Nanotechnology Environmental, Health, and Safety Research

Assess the *in vitro* and *in vivo* toxicological pathways induced after exposure to nanoparticles and the components of nano-enabled devices

September 15, 2011
Summary of All Objectives of the Work Project

To produce a comprehensive technology landscape and review of the state-of-the-art and state-of-the-science relevant to nanobiotechnology

- **OBJECTIVE 1.** Review the currently used and soon to be used nanoparticles and nano-enabled devices in pharmaceutical and other biomedical industries
  - Describe the various techniques and tools used to characterize the nanomaterials

- **OBJECTIVE 2.** Assess the *in vitro* and *in vivo* toxicological pathways induced after exposure to nanoparticles and the components of nano-enabled devices

- **OBJECTIVE 3.** Survey the current efforts in the emerging field of predictive toxicology and the regulatory policies that may result from mathematical and statistical based tools in nanotoxicology
  - Examine the gaps in regulation, policies, and standards relevant to the incorporation of NPs in nano-enabled devices
THIS REPORT INCLUDES:

**OBJECTIVE 2.** Assess the *in vitro* and *in vivo* toxicological pathways induced after exposure to nanoparticles and the components of nano-enabled devices
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The inclusion of nanoparticles into consumer-based products is increasing at an exponential rate. These particles, which include colloids, metal oxides, and carbonaceous particles, are at the forefront of basic and applied research, product development, and environmental health and safety assessments. The nanotechnology sector, when combined with the biotechnology sector, is expected to generate revenue upwards of $2.6 trillion by the year 2014 (Dai 2010). Nanomaterials hold the potential to generate added value to consumer products through the addition of novel properties such as increased scratch resistance, insulating capacity, or antibacterial abilities (Richardson 2010).

The incorporation of nanoparticles into consumer products elicits the need to develop a wide range of information that is needed for risk assessment and ultimately risk management. Course variables needed for these types of assessments include material physicochemical characterization, hazard identification, and exposure analyses. Finer variables needed for each of these course variables include characterization over time and concentration in a variety of matrices, mechanisms of uptake and subsequent action on both the organism and cellular levels, and life-cycle analyses of materials from cradle to grave. This white paper describes some of the mechanisms of action and pathway-specific toxicities, at the molecular level, induced after nanoparticle exposure.

The following table includes a literature review on the currently identified engineered nanoparticle-induced toxicological pathways in either *in vitro* (i.e. cell or tissues culture systems) or *in vivo* (i.e. whole animal systems). While there is very little evidence of any components of nano-enabled devices inducing pathway-specific toxicities, it is reasonable to postulate that the nanomaterial-induced pathways described below may be relevant to nano-enabled devices.

It is noteworthy to define engineered nanomaterials, nanoparticles, and nano-enabled devices:

An **engineered nanomaterial** is a substance deliberately designed and produced by man that includes both engineered nanoparticles and incidental nanoparticles, as well as membranes, fibers, wires, and other objects in the nanometer size scale ranging 1 to 250 nm only because specific mechanical, electronic, optical, or other property are unique to that nanometer size scale.

A **nanoparticle** is a three dimensional particulate engineered, often in isometric in shape, to have a diameter measuring between 1 and 250 nm and exhibits novel properties that differ from their larger sized (e.g. micrometer size scale) counterpart of identical composition. Sometimes a particulate in other shapes (such as cylinders, pyramids, cubes, boxes, or cones) are also referred to as **nanoparticle**. In these examples, only one dimension measuring less than 250 nm is required to be called **nanoparticle**.

A **nano-enabled device** is an engineered sensor, implant, drug, or other tangible object that contains an embedded component material on the nanometer size scale that was intentionally synthesized, self-assembled, assisted to self-assemble, or structured to create novel properties, processes, or principles.
Table 1. Mechanisms of action and pathway-specific toxicities, at the molecular level, induced after nanoparticle exposure. The table is sub-divided into the following categories: NF-κB, NF-κB and Nrf-2, MAPKs, MAPKs and Nrf-2, and Cell Cycle. Each category contains information on the particle-type used in the study, the genes or proteins perturbed, the test system and doses used in the study, and the literature reference.

<table>
<thead>
<tr>
<th>Pathway Identified</th>
<th>Particle-Type (and Size)</th>
<th>Genes or Proteins</th>
<th>Test System (and Doses)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB</td>
<td>ZnO (24-70 nm)</td>
<td>NF-κB, IL-8, p-p65, p-IκB</td>
<td>BEAS-2B cells (2-8 μg/mL)</td>
<td>[1]</td>
</tr>
<tr>
<td>NF-κB</td>
<td>ZnO (&lt;100 nm) and CdS (10 nm)</td>
<td>NF-κB</td>
<td>IP15 (glomerular mesangial) and HK-2 (epithelial proximal) cell lines (5 μg/cm²)</td>
<td>[7]</td>
</tr>
<tr>
<td>NF-κB</td>
<td>TiO₂, P25 (170 nm)</td>
<td>NF-κB, TLR4</td>
<td>NIH/3T3 (10 ng/mL)</td>
<td>[8]</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Poly(acrylic acid)-coated gold in the presence of fibrinogen (5, 10, and 20 nm)</td>
<td>Mac-1 integrin receptor, NF-κB</td>
<td>THP-1 (10 μg/mL)</td>
<td>[14]</td>
</tr>
<tr>
<td>NF-κB</td>
<td>CdSe/ZnS-COOH QD (15 nm)</td>
<td>NF-κB, TNF-α, IL-1B and IL-10, HMOX-1, IL-6</td>
<td>HDF (30 and 60 nM)</td>
<td>[15]</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Ag, Au, Pt, Al2O3, Fe3O4, SiO2, and ZnO (12 to 20 nm)</td>
<td>NF-κB, CRE, HIF-1α, AP-1, NFAT, SMAD, SRF, E2F, MyC, p53</td>
<td>RAW 264.7 macrophages (0.375-100 μg/mL)</td>
<td>[16]</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Tungsten carbide cobalt (62 nm)</td>
<td>SOX2, ID2, FOXO4 and others related to hypoxia response, carbohydrate metabolism, and endocrine pathways</td>
<td>HaCaT keratinocytes (33 μg/mL)</td>
<td>[6]</td>
</tr>
<tr>
<td>General ROS</td>
<td>Cu₂O (10-20 nm)</td>
<td>MT, MTF1, CTR1, SOD, ATP7A &amp; 7B</td>
<td>Zebrafish larvae, zebrafish hepatocytes (28-55 ppm)</td>
<td>[10]</td>
</tr>
</tbody>
</table>

NF-κB and Nrf-2

| 6 combustion-derived NPs: UfCP, SootL, SootH, Ptx90, DEP, PtxG (800-43 m²/g) | CSF2, CXCL1/5, IL-6, MT1/2, CYP1A1, NQO1, OGG1 | BALB/cJ mice (0.5-50 μg/μL) | [3]        |
MAPKs

<table>
<thead>
<tr>
<th>MAPKs</th>
<th>CDSe/ZnS QD (10.5 nm)</th>
<th>TNF-α, CXCL8, p47phox, p-p38</th>
<th>PBMC, THP-1 cells (0.2-25 nM) and BALB/c mice (2 nmol/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAPK, EGF-R</td>
<td>Printex 90 (14 nm)</td>
<td>ERK1/2, JNK1/2, Casp3, Phosphotyrosine</td>
<td>RLE-6TN rat lung epithelial cells (1-10 μg/cm²) [12]</td>
</tr>
<tr>
<td>Akt, ERK1/2</td>
<td>Printex 90 (14 nm)</td>
<td>Akt, ERK1/2, GSK-3 fusion protein</td>
<td>RLE-6TN rat lung epithelial cells (1-10 μg/cm²) [11]</td>
</tr>
</tbody>
</table>

MAPKs and Nrf-2

<table>
<thead>
<tr>
<th>MAPK (ERK), Nrf-2</th>
<th>Si, porous (5-15 nm, 349.71 m³/g) and Si, fumed (7 nm, 644.44 m³/g)</th>
<th>SOD, HO-1</th>
<th>BEAS-2B cells (1 mg/L) [4]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK, p-38, Nrf-2, JNK</td>
<td>CeO₂ (15, 30, and 45 nm)</td>
<td>Nrf-2, HO-1, p-38, JNK, SOD</td>
<td>BEAS-2B cells (1 mg/L) [5]</td>
</tr>
</tbody>
</table>

Cell Cycle

<table>
<thead>
<tr>
<th>Cell cycle, MAPK, Nrf-2, PI3K/AKT, glycolysis</th>
<th>TiO₂, P25 (125 nm)</th>
<th>SOD2, YWHA2, PRDX6, YWHAB, YWHA3, YWHA2, AMPK2, and GSK-3α/β</th>
<th>BEAS-2B (10 μg/mL) [13]</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>Nickel ferrite</td>
<td>p53, survivin, Bax/bcl-2, and casapase</td>
<td>A549 (25-100 μg/mL) [9]</td>
</tr>
</tbody>
</table>

REFERENCES


7. Igor Pujalté, I.P., Brigitte Brouillaud, Mona Tréguer, Etienne Durand, Céline Ohayon-Courtès, and Béatrice L'Azou, Cytotoxicity and oxidative stress induced by different metallic nanoparticles on human kidney cells. Particle and Fibre Toxicology, 2011. 8(10).


The following engineered nanomaterial-induced toxicological pathways have been identified in the current peer-reviewed literature. Detailed descriptions of the NF-κB and Nrf-2 pathways are described below.

INTRODUCTION

There has been rapid progress in the field of nanobiotechnology in recent years; however, information regarding the potential for adverse health outcomes arising from nanoparticle exposures is still in its infancy. For example, cellular immunoregulatory response to nanoparticles is not well understood. Various exposures (such as dermal, oral, inhalation, and intentional intravenous) to such materials are and will continue to be a relevant and, thus require further investigation. Engineered nanomaterials and particles within nano-enabled devices are thought to induce different biological effects on organisms when compared to materials of the same composition but on the micrometer size scale. This hypothesis has been proposed because a nanoparticle has a smaller particle diameter and corresponding larger surface area per unit mass [1-3]. The smaller size would allow for increased translocation and penetration within and around cells and tissues and the larger surface area would allow for greater surface reactivity with micro and macromolecules resulting in changes in structure and function.

However, it is largely unknown if specific pathways or subcellular mechanisms of action are triggered as a result of engineered nanomaterial exposure. Some reports have shown that nanomaterials can be internalized into cells and others have speculated on specific routes of entry for nanometer sized particles [4-6]. But if there is internalization, what is the consequence? Are there any particle-induced mechanisms of injury? If so, who are the key participants on the molecular level inside the cell? And what do those processes develop into?

There have been reports in the scientific literature that found that immune mediators of inflammation, such as NF-κB, ERK1/2, and Nrf-2 transcription factors, may be initiated after nanomaterial exposure in exposed tissues and cells. This initiation is then followed by upregulation of inflammatory response proteins. Both the initiation of mediators and response of the cell is dependent, however, on nanoparticle-type, dose, and cell-type.

NF-κB

NF-κB is a major transcription factor responsible for regulating genes of both the innate and adaptive immune response [8]. NF-κB becomes activated through distinct signaling components: inactivated, cytosolic NFκB is complexed with the inhibitory IκBα (NFKBIA) protein. A variety of extracellular signals can be recognized via integral membrane receptors, which can lead to the activation of the enzyme IκB kinase (IKK or IKBKB). The role of IKK is to phosphorylate the NF-κB-associated IκBα protein, resulting in ubiquination and dissociation of IκBα from NF-κB. IκBα is degraded by the proteosome and the liberated NF-κB is then translocated into the nucleus where it binds to specific DNA motifs in promoters, termed response elements. Here, it can upregulate genes involved in immune cell development, and maturation, as well as those dedicated to survival, inflammation, and lymphoproliferation [9].

Certain cell types have also been shown to be capable of phagocytosing nanoparticles and setting off inflammatory responses [18]. Hoshino et al. (2009) found that direct injections of QD/nucleotide complexes into the peritoneal cavity of mice resulted in inflammation with the infiltration of inflammatory cells [19]. Lee et al. found that upregulation of ERK 1/2 occurred in monocytes, THC-1 cells, and BALB/c mice after exposures to CdSe/ZnS QDs [20]. There are conflicting data in the literature reporting both pro- and anti-oxidant properties after fullerol exposures. C60 fullerenes have been shown to be toxic to HDF cells, but hydroxylated fullerenes have not been tested in dermal cells [21-22]. For silver, Rolla et al. found in a multi-nanoparticle study assessing possible modulation of many cellular signaling pathways that silver significantly downregulated the SMAD/TGF-β
pathways, but upregulated the HIF-1α pathway [23]. Titanium dioxide has been shown to modulate the NF-kB pathway in both NIH-3T3 and BAES-2B cells exposed to 10 ng/mL and 10 μg/mL, respectively [24-25]. These studies tested aggregated P25 Degussa particles however, unlike the present study. Since such findings of cellular perturbation have been presented in the literature, further inquiry of the mechanisms of gene induction or suppression is necessary.

A theme that emerges in the immune-transcriptional profile of cells treated with nanoparticles is inflammation. Up-regulated genes are pattern recognition receptors (PRRs) and their signal transduction machinery, inflammatory cytokines, and receptors are responsible for producing the cell signals. PRR upregulation induces inflammation by preparing transcripts that put the cell on a hair-trigger that escalates (and in some cases heightens) this response. Two biomarkers for this inflammation are the increase in MYD88, which conveys recognition of a threat by a PRR, and the onset in MAP kinase cascade. In evaluating early transcription factor activity, it is evident that the MAP kinase protein ERK1/2 is often upregulated very early (i.e. within a few minutes of exposure). Phosphorylation of IκB is usually immediately follows ERK1/2 activation.

**Research Recommendations:**

1. Specific, pathway-driven cellular responses to nanoparticle exposures need to be further elucidated for improved drug delivery, immune/inflammatory response mechanism elucidation, and gathering of basic toxicological response data. Comparative nanoparticle studies are needed to assess the effects of altering different physicochemical properties, such as chemical composition, surface charge and crystalline structure on appropriately dosed cells, tissues, and animals.

2. In assessing the extent of NF-kB dependency in cells exposed to nanoparticles, an experimental design comparing normal primary cells with IκB-overexpressed cells is needed. Endpoints such as differences in viability, antioxidant depletion, gene up and downregulation, and downstream protein upregulation should be included.

3. Nanomaterial physicochemical properties, reactive oxygen species generation, and cellular viability should be linked, systematically, to antioxidant ability, pathway-specific mechanisms of toxicity, and apoptosis, and DNA damage.

4. To evaluate the response to nanoparticles in normal cells, a two-fold approach must be used (at minimum). First, the transcriptional response should be probed via a RT-PCR gene array. Second, the translational activity should be studied by way of protein analysis.

**NRF2**

Expression of enzymes and low molecular weight antioxidants present in the intracellular environment remains a critical aspect of toxicology when dealing with potential insults, such as small particles, that act through an oxidative stress mechanism. When cells are exposed to nanoparticles, reactive oxygen species are sometimes produced. While these cells are sometimes exposed to reactive oxygen species through their aerobic environment as well as from byproducts of normal cellular metabolism, an increase in intracellular oxidants due to exposure to nanoparticles may lead to damage of protein, lipids, and DNA. This event becomes extremely important when comparing the effects of nanoparticles from one cell type to another. Not all cells are the same and not all cells respond to particles in the same way.
There are many different biomarkers that can be probed when an oxidative stress pathway is suspected to be perturbed. Catalase, heme oxygenase (decycling) 1, glutathione, superoxide dismutase, cyclooxygenase, nuclear factor kB and toll-like receptors are all genes, enzymes, and proteins commonly associated with oxidative stress. Specific to glutathione, GSH may play a role in the metabolism of oxidants, such as those produced after the exposure to nanoparticles, by acting through the enzyme glutathione peroxidase. Glutathione peroxidase catalyzes the oxidation of GSSG to GSH and water (Heffner and Repine 1989). Studies have shown that differences in glutathione levels may be due to differences in the rate of GSH synthesis. For example, Jarvin and others (2000) have shown that expression of γ-GCS, the rate limiting enzyme in glutathione synthesis, has been shown to be higher in epithelial cells than mesothelial cells (Jarvinen, Pietarinin-Runtti et al. 2000). Similarly, in vivo experiments dictate that normal bronchial epithelium contains increased γ-GCS over normal mesothelium (Puhakka, Ollikainen et al. 2002).

Treatment of cells with electrophilic xenobiotics often invokes the transcriptional activation of genes involved in both detoxification and metabolism. This reaction, often known as the electrophile counterattack response, is, in part, mediated by the binding of sensitive transcription factors to the antioxidant response element located in the promoter regions of genes involved in reactive oxygen species detoxification (Prestera, Zhang et al. 1993; Ishii, Itoh et al. 2000). Central to this response is the NRF2-KEAP1 transcription factor. NRF2, a cap ‘n’ collar basic leucine zipper transcription factor, remains bound to KEAP1 in the cytosol under normal redox conditions. In this state, forced proteosomal degradation of NRF2 limits intracellular accumulation. However, under conditions of oxidative stress, the NRF2-KEAP1 bond is cleaved resulting in increased overall NRF2 levels and subsequent nuclear translocation. Recent literature suggests that a variety of cell types harbor loss of function and mutations in KEAP1, which increase basal stabilization and translocation of NRF2 (Singh, Misra et al. 2006). NRF2 has been reported play a role in the regulation of both catalase and glutathione synthesis, however; NRF2-null cells demonstrate no change in superoxide dismutase enzymatic activity (Zhu, Itoh et al. 2005). Exposure to nanoparticles is of no exception – decomplexing of the NRF2-KEAP1 bond following translocation of NRF2 across the nucleus.

An increase in overall NRF2, combined with nuclear translocation, is believed to occur after treatment with some nanoparticles. These types of results have been seen for CeO$_2$ nanoparticles (Eom and Choi 2009). While NRF2 stabilization and nuclear translocation were documented, confirmation of a protein with a gene that lies downstream of the NRF2 consensus binding sequence was not confirmed. Catalase, for which the basal expression was earlier assessed, has previously been reported to be regulated by NRF2 and was thus identified as an ideal candidate (Zhu, Itoh et al. 2005). Following nanoparticle exposure, catalase is often induced – at different time points depending on cell types. The induction of catalase is part of a NRF2 pathway-specific response. However, increased expression of various antioxidant phase II genes may also play an important role in the response to oxidative stress driven by nanoparticles.

As the field of nanotoxicology increases its breadth and depth in human and environmental health research, the utilization of in vitro toxicological tests will play a central role in the identification of potential cellular targets. The goal of this line of research is to supplement the current particle toxicology literature and serve as a basis for future in vivo hypothesis-driven research. Additionally, the use of multiple cell types in a mechanistic toxicity study could further strengthen hazard identification and exposure evaluations, thereby contributing to future comprehensive nanomaterial risk assessments.
Dr. Christie M. Sayes is the Program Manager for Nanotoxicology & Nanopharmacology in the Center for Aerosols and Nanomaterials Engineering at RTI International. She was formerly a professor of toxicology at Texas A&M University. Dr. Sayes maintains her adjunct faculty appointment at Texas A&M in the Department of Biomedical Engineering and the Interdisciplinary Program in Toxicology. She has more than a decade of experience in the fields of nanotechnology and nanotoxicology. She has authored numerous publications, including original research, invited reviews and book chapters. She is a member of the Society of Toxicology, the American Chemical Society, and the Society of Environmental Toxicology and Chemistry. She serves on the Scientific Advisory Board for the EPA’s FIFRA Program and on the Editorial Board of the journals *Nanotoxicology* and *Toxicology Letters*. Recently, she was elected onto the Executive Committee of the North Carolina Chapter Society of Toxicology. Dr. Sayes has proven abilities in providing technical guidance and leadership to students, technicians, and colleagues; a high aptitude for development of complex particle toxicological and biocompatibility basic and applied research projects in cell culture based and animal based models; substantial training in nanomaterial & nanotoxicology research techniques & instruments; significant experience working independently & collaborating across disciplines and organizations; excellent communication and interpersonal skills with colleagues in science and engineering, senior management, and new and existing clients and other funding sources.