NM as well as on the route of exposure. Adsorption of biomolecules to the NM surfaces influences the interactions at the NM-bio interface (Aggarwal

et al., 2009). Protein binding (forming protein corona) and opsonization are additional processes that change the surface properties of NMs, and thus uptake. Experimental studies revealed that most NMs are actively incorporated into the cell via endocytotic pathways (Conner and Schmid, 2003; Rothen-Rutishauser et al., 2006). Alternative routes include clathrin- and caveolin-independent pathways, but these mechanisms are still poorly understood (Mayor and Pagano, 2007).

Once NMs have been taken up by the target cells, they are mainly directed to the endosomal/lysosomal pathway where they are degraded. The phenomenon of NMs escaping endosomes is called the "proton sponge effect" (ur Rehman et al., 2013). Certain types of NMs penetrate the vesicle (or cell) membrane directly and enter the cytosol, where they can induce oxidative stress. In addition, interaction with organelles, e.g. mitochondria or cell nucleus, can occur (Huk et al., 2015). The presence of tight junctions prevents NMs larger than 2 nm from leaving the circulation (Garnett and Kallinteri, 2006). Bigger NMs are mainly found in the liver and spleen, whereas smaller particles extravasate into the bone marrow. From the circulation, NMs are cleared by liver, spleen and through glomerular filtration (Volkovova et al., 2015). Chemical reactivity and composition play an important role in NM stability. While some are rapidly degraded, others can remain in the organism for weeks to months, and their accumulation in the body can cause severe damage (Ye et al., 2012).

3.1. Testing methods

The methods used for studying uptake are TEM, confocal microscopy, flow cytometry (light scattering or fluorescence) and ICP-MS (Garza-Ocañas et al., 2010). Some relatively large particles may be visible with a regular light microscope. For confocal imaging, NMs are often labeled with a fluorescent tag which allows real time tracking of particles (Seo et al., 2011). Both confocal and electron microscopy methods require *ex situ* sample preparation that may be prone to artifacts. Particle agglomerates can be induced through transition of particles from dispersed to dried state in a sample. Additionally, it is difficult to distinguish between internalized particles and those adhering to the cell surface. A promising tool to overcome these issues is Raman spectroscopy, which is more often associated with determination of molecular structure and qualitative chemical analysis. A combination of Raman spectroscopy with optical microscopy allows fast, non-invasive, and label-free study of cellular binding and uptake of NMs (Collins et al., 2017). This direct detection method may lead to a more accurate assessment of NM behavior over both the short and long term. New techniques for tissue clearing may allow for a more refined observation of particle distribution in the near future (Susaki and Ueda, 2016). For *in vivo* imaging of particle distribution magnetic resonance imaging (MRI) may be used (Al Faraj et al., 2011).

Analytical chemistry on cell supernatants can be used to study the release of NMs (Dos Santos et al., 2011; Elsaesser et al., 2011). The use of radiolabeled NMs also provides a quantitative approach to *in vivo* biodistribution studies. It is possible to determine NM uptake in the key organs as well as in other tissues, thus enabling total mass balance calculations. Dependent upon the isotope selected, further tissue processing may be required after sample removal, e.g. tissue solubilization or ashing. It is also possible to undertake real-time imaging and to follow the biodistribution *in vivo* using techniques such as positron-emission tomography (PET), computed tomography (CT) and single photon emission computed tomography (SPECT) with appropriate labeling of NMs (Chen et al., 2011).

4. Immunotoxicity testing of NMs

4.1. Mechanisms of immunotoxicity

NMs can interact with immunocompetent cells and induce immunotoxicity (Zolnik et al., 2010; Di Gioacchino et al., 2011; Hussain et al., 2012; Dobrovolskaia and McNeil, 2016). Direct damage to immune cells by NMs leads to apoptosis and necrosis, while interactions of NMs with the immune response itself can change immune-specific signaling pathways, resulting in changes in immune cell function measured by expression of surface markers, cytokine production, cell differentiation and immune activation (Hartung and Corsini, 2013). The key elements in the identification of NM-induced immunotoxicity are duration and deregulation of the inflammatory response. Therefore, reliable testing requires relevant *in vitro* and *in vivo* models, that can distinguish between normal and pathological responses (Boraschi et al., 2017). Due to their small size, NMs may escape the particle-clearing defensive mechanisms (e.g.phagocytosis), and so many of them do not trigger a direct inflammatory response (Kunzmann et al., 2011). The increased aggregation that tends to occur on contact with the biological environment may cause effective clearing or sequestering by immune cell which tend to recognize preferentially larger particles (>0.5 μm) (Geiser, 2010). Moreover, inflammation may be caused by bacterial components adsorbed to the NMs. The reactions of self-proteins with NMs and their persistence in the organism can cause autoimmune reactions. Another factor contributing to autoimmunity is the defective clearance of apoptotic cells by scavenger phagocytes (Muñoz et al., 2010). Activation of inflammasomes can occur via several mechanisms (rupture of lysosomes, direct recognition of NMs by toll-like receptors) (Boraschi et al., 2017). Neutrophils or polymorphonuclear granulocytes play a key role in NM-induced inflammation (Bhattacharya et al., 2013). Activation of mast cells can lead to production of histamines and other substances causing airway inflammation. One factor suspected to contribute to the recent dramatic increase in incidence of allergies, lung diseases and asthma is environmental pollution and inhalation of ultrafine particles (Heinrich, 2011).

4.2. Testing methods

Assessment of immunotoxicity is essential for safe use of NMs and therefore development of appropriate *in vitro* tests and cellular models is required (Dusinska et al., 2009, 2015; Tulinska et al., 2015). Although not specifically developed to test NMs, OECD methods for testing of chemicals, EFSA recommendations, and guidance for industry (ICH, 2005) can help to identify possible effects on the immune system. Guidance is specifically focused on non-clinical testing for immunotoxicity induced by human pharmaceuticals, restricted to unintended immunosuppression and immunoenhancement, and excluding allergenicity or drug-specific autoimmunity. Different *in vivo* and *in vitro* tests for immunotoxicity are available (Table 1).

Immunotoxicity can be tested *in vivo* in experimental animal models for human risk assessment (Smith et al., 2016). An advantage of *in vivo* models is the ability to fully study adsorption, distribution, metabolism and excretion (ADME) of NMs, and these factors play a critical role in the immunological response (Oostingh et al., 2011). However, to meet the 3Rs requirement, and to increase efficiency, alternative *in vitro* testing strategies need to be developed (Dobrovolskaia and McNeil, 2013). Validation of an *in vitro* method to detect immunotoxicity must rely on high quality *in vivo* data. In this regard, it is essential that a sufficiently large number of positive and negative reference compounds, including both drugs

Table 1
Immunotoxicity tests.

Endpoint	Existing method	Recommended for NM testing/notes	Validated/Reference
Direct Immunotoxicity	*In vivo* repeated dose 28-day oral toxicity study in rodents including clinical signs of infections, hematology, serum immunoglobulin levels and albumin/globulin ratio, organ weights (thymus, spleen) enhanced histopathology evaluation of primary and secondary lymphoid organs, i.e., bone marrow, thymus, spleen, draining and distant lymph nodes	Yes	OECD 407, ICH S8, 2005[a]
	In vivo Host resistance models (viruses, bacteria, parasites, tumors)	Yes	(Burleson and Burleson, 2010); (Freebern, 2010); (Luebke, 2010); (Ng et al., 2010)
	In vivo/In vitro T-dependent antibody response	Yes	(Plitnick and Herzyk, 2010; White et al., 2010; Fischer et al., 2011; Lebrec et al., 2014)
	In vivo/In vitro The Cytotoxic T-lymphocyte assay	Yes	(Burleson et al., 2010)
	In vivo/In vitro NK cell assays	Yes	(Li and Huang, 2010)
	In vivo Immunophenotypic analysis of peripheral blood leukocytes	Yes	(Lappin and Black, 2003; Zeigler et al., 2013)
	In vivo/In vitro Lymphocyte proliferation assay	Yes (with limitations)	(Dobrovolskaia and McNeil, 2007; Dobrovolskaia et al., 2008; Hartung and Corsini, 2013)
	In vivo/In vitro Expression of cytokines	Yes (with limitations)	(Corsini and House, 2010)
	In vivo/In vitro Function of phagocytes	Yes	(Barnett and Brundage, 2010)
	In vitro Myelotoxicity - CFU-GM assay	Yes (with limitations)	(Dal Negro et al., 2001; Pessina et al., 2001; Dobrovolskaia et al., 2009)
	In vitro Lymphotoxicity, apoptosis, necrosis	Yes	(Nagarkatti et al., 2010)
	In vitro dendritic cell maturation	Yes	(Gao and Lawrence, 2010)
Delayed type hypersensitivity reaction	*In vivo* The delayed type hypersensitivity assay using protein and xenogenic cell antigens	Yes	(Dietert et al., 2010)
	In vivo Lymph node proliferation assay	Yes (for NMs < 100 nm)	(Weaver et al., 2005; Dobrovolskaia et al., 2009)
Complement activation	*In vitro* Complement activation	Yes	(Dobrovolskaia et al., 2009)

[a] http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S8/Step4/S8_Guideline.pdf; OECD methods: http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm.

and chemicals, are tested (Gennari et al., 2005; Dusinska et al., 2015). The suitability of different *in vitro* immunotoxicity methods for the analysis of NM-induced immunotoxicity is widely discussed (Gennari et al., 2005; Pfaller et al., 2010; Oostingh et al., 2011; Luebke, 2012; Hartung and Corsini, 2013; Boverhof et al., 2014; Giannakou et al., 2016). Most *in vitro* models for immunotoxicity detect immunosuppression, an unspecific immune response that can be induced by a wide range of events. However, some *in vitro* models include innate and adaptive immune system cells as well as cellular markers associated with immune function, such as gene expression, protein synthesis and proliferation (Luebke, 2012; Hartung and Corsini, 2013).

Selection of appropriate cell models for *in vitro* screening for immunotoxicity is of strategic importance. For maximizing human relevance, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) recommends the use of human cells for all *in vitro* test systems. Primary human cells will be of the highest clinical relevance. With the exception of bone marrow assays, the source of cells should be peripheral blood leukocytes which are easily accessible from donors (prescreened for health, immune reactivity, etc.) (Gennari et al., 2005; Tulinska et al., 2015). Blood is a first target model for intravenous administration of NMs used in medical diagnostics and therapy, and a surrogate target model for other routes of exposure. The blood cell model gives information on the overall body response to NMs, as well as being applicable to environmental and industrial pollutants (Dusinska and Collins, 2008; Oostingh et al., 2011). Further, the main strength of the human peripheral blood cells as an *in vitro* testing model is its complexity, with several cell components present in a relatively intact environment. Primary cells are often more sensitive compared with continuous cell lines, with a qualitatively different response (Oostingh et al., 2011). Challenges related to this cell model are high inter-individual differences between human blood donors and the relatively short survival time of primary blood cells in culture. Besides primary cells, well-characterized and validated cell lines (human or animal) can be used for immunotoxicity testing of NMs (Gennari et al., 2005). Recently, several research groups published alternative approaches, using various cell lines - human Jurkat T-cell, human lymphoid T-cell (MOLT-4) or B-cell (IM-9), human acute myeloid leukemia HL-60 cell, murine T-cell (CTLL-2) or THP-1 (human monocytic cell line derived from acute monocytic leukemia patient) (Minervini et al., 2005; Schmeits et al., 2013, 2015; Song et al., 2014; Markovič et al., 2015). Precision-cut tissue slices are also being used (Sewald and Braun, 2013).

An *in vitro* tiered approach was originally proposed to screen for adverse effects of chemicals on immune cells (Gennari et al., 2005). In the first tier, evaluation of myelotoxicity should be performed. In the second tier, lymphotoxicity testing should be carried out since lymphocytes are the primary effector and regulatory cells of acquired immunity. In tier 3, immune cell functionality should be assessed by performing specific functional assays, i.e., proliferative

responses, cytokine production, NK-cell activity, using non-cytotoxic concentrations of the tested chemicals (viability >80%).

Immune function assays are reliable tests to monitor the action of immunocompetent cells. They are widely used to determine congenital or acquired immune disorders in clinical diagnostics. Adaptation of such tests allows evaluation of the effect of NMs on specific (acquired) and non-specific (natural) immune responses. Specific immune response can be monitored *in vitro* by a proliferative response of lymphocytes stimulated with mitogens and antigens using for instance ^{3}H-thymidine incorporation or flow cytometry. For T-cells, mitogens such as concanavalin A (ConA) and phytohemagglutinin (PHA) or antigens such as anti-CD3 and anti-CD28 can stimulate the cells. For T-dependent B-cell response, mitogen pokeweed can be used (Tulinska et al., 2015). However, an optimal system still needs to be developed.

The function of phagocytes can easily be determined by measuring the phagocytic activity of granulocytes and monocytes; for example, using fluorescein-labelled *Staphylococcus aureus* with flow cytometry. Moreover, the respiratory burst of phagocytes can be measured (using hydroxyethidine) in the same tube. Natural killer cell activity is a reliable method for monitoring tumor defence and has been recommended for assessment of immunotoxic potential (Lankveld et al., 2010). Cytolytic function of NK-cells can be measured by either radiolabel release or flow cytometry.

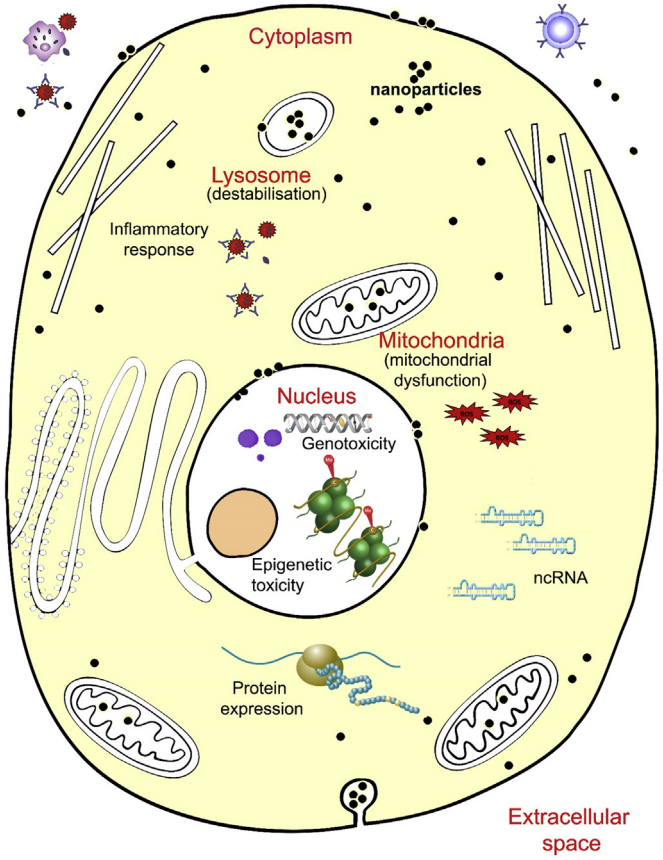

Fig. 1. Schematic illustration of a link between NM-induced epigenetic changes and other forms of NM toxicity.
NMs taken up by cells can either directly interact with the genetic material or indirectly affect intermediate molecules which are involved in normal genome function or cell division. Oxidative stress is considered to be the key indirect mechanisms of NM-induced genotoxicity. Induced epigenetic alterations can mediate chromosomal instability or changes in locus specific gene expression, possibly affecting multiple cellular pathways and targets. nc, non-coding.

Potential effects of NMs on cytokine expression should be determined by enzyme-linked immunosorbent assay (ELISA), flow cytometry or molecular biology techniques, such as real-time polymerase chain reaction (RT-PCR). The cytokines to be measured, such as proinflammatory or specific immunoregulatory cytokines, need to be carefully considered to obtain the most appropriate information. Both basal and activated cytokine production should be measured, and for activated cytokine production, anti-CD3 and anti-CD28, lipopolysaccharides (LPS), or allergens should be used (Hartung and Corsini, 2013). An assessment of the functionality of immune cells could also include the measurement of other mediators, e.g., histamine, cytokines or activation of the complement cascade leading to hypersensitivity reactions.

The systems and methods currently in use for testing immunotoxicity, described in Table 1, are reliable and reproducible. However, implementation for testing the effects of NMs requires additional development (Luebke, 2012; Hartung and Corsini, 2013).

5. Genotoxicity testing of NMs

5.1. Mechanisms of genotoxicity

Genotoxicity testing is crucial in safety assessment of new compounds and products including NMs (Dusinska et al., 2015; Doak and Dusinska, 2017) as it may have implications for risks of cancer and possibly other chronic diseases. If the genetic event occurs in germ cells, it may cause genetic disease or reproductive toxicity and thus may influence the health not only of individuals but also of the next generation. Published data show that there are direct and indirect mechanisms of NM-induced genotoxicity (Bhabra et al., 2009; Donaldson et al., 2010; Sood et al., 2011; Magdolenova et al., 2014; Doak and Dusinska, 2017). NMs taken up by cells can come into direct contact with the genetic material causing physical or chemical damage (Fig. 1). However, the most likely mechanism of NM-induced genotoxicity is indirect, via intermediate biomolecules that either are involved in normal genome function or cell division, or attack DNA, causing DNA injury or chromosomal malformations. Oxidative stress is considered to be the key indirect mechanism of NM-induced genotoxicity. Induction of reactive oxygen species (ROS) by NMs can cause DNA injury as well as damage to lipids, proteins and other cellular components. The secondary mechanisms of NM-induced genotoxicity *in vivo* are also mediated by extracellular ROS via the inflammatory responses of macrophages and neutrophils (Stone et al., 2009; Magdolenova et al., 2014).

Genotoxicity covers different endpoints depending on which level of the genome is targeted and where injury takes place. It includes different DNA lesions as well as mutagenicity at the gene level, chromosome breakage and/or rearrangements (clastogenicity), and numerical chromosome aberrations (aneuploidy).

5.2. Testing methods

Genotoxicity testing of NMs can be carried out *in vitro* and *in vivo* (Table 2). The *in vitro* approach is appropriate for testing primary genotoxicity, while *in vivo* models can also give information on secondary effects such as inflammation (Arora et al., 2012; Dusinska et al., 2011). However, novel *in vitro* techniques (such as 3D co-culture of different cell types) are under development and should lead to model systems simulating *in vivo* conditions (Evans et al., 2017). Testing with cultured mammalian cells (either permanent cell lines or primary cultures) employs various endpoints: DNA damage, gene mutations and chromosomal damage or aneuploidy. *In vivo*, DNA damage, chromosome aberrations (Dandekar et al., 2010) and micronuclei can be measured in different tissues;

Table 2
Genotoxicity and carcinogenicity tests.

Endpoint	Existing method	Recommended for NM testing/notes	Validated/Reference
Genotoxicity			
Mutations	Bacterial reverse mutation test (Ames test)	No (Cell wall different from mammalian cell membrane, limited uptake)	OECD 471
	In vitro mammalian gene mutation test (*HPRT* or *TK*)	Yes	OECD 476
	In vitro mammalian gene mutation test (*TK*)		OECD 492
	In vitro Pig-a mammalian gene mutation test	Under development	
	In vivo Pig-a mammalian gene mutation	Yes	
	In vivo mouse heritable translocation assay	Yes	OECD 485
Chromosomal damage	*In vivo* mammalian erythrocyte micronucleus test	Yes	OECD 474
	Mammalian bone marrow chromosome aberration test	Yes	OECD 475
	In vitro mammalian chromosome aberration test	Yes	OECD 473
	In vivo mammalian spermatogonial chromosome aberration test	Yes	OECD 483
	In vitro mammalian cell micronucleus test	Yes (with modifications for NMs)	OECD 487
	Rodent dominant lethal test	Yes	OECD 478
DNA damage and repair	*In vivo* comet assay	Yes	OECD 489
	In vitro comet assay	Yes	(Dusinska and Collins, 1996; El Yamani et al., 2017) JaCVAM EURL-ECVAM/ICVAM
	The γH2AX assay	Yes	(Ismail et al., 2007; Collins et al., 2017)
	In vitro DNA unwinding	Yes	(Semisch et al., 2014)
	Unscheduled DNA synthesis (UDS) test with mammalian liver cells *in vivo*	No	OECD 486
Carcinogenicity			
	Carcinogenicity studies (*in vivo*)	Yes	OECD 451
	Combined chronic toxicity/carcinogenicity studies (*in vivo*)	Yes	OECD 453
	In vitro carcinogenicity: Syrian hamster embryo (SHE) cell transformation assay	Yes (used for NM)	OECD draft (No 31); EU B.21; OECD Guidance document; Series on Testing & Assessement No. 214, May 2015
	In vitro carcinogenicity: Bhas 42 cell transformation assay	Yes (used for NM)	OECD Guidance document; Series on Testing & Assessment No. 231, January 8, 2016

OECD methods: http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm.

and transgenic rodents are available that allow detection of mutations at a specific locus in cells from different organs (Magdolenova et al., 2014).

5.2.1. DNA damage

DNA damage can be measured with various assays such as alkaline elution, neutral elution, DNA unwinding chromatography (Semisch et al., 2014), fluorometric detection of alkaline DNA unwinding (Moreno-Villanueva et al., 2011), or γ-H2AX double strand break assay, but the most common test for detecting DNA damage after NM exposure is the comet assay (Magdolenova et al., 2014; Cowie et al., 2015; El Yamani et al., 2017).

The comet assay (single cell gel electrophoresis) is one of the most common tests for genotoxicity, detecting DNA breaks and alkali-labile sites, but also damaged bases by incubating nucleoids with lesion-specific endonucleases, such as endonuclease III and formamidopyrimidine DNA glycosylase (Fpg) that recognize oxidized pyrimidines and purines respectively (Huk et al., 2014; Dusinska and Collins, 1996). Photogenotoxic effects of NMs in combination with ultraviolet radiation have been measured with the comet assay (Jha, 2008). The possibility of interference of NMs in the assay - causing additional damage to DNA during the performance of the assay, or inhibiting enzyme activity - has been discussed (Magdolenova et al., 2012; Karlsson et al., 2015) but the evidence to date suggests that these concerns are unfounded and the comet assay is a reliable test, suitable for adaptation as a high-throughput assay.

A relatively specific and sensitive assay for double strand breaks is based on the fact that a cellular response to these breaks is phosphorylation of one of the core nucleosomal histones, H2AX. The phosphorylated form is known as γ-H2AX, and the concentration in the proximity of a double strand break is sufficient to form a focus, visible by immunohistochemistry using a fluorescence-tagged antibody to the phosphorylated form. Alternatively, γ-H2AX can be measured by flow cytometry (Ismail et al., 2007; Lewis et al., 2010). The γ-H2AX assay is an interesting endpoint for automated procedures, but little information so far exists about using this test to predict NM-induced genotoxicity.

5.2.2. Gene mutations

Initial testing for genotoxicity of chemicals is usually done with the bacterial Ames test (Warheit et al., 2007). The Ames test is based on induction of reverse-mutations in a defective histidine synthetase gene. Reversal of this mutation will restore function of the synthetase, enabling the bacterium to form a visible colony when plated in minimal histidine medium. However, this assay has serious limitations for NM mutagenicity testing on account of the size of bacteria (not much bigger than some NMs), and the presence of a cell wall, which limits or prevents uptake of NMs (Doak et al., 2012). If they do enter the cell, NMs could possibly interfere with histidine synthesis and induce false negative or positive results. The Ames test has nevertheless been used to assess genotoxicity of a variety of NMs, and has so far given largely negative results (Landsiedel et al., 2009; Doak et al., 2012; Magdolenova et al., 2014).

Mammalian cells are more suitable to test mutagenicity of NMs. Mutations are assessed at a specific locus - often the *HPRT* (hypoxanthine phosphoribosyltransferase) or *TK* (thymidine kinase) genes (Wang et al., 2007; Huk et al., 2014, 2015; Gabelova et al., 2017). These assays can detect a range of mutations, including

deletions; however, the mouse lymphoma assay (based on the *TK* gene) detects deletions with higher efficiency compared with the *HPRT* gene mutation test (Johnson, 2012). HPRT is a purine salvage enzyme, which phosphorylates 'waste' purines and adds them to the cellular DNA precursor nucleotide pool (Wang et al., 2011; Huk et al., 2014). The *HPRT* gene is X-linked, with only one active copy per cell, so that a mutation in only one allele is needed for phenotypic expression. Recently the *in vitro* Pig-a (phosphatidylinositol glycan anchor biosynthesis, class A) gene mutation assay was used; it could complement the *in vivo* Pig-a assay and also be included among *in vitro* mammalian mutagenicity tests (Krüger et al., 2015).

5.2.3. Clastogenicity and aneugenicity

The chromosomal aberration (CHA) test identifies agents that cause structural chromosome or chromatid breaks, dicentrics and other abnormal chromosomes, notably translocations which are implicated in the aetiology of various human genetic diseases and cancers. For *in vitro* testing, cell cultures are exposed to the test substance and incubated with a metaphase-arresting substance (e.g. colcemid) to accumulate metaphase cells, which are analysed microscopically (Bonassi et al., 2008; Aoshima et al., 2010). Chromosomal damage is scored either in mitotic cells as chromosome aberrations or in interphase cells as micronuclei.

Micronuclei (MN) are formed during anaphase from chromosomal fragments or whole chromosomes that are left behind when the nucleus divides. Excluded from the nuclei of daughter cells, they form single or multiple micronuclei in the cytoplasm, which are detected by visual (or automated) examination after staining. Formation of nucleoplasmic bridges and binucleated cells provides a complementary assay for chromosome rearrangement. Increased assay sensitivity can be obtained (in the *in vitro* assay) by incubating treated cells with cytochalasin B, which blocks cell division, but not mitosis, so that binucleated cells accumulate. However, cytochalasin B inhibits endocytosis and may thus prevent uptake of NMs. Therefore, the cells need to be exposed to NMs several hours before adding cytochalasin B (Magdolenova et al., 2012; Dusinska et al., 2015). Additionally, at least 24 h treatment is recommended to cover 1–1.5 cell cycles, as some compounds including NMs might be active only at a specific cell cycle stage and also access to nuclear DNA will be facilitated by the absence of the nuclear membrane during mitosis (Dusinska et al., 2015). The MN assay is less time-consuming and thus more used for NM testing than the CHA assay. Histological staining with labelled DNA probes reduces the risk of falsely identifying NM aggregates as MN fragments (Doak et al., 2009; Fenech et al., 2011; Li et al., 2012).

5.2.4. Cell transformation

Recently, cell transformation assays (CTAs) have been used to test NM**s** (and larger particles and fibres) (Ponti et al., 2009; Ohmori et al., 2013). CTAs are *in vitro* tests measuring the conversion from normal to transformed phenotype of mammalian cells (primary Syrian hamster embryo (SHE) cells, or stable cell lines such as mouse BALB/c-3T3 or C3H/10T1/2) when exposed to test chemicals. SHE and BALB/c 3T3 CTA have the potential to detect non-genotoxic as well as genotoxic carcinogens. The most frequently used endpoint is morphological transformation; morphologically transformed cells are characterized by the loss of density-dependent regulation of growth and the formation of colonies with criss-crossed cells or foci of piled-up cells that are not observed in untreated control cultures (Gabelova et al., 2017; Sasaki et al., 2014).

5.2.5. Testing battery for genotoxicity

OECD test guidelines exist for several genotoxicity assays *in vivo* and *in vitro*. Table 2 gives an overview of available tests for genotoxicity and carcinogenicity testing of NMs, with the OECD test guideline reference to each test, where available. However, these OECD test guidelines are formulated for testing chemicals (Warheit and Donner, 2010), and their suitability for NM testing is now under discussion in the OECD working group on genotoxicity. For genotoxicity testing of NMs a combination of the comet assay, mammalian gene mutation assays (in either *TK* or *HPRT* locus), and (for clastogenicity and aneugenicity) the cytokinesis-block MN assay modified for NM genotoxicity testing, seems most appropriate. CTAs are promising tests for predicting NM-induced cell transformation as one of the crucial carcinogenicity endpoints (Table 2).

6. A novel endpoint in toxicity testing of NMs – epigenetics

Epigenetics refers to heritable changes in gene expression that occur without alterations in DNA sequence. The epigenetic landscape depends on the interplay among three basic mechanisms - DNA methylation, histone modifications and RNA-mediated post-transcriptional regulation (Fig. 2). Collectively they enable sophisticated, time- and tissue-specific control of gene expression during development and differentiation or in response to internal and external stimuli, including exposure to various toxicants. Indirectly, NMs can influence DNA methylation by their pro-oxidative properties. Oxidatively damaged DNA can affect the ability of DNA methyltransferases, a family of enzymes that catalyze the transfer of a methyl group to DNA, to interact with DNA (Simkó et al., 2011). Down- or up-regulation of epigenetic modifiers (enzymes that catalyze epigenetic modifications), were repeatedly found after NM-exposure (Sule et al., 2008; Qian et al., 2015; Ma et al., 2016; Patil et al., 2016; Choudhury et al., 2017). These changes can cause large-scale disruptions of DNA methylation and histone modification patterns. However, there is still a lack of information about NM-induced modifications to histone proteins (Choi et al., 2008; Gao et al., 2016). Several NMs exhibited epigenetic effects in terms of deregulation of microRNA (miRNA) expression profiles (Li et al., 2011; Chew et al., 2012; Balansky et al., 2013; Eom et al., 2014; Sun et al., 2015; Alinovi et al., 2017). Despite an increasing number of studies, showing epigenetic changes resulting from NM exposure, their potential health risk remains controversial, mainly due to the lack of human epidemiological data. Currently, the evidence for epigenetic toxicity of NMs is mostly based on *in vitro* and animal models. Development of effective testing strategies can help us to distinguish between adverse health effects of NM exposure in contrast to adaptive changes.

6.1. Model systems for testing NM-induced epigenetic toxicity

Epigenetic endpoints such as global hypomethylation, gene-specific hypermethylation, changes in chromatin structure and aberrant miRNA-regulated gene expression are difficult to study in human subjects, because of their cellular and tissue specificity and the complex biological environment (Mytych and Wnuk, 2013). They are insufficient in themselves as indicators of an adverse response without connection with phenotype, for example disease-associated aberrant gene expression (Rasoulpour et al., 2011). Our current lack of knowledge about the interplay between individual members of the epigenetic machinery and the precise participation of each in the regulation of gene expression is the main reason that no hazard assessment test for epigenetic toxicity of NMs is available. Testing is challenging also because the epigenetic effects mediated by NMs are likely to be species-, tissue-, exposure- and time-specific. To test epigenetic effects of NM exposure, it is important to develop long-term impact assessment, in contrast with classical toxicology tests, which are focused primarily on acute

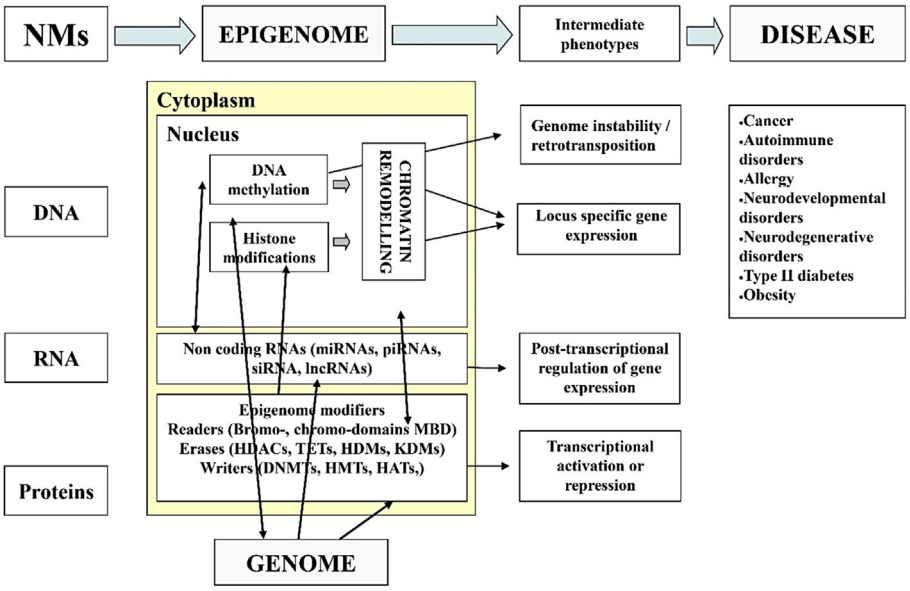

Fig. 2. Schematic illustration of a link between NM-induced epigenetic changes and disease risk.
NMs can induce epigenetic changes on DNA, RNA and protein level. In nucleus, global as well as gene specific shift in DNA methylation pattern has been reported, having impact on chromatin remodeling and subsequently affecting locus specific gene expression. Global DNA hypomethylation was associated with increased genomic instability. NM-induced variations in gene expression of ncRNAs are the second mechanism that participates in aberrant expression pattern potentially leading to pathological development. Genetic variability in epigenetic modulators can account for large spectrum of epigenetic deregulation with a possible impact on DNA methylation and histone modification, as well as ncRNA expression. Aberrant DNA methylation can in turn affect gene expression of ncRNAs or epigenetic regulators. Disruption of the equilibrium in these high order regulations can have an impact on disease development. Listed diseases were associated with aberrant epigenetic regulations and can be induced via NM and other environmental exposures. nc, non-coding; lnc, long non-coding.

or sub-acute toxicological effects. In addition to the use of traditional *in vitro* models, rat and/or rabbit models were proposed to assess *trans*-generational epigenetic effects, as well as zebrafish, *Drosophila* and *Caenorhabditis elegans* (Lyko et al., 2006; Zhang et al., 2007; Elango et al., 2009). However, there are many difficulties in interpreting the results from animal models for hazard identification because of issues such as species differences in epigenetic control mechanisms, metabolism and tissue-specific responses (Goodman et al., 2010). The use of induced pluripotent stem cells (iPSC) (Greally and Jacobs, 2013) is one of the promising *in vitro* approaches to studying NM-mediated epigenetic deregulation. This system allows generation of cell culture models that may be able to generate human cell types, normally very difficult to obtain. However, several issues remain as significant hurdles as discussed elsewhere (Scott et al., 2013). Improvements in iPSC production and differentiation processes are required before cell-based toxicity assays, that accurately reflect mature tissue phenotypes, can be implemented in a cost-effective manner (Scott et al., 2013).

6.2. Methods for testing NM-induced epigenetic toxicity

Epigenetic changes can be tested using both standard molecular biology techniques and whole-genome approaches (WGA) (Ren et al., 2017) (Table 3). Bisulfide treatment and chromatin immunoprecipitation (ChIP) are crucial steps for investigating DNA methylation and histone modifications as well as protein-chromatin interactions. Additionally, there are several methylation-sensitive restriction endonucleases with the ability to recognize methylation specific restriction sites on DNA. Together with common molecular biological methods e.g. PCR, sequencing, Southern and Western blotting, high performance liquid chromatography (HPLC), MS and others, they provide powerful tools for investigation of epigenetic changes. Recently, a modified, methylation-sensitive comet assay has been developed as a tool for high throughput screening of DNA methylation changes (Wentzel et al., 2010; Lewies et al., 2014) and is being standardized for NM testing under the Horizon 2020 HISENTS project (http://hisents.eu/). Most epigenetic studies focus on DNA methylation, largely because this modification is the easiest to be studied. The samples are easily prepared compared to more complex preparation required for ChIP-based assays. Methylated DNA immunoprecipitation (MeDIP) allows immunoprecipitation of methylated DNA which can be identified by traditional methods or WGA (massive parallel sequencing (MPS) and microarray hybridization). DNA methylation analyses enable quantitative evaluation in contrast to ChIP assays. This is a major disadvantage limiting larger use of ChIP and the reason for low availability of data on NM-induced changes of histone modifications. DNA methylation can be studied at global or gene-specific levels. Global DNA methylation changes are studied mostly by analysis of repetitive sequences LINE-1 or Alu and measured as global 5-methylcytosine (5-mC) and 5-hydroxymethyl cytosine (5-hmC) levels. Gene-specific DNA methylation analyses are performed using PCR based methods – pyrosequencing, methylation specific PCR, RT-PCR, matrix assisted laser desorption ionization–time of flight MS (Maldi-TOF MS), Sequenom MassArray and others or by high-coverage methods (MPS, arrays). After purification, the histone modifications can be analysed either by ELISA, to study global genomic content of certain modification, or using modification-specific antibodies. They allow identification of non-histone proteins such as chromatin-remodeling or histone modifying factors associated with chromatin. Downstream analyses can be performed by ChIP qPCR (gene-specific measure) or across the genome by hybridization to microarray (ChIP-Chip) or using MPS (ChIP-Seq). Methods used to study dynamic chromatin processes were recently reviewed in depth (Cuvier and Fierz, 2017). MiRNA changes can be measured by the same methods as for mRNA analysis (RT-PCR, microarray, deep

Table 3
Methods for testing epigenetic endpoints.

Endpoint	Existing method	Applications	Reference
DNA methylation	Whole-genome approaches (WGA) massively parallel DNA sequencing (MPS), microarrays	Gene specific DNA methylation, differentially methylated regions	(Ren et al., 2017)
	HPLC	Global level of 5-mC, 5-hmC	(Ren et al., 2017)
	Methylation sensitive comet assay	Toxicology screenig	(Wentzel et al., 2010; Lewies et al., 2014)
	Pyrosequencing	Global DNA methylation - repetitive sequences LINE-1 or Alu; gene specific DNA methylation	(Ren et al., 2017)
	Molecular biology and mass spectrometry techniques: methylation specific PCR, real-time PCR (RT-PCR, Maldi-TOF MS, Sequenom MassArray	Semi-quantitative or quantitative	(Ren et al., 2017)
	Methylated DNA immunoprecipitation (MeDIP)	Downstream analyses by traditional methods or WGA (massive parallel sequencing (MPS) and microarray hybridization) or molecular biology	(Ren et al., 2017)
Histone modifications	ChIP-qPCR, Chromatin immunoprecipitation (ChIP) coupled to detection by quantitative RT-PCR	Transcription factor binding to DNA	(Cuvier and Fierz, 2017)
	ChIP-Chip, chromatin immunoprecipitation with DNA microarray	Interactions between proteins and DNA *in vivo*	(Cuvier and Fierz, 2017)
	ChIP-Seq, chromatin immunoprecipitation with MPS	Mapping global binding sites precisely for any protein of interest	(Cuvier and Fierz, 2017)
Non-coding RNAs	RT-PCR	Selected miRNAs, small scale experiments	(Tian et al., 2015)
	Microarrays	Thousands of miRNAs	(Tian et al., 2015)
	RNA-seq	Whole genome analysis	(Tian et al., 2015)

sequencing). Recently developed single cell genomic approaches will allow us to gain insight into the dynamics of molecular changes induced by NMs. The recent review of Clark and colleagues focuses on single cell epigenomic methods and their application for understanding gene regulation (Clark et al., 2016). The emerging evidence shows that NM-induced changes in gene expression do not occur at predictable locations (reviewed in Smolkova et al., 2015; Smolkova et al., 2017)). This is the reason why WGA are, despite their costs, the most promising approach to test epigenetic toxicity of NMs. While microarray platforms are mostly focused on analysis of DNA methylation in promoters of genes or CpG islands, MPS have no such limitations. On the other hand, changes occurring in areas outside known genes could be difficult to interpret in terms of their functional consequences. The generation of large datasets calls for the development of novel bioinformatic instruments for their functional annotation and consequences. System biology can help to decipher this complexity and provides a valuable tool for predicting adverse outcomes of NM-exposure.

7. High throughput screening of NMs

With the growing number and complexity of the new generation of manufactured NMs, there is a huge demand to come up with rapid and reliable ways of testing their safety, preferably using *in vitro* approaches so as to avoid the ethical dilemmas associated with animal-based research. In developing intelligent testing strategies for risk assessment of NMs based on grouping and read-across approaches, and to satisfy regulatory needs, predictive models should be developed based on a wide pool of reliable data. This can be achieved with HTS and high content analysis (HCA) approaches. Adoption of the HTS/HCA methods in the hazard assessment of NMs allows the testing of large numbers of different materials, at different concentrations and time points, and on different types of cells, addressing many toxicological endpoints. It also reduces the effect of inter-experimental variation, and makes substantial savings in time and cost. HTS/HCA approaches facilitate the classification of the key biological indicators of MNM-cell interactions. Within the EC FP7 NANoREG project (www.nanoreg.eu/) HTS/HCA, methods have been reviewed and their adaptation for NM testing was thoroughly assessed. Advantages and challenges pertaining to HTS/HCA approaches in NM safety evaluation have been identified and are summarised in the paper by Collins et al. (2017).

Prediction of the toxicity of compounds can be optimized using *in vitro* toxicity screening methods by means of a battery of tests for different kinds of toxicity. Such a battery of tests will be based on the identification of a certain set of critical issues for specific compounds including cytotoxicity, immunotoxicity, genotoxicity, cell transformation and epigenetic toxicity. According to Schoonen et al. (2013) these tests preferentially should meet criteria such as: a) the amounts of compound for testing should be limited; b) the purity of the compound should already be high enough for toxicity testing; c) the solubility of the compound should be checked at all tested concentrations; d) the times of the assays should be short, preferentially between 1 and 5 days; e) the throughput should be high, preferentially at least 40–80 compounds per assay; f) the assays should identify cell-, tissue-, and species-specific effects.

One uniquely challenging aspect of nanotoxicology is that it is highly property- and structure-dependent (Landsiedel et al., 2009; Verma and Stellacci, 2010). The challenges in evaluating the large number of NMs and their complex variations using resource-intensive and time-consuming *in vivo* assays, are a motivating factor for developing faster, more economical and reliable assays (Lan et al., 2014; Collins et al., 2017). Several methods have been developed and adapted for HTS applications during recent years. The modifications can involve improved methods of cell cultivation and manipulation (e.g. using robotics), accumulation of samples at low temperature for bulk processing, miniaturization of assays so that more samples are processed together in a shorter time, and accelerated processing and analysis of results. HTS methods save time and money and large-scale experiments may be performed which are otherwise not practicable, and automation gives more uniform sample treatment and less dependence on operator performance. The HTS modifications now available vary largely in their versatility, capacity, complexity, validation status, and costs. Given reliable *in vitro* tests, animal toxicity testing can be postponed or abandoned and the amounts of compound needed for testing are minimized by miniaturization of the test models into 96- or 384-

well plates (Schoonen et al., 2013; Collins et al., 2017). There are several technical challenges related to HTS/HCA for NM testing, such as possible interference of NMs with HTS/HCA techniques. A common bottleneck results from the fact that ideally toxicity screening should be coupled with characterization of NMs in exposure medium *in situ*, prior to and at the end of the test. However, characterization methods are not yet developed for HTS. This obstacle can be partially overcome by using a freezing dispersion medium (Vila et al., 2017) or by experimental design to test several cell lines, times of exposure and toxicity endpoints (El Yamani et al., 2017).

Several cytotoxicity, immunotoxicity and genotoxicity assays have been adapted to HTS screening (Collins et al., 2017). An example of the adaptation of a standard assay to HTS screening and miniaturization is the comet assay that can measure DNA breaks and specific DNA lesions (as well as, perhaps, global DNA methylation) (Watson et al., 2014; Dusinska et al., 2015; Harris et al., 2015; El Yamani et al., 2017). As well as saving time, the ability to run several hundred sample gels in one experiment means that inter-experimental variation is less of an issue. The problem with increasing the throughput of samples is that the amount of scoring is similarly increased, and this can become a serious bottleneck (Brunborg et al., 2014). Therefore, attention is being given to developing and, importantly, validating fully automated scoring systems (Azqueta et al., 2011). A HTS CometChip screening assay based on a microfabricated 96-well design with automated processing was also used to evaluate DNA damage caused by ZnO, Ag, Fe_2O_3, CeO_2 and SiO_2 NMs in human lymphoblastoid (TK6) cells in suspension, and adherent Chinese hamster ovary (H9T3) cells (Watson et al., 2014). HTS comet assay approaches, whether 12 mini-gels on a slide, 48 or 96 mini-gels on a GelBond film (Shaposhnikov et al., 2010; Gutzkow et al., 2013; Harris et al., 2015; El Yamani et al., 2017) or microfabricated 96-well CometChips (Watson et al., 2014) all show robustness, improved efficiency and reduced processing time, and in addition they reduce the risk of user bias in comparison with the standard comet assay.

In a recent study, an automated platform with high content imaging endpoints for cell viability, oxidative stress and DNA damage (double strand breaks assessed by γ-H2AX-staining), in combination with the HTS comet assay, was successfully employed to measure DNA damage caused by coated and uncoated iron oxide NMs in two mammalian fibroblast lines, showing that these methods provide a fast way to determine NM toxicity (Harris et al., 2015).

8. Testing strategy for hazard assessment of NMs

A strategy for *in vitro* toxicity testing of NMs in a regulatory context requires a battery of tests addressing different mechanisms and covering all main important toxicity endpoints. Thus, to identify relevant short-term hazard models, standard toxicity assays for different markers such as cell uptake and transport, cell viability, oxidative stress, pro-inflammatory response, immunotoxicity, genotoxicity and also epigenetic toxicity have to be considered. It is especially important, as epigenetic toxicity has been induced by NMs and occurred at sub-cytotoxic and sub-genotoxic concentrations (Ghosh et al., 2017). OECD-recommended methods should be preferred where possible, but new tests such as for epigenetic toxicity endpoints (Smolkova et al., 2015, 2017) need to be developed as OECD methods for these endpoints do not exist. Possible interference with the standard assays should be tested (Guadagnini et al., 2015).

The increasing number of new nanotechnological applications calls for the development of a test paradigm for NMs that is relevant to both pristine particles, in their intended use, and also for accidental or unintended exposure. Thus, information is urgently needed on how to evaluate the potential hazards associated with exposure to NMs, on general mechanisms of toxicity, and how to group and rank NMs according to their toxicity. Safety assessment of NMs depends on knowledge of their effects at different levels - cells, organs, animals, and humans. Such knowledge will help with the introduction of guidelines for the safe production, use, and disposal of NMs. There is presently no consensus on the most suitable toxicity tests, models for exposure assessment, and standardized testing strategies to evaluate possible hazards of therapeutic NMs. The number and diversity of engineered NMs makes it challenging to assess toxicological effects and thus new test strategies must be developed to minimize workload (preferably HTS assays).

The classification of NM hazards requires information about their mechanism of action. Recently, a research strategy was proposed for the development of an intelligent testing strategy for NMs (Stone et al., 2014) based on several steps: identification of the biological mechanism underlying the toxic effect and the physicochemical properties of the NMs that drive the toxic effects; design of *in vitro* and HTS screening tools that target these key biological processes; development of robust *in silico* approaches for risk assessment (Oomen et al., 2015; Dekkers et al., 2016). An important aspect of impact assessment of NMs is the grouping and ranking of NMs and NM-containing products according to their inherent toxicity.

Keeping in mind the 3Rs, the testing strategy should reduce *in vivo* experiments. The development of alternative testing strategies and HTS methods for hazard assessment of NMs has been proposed in the NanoTEST project (www.nanotest-fp7.eu) (Dusinska et al., 2009, 2015; Dusinska and Tran, 2015). Cytotoxicity, oxidative stress, immunotoxicity and genotoxicity were investigated in various cell culture models, representing eight different organs (blood, vascular system, lung, brain, liver, kidney, gastrointestinal system and placenta). All *in vitro* studies were harmonized, using NMs from the same batch, and identical dispersion protocols, exposure time, concentration range, culture conditions, and time-courses. The *in vitro* methods were critically evaluated, and where appropriate, standard methods were adapted (Dusinska et al., 2015). The suitability of human and mammalian primary cells and cell lines derived from blood, vascular/central nervous system, liver, kidney, lung and placenta for the assessment of NM genotoxicity (DNA strand breaks and oxidized DNA lesions) was investigated by Cowie et al., (2015). The results from the statistical evaluation showed that all of the cell types can be used to assess the genotoxic potential of NMs, but with different sensitivities. This work also assessed the effect of changes in experimental conditions on the toxic impact of NMs in different cell culture models. The results of these studies can be used to generate recommendations for a suitable and robust testing strategy that can be applied to new medical NMs as they are developed. The recommendations include needs for standardized characterization of NMs, measurement of NM uptake and for each type of toxicity endpoint at least two different methods that are not prone to interfere with *in vitro* assays (Dusinska et al., 2015; Guadagnini et al., 2015).

Robust and standardized methods are of great importance in nanotoxicology, with comprehensive material characterization as an integral part of the testing strategy. It is also important to critically evaluate the quality of the utilized assays and their predictive value for the endpoints of interest such as cancer (Møller et al., 2011). Evaluating the effect of a specific type of NM should emphasize mechanistic steps that are close to the adverse outcome endpoint (for example, mutations in oncogenes or tumor suppressor genes provide stronger mechanistic evidence than inflammation) and on specific endpoints in the presumed causal

pathway from exposure to disease endpoint (measurement of DNA damage, DNA repair, epigenetic changes).

Regarding the likelihood of biomolecular corona formation, it is also important to set up experimental conditions that can mimic exposure in humans. As NMs change their properties depending on the surrounding milieu we recommend at least two different exposure conditions for testing NM effects (Magdolenova et al., 2012; Corbo et al., 2016). Tests for assessing long-term effects of chronic exposure of humans and new tools for detecting NMs *in situ* must be developed. Biotransformation of NMs in the human body, their interaction with biological systems as well as ADME in living systems should be studied in conjunction with the characterization of NMs in terms of size distribution, surface properties, biopersistence, and stability of original and modified NMs in different biological media.

9. Future perspective

The main challenge in safety assessment of NMs remains development of alternative models and methods that mimic *in vivo* conditions. The demand to move from animal models to novel technologies arises from various concerns, including the need to evaluate large numbers of new NM products, the limited predictivity of traditional tests for human health effects, duration and costs of current approaches, and animal welfare considerations. Existing *in vitro* test methods need further modifications taking into considerations challenges related to NM physicochemical properties and their interaction with surrounding and target environments, cellular uptake and novel endpoints, particularly epigenetics (Stefanska et al., 2012; Smolkova et al., 2017).

A future strategy for cytotoxicity, immunotoxicity, genotoxicity and epigenetic toxicity testing includes the optimization of existing test systems via extension of metabolic capacity, the development of organotypic 3-D (co)-cultures, a transition from cell lines to primary cells or stem cell-derived systems, refinement and expansion of endpoints measured. Increased use of HTS technologies, automation and standardization are crucial (Dusinska et al., 2015; Collins et al., 2017). Tools for detecting NMs *in situ* must be developed. *In silico* estimation of toxicity based on toxicogenomics databases, monitoring many biomarkers simultaneously, will be increasingly important, providing a rapid and sensitive approach for immunotoxicity and genotoxicity identification and adverse health effects prediction (Lan et al., 2014; Dusinska et al., 2015).

Toxicogenomics represents a novel approach to investigate immunotoxicity and genotoxicity using a large number of genes to assess genotoxic and immunotoxic adverse outcome pathways. To distinguish immunotoxic from non-immunotoxic compounds, human peripheral blood mononuclear cells should be preferably used. Identification of the gene signature of direct immunotoxicants should include testing the effects of large number of compounds on the transcriptome of human cells such as Jurkat T-cells (Hochstenbach et al., 2010). Diverse modes of action are involved in direct immunotoxicity and a set of pathways or genes, rather than one single gene, should be used to screen compounds for immunotoxicity and genotoxicity (Hartung and Corsini, 2013; Shao et al., 2013).

A promising new assay is the HuLA (human lymphocyte activation) assay which uses cryopreserved peripheral blood mononuclear cells isolated from humans after immunization with a standard influenza vaccine (Collinge et al., 2010). The clear advantage of this approach is the use of human cells from individual donors to measure a highly relevant function (Luebke, 2012). Similarly, primary human cell data can be generated using T-cell models developed to detect allergenicity of drugs, proteins, and chemicals. Co-culture techniques can be used to mimic and detect antigen presentation to and activation of naive T-cells. Test substance can be added directly to T-cells, bound to proteins before addition, or used to pulse dendritic cells prior to co-culture. Multiple options are available to assess T cell activation, including upregulation of cell surface markers, production of cytokines, and cell proliferation (Martin et al., 2010; Luebke, 2012).

As immunotoxicity and genotoxicity share, at least partially, the same adverse outcome pathways, the testing should include both endpoints simultaneously and where relevant with the same *in vitro*/*ex vivo* co-culture models. Future strategies also should address effects of chronic exposure to medical NMs; in particular there is a need for assays focused on the endpoint of carcinogenicity, in addition to existing *in vitro* cell transformation assays which have yet to be validated.

Accumulating evidence demonstrate an eligibility of epigenetic endpoints for their incorporation into NM risk and safety assessment. However, better understanding of the epigenetic landscape in normal conditions and its deregulation in complex diseases remains a prerequisite for achieving this goal. The technological advances in epigenomics will soon enable identification of cell-type specific epigenetic profiles and will allow us to distinguish between adverse effects and adaptive response. Only after accumulation of a sufficient amount of reproducible data on epigenetic changes induced by NMs and convincing evidence for their association with disease risk, we will be able to select the most representative model systems, endpoints to be evaluated and techniques to be employed for reliable testing of epigenetic toxicity of NMs. Integration of epigenetic endpoints into NM safety assessment represent a major challenge for biomedical research and requires considerable attention.

Conflict of interest

The authors declare that there are no conflicts of interest.

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