Published in final edited form as:

Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2010 May ; 2(3): 219–231. doi:10.1002/wnan.54.

Assessing nanotoxicity in cells in vitro

Jedd M. Hillegass,

jedd.hillegass@uvm.edu University of Vermont College of Medicine

Arti Shukla,

arti.shukla@uvm.edu University of Vermont College of Medicine

Sherrill A. Lathrop,

sherrill.lathrop@uvm.edu University of Vermont College of Medicine

Maximilian B. MacPherson,

maximilian.macpherson@uvm.edu University of Vermont College of Medicine

Naomi K. Fukagawa, and

naomi.fukagawa@uvm.edu University of Vermont College of Medicine

Brooke T. Mossman

brooke.mossman@uvm.edu University of Vermont College of Medicine

Abstract

Nanomaterials are commonly defined as particles or fibers of less than 1 micron in diameter. For these reasons, they may be respirable in humans and have the potential, based upon their geometry, composition, size and transport or durability in the body, to cause adverse effects on human health, especially if they are inhaled at high concentrations. Rodent inhalation models to predict the toxicity and pathogenicity of nanomaterials are prohibitive in terms of time and expense. For these reasons, a panel of *in vitro* assays is described below. These include cell culture assays for cytotoxicity (altered metabolism, decreased growth, lytic or apoptotic cell death), proliferation, genotoxicity, and altered gene expression. The choice of cell type for these assays may be dictated by the procedure or endpoint selected. Most of these assays have been standardized in our laboratory using pathogenic minerals (asbestos, silica) and nonpathogenic particles (fine titanium dioxide or glass beads) as negative controls. The results of these *in vitro* assays should predict whether testing of selected nanomaterials should be pursued in animal inhalation models that simulate physiologic exposure to inhaled nanomaterials. Conversely, intrathoracic or intrapleural injection of nanomaterials into rodents can be misleading as they bypass normal clearance mechanisms, and nonpathogenic fibers and particles can test positively in these assays.

Keywords

Nanoparticles; Nanofibers; Nanotubes; Nanospheres; Asbestos

With the advent of nanotechnology, concerns about the potential adverse health effects of nanomaterials have been expressed, especially to workers and users [1,2]. For these reasons, screening assays are needed to assess a myriad of chemically and physically diverse nanomaterials. Because of the expense of *in vivo* experiments and public and governmental urging to develop alternatives to animal testing, *in vitro* models may be more attractive for preliminary testing of nanomaterials to assess their potential toxicologic effects and ability to elicit disease.

Human health concerns for nanomaterials are predicated historically by epidemiologic and clinical studies on naturally occurring fibers and particles such as asbestos and silica, respectively. Whereas inhalation of asbestos fibers is associated with the development of nonmalignant (pleural and pulmonary fibrosis or asbestosis) and malignant diseases (lung cancers and mesotheliomas) [3,4], silica is associated primarily with the development of silicosis, an occupationally-linked pulmonary fibrosis [5]. After decades of research, the complex mechanisms of disease by these minerals are still incompletely understood, but several properties appear important in the long-term health effects of asbestos fibers. These include: 1) respirability or ability to enter the lung; 2) durability, due to intrinsic lack of solubility and/or inability to be cleared by macrophages in the lung, pleura or peritoneum; 3) fibrous geometry; 4) length to width ratio, i.e., longer ($> 5 \mu\text{m}$) and thinner fibers are more carcinogenic and fibrogenic; and 5) surface properties which play a role in the generation of reactive oxygen or nitrogen species (ROS/RNS) [6]. In addition, both ROS and RNS have been linked to the generation and augmentation of the inflammatory responses to asbestos and silica, and inflammation is thought to be key to the development of fibrosis and many cancers [7]. We recently have shown that stimulation of the inflammasome of human macrophages via NADPH oxidase acts as a sensor for the production of proinflammatory cytokines such as interleukin- 1β by asbestos, suggesting that inflammation mediates responses of target cells of lung disease [8]. These studies underscore the importance of effective screening strategies for nanomaterials using multiple cell types, especially since nano-sized particles and fibers may be similar to ultrafine (UF) particles that can penetrate the endothelium of the lung and be transported to distal organs such as the heart and brain [1,2].

For testing of the pathogenic effects of asbestos and asbestos-like fibers, most *in vitro* assays have been designed using target cells of the lung and pleura with endpoints such as cytotoxicity, proliferation, and genotoxicity. These phenomena are related to the multiple stages of cancer development which may involve genotoxic (changes to DNA) as well as proliferative events that can lead to the selective expansion of an asbestos-mutated cell population. In this chapter, we review *in vitro* assays for cytotoxicity, proliferation, genotoxicity, and more robust toxicogenomic approaches that can be used to screen nanomaterials for their potential pathogenic effects. Since the majority of these assays have been standardized in our laboratory using a variety of pathogenic minerals (asbestos, silica) and nonpathogenic particles (fine titanium dioxide [TiO_2] or glass beads), we will frequently supplement our discussion of nanomaterials with mention of other mineral/particle types to demonstrate each assay's utility in predicting toxicity. Although cell-free *in vitro* assays to predict dissolution of nanomaterials in the body are not discussed in detail, they are recommended to predict nanomaterial durability over time, especially since it has been shown that diseases resulting from exposure to other pathogenic materials, such as asbestos, require decades to develop [3,4].

I. ASSAYS FOR CYTOTOXICITY

Before assessing the cytotoxic effects of nanoparticles or other compounds of interest on a given cell type, standard growth curve data should be collected to determine baseline growth properties of selected cells. When comparing cells with each other using this method, they can be classified according to their growth rates, which may help to explain results of cytotoxicity experiments. In general, nonmalignant cell lines undergo lag, log, and stationary growth phases, each of which may reflect different responses to the same concentration of nanomaterials. Growth curves can also illustrate the doubling times of different cell types. Most of the work in our laboratory uses normal epithelial or mesothelial cells at 80-90% confluency that resembles their contiguous architecture in the lung *in situ*.

A. Trypan Blue Exclusion Assay

In this assay cells are treated with agents, trypsinized, and subsequently stained with trypan blue, a diazo dye which is taken up by dead cells, but excluded by viable cells. Unstained cells reflect the total number of viable cells recovered from a given dish. This method is advantageous because it conveys the actual number of viable cells and increases (cell proliferation) or decreases (cytotoxicity) in comparison to control, untreated cells.

Recently we have used this assay to assess cytotoxicity of crocidolite asbestos as well as other minerals including talc, TiO_2 , and glass beads on a TERT-1 immortalized, contact-inhibited human mesothelial cell line, LP9/TERT-1 [9]. These studies reveal that at the same surface area concentration ($75 \mu\text{m}^2/\text{cm}^2$), crocidolite asbestos is cytotoxic ($\leq 50\%$ cell viability compared to control), whereas other nonpathogenic minerals (e.g. glass beads or fine TiO_2) show no significant toxic effects. Bejjani et al [10] provide an example of this assay in which they illustrated that poly(lactic) acid nanoparticles (PLA) for gene delivery in human and bovine retinal pigment epithelial cells do not reduce cell viability at concentrations up to 4 mg/ml PLA. An additional study utilizing trypan blue to evaluate the toxicity of various metal oxide nanoparticles and multiwalled carbon nanotubes (MWCNT) to the human lung epithelial tumor cell line A549 demonstrated that $\text{CuZnFe}_2\text{O}_4$, ZnO, and CuO nanoparticles, as well as MWCNT, caused a significant increase in nonviable cells at concentrations of $20 \mu\text{g}/\text{cm}^2$ (CuO nanoparticles only) and $40 \mu\text{g}/\text{cm}^2$ [11].

B. Microculture Tetrazolium Assay (MTA)

Short-term MTAs are metabolic assays which do not provide direct information about total cell numbers, but measure the viability of a cell population relative to control, untreated cells. Cells are treated with particulates for various times before addition of soluble yellow tetrazolium salts such as MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; Promega) or MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; R&D Systems) for 2-4 hr at 37°C . During this process, viable cells with active respiratory mitochondrial activity bioreduce MTS or MTT into an insoluble purple formazan product via mitochondrial succinic dehydrogenases, which is subsequently solubilized by dimethyl sulfoxide (DMSO) or detergent and quantitated on a visible light spectrophotometer. Data are represented as optical density (OD)/control group. When considering this method for the determination of cell viability, it is important to note that since it measures respiratory activity, cells that possess low metabolism must be utilized in high numbers. In addition, this assay has a number of inherent shortcomings. First, certain human cell lines are inefficient at processing the tetrazolium salt reagents [12]. Second, the requirement of DMSO to solubilize the formazan product generated by reduction of the tetrazolium salts is problematic since this step not only lengthens the protocol, but also exposes lab personnel and equipment to potentially hazardous amounts of solvent [12]. As a result, a number of modifications to this protocol have been established, including the use of the tetrazolium derivative XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide), which is metabolized to a water soluble formazan product and thereby eliminates the solubilization step required with MTS or MTT [12-14].

We have shown using the MTS assay that cytotoxicity of the chemotherapeutic agent doxorubicin (DOX) is increased in human mesothelioma cells when it is loaded into synthetically created acid-prepared mesoporous spheres (APMS), amorphous silica-based nanoporous particles which enhance intracellular delivery and efficacy of DOX [15]. The MTS/MTT assay to assess cell viability has become a widely used, standard technique in recent nanoparticle research. The cell viability of a human small cell lung cancer line, NCIH69, was reduced significantly when cells were treated with the anti-tumor agent, paclitaxel, loaded into poly(lactic glycolic acid) (PLGA) nanoparticles compared to empty PLGA or commercially

available Taxol® at 2.5 µg/ml [16]. Kommareddy et al [17] have shown increased cytotoxicity of thiolated gelatin nanoparticles designed to release their contents in a reducing environment. Using murine fibroblasts (NIH-3T3 line), treatment with thiolated gelatin nanoparticles (200 µg/ml medium) incorporating 100 mg of 2-iminothiolane resulted in 79% viability compared to controls, while 20 and 40 mg resulted in 92% and 88% viability, respectively. Long circulating monensin nanoparticles (LMNP) were shown to potentiate the *in vitro* cytotoxic effects of anti-My9, a ricin-based immunotoxin, in HL-60 sensitive (500x potentiation) and resistant (5x potentiation) human tumor cell lines [18].

C. Clonogenic Assay or Colony Forming Efficiency (CFE)

The clonogenic assay or CFE assay allows assessment of decreased or increased survival and proliferation over extended periods of time (weeks). After plating at a very low density, cells are allowed to grow until colonies are observed, i.e. from 10 days to 3 weeks. Cells can either be pretreated with particulates of interest or treated following plating. It is assumed that each colony originates from a single plated cell, hence the name “clonogenic assay”. Colonies can be stained with crystal violet or nuclear stains and quantitated according to numbers and/or size. We have used this method to show that hamster tracheal epithelial cells have increased numbers of colonies, interpreted as increased survival and/or proliferation, after exposure to low concentrations of crocidolite asbestos fibers [19].

Herzog et al [20] have recently illustrated the effects of HiPco® SWCNT, arc discharge (AD) SWCNT, and Printex 90 carbon black nanoparticles on A549, normal human bronchial epithelial (Beas-2B), and human keratinocyte (HaCat) cells. The clonogenic assay was utilized because carbon can interfere with many other colorimetric viability assays. Increasing doses of all nanomaterials resulted in decreased numbers of colonies, but more so in cells exposed to the two carbon nanotube preparations (the HiPco® nanotubes causing the strongest response) as compared to the carbon black nanoparticles. The Beas-2B cells were the most responsive cell type to nanomaterials [20]. An additional study used the clonogenic assay to assess cytotoxicity in A549 cells exposed to medium “depleted” by two types of SWCNT (HiPco® and AD) in order to determine if these carbonaceous nanoparticles are capable of reducing the availability of medium components, thereby leading to false positive results in cytotoxicity assays [21]. Indeed, significant ($p \leq 0.05$), dose-dependent reductions in colony size were observed following incubation in medium depleted by HiPco® SWCNT (0.025- 0.4 mg/ml) and AD SWCNT (0.1- 0.4 mg/ml) [21].

D. Lactate Dehydrogenase (LDH) Assay

LDH is a soluble cytosolic enzyme which serves as an indicator of lytic cell death since it is readily released into extracellular medium following cellular membrane damage resulting from apoptosis or necrosis. Although widely accepted as a marker of cell death, it should be noted that this test is simply an index of cell membrane integrity, and in certain circumstances can be positive even when the cell count is not significantly modified. The original assay was designed to measure the oxidation of β -NADH to β -NAD⁺ when LDH reduced pyruvate to lactate, a phenomena which could be measured as a decrease in absorbance at 340 nm [22]. Subsequent modifications of this method have focused on both making the assay more time- and cost- effective, and increasing sensitivity through use of a fluorometer [23]. A number of commercially available kits have also been developed. In a kit from Promega, an aliquot of cell medium reacts with a tetrazolium salt which, through NADH generated by LDH release, is converted to a red formazan endproduct that is read on a spectrophotometer. Complete lysis of cells using a lysis buffer is a positive control in this assay. The OD values of treatment groups are expressed as percent LDH release relative to LDH values from completely lysed cells. The amount of LDH per sample can also be assessed quantitatively by generating a standard curve using standards containing known LDH amounts. We have routinely used this

assay *in vitro* and in bronchoalveolar lavage samples from rodents to demonstrate cytotoxicity following asbestos exposure [24].

The cytotoxicity of nanomaterials to be used for drug and/or gene delivery has been evaluated using the LDH assay. For example, LDH release studies were conducted on human lung epithelial (16HBE14o) cells treated with nanoparticles consisting of porcine gelatin, human serum albumin (HSA), and polyalkylcyanoacrylate. The gelatin and HSA nanoparticles showed no dose-related increases in LDH release, while the polyalkylcyanoacrylate nanoparticles caused cytotoxicity, suggesting gelatin and HSA nanoparticles were more suitable for use in drug delivery or gene therapy studies [25]. Nanoparticles containing different metal/metal oxide groups have recently been analyzed by the LDH assay for their toxic effects on rat liver BRL3A cells [26]. Among the metals (silver, molybdenum, aluminum, iron oxide, TiO₂, manganese oxide, and tungsten) incorporated into differently sized nanoparticles, silver was the most toxic at concentrations from 10-50 µg/ml medium. Moreover, large diameter nanoparticles (100 nm) elicited significantly more LDH release than smaller diameter nanoparticles (15 nm). These results were confirmed by MTT assays. In a similar study by Jeng et al [27], nanoparticles containing zinc oxide (as compared to TiO₂, chromate, iron oxide, and aluminum oxide) elicited the strongest LDH release in a dose-dependent manner in Neuro-2A cells.

E. TdT dUTP Nick End Labeling (TUNEL) and Apostain Assays

Apoptosis, a form of programmed cell death, is characterized by cell membrane blebbing, mitochondrial DNA damage, nuclear and cytoplasmic shrinkage, fragmentation into apoptotic bodies, chromatin condensation, and DNA fragmentation. Morphological indicators of apoptosis in response to hydroxyapatite (HAP) nanoparticles were illustrated by Liu et al [28] using a human hepatoma cell line (BEL-7402). Cell nuclei were fluorescently stained with Hoechst 33258 dye, and while control cells had large and round nuclei, increasing concentrations of HAP nanoparticles from 50-200 mg/l medium resulted in smaller and more fragmented nuclei, as well as more condensed chromatin.

There are several immunohistochemical techniques to visualize apoptotic cells *in vitro*. The two most common of these are the TUNEL and Apostain techniques. The TUNEL assay labels the ends of DNA that have been fragmented by endonucleases as a result of apoptosis, resulting in biotinylated dUTP at the 3'-OH end which can be detected using streptavidin-horseradish peroxidase and a diaminobenzidine chromogen by light microscopy. Alternatively, the incorporated dUTP nucleotides can be labeled with a fluorescent dye and visualized using fluorescent microscopy. TUNEL has been used to illustrate the enhanced apoptosis of A549 cells exposed to the anti-tumor agent paclitaxel after loading into PLGA nanoparticles. A wheat germ agglutinin (WGA) group was attached externally to increase affinity to tumor cells. TUNEL positive cells, as measured by fluorescein isothiocyanate (FITC) fluorescence, were not seen in control groups and were slightly higher in paclitaxel-loaded PLGA with WGA than in paclitaxel-loaded PLGA without WGA. Paclitaxel alone elicited a small apoptotic response, but not as great as the PLGA-loaded groups [29].

Whereas the TUNEL method detects fragmented DNA (a feature of both necrosis and apoptosis), Apostain is thought to be a specific marker of apoptosis as it labels condensed chromatin. Apoptotic nuclei are more sensitive to thermal DNA denaturing, so after cells are heated in the presence of MgCl₂, the Apostain antibody targets the resulting single-stranded DNA of condensed chromatin from apoptotic cells. Several publications from our group have used the Apostain technique to assess apoptosis in various cell types induced by asbestos. For example, crocidolite asbestos at 5 µg/cm² dish induces apoptosis in mouse alveolar type II (C10) cells [30,31] that is inhibited when cells are pretreated with rottlerin, a PKCδ inhibitor [30], PKA (H89), or MEK1/2 (U0126) inhibitors [31]. To date, this technique has not been

used to detect apoptotic processes following nanoparticle administration, although its ability to identify apoptosis in asbestos-treated cells supports its future potential. Apoptosis may also be assessed using flow cytometry and a variety of nuclear stains or stains for early apoptotic events. Specifically, Annexin V, a marker of the externalization of phosphatidylserine on the outer surface of the plasma membrane, is an early sign of apoptosis. Nuclear stains such as propidium iodide (PI) or 7-amino actinomycin D (7AAD) can also be used to measure apoptosis in its later phases when the cell membrane loses integrity. Such methods have not been used to determine nanoparticle cytotoxicity *in vitro*, although nanoparticles have been labeled with these stains to target apoptotic cells [32-34].

F. Other Methods for Determining Cytotoxicity

Although several fundamental cytotoxicity assays are described above, this list is far from inclusive. A number of other assays exist for determining cytotoxicity, including a plethora of dye-based assays (non-fluorescent and fluorescent) similar to trypan blue and MTS/MTT/XTT mentioned previously. These include calcein AM, neutral red, Live/Dead® (Invitrogen), CellTiter 96® Aqueous One (Promega), alamar Blue® (Invitrogen), and CytoTox One™ Homogenous Membrane Integrity (Promega). One study utilized a large number of these viability dyes to assess the toxicity of four carbon-based (SWCNT, carbon black, fullerenes, and fullerene crystalline aggregates) and one noncarbon-based (quantum dots) nanomaterial [35]. Results were highly variable due to interactions of the carbon nanomaterials with dye/dye product and the authors recommended implementing more than one assay to accurately determine toxicity [35].

Other *in vitro* nanotoxicity assays include the examination of lipid peroxidation to elucidate the role played by oxidative stress, as well as methods to investigate apoptosis including cytochrome c release from mitochondria and caspase activation. Lipid peroxidation is the oxidative degradation of cell membranes initiated by the presence of ROS, and is most commonly measured by assaying the presence of malondialdehyde (MDA) or other thiobarbituric acid reactive substances (TBARS) [36-38]. This assay has been used extensively to demonstrate the ability of a variety of nanomaterials to elicit lipid peroxidation in multiple cell types, such as: fullerenes in human dermal fibroblasts (HDF) and human liver carcinoma (HepG2) cells [37], realgar nanoparticles in promyelocytic leukemia (HL-60) cells, silver nanoparticles in human skin carcinoma (A431) and human fibrosarcoma (HT-1080) cells [13], cerium oxide (CeO₂) and crystalline silica nanoparticles in A549 cells [39,40], and co-exposure of carbon black and Fe₂O₃ nanoparticles in A549 cells [41].

Disruption of mitochondrial function plays a fundamental role in the initiation of apoptosis; therefore, one can assay the release of various proteins normally present in the inner membrane of these organelles, thus signaling the early stages of apoptotic cell death. One such protein is cytochrome c, a small heme protein whose release leads to a signaling cascade eventually resulting in the activation of multiple caspases including caspase-9, caspase-7, and caspase-3 [42]. Detection of these proteins can be accomplished through a variety of methods including Western blotting, immunofluorescence confocal microscopy, and utilization of commercially available kits. Considerable evidence exists that these methods are capable of detecting nanotoxicity *in vitro*. For example, mouse fibroblasts (NIH3T3) exposed to 50 µg/ml of a nanosilver powder for 24 hr exhibited an increase in cytochrome c release [43]. Nanoscale HAP, when administered to human gastric cancer cells (SGC-7901) at 100 µg/ml for 12-48 hr, caused release of cytochrome c and activation of caspases-3 and -9 [44]. Finally, it has been demonstrated that both CeO₂ (5-40 µg/ml) and TiO₂ (5-40 µg/ml) nanoparticles trigger the activation of caspase-3 in Beas-2B cells following 24 hr of exposure [45,46].

II. ASSAYS FOR CELL PROLIFERATION

Cell proliferation can be a compensatory response of surrounding cells to necrosis or apoptosis and a critical mechanism in tumor promotion and progression. There are currently several established methods for determining cell proliferation, each with its own specificity and limitations. Current methods include histochemical, immunohistochemical, and flow cytometric approaches. Histochemical procedures include direct observation of mitosis by staining for DNA content, incorporation of tritiated thymidine ($[^3\text{H}]$ thymidine), and uptake of the DNA analog, bromodeoxyuridine (BrdU). Current antibodies of interest in immunohistochemistry are Ki-67 and antibodies that recognize proliferating cell nuclear antigens (PCNAs) [47].

A. DNA Content

Observing and counting cells in mitosis is a direct way of quantifying proliferation and identifying agents that inhibit or induce mitotic progression. Results are typically expressed as a mitotic index, calculated by dividing the number of cells undergoing mitosis by the total number of cells in a given population. This method may employ the nuclear antigen Ki-67 and/or a compound capable of arresting cells in metaphase such as colchicine or Colcemid®. A decrease in mitotic index was observed in rat pleural mesothelial cell following crocidolite asbestos exposure [48]. Dong et al [49] looked at mitotic cells to evaluate differential effects on proliferation of SWCNTs conjugated to various surfactants in human astrocytoma cells. In addition to the mitotic index, the DNA content of cells in other phases of the cell cycle can be evaluated as a measure of the proliferative state. In normal somatic cells undergoing S phase, the DNA within each cell doubles from a diploid state to a tetraploid state that can be identified through several staining techniques.

B. $[^3\text{H}]$ thymidine Incorporation

Incorporation of $[^3\text{H}]$ thymidine into the DNA of viable cells during S phase is indicative of the number of cells undergoing proliferation. The use of $[^3\text{H}]$ thymidine is complicated by the fact that dividing (nonquiescent) cells are required to take up the label, which is not always possible in confluent cells *in vitro*. In addition, use of radioactive material is expensive and requires special training and facilities. Moreover, this technique often requires a lengthy incubation period (24-48 hr) with $[^3\text{H}]$ thymidine [24]. This method has been used to demonstrate the ability of nitric oxide-releasing nanofiber gels to inhibit vascular smooth muscle cell proliferation *in vitro* [50].

C. Incorporation of Bromodeoxyuridine (BrdU)

More recently, incorporation of BrdU has been used to circumvent the complications of using a radioactive material since its presence can be detected using specific antibodies or by flow cytometry. BrdU also shows increased specificity for cells undergoing DNA synthesis in contrast to $[^3\text{H}]$ thymidine, which can be incorporated into DNA during unscheduled DNA synthesis in a non-specific manner [24,51]. However, challenges similar to those seen with $[^3\text{H}]$ thymidine incorporation (need for viable cells and lengthy incubations) exist [47]. The BrdU incorporation assay has been employed to show the proliferative effects of nontoxic concentrations of particulate matter (PM) in pulmonary epithelial cells [52], as well as the anti-proliferative effects of heparin-deoxycholic acid nanoparticles on squamous cell carcinoma and human umbilical vascular endothelial cells [53].

D. Ki-67

Ki-67 is a nuclear antigen present at all stages of the cell cycle except G_0 , when cells are in a resting state. This immunohistochemical technique for assessing cell proliferation has been

used to confirm the effects of asbestos on proliferating bronchiolar epithelial cells *in vitro* and *in vivo*, and shows results congruent with PCNA staining [54].

E. Proliferating Cell Nuclear Antigen (PCNA)

PCNA, a protein synthesized in the nucleus in the early G₁ and S phases of the cell cycle, is associated with DNA synthesis and repair. Use of antibodies to detect PCNA correlates well with both the incorporation of [³H]thymidine and the detection of BrdU through immunoassay and flow cytometry detection [47,53,55]. However, due to the long half life of PCNA (~20 hr), detection of PCNA in non-proliferating cells may occur through association with lingering molecules of the protein. This technique has yet to be used to assess the proliferative effects of nanoparticles *in vitro*, although our laboratory has demonstrated an increase in PCNA-positive lung epithelial cells following exposure to crocidolite asbestos [56].

III. ASSAYS FOR GENOTOXICITY

Engineered nanomaterials possess distinct physicochemical properties as a result of their nanometer-scale size, increased surface area, variable chemical composition, surface structure, and shape [57]. These unique properties may allow nanomaterials to directly interact with biological systems and subsequently alter cell signaling and function. Although the interaction of nanomaterials with lipid membranes and their subsequent intracellular transport is poorly understood, it has been demonstrated that they can enter cells using various endocytotic processes [57,58]. These processes are most likely dependent on surface properties that may be directly related to their genotoxic potential. It is therefore imperative that direct effects on DNA be examined to provide preliminary information on the potential genotoxicity of these materials. Subsequent sections will describe a battery of *in vitro* assays in both prokaryotic and eukaryotic systems that can be employed to accomplish this task.

A. Determination of Gene Mutations Using the Ames Assay in *Salmonella typhimurium* and *Escherichia coli*

The reverse mutation (Ames) assay in *Salmonella typhimurium* employs bacteria deficient in DNA repair mechanisms that are unable to grow in the absence of histidine [59]. Following exposure to compounds of interest, reversion to a histidine-positive phenotype (indicating a reverse mutation in the histidine locus) is established by counting colonies that have been grown in histidine-free media. Inclusion of an exogenous metabolizing system (Aroclor-induced rat liver S9 microsomal fraction) allows for the detection of mutagens requiring metabolic activation to form DNA-reactive intermediates. In addition to several strains of *S. typhimurium* (e.g. TA98, TA100, TA102, TA1535, TA1537, TA1538), each allowing detection of different mutation types, this assay has been adapted in a strain of *Escherichia coli* (WP2uvrA) to identify base-pair substitutions based on reversion at the tryptophan locus.

The Ames assay has been utilized in several studies to determine the mutagenicity of various types of nanomaterials. In one study, UF TiO₂ particles having a median particle size of 140 nm were exposed to *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2uvrA at concentrations ranging from 100-5000 µg/plate. Since no positive mutagenic responses or compound-related toxicity were detected, either in the presence or absence of S9 metabolic activation, this nanomaterial was considered non-mutagenic [60]. Similarly, a study examining the genotoxicity of fullerenes to the same strains of *S. typhimurium* and *E. coli* at concentrations ranging from 39.1-5000 µg/plate found that they were non-mutagenic regardless of the presence or absence of S9 metabolic activation [61]. Despite the fact that the mutagenicity studies with nanomaterials were all negative, studies by Faux and colleagues [62] show positive correlation between iron-dependent crocidolite asbestos exposure and mutagenicity in *S. typhimurium* TA102, indicating this assay is capable

of identifying pathogenic particulates. Given the possible differences in cellular uptake of particulates and genomic complexity between prokaryotes and eukaryotes, genotoxicity data for nanomaterials obtained from the Ames assay should be interpreted carefully. The Ames assay should not be considered as a stand alone assay for identifying genotoxicity elicited by nanomaterials in humans and other vertebrates, and should instead be supplemented with additional studies as described hereafter.

B. Identifying DNA Base Modifications via Measurement of Oxidized Guanine Bases

Point mutations represented by single base changes within a particular gene can be identified by assaying any one of several oxidized guanine bases, the most common of which include 8-hydroxydeoxyguanosine (8-OHdG) and 7,8-dihydro-oxodeoxyguanine (oxo-dG). These base modifications are often a consequence of oxidative injury, and measurement of these various oxidized bases (via immunohistochemistry or HPLC) represents a logical step to better understanding the prospective genotoxicity of nanomaterials, especially given their potential to generate ROS [63].

Our laboratory has employed this method to examine the propensity of crocidolite asbestos to cause oxidative DNA damage in rat and human pleural mesothelial cells [64]. A similar study looked at additional markers of oxidative DNA damage following exposure of human mesothelial cells to crocidolite asbestos including 8-oxo-2'-deoxyguanosine, 8-oxoguanine, and 8-oxoguanosine [65]. However, few studies have employed this method to determine whether nanomaterials can cause DNA base modifications. Data obtained following treatment of human fibroblasts with 50-500 $\mu\text{m}^3/\text{cell}$ of nano-sized particles of cobalt-chromium alloy (~ 30 nm) for 3 or 24 hr did not show a significant increase in 8-OHdG staining [66]. Also, a study of A549 cells exposed to luminescent silica nanoparticles (~ 50 nm) at 0.1-500 $\mu\text{g}/\text{ml}$ found no significant increases in DNA base modification as indicated by similar levels of oxo-dG between exposed and control groups [67].

C. Cytogenetic Assessment of Chromosome Damage through Analysis of Chromosomal Aberration Induction and Micronuclei

In addition to determining mutations of a particular gene, it is important to evaluate effects on the number and integrity of chromosomes via karyotype analyses. Such analyses can be carried out directly via simple staining techniques (5% Giemsa) and microscopy, and entail evaluation of changes in the morphological appearance of chromosomes (chromosomal aberrations representing clastogenicity) and the presence of micronuclei. Protocols typically involve treatment of cells during S-phase (due to the sensitivity of cells at this point in the cell cycle) followed by treatment at predetermined intervals with a substance such as Colcemid® or colchicine that is capable of arresting the cells in metaphase.

Alternatively, assessment of chromosomal breakage and chromosome loss events can be carried out by identifying the presence of micronuclei. Micronuclei are chromosomal fragments or whole chromosomes that are not incorporated into the nucleus of either daughter cell at anaphase, and are therefore bound by a membrane and remain in the cytoplasm through subsequent cell cycles. Micronucleus assays typically employ a cytokinesis-block technique in which cytochalasin B is used to inhibit cytokinesis, thus allowing micronuclei to be assessed in binucleated cells. This is important since micronuclei are most accurately quantified in binucleated cells that have undergone only one cell division.

Karyotypic analyses as described above have been carried out for a number of nanomaterials. One study examined the potential photo-clastogenicity of eight different classes of UF TiO_2 (≤ 60 nm) in Chinese hamster ovary (CHO) cells in the absence and presence of 750 mJ/cm^2 UV light. Concentrations of UF TiO_2 ranged from 209.7-5000 $\mu\text{g}/\text{ml}$, and it was determined

that none of these concentrations, either with or without UV exposure, were photo-clastogenic [68]. Another study tested the ability of UF TiO₂ (~140 nm) to induce chromosomal aberrations in CHO cells at concentrations ranging from 25-2500 µg/ml, and found that this material did not induce increases in chromosome number or morphological aberrations over the vehicle control at any concentration tested, either in the presence or absence of metabolic activation [60]. A third study evaluating the genotoxicity of fullerenes at concentrations of up to 5000 µg/ml (+/- S9 microsomal fraction), revealed that this nanomaterial induced chromosomal numerical or structural aberrations in only 5% of the cells tested [61].

Several studies have demonstrated the ability of UF TiO₂ to generate micronuclei in various cell types. A study by Rahman et al [69] showed that an UF TiO₂ (≤ 20 nm) concentration of 1.0 µg/cm² significantly increased micronuclei induction following treatment for 12-72 hr in Syrian hamster embryo fibroblasts. Similarly, human peripheral blood lymphocytes treated with 20-50 µg/ml of UF TiO₂ (<100 nm) displayed significant increases in micronuclei formation [70]. Finally, a ~2.5-fold increase in micronucleated cells was observed in a human B-cell lymphoblastoid cell line (WIL2-NS) treated with 130 µg/ml of UF TiO₂ (<100 nm) [71]. Outside of TiO₂, cobalt-chromium alloy nanoparticles (~30 nm) at concentrations ranging from 5-500 µm³/cell caused a dose-dependent increase in micronuclei, nuclear blebs, and nucleoplasmic bridges in human fibroblasts [66].

D. Evaluating DNA Strand Breaks Using the Single Cell Gel Electrophoresis (Comet) Assay

The single cell gel electrophoresis assay (SCGE), also referred to as the comet assay, allows single and double DNA strand breaks and alkaline labile sites to be detected in individual cells. This assay is based on the simple principle that DNA containing strand breaks is capable of migrating more rapidly in agarose gel than intact DNA upon application of an electric field. Therefore, the extent of DNA damage is directly related to the length of the comet “tail” that is visualized following exposures to test materials. Staining with a fluorescent dye allows for the comet tails to be visualized, and various parameters of these structures are typically determined using image analysis software and subsequently used to calculate an olive tail moment (OTM), which is defined by both the tail length and distribution of DNA in the tail parameters [72].

This assay has proven useful in determining the ability of UF TiO₂ to induce DNA strand breaks. Kang et al [70] has shown that human peripheral blood lymphocytes treated with 20-100 µg/ml of TiO₂ (<100 nm) for 0-24 hr possess dose- and time-dependent increases in OTM over untreated controls, suggesting a significantly higher prevalence of DNA strand breaks. Another study demonstrated that treatment of WIL2-NS cells with 65 µg/ml UF TiO₂ (<100 nm) induced an increase in OTM of approximately 5-fold, as well as a 3-fold increase in the percentage of DNA in the comet tail [71]. Other nanomaterials evaluated using the comet assay include aqueous suspensions of colloidal C60 fullerenes in water at concentrations as low as 2.2 µg/l that produced statistically significant increases in OTM compared to a negative control [73], and cobalt-chromium alloy nanoparticles (~30 nm) that showed a dose-dependent increase in OTM following 24 hr exposures of human fibroblasts to 5×10⁻⁴-5000 µm³/cell [66]. However, studies of luminescent silica nanoparticles (~50 nm) in A549 cells at concentrations of up to 500 µg/ml did not cause any significant change in the number of DNA strand breaks as determined by comet tail length measurements [67].

IV. ASSAYS FOR ALTERATION IN GENE EXPRESSION

Gene expression assays, i.e., gene profiling, are an important tool for screening different environmental particles, including nanoparticles. Techniques used to assess gene expression include: Northern blot analysis, ribonuclease protection assays (RPA), quantitative real-time polymerase chain reaction (qRT-PCR), PCR arrays and microarrays.

A. Northern Blot Analyses and Ribonuclease Protection Assays (RPA)

Northern blot analysis remains a standard method for detection and quantitation of mRNA levels despite the advent of more robust techniques. Northern blot analysis provides a direct relative comparison of message abundance between samples on a single membrane and is a preferred method for determining transcript size and for detecting alternatively spliced transcripts. Northern hybridization is exceptionally versatile in that radiolabeled or nonisotopically labeled DNA, *in vitro* transcribed RNA, oligonucleotides, and sequences with only partial homology can be used as hybridization probes.

The RPA is a sensitive method used to detect and quantify specific mRNA transcripts in a complex mixture of total RNA or mRNA molecules. It utilizes a synthetic RNA probe (incorporating either radioactive or biotinylated nucleotides) complementary to the target of interest. Following hybridization, the mixture of single-stranded RNA and double-stranded probe:target hybrid is treated with ribonuclease, which digests all single-stranded RNA but no double-stranded RNA molecules leaving only the double-stranded gene target. Usually, the sample is electrophoresed on a denaturing TBE-urea polyacrylamide gel and detected by methods specific to the label on the probe.

B. Polymerase Chain Reaction (PCR)

Real-time PCR is a quantitative method for the determination of copy number of PCR templates, such as DNA or cDNA and consists of two types: probe-based and intercalator-based. Probe-based real-time PCR, also known as TaqMan PCR, requires a pair of PCR primers (as regular PCR does), and an additional fluorogenic oligonucleotide probe with both a reporter fluorescent dye and a quencher dye attached. The intercalator-based (SYBR Green) method requires a double-stranded DNA dye in the PCR reaction which binds to newly synthesized double-stranded DNA and renders fluorescence. Both methods require a special thermocycler equipped with a sensitive camera that monitors the fluorescence in each well of a 96-well plate at frequent intervals during the PCR reaction.

PCR arrays are important tools for analyzing the expression of a focused panel of genes. Each 96-well plate includes SYBR Green-optimized primer assays for a thoroughly researched panel of relevant, pathway- or disease-focused genes. In PCR arrays, 96 different gene-specific products are simultaneously amplified under uniform cycling conditions using specific master-mix formulation and subsequently detected.

C. Microarray Analyses

Gene expression profiling by microarray analysis has enabled the measurement of mRNA levels of thousands of genes in a single RNA sample. In this technique, a glass slide or membrane is spotted or “arrayed” with DNA fragments or oligonucleotides that represent specific gene coding regions. Purified RNA is then fluorescently or radioactively labeled and hybridized to the slide/membrane. After thorough washing, the raw data is obtained by laser scanning or autoradiographic imaging and subsequently entered into a database and analyzed by a number of statistical methods.

Although we and others have used the techniques described above for screening the effects of pathogenic fibers and particles, including ambient PM, diesel exhaust, silica and coal dust, metals, and asbestos, we focus below on the more robust and state-of-the-art microarray technique for screening of nanoparticles.

In a comparative study using human transferrin-derived magnetic particles and underivatized particles in human fibroblasts, microarrays detecting 1718 mRNAs and different microscopy procedures were utilized. Results indicated that the transferrin-derivatized particles induced

upregulation of many genes, particularly those regulating cytoskeletal function and cell signaling [74]. Microarray analysis also has been used to understand the mechanisms underlying nano-sized air pollution (carbon black)-mediated progression of atherosclerosis, revealing primarily the induction of proinflammatory molecules [75]. Exposure of mouse hepatic cells to poly(ethylene glycol)-block-poly(lactide) (PLA-PEG) nanoparticles resulted in over-expression of many ATP-binding cassette transporters and down-regulation of Glutathione-s-transferase P1 as studied using a mouse cDNA microarray and validated by RT-PCR [76]. Inhalation of TiO₂ nanoparticles by mice results in emphysema-like lung injury and the differential induction of hundreds of genes related to cell cycle, apoptosis, chemokines and the complement cascade when assessed by microarray [77]. A combined proteomic and RT-PCR approach also showed the expression of macrophage migration inhibitory factor in lung epithelial cells and lung tissues after exposure to BSA-coated TiO₂ particles (0.29 μ m mean diameter) [78]. Submicron-sized titanium particles can also induce macrophage colony stimulating factor expression in osteoblasts as demonstrated by RT-PCR, and thus may have a significant role in contributing to the onset of periprosthetic osteolysis [79]. Increased transcription of heme oxygenase-1, a sensitive antioxidant and stress response, was observed in A549 cells after exposure to UF carbonaceous model particles (count median mobility diameter $\sim$ 95 $\pm$ 5 nm) [80]. Cobalt nanoparticles activate cellular pathways of defense and repair mechanisms in BALB3T3 fibroblasts, including 10 differentially expressed sequences [81] that might represent candidate biomarkers of exposure.

Conclusion

The techniques described above can be used to evaluate different endpoints or types of cell injury by nanomaterials. Since it is clear that pathogenic fibers and particles such as asbestos and silica have multiple mechanisms of cell type-specific injury, more than one assay and cell type should be employed for an uncharacterized material. An integrated approach investigating multiple parameters over a range of concentrations may be necessary. It is also imperative that proper positive (asbestos fibers) and negative (amorphous particles such as glass beads) controls are incorporated. In addition to the traditional assays described above (see Table I), a number of companies are marketing more rapid, commercially available assays for cell viability that should be properly validated with pathogenic and nonpathogenic particulates before they are used to evaluate nanomaterials.

Traditionally, fibers and particles have been added to cells and evaluated on an equal weight basis per volume of medium or surface area of plate. This may reflect vastly different numbers and surface areas of materials per unit weight. Several recent studies suggest that introduction of equal surface areas of particles or fibers may reflect a more accurate basis for comparisons between groups as this parameter is a more accurate predictor of toxic responses, such as oxidative stress, to particles [82]. Regardless, complete characterization of size range, surface area, and chemical composition of test materials is advocated to draw conclusions.

References

1. Long TC, Tajuba J, Sama P, Saleh N, Swartz C, Parker J, Hester S, Lowry GV, Veronesi B. Nanosize titanium dioxide stimulates reactive oxygen species in brain microglia and damages neurons in vitro. *Environ Health Perspect* 2007;115:1631–1637. [PubMed: 18007996]
2. Balbus JM, Maynard AD, Colvin VL, Castranova V, Daston GP, Denison RA, Dreher KL, Goering PL, Goldberg AM, Kulinowski KM, et al. Meeting report: hazard assessment for nanoparticles—report from an interdisciplinary workshop. *Environ Health Perspect* 2007;115:1654–1659. [PubMed: 18007999]
3. Mossman BT, Bignon J, Corn M, Seaton A, Gee JB. Asbestos: scientific developments and implications for public policy. *Science* 1990;247:294–301. [PubMed: 2153315]

4. Robinson BW, Lake RA. Advances in malignant mesothelioma. *N Engl J Med* 2005;353:1591–1603. [PubMed: 16221782]
5. Mossman BT, Churg A. Mechanisms in the pathogenesis of asbestosis and silicosis. *Am J Respir Crit Care Med* 1998;157:1666–1680. [PubMed: 9603153]
6. Shukla A, Gulumian M, Hei TK, Kamp D, Rahman Q, Mossman BT. Multiple roles of oxidants in the pathogenesis of asbestos-induced diseases. *Free Radic Biol Med* 2003;34:1117–1129. [PubMed: 12706492]
7. O'Neill LA. Immunology. How frustration leads to inflammation. *Science* 2008;320:619–620. [PubMed: 18451288]
8. Dostert C, Pettrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 2008;320:674–677. [PubMed: 18403674]
9. Shukla A, Macpherson MB, Hillegass J, Ramos-Nino ME, Alexeeva V, Vacek PM, Bond JP, Pass HI, Steele C, Mossman BT. Alterations in Gene Expression in Human Mesothelial Cells Correlate with Mineral Pathogenicity. *Am J Respir Cell Mol Biol*. 2008
10. Bejjani RA, BenEzra D, Cohen H, Rieger J, Andrieu C, Jeanny JC, Gollomb G, Behar-Cohen FF. Nanoparticles for gene delivery to retinal pigment epithelial cells. *Mol Vis* 2005;11:124–132. [PubMed: 15735602]
11. Karlsson HL, Cronholm P, Gustafsson J, Moller L. Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. *Chem Res Toxicol* 2008;21:1726–1732. [PubMed: 18710264]
12. Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res* 1988;48:4827–4833. [PubMed: 3409223]
13. Arora S, Jain J, Rajwade JM, Paknikar KM. Cellular responses induced by silver nanoparticles: In vitro studies. *Toxicol Lett* 2008;179:93–100. [PubMed: 18508209]
14. Arora S, Jain J, Rajwade JM, Paknikar KM. Interactions of silver nanoparticles with primary mouse fibroblasts and liver cells. *Toxicol Appl Pharmacol*. 2009
15. Hillegass, JM.; Blumen, SR.; Cheng, K.; Macpherson, MB.; Alexeeva, V.; Butnor, KJ.; Ramos-Nino, ME.; Shukla, A.; James, TA.; Weiss, DJ., et al. Drug delivery by acid-prepared mesoporous spheres for cancer treatment. Submitted
16. Fonseca C, Simoes S, Gaspar R. Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity. *J Control Release* 2002;83:273–286. [PubMed: 12363453]
17. Kommareddy S, Amiji M. Preparation and evaluation of thiol-modified gelatin nanoparticles for intracellular DNA delivery in response to glutathione. *Bioconjug Chem* 2005;16:1423–1432. [PubMed: 16287238]
18. Shaik MS, Ikediobi O, Turnage VD, McSween J, Kanikkannan N, Singh M. Long-circulating monensin nanoparticles for the potentiation of immunotoxin and anticancer drugs. *J Pharm Pharmacol* 2001;53:617–627. [PubMed: 11370701]
19. Mossman BT, Sesko AM. In vitro assays to predict the pathogenicity of mineral fibers. *Toxicology* 1990;60:53–61. [PubMed: 2156357]
20. Herzog E, Casey A, Lyng FM, Chambers G, Byrne HJ, Davoren M. A new approach to the toxicity testing of carbon-based nanomaterials--the clonogenic assay. *Toxicol Lett* 2007;174:49–60. [PubMed: 17920791]
21. Casey A, Herzog E, Lyng FM, Byrne HJ, Chambers G, Davoren M. Single walled carbon nanotubes induce indirect cytotoxicity by medium depletion in A549 lung cells. *Toxicol Lett* 2008;179:78–84. [PubMed: 18502058]
22. Bergmeyer, HU.; Bernt, E. *Methods of Enzymatic Analysis*. Academic Press; London: 1963.
23. Moran JH, Schnellmann RG. A rapid beta-NADH-linked fluorescence assay for lactate dehydrogenase in cellular death. *J Pharmacol Toxicol Methods* 1996;36:41–44. [PubMed: 8872918]
24. Robledo RF, Buder-Hoffmann SA, Cummins AB, Walsh ES, Taatjes DJ, Mossman BT. Increased phosphorylated extracellular signal-regulated kinase immunoreactivity associated with proliferative

- and morphologic lung alterations after chrysotile asbestos inhalation in mice. *Am J Pathol* 2000;156:1307–1316. [PubMed: 10751356]
25. Brzoska M, Langer K, Coester C, Loitsch S, Wagner TO, Mallinckrodt C. Incorporation of biodegradable nanoparticles into human airway epithelium cells-in vitro study of the suitability as a vehicle for drug or gene delivery in pulmonary diseases. *Biochem Biophys Res Commun* 2004;318:562–570. [PubMed: 15120637]
 26. Hussain SM, Hess KL, Gearhart JM, Geiss KT, Schlager JJ. In vitro toxicity of nanoparticles in BRL 3A rat liver cells. *Toxicol In Vitro* 2005;19:975–983. [PubMed: 16125895]
 27. Jeng HA, Swanson J. Toxicity of metal oxide nanoparticles in mammalian cells. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 2006;41:2699–2711. [PubMed: 17114101]
 28. Liu ZS, Tang SL, Ai ZL. Effects of hydroxyapatite nanoparticles on proliferation and apoptosis of human hepatoma BEL-7402 cells. *World J Gastroenterol* 2003;9:1968–1971. [PubMed: 12970886]
 29. Mo Y, Lim LY. Paclitaxel-loaded PLGA nanoparticles: potentiation of anticancer activity by surface conjugation with wheat germ agglutinin. *J Control Release* 2005;108:244–262. [PubMed: 16213056]
 30. Shukla A, Stern M, Lounsbury KM, Flanders T, Mossman BT. Asbestos-induced apoptosis is protein kinase C delta-dependent. *Am J Respir Cell Mol Biol* 2003;29:198–205. [PubMed: 12626342]
 31. Barlow CA, Barrett TF, Shukla A, Mossman BT, Lounsbury KM. Asbestos-mediated CREB phosphorylation is regulated by protein kinase A and extracellular signal-regulated kinases 1/2. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L1361–1369. [PubMed: 17322281]
 32. Song EQ, Wang GP, Xie HY, Zhang ZL, Hu J, Peng J, Wu DC, Shi YB, Pang DW. Visual recognition and efficient isolation of apoptotic cells with fluorescent-magnetic-biotargeting multifunctional nanospheres. *Clin Chem* 2007;53:2177–2185. [PubMed: 17962366]
 33. Quinti L, Weissleder R, Tung CH. A fluorescent nanosensor for apoptotic cells. *Nano Lett* 2006;6:488–490. [PubMed: 16522048]
 34. Schellenberger EA, Reynolds F, Weissleder R, Josephson L. Surface-functionalized nanoparticle library yields probes for apoptotic cells. *Chembiochem* 2004;5:275–279. [PubMed: 14997519]
 35. Monteiro-Riviere NA, Inman AO, Zhang LW. Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. *Toxicol Appl Pharmacol* 2009;234:222–235. [PubMed: 18983864]
 36. Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol* 1978;52:302–310. [PubMed: 672633]
 37. Sayes CM, Fortner JD, Guo W, Lyon D, Boyd AM, Ausman KD, Tao YJ, Sitharaman B, Wilson LJ, Hughes JB, et al. The differential cytotoxicity of water-soluble fullerenes. *Nano Letters* 2004;4:1881–1887.
 38. Yang CF, Shen HM, Shen Y, Zhuang ZX, Ong CN. Cadmium-induced oxidative cellular damage in human fetal lung fibroblasts (MRC-5 cells). *Environ Health Perspect* 1997;105:712–716. [PubMed: 9294717]
 39. Lin W, Huang YW, Zhou XD, Ma Y. Toxicity of cerium oxide nanoparticles in human lung cancer cells. *Int J Toxicol* 2006;25:451–457. [PubMed: 17132603]
 40. Lin W, Huang YW, Zhou XD, Ma Y. In vitro toxicity of silica nanoparticles in human lung cancer cells. *Toxicol Appl Pharmacol* 2006;217:252–259. [PubMed: 17112558]
 41. Guo B, Zebda R, Drake SJ, Sayes CM. Synergistic effect of co-exposure to carbon black and Fe₂O₃ nanoparticles on oxidative stress in cultured lung epithelial cells. *Part Fibre Toxicol* 2009;6:4. [PubMed: 19203368]
 42. Ott M, Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S. Cytochrome c release from mitochondria proceeds by a two-step process. *Proc Natl Acad Sci U S A* 2002;99:1259–1263. [PubMed: 11818574]
 43. Hsin YH, Chen CF, Huang S, Shih TS, Lai PS, Chueh PJ. The apoptotic effect of nanosilver is mediated by a ROS- and JNK-dependent mechanism involving the mitochondrial pathway in NIH3T3 cells. *Toxicol Lett* 2008;179:130–139. [PubMed: 18547751]
 44. Chen X, Deng C, Tang S, Zhang M. Mitochondria-dependent apoptosis induced by nanoscale hydroxyapatite in human gastric cancer SGC-7901 cells. *Biol Pharm Bull* 2007;30:128–132. [PubMed: 17202672]

45. Park EJ, Choi J, Park YK, Park K. Oxidative stress induced by cerium oxide nanoparticles in cultured BEAS-2B cells. *Toxicology* 2008;245:90–100. [PubMed: 18243471]
46. Park EJ, Yi J, Chung KH, Ryu DY, Choi J, Park K. Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. *Toxicol Lett* 2008;180:222–229. [PubMed: 18662754]
47. Hall PA, Levison DA. Review: assessment of cell proliferation in histological material. *J Clin Pathol* 1990;43:184–192. [PubMed: 2185282]
48. Wyder M, Maier P, Zbinden G. Differential cytotoxic, growth-inhibiting and lipid-peroxidative activities of four different asbestos fibres in vitro. *Toxicology in Vitro* 1988;2:297–302.
49. Dong, L.; Joseph, KL.; Witkowski, CM. Nanotechnology. Vol. 19. 2008. Cytotoxicity of single-walled carbon nanotubes suspended in various surfactants..
50. Kapadia MR, Chow LW, Tsihlis ND, Ahanchi SS, Eng JW, Murar J, Martinez J, Popowich DA, Jiang Q, Hrabie JA, et al. Nitric oxide and nanotechnology: a novel approach to inhibit neointimal hyperplasia. *J Vasc Surg* 2008;47:173–182. [PubMed: 18178471]
51. Quinlan TR, Berube KA, Marsh JP, Janssen YM, Taishi P, Leslie KO, Hemenway D, O'Shaughnessy PT, Vacek P, Mossman BT. Patterns of inflammation, cell proliferation, and related gene expression in lung after inhalation of chrysotile asbestos. *Am J Pathol* 1995;147:728–739. [PubMed: 7677184]
52. Timblin C, Berube K, Churg A, Driscoll K, Gordon T, Hemenway D, Walsh E, Cummins AB, Vacek P, Mossman B. Ambient particulate matter causes activation of the c-jun kinase/stress-activated protein kinase cascade and DNA synthesis in lung epithelial cells. *Cancer Res* 1998;58:4543–4547. [PubMed: 9788597]
53. Park K, Lee GY, Kim YS, Yu M, Park RW, Kim IS, Kim SY, Byun Y. Heparin-deoxycholic acid chemical conjugate as an anticancer drug carrier and its antitumor activity. *J Control Release* 2006;114:300–306. [PubMed: 16884806]
54. Manning CB, Sabo-Attwood T, Robledo RF, Macpherson MB, Rincon M, Vacek P, Hemenway D, Taatjes DJ, Lee PJ, Mossman BT. Targeting the MEK1 cascade in lung epithelium inhibits proliferation and fibrogenesis by asbestos. *Am J Respir Cell Mol Biol* 2008;38:618–626. [PubMed: 18192500]
55. Hall PA, Woods AL. Immunohistochemical markers of cellular proliferation: achievements, problems and prospects. *Cell Tissue Kinet* 1990;23:505–522. [PubMed: 2276170]
56. Buder-Hoffmann S, Palmer C, Vacek P, Taatjes D, Mossman B. Different accumulation of activated extracellular signal-regulated kinases (ERK 1/2) and role in cell-cycle alterations by epidermal growth factor, hydrogen peroxide, or asbestos in pulmonary epithelial cells. *Am J Respir Cell Mol Biol* 2001;24:405–413. [PubMed: 11306433]
57. Kabanov AV. Polymer genomics: an insight into pharmacology and toxicology of nanomedicines. *Adv Drug Deliv Rev* 2006;58:1597–1621. [PubMed: 17126450]
58. Lanone S, Boczkowski J. Biomedical applications and potential health risks of nanomaterials: molecular mechanisms. *Curr Mol Med* 2006;6:651–663. [PubMed: 17022735]
59. Ames BN, Durston WE, Yamasaki E, Lee FD. Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. *Proc Natl Acad Sci U S A* 1973;70:2281–2285. [PubMed: 4151811]
60. Warheit DB, Hoke RA, Finlay C, Donner EM, Reed KL, Sayes CM. Development of a base set of toxicity tests using ultrafine TiO₂ particles as a component of nanoparticle risk management. *Toxicol Lett* 2007;171:99–110. [PubMed: 17566673]
61. Mori T, Takada H, Ito S, Matsubayashi K, Miwa N, Sawaguchi T. Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis. *Toxicology* 2006;225:48–54. [PubMed: 16782258]
62. Faux SP, Howden PJ, Levy LS. Iron-dependent formation of 8-hydroxydeoxyguanosine in isolated DNA and mutagenicity in *Salmonella typhimurium* TA102 induced by crocidolite. *Carcinogenesis* 1994;15:1749–1751. [PubMed: 8055658]
63. Nel A, Xia T, Madler L, Li N. Toxic potential of materials at the nanolevel. *Science* 2006;311:622–627. [PubMed: 16456071]

64. Fung H, Kow YW, Van Houten B, Mossman BT. Patterns of 8-hydroxydeoxyguanosine formation in DNA and indications of oxidative stress in rat and human pleural mesothelial cells after exposure to crocidolite asbestos. *Carcinogenesis* 1997;18:825–832. [PubMed: 9111221]
65. Chen Q, Marsh J, Ames B, Mossman B. Detection of 8-oxo-2'-deoxyguanosine, a marker of oxidative DNA damage, in culture medium from human mesothelial cells exposed to crocidolite asbestos. *Carcinogenesis* 1996;17:2525–2527. [PubMed: 8968073]
66. Papageorgiou I, Brown C, Schins R, Singh S, Newson R, Davis S, Fisher J, Ingham E, Case CP. The effect of nano- and micron-sized particles of cobalt-chromium alloy on human fibroblasts in vitro. *Biomaterials* 2007;28:2946–2958. [PubMed: 17379299]
67. Jin Y, Kannan S, Wu M, Zhao JX. Toxicity of luminescent silica nanoparticles to living cells. *Chem Res Toxicol* 2007;20:1126–1133. [PubMed: 17630705]
68. Theogaraj E, Riley S, Hughes L, Maier M, Kirkland D. An investigation of the photo-clastogenic potential of ultrafine titanium dioxide particles. *Mutat Res* 2007;634:205–219. [PubMed: 17855159]
69. Rahman Q, Lohani M, Dopp E, Pemsel H, Jonas L, Weiss DG, Schiffmann D. Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts. *Environ Health Perspect* 2002;110:797–800. [PubMed: 12153761]
70. Kang SJ, Kim BM, Lee YJ, Chung HW. Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes. *Environ Mol Mutagen*. 2008
71. Wang JJ, Sanderson BJ, Wang H. Cyto- and genotoxicity of ultrafine TiO₂ particles in cultured human lymphoblastoid cells. *Mutat Res* 2007;628:99–106. [PubMed: 17223607]
72. Kumaravel TS, Jha AN. Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. *Mutat Res* 2006;605:7–16. [PubMed: 16621680]
73. Dhawan A, Taurozzi JS, Pandey AK, Shan W, Miller SM, Hashsham SA, Tarabara VV. Stable colloidal dispersions of C60 fullerenes in water: evidence for genotoxicity. *Environ Sci Technol* 2006;40:7394–7401. [PubMed: 17180994]
74. Berry CC, Charles S, Wells S, Dalby MJ, Curtis AS. The influence of transferrin stabilised magnetic nanoparticles on human dermal fibroblasts in culture. *Int J Pharm* 2004;269:211–225. [PubMed: 14698593]
75. Yamawaki H, Iwai N. Mechanisms underlying nano-sized air-pollution-mediated progression of atherosclerosis: carbon black causes cytotoxic injury/inflammation and inhibits cell growth in vascular endothelial cells. *Circ J* 2006;70:129–140. [PubMed: 16377937]
76. Zhang Y, Hu Z, Ye M, Pan Y, Chen J, Luo Y, Zhang Y, He L, Wang J. Effect of poly(ethylene glycol)-block-poly(lactide) nanoparticles on hepatic cells of mouse: low cytotoxicity, but efflux of the nanoparticles by ATP-binding cassette transporters. *Eur J Pharm Biopharm* 2007;66:268–280. [PubMed: 17182234]
77. Chen HW, Su SF, Chien CT, Lin WH, Yu SL, Chou CC, Chen JJ, Yang PC. Titanium dioxide nanoparticles induce emphysema-like lung injury in mice. *Faseb J* 2006;20:2393–2395. [PubMed: 17023518]
78. Cha MH, Rhim T, Kim KH, Jang AS, Paik YK, Park CS. Proteomic identification of macrophage migration-inhibitory factor upon exposure to TiO₂ particles. *Mol Cell Proteomics* 2007;6:56–63. [PubMed: 17028300]
79. Seo SW, Lee D, Cho SK, Kim AD, Minematsu H, Celil Aydemir AB, Geller JA, Macaulay W, Yang J, Young-In Lee F. ERK signaling regulates macrophage colony-stimulating factor expression induced by titanium particles in MC3T3.E1 murine calvarial preosteoblastic cells. *Ann N Y Acad Sci* 2007;1117:151–158. [PubMed: 18056041]
80. Bitterle E, Karg E, Schroepel A, Kreyling WG, Tippe A, Ferron GA, Schmid O, Heyder J, Maier KL, Hofer T. Dose-controlled exposure of A549 epithelial cells at the air-liquid interface to airborne ultrafine carbonaceous particles. *Chemosphere* 2006;65:1784–1790. [PubMed: 16762398]
81. Papis E, Gornati R, Prati M, Ponti J, Sabbioni E, Bernardini G. Gene expression in nanotoxicology research: analysis by differential display in BALB3T3 fibroblasts exposed to cobalt particles and ions. *Toxicol Lett* 2007;170:185–192. [PubMed: 17412531]
82. Mossman BT, Shukla A, Fukagawa NK. Highlight Commentary on “Oxidative stress and lipid mediators induced in alveolar macrophages by ultrafine particles”. *Free Radic Biol Med* 2007;43:504–505. [PubMed: 17640559]

TABLE I**Assays for Assessing the Pathogenic Potential of Nanomaterials**

Endpoints:	
Cytotoxicity:	Trypan blue exclusion assay, MTA assays, clonogenic (CFE) assay, LDH assay, TUNEL assay, Apostain technique, flow cytometry with PI, 7AAD, and/or Annexin V, lipid peroxidation, cytochrome c release from mitochondria, caspase activation
Proliferation:	DNA content, [³ H]thymidine incorporation, BrdU incorporation, Ki-67, and detection of PCNA
Genotoxicity:	Ames assay (<i>S. typhimurium</i> or <i>E. coli</i>), detection of DNA base modifications, karyotype analyses (induction of chromosome aberrations and micronuclei), comet assay
Gene Expression:	Northern blot analyses, ribonuclease protein assays (RPA), real-time PCR, PCR arrays, microarrays

*All abbreviations are described in the text.