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Review article

In vivo effects: Methodologies and biokinetics of inhaled nanomaterials

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ABSTRACT

Inhalation is the prevailing route of inadvertent exposure for manufactured nanomaterials (MNs). For assessing potential adverse effects, indepth knowledge about Exposure-Dose-Response relationships is required to define a risk as a function of hazard and relevant exposure. Intrinsic (physico-chemical) and extrinsic (functional) MN properties determine the biological/toxicological properties (effects) of MNs. Predictive testing strategies are useful for comparative hazard and risk characterization against toxicologically well-defined positive and negative benchmark materials involving studies in rodents, cells, and cell-free (abiotic) assays.

Inhalation studies can be used for hazard identification as well as for hazard and risk characterization of inhaled MNs. A design to provide dose-response data is ideal, but less so if only exposure-response data are available. Information should also be provided for biokinetics and for identifying secondary targets. Bolus-type dosing (intratracheal instillation; oropharyngeal aspiration) can be useful for hazard identification and characterization, but not for risk characterization. Combining results from bolus dosing or *in vitro* tests with results of a subchronic inhalation study of the same group of MNs can be a suitable predictive bridging approach.

In vitro cellular assays designed to determine *in vivo* effects and underlying mechanisms present additional challenges. Cellular dose equivalency to *in vivo* is difficult to achieve because of static, mostly acute *in vitro* systems with no MN clearance. The dose dependency of mechanisms has to be considered as well. Still, *in vitro* tests are suitable for toxicity ranking against well-characterized benchmarks (Hazard ID). Regarding abiotic assays, predictive toxicity ranking using the metric of specific MN surface reactivity (ROS assays) is a promising screening tool, but requires further validation and standardization. Dynamic abiotic dissolution assays are also a promising tool for predicting *in vivo* dissolution rates but require standardization.

Information about MN dissolution using static (equilibrium solubility, μg/L) and dynamic (dissolution rate, ng/cm^2/day) abiotic *in vitro* assays provide different information about the solubilization of MNs reflecting either static *in vitro* or dynamic *in vivo* conditions. Results of both assays may be useful for categorization if performed in physiologically relevant fluids. Because the *in vivo* dissolution rates of MNs can differ widely, it is too simplistic to group MNs just into soluble and poorly soluble materials. Static (equilibrium solubility) and dynamic (dissolution rate) abiotic assays are based on different concepts. Results from dynamic dissolution in relevant physiological fluids - rather than just water - add valuable information about the extrinsic functional characteristics of MNs, which may be considered as a grouping tool into high, moderate, low and insoluble MNs.

Systemic biodistribution of MNs depends on the point-of-entry. For example, MNs deposited by inhalation or instillation in the respiratory tract distribute differently than intravenously administered MNs; thus, biokinetic models based on data from intravenous MN administration should not be used to model biodistribution following inhalation. The significance of biodissolution for biokinetics, effects and underlying mechanisms has to be assessed in separate *in vivo* studies, involving biopersistence/biodurability and ultra-high resolution imaging for analysing bioprocessing and biotransformations at a sub-cellular level.

With respect to grouping, several strategies are necessary to cover all classes of MNs of different compositions and for different exposure routes, all of which are to be considered in regulatory decision-making. The suggested grouping and extrapolation framework presented in this paper could be pivotal in leveraging subchronic inhalation data with data from alternative test methods, thus leading to more efficient, cost-effective, and – in the long run – animal and cost saving methods to obtain needed input data for regulatory use.

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1. Introduction

The topic of *in vivo* effects and biokinetics of inhaled nanomaterials, as well as the other topics in this special issue of NanoImpact, is specifically focusing on regulatory needs for nanomaterials in the context of answering regulatory risk assessment questions. Sayre et al. (2017) provided detailed background information to introduce this series of papers including well-thought-through regulatory questions for each topic. The following critical review and discussions are in response to the questions for the topic *in vivo* effects and may serve as input for the regulatory decision process directed at the human health protection from potential adverse effects in inhaled MNs.

The three common exposure routes for manufactured nanomaterials (MNs) are inhalation, oral and dermal exposure. The latter exposure route is most often viewed as the one of least concern due to the efficient barrier function of healthy skin (Prow et al., 2011). To our knowledge, no quantitative comparable exposure assessments for inhalational and oral exposure have been conducted and published. Exposure of the respiratory tract as point-of-entry includes also exposure *via* the gastro-intestinal (GI) tract with those MNs that are cleared by mucociliary clearance towards the oro-pharynx, to be swallowed into the GI-tract. Overall, most publications deal with exposure of the respiratory tract and its cells and view this as the exposure route of highest concern due to direct interactions in the lung and because of subsequent significant transport into the human body.

Whereas inhalation of MNs is the only physiological mode of exposure for the respiratory tract, bolus-type intratracheal inhalation or oro-pharyngeal aspiration as less expensive and easy to execute dosing methods are used as alternatives to evaluate effects of MNs in the respiratory tract. However, it has been shown that a dose administered to the respiratory tract as bolus within less than 1 s will induce significantly greater lung inflammation compared to the same dose administered over several hours or days. This is due to the huge difference in the dose rate which has to be considered (Baisch et al., 2014). Nonetheless, bolus-type delivery for establishing dose-response relationships is still useful for hazard identification and ranking as shown by Warheit et al. (2005), but the results cannot be used for risk characterization (Driscoll et al., 2000).

The National Academy of Sciences suggested four steps in the risk assessment paradigm (NAS, 1983): Hazard Identification; Hazard Characterization; Exposure Assessment; and Risk Characterization. Fig. 1 shows the inter-relationships between these steps specifically adapted for manufactured nanoparticles (NPs), and with the added step of Risk Management.

Other important determinants for regulatory decision-making include knowledge about the correlation between physico-chemical MN properties and biological/toxicological properties, including also results of functional assays determined using *in vivo* as well as cellular and non-cellular *in vitro*[1] studies such as dissolution and inherent ROS-inducing capacity of MNs (see Gao and Lowry, 2017). Such results are valuable for an initial categorization of MNs to inform regulators or manufacturers about a potential hazard so as to guide decisions regarding either additional testing requirements or no further action for additional testing. The difficulties associated with establishing a perfect grouping were summarized by participants of a multi-disciplinary workshop on nanomaterial risk potential and regulatory decisions: *"Although no single categorization strategy is likely to work for all classes of engineered nanomaterials (ENMs) in all regulatory situations, it may be possible to develop a general framework that can be adapted and customized for specific ENM compositions and specific regulatory contexts"* (Godwin et al., 2015). Of great consequence for the design of any study assessing the toxicity of inhaled MNs, *in vitro* or *in vivo*, is the selection of relevant doses. For example, it is not appropriate and scientifically not justifiable to perform an acute short-term study, *in vivo* or *in vitro*, with a single dose suggesting that this is realistic because it is equivalent to the predicted total dose, accumulated over 45 years of inhalation exposure at a workplace (Gangwal et al., 2011). This is highly misleading and results cannot be considered as relevant (Oberdörster, 2012). High, irrelevant experimental doses not only "make the poison", but also determine the mechanism, as pointed out by Slikker et al. (2004). Validation and scientific acceptance of toxicological results is essential for regulatory acceptance.

An understanding of dosimetry and extrapolation modelling is essential for translating results of inhalation tests with MNs to be applied for regulatory purposes. Fig. 2 shows the complexity of respiratory tract dosimetry to emphasize the importance of expressing and analysing data in the form of Exposure-Dose-Response relationships.

Most often, results are only reported as Exposure-Response correlations. This is insufficient, it does not consider the fundamental importance of Dose in toxicology, making it difficult to use results of an inhalation study as input for extrapolation modelling where the deposited and long-term retained doses are needed to characterize effects.

While this document focuses on regulatory processes using established guidelines (*e.g.*, OECD, 2016c, d) and presents suggestions for expansion and new to be established testing protocols, it should not be forgotten that a wide spectrum of scientific data is used for setting limits for inhalation exposure, including *e.g.*, classification as carcinogens, irritants, allergens. Importantly, clinical and epidemiological data should be of highest preference, these are dealing with the ultimate species of interest, humans.

1.1. Inhalation protocols for risk and hazard characterization

The OECD recommends recording toxicity and ecotoxicity relative to at least three metrics, mass, particle number and surface area (OECD, 2012). However, for mammalian toxicity, solubility (dissolution rates) and specific surface reactivity, should also be assessed as functional endpoints. Necessary information before conducting a test, *e.g.*, 90-day inhalation study, include MMAD, GSD, mass concentration, agglomeration/aggregation state, shape, chemistry, density, and several others as shown in Table 1 (Oberdörster et al., 2015). Although Table 1 is based on a paper with focus on CNT/CNF, it serves generally as guidance for other MNs as well.

The authors discuss objectives for acute/subacute, subchronic and chronic inhalation studies, concluding that acute inhalation studies will give important information for the design of subsequent subchronic 90-day studies. However, chronic effect studies are often more of a priority for evaluating toxicity because - if appropriately designed - a full risk assessment including risk characterization for regulatory actions to prevent long-term effects can be performed. Acute studies, though, should be designed with the same attention to detail. However, subchronic data are currently thought to reflect better the potential responses in workers producing or handling MNs who are exposed to low concentrations of aerosolized nanomaterials over long periods of time.

The question as to whether data from different animal models allow reliable interspecies extrapolations needs to recognize that differences between animal species and subsequent extrapolation to humans will not be straightforward. For example, it is well-known that the overload-induced lung tumours in rats resulting from chronic inhalation exposure of poorly soluble particles of low toxicity (PSLT particles) are not even extrapolatable to mice or hamsters, which makes it very questionable as to whether overload-induced lung tumours in rats caused by inhaled PSLT particles can be extrapolated to humans. Thus, the concept for deriving a "safe" human exposure level should be based on the retained lung burden that did not induce inflammation or fibrosis in a long-term rat inhalation study with PSLT particles, since these effects are preconditions for overload induced tumours in rats.

Currently, when lacking chronic 2-year inhalation studies, a

[1] The term "*in vitro*" is used in this article in its literal sense to include both cellular and non-cellular (cell-free; acellular; abiotic) assays (see also Fig. 1, Hazard Characterization).