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REVIEW ARTICLE

Development of *in vitro* systems for nanotoxicology: methodological considerations

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Abstract

Due to the rapid development of a diverse array of nanoparticles, used in a wide variety of products, there are now many international activities to assess the potential toxicity of these materials. These particles are developed due to properties such as catalytic reactivity, high surface area, light emission properties, and others. Such properties have the potential to interfere in many well-established toxicity testing protocols. This article outlines some of the most frequently used assays to assess the cytotoxity and biological reactivity of nanoparticles *in vitro*. The article identifies key issues that need to be addressed in relation to inclusion of relevant controls, assessing particles for their ability to interfere in the assays, and using systematic approaches to prevent misinterpretation of data. The protocols discussed range from simple cytotoxicity assays, to measurement of reactive oxygen species and oxidative stress, activation of proinflammatory signaling, and finally genotoxicity. The aim of this review is to share knowledge relating to nanoparticle toxicity testing in order to provide advice and support for guidelines, regulatory bodies, and for scientists in general.

Keywords: *Nanoparticles; protocols;* in vitro*; cytotoxicity; genotoxicity; inflammation*

Contents

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Introduction

Nanotechnology involves the development and production of a vast array of different nanomaterials, including nanoparticles and nano-objects such as nanotubes. There are a number of definitions of nanoparticles that have evolved over time. A British Standards Institution (BSI) document of 2005 (PAS 71) defined nanoparticles as a "particle with one or more dimensions at the nanoscale," but this has been more recently updated to a "nano-object with all three external dimensions in the nanoscale" (PAS 136; British Standards Institution (2007)). Within this definition a nano-object is a "discrete piece of material with one or more external dimensions in the nanoscale," while nanoscale refers to a "size range from approximately 1 nm to 100 nm" (PAS 136; British Standards Institution (2007)). This definition relates mainly to approximately spherical or cuboid particles, and would therefore not include some fiber-like particles such as long nanotubes. Due to this ever-expanding array of diverse nanoparticles and nano-objects under development and production, and due to the necessity to ensure their safety, there is an urgent requirement by toxicologists, industry, regulators, and advisory bodies to establish *in vitro* toxicity tests that can be used to screen nanomaterials (Maynard et al., 2006). This article describes a selection of some of the most common *in vitro* toxicity assays used to examine nanoparticles and nano-objects, and discusses some of the potential advantages and pitfalls of each technique. Furthermore we provide an outline of suggested strategies or frameworks in which these protocols might be used as a screening tool for such nanomaterials, to provide candidates either suitable for or in need of further testing. Note that we have not included a discussion of the particle characterization techniques for nanoparticles that could also be conducted in relation to such studies. A detailed assessment is outwith the scope of this review, but it is important to stress that thorough characterization of factors such as size, surface area, shape, composition/contamination, solubility, and aggregation/agglomeration is essential. For recommendations on the characterization strategies of nanoparticles refer to reviews on this topic (e.g. Monteiro-Riviere and Tran, 2007; Powers et al., 2007).

The advantages and disadvantages of *in vitro* systems

There are a number of obvious advantages to *in vitro* toxicity testing of any chemical or particle, including the ethical desire to reduce animal testing, the speed of results, and the relatively lower cost compared to *in vivo* studies. In general, researchers tend to use relatively simple *in vitro* systems, which are therefore relatively easy to perform, control, and interpret. There are a large number of different tumor and transformed cell-derived cell lines available, but discussion of their relative merits is beyond the scope of this review. It is also possible to increase the complexity of these *in vitro* systems to include multiple cell types, with the aim to more closely mimic the *in vivo* situation. For example, Rothen-Rutishauser et al. (2005) have developed a culture of dendritic cells and epithelial cells that mimic the lung surface, while Jepson and Clark (1998) have developed an M-cell model that mimics the Peyer's patches of the gastrointestinal tract. In addition, there are well-developed and -characterized skin models such as EpiDerm® and EpiSkin® (Netzlaff et al., 2005) which are likely to prove useful for *in vitro* screening of nanoparticles. With this increase in system complexity comes the potential to generate more meaningful data, but also data that might be more difficult to interpret. One of the great benefits of an *in vitro* system is the ability to manipulate parameters using interventions such as pharmaceutical agents in order to investigate mechanisms. For example, antioxidants can be used to investigate the role of reactive oxygen species in particle-induced cytokine expression (Brown et al., 2004), and there are a wide array of endocytosis inhibitors (e.g. cytochalasin D; Geiser et al., 2005), as well as inhibitors of cell signaling pathways (e.g. mitogen-activated protein (MAP) kinase inhibitors, caspase inhibitors, calcium blockers, etc.; Brown et al., 2004; Sydlik et al., 2006). Mechanisms can of course also be investigated *in vivo*, but such a study is more complex, time consuming, and expensive.

There are, of course, also a number of disadvantages to *in vitro* systems. The main disadvantage is that every *in vitro* system is limited to either one cell type, or a combination of just a few cell types (two are common, but three or more are unusual). Therefore, an *in vitro* system is not able to fully replicate the complex interactions that occur between multiple cell types *in vivo*, both within an organ and also between organs (for example via humoral mediators). An *in vitro* system can investigate the potential for particles to cross cell boundaries, but it cannot be used for true pharmacokinetic or toxicokinetic studies in order to identify the targets of exposure within the body. The responses measured *in vitro* can often reflect those measured *in vivo*, for example with respect to cellular morphology, uptake of particles, cell signaling, gene expression, and protein production. However, other endpoints such as histological changes and effects on the immune system are more limited and difficult. As data accumulate with respect to the toxicological impacts of nanoparticles in animal and cellular models, scientists should be able to develop a battery of *in vitro* tests that can be used as alternatives to animal testing. It is unlikely that one test will be sufficient in the long term to assess hazard for risk assessment purposes. Furthermore, different nanoparticle types might warrant different batteries of tests.

In vitro systems for nanotoxicology

There is currently a need to develop and validate *in vitro* assays for assessing the potential toxicity of the ever-expanding range of nanoparticles. If all new nanoparticles were to be tested in animals, taking into consideration manipulations in composition, size, formulation, contaminants, and routes of exposure, then hundreds of thousands of animals would

be required to fully assess the potential hazard of these materials. A "key goal" for toxicologists is therefore to identify *in vitro* assays that accurately reflect the ability of nanoparticles to induce toxic effects in humans.

However, one of the major problems in this field is that we are currently unaware of the potential health effects of this diverse array of nanoparticles that are already on the market and are under development. Instead, our current knowledge regarding the health effects of particles on humans are in general limited to particulate air pollution (PM_{10}) and a number of occupational dusts (e.g. silica and asbestos). PM_{10} consists of a wide range of particle sizes (approximately 10 μm and smaller aerodynamic diameter) and composition (combustion-derived carbon, metals, organics, pollen, secondary sulfates and nitrates). Elevated PM_{10} is associated with a number of adverse health effects such as acute increased morbidity and mortality due to respiratory and cardiovascular disease, as well as chronic effects such as cancer. Much work has been conducted to identify the mechanism of these acute health effects, and much attention has focused on the ultrafine fraction (diameter less than 100 nm) (Donaldson et al., 2005). The ultrafine hypothesis, originally proposed by Seaton et al. (1995), suggests that smaller particles are more prone to induce inflammation in the lung, leading to the cardiovascular effects measured by epidemiology studies. Epidemiology (Peters et al., 1997) and human exposure studies (Mills et al., 2007) have also provided evidence to support this hypothesis. A more detailed description of the adverse health effects and mechanisms of toxicity of combustion-derived nanoparticles is provided by Donaldson et al. (2005).

There is therefore much work required to identify the true effects of engineered nanoparticles on human health. In the absence of this information, our alternative *in vitro* tests have to be driven by the effects of nanoparticles observed in animal models. Mechanistic *in vivo* work suggested that particle size (Ferin et al., 1990; Oberdoerster et al., 1990) and surface area (Duffin et al., 2002; Stoeger et al., 2006; Duffin et al., 2007) were key factors in driving inflammation and oxidative stress (Li et al., 1999) following exposure via the lungs. Much of this work was conducted using low solubility, low toxicity particles such as carbon black, TiO_2, and polystyrene beads. These results therefore provide an indication of the potential toxicity of a range of engineered nanoparticles, but this is certainly not the complete picture. However, in the absence of these wider *in vivo* data, and due to the urgent need for *in vitro* models, one suggestion is to prioritize the *in vitro* tests developed to reflect the *in vivo* knowledge of which we are currently most confident, i.e. inflammation and oxidative stress, but also to expand this to include standardized *in vitro* tests that are already currently available (e.g. genotoxicity) that might be developed further to make them appropriate for nanoparticles.

One of the first steps required in an *in vitro* study is to establish the cytotoxic potential of nanoparticles, in order to allow benchmarking (e.g. LC_{50}: lethal concentration, 50%) and appropriate interpretation within genotoxicity testing, but probably more important, to establish sublethal concentrations for use in tests of mechanistic endpoints. These mechanistic endpoints are thought to be more appropriate than cytotoxicity assays, since the sublethal exposure concentrations are likely to be more reflective of potential human exposure concentrations, although data on human exposure are currently also lacking.

Toxicity or viability assays

There are a wide variety of assays that are used to assess toxicity or cell viability. One of the most common is the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mossman, 1983), or variations of this assay (e.g. MTS, XTT, WST-1, etc.). These assays principally determine cell viability through determination of mitochondrial function by measuring the activity of mitochondrial enzymes such as succinate dehydrogenase. The assay generates a colored product (e.g. a purple formazan), which can be quantified by light absorbance at a specific wavelength. The absorbance value generated is representative of both the cell number and the functional viability of those cells. Such assays can therefore detect proliferation as well as cytotoxicity. There are a wide range of non-particulate positive control substances that should induce cell death via the MTT assay. We consider it very useful to include a positive control that is linked to the hypothesis being tested. For example, an oxidant such as *tert*-butyl hydroperoxide has been used as a positive control when assessing the ability of nanoparticles to induce cytotoxicity via oxidative stress (Brown et al., 2007a).

When testing nanoparticles, it is important to realize that protocols used to assess endpoints such as the MTT assay can vary between groups. In the final stages of the MTT assay, solubilization of the cells and the formazan product is required using a solvent such as dimethylsulfoxide (DMSO) or isopropanol. When testing nanoparticles, this generates a suspension containing cell debris, the dissolved formazan, and particulates. In our experience it is advantageous to centrifuge the sample at this stage, to transfer the supernatant to a fresh 96-well plate, and therefore to read the absorbance of the supernatant devoid of particles and cell debris. This reduces background interference due to the inclusion of particles.

In fact, there are a number of additional control experiments that should be conducted before embarking upon a full MTT (or equivalent) assay. First, a number of particles may generate an absorbance at the same wavelength as that used to quantify the colored product, leading to an overestimation of the cell viability. This interference can often be controlled for by subtraction of the background absorbance of the cells in the presence of the particles, but without the assay reagents. Second, the large surface area or other surface properties can result in a high adsorptive capacity which allows the nanoparticles to effectively extract the colored product from the cell extract, leading to an underestimation of cell viability (Worle-Knirsch et al., 2006). This is more

difficult to control for, and therefore if adsorption is found to occur, an alternative assay may need to be considered. The addition of protein as a dispersant, leading to coating of the particle surface, may help to reduce interference by adsorption, but of course the role of the adsorbed protein must be taken into consideration when interpreting the toxicity data. Third, nanoparticles can exhibit oxidative surface properties, and the color production occurs via an oxidative reaction. It is therefore necessary to assess whether the particles, in the absence of cells, can trigger an increase in absorbance. However, if these potential confounders are controlled for, the MTT and derived assays can be used successfully to address nanoparticle-induced toxicity (Stone et al., 1998). In some specific cases, however, it may be preferable to consider alternative viability assays. Of course, it would also be useful to include a positive and negative control particle to benchmark against the particles under investigation. A positive control could include alpha quartz, or a relatively toxic nanoparticle such as copper oxide (Karlsson et al., 2008). A negative control could include a larger version of the test particle under investigation, or perhaps a polystyrene nanoparticle (negatively charged) or TiO_2.

Another equally common measure of cytotoxicity is the lactate dehydrogenase (LDH) assay. LDH is an enzyme that is normally found within the cell cytoplasm. Reduced cell viability leads to an increase in the leakiness of the plasma membrane and therefore release of the LDH enzyme into the cell culture medium. Again, the large surface area of nanoparticles provides the possibility of interference due to adsorption of the LDH protein on the particle surface. It is necessary to centrifuge the cell supernatant to remove any contaminating cell debris and particles, therefore leading to the removal of particle-adsorbed protein from the supernatant. Even if the particles are not removed by centrifugation, there is a possibility that the adsorbed protein is no longer functional as an enzyme. Enzyme or protein adsorption is not specific to LDH, and has been observed with other enzymes such as myeloperoxidase (Hohr et al., 2002). LDH adsorption can therefore lead to an underestimation of nanoparticle-induced cytotoxicity. Again, if the relevant controls are conducted, and adsorption is found to not be a problem, the LDH assay can successfully be used to determine cytotoxicity of nanoparticles. The detergent Triton X-100 is commonly used as a positive control in the LDH assay, as well as to determine the maximal LDH release from the cells. In addition, well-known membranolytic particles such as crystalline silica can be included as a positive control or benchmark (Schins et al., 2002). There is a possibility that the particle treatment could reduce cell proliferation and therefore cell number. Fewer cells are obviously capable of releasing less LDH should toxicity be induced, therefore potentially leading to an underestimation of toxicity. Such an effect could be checked via a measure of cell number, including total cellular protein or total releasable LDH from treated cells (assessed following particle and then Triton X-100 treatment) compared to the control cells.

Trypan Blue exclusion has been used in a small number of studies to assess the toxicity induced by particles. Trypan Blue is a large negatively charged molecule. Cells with an intact cell membrane are able to prevent Trypan Blue uptake and therefore appear clear by light microscopy. In contrast, dead cells, which are unable to maintain an intact plasma membrane, are colored blue within seconds of exposure to the dye. While this assay is a useful quick check of the viability of cells following isolation from an organ, or prior to seeding for cell culture, it is not sufficiently sensitive or reliable to use for *in vitro* toxicity testing, and not appropriate for high throughput testing, when compared to the aforementioned assays, mainly due to the requirement for manual counting of cells (da Costa et al., 1999).

The fluorescent dye propidium iodide (PI) works in a similar way to Trypan Blue, staining the DNA/nucleus of dead cells due to the heightened plasma membrane permeability. This staining is used as an indicator of cell death via necrosis. It is relatively common to combine PI staining with annexin V–FITC (fluorescein isothiocyanate). Annexin V (AV) binds to phosphatidyl serine on the surface of apoptotic cells. Using flow cytometry of dual-stained cells allows the identification of both apoptotic and necrotic cell death within the same cell population. This is a relatively easy technique to conduct. Staurosporin can be used as a positive control to induce apoptosis, while a range of substances can be used to generate necrosis (which again could be linked to the hypothesis being tested). Of course, there are additional control experiments that need to be considered. This assay measures fluorescence, and it is conceivable that the particles could interfere in assessment of the light emitted. There are a variety of ways in which this interference might occur, including physical blocking of the light emitted (e.g. carbon), reflection of the excitation light (e.g. TiO_2), and particle-induced fluorescence (e.g. quantum dots or polystyrene beads), and so potential interference needs to be assessed. It is probably a good idea to wash the cells prior to loading into the flow cytometer, because once the machine is contaminated or even blocked, it might be troublesome to clean. It is possible to also detect changes in the staining of PI- and AV-treated cells using other techniques such as fluorescence or confocal microscopy. Other dyes are also available as alternatives to PI or AV–FITC (see, for example, Molecular Probes® catalog), and these can be used if interference occurs with these dyes with specific particles, for example due to fluorescence emission or excitation wavelength overlaps.

The assessment of cellular adenosine triphosphate (ATP) content is a relatively sensitive assessment of cell viability. Kits using luminescence to assess the ATP content of cell extracts are available, and the assay can be conducted in a 96-well plate format. If the ATP content is extracted using perchloric acid, followed by neutralization, it is possible that particles might be removed during the extraction protocol. Use of this assay with particles such as carbon black has not been problematic in our hands, but we have not assessed this assay with a wide array of nanoparticles. One of the

advantages of this assay is that the same extract can be used to measure reduced and oxidized glutathione (see below).

There are many other commercially available assays, such as the Live/Dead® assay used by Sayes et al. (2006). Most of these assays can be confounded by the issues that have been highlighted above, and therefore should be controlled appropriately. These observations are confirmed by Monteiro-Riviere et al. (2009), who found that for carbonaceous nanomaterials, they had the potential to interfere in a wide range of toxicity assays when assessed in human epidermal keratinocytes. It is therefore appropriate to design and conduct a series of control pilot studies before embarking upon a full cytotoxicity assessment. When interpreting data, it is useful to critically assess the data generated and not to take it for granted that the data generated are a true reflection of the actual toxicity. A good understanding of the assay and how it works, as well as a good understanding of how the nanoparticles might behave in the assay system and with respect to the parameters measured, should lead to appropriate experimental design and data interpretation, especially if approached in a logical and systematic manner. We agree with the conclusion of Monteiro-Riviere et al. (2009) in that the best way to minimize interpretation is to use a combination of at least two different cytotoxicity assays, taking into consideration that they measure different endpoints and therefore should not be expected to generate identical results.

Assays of reactive oxygen species production: cell-free

Reactive oxygen species (ROS) and/or free radicals have been shown to be produced by a variety of pathogenic particles (e.g. alpha quartz; Albrecht et al., 2005) and nanoparticles (Stone et al., 1998; Foucaud et al., 2007) Free radicals are molecules containing an unpaired electron that are usually neutral in charge. They are often generated by homolytic cleavage of a covalent bond, or removal of a hydrogen atom leading to the production of a highly electrophilic, reactive species capable of damaging macromolecules such as DNA, proteins, and lipids. ROS are electrophilic molecules (e.g. H_2O_2) or free radicals (e.g. OH·) containing an oxygen atom. Both free radicals and ROS can be made naturally in the body as intermediates in metabolic reactions, as well as a result of physical (radiation) and chemical toxic insults. It is therefore useful to discuss potential assays that can be used to measure ROS production by nanoparticles in a variety of environments. Different ROS differ in their reactivity and toxicity. For example, hydroxyl radicals (OH·) are considered to be more toxic than superoxide anion radicals or hydrogen peroxide (H_2O_2). On the other hand, H_2O_2 is rather stable and may therefore "act at a distance," while the damaging effects of OH· will occur at or very near to its site of generation, due to its extremely high reactivity (Marnett, 2000). It may therefore be useful in the future to develop improved methods to determine between different ROS species produced by particles.

The methods used to measure ROS production vary in their specificity and sensitivity. Some assays appear to measure a variety of ROS species (e.g. plasmid assay, DCFH, luminol-enhanced chemiluminescence), while others may be more defined (e.g $O_2\cdot^-$ detection by the cytochrome C reduction assay or by lucigenin-enhanced chemiluminescence; H_2O_2 detection using horseradish peroxidase) (Faulkner and Fridovitch, 1993; Dikalov et al., 2007). The following section outlines some of the protocols that have been most frequently used with nanoparticles, and is not a comprehensive assessment of the numerous assays and specific detection techniques that could be used for this purpose. It should be noted that assessment of ROS production by particles alone is not sufficient to determine potential toxicity, but instead such assessments should be used in combination with other assessments of molecular or cellular impacts. In fact, a method of assessment of ROS production by particles could even be listed as a "characterization" assay, and not as an *in vitro* test.

ROS production can be measured in a cell-free environment, or in the presence of cells, as will be discussed in more detail in the next section. ROS production has been measured by a number of assays including the fluorescent dye 2,7-dichlorofluorescin (DCFH) originally by Wilson et al. (2002), and further developed by Foucaud et al. (2007) for nanoparticles. In this assay the dye is obtained as a diacetate precursor, which is cleaved by high pH to make the non-fluorescent product DCFH. The presence of ROS converts DCFH to a fluorescent product, 2,7-dichlorofluorescein, which can be measured by fluorimetry. Again, nanoparticles can produce a background fluorescence/interference in the absence of the dye, which needs to be assessed, and if possible deducted from the experimental reading. ROS production tends to change with time, and so it is useful to conduct an assessment of fluorescent change over time and to choose a time point at which the reaction has not gone to completion, for comparison between particles.

Electroparamagnetic resonance (EPR) is also a technique that has been widely used to assess nanoparticles and particle-induced ROS generation (Figure 1). The use of specific spin traps or probes in combination with specific reagents can allow for the quantification as well as specific identification of the free radical species generated, whereas this level of specificity is not possible with the DCFH assay. Examples of EPR methods used in conjunction with nanoparticles and particles are measurement of the H_2O_2-dependent formation of hydroxyl radicals with the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) (e.g. Schins et al., 2002), or the formation of superoxide anion using the spin probe 1-hydroxy-4-phosphonooxy-2,2,6,6-tetramethylpiperidine (PP-H) (Papageorgiou et al., 2007). In some cases, EPR has also been used to demonstrate that specific nanoparticles can also quench rather than generate ROS in cell-free environments (e.g. Fenoglio et al., 2008). Potential pitfalls of EPR-based measurements of ROS formation by nanoparticles may result from chemical or physical interference with spin-trapping agents, and could be checked by

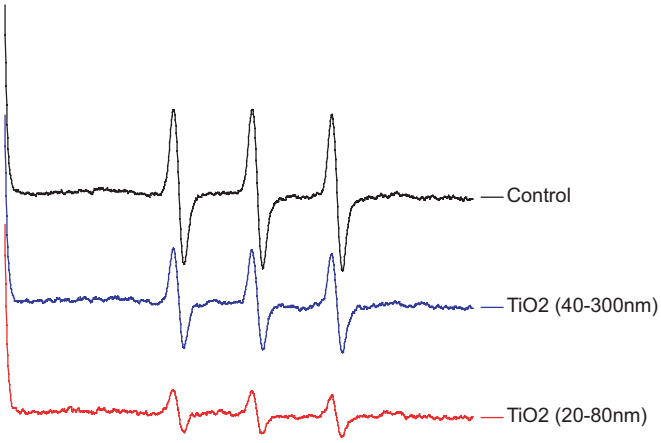

Figure 1. ROS generation from A549 human lung epithelial cells upon 4 h treatment with fine or ultrafine TiO_2, measured by electron paramagnetic resonance. Shown are the spectra of the spin-probe TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), a stable radical which is progressively blunted upon contact with radicals.

the analysis of specific ROS donor systems (e.g. xanthine/xanthine oxidase, H_2O_2/Fe) spiked with nanoparticles.

The plasmid assay has been used in a few studies to assess ROS production (Gilmour et al., 1997; Stone et al., 1998; Dick et al., 2003). In this assay, unwinding and linearization of a coiled bacterial DNA plasmid is used to estimate free radical and/or ROS exposure. This technique is not particularly sensitive, and may be subject to DNA binding to the nanoparticle surface. However, this assay has been used to demonstrate that for a panel of metal oxide nanoparticles, those that were able to induce inflammation in the rat lung were also able to generate ROS production (Dick et al., 2003). Another similar approach to measure ROS formation by (nano)particles involves measurement of the oxidation of naked DNA probes. Exemplary, specific particles have been shown in cell-free systems to induce the hydroxyl radical-specific DNA lesion 8-hydroxydeoxyguanosine (8-OHdG) (Prahalad et al., 2001). Some researchers have interpreted treatment of naked DNA with (nano)particles as a measure of their genotoxicity, but this is ambiguous, since these protocols involve simply exposing DNA directly to these materials. Such an approach does not reflect the true potential for particles to generate genotoxicity, which is defined as a measure of the potential of a chemical to cause damage to a cell's DNA. Although naked DNA experiments may identify whether a nanoparticle possesses intrinsic DNA damaging properties, they do not take into account the importance of the cellular functions and their microenvironment, e.g. nanoparticle uptake and nuclear penetration, antioxidant effects, DNA repair processes, etc.

The assays described for measuring ROS production in a cell-free environment also have the potential to measure interactions between nanoparticles and other substances. For example, Wilson et al. (2002) demonstrated that ROS production by carbon-black nanoparticles, according to the DCFH assay, was potentiated in the presence of metal salts such as $FeCl_3$, $FeSO_4$, and $CuSO_4$, suggesting that nanoparticles and metal ions interact to enhance ROS production. This was reflected *in vivo* by potentiation of the particle-induced inflammation in the rat lung.

It is important to note that the measurement of ROS production by particles in a cell-free environment is not a measure of oxidative stress. Oxidative stress can only occur in biological cells/organisms and is defined as the result of an imbalance between prooxidants (e.g. ROS) and antioxidant defense mechanisms of the body.

Assays of reactive oxygen species production: cellular ROS

For the evaluation of ROS production in the presence of cells, various methods are available, which may considerably differ in their sensitivity and specificity as well as their ability to detect intra- and/or extracellular species (Bartosz, 2006; Dikalov et al., 2007). Again, the fluorescent assay using DCFH can be used to measure ROS by fluorimetric or by flow-cytometric techniques. In this case the dye is delivered to the cells with the diacetate group intact, as this chemical moiety renders the molecule relatively more lipophilic, allowing it to gain access to cells. As described above, background caused by the particles in the absence of the dye needs to be controlled for and subtracted. In fact, there are a range of commercially available fluorescent probes available that measure ROS production in different cellular compartments such as the mitochondria (e.g. dihydrorhodamine). There have not been many publications using these reagents and nanoparticles, and such assays at this time might not be suitable for use in test guidelines.

As well as measuring intracellular ROS, it is also possible to measure extracellular ROS production by cells, for example, the phagocytic burst by neutrophils and macrophages. The cytochrome C assay measures superoxide anion production by cells. This assay measures the reduced and oxidized form of cytochrome C each at a specific wavelength in order to ascertain the extent of oxidation. Another approach involves the quantification of extracellular H_2O_2 by spectrophotometric determination of horseradish peroxidase-catalyzed oxidation of a specific probe (Dikalov et al., 2007). There are also luminescence assays that measure the phagocytic burst using chemical enhancers such as lucigenin or luminol (Faulkner and Fridovitch, 1993; Myhre et al., 2003). Such assays are especially relevant for fiber-like (high aspect ratio) nanoparticles (HARN) that have the potential to induce frustrated phagocytosis. Frustrated phagocytosis has been reported to occur for pathogenic fibers and is related to fiber length (Davis et al., 1986). Fibers greater than 10–20 μm in length are longer than a macrophage can engulf. During phagocytosis, the macrophage makes ROS such as superoxide anions in order to "kill" the particle ingested. During frustrated phagocytosis the phagosome is unable to close, and therefore remains open to the surrounding environment, allowing continual release of damaging ROS (Hill et al., 1995). The cytochrome C assay has been successfully used to measure ROS production by monocyte-derived

macrophages exposed to a range of nanotubes (Brown et al., 2007b). This study clearly demonstrated that longer nanotubes were more likely to result in frustrated phagocytosis and superoxide anion production than entangled nanotubes.

Cellular ROS generation can also be measured with EPR by employing low-toxicity spin traps or spin probes such as TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) to cell cultures. As such, it could be demonstrated that lung epithelial cells generate ROS, when treated with high concentrations of ultrafine, but not fine TiO_2 particles (Singh et al., 2007). Similarly, EPR has been used to measure the phagocytic burst from macrophages and neutrophils (e.g. Haberzettl et al., 2008).

Oxidative stress assays

Glutathione is an antioxidant that is found in cells and biological fluids throughout the body. In its reduced form (GSH), glutathione acts as an antioxidant by reacting directly with ROS to neutralize them. In doing so, oxidized glutathione (GSSG) is made by the combination of two GSH molecules. The body is able to rapidly convert GSSG back to GSH using NADPH (reduced nicotinamide adenine dinucleotide phosphate) as a reducing source. However, during exposure to large amounts of ROS, starvation, or ill health, NADPH can become depleted, leading to an accumulation of GSSG and a depletion of GSH. Therefore, changes in the GSH:GSSG ratio can be used as an indicator of oxidative stress. However, in reality, GSSG concentrations are often very low, especially *in vitro*, making GSSG difficult to detect. This is confounded by the fact that cells will often actively export GSSG as a protective mechanism, decreasing further the ability to measure GSSG in cells. This can be improved by measurement of GSSG in the cells and culture medium, but still the assays available can struggle to detect the relatively low concentrations available. It is therefore often more common to measure GSH nmol/mg protein, or GSH nmol/10^6 cells. There are a number of assays available to measure GSH, such as the *o*-phthalaldehyde (OPT) method, which uses the same cellular extract required for the ATP assay, generates a fluorescent signal, and in the limited number of studies conducted does not appear to be affected by nanoparticles (e.g. Stone et al., 1998). An alternative is to reduce the total glutathione of the cell extract using a reducing agent such as β-mercaptoethanol, therefore allowing measurement of the ratio of GSH to total glutathione.

Other markers of oxidative stress include measurement of lipid peroxidation (e.g thiobarbituric acid reactive substances (TBARS) assay) and the Trolox equivalent antioxidant capacity assay (TEAC). For the TEAC assay we have encountered problems due to particle interference, especially in the presence of organic material (e.g. homogenized tissue), which seems to aid particle dispersion (Rosenkranz et al., manuscript in preparation).

Measurement of mRNA expression changes of oxidative stress-dependent genes has also been put forward as a sensitive marker of oxidative stress induced by particles and nanoparticles; among these, the best-described is heme oxygenase-1 (HO-1) (Xiao et al., 2003; Li et al., 2008). HO-1 is known to have antioxidative and antiinflammatory properties, and its enhanced protein expression in the lung in response to oxidative stress is widely regarded as a protective mechanism against oxidative tissue injury.

Particle dispersion

As described in many publications, nanoparticles have the tendency to both aggregate and agglomerate (Oberdoerster et al., 2007). An agglomerate is a "collection of loosely bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components," while an aggregate is defined as a "particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of the calculated surface areas of the individual components" (British Standards Institution, 2007). This means that agglomerates might be easily separated by dispersants or a small amount of energy (e.g. vortex or short sonication), while further dispersion of aggregates is unlikely. A number of studies have now demonstrated that small concentrations of protein (usually albumin below 1% final concentration) improve particle dispersion and the stability of that dispersion over time (e.g. Foucaud et al., 2007; Porter et al., 2008), especially if incorporated in the medium prior to particle addition, and if combined with a short sonication (e.g. 10 min). In addition, some studies, especially those relating to respiratory exposure, have used the lung lining fluid component phospholipid dipalmatyl phosphatidyl choline (DPPC) as a surfactant to aid dispersion. In the study by Foucaud et al. (2007), DPPC (0.025%) did not dramatically improve dispersion according to light microscopy images, but like bovine serum albumin (BSA; 1%), it enhanced ROS production by carbon-black nanoparticles (measured by DCFH). Mixing both dispersants together induced an additive increase in ROS production, suggesting that increasing particle dispersion enhances surface reactivity in terms of ROS production. Porter et al. (2008) also demonstrated that a combination of BSA (0.6 mg/mL) and DPPC (0.01 mg/mL) could be instilled into the rat lung without inducing any significant increase in lung background inflammation. They further showed that such a dispersant did not prevent silica (alpha quartz)-induced inflammation, suggesting that although the particles were coated with protein/DPPC, this did not prevent the surface reactivity-induced lung response. If anything, the improved dispersion again helped to increase the particle reactivity. It remains to be seen whether the particles react directly with the proteins or lipids to induce the production of cytotoxic or bioactive components such as lipid peroxides.

Of course, adding protein or other dispersants to the nanoparticles could influence their surface properties and therefore their interaction with cells and other biological molecules. Our own research has identified that polystyrene

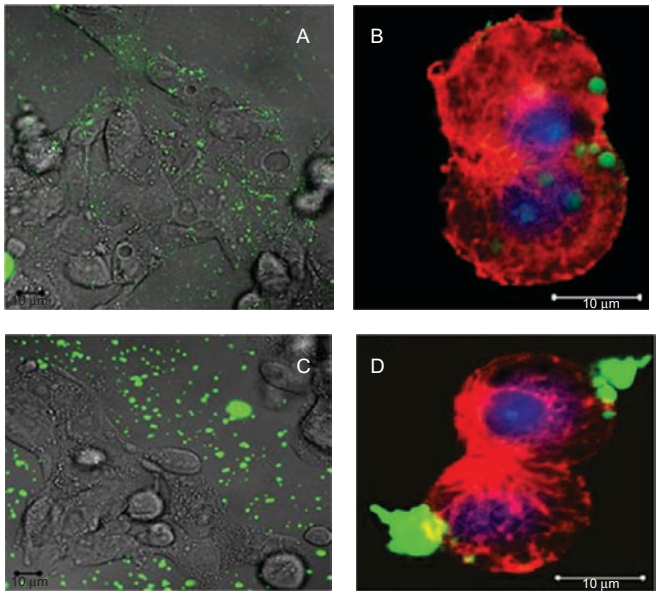

Figure 2. Human hepatocyte cell line C3A (A and C) and rat primary hepatocyte couplets (B and D) treated with fluorescent polystyrene beads (green) of 20 μm (A and B) or 200 μm (C and D) diameter for 1 h *in vitro*. Images A and C are viewed by phase contrast bright field microscopy overlaid with a fluorescent image. Images B and D are viewed by confocal microscopy where red represents the F-actin cytoskeleton and blue the nuclear region. Black bars (A and C) and white bars (B and D) all represent 10 μm.

nanoparticles dispersed in heat-inactivated fetal calf serum (10%) are rapidly taken up into the C3A hepatocyte cell line and into primary rat hepatocytes, but in contrast, uptake does not occur when the particles are dispersed in serum-free medium (Figure 2). This suggests that either the improved dispersion aids uptake of the smaller agglomerates and/or individual nanoparticles, or alternatively that the proteins adsorbed to the particle surface interact with cell surface receptors that facilitate uptake into the cells. Obviously, increased uptake into cells of this type might be expected to be associated with increased risk of toxicity.

While there is no doubt that improving particle dispersion aids our understanding of dose and particle behavior within an *in vitro* system, it is important to ensure that dispersion is reflective of the *in vivo* situation. For example, in the lung, inhaled nanoparticles deposit into lung lining fluid and are immediately immersed, coming rapidly into contact with the epithelial cells below (Gehr et al., 2006). They do not remain as a stable suspension that does not change with time. In the blood and other biological fluids, nanoparticles will interact with different blood components that will also change the way that they behave over time. Therefore, our desire to generate a stable suspension must not override the need to generate a representative exposure scenario.

Inflammation

It is not possible to measure "inflammation" *per se in vitro*, as this involves a complex interaction of multiple cell types. However, it is possible to measure markers of proinflammatory signaling and gene expression that, if they occurred *in vivo*, would be anticipated to drive inflammation. One of the most common techniques for measuring proinflammatory signaling is to measure cytokine and/or chemokine protein production by cells. Examples of cytokines and chemokines associated with inflammation include tumor necrosis factor alpha (TNFα), interleukin (IL)8, IL1α, IL1β, IL6, and granulocyte macrophage colony-stimulating factor (GM-CSF). Each cytokine plays a specific role in promoting or controlling inflammation. For example, both TNFα and IL1 are potent proinflammatory cytokines that are made predominantly by macrophages, monocytes, and dendritic cells. They act as proinflammatory molecules activating other inflammatory cells, including macrophages, resulting in the further production of other proinflammatory mediators. TNFα has been demonstrated to be up-regulated by a number of nanoparticles such as carbon (Brown et al., 2004). IL8 (CXCL8) is an example of a chemokine which is also produced by macrophages as well as other cell types. With respect to pulmonary toxicology this chemokine has been measured in response to epithelial treatment with particles (e.g. Schins et al., 2000; Singh et al., 2007; Donaldson et al., 2008) due to its role in attracting and therefore recruiting other inflammatory cells to the site of inflammation.

Cytokine protein production results in release of the protein into the cell culture medium. The medium can therefore be harvested, and centrifuged to remove cellular debris and particles, and then the cytokine protein content assessed by enzyme linked immunosorbent assay (ELISA). ELISA techniques are well-established, reliable, and usually relatively sensitive. However, due to the propensity for proteins to bind to the particle surface, there is a tendency for cytokine proteins to adsorb to the particle surface and therefore cytokine protein production to be underestimated. Cytokine mRNA expression can be measured as an indicator of alterations at the gene expression level, but mRNA content is not always reflective of the protein production, due to posttranscriptional modification, changes in protein stability, mRNA stability, etc. (e.g. Brown et al., 2004). Some of these posttranscriptional mechanisms are more important for some cytokines than for others, e.g. tumor growth factor beta (TGFβ), since a large proportion of this protein is latent (Yoshinaga et al., 2008).

It is worth noting that actions that decrease the availability of the particle surface are likely to reduce the interference in cytokine protein determination by preventing protein binding. For example, in our experience, cytokine adsorption to nanoparticles is less of a problem in macrophage studies than for epithelial cell studies. This is because the macrophages rapidly ingest the nanoparticles (Clift et al., 2008), preventing continued exposure of the particle surface, whereas for epithelial cells the proportion of particles removed from the medium is much smaller.

Cell types

As mentioned previously there are too many different cell types to discuss in full in this review. The cell type chosen will depend upon the route of exposure considered, and the potential target organs of concern. However, as stated above, the type of cell chosen can influence the results greatly by increasing or reducing the chance of generating a measurable and reliable signal. Macrophages appear to be relatively sensitive to nanoparticles in terms of ROS production, oxidative stress, cell signaling (e.g. calcium and NF-κB), and cytokine production (Stone et al., 2000; Brown et al., 2004). They can also distinguish between pathogenic fibers, suggesting that they might be useful for comparing high aspect ratio nanoparticles such as carbon nanotubes (Brown et al., 2007b). Macrophages are found throughout the body, and play a key role in the body's defense to particles, as well as in inflammatory-related health effects. However, they will not provide a full indication of the potential effects of nanoparticles, and therefore it would be advisable to substitute such studies with an organ-specific cell type. Macrophages are very sensitive to bacterial endotoxin; it is therefore also important to either assess the endotoxin content (see below) of particles before exposure to macrophages, or to generate them via a system that is likely to be sterile and endotoxin-free. Apart from these distinctions among cell types, it is also important to consider differences in primary cells versus cell lines. This is especially important when addressing the role of oxidative stress and ROS-dependent signaling, as both immortalization and differentiation are well known to affect the sensitivity of cells. When using primary cells, effects of "cell isolation stress" should also be taken into account.

It is also worth noting that the culture status or conditions of the cells might greatly influence their response to particles. For example, cells that are contact-inhibited will stop proliferating when confluence is achieved. The cell metabolism will subsequently be greatly altered and therefore susceptibility to toxicants might be altered. It might therefore be worth considering limiting exposures to either the logarithmic phase of growth or confluence. At this time we are not aware of any evidence to suggest which protocol might be most appropriate, and so perhaps this is a research question that needs to be addressed.

Endotoxin determination

Assessing particles for endotoxin (lipopolysaccharide) can be achieved through the use of a number of commercially available assays, but at the time of publication there is no clear information to ascertain which tests might be best suited for nanoparticles. One widely used assay includes the *Limulus* amebocyte lysate (LAL) chromogenic endpoint assay. The assay is based upon the observation that amebocytes (blood cells) from the American horseshoe crab, *Limulus polyphemus*, clot in response to endotoxin (see e.g. cellsciences.com). Treatment of the *Limulus* amebocyte lysate with endotoxin stimulates an enzymatic reaction that results in a change in opacity and gelation of the formulation, which can be measured spectrophotometrically. Of course, any particle could generate a background signal that might overlap with that measured in the assay, and so background absorbance should be determined. In a recent review by Jones and Grainger (2009), the authors point out that such assays may not be able to measure endotoxin bound to a particle surface, but instead may be limited to free endotoxin in solution.

Controls within an experiment

There is a relatively long list of controls that should be considered for an *in vitro* nanoparticle study; these controls are outlined in Table 1.

Dose

Dose in particle studies is often expressed in mass per unit volume (e.g. μg/mL). While many attempts have been made to generate stable suspensions, it is often the case that the particles will settle due to agglomeration and gravity over time in culture. It is probably therefore more appropriate to also express dose in terms of mass per unit surface area of the culture dish (μg/cm^2). Expression dose in both forms

Table 1. Controls for consideration when investigating nanoparticles using *in vitro* assay systems.

Control	Example	Purpose
A reagent known to induce a positive effect in the chosen assay	Lipopolysaccharide (LPS) induces TNFα protein production by macrophages	To ensure that the cells are able to generate the response measured
A particle know to induce a positive effect in the chosen assay and *in vivo*	Silica (alpha quartz) induces TNFα protein production by macrophages	To ensure that the cells are able to generate the response measured and to provide a benchmark for comparison
A particle known to induce a negative effect in the assay and *in vivo*	250 nm diameter TiO_2 does not stimulate TNFα protein production by macrophages	To ensure that the effects measured are specific and not simply a response to any particle
Any particle which allows a specific hypothesis to be tested (e.g. size)	A larger version of the nanoparticle investigated, identical apart from size	To allow identification of a nanoparticle-specific effect
Vehicle-only treatment	Dispersion medium	To investigate whether the dispersion medium alone has any impact on the endpoint measured
Soluble components	1. Bioavailable iron released from some nanotubes 2. Silver ions released from colloidal silver	To identify whether soluble components contribute to the biological response measured

allows the extrapolation of findings between studies. There is currently no established standard cell number per unit area of culture dish that should be used for each cell type. Cell density can influence cell behavior and obviously impacts on the dose of particles received by each cell. It might therefore be useful to routinely seed cells at the same cell density per unit area of culture dish (number of cells per cm^2), and therefore dose could also be expressed as mass per cell number ($\mu g/10^6$ cells). This would improve the ability to compare dose between studies. The advantage of expressing dose as the mass per volume is that it provides information on the particle density of the suspension. As such, it provides clues for the potential likelihood of artifacts such as particle binding, excessive aggregation, or adsorption or scattering of light.

The physiological relevance of concentrations or doses used *in vitro* is frequently questioned. Donaldson et al. (2008) have recently attempted to relate the *in vitro* dose of particles to an epithelial cell line to the inhalation dose by rats. This study is based upon the observation that particles are more likely to deposit at bifurcations within the respiratory system, leading to focal (relatively high) concentration of particles in the proximal alveolar region (PAR). The surface area of the PAR can therefore be calculated and related to the area of a culture dish *in vitro*, therefore allowing a comparison per unit surface area *in vivo*. Using inhalation data for TiO_2 and $BaSO_4$ particles, Donaldson et al. (2008) calculated that the threshold concentration (surface area units) for initiation of inflammation is in the order of magnitude of 1 cm^2 per cm^2 of the PAR surface. Assessment of IL8 production by A549 epithelial cells was also found to require a threshold dose of 1 cm^2/cm^2 for TiO_2, suggesting that the *in vitro* dose and study protocol are relevant in comparison to animal studies.

A thorough physicochemical characterization of nanoparticles, including parameters such as size, shape, surface area, and composition, is also important, as this can lead to innovative ways to express dose, such as via surface area (Donaldson et al., 2008). Suppliers often provide information on particle size and composition, but due to variations between batches it is essential that this information is verified independently.

Genotoxicity

Genotoxicity assays have been introduced to allow for improved cancer risk assessment strategies. Many tests are nowadays available to screen for gene mutations, chromosomal mutations, and aneugenic effects (aneuploidy), as well as to measure formation of DNA strand breaks, DNA adducts, and the induction of DNA damage repair (Vainio et al., 1992; McGregor et al., 2000). However, particles are known to form a rather specific group of compounds when genotoxicity testing is considered, especially those that are poorly soluble. Because of their physicochemical properties, they show rather specific mechanisms of DNA damage induction. Moreover, they may also introduce artifacts in specific genotoxicity assays. A detailed review on the mechanisms whereby particles can induce genotoxicity is provided elsewhere (Schins and Knaapen, 2007). For a recent overview of genotoxicity studies performed with nanoparticles we refer to Landsiedel et al. (2009).

For (poorly soluble) particles, two principal modes of genotoxic action are discussed, referred to as primary and secondary genotoxicity. Secondary genotoxicity is considered to result from oxidative DNA attack by reactive oxygen/nitrogen species (ROS/RNS) generated during particle-elicited inflammation, whereas primary genotoxicity is defined as genetic damage elicited by particles (Figure 3). Of major importance for risk assessment, secondary genotoxicity is considered to involve a threshold; its value is determined by the exposure concentration that will trigger inflammation and overwhelm antioxidant and DNA damage repair capacities in the lung (Greim et al., 2001). This discrimination is also important for nanoparticles, because of the established association between the surface area and inflammatory potency of inhaled particles (Duffin et al., 2002; Stoeger et al., 2006; Duffin et al., 2007) and the observed increased carcinogenicity of specific nanoparticles when applied in large concentrations in rodents (reviewed in Borm et al. (2004)). To exclude whether nanoparticles are primary-genotoxic, it is therefore crucial to identify valid genotoxicity assays for these specific materials.

Currently, however, only limited data are available with regard to *in vitro* genotoxicity testing strategies for nanoparticles. Among the various genotoxicity assays that are available to screen for potential chemical carcinogens, only a few have been used to considerable extent with nanoparticles, i.e. the salmonella reverse mutation assay, the micronucleus test, and the alkaline comet assay (reviewed in Landsiedel et al. (2009)). Moreover, many of these studies

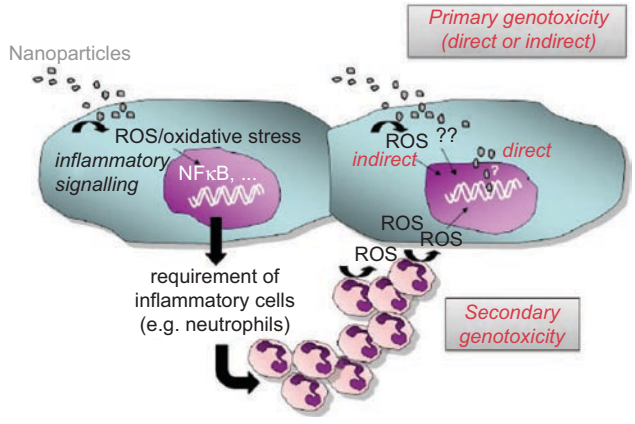

Figure 3. Schematic diagram of the mechanisms of "primary" and "secondary" genotoxicity in nanoparticle exposed cells. *In vitro* genotoxicity testing allows for the identification of primary genotoxicity of nanoparticles, which may result from either direct (e.g. physical interaction between nanoparticles and genomic DNA) or indirect pathways (e.g. formation of ROS by nanoparticle-activated target cells). Secondary genotoxicity implies a pathway of genetic damage resulting from oxidative DNA attack by ROS, generated from activated phagocytes (neutrophils, macrophages) during particle-elicited inflammation.

have employed only limited genotoxicity measurements in relation to mechanistic investigations, rather than obeying recommended guideline criteria. Finally, it should be considered that testing of nanoparticles may result in false positive or negative findings because of assay interference through similar mechanisms as discussed for cytotoxicity.

The salmonella reverse mutation assay, also known as the Ames test, represents the most widely applied *in vitro* assay subject to well-defined international recommendations (i.e. Organisation for Economic Co-operation and Development (OECD) Guideline 471), and has been used for genotoxicity testing of various nanoparticles, such as TiO_2, fullerenes, or carbon nanotubes (Mori et al., 2006; Kisin et al., 2007; Warheit et al., 2007). However, because of the well-known differences in membrane structure and composition of bacteria, and the discussed importance of endocytosis in nanoparticle toxicity (reviewed in Unfried et al. (2007)), it remains to be investigated to what extent bacterial mutagenicity tests identify the true genotoxic potency of nanoparticles in mammalian cells. Mutagenicity assays in mammalian cells provide relevant additions or alternatives to these bacterial assays (e.g. Driscoll et al., 1997; Jacobsen et al., 2008), but will require further standardization.

For the evaluation of clastogenic (chromosome breaking) effects of nanoparticles in mammalian cells, the micronucleus assay and the alkaline comet assay have been applied. For both assays, international recommendations are available, albeit not yet OECD-approved (Tice et al., 2000; Kirsch-Volders et al., 2003). Examples of investigated materials include titanium dioxide, carbon black, cobalt–chromium alloy nanoparticles, and nanotubes (e.g. Rahman et al., 2002; Gurr et al., 2005; Kisin et al., 2007; Papageorgiou et al., 2007; Mroz et al., 2008; Muller et al., 2008). Earlier, both the micronucleus assay and the alkaline comet assay have proved to be adequate for the *in vitro* genotoxicity testing of fine particles as well as fibers (Greim et al., 2001; Speit, 2002; Schins and Knaapen, 2007).

The micronucleus assay is based on the microscopic detection of a chromosome or chromosome fragment from a cell which has failed to integrate into the nucleus of its daughter cell after division. In the so-called cytokinesis block micronucleus assay, the actin-inhibitor cytochalasin B is applied to the cell culture after treatment with the test compound. This approach allows for the quantification of background micronuclei levels as well as cell proliferation, by distinguishing mononuclear from binucleated cells, i.e. cells that did not, or respectively, did undergo division during the cell culture. Using fluorescent *in situ* hybridization (FISH) with probes targeted to the centromere region, one can determine whether a specific micronucleus represents an acentric chromosome fragment (i.e. resulting from a clastogenic event), or whether it holds an entire chromosome (i.e. aneugenic effect). The comet assay, also known as single cell gel electrophoresis, is based on the microscopic dectection of damaged DNA fragements of individual cells, appearing as "comets" upon cell lysis and subsequent DNA denaturation and electrophoresis. The most common method used is the alkaline version, which allows for the detection of single and double DNA strand breaks, DNA cross-links, and alkali-labile sites. Various modifications of the comet assay have been developed, e.g. for the specific quantification of DNA double strand lesions (neutral comet assay), oxidative DNA adducts such as 8-OHdG (by the detection of formamidopyrimidine DNA glycosylase-sensitive sites), or damage repair effects.

Advantages and limitations of the micronucleus and comet assay have been described in detail elsewhere (Fenech, 1997; Tice et al., 2000; Kirsch-Volders et al., 2003). The major strength of the micronucleus assay is that it can detect both chromosomal and genomic mutations. Its main limitation with regard to *in vitro* genotoxicity testing is that it can only be applied to dividing cells, in contrast to the comet assay. The principal disadvantage of the comet assay, on the other hand, is that it does not measure fixed mutations, unlike the micronucleus assay. Apart from the general advantages and limitation for both tests, a number of aspects are to be considered when working specifically with (nano)particles. In general, when applying nanoparticles to genotoxicity testing, several principal criteria should be obeyed. Most important, as addressed in the various guidelines and recommendations, relevant (positive) controls should be included, and testing should be performed in the appropriate dose–response range and in relation to cytotoxicity evaluation. Alongside the recommended assay-specific non-particulate positive controls, respirable crystalline silica can serve as a relevant particle control (Schins and Knaapen, 2007). Of course, the aforementioned aspects of nanoparticle dispersion, sonication, and cell-type selection are also important for genotoxicity testing. Since genotoxicity guidelines recommend specific exclusion of too-strong cytotoxicity in their assays, it is crucial to select the most appropriate cytotoxicity assay for comparison.

Lastly, as described earlier for cytotoxicity measurements, also for genotoxicity tests it is crucial to identify, and ideally to exclude, potential artifacts. For instance, when using the comet assay with automated software one should be aware that particles or aggregates can localize at or near comet appearances, and affect their quantification due to their fluorescence or ability to quench DNA-staining agents such as ethidium bromide. It is also important to consider that during the final processing steps of the comet assay, nanoparticles may come into direct contact with the nuclear DNA and thereby have the potential to induce artificial damage. We have observed both such effects when working with high concentrations of TiO_2 particles (unpublished observations). It also remains to be tested whether artifacts may occur in other genotoxicity assays, e.g. the micronucleus assay, for which automated imaging analysis protocols are also under continuing development. Exemplary, when using the cytokinesis block micronucleus assay, one should also consider potential interactions of the tested nanoparticles with cytochalasin B, as recently discussed by Landsiedel et al. (2009). Investigations should be devoted to the critical validation and further development of testing

protocols for the specific purpose of genotoxicity screening of nanoparticles.

Conclusions and suggested strategies for *in vitro* toxicity testing

In conclusion, there is an urgent need to agree upon and establish a range of *in vitro* tests that allow nanoparticles to be assessed for their potential hazard. There are in fact a number of international efforts to discuss and determine which protocols might be standardized. For example, this review was written in response to a request for information from the OECD in relation to their consideration of alternative methods. Due to our current lack of understanding of nanoparticle-induced disease, it is unlikely that these assays, at this time, will be fully predictive of toxicity, but they can be used as a first screen to prioritize those particles that should be tested in more detail for toxicity. Again, due to our current lack of understanding of nanoparticle-induced disease, the easiest assays to develop first are those that we know relate to *in vivo* effects in animal models, and therefore those that we best understand. Coupled with modified existing standard protocols, this provides a relatively powerful battery of tests that can be used for nanoparticle toxicity testing. Probably the most important aspect of such studies is an understanding of the way in which each assay works, an understanding of the potential ways in which nanoparticles might interfere in the assay, and therefore an intelligent, systematic approach to study design and data interpretation.

Our suggestion at this time would be to develop a tiered strategy of tests that start with an assessment of viability in order to determine values for regulatory toxicity (e.g. LC_{50} or no observable effect level (NOEL)), but also to determine sublethal concentrations for the assessment of more relevant mechanistic endpoints. Such concentrations could then be used in subsequent experiments, but the nature of these tests might depend upon the particle type. For most particles it appears to be relevant to assess ROS production in a cell-free and cellular environment, due to the role of particle-derived ROS in inducing both lethal and sublethal affects associated with toxic responses. Cellular ROS could be backed up by a measure of oxidative stress, such as glutathione depletion, in order to assess whether the particle-derived ROS are able to result in a cellular effect. Again, the measurement of oxidative stress is relevant due to its role in controlling responses such as proinflammatory gene expression.

There are a number of cell types that could of course be used for this test. Macrophages are a useful cell type to consider due to their relevance in all tissue types, their role in clearing particles from the body, and their role in inflammation and disease, as well as their relative sensitivity to particles. The choice of macrophages as a target cell type is therefore independent of the route of entry of the particles into the body. If macrophages are used in the initial phase of the investigations, subsequent phases could employ alternative cell types that are determined by the route of entry into the body of the particles, as well as the expected target sites of exposure/accumulation. Macrophage responses could then be compared with those of these organ-specific cell types. With macrophages it might also be useful to assess cell-generated ROS in addition to particle-derived ROS, especially in relation to fiber-like nanoparticles that might elicit "frustrated phagocytosis." Once ROS or oxidative stress has been determined, the assessment of proinflammatory gene expression is very useful due to the role of inflammation in the initiation and progression of disease. Proinflammatory molecules measured can be determined by the cell type under investigation. For example, TNFα is particularly relevant for macrophages, while IL8 or IL6 might be more relevant for epithelial cells. If possible, it would be more appropriate to measure a panel of cytokines rather than targeting an individual protein, as they can be differentially up-regulated.

Macrophages are less appropriate when evaluating genotoxicity of nanoparticles, as they may not be representative of typical target cells for carcinogenesis (e.g. lung epithelial cells). DNA-damaging potencies of nanoparticles may be considerably different in professional phagocytes versus other cell types, as a result of contrasting mechanisms of particle uptake and ROS-generating capacities. Most important, an initial genotoxicity screening of nanoparticles should not be limited to a single assay, in concordance with general genotoxicity testing guidelines, and to avoid false negative or positive outcomes. Appropriate candidates for such first-stage screening are the comet assay and micronucleus assay. Both assays are relatively easy to perform, allow for the detection of global DNA damage and mutations on the chromosomal and genomic level, and are also applicable in an eventual subsequent *in vivo* testing. Subsequent investigations could involve *in vitro* mammalian mutagenicity tests and eventual *in vivo* genotoxicity testing. In every study a range of particle concentrations and time points would be advantageous, as well as the inclusion of a range of controls, as outlined previously for different techniques. A strategy such as this could provide a first-stage attempt at screening particles, allowing their benchmarking, before choosing specific particles for further testing, perhaps *in vivo*.

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References

Albrecht C, Knaapen AM, Becker A, Hohr D, Haberzettl P, van Schooten FJ, Borm PJ, Schins RP (2005). The crucial role of particle surface reactivity in respirable quartz-induced reactive oxygen/nitrogen species formation and APE/Ref-1 induction in rat lung. Respir Res 6:129.

Bartosz G (2006). Use of spectroscopic probes for detection of reactive oxygen species. Clin Chim Acta 368:53–76.

Borm PJ, Schins RP, Albrecht C (2004). Inhaled particles and lung cancer, part B: paradigms and risk assessment. Int J Cancer 110:3–14.

British Standards Institution (2007). Terminology for nanomaterials. PAS 136. BSI, London.

Brown DM, Donaldson K, Borm PJ, Schins RP, Denhart M, Gilmour P, Jimenez LA, Stone V (2004). Calcium and reactive oxygen species-mediated activation of transcription factors and TNFα cytokine gene expression in macrophages exposed to ultrafine particles. Am J Physiol Lung Cell Mol Physiol 286:L344–L353.

Brown DM, Hutchison L, Donaldson K, MacKenzie SJ, Dick CA, Stone V (2007a). The effect of oxidative stress on macrophages and lung epithelial cells: the role of phosphodiesterases 1 and 4. Toxicol Lett 168:1–6.

Brown DM, Kinloch IA, Bangert U, Windle AH, Walter DM, Walker GS, Scotchford CA, Donaldson K, Stone V (2007b). An in vitro study of the potential of carbon nanotubes and nanofibres to induce inflammation mediators and frustrated phagocytosis. Carbon 45:1743–1756.

Clift MJ, Rothen-Rutishauser B, Brown DM, Duffin R, Donaldson K, Proudfoot L, Guy K, Stone V (2008). The impact of different nanoparticle surface chemistry and size on uptake and toxicity in a murine macrophage cell line. Toxicol Appl Pharmacol 232:418–427.

da Costa AO, de Assis MC, Marques EA, Plotkowski MC (1999). Comparative analysis of three methods to assess viability of mammalian cells in culture. Biocell 23:65–72.

Davis JM, Addison J, Bolton RE, Donaldson K, Jones AD, Smith T (1986). The pathogenicity of long versus short fibre samples of amosite asbestos administered to rats by inhalation and intraperitoneal injection. Br J Exp Pathol 67:415–430.

Dick CA, Brown DM, Donaldson K, Stone V (2003). The role of free radicals in the toxic and inflammatory effects of four different ultrafine particle types. Inhal Toxicol 15:39–52.

Dikalov S, Griendling LK, Harrison DG (2007). Measurement of reactive oxygen species in cardiovascular studies. Hypertension 49:717–727.

Donaldson K, Tran CL, Jimenez LA, Duffin R, Newby D, Mills N, MacNee W, Stone V (2005). Combustion-derived nanoparticles: a critical review of their toxicology following inhalation exposure. Part Fibre Toxicol 2:1–14.

Donaldson K, Borm PJ, Oberdorster G, Pinkerton KE, Stone V, Tran CL (2008). Concordance between in vitro and in vivo dosimetry in the proinflammatory effects of low-toxicity, low-solubility particles: the key role of the proximal alveolar region. Inhal Toxicol 20:53–62.

Driscoll KE, Deyo LC, Carter JM, Howard BW, Hassenbein DG, Bertram TA (1997). Effects of particle exposure and particle-elicited inflammatory cells on mutation in rat alveolar epithelial cells. Carcinogenesis 18:423–430.

Duffin R, Tran CL, Clouter A, Brown DM, MacNee W, Stone V, Donaldson K (2002). The importance of surface area and specific reactivity in the acute pulmonary inflammatory response to particles. Ann Occup Hyg 46(Suppl 1):242–245.

Duffin R, Tran L, Brown D, Stone V, Donaldson K (2007). Proinflammogenic effects of low-toxicity and metal nanoparticles in vivo and in vitro: highlighting the role of particle surface area and surface reactivity. Inhal Toxicol 19:849–856.

Faulkner K, Fridovich I (1993). Luminol and lucigenin as detectors for O2.-. Free Radic Biol Med 15:447–451.

Fenech M (1997). The advantages and disadvantages of the cytokinesis-block micronucleus method. Mutat Res 392:11–18.

Fenoglio I, Greco G, Tomatis M, Muller J, Raymundo-Pinero E, Beguin F, Fonseca A, Nagy JB, Lison D, Fubini B (2008). Structural defects play a major role in the acute lung toxicity of multiwall carbon nanotubes: physicochemical aspects. Chem Res Toxicol 21:1690–1697.

Ferin J, Oberdoster G, Penney DP, Soderholm SC, Gelein R, Piper HC (1990). Increased pulmonary toxicity of ultrafine particles? I. Particle clearance, translocation, morphology. J Aerosol Sci 21:381–384.

Foucaud L, Wilson MR, Brown DM, Stone V (2007). Measurement of reactive species production by nanoparticles prepared in biologically relevant media. Toxicol Lett 174:1–9.

Gehr P, Blank F, Rothen-Rutishauser BM (2006). Fate of inhaled particles after interaction with the lung surface. Paediatr Respir Rev 7 (Suppl 1):S73–S75.

Geiser M, Kapp N, Schurch S, Kreyling W, Schulz H, Semmler M, Im Hof V, Heyder J, Gehr P (2005). Ultrafine particles cross celullar membranes by non-phagocytic mechanisms in lungs and in cultured cells. Environ Health Perspect 113:1555–1560.

Gilmour PS, Brown DM, Beswick PH, MacNee W, Rahman I, Donaldson K (1997). Free radical activity of industrial fibers: role of iron in oxidative stress and activation of transcription factors. Environ Health Perspect 105(Suppl 5):1313–1317.

Greim H, Borm P, Schins R, Donaldson K, Driscoll K, Hartwig A, Kuempel E, Oberdorster G, Speit G (2001). Toxicity of fibers and particles. Report of the workshop held in Munich, Germany, 26–27 October 2000. Inhal Toxicol 13:737–754.

Gurr JR, Want AS, Chen CH, Jan KY (2005). Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells. Toxicology 213:66–73.

Haberzettl P, Schins RP, Hohr D, Wilhelmi V, Borm PJ, Albrecht C (2008). Impact of the FcgammaII-receptor on quartz uptake and inflammatory response by alveolar macrophages. Am J Physiol Lung Cell Mol Physiol 294:L1137–L1148.

Hill IM, Beswick PH, Donaldson K (1995). Differential release of superoxide anions by macrophages treated with long and short fibre amosite asbestos is a consequence of differential affinity for opsonin. Occup Environ Med 52:92–96.

Hohr D, Steinfartz Y, Schins RP, Knaapen AM, Martra G, Fubini B, Borm PJ (2002). The surface area rather than the surface coating determines the acute inflammatory response after instillation of fine and ultrafine TiO2 in the rat. Int J Hyg Environ Health 205:239–244.

Jacobsen NR, Pojana G, White P, Moller P, Cohn CA, Korsholm KS, Vogel U, Marcomini A, Loft S, Wallin H (2008). Genotoxicity, cytotoxicity, and reactive oxygen species induced by single-walled carbon nanotubes and C(60) fullerenes in the FE1-Mutatrade mark Mouse lung epithelial cells. Environ Mol Mutagen 49:476–487.

Jepson MA, Clark MA (1998). Studying M cells and their role in infection. Trends Microbiol 6:359–365.

Jones C, Grainger DW (2009). In vitro assessments of nanomaterial toxicity. Adv Drug Deliv Rev 61:438–456.

Karlsson HL, Cronholm P, Gustafsson J, Möller L (2008). Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. Chem Res Toxicol 21:1726–1732.

Kirsch-Volders M, Sofuni T, Aardema M, Albertini S, Eastmond D, Fenech M, Ishidate M Jr, Kirchner S, Lorge E, Morita T, Norppa H, Surralles J, Vanhauwaert A, Wakata A (2003). Report from the in vitro micronucleus assay working group. Mutat Res 540:153–163.

Kisin ER, Murray AR, Keane MJ, Shi XC, Schwegler-Berry D, Gorelik O, Arepalli S, Castranova V, Wallace WE, Kagan VE, Shvedova A (2007). Single-walled carbon nanotubes: geno- and cytotoxic effecs in lung fibroblast V79 cells. J Toxicol Environ Health 70:2071–2079.

Landsiedel R, Kapp MD, Schulz M, Wiench K, Oesch F (2009). Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations—many questions, some answers. Mutat Res 681:241–258.

Li H, van Berlo D, Shi T, Speit G, Knaapen AM, Borm PJ, Albrecht C, Schins RP (2008). Curcumin protects against cytotoxic and inflammatory effects of quartz particles but causes oxidative DNA damage in a rat lung epithelial cell line. Toxicol Appl Pharmacol 227:115–124.

Li XY, Brown D, Smith S, MacNee W, Donaldson K (1999). Short-term inflammatory responses following intratracheal instillation of fine and ultrafine carbon black in rats. Inhal Toxicol 11:709–731.

Marnett LJ (2000). Oxyradicals and DNA damage. Carcinogenesis 21:316–370.

Maynard AD, Aitken RJ, Butz T, Colvin V, Donaldson K, Oberdorster G, Philbert MA, Ryan J, Seaton A, Stone V, Tinkle SS, Tran L, Walker NJ, Warheit DB (2006). Safe handling of nanotechnology. Nature 444:267–269.

McGregor DB, Baan RA, Partensky C, Rice JM, Wilbourn JD (2000). Evaluation of the carcinogenic risks to humans associated with surgical implants and other foreign bodies – a report of an IARC Monographs Programme Meeting. International Agency for Research on Cancer. Eur J Cancer 36:307–313.

Mills NL, Tornqvist H, Gonzalez MC, Vink E, Robinson SD, Soderberg S, Boon NA, Donaldson K, Sandstrom T, Blomberg A, Newby DE (2007). Ischemic and thrombotic effects of dilute diesel-exhaust inhalation in men with coronary heart disease. N Engl J Med 357:1075–1082.

Monteiro-Riviere NA, Tran CL (2007). Nanotoxicology. Characterization, Dosing and Health Effects. Informa Healthcare, London.

Monteiro-Riviere NA, Inman AO, Zhang LW (2009). Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. Toxicol Appl Pharmacol 234:222–235.

Mori T, Takada H, Ito S, Matsubayashi K, Miwa N, Sawaguchi T (2006). Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis. Toxicology 225:48–54.

Mossman T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63.

Mroz RM, Schins RP, Li H, Jimenez LA, Drost EM, Holownia A, MacNee W, Donaldson K (2008). Nanoparticle-driven DNA damage mimics irradiation-related carcinogenesis pathways. Eur Respir J 31:241–251.

Muller J, Decordier I, Hoet PH, Lombaert N, Thomassen L, Huaux F, Lison D, Kirsch-Volders M (2008). Clastogenic and aneugenic effects of multi-wall carbon nanotubes in epithelial cells. Carcinogenesis 29:427–433.

Myhre O, Andersen JM, Aarnes H, Fonnum F (2003). Evaluation of the probes 2',7'-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation. Biochem Pharmacol 65:1575–1582.

Netzlaff F, Lehr CM, Wertz PW, Schaefer UF (2005). The human epidermis models EpiSkin, SkinEthic and EpiDerm: an evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport. Eur J Pharm Biopharm 60:167–178.

Oberdoerster G, Ferin J, Finkelstein J, Wade P, Corson N (1990). Increased pulmonary toxicity of ultrafine particles? II. Lung lavage studies. J Aerosol Sci 21:384–387.

Oberdoerster G, Stone V, Donaldson K (2007). Toxicology of nanoparticles: a historical perspective. Nanotoxicology 1:2–25.

Papageorgiou I, Brown C, Schins R, Singh S, Newson R, Davis S, Fisher J, Ingham E, Case CP (2007). The effect of nano- and micron-sized particles of cobalt-chromium alloy on human fibroblasts in vitro. Biomaterials 28:2946–2958.

Peters A, Wichmann HE, Tuch T, Heinrich J, Heyder J (1997). Respiratory effects are associated with the number of ultrafine particles. Am J Respir Crit Care Med 155:1376–1383.

Porter D, Sriram K, Wolfarth M, Jefferson A, Schwegler-Berry D, Andrew ME, Castranova V (2008). A biocompatible medium for nanoparticle dispersion. Nanotoxicology 2:144–154.

Powers KW, Palazuelos M, Moudgil BM, Roberts SM (2007). Characterization of the size, shape, and state of dispersion of nanoparticles for toxicological studies. Nanotoxicology 1:42–51.

Prahalad AK, Inmon J, Dailey LA, Madden MC, Ghio AJ, Gallagher JE (2001). Air pollution particles mediated oxidative DNA base damage in a cell free system and in human airway epithelial cells in relation to particulate metal content and bioreactivity. Chem Res Toxicol 14:879–887.

Rahman Q, Lohani M, Dopp E, Pemsel H, Jonas L, Weiss DG, Schiffmann D (2002). Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts. Environ Health Perspect 110:797–800.

Rothen-Rutishauser BM, Kiama SG, Gehr P (2005). A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. Am J Respir Cell Mol Biol 32:281–289.

Sayes CM, Liang F, Hudson JL, Mendez J, Guo W, Beach JM, Moore VC, Doyle CD, West JL, Billups WE, Ausman KD, Colvin VL (2006). Functionalization density dependence of single-walled carbon nanotubes cytotoxicity in vitro. Toxicol Lett 161:135–142.

Schins RP, McAlinden A, MacNee W, Jimenez LA, Ross JA, Guy K, Faux SP, Donaldson K (2000). Persistent depletion of I kappa B alpha and interleukin-8 expression in human pulmonary epithelial cells exposed to quartz particles. Toxicol Appl Pharmacol 167:107–117.

Schins RP, Duffin R, Hohr D, Knaapen AM, Shin T, Weishaupt C, Stone V, Donaldson K, Borm PJ (2002). Surface modification of quartz inhibits toxicity, particle uptake, and oxidative DNA damage in human lung epithelial cells. Chem Res Toxicol 15:1166–1173.

Schins RP, Knaapen AM (2007). Genotoxicity of poorly soluble particles. Inhal Toxicol 19(Suppl 1):189–198.

Seaton A, MacNee W, Donaldson K, Godden D (1995). Particulate air pollution and acute health effects. Lancet 345:176–178.

Singh S, Shi T, Duffin R, Albrecht C, van Berlo D, Hohr D, Fubini B, Martra G, Fenoglio I, Borm PJ, Schins RP (2007). Endocytosis, oxidative stress and IL-8 expression in human lung epithelial cells upon treatment with fine and ultrafine TiO2: role of the specific surface area and of surface methylation of the particles. Toxicol Appl Pharmacol 222:141–151.

Speit G (2002). Appropriate in vitro test conditions for genotoxicity testing of fibers. Inhal Toxicol 14:79–90.

Stoeger T, Reinhard C, Takenaka S, Schroeppel A, Karg E, Ritter B, Heyder J, Schulz H (2006). Instillation of six different ultrafine carbon particles indicates a surface area threshold dose for acute lung inflammation in mice. Environ Health Perspect 114:328–333.

Stone V, Shaw J, Brown DM, MacNee W, Faux SP, Donaldson K (1998). The role of oxidative stress in the prolonged inhibitory effect of ultrafine carbon black on epithelial cell function. Toxicol In Vitro 12:649–659.

Stone V, Tuinman M, Vamvakopoulos JE, Shaw J, Brown D, Petterson S, Faux SP, Borm P, MacNee W, Michaelangeli F, Donaldson K (2000). Increased calcium influx in a monocytic cell line on exposure to ultrafine carbon black. Eur Respir J 15:297–303.

Sydlik U, Bierhals K, Soufi M, Abel J, Schins RP, Unfried K (2006). Ultrafine carbon particles induce apoptosis and proliferation in rat lung epithelial cells via specific signaling pathways both using EGF-R. Am J Physiol Lung Cell Mol Physiol 291:L725–L733.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000). Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35:206–221.

Unfried K, Albrecht C, Klotz LO, von Mikecz A, Grether-Beck S, Schins RPF (2007). Cellular responses to nanoparticles: target structures and mechanisms. Nanotoxicology 1:52–71.

Vainio H, Magee P, McGregor D, McMichael A, eds. (1992). Mechanisms of Carcinogenesis in Risk Identification. IARC Scientific Publications No. 116. IARC, Lyon.

Warheit DB, Hoke RA, Finlay C, Donner EM, Reed KL, Sayes CM (2007). Development of a base set of toxicity tests using ultrafine TiO2 particles as a component of nanoparticle risk management. Toxicol Lett 171:99–110.

Wilson MR, Lightbody JH, Donaldson K, Sales J, Stone V (2002). Interactions between ultrafine particles and transition metals in vivo and in vitro. Toxicol Appl Pharmacol 184:172–179.

Worle-Knirsch JM, Pulskamp K, Krug HF (2006). Oops they did it again! Carbon nanotubes hoax scientists in viability assays. Nano Lett 6:1261–1268.

Xiao GG, Wang M, Li N, Loo JA, Nel AE (2003). Use of proteomics to demonstrate a hierarchical oxidative stress response to diesel exhaust particle chemicals in a macrophage cell line. J Biol Chem 278:50781–50790.

Yoshinaga K, Obata H, Jurukovski V, Mazzieri R, Chen Y, Zilberberg L, Huso D, Melamed J, Prijatelj P, Todorovic V, Dabovic B, Rifkin DB (2008). Perturbation of transforming growth factor (TGF)-beta1 association with latent TGF-beta binding protein yields inflammation and tumors. Proc Natl Acad Sci USA 105:18758–18763.