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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