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Review

Biomonitoring of genotoxic effects for human exposure to nanomaterials: The challenge ahead

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ABSTRACT

Exposures to nanomaterials (NMs), with their specific physico-chemical characteristics, are likely to increase over the next years, as their production for industrial, consumer and medical applications is steadily rising. Therefore, there is an urgent need for the implementation of human biomonitoring studies of genotoxic effects after NM exposures in order to monitor and assure safety for workers and the general population. In this review, most commonly used biomarkers of early genetic effects were analyzed for their adequacy after NM exposures. A more in depth analysis of the *ex vivo/in vitro* lymphocyte MN assay was performed, although, in literature no studies are available using this assay for NM exposures. Therefore, the known factors determining the NMs tissue/cellular targets and the multiplicity of modes of action of NMs were summarized. The main pending questions are whether (1) lymphocytes are a NM target or an adequate surrogate tissue, (2) whether the buccal MN assay might be more suitable for NM exposures via inhalation or ingestion, as buccal cells might be exposed more directly. While the current state-of-the-art does not allow for drawing firm conclusions, major research gaps are identified and some cautious recommendations can be formulated. Therefore *in vitro* and *in vivo* studies should be conducted comparing methodologies side-by-side in the same subjects and for different types of NMs. The *ex vivo/in vitro* MN assay in its automated version, allowing objective analysis of large cohorts and detection of direct and indirect genotoxic effects, remains a valuable candidate for human biomonitoring to NM exposure. Considering the potential cancer risk from exposure to NMs and previous dramatic experiences with too late surveillance of occupational exposures to similar substances (e.g. to asbestos), there is an urgent need to define and implement adequate scientifically sound biomonitoring methods and programme for exposure to NMs.

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1. Introduction

Nanomaterials (NMs) have been defined in 2011 as natural, incidental or engineered materials containing particles (in an unbound state or as an aggregate or as an agglomerate) of which 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range of 1–100 nm (EU recommendation 2011/696/EU). Human biomonitoring, as an integral part of the safety assessment of NMs, is merely initiating as can be evidenced by the scarcity in biomonitoring data for NMs up to date. Because these NMs are gaining a lot of interest due to their specific physico-chemical characteristics, making them very interesting for a great number of industrial and bio-medical applications, increased occupational exposure is expected. In addition to occupational exposure and exposure through consumer products, NM exposures are likely to rise as a consequence of their release into the environment. Therefore, the design of sound procedures for human biomonitoring specifically adapted for the assessment of NMs is essential.

The specific characteristics of NMs, making them interesting for numerous applications in different fields, introduce new challenges for hazard identification and risk assessment. In absence of experimental data for human biomonitoring of early genetic effects, knowledge acquired with *in vitro* and *in vivo* animal experiments with NMs can serve as support when designing and performing adequate biomonitoring studies of early genetic effects.

In this review, an overview will be given of NM-specific issues that need to be taken into consideration when performing biomonitoring studies for early genetic effects. An analysis of the biomarkers of exposure and early genetic effects for NM exposures, with particular focus on the lymphocyte Cytokinesis-Block Micronucleus (CBMN) assay, will be performed. Based on the current knowledge obtained from *in vitro* and *in vivo* genotoxicity studies, research gaps will be identified and recommendations for the design of adequate biomonitoring studies will be formulated, in order to avoid the pitfalls that have been encountered performing experimental *in vitro* and *in vivo* studies. Bringing together knowledge acquired in the past decade on genotoxicity of NMs, their genotoxic modes of action and *in vivo* biodistribution allows to avoid mistakes made in the past (f.e. performing *in vivo* genotoxicity assays using non-target tissues, possibly leading to false negative results).

2. Lessons learned from in vitro and in vivo cell toxicity and genotoxicity studies after NM exposure

A recurrent issue with regards to NM testing is the interference of NMs with specific assay compounds, such as colorimetric compounds and enzymes. This has been previously demonstrated when testing the cytotoxic properties of NMs with classic cytotoxicity assays or when assessing their potential to induce specific DNA lesions using the alkaline comet assay in combination with lesion-specific enzymes [1,2]. In both cases, the assay outcome was altered because of interferences between the NMs and assay compounds and therefore necessitates additional controls in order to allow assay interpretation. Besides these interferences with colorimetric compounds or enzymes, NM-specific issues need to be considered at additional levels, i.e. the assay protocol/experimental setup and the experimental system.

The *ex vivo*/*in vitro* MN assay applied for biomonitoring studies assesses MN after *in vitro* culture of *in vivo* exposed T lymphocytes; the *in vitro* MN genotoxicity assay assesses MN in T lymphocytes exposed during the culture period. They both use phytohaemagglutinin (PHA) to stimulate proliferation of T lymphocytes and cytochalasin B to identify the lymphocytes which divided and express MN during the *in vitro* culture period. Knowledge gained with the *in vitro* MN assay for NMs will therefore be helpful for the ex *vivo*/*in vitro* MN assay. Specific adaptations to the protocol have been proposed for the *in vitro* micronucleus (MN) assay by us and others. In particular, the use of cytochalasin-B and the exposure period need careful consideration. Since cytochalasin-B is an actin inhibitor, it inhibits, besides the cytoplasmic cell division, also the process of actin-dependent endocytosis, which is one of the routes for NMs to be taken up by cells, leading to decreased cellular uptake and an underestimation of the NMs potential to induce MN. For this reason, the simultaneous addition of NMs and cytochalasin-B has been discouraged allowing at least a period of exposure to solely NMs. The length of the NM exposure is of major importance as well. Nuclear uptake of NMs has been reported, but only for a limited number of NMs. For NMs that are unable to cross the nuclear barrier in interphase cells, an exposure period comprising mitosis might allow them to come into contact with the chromatin. Therefore very short exposure periods might not consent the NMs to reach their target and again lead to an underestimation of their adverse effect. Awareness of these protocol concerns has led to the organization of an OECD project to tackle these issues and generate a guidance document specific for nanomaterial testing.

When performing *in vivo* experiments, the choice of experimental system and in particular the selection of the tissues for analysis require careful consideration of the NMs biokinetics and biodistribution. Genotoxic effects, including the induction of MN, should be evaluated in primary and secondary target tissues as analysis of non-target tissues might lead to false negative effects.

Although some insights into *in vitro and in vivo* NM genotoxicity have been gained in the last decade, several knowledge gaps persist in order to assure a robust framework for hazard and risk assessment. One of the main research gaps is the lack of information on NMs mode(s) of action. Until now, studies were essentially based on expertise with soluble chemicals and ROS production considered as the major mode of action of small particles. However NMs can translocate through cell membranes, act via multiple pathways, directly and indirectly, and require therefore multi-endpoint approaches/assays to ensure safety. Fully elucidating these would benefit the understanding of the complex dose-response relationships that are often observed (f.e. for MN frequencies) and currently hampering the definition of threshold doses and exposure limits for NMs. In addition, thorough evaluation and validation for NMs of the existing test methods is still lacking. Small studies, addressing specific questions in a systematic way, are needed to address urgent issues such as (1) the identification of both sensitive and robust cell and molecular models for NM genotoxicity evaluation, (2) identification of adequate protocols eliminating possible interferences and in particular interactions/interferences leading to underestimation of the potential effects, (3) development or validation of nano-specific assays, in particular for the assessment