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Review

# Keeping it real: The importance of material characterization in nanotoxicology

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ABSTRACT

Nanomaterials are small and the small size and corresponding large surface area of nanomaterials confers specific properties, making these materials desirable for various applications, not least in medicine. However, it is pertinent to ask whether size is the only property that matters for the desirable or detrimental effects of nanomaterials? Indeed, it is important to know not only what the material looks like, but also what it is made of, as well as how the material interacts with its biological surroundings. It has been suggested that guidelines should be implemented on the types of information required in terms of physicochemical characterization of nanomaterials for toxicological studies in order to improve the quality and relevance of the published results. This is certainly a key issue, but it is important to keep in mind that material characterization should be fit-for-purpose, that is, the information gathered should be relevant for the end-points being studied.



``When things are large, they are what they are. When they are small, it's a different game: they are what our measurements make them.''

George M. Whitesides, No Small Matter. Science on the Nanoscale. [2009]

## 1. Introduction

Engineered nanomaterials have become the focus of extensive research in many areas including biomedical applications due to the novel and unique properties arising at the nanoscale. In addition, during the past decade, there has been an exponential increase in the number of papers on the toxicological effects of nanomaterials. However, while this certainly shows that the potential risks of nanomaterials are being considered, it has been argued that numerous poorly controlled studies have been published, offering little insight into any `nanospecific' effects [1]. Indeed, Krug concluded in a recent overview of the field that while 10.000 papers have been produced on environmental and health effects of nanomaterials in the last 15 years, we are left with ``a plethora of low-value results'' due to the lack of harmonized experimental protocols, poor or nonexistent characterization of the nanomaterials, a lack of reference materials, the frequent reliance on unrealistically high doses both for *in vitro* and *in vivo* studies, and so on [1]. Others have also complained, on the basis of a meta-analysis of several dozen papers focused on silica particles, that ``after over a decade of research, answers for the most basic questions are still lacking'' and suggested that more coherence in the experimental methods and materials used is needed [2].

Ten years have passed since the review by Oberdorster et al. defining and outlining the emerging discipline of nanotoxicology [3]; in their review, which is now a `citation classic', the authors defined nanotoxicology as a ``science of engineered nanodevices and nanostructures that deals with their effects in living organisms'' and they pointed out that nanotoxicology research also will advance the field of nanomedicine by providing information on the undesirable properties of nanomaterials and means to avoid them. Indeed, this is sometimes referred to as `safe-by-design' [4].

In another very pertinent review, also published in 2005, Oberdorster et al. summarized the views of an international expert group convened to develop a screening strategy for the hazard identification of engineered nanomaterials [5]. Hence, the authors stated that ``there is a strong likelihood that biological activity of nanoparticles will depend on physicochemical parameters not routinely considered in toxicity screening [of chemicals]'' and put forward a list of physicochemical properties that may be important in understanding the toxicity of nanomaterials namely: particle size and size distribution, agglomeration state, particle shape,

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crystal structure, chemical composition, surface area, surface chemistry, surface charge, and porosity [5]. Similar suggestions for minimal material characterization requirements in nanotoxicology have been proposed in recent years, as we shall discuss in the present essay. The question is: are we ready to adopt such requirements as an international standard(s)? Indeed, can we afford not to do so? Furthermore, are there any examples of `good' nanotoxicological studies, or has all been for naught?

## 2. International harmonization efforts in nanotoxicology

Warheit asked in an editorial several years ago ``how meaningful are the results of nanotoxicity studies in the absence of adequate material characterization?'' [6]. He also noted that while most nanotoxicological studies are conducted under *in vitro* conditions (*i.e.*, in the wet phase), the physicochemical characterization is frequently carried out on the ``just-received'' nanomaterials in the dry phase, which has limited relevance for the test conditions. He also professed a list of minimal characterization requirements prior to conducting hazard assessment studies, similar to the one cited above. In a follow-up, Sayes and Warheit discussed three phases of material characterization [7]. Primary characterization is performed on particles as-synthesized or as-received, in its dry state or powder form. Secondary characterization, on the other hand, is performed on particles in the wet phase as a solution or suspension in aqueous media, *eg.* in water or cell culture medium. Finally, tertiary characterizations are performed on particles following interactions with biological systems *in vitro* or *in vivo*, and may include characterization of particles in blood, or lung fluid. The tertiary characterization of particles in the actual test system is certainly non-trivial, but it is the most relevant for the interpretation of the toxicological data [7]. The authors offered a list of physicochemical properties relevant to nanotoxicological testing, and they concluded that ``no single technique can accurately describe a specific property of a material'' [7]. Thus, all material characterization should be performed using more than one method.

The journal *Nature Nanotechnology* recently invited the nanotoxicology community to `join the dialogue' on whether guidelines should be implemented on the types of information that are required to improve the quality and relevance of the published papers [8]. It was further stated in the editorial that nanomaterial characterization ``should be done based on relevance to the study'' [8]. Indeed, different types of information (and consequently, the use of different methods) may be needed depending on the purpose of the study [9]. Hence, nanomaterial characterization should be fit-for-purpose. Furthermore, the methods that are used for characterization need to be standardized and validated. Fubini et al. emphasized that while there are obvious properties that should be assessed before any *in vivo* or *in vitro* testing is conducted, the ``choice of characteristics to be measured more accurately should be tailored to the end-point investigated in that particular study'' [10]. Others have highlighted that, ``for regulatory purposes, the standards applied and data generation required must be more prescriptive, whereas for research, these must be primarily based on the hypothesis to be tested'' [11]. Furthermore, while it would be laudable to characterize every aspect of a test material, both at synthesis and in the test system, this is certainly impractical [5] and it would therefore be advisable for the scientific community to agree on a number of common parameters that should be measured for all studies, in order to describe what the material looks like, what it is made of, and what factors govern how it interacts with its biological surroundings [12], while other characteristics should be based on relevance to the study [13].

The Seventh Framework Programme of the European Commission started in 2007 and projects funded in the final round will run until 2017. Among these projects, 50 have focused on nanosafety. The NanoImpactNet project played an important role in the integration of nanosafety research in the early phase of FP7 through the organization of workshops and conferences. In one such workshop, experts provided recommendations for minimal characterization of nanomaterials, and a distinction was made between `essential metrics', including size and size distribution, chemical composition and surface charge, and `often important metrics', including shape and solubility [14]. The need for methods to determine interactions of nanomaterials with the surrounding biological matrix was also highlighted. The research infrastructure project QualityNano has emphasized the importance of standardization and harmonization of procedures in all aspects of nanosafety assessment and also highlighted the need for *in situ* characterization [15]. The NANOREG project, with close to 70 partner institutes and a total of 50 million Euro in funding (provided by the European Commission and the participating member states), is designed to facilitate a common approach to regulatory testing of nanomaterials and aims to develop a regulatory framework for nanomaterials, in close cooperation with international organisations involved in standardization and regulation of nanomaterials, such as ECHA, OECD, CEN and ISO. In the NANOREG project, several characterization protocols based on OECD guidelines for analysis of physicochemical properties of nanomaterials are being verified and validated, including quantitative analysis of surface coatings, and size distribution analysis in the dry state or powder form as well as in the wet state, *i.e.*, in liquids for compliance with the EU definition [16].

The FP7 project ITS-NANO (for `intelligent testing strategy') developed a framework of future research priorities in cooperation with all the major stakeholders (*i.e.*, government, industry, academia, funding agencies and NGOs), and emphasized the importance of physicochemical characterization, along with exposure identification, hazard identification and modelling approaches [17]. In the large FP7 project, MARINA (for `managing risks of nanomaterials'), first steps are taken towards developing an intelligent testing strategy for nanomaterials. Notably, in the project, a common panel of representative nanomaterials from the Joint Research Centre (JRC) nanomaterial repository are being applied. Each type of material in the repository has been sourced as a large single batch which has been sub-sampled into individual vials to produce a collection of thoroughly characterized nanomaterials available for benchmarking in research and regulatory studies. In a recent study, 6 metal oxide nanomaterials were evaluated using 10 different toxicity assays in 9 different laboratories using 12 cellular models representing 6 different target organs [18]. The nanomaterials were all subjected to detailed physicochemical characterization. With this approach, a hazard ranking of the metal oxides could be established and cell-specific responses were noted. The NANOREG project also uses nanomaterials from the JRC repository. In another recent study, US researchers belonging to the `engineered nanomaterials grand opportunity' (Nano GO) consortium conducted so-called round robin or interlaboratory comparisons of a total of 7 metal oxides and multi-walled carbon nanotubes using 3 different cell lines [19]. These and other studies [20] point to the importance of conducting studies with multiple relevant cell types in order to perform accurate *in vitro* evaluations of nanomaterials. Moreover, applying extensively characterized nanomaterials for benchmarking will allow for comparisons across studies and may prevent the generation of ``low-value'' results [1].

For a discussion of material characterization in the context of nanomedicines, see the excellent papers by McNeil and co-