Importance of robust nanomaterial test substance characterization as a necessary prerequisite for evaluating the results of in vivo nanotoxicity studies

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Personal Care Products Council
Safety Workshop – Newark, NJ
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Outline
• Definitions of Nanoparticles – scientific vs. regulatory
• Why is NM physicochemical characterization important?
• What are some recommended minimal essential physico-chemical particle characteristics before undertaking a nanotoxicity study?
• Relating physchem issues to pulmonary toxicity
• 3 examples using pulmonary bioassay methodologies as a measure of lung toxicity - hazard assessment
• Pulmonary bioassay studies in rats with –
  1) Fine/ultrafine (Nano) TiO₂ particle types;
  2) Fine and Nano-sized zinc oxide particulates;
  3) Inhalation of carbon nanofibers

What are the issues with Nanogrouping/ Nanocategorization and why are they important?

Definitions- Particle Size

• Nano = Ultrafine = < 100 nm
• Fine = 100 nm - 3 μm

• An ultrafine particle is defined as a particle of average primary size of roughly 100 nm and exhibits a property that is uniquely different than that of its bulk counterpart.
European Commission Definition
October 18, 2011- Nanomaterial

- Natural, incidental or manufactured nanomaterials;
- Particles in an unbound state, an aggregate or agglomerate;
- Where 50% of particles based on a number size distribution, have one or more external dimensions in the size range 1 nm to 100 nm.

Category 1: size > 500 nm
Category 2: 500 nm > size > 100 nm
A nanospecific risk assessment should be undertaken if the characterisation demonstrates that >0.15% of the number size distribution is <100 nm.
Category 3: 100 nm > size > 1 nm
The material is considered a nanomaterial and nanospecific risk assessment has to be performed. A VSSA above the threshold (e.g. >60 m2/cm3) may be used as an additional qualifier to indicate a size below 100 nm.

Why is Material Characterization Important?
- You have to know what you working with – otherwise the results of studies are meaningless.
- Confirmation/validation of results by others with any material is important – Others (as well as you) cannot repeat studies and obtain results – without knowing that one is using the same nanomaterial-type.
- (Manuscripts submitted to Toxicological Sciences & other journals will not be processed/reviewed.)
Particle Scale

Nanoparticles

Ultrafine

Respirable PM 2.5

PM 10

1 nm

10 nm

100 nm

1 μm

10 μm

Unique structures and morphologies

Carbon Nanotubes
Studies to Assess Pulmonary Hazards to Nanoparticulates

• Key Features of Nanotoxicology Studies

  1) Rigorous physicochemical characterization of particle-types
  2) Dose response characteristics
  3) Time course experimental protocol
  4) Utilization of benchmark particulate controls (positive and/or negative)
Ultrafine TiO$_2$
Studies

Pulmonary Instillation Studies with Nanoscale TiO$_2$ Rods and Dots in Rats: Toxicity is not Dependent upon Particle Size and Surface Area

Warheit et al., Tox Sci, 91: 227-236, 2006

Protocol for Nanoscale TiO$_2$ Pulmonary Bioassay Study

- Exposure Groups
  - PBS (control)
  - Particulate Types (1 and 5 mg/kg)
    - Fine-sized TiO$_2$ particles
    - Nanoscale TiO$_2$ rods
    - Nanoscale TiO$_2$ dots
    - Quartz Particles (positive control)

- Instillation Exposure
- Postexposure Evaluation via BAL and Lung Tissue

- 24 hr
- 1 wk
- 1 mo
- 3 mo
Characterization of Nanoscale TiO\textsubscript{2} Particles

<table>
<thead>
<tr>
<th>XRD particle size</th>
<th>Surface Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Fine TiO\textsubscript{2} rutile d\textsubscript{50} = 300 nm</td>
<td>6 m\textsuperscript{2}/g</td>
</tr>
<tr>
<td>• TiO\textsubscript{2} Nanorods anatase</td>
<td>26.5 m\textsuperscript{2}/g</td>
</tr>
<tr>
<td>• length = 90-233 nm</td>
<td></td>
</tr>
<tr>
<td>• width = 20-35 nm</td>
<td></td>
</tr>
<tr>
<td>• TiO\textsubscript{2} Nanodots anatase d\textsubscript{50} = 6 nm</td>
<td>169.4 m\textsuperscript{2}/g</td>
</tr>
<tr>
<td>• Min-U-Sil αQ d\textsubscript{50} = 1.3 µm</td>
<td>4.0 m\textsuperscript{2}/g</td>
</tr>
</tbody>
</table>

RESULTS

Biomarkers = Pulmonary Inflammation

Collaborative Studies with Rice University: TiO\textsubscript{2}

Pigmentary & Nano-TiO\textsubscript{2} are not different
Cytocentrifuge Prep of BALF Cells - Rat Exposed to Nanoscale TiO$_2$ Dots – 24 hr pe

Cytocentrifuge Prep of BALF-derived Cells From a Rat Exposed to Nanoscale TiO$_2$ Dots – 1 wk pe

Pulmonary Toxicity Study in Rats with Three Forms of ultrafine-TiO$_2$ Particles: Differential Responses related to Surface Properties

Warheit et al., Toxicology 230: 90-104, 2007
Characterization of Ultrafine TiO₂ Particle-types - 1

Characterization of Ultrafine TiO₂ Particle-types - 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Crystalline phase</th>
<th>Median size and width distribution (nm) in water*</th>
<th>Surface area (m²/g)</th>
<th>pH</th>
<th>Chemical reactivity in water* in PBS</th>
<th>deionized water</th>
<th>PBS</th>
<th>delta b*</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-1</td>
<td>rutile</td>
<td>382.0 ± 36%</td>
<td>2667.2 ± 35%</td>
<td>5.8</td>
<td>7.49</td>
<td>6.75</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>uf-1</td>
<td>rutile</td>
<td>136.0 ± 35%</td>
<td>2144.3 ± 45%</td>
<td>18.2</td>
<td>5.64</td>
<td>6.78</td>
<td>10.1</td>
<td></td>
</tr>
<tr>
<td>uf-2</td>
<td>rutile</td>
<td>149.4 ± 50%</td>
<td>2990.7 ± 31%</td>
<td>35.7</td>
<td>7.14</td>
<td>6.78</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>uf-3</td>
<td>80/20 anatase/rutile</td>
<td>129.4 ± 44%</td>
<td>2691.7 ± 31%</td>
<td>53.0</td>
<td>3.28</td>
<td>6.70</td>
<td>23.8</td>
<td></td>
</tr>
</tbody>
</table>

Protocol for ultrafine TiO₂ Pulmonary Bioassay Study

- Exposure Groups
  - PBS (vehicle control)
  - Particle-types (1 and 5 mg/kg)
    - rutile-types uf-1 TiO₂
    - rutile-type uf-2 TiO₂
    - anatase/rutile-type uf-3 TiO₂
    - rutile-type F-1 fine TiO₂ (negative control)
    - α-Quartz particles (positive control)

- Instillation Exposure
- Postexposure Evaluation via BAL and Lung Tissue
RESULTS

Biomarkers
Pulmonary Inflammation
Pulmonary Cytotoxicity
Lung cell Proliferation
Lung Morphology

Pulmonary Inflammation

BAL Fluid LDH Values (cytotoxicity)
**BAL Fluid Micro Protein Values (permeability)**

![Graph showing BAL Fluid MTP Values in Rats exposed to Fine or Ultrafine-TiO₂ Particulates](image)

**Pulmonary Cell Proliferation Rates**

![Graph showing Lung Parenchymal Cell Proliferation rates of rats exposed to Fine or Ultrafine-TiO₂ Particulates](image)

**Lung Sections of Rats exposed to uf-1 (A); uf-2 (B); or F-1 (C)- 3 months pe**

![Images of lung sections](image)
Lung Section of Rat exposed to uf-3 @ 3 months postexposure

Lung Section of Rat exposed to Quartz particles @ 3 months postexposure

Summary - Important Particle Characteristics

- Primary particle size
- Particle shape (SEM)
- Surface area
- Surface charge
- Composition - e.g., crystalline vs. amorphous, crystal structures
- Surface Coatings
- Aggregation status
- **Particle surface reactivity**
Nanoscale and fine zinc oxide particles: can in vitro assays accurately forecast lung hazards following inhalation exposures?


TEM - Fine Zinc Oxide Particles

TEM – “Nano” Zinc Oxide Particles
Table 1. Physicochemical Characteristics of Fine and Nano ZnO Particles

Particle characterization in the wet state

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Avg. Particle Size in soln. (nm) DLS</th>
<th>Surface charge (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nano ZnO water</td>
<td>168 ± 16%</td>
<td>-34.5</td>
</tr>
<tr>
<td>PBS</td>
<td>314 ± 31%</td>
<td>-28.5</td>
</tr>
<tr>
<td>Fine ZnO water</td>
<td>243 ± 19%</td>
<td>-55.76</td>
</tr>
<tr>
<td>PBS</td>
<td>319 ± 35%</td>
<td>-14.9</td>
</tr>
</tbody>
</table>

Particle characterization in the dry state

<table>
<thead>
<tr>
<th>Report primary Particle size (nm) by supplier</th>
<th>Surface area (m²/g)</th>
<th>Density (g/ml)</th>
<th>Calculated size in dry state (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nano ZnO 50 – 70</td>
<td>12.1</td>
<td>5.6</td>
<td>90</td>
</tr>
<tr>
<td>Fine ZnO &lt;1000</td>
<td>9.6</td>
<td>5.6</td>
<td>111</td>
</tr>
</tbody>
</table>

Mean Particle Size Determinations in the ZnO Inhalation Studies

MMAD (cascade impactor analyses)

| Fine ZnO 25 mg/m³ | 3.3 μm |
| Fine ZnO 50 mg/m³ | 3.2 μm |
| Nano ZnO 25 mg/m³ | 2.8 μm |
| Nano ZnO 50 mg/m³ | 2.6 μm |

Ninety-Day Inhalation Toxicity Study with Vapor Grown Carbon Nanofibers in Rats

MP DeLorme, Y Muro, T Arai, DA Banas, SR Frame, KL Reed, and DB Warheit

Question: What physicochemical features distinguish the pulmonary toxicity of carbon nanofibers from carbon nanotubes or asbestos fibers?

1. Specific surface area aspects (e.g., 250 m²/g vs. 13.8 m²/g)
2. Catalyst metals (Fe or other metals → ROS formation – cell injury)
3. Aspect ratio (length / diameter)
4. Fibre paradigm issues [e.g., dose, dimension, durability]
Schematic of Experimental Protocol for Carbon Fiber 90-Day Inhalation Study

- 90 Day Exposure Groups (mg/m^3)
  - 0
  - 0.5
  - 2.5
  - 25
- 3 Month P.E. Recovery Period (groups)
  - 0
  - 25

- Traditional tox endpoints
- Clin Path
- Histopath
- BALF Analysis
- Cell Proliferation

90-Day Inhalation Exposure Study with Carbon Nanofibers

- Histopathology
- BAL fluid endpoints
  - Total cell counts and cellular differentials
  - BAL Fluid LDH (cytotoxicity)
  - BAL Fluid Microprotein (permeability)
  - BAL Fluid Alkaline Phosphatase
    (Type II cell cytotoxicity)
- Cell Proliferation studies – BrdU
  - Terminal bronchiolar (Airway)
  - Lung parenchymal cell
  - Subpleural/(Mesothelial)

90-Day Inhalation Exposure Study with Carbon Nanofibers – VGCF®-H characterization

Chemical composition
- C > 99.5%; O = 0.03%; Fe = 0.003 (ICP-AES)
- Purity = 99.7%
- Surface area = 13.8 m^2/g (BET)
- Median lengths = 5.8 μm; diameters = 158 nm
- Fiber counts (NIOSH 7400 method)

<table>
<thead>
<tr>
<th>MMAD (μm)</th>
<th>Fiber counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.9 (3.1)</td>
<td>4.9 ± 3.5</td>
</tr>
<tr>
<td>3.2 (2.1)</td>
<td>56 ± 31</td>
</tr>
<tr>
<td>3.3 (2.0)</td>
<td>252 ± 143</td>
</tr>
</tbody>
</table>
Aerosol sample taken from filter in high exposure conc. chamber - TEM

Aerosolized VGCF®-H nanofibers counts

Percent Neutrophils in BAL Fluids of Rats exposed to VGCF-H Inhalation Exposed Rats (Main)

Exposure Levels

PMN’s

% PMNs

Males Females

* #
Cytocentrifuge cellular preparation
25 mg/m³ (BALF cells)

**BAL Fluid LDH Values in VGCF-H Inhalation Exposed Rats**

Exposure Levels:
- 0 mg/m³
- 0.5 mg/m³
- 2.5 mg/m³
- 25 mg/m³

**Terminal Bronchiolar Epithelial Cell Proliferation rates of Rats exposed to VGCF-H**

Exposure Groups:
- 0 mg/m³
- 0.5 mg/m³
- 2.5 mg/m³
- 25 mg/m³
Lung tissue from a rat exposed to 25 mg/m³ VGCF®-H particulates (#410)

**VGCF®-H study results**
- Small numbers of extrapulmonary fibers observed in organs—no adverse effects
- **NOAEL = 0.54 mg/m³ (4.9 f/cc)**
- **2.5 mg/m³** – (histopathology) minimal inflammation of terminal bronchiole and alveolar ducts in male and female rats. No CP or BALF.
- **25 mg/m³** – (histopathology) slight inflammation of the TB and AD regions in male and female.
- ↑ in female lung weights
  - ↑ BALF endpoints – PMNs, LDH, MTP, AlkPhos
  - ↑ Cell Proliferation – TB, Lung parenchyma
  - subpleural/(mesothelial) [↑ subpleural–No meso]
VGCF™-H carbon nanofibers do not have toxicity effects similar to carbon nanotubes or asbestos fibers

• Likely due to differences in physicochemical characteristics –
• Low surface area metrics
• Reduced length fibers (easily phagocytized by alveolar macrophages in the lung)
• Reduced catalyst metal content
• Does not meet the fibre paradigm criteria

Summary - Important Particle Characteristics

• Primary particle size
• Particle shape (SEM)
• Surface area
• Surface charge
• Composition - e.g., crystalline vs. amorphous – crystal structures
• Surface Coatings
• Aggregation status in testing media
• Particle surface reactivity

Nanocategorization

• What is Nanogrouping/Nanocategorization and why is it important?
Human Health Breakout Group 2

OECD Expert Meeting on Categorization of Manufactured Nanomaterials

18 September 2014

Session Co-Chairs:
- Dr. David Warheit (BIAC/DuPont)
- Dr. Phil Sayre (EPA/OPPT)
Rapporteur: Dr. Agnes Oomen (RIVM/Netherlands)

Overview

- Charge to Health Session 2, & Overview of Structure
- Review of Health Session 1: Structure for ENM Categories
- Specific Questions to be Addressed
- Possible Approaches to Initiate Discussions

Structure for ENM Categories:
Physicochemical Groupings from Thought Starter

- Inorganic: Carbon Based Materials
  - Fullerenes other than CNTs
    - Fullerenes – with or without modification of functional groups
  - Carbon nanotubes
    - Multi-walled Carbon nanotubes
      - Number of walls - Functionalized or unfunctionalized
    - Single-walled Carbon nanotubes
      - Functionalized - Complex arrays of carbon nanotubes
  - Carbon Nanofibers
  - Graphite and graphitic sheets
  - Carbon black derivatives
- Metalloids
  - Coating
    - Coated/Treated - Uncoated/Untreated
- Metalloid Oxides and other metalloid compounds
  - Coating - Coated/Treated - Uncoated/Untreated
- Metals
  - Coating
    - Coated/Treated - Uncoated/Untreated
- Metal oxides and other metal compounds
  - Coating - Coated/Treated - Uncoated/Untreated - Solubility
- Quantum dots
- Organic Compounds
Physicochemical Factors, in Context of Inhalation Toxicity

Inhalation of MNs

Reference: http://www.ec.gc.ca/scitech...

Further Approaches to Initiate Discussion on Pulmonary Toxicity Categories

• Mode of Action Considerations
  • Long-term lung inflammation
  • Fibrosis
  • Other MOAs and/or Biokinetics Anchors

• Targeted Testing within a Category:
  • Use short-term study results, anchored by longer-term * in vivo studies, to estimate toxicity of new MN within the Category

• Focus on an Individual data-rich Group of MNs, and Subcategories:
  • Toxicity Outcomes, based on:
    • varied Metal Oxide crystallinities
    • varied Carbon allotrope forms

Characterization of Ultrafine TiO₂ Particle-types - 2

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Pulmonary Inflammation

Differences in Inhalation Toxicity
Effects for various Carbon Allotropes

- Subchronic inhalation data (several sources):
  - MWCNT \( \times 2 \) \( \rightarrow \) NOAEL = < 0.1 mg/m\(^3\)
  - CNF \( \rightarrow \) NOAEL = 0.5 – 2.5 mg/m\(^3\)
  - Carbon black \( \rightarrow \) NOAEL = 1.0 mg/m\(^3\)

- 5-day Inhalation Screen (Ma-Hock, et al., 2013):
  - MWCNT \( \rightarrow \) NOAEL < 0.5 mg/m\(^3\)
  - Graphene \( \rightarrow \) NOAEL 2.5 mg/m\(^3\)
  - CB, or graphite nanoplatelets \( \rightarrow \) NOAEL = 10 mg/m\(^3\)