In-vitro and In-vivo toxicity of nanoparticles

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Outline

- Nanotoxicology
- Physicochemical properties of nanomaterials: biological effects
- Methods for assessing toxicity of nanomaterials
- Conclusion
Nanotoxicology was proposed as a new branch of toxicology to address the adverse health effects likely to be caused by nanomaterials.

Donaldson et al. 2004 quoted “discipline of nanotoxicology would make an important contribution to the development of a sustainable and safe nanotechnology”.

Arora et al. 2012
Nanotoxicology

- Physiochemical determinants
  - Surface area
  - Size/Shape

- Regulatory issue

- Routes of exposure
  - Respiratory tract
  - Skin
  - Gastrointestinal tract

- Biodistribution
  - Clearance
  - Opsonization

- Genotoxicity

- Molecular determinant

- Inflammation

- Oxidative stress

- Chromosomal aberrations

- Mutagenesis

- Government

- Industry

- Academy

Arora et al. 2012
Properties of Nanoscale Materials

The same properties making nanomaterials so interesting can make them potentially harmful:

• Large surface area
• Catalytic reactivity
• Enhanced permeation
• Light emission properties
Biological effects due to physicochemical properties of nanoparticles

<table>
<thead>
<tr>
<th>Physicochemical property</th>
<th>Toxicokinetic finding</th>
<th>Biological effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Size</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 nm gold NPs</td>
<td>Most widespread organ distribution including blood, liver, lung, spleen, kidney, brain,.....</td>
<td>Biodistribution of the nanoparticles</td>
</tr>
<tr>
<td>50 nm gold NPs</td>
<td>Pass blood-brain barrier in mice</td>
<td>Blood brain barrier permeability</td>
</tr>
<tr>
<td><strong>Shape</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spherical gold NPs</td>
<td>Higher uptake by Hela cell</td>
<td>Rode shape gold NPs showed less uptake</td>
</tr>
<tr>
<td>Open ended single-walled carbon nanotube</td>
<td>Blocking of ion channels in CHO cells</td>
<td>Close-ended SWNTs are comparatively less reactive</td>
</tr>
<tr>
<td><strong>S/V ratio</strong></td>
<td></td>
<td></td>
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<tr>
<td>TiO₂ (300cm² surface area)</td>
<td>Increased lymph-node burdens and inflammation</td>
<td>More reactives in rats as compared to BaSO₄ inflammation</td>
</tr>
<tr>
<td>TiO₂ and BaSO₄ with same surface area</td>
<td>Inflammatory effects were similar</td>
<td></td>
</tr>
</tbody>
</table>

Arora et al. 2012
What is special with small particles?

1. Deposition

2. Surface area

higher energy
3. Cell entry

What is special with small particles?

nano- Trojan horse

metal oxide nanoparticle
metal ions

intracellular dissolution, damage through ions
catalytic generation of ROS by undissolved nanoparticles

metal oxide microparticles

pH = 5.5

1 µm

Picture from Limbach et al, CHIMIA, 2009
General approach for nanoparticles

Particles

Generated in the laboratory

Characterization

Commercially available/ provided by collaborators

Particle size in solution (DLS)

TEM analysis

50 nm

Metal release

Zeta potential

Toxicity

Cell death

DNA damage

Mitochondrial damage

Reactive oxygen species
We are exposed to (nano)particles
How Might We be Exposed?

• Routes of exposure:
  – Injection
  – Ingestion
  – Inhalation
  – Dermal exposure
Olfactory nerve exposure and central nervous system effects
Regional Lung Deposition

particle density: 1 g cm$^{-3}$
respiratory flow rate: 300 cm$^{3}$s$^{-1}$
respiratory cycle period: 5 s

Michael Ellenbecker, 2012
Dermal Exposures

Michael Ellenbecker, 2012
Biodistribution

Physical clearance processes

- Mucociliary movement
- Epithelial endocytosis
- Interstitial translocation
- Blood circulation translocation

Chemical clearance processes

- Dissolution
- Leaching and protein binding

(S. Arora et al., 2012)
Biodistribution

Certain kinds of nanoparticles
- Absorption across the GIT barrier and entry into the systemic circulation

Some nanoparticles
- Accumulate in the liver during first-pass metabolism

After intravenous administration
- Nanoparticles get distributed to colon, lungs, bone marrow, liver, spleen and lymphatics

(Curtis et al., 2006; Oberdorster et al., 2005)
(Oberdorster et al., 2005)
(Fabian et al., 2008)
Biodistribution

Clearance of nanoparticles depends on:

- Size                   Gold nanoparticles
- Surface characteristics hydrophobic particles

Garnett and Kallinteri, 2006
Jong et al., 2008
Understanding the relevance of physicochemical Properties (size, surface charge, hydrophilicity) of Nanoparticles and their ADME is critical:

ADME

Li and Huang 2008
Molecular determinants

• When exposed to light or transition metals, nanoparticles may promote the formation of pro-oxidants destabilizes the delicate balance between the biological system's ability to produce and detoxify the reactive oxygen species (ROS)

S. Arora et al 2012
Molecular determinants are nanomaterial characteristics that can culminate in ROS generation.
Possible pathophysiological outcomes due to various nanomaterials

**Experimental NM effects**

1. ROS generation
2. Oxidative stress
3. Inflammation
4. Uptake in neuronal tissue
5. DNA damage
6. Protein denaturation
degradation

**Possible pathophysiological outcomes**

- Protein, DNA and membrane injury
- Inflammation, mitochondrial perturbation
- Fibrosis, granulomas
- Brain and peripheral nervous system injury
- Mutagenesis, metaplasia, carcinogenesis
- Loss of enzyme activity

Nel et al 2006
Oxidative stress - damage to mitochondria

- Effects on mitochondrial e⁻ transfer chain
- Indirect effects on mitochondrial function
  - $\text{H}_2\text{O}_2$ and increase $\text{Ca}^{2+}$ in cytosol
- Structural damage

Genotoxicity and immunogenic potential

• ROS (Reactive oxygen species) due to their high chemical reactivity can react with DNA, protein, carbohydrates and lipids in a destructive manner causing cell death either by apoptosis or necrosis.

• The most frequently affected macromolecules are those genes or proteins which have roles in oxidative stress, DNA damage, inflammation or injury to the immune system.

S. Arora et al 2012
Genotoxicity and immunogenic potential

- Sub Micronic to nanometer-sized preparations of SiO$_2$ were found to increase arachidonic acid metabolism eventually leading to lung inflammation and pulmonary disease as well as genes expression directly related to inflammation.

Driscoll et al 1996
Genotoxicity and immunogenic potential

Primary Genotoxicity

Directly related to the exposure of the ‘substance’

Secondary Genotoxicity

• Substance’ interacting with cells or tissues and releasing factors, which, in turn, cause adverse effects such as inflammation and oxidative stress

Most investigations on genotoxicity and cellular interactions of engineered nanomaterials are limited to screening for cytotoxicity. A few studies have focused on immunological responses of nanoparticles

S.Arora et al 2012
Mechanisms for DNA damage

Primary genotoxicity

- Non-uptake
- Lipid peroxidation?

Cellular uptake

- Direct DNA damage
- Indirect DNA damage

- Damage to:
  - Spindle apparatus
  - DNA repair enzymes
  - Cell cycle control proteins

- DNA lesions
- Strand breaks

Indirect DNA damage

- Mitochondrial damage
- Antioxidant depletion
- ROS

DNA lesions
- Strand breaks
- Aneuploidy

Mitochondrial damage
- Antioxidant depletion
- ROS
- Oxidative DNA damage

- DNA lesions
- Strand breaks
- Aneuploidy

Inflammatory response

- ROS
- Oxidative DNA damage

Genotoxicity

- Mutations
- Cancer

(S. Arora et al., 2012)
Methods for assessing toxicity of nanomaterials

- Both in vitro and in vivo studies on biological effects of nanoparticles need to be performed.

*Donaldson et al., 2009*
Limited to one cell type or combination of just a few cell types

Effects on immune system are more limited and difficult

Can not be used for true pharmacokinetic or toxicokinetic studies

Disadvantages of in vitro systems

(S. Arora et al., 2012)
Ethical desire to reduce animal testing

The advantages of in vitro systems

Speed of results

Lower cost compared to in vivo studies

(S. Arora et al., 2012)
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.
General In vitro methods for nanotoxicity assessment

- Cell viability assay
  - Proliferative assay
  - Apoptosis assay
  - Necrosis assays

- Oxidative stress assay
  - Lipid peroxidation
  - Plasmid assay
  - Oxidative stress

- Inflammatory assay

ELIZA

Poonam Takhar et al 2011
Proliferative assay

- MTT assay (Fullerenes, carbon nanoparticles)
- Alamar Blue (Quantum dots)
- Incorporation of (3H)thymidin into DNA (carbon nanoparticles)
- Colony formation assay (Carbon based nanomaterials)
Cell viability - mitochondrial activity MTT assay

- Measures activity of enzymes that reduce MTT (yellow) to formazan (purple)
- Analyze absorbance of this colored solution by a spectrophotometer
MTT assay

1. Particles may generate an absorbance at the same wavelength as that used to quantify the colored product → Overestimation of cell viability

2. Surface properties can result in a high adsorptive capacity which allows the NPs to effectively extract the colored product from cell extract, → underestimation of cell viability

3. NPs can exhibit oxidative surface properties and the color production occurs via an oxidative reaction

Viki Stone et al 2012
DNA laddering (Silver nanoparticles)

Caspase assays (Silver nanoparticles)

Comet assay (metal nanoparticles, Magnetic nanomaterials)

Annexin V (Chitosan nanomaterials)

Apoptosis assay
Annexin V (propidium iodide staining)

This assay measures fluorescence and particles could interfere by variety of ways

- Physical blocking of the light emitted
  - carbon
- Reflection of the excitation light
  - TiO$_2$
- Particle-induced fluorescence
  - QDs, polystyrene beads
Annexin V (propidium iodide staining)

- Gold nanoparticles have been shown to bind propidium iodide and to be taken up by intact cell culture cells. So False positive in the detection of necrotic cells.
Trypan blue assay
(Gold nanoparticles)

LDH
(Zinc oxide nanoparticle)

Neutral red uptake
(iron oxide and TiO$_2$ nanoparticles)

Necrosis assay
Trypan blue staining

It is not sufficiently sensitive or reliable to use for in vitro toxicity testing and not appropriate for high throughput testing mainly due to the requirement for manual counting of cell.

Viki Stone et al 2012
Large surface area of nanoparticles provides the possibility of interference due to adsorption of the LDH protein on the particle surface. So LDH adsorption can contribute to underestimation of nanoparticle-induced cytotoxicity.

Viki Stone et al 2012
Trypan blue staining and LDH – assay (membrane damage)

- Cu nano inhibit LDH
- TiO$_2$ nano adsorb LDH

Validation of an LDH assay for assessing nanoparticle toxicity

Xianglu Han$^{a,*}$, Robert Gelein$^a$, Nancy Corson$^a$, Pamela Wade-Mercer$^a$, Jingkun Jiang$^d$, Pratim Biswas$^e$, Jacob N. Finkelstein$^{a,b,c}$, Alison Elder$^a$, Günter Oberdörster$^a$
Neutral red uptake

• Some nanoparticles like single wall carbon nanotubes have been shown to interact with neutral red and deplete the dye from the cell supernatant leading to false positive results.
Genotoxicity assays which uses for nanoparticles

- Salmonella reverse mutation assay
- Micronucleus test
- Alkaline comet assay

Viki Stone et al, 2012
Ames Test

- This test has been used for genotoxicity of various nanoparticles, such as TiO$_2$, fullerenes or carbon nanotubes.
Salmonella reverse mutation assay (Ames Test)

This test is usually employed as an adjunct technique because it is difficult to interpret the data generated in a prokaryotic system to a eukaryotic genotoxicity testing. Results could be ambiguous in some instances when some NPs are not able to cross the bacterial wall or in situations where the nanomaterials are bactericidal.

S. Arora et al 2012
Micronucleus test & Comet assay

• They have been applied for evaluation of chromosome breaking effects of nanoparticles in mammalian cells.

• Examples of investigated materials include TiO2, carbon black, nano tubes and cobalt-chromium alloy nanoparticles.
Genotoxicity of nanoparticles depends on chemical composition

Measuring oxidative stress

• Measuring free radicals directly - Electron Spin Resonance
  - Detects unpaired electrons
  - Use of “traps” for detection (e.g. DMPO) for generating more stable radicals

• Fluorescence dyes
  - 2’,7’-dichlorofluorescein diacetate (DCFH-DA
  - Dihydrorhodamine123

• Effects on cellular antioxidants
  - SOD
  - Glutathione (GSH)

• Lipid peroxidation
  - Malondialdehyde (MDA) e.g. using GC-MS
  - Isoprostanes, F2-isoprostanes

• Oxidative DNA damage
  - Comet assay in combination with restriction enzymes (FPG, ENDO, hOGG1)
  - 8-oxodG
Conclusion

A critical step in nanotoxicology is to characterize the nanomaterial under examination and this is much more difficult than is the case in classical toxicology. These include: particle size, roughness, shape, charge, composition, and surface coating.
the need for more toxicology research on manufactured nanomaterials is clear. In addition to standard tests, there is a need to develop better and rapid screening methods and to move into more predictive toxicology
Conclusion

• The best way to minimize interpretation is to use a combination of at least two different cytotoxicity assays.
Thank you!