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Limited Usefulness of *In Vitro* Toxicity Data in Hazard Identification of Nanomaterials

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Abstract

Short-term in vitro tests of toxicity can provide a rapid and relatively inexpensive way to assess the potential toxicity of large numbers of untested nanoparticles, and therefore, the number of *in vitro* studies of nanomaterials in the literature has sky rocketed in recent years. However, there are a number of inherent issues of *in vitro* test systems that result in false positives and false negatives, and recent studies have shown little correlation between *in vitro* and *in vivo* toxicity of nanomaterials. Some generic and specific issues of *in vitro* toxicity testing of nanomaterials are discussed.

Keywords: *In vitro* tests; Toxicity studies; Nanomaterials; Nanoparticles

Abbreviations: LDH: Lactose Dehydrogenase; TGF-B: Transforming Growth Factor-B; MTT: Mitochondrial Reduction of Tetrazolium

Introduction

With the increasing use and development of engineered nanomaterials in electronics, pesticides, consumer products, and chemical and pharmaceutical industries, there is a growing concern about their potential risks to humans and the environment. The toxic effects of most nanomaterials have not been characterized, but it is generally believed that nanoparticles can have toxicological properties that differ from their bulk materials. A challenge facing hazard identification and safety assessment of engineered nanomaterials is the diversity and complexity of the types of materials with varying physicochemical properties, many of which can affect their toxicity by different mechanisms [1].

Inhalation is the "gold standard" in regard to method of exposure of the respiratory tract for hazard identification of fibers and particles and to obtain dose-response data in quantitative risk assessment since inhalation is the normal physiological route for delivery of particles in the lungs. However, inhalation studies are costly, use large number of animals, take long time to complete, and require sophisticated exposure facilities. As a result, very few *in vivo* studies are available for engineered nanomaterials. On the other hand, short-term *in vitro* tests of toxicity can provide a rapid and relatively inexpensive way to assess the potential toxicity of large numbers of untested nanoparticles. Hence, the number of *in vitro* studies of nanomaterials in the literature has sky rocketed in recent years.

Issues of *In Vitro* Systems

A review of the toxicity studies of fibers by an expert panel has concluded that no single short-term *in vitro* test (or battery of tests)can be used to predict the carcinogenicity potential of fibrous particles [2]. At present, *in vitro* test systems also appear to have limited usefulness for hazard identification of nanoparticles due to a number of inherent issues resulting in false positives and false negatives. Some generic issues associated with the *in vitro* approach include:

(i) High-dose effects - effects observed at high-dose levels used in *in vitro* as says may not extrapolate to low-dose effects *in vivo*.

(ii) Time course effects - short-term *in vitro* endpoints (e.g. release of inflammatory mediates. cell proliferation) may not be predictive of long-term physiological effects.

(iii) Cell line effects - toxic responses may differ using different cell lines.

In addition, there are a number of issues specific to *in vitro* toxicity testing of nanoparticles:

(i) A number of end points employ the measurement of a cellular product, such as release of a protein. Recent data from several research groups [3-5] have demonstrated that various types of nanoparticles can absorb key proteins

such as albumin, LDH, fibronectin, and T GF-β, leading to confounding end point measurements.

(ii) Some nanomaterials such as carbon nanotubes have been shown to interfere with the MTT cytotoxicity assay by absorbing the reduced formazandye, resulting in an underestimation of cytotoxic potency [6].

(iii) Many assays employ the measurement of a colored or fluorescent product. For instance, fluorescent nanoparticles such as quantum dots may interfere with the product used to quantity specific cellular responses [7].

(iv) Under *in vitro* cell culture conditions ('wet phase'), physicochemical characterization of particles including particle size are likely to change from the powder form ('dry phase'). The type and composition of culture medium (e.g. addition of serum) can affect toxicity measurements - probably due to influences affecting agglomeration and/or surface chemistry of nanoparticle [8].

(v) Use of organic solvents for creating suspensions or dispersive agents/surfactants to maintain the nanoparticles from forming aggregates may not be relevant to normal exposure conditions, and these agents may have biological activity that can confound the findings. Incomplete removal of the organic solvent tetrahydrofuran used to create water-soluble suspensions of C_{60} is believed to contribute to the cytotoxicity of C_{60} in human cells [9].

(vi) The particokinetics of nanomaterials in culture media is often not considered, resulting in erroneous dose-responses [10].

(vii) New mechanisms may be missed leading to false negatives. For instance, while inflammation and oxidative stress have been identified as possible mechanisms underlying the etiology of nanoparticles, the toxicity of cationic dendrimers appears to be related not to oxidative stress generation, but to disruption of cell membrane integrity through interaction of the positive charge terminal group with the anionic lipids of the cell membrane [11].

Therefore, depending on the type of cells, the duration of exposure, the concentration of nanoparticles and the composition of the culture media, testing of the same nanomaterial can have different outcomes. Recent studies have shown little correlations between *in vitro* and *in vivo* toxicity of some nanomaterials. For instance, Sayes et al. [12] assessed the capacity of *in vitro* screening studies to predict *in vivo* pulmonary toxicity of several fine or nano-sized particles in rats, including carbonyl iron, crystalline and amorphous silica and zinc oxide. For the *in vitro* component of the study, different culture conditions were utilized. In the *in vivo* component of the study, rats were exposed by intratracheal instillation to each of the materials. Following exposures, the lungs of exposed rats were lavaged and end points were measured at numerous time points post-exposure. When considering the range of toxicity endpoints, the comparisons of *in vivo* and *in vitro* measurements demonstrated little correlation. Similarly, whereas nano-C60 and C_{60} $(OH)_{24}$ were reported to be toxic to a number of cell types *in vitro* [13], there was no evidence of adverse effects in lung tissues at three months post-instillation exposure to doses up to 3mg/kg of the two types of fullerenes in rats [14]. *In vitro* assays of oxidant stress also failed to predict the progressive interstitial fibrotic response to inhalation exposure to single-walled carbon notubes (SWCNTs) [15,16].

Low-throughput *in vitro* testing methods for nanomaterials have improved in recent years. For instance, interaction with colorimetric indicator dyes confounding the measurement when testing the toxicity of carbon nanomaterials could be avoided by employing the clonogenic assay which does not use any dye or stain [17]. A nanoparticle dispersion system using an electrospray method to deliver nanoparticles for *in vitro* nanotoxicity studies has been developed [18]. Nonetheless, the poor correlations between *in vitro* and *in vivo* toxicity data of nanomaterials can also be due to the toxicokinetic of nanoparticles in animals. All nanoparticles, upon exposure to tissues and fluids of the body, will immediately adsorb on to the surface of some of the macro molecules that they encounter. The specific features of this adsorption process will depend upon the size and surface characteristics of the particles, including surface chemistry and surface energy [19]. One paradigm of nanoparticle toxicity is the ability of some nanoparticles to form acorona with proteins, which leads to adverse biological effects through protein unfolding, fibrillation, thiol cross-linking and loss of enzyme activity [20].

It has also been found that for certain nanoparticles the clearance mechanism may be less effective than for larger particles after deposition in the respiratory tract. Their small size helps them to enter the cells by endocytosis and reach the circulating system, eventually reaching various potential target sites [21,22]. Dissolution appears to be one of the key elements for determining the biological fate and effects of some nanoscale materials [23]. Other nanoparticle characteristics and agglomeration/aggregation state can also affect their deposition, distribution, metabolism and excretion [1].

Conclusion

Due to the issues of *in vitro* systems and the toxicokinetics issues discussed above, many of the *in vitro* toxicity data reported in the literature are of limited value in hazard identification of nanomaterials. For the same reasons, screening strategies [24-26] developed for the hazard identification process of nanomaterial risk assessment which start with using short-term *in vitro* screening assays, are prone to fail and unlikely to be validated later by animal studies. Adequate *in vivo* toxicity studies on nanomaterials are scarce [27]. Therefore, the scientific community has not been able to definitely determine which nanomaterials and which are not, hazardous to humans or the environment. Conflicting results are often reported for

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nanomaterials of the same class/subclass or seemingly identical materials. The challenges in toxicity testing and risk assessment of nanomaterials have been discussed.

Testing every nanomaterial in animals is impractical, if not impossible. As toxicity testing of nanoparticles by the traditional approach appears problematic, a paradigm setting a stage for "toxicity testing of nanomaterials in the 21st century" has been proposed [28]. In this paradigm, only a small number of short-term *in vivo* studies in rodents are necessary to first characterize the toxicological properties of reference materials of each class/subclass of nanoparticles. *In vivo* and *in vitro* high-throughput genomics and/or proteomics studies are then performed to investigate the underlying molecular mechanisms/ toxicity pathways and biomarkers of the toxic responses. As *in vitro* studies allow specific biological and mechanistic pathways to be isolated and test edunder controlled conditions, mechanism-based short-term in vitro assays in appropriate cell lines (preferably of human origin and at target tissues) may be conducted to aid in elucidation or interpretation of mechanisms, toxicity pathways and biomarkers data derived from the *in vivo* animal studies. Once these mechanistic data on reference materials are obtained, they can be used to bench mark the effects and the hazard potential of any nanoparticle belonging to the same class/subclass by comparing data of their high-throughput *in vitro* and/or mechanism-based short-term *in vitro* assays. In other words, animal studies are no long needed and hazard potential of any nanomaterial can be semi-qualitatively evaluated by toxicity testing under this paradigm [28].

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