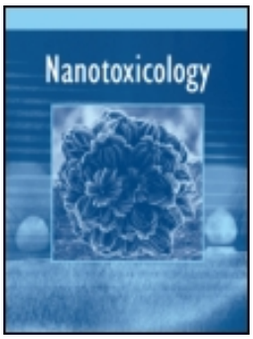

Nanotoxicology

ISSN: 1743-5390 (Print) 1743-5404 (Online) Journal homepage: informahealthcare.com/journals/inan20

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To cite this article: Maria Dusinska & Lang Tran (2015) NanoTEST in a Nutshell, Nanotoxicology, 9:sup1, 1-4, DOI: 10.3109/17435390.2014.991774

To link to this article: https://doi.org/10.3109/17435390.2014.991774

http://informahealthcare.com/nan
ISSN: 1743-5390 (print), 1743-5404 (electronic)

Nanotoxicology

Nanotoxicology, 2015; 9(S1): 1–4
 DOI: 10.3109/17435390.2014.991774

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EDITORIAL

NanoTEST in a Nutshell

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There is a huge effort underway in nanotechnology with the development of nanomaterials which have better properties and are more effective than their parent bulk materials, and also are safe to use. In nanomedicine safety aspects can largely be addressed with the same methodologies and tools as are needed for testing efficacy. The impact of medical nanomaterials is also easier to investigate because human exposure is already known. Since in nanomedicine, nanoparticles (NPs) enter the body, their interaction with cells and tissue is inevitable and must be investigated. The main aim of the FP7 NanoTEST project (www.nanotest-fp7.eu, Dusinska et al., 2009) was to study the biological impact of NPs in nanomedicine as a basis for understanding molecular and cellular pathways that can lead to toxic effects, together with the development of appropriate methods to test them. This supplement consists of 13 scientific papers including a commentary, all of them from the NanoTEST project, showing the approach towards development of testing strategies and high throughput methods for hazard assessment of nanomaterials used in nanomedicine. All *in vitro* studies were harmonized with NPs from the same batch with identical dispersion protocols, exposure time, concentration range, culture conditions and time-courses.

The first paper addresses critical aspects of assessing the safety of nanomaterials in medicine: the balance between risks and benefits; and the major challenges encountered when studying biological impact, biocompatibility, distribution in the human body, biodegradation and excretion routes, and dispersion in the environment (Juillerat et al., 2015). A final goal of NanoTEST was the development and validation of tools for evaluating the biological impact of NPs. This could only be achieved by addressing interactions of NPs with cells, living tissues and their possible effects in the human (and animal) body.

For hazard assessment of NPs, specific characteristics related to size and surface properties, that might influence their behaviour and adverse effects, must be taken into consideration. In NanoTEST titanium dioxide (TiO_2) NPs of nominal size 20 nm, iron oxide (8 nm) both uncoated (U-Fe_3O_4) and oleic acid coated (OC-Fe_3O_4), rhodamine-labelled 25 nm (Fl-25 SiO_2) and 50 nm (Fl-50 SiO) amorphous silica and polylactic glycolic acid polyethylene oxide polymeric (PLGA-PEO) NPs, were investigated. The main characteristics of these NPs are described in Guadagnini et al. (2015a). The major goal of this paper was to evaluate the available toxicity tests and to investigate possible interference with tested nanomaterials. The panel of NP suspensions used in this project showed that many NP characteristics (composition, size, coatings and agglomeration) interfere with a range of *in vitro* cytotoxicity assays. The paper also proposes how to avoid interference of NPs with testing systems as the first step of a screening strategy for biomedical NPs. The information and recommendations provided by the authors are also valuable for NP safety assessment generally.

NanoTEST addressed the main toxicity endpoints – cytotoxicity, oxidative stress, immunotoxicity and genotoxicity – using various *in vitro* cell culture models representing eight different organs. Results from vascular system, placenta, brain, kidney, gastrointestinal system and (partially) blood have already been published elsewhere (Aranda et al., 2013; Cartwright et al., 2011; Halamoda Kenzaoui et al., 2012a,b,c, 2013a,b; Kazimirova et al., 2012; Magdolenova et al., 2012a,b). One of the main routes of exposure to NPs is through the lungs. Lung epithelial cells are the first target cells after inhalation but also secondary targets after injection of NPs due to the small distance between the epithelial cells and the blood capillaries. Several nanomaterials are already used for lung therapeutics and diagnostic purposes. Guadagnini et al. (2015b) studied possible adverse pulmonary responses by evaluating cytotoxicity, reactive oxygen species (ROS) production and pro-inflammatory responses. The effects of PLGA, silica, iron oxide and TiO_2 NPs were studied using human bronchial (16HBE) and alveolar epithelial cells (A549) with different sensitivity depending on cell type, toxicity endpoint and NPs used. PLGA NPs were proposed as good candidates for negative control NPs and SiO_2 NPs were revealed to be the best benchmark NPs. The authors concluded that measurement of oxidative stress does not systematically allow the prediction of cellular responses and proposed that a battery of assays and cell lines are necessary to evaluate the pulmonary effects of NPs.

Blood is the main route for biodistribution of therapeutic NPs, and NPs that pass through the lungs or gastrointestinal tract are also distributed to other organs through the blood circulation. NPs were therefore studied *in vitro* in a blood cell model, using both stable cell lines as well as primary human blood cells (Magdolenova et al., 2015; Tulinska et al., 2015). A human blood cell model was used for immunotoxicity and genotoxicity testing to measure the response to PLGA-PEO NPs in fresh peripheral whole blood cultures and in isolated peripheral blood mononuclear cell cultures from 13 human volunteers, showing that primary blood cells are suitable for detecting the response to NPs (Tulinska et al., 2015). Using several immunotoxicity tests, proliferative activity of T-lymphocytes and T-dependent B-cell response in cultures stimulated with mitogens, cytotoxicity of natural killer cells, phagocytic activity of granulocytes and

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monocytes, and the respiratory burst of phagocytes were investigated. Genotoxicity was assessed using the micronucleus test and the comet assay; there was no increased frequency of cells with micronuclei and no induction of strand breaks or oxidized DNA bases in PLGA-PEO-treated cells.

Magdolenova et al. (2015) investigated the coating-dependent induction of cytotoxicity and genotoxicity of iron oxide NPs *in vitro* and showed that human lymphoblastoid TK6 cells and primary human blood cells are sensitive *in vitro* models for genotoxicity. Surface coatings of NPs are known to influence advantageous features of NPs as well as potential toxicity. The authors investigated the potential cytotoxicity and genotoxicity of U-Fe_3O_4 NPs in comparison with OC-Fe_3O_4 NPs. Genotoxicity was evaluated by the alkaline comet assay for detection of strand breaks and oxidized purines. U-Fe_3O_4 NPs were found not to be cytotoxic or genotoxic, in contrast with OC-Fe_3O_4 NPs which showed cytotoxicity in a dose-dependent manner, and also induced DNA damage, indicating their genotoxic potential. Coating clearly changed the behaviour and cellular uptake of the NPs, inducing pathological morphological changes in the cells.

Genotoxicity is a crucial endpoint in safety testing of nanomaterials as it addresses potential mutagenicity which has implications for risks of both genetic disease and carcinogenesis. The suitability of human and mammalian cells of different origin for the assessment of genotoxicity of all NanoTEST NPs with the alkaline comet assay was investigated by Cowie et al. (2015). Using primary cells and cell lines derived from blood (human lymphocytes and lymphoblastoid TK6 cells), vascular/central nervous system (human endothelial HCEC cells), liver (rat hepatocytes and Kupffer cells), kidney (monkey Cos-1 and human HEK293 cells), lung (human bronchial 16HBE14o cells) and placenta (human BeWo b30 cells), the authors investigated which *in vitro* cell model is the most sensitive (and likely to represent what happens *in vivo)* to detect strand breaks and oxidized DNA lesions. The results from the statistical evaluation show that OC-Fe_3O_4 and TiO_2 NPs are genotoxic in the experimental conditions used and that all cells can identify genotoxic and non-genotoxic NPs with differing sensitivity. TK6 cells, human lymphocytes, BeWo b30, and kidney cells seem to be the most reliable for detecting a concentration-response *in vitro*.

When testing toxicity of NPs, their bioavailability, i.e. uptake and transport of NPs across biological barriers, is important. The study of Correia Carreira et al. (2015) utilized a human placental choriocarcinoma BeWo b30 *in vitro* cell barrier model of the placenta to explore toxicity, uptake and transport of iron oxide and silica NPs. The study demonstrated that iron oxide NPs were more cytotoxic than silica NPs which may be of clinical relevance when considering fetal exposure *in utero*. Both NPs can transfer extensively across the placental barrier model but physico-chemical characteristics such as surface chemistry impact on both uptake and transport. The NP coating strongly influenced transport with the OC-Fe_3O_4 NPs transferring across the barrier, whereas U-Fe_3O_4 NPs aggregated and precipitated onto the cell surface. Fl-SiO_2 NP transport across the cell barrier was broadly unaffected by size or NP concentration. Transport across the cell barrier was reduced for all NPs compared with transport across the insert in the absence of cells, indicating that the placental cells can act as a partial but not complete barrier to NP transport.

The transport of silica NPs across the placenta model of maternal-fetal transfer was also investigated by Poulsen et al. (2015) using the *ex vivo* perfused human placenta model as well as BeWo b30 cells with slightly different results. In the placental perfusion experiments and BeWo b30 model, silica NP uptake and transport were limited – though with confocal microscopy, there was visual confirmation of particle accumulation in both systems. Both studies demonstrate the importance of developing sound models for uptake and transport of NPs across biological barriers and the need for quantitative reliable tests that should be an integral part of hazard assessment of nanomaterial.

For nanomaterial safety assessment validated *in vitro* assays that are suitable for nanomaterial need to be developed and further adapted to increase their throughput. Harris et al. (2015) addressed the need to test NP toxicity using high throughput analysis and high content imaging. They tested automated platform and high throughput technologies and high content imaging end-points for cell viability, oxidative stress and DNA damage (double strand breaks). At the same time, the medium throughput comet assay was employed to measure DNA strand breaks and oxidized bases showing that these methods provide a fast way to determine the toxicity of NPs and that they can be used to define the mechanism of NP toxicity *in vitro*.

In vitro studies performed on human and mammalian cells were accompanied by *in vivo* experiments to validate *in vitro* data. Volkovova et al. (2015) administered TiO_2 and OC-Fe_3O_4 NPs intravenously to female outbred Wistar rats, and determined LD50 values of 59.22 and 36.42 mg/kg for TiO_2 and OC-Fe_3O_4 NPs, respectively. In the main experiment, animals were treated with OC-Fe_3O_4 NPs in doses equivalent to 0.1, 1 or 10% of the LD50 respectively and sampled 24 h, 1, 2, and 4 weeks post-injection for adverse effects. Various parameters were measured to assess liver toxicity. OC-Fe_3O_4 NP deposits were clearly seen in whole liver parenchyma in contrast to Magnetic Resonance Imaging (MRI) of the kidney, confirming that liver is the main target organ of these NPs. Highest TiO_2 NPs levels were revealed also in liver, followed by spleen, and lung (Sebekova et al., 2014). The data reported by Volkovova et al. (2015) suggest that the liver likely retains functional integrity with sub-lethal doses of OC-Fe_3O_4 NPs, albeit with some stimulation of redox defences and evidence of some tissue injury shortly after the injection. Data on nephrotoxicity were published previously (Sebekova et al., 2014) showing mild or no effect and the majority of results addressing other organ and tissue toxicity are under preparation.

Additionally to *in vivo/in vitro* comparisons, the NanoTEST project also focused on computational modeling and its possible application as part of an alternative testing strategy. Pilou et al. (2015) presented an integration of two existing numerical models in order to connect external exposure to NPs with internal dose through inhalation, and to use computational fluid-particle dynamics (CFPD) to analyse the behaviour of NPs in the respiratory and cardiovascular systems. The authors combined a lung transport and deposition model with a lung clearance/retention model to estimate NP doses in the different regions of the human respiratory tract and some adjacent tissues. These studies showed that with proper refinement the developed computational models and methodologies could serve as an alternative testing strategy, eventually replacing transport/deposition experiments that are expensive both in time and resources, and could make a contribution to risk assessment.

The quantitative structure-activity relationship ((Q)SAR) models also promise to be valuable tools for future testing strategies. Burello & Worth (2011) proposed a theoretical model predicting oxidative stress potential that could be used to guide the development of more rational and efficient screening strategies. The authors describe an approach in their commentary for designing safer nanomaterials, focusing on the oxidative stress mechanism as probably the best-developed toxicological pathway for explaining NP toxicity. This mechanistic approach relies on the principle that redox couples with standard redox potentials near the conduction or valence bands of NPs can exchange electrons with these particles. All this information points towards

the emergence of an effective rule for designing safer NPs: namely, design NPs whose energy structure will not interfere with the cellular redox equilibrium (Burello & Worth, 2015). In addition, the theoretical model predicting oxidative stress potential can create a more coherent conceptual framework when additional toxicity-related physicochemical properties (e.g. agglomeration and solubility in water) are included.

The final paper (Dusinska et al., 2015) summarizes the efforts of the entire consortium to develop an alternative testing strategy for hazard and risk assessment of nanomaterials with stress on alternative testing strategies and high throughput toxicity testing. All methods used in the NanoTEST project were critically evaluated, and where appropriate, standard methods were adapted. The work has also assessed a broad range of cell models taking into account the inherent impact of NP properties and the effects of changes in experimental conditions. The results of these studies have been used to generate recommendations for a suitable and robust testing strategy which can be applied to new medical NPs as they are developed, suggesting for each type of toxicity at least two different methods.

The driving force of the NanoTEST project was the belief that a better understanding of NP kinetics, molecular and cellular mechanisms, and pathways of action would benefit nanotechnology, providing key knowledge required for risk assessment of NPs, especially those in clinical use, for instance as diagnostic or delivery tools. Responsible development of this promising new technology depends on a knowledge of the effects of NPs at different levels - cells, organs, animals, individuals, and human populations. Such knowledge – including the novel findings reported in this special issue – will help with the introduction of guidelines for the safe production, use and disposal of NPs. We have devised and used *in vitro* and *ex vivo* models to investigate the impact of NPs on cell and tissue functions, focusing on the main toxicity endpoints – cytotoxicity, oxidative stress, immunotoxicity and genotoxicity. Our approach is consistent with the requirement to reduce, refine and replace animal testing in experimental science (the '3Rs' principle). The set of studies performed within NanoTEST contributes to a better understanding of how the 3Rs can be achieved. However, there is still need to validate the findings with respect to what happens *in vivo* at relevant doses. Within NanoTEST only two of the tested NPs were investigated both *in vitro and in vivo* and thus more *in vitro–in vivo* comparison is needed. We sincerely hope that after *in vivo* validation and dosimetric corroboration, approaches described here will be incorporated in an evolving regulatory framework which will ensure the safe, integrated and responsible introduction of nanotechnology into clinical practice.

There is still a lot to be done, but NanoTEST has set the scene for further investigations into NP toxicity, and for a systematic and logical strategy of risk assessment.

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