

# Nanotoxicology: a perspective and discussion of whether or not *in vitro* testing is a valid alternative

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**Abstract** Despite the many proposed advantages related to nanotechnology, there are increasing concerns as to the potential adverse human health and environmental effects that the production of, and subsequent exposure to nanoparticles (NPs) might pose. In regard to human health, these concerns are founded upon the plethora of knowledge gained from research relating to the effects observed following exposure to environmental air pollution. It is known that increased exposure to environmental air pollution can cause reduced respiratory health, as well as exacerbate pre-existing conditions such as cardiovascular disease and chronic obstructive pulmonary disease. Such disease states have also been associated with exposure to the NP component contained within environmental air pollution, raising concerns as to the effects of NP exposure. It is not only exposure to accidentally produced NPs however, which should be approached with caution. Over the past decades, NPs have been specifically engineered for a wide range of consumer, industrial and technological applications. Due to the inevitable exposure of NPs to humans, owing to their use in such applications, it is therefore imperative that an understanding of how NPs interact with the human body is gained. *In vivo* research poses a beneficial model for gaining immediate and direct knowledge of human exposure to such xenobiotics. This research outlook however, has numerous limitations. Increased research using *in vitro* models has therefore been performed, as these models provide an inexpensive and high-throughput alternative to

*in vivo* research strategies. Despite such advantages, there are also various restrictions in regard to *in vitro* research. Therefore, the aim of this review, in addition to providing a short perspective upon the field of nanotoxicology, is to discuss (1) the advantages and disadvantages of *in vitro* research and (2) how *in vitro* research may provide essential information pertaining to the human health risks posed by NP exposure.

**Keywords** Nanotechnology · Nanoparticle · *In vitro* testing strategies · Human health · Toxicity · Nanotoxicology · Environmental air pollution · NP exposure

## Introduction

Over the past twenty years, the increase in nanotoxicology research has been concomitant with the overwhelming increase in the level of nanotechnology-related products being produced (Maynard 2007). This new industrial revolution promises to provide an advantageous basis for numerous applications, including medicine, consumer products (such as cosmetics and sporting equipment), environmental remediation and information technology (Maynard 2007), and is proposed to have a net worth of \$15 billion by 2015 (Service 2004).

In order to realise these proposed benefits, heightened research has been performed over the past two decades in order to determine whether the potential benefits of nanotechnology could be recognised without any adverse impact upon human health, as well as, most recently, the environment. This has been aptly termed nanotoxicology. Based on the principles of toxicology (Timbrell 1999), nanotoxicology can be defined as ‘the study of the effects

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of nanoparticles (NPs) (either accidentally produced or engineered) on living organisms' and described as a multidisciplinary science including material science, chemistry, physics and medicine (Donaldson et al. 2004). The field of nanotoxicology focuses upon gaining a thorough understanding of the relationship between properties such as particle size, surface area and reactivity, dose (concentration), material composition and the potential toxicity of NPs. In relation to this, it is also proposed that nanotoxicology will develop and implement 'nano-specific' protocols in order to investigate and gain the knowledge necessary in order to determine the potential toxicity of the plethora of different NPs. In addition to this, it is important to note that over the past decade, there has been increased confusion as to the definition of a NP. The International Organization for Standardization (ISO), has provided specific definitions in their recent document entitled "Nanotechnologies – Terminology and definitions for nanoobjects—Nanoparticle, nanofibre and nanoplate". For the basis of this review, the following definition for a NP; a nano-object [a material with one, two or three external dimensions in the nanoscale (1–100 nm)] with all three external dimensions in the nanoscale (ISO/TS: 27687:2008); will be used.

The requirement for gaining an understanding of the potential adverse effects of NP exposure is, in part, based upon the known effects of exposure to environmental air pollution. It is well understood that the NP component contained within environmental air pollution, specifically particulate matter with a size of 10  $\mu\text{m}$  or less ( $\text{PM}_{10}$ ), can increase the potential for humans to exhibit increased pulmonary diseases. However, due to the rapid advent of new, engineered NPs, as well as what is already known in regard to exposure to (accidentally produced) NPs within environmental air pollution, the need to understand the potential effects of exposure to these nano-sized materials requires efficient, effective and speedy assessment. This, of course, would be possible using *in vitro* testing strategies; however, the questions remains whether or not such testing strategies are enough to define what the exact effect would be *in vivo*. The aim of this review therefore in addition to providing a short perspective upon the field of nanotoxicology is to discuss (1) the advantages and disadvantages of *in vitro* research and (2) how *in vitro* research may provide essential information pertaining to the human health risks posed by NP exposure.

### Nanotoxicology: a brief perspective

Recently, the field of nanotoxicology has been described as a multi-interdisciplinary field, consisting of biologists (including toxicologists), chemists (including biochemists),

physicists, mathematicians and epidemiologists (European Science Foundation (ESF) Report 2005). This, however, is not a new concept to the field. Since the first studies that showed NPs to induce a heightened adverse effect on biological systems compared to their larger sized counterparts at the same mass dose (Ferin et al. 1992; Oberdorster et al. 1992), the understanding that NPs require a multidisciplinary approach (i.e. biology, physics, chemistry and medicine) has been accepted. Due to the constant increase in the number, type and sensitivity of equipment being used to study NPs and their subsequent effects, this multi-interdisciplinary approach has been significantly improved in the past few years (Bouwmeester et al. 2010). A complete review of the history and current status of the field of nanotoxicology is beyond the remit of this overview. In order to obtain a clear understanding of the field up to the current moment, it is suggested that such publications as Oberdorster et al. (2005, 2007), Donaldson et al. (2006), Stone et al. (2009) and Knol et al. (2009) are considered. Briefly, however, concern surrounding the exposure of humans to NPs (mainly) derives from their small size and emanates from two independent findings that separately recognised that as particle size decreases, toxicity generally increases.

One of the initial studies to demonstrate the potentially harmful effects of exposure to NPs was by Ferin et al. (1992). In this study, rats were exposed, via inhalation, to both NP (21 nm diameter) and fine (250 nm diameter) titanium dioxide ( $\text{TiO}_2$ ) particles, as well as intratracheally instilled with  $\text{TiO}_2$  particles of various sizes (12, 21, 230 and 250 nm in diameter) over a period of 12 weeks. Examination of the effects of treatment with each particle size was then performed over a 70-week post-exposure period. It was demonstrated that  $\text{TiO}_2$  NPs promoted an acute inflammatory response following both intratracheal instillation and sub-chronic inhalation techniques compared to the larger particles (230 and 250 nm). The inflammation observed in exposure animals was subsequently found to reduce to control levels post-exposure (64 weeks), with a noted decrease (from peak levels) in the number of neutrophils present in the lung at this time. NPs were also found to remain within the lung longer (501 days) than fine particles (174 days). The prolonged retention of  $\text{TiO}_2$  NPs in the lung was suggested to be an effect of the finding that at equivalent masses, NPs were able to translocate to the pulmonary interstitium more efficiently than the larger  $\text{TiO}_2$  particles. It was suggested that the translocation of NPs to the interstitium was due to the smaller particles (12 and 21 nm) not being taken up by alveolar macrophages and undergoing clearance from the alveoli via uptake by alveolar type-1 epithelial cells instead. In addition to this, it was found that an increased dose (increased number of particles and decreased particle

size) promoted movement of particles within the pulmonary system. It was also observed that the number of particles present, particle size, delivered dose and the delivered dose rate also had an effect on the translocation process. Ferin et al. (1992) concluded that the observed inflammation was due to exposure of the NPs to the rat lung, impaired lung clearance and NP redistribution.

In a subsequent publication, these findings were supported by Oberdorster et al. (1992), who showed increased levels of inflammation to be present in the alveolar space of rats after instillation with 500 µg TiO<sub>2</sub> NPs (20 nm) over 24 h, compared to TiO<sub>2</sub> fine particles (250 nm) at the same mass dose. It was also found, in comparison with Ferin et al. (1992), that NPs were able to enter the interstitium more readily than the larger TiO<sub>2</sub> particles. Oberdorster et al. (1992) further suggested that the increased inflammatory response to acute NP exposure could not be explained fully by the movement of particles to the interstitium but could be related to the larger surface area of the particles and their interaction with alveolar macrophages and interstitial cells. Focusing on alveolar macrophages, it was noted that they were effective inhibitors of the inflammatory response to NPs due to the active uptake of these particles. The findings of both Ferin et al. (1992) and Oberdorster et al. (1992) prompted increased interest into the effects of NPs on the lung, as well as the possible health effects that exposure to NPs might pose to respiratory and cardiovascular functions (Li et al. 1997, 1999) and why these effects are observed (Brown et al. 2001; Duffin et al. 2002, 2007; Stoeger et al. 2006).

In addition to these laboratory investigations, epidemiological studies conducted over the last two decades have shown a positive correlation between the level of particulate air pollution and increased adverse health effects (Dockery et al. 1993; Bremner et al. 1999; Braga et al. 2000), including increased pulmonary diseases (Choudhury 1997; Pope and Dockery 1999; Schwartz 2004), as well as a rise in the number of deaths from cardiovascular disease (Abbey et al. 1999; Aga et al. 2003; Zanobetti et al. 2003; Medina et al. 2004; Pope et al. 2009). Additional epidemiological studies have also reported a direct relationship between the exposure of NPs in air pollution and adverse health effects (Peters et al. 1997; Wichmann et al. 2000; Schulz et al. 2005). Specifically, Peters et al. (1997) reported that human respiratory ill health was associated with the number of ambient NPs inhaled. In this study, it was observed that twenty-seven non-smoking asthmatics, who resided within a highly polluted city in central Europe, had a significant decrease in the peak expiratory flow from their lungs over a 6-month period. It was concluded by Peters et al. (1997) that the adverse health effects observed following exposure to PM<sub>10</sub> could be related to the size distribution of environmental air

pollution and therefore supporting the theory originally stated by Seaton et al. (1995) that NPs could potentially drive toxicity in the lung.

Progress in the field of nanotoxicology has gained increased intensity since the aforementioned laboratory-based and epidemiological studies. A prime example of how the field has changed in the past decade is that it is now ingrained into researchers within the field that they must fully understand not only the cellular system they are using but also the NP suspensions they are using. In the studies by Ferin et al. (1992) and Oberdorster et al. (1992), the concept of particle characterisation was highlighted in their subsequent conclusions (i.e. particle size); however, understanding the fundamental particle characteristics was not so pertinent at that time compared to understanding the effects that the smaller particles had. Nowadays, however, it is well understood that particle size and, particularly, the surface area and reactivity (Brown et al. 2001; Duffin et al. 2002, 2007) are relative to their effects. It is no longer sufficient to simply suspend a dry or wet sample of NPs in the suspension vehicle of choice and then expose the chosen model (i.e. tissue or cell cultures) to these NPs. The concept for increased NP characterisation has been based upon reports showing that NPs can alter their characteristics, such as size, shape, zeta potential, surface area and reactivity, length, aspect ratio, chemical composition and surface attachments (such as the attachment of additional proteins to the surface of NPs when suspended in cell culture media containing serum proteins) when in different suspension media and after alternative preparation techniques. Thus, the particle suspension originally used and exposed to the experimental model is perhaps not the one interacting with the model, and so the hypothetical effect observed is possibly not relative to the original NP suspension used. Therefore, it is essential that information pertaining to the characteristics of NPs is obtained relative to the experimental set-up being used.

Although increased attention is now being given to the specific characteristics of the NPs being used, questions still arise concerning the specific exposure method employed relative to the potential exposure route for the cells used. For example, are suspension cultures suitable for assessing the effects of inhaled NPs, when exposure systems are available that can deposit defined doses of NPs at the air–liquid interface (Tippe et al. 2002; Diabaté et al. 2008; Lenz et al. 2009; Brandenberger et al. 2010). It may be argued, however, that suspension cultures are more beneficially used for the investigation of NPs that are for use in applications such as medicine and food, which could enter the human body via injection and ingestion (Knol et al. 2009). Additionally, suspension cultures would be beneficial for exposing cells of secondary target

organs in the human body following their inhalation. Knowledge of the translocation of NPs following their inhalation (regarded as the primary exposure route of NPs) is in its infancy however, and the precise methods needed to understand this increasing area of nanotoxicology are yet to be confirmed (Knol et al. 2009). In addition to using realistic exposure systems, it is also pertinent to highlight the necessity to use realistic doses. Recent research into the toxicity of NPs (Ryman-Rasmussen et al. 2009) has shown detrimental effects of nano-objects over time; however, the concentrations used have been significantly higher than what any human would be exposed to. This parameter is of the utmost importance if the field of nanotoxicology is going to understand and provide a realistic knowledge base of NP effects to human health, which will help the production and use of nanotechnology applications within both an occupational and consumer setting.

Despite the need to measure the previously highlighted parameters and to maintain a realistic aspect to nanotoxicology research, the precise mechanism of NP toxicology is still not understood. Currently, the hypothesis that NPs induce adverse cellular effects via oxidative means (oxidative stress paradigm) (Donaldson et al. 2003) is used as a basis for many NP investigations. Recently, however, additional paradigms have been suggested for NPs, such as the fibre paradigm (Dörger et al. 2001; Donaldson and Tran 2004) and the theory of genotoxicity (Schins and Knaapen 2007). The fibre paradigm was recently and perhaps most notably highlighted in the paper by Poland et al. (2008), in which it was shown that multi-walled carbon nanotubes caused granulomas in the peritoneal cavity *in vivo*. This paradigm, however, can only be attributed to nanofibres and especially those relating to the specific characteristics of HARN (high aspect ratio nanoparticles) (Donaldson et al. 2006). Thus, the plethora of spherical NPs available cannot fit this paradigm. They may, however, in addition with the fibrous nano-objects fit into the theory of genotoxicity. This theory has been based upon numerous testing strategies using NPs >100 nm in size, with a few actually based upon NPs <100 nm and so requires increased, in-depth research to fully understand the ability for NPs to be genotoxic, mutagenic and potentially carcinogenic.

Due to the increased attention and funding opportunities provided for nanotoxicology research over the past 5–10 years, there has been an abundance of published studies claiming to be assessing the effects of NPs in relation to human health (Oberdorster et al. 2007). In the majority of these publications, there have been the advantages of numerous time points, NP types and doses as well as end-points measured. This has only been possible due to the use of an *in vitro*-based testing strategy.

## Nanotoxicology testing *in vitro*

As highlighted earlier, the ability to perform such a magnitude of research, which is required in order to maintain an even balance of knowledge and understanding of the effects of NPs compared to the augmented production of NP-based products and applications, has been specifically due to the availability of *in vitro* testing strategies. Similarly, the European Union has promoted the use of *in vitro* testing strategies for this reason to investigate the effects of new cosmetics (European Cosmetics directive; 76/768/EEC), suggesting specific cell types, such as the human promyelomonocytic cell U937 (a surrogate dendritic cell line) for use in exposure and toxicological analyses.

Although any experimental analysis can be performed with cells obtained from either *in vivo* experimentation (*ex-vivo*) as well as *in vitro*, it is pertinent to highlight the constant debate of the advantages and disadvantages of both *in vitro* and *in vivo*. Although *in vivo* (specifically mice and rat models, however, also considering research on hamster, guinea pig and monkey models) research is known to enable scientific research to observe almost ‘first-hand’ the effects of a substance as they would occur in a hominid, *in vitro* models provide the possibility to investigate toxic effects on human cells extensively, which cannot be conducted *in vivo* (Rothen-Rutishauser et al. 2008a). Cultured human and animal cells can be better controlled and therefore yield more reproducible data than *in vivo* systems; however, they require a high standardisation to maximise reproducibility.

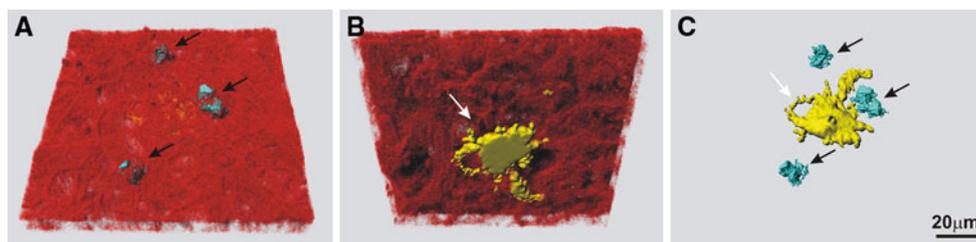
Commonly, nanotoxicology studies have used monocultures of cells that are specific to organs of the body. For example, epithelial cells are commonly used, as these form the basis for many of the natural barriers formed within the human body. Macrophage cells are used as a model to study the effects of NPs on the human immune system. Dendritic cells are also used, however, not as commonly as macrophages. This is interesting as dendritic cells would, arguably, provide a more beneficial model for NP-immune system interactions due to their natural role within the human body and its innate/adaptive immune systems. Additionally, specific cells such as C3A and HepG2 cells are also used for example for the liver, the PC12 cell line is used for analysis of NPs with the brain, whilst there are also numerous tumour cell lines that are used, such as mesothelioma cell lines (e.g. IST-Mes3/2P). In fact, there are many different cell lines used for every different organ of the human body. Table 1 provides an example of the most commonly used human cell lines used in studies concerning nanotoxicology.

Although monoculture systems provide the basis for high-throughput analysis for nanotoxicology, they do not represent a realistic model of how NPs will interact with a

**Table 1** Human cell culture models used in studies for nanotoxicity

Primary cultures	References
Monocyte-derived macrophages	(Waldman et al. 2007)
Monocyte-derived dendritic cells	(Blank et al. 2007; Rothen-Rutishauser et al. 2007, 2005)
Cell lines	
Airway epithelial cells	
Calu-3	(Bivas-Benita et al. 2004; Grenha et al. 2007; Rotoli et al. 2008)
16HBE14o-	(Brzoska et al. 2004; Holder et al. 2008)
BEAS-2B	(Herzog et al. 2007; Jang et al. 2006; Park et al. 2007; Veranth et al. 2007)
Alveolar epithelial cells	
A549	(Duffin et al. 2007; Park et al. 2007; Stearns et al. 2001)
Immortalised human alveolar type 2 cells with alveolar type 1 phenotype	(Kemp et al. 2008)
Macrophages	
THP-1	(Chen et al. 2006; Ece et al. 2008; Goulaouic et al. 2008; Wottrich et al. 2004)
Fibroblasts	
MRC-9	(Limbach et al. 2005)
Mesothelial cells	
MSTO-211H	(Kaiser et al. 2008; Wick et al. 2007)
3D cultures	
3D aggregates of A549 cells	(Carterson et al. 2005)
Triple cell co-culture model (epithelial cells, macrophages, dendritic cells)	(Rothen-Rutishauser et al. 2007, 2008a, b)

Adapted from Blank et al. (2009)



**Fig. 1** Laser scanning microscopy images of the triple cell co-culture model established by Rothen-Rutishauser et al. (2005). Epithelial cells (red, volume rendering), human monocyte-derived macrophages (light blue, surface rendering; black arrows) and human monocyte-

derived dendritic cells (yellow, surface rendering; white arrow) are shown. The same data set is shown from top (a), from bottom (b) and without epithelial cells from top (c). Reproduced with permission from ALTEX (Rothen-Rutishauser et al. 2008b)

specific organ of the body. Over the past 5 years, there have been increased efforts to establish more realistic models to study the toxic potential of NPs. An example of such an effort is the triple cell co-culture system composed of epithelial cell line (A549 or 16HBE14o<sup>-</sup>), human monocyte-derived macrophages and dendritic cells that has been established, simulating the most important barrier functions of the epithelial airway by Rothen-Rutishauser et al. (2005). This model provides a clear basis for investigating the interaction of NPs with the lung (Rothen-Rutishauser et al. 2005, 2008b) as well as at the air–liquid interface (Blank et al. 2006, 2007) (Fig. 1). It is also important to point out that in order to further mimic the situation in vivo, primary alveolar epithelial cells have

been harvested from human lung biopsies and employed in place of the epithelial cell lines commonly used in the triple cell co-culture model (Lehmann et al. 2010).

There are additional co-culture systems also available, such as the ones reported by Alfaro-Moreno et al. (2008) and Bhabra et al. (2009). The co-culture system of Alfaro-Moreno et al. (2008) is in fact a ‘quad-culture’, containing epithelial, endothelial, macrophage and mast cells, whilst that of Bhabra et al. (2009) is a bi-culture of BeWo (placental) and human fibroblast cells. The latter study reported that cobalt-chromium (CoCr) NPs can indirectly affect the homeostasis of fibroblast cells despite being located behind a confluent layer of BeWo cells. It was suggested by Bhabra and colleagues that increased DNA damage

observed in the human fibroblasts cells following CoCr NP exposure at  $0.036\text{--}0.36\text{ mg cm}^{-2}$  was due to a novel mechanism in which purine nucleotides are transmitted through gap junctions, or hemichannels, in the BeWo cell layer. The study of Bhabra et al. (2009) highlights the advantages of using in vitro cell co-culture systems, as the indirect effects reported would not be observed using monoculture testing strategies, and so therefore without using such a co-culture system, incorrect information could be gathered concerning the potential risks of NPs. Despite these specific examples, numerous laboratories use such co-culture systems, whether bi-, triple- or quad-cultures. Additionally, laboratories also use the supernatant taken from exposed monocultures of one cell type and then use this to treat a different monoculture cell type. Although this is not an advantageous co-culture technique, it is a performed practice to gain an understanding of how NPs interact with different cells over time. It is, however, whilst there are suitable co-culture systems available that are cheap (up to 1/10th of the total cost of any in vivo testing strategy), efficient and easy to construct/use, perhaps a dated methodology.

One aspect emphasised by the model of Rothen-Rutishauser et al. (2005) is that the architecture is specific to that as it is in the human lung (i.e. macrophages on the apical side, a layer of epithelial cells and dendritic cells on the basolateral side) (Fig. 1). This type of detail is absolutely essential as it provides a clear sign as to the interaction of NPs at the epithelial airway wall. Studies using co-culture cell systems have reported that they observe different reactions compared to monoculture analysis (Mueller et al. 2010; Lehmann et al. 2009); however, such reactions observed from a culture containing two, three or four different types of cells merely cultured in the same dish, (although providing data showing that NPs interact with different cells in an opposite manner to each cell monoculture) is not specific to that as it would occur in the human body. Thus, the architecture of the in vitro cell co-culture model in regard to the specific organ they represent is essential. Understanding how the organ works, taking time to prepare and perform series, upon series of baseline testing strategies, such as trans-epithelial electrical resistance (for epithelial layers), understanding the manner in which the cells interact with one another, the ratio of different cells to each other cell type in the co-culture, as well as if the cells remain viable in the co-culture are essential questions that must be thoroughly investigated prior to any form of nanotoxicology testing.

As previously highlighted in this review and the studies referenced, there are a number of different toxicological endpoints that researchers have used to assess the potential adverse effects that NPs may have on organs of the human body. These include numerous different biochemical- and

molecular-based testing strategies, investigating the potential for NPs to cause cytotoxicity, inflammation, oxidative stress, cell proliferation and genotoxicity. It is also important to note that when assessing the potential toxicity of any form of NP, it is important to investigate how the particles enter the specific cells being examined and in which compartments they may be found within (or, which is also possible, if the NPs stay attached to the cell membrane). This is of great importance, as it has been shown that the uptake behaviour can influence the cellular response following NP exposure (Lovric et al. 2005; Unfried et al. 2007; Maysinger et al. 2007). As it is not possible to go into sufficient detail regarding the entry mechanisms of NPs into cells, it is suggested that Unfried et al. (2007), as well as Rothen-Rutishauser et al. (2007) and Muehlfeld et al. (2008a; b) which provide an extensive overview of these processes, are considered.

In addition to all of the previously mentioned parameters highlighted in this review that should be taken into consideration when assessing the potential toxicity of NPs (in relation to the specific experimental question), it is also vital that additional and appropriate (positive and negative) controls are used. These are of course relative to the specific methodology being employed. Although negative controls are easily identified (usually cell culture medium/buffer only), determination of the correct positive control must be given more thought. For example, a low concentration of Triton X100 (such as 0.1 or 0.2%) is able to provide a clear positive reaction in any lactate dehydrogenase (LDH) assay (either diagnostic kit, or laboratory protocol); however, this form of compound would not be useful when assessing the potential production of reactive oxygen species by NPs within cells. It is therefore pertinent that the reaction that the test is causing is debated and the most adequate and specific positive controls are used.

In addition, testing for false-positive and false-negative effects within many of the biochemical tests commonly used in NP toxicology testing is essential to obtain a clear answer as to their potential adverse effects upon cellular-based systems. These issues have previously been discussed in detail by Stone et al. (2009), and it is suggested that this in-depth review of in vitro toxicology testing strategies is considered. Examples of such false positive/negative effects are, however, the ability for proteins to adsorb to the surface of NPs is realistic and can significantly affect the ability for NPs to interact with cellular systems. It is therefore also essential that the ability for proteins to adsorb to the surface of particles during toxicological tests is assessed [such as when using an enzyme-linked immunosorbent assay (ELISA)]. This is also true of enzymes, such as LDH. It is known that this enzyme can also adsorb to the surface of NPs, masking their toxicity and thus providing a false-negative toxic result. As with

protein adsorption, enzyme adsorption must also be investigated to correlate valid and representative toxicity data. Determination of these aspects is easily performed. Generally, the protocol consists of incubation of the NPs with the protein/enzyme for a period of time followed by the specific toxicological test (Clift et al. 2008). Any adsorption of the proteins or enzymes to the NPs is then apparent via a loss or increase in the specific protein/enzyme being measured. Additionally, it is also necessary to determine the ability for the NPs used to interact with the assays in regard to the fluorescent dyes or formazans that are used. An example of this was reported by Worle-Knirsch et al. (2006), where it was reported that carbon nanotubes interacted with the MTT formazan (tetrazolium salt) used and provided a false-negative toxicity. In addition to this, it is also pertinent that an assessment of the toxicity of the suspension media/buffer is performed. Increasingly, NPs are suspended in such buffers as Pluronic F127 and Tween80 in order to obtain a well-dispersed and characteristic NP suspension. If, however, a toxic response is observed following cellular exposure with NPs suspended in such buffers, it is essential that the toxicity of these buffers is known in order to assess the specific effects of the NPs only (Wick et al. 2007).

## Conclusion

In summary, although the parameters highlighted in this review can be performed using either *in vivo* or *in vitro* models, due to the heightened level of control/baseline analysis needed, the advantages of *in vitro* research enable such experimental testing strategies beneficial to nanotoxicology. This, however, will not be sufficiently covered by only performing monoculture analyses. It is essential that co-culture systems mimicking the *in vivo* system are established and used for the specific organs that are in danger of interacting with NPs. These systems, however, will not be able to be completely definitive of the *in vivo* situation until further *in vivo* analyses are performed in order to confirm the findings of *in vitro* investigations. Thus, in conclusion, the future of nanotoxicology testing lays with *in vitro* research; however, increased acute and chronic *in vivo* research is necessary in order to fulfil this possibility and to subsequently reduce, refine and replace all animal experimentation.

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