Cytotoxicity and Inflammatory Effect of Silver Nanoparticles in Human Cells

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1. Approaches to practical toxicology tests to assess nanoparticles

2. Cytotoxicity and inflammatory effects of silver nanoparticles
Nanoparticles and toxicity assay

- The rapidly developing field of nanotechnology will result in exposure of nanoparticles to humans via several routes (e.g., inhalation, ingestion, skin, etc.). Nanoparticles can translocate from the route of exposure to other vital organs and penetrate cells.

- Toxicity studies to determine the deleterious effects of nanoparticles on living cells are required.

- Due to the nanosize and the nature of agglomeration, simple standard methods to characterize the biological effects of nanoparticles are currently unavailable.

- In this study, practical information regarding the optimal *in vitro* tests for nanotoxicity were evaluated.
Silver nanoparticles

- Antimicrobial reagents, detergents, water purificants, wall paints, textiles

![Silver nanoparticles](image)
Establishment of *in vitro* toxicity assay

Identification of mechanisms for toxicity and inflammation

MTT/CCK-8

Annexin staining, caspase activation

Cytokine production, activation of signaling molecule

ROS

Production & characterization of physical and chemical properties

Cytotoxicity

Inflammation

Biological tests

Synthesis
In vitro tests for nanoparticles

- Production of diverse particles (size, surface)
- Assess biological activities

ISO/TC229
OECD
U.S. NCL

Review in vitro methods

Assess toxicity tests

Establish proper methods

Understanding of proper methods for nanoparticles
### Exposure routes of nanomaterials

#### Skin
- **Cell line**: SK-Mel
- **Origin**: Skin epithelial
- **Characteristics**: Proper for cytotoxicity and cytokine production

#### Respiratory tract
- **Cell line**:
  - A549: Lung epithelial
  - BEAS-2B: Bronchial epithelial
- **Characteristics**:
  - A549: Proper for cytotoxicity
  - BEAS-2B: Proper for cytokine production

#### Immune system
- **Cell line**: U937
- **Origin**: Macrophage
- **Characteristics**: Proper for cytotoxicity and cytokine production

#### Skin
- **Cell line**:
  - A375: Skin epithelial
- **Characteristics**: Too fast growing

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<table>
<thead>
<tr>
<th></th>
<th>Cell line</th>
<th>Origin</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Respiratory</strong></td>
<td>A549</td>
<td>Lung epithelial</td>
<td>Proper for cytotoxicity</td>
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<tr>
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</table>
## Standard toxicology tests and silver nanoparticles

<table>
<thead>
<tr>
<th>Category</th>
<th>Tests</th>
<th>Mechanism</th>
<th>Method</th>
<th>Suggestion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In Vitro Immunology (Blood contact Properties)</strong></td>
<td>Hemolysis</td>
<td>Release of hemoglobin</td>
<td>Standard</td>
<td>Proper</td>
</tr>
<tr>
<td></td>
<td>Complement activation</td>
<td>Activation of C3 complement</td>
<td>Standard</td>
<td>Inappropriate</td>
</tr>
<tr>
<td><strong>In Vitro Immunology</strong></td>
<td>Leukocyte proliferation</td>
<td>Leukocyte proliferation with mitogen stimulation</td>
<td>Standard</td>
<td>CCK-8</td>
</tr>
<tr>
<td>(Cell-based assays)</td>
<td>Phagocytosis</td>
<td>Zymosan assay</td>
<td>Standard</td>
<td>Proper</td>
</tr>
<tr>
<td></td>
<td>Cytokine induction</td>
<td>Cytokine production</td>
<td>Standard</td>
<td>Proper</td>
</tr>
<tr>
<td><strong>Toxicity</strong></td>
<td>Oxidative stress</td>
<td>Detection of ROS</td>
<td>Standard</td>
<td>Proper</td>
</tr>
<tr>
<td></td>
<td>Cytotoxicity (necrosis)</td>
<td>Cell viability and mitochondrial integrity</td>
<td>Standard</td>
<td>CCK-8</td>
</tr>
<tr>
<td></td>
<td>Cytotoxicity (apoptosis)</td>
<td>Activation of caspase 3</td>
<td>Standard</td>
<td>Annexin-V</td>
</tr>
<tr>
<td><strong>Targeting</strong></td>
<td>Cell binding/internalization</td>
<td>N/S</td>
<td>N/S</td>
<td>TEM, confocal microscope or other methods</td>
</tr>
</tbody>
</table>

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Characteristics specific to metal nanomaterials

- Nanoparticles larger than 100 nm tend to aggregate relatively quickly *in vitro* when compared to nanoparticles smaller than 100 nm. Fresh samples within two weeks after synthesis is recommended for tests.

- Each standard toxicology method must be verified before use. (ex. interference with a specific wavelength, electrophoresis)
Flow chart for nanotoxicity tests

Analysis of biological properties:
- Cytotoxicity
- Apoptosis
- Cytokine production
- Hemolysis
- Leukocyte proliferation
- ROS production

Analysis of chemical/physical properties:
- Aggregation
- Particle size

Small particle size:
- 100 nm

Large particle size
Biological reactivity of silver nanoparticles
Cytotoxicity of silver nanoparticles

20 nm

80 nm

Cell viability (%) vs. Concentration (µg/mL) for different cell lines:
- SK-Mel28 (skin)
- A375 (skin)
- A549 (lung)
Cytotoxicity of silver nanoparticles

5 nm

Cell viability (%)

Conc. (µg/mL) 0 0.75 1.5 3.125 6.25 12.5 25

80 nm

Cell viability (%)

Conc. (µg/mL) 0 0.75 1.5 3.125 6.25 12.5 25

U937 cells (macrophage)
Induction of apoptosis by silver nanoparticles

- **Annexin**
- **Propidium iodide**

: U937 cells (macrophage)
: 25 µg/mL for 15 hrs
Lysosomal aggregation by silver nanoparticles

: U937 cells (macrophage)
: 20 nm, 25 µg/mL for 24 hrs
**ROS production by silver nanoparticles**

- **H₂O₂**
- Silver nanoparticle (20 nm)
- Unstained control
- Stained control

BEAS-2B (lung) BEAS-2B (lung)
- 20 nm, 30 µg/mL, for 3 hrs
- stained with CM-H₂DCFDA
Cytokine production by silver nanoparticles

- Cytokine array

Positive control

Positive: chemokines (IL-8, MIF, RANTES), Serpin E1, IL-16
Negative: TNF-α, IL-6, IL-1
Cytokine production by silver nanoparticles

- ELISA (IL-8)

: U937 cells
(macrophages)
: 20 nm for 24 hrs
Activation of signaling molecule by silver nanoparticles

- MAP kinase (ex. ERK) activation

: Protein 30 µg loading
: LPS (*E. coli* lipopolysaccharide) 50 ng/mL
: 5 nm silver nanoparticles, 1.5 µg/mL
In human cells, epithelial cells from skin or lung, and macrophages, 5 nm and 20 nm silver particles induced stronger cytotoxicity and ROS synthesis than 80 nm particles did.

5 nm and 20 nm silver particles induced chemokine production, mainly IL-8, MIF and RANTES, while proinflammatory cytokines, IL-1, IL-6 and TNF-α were not induced significantly in the same conditions.

Some MAP kinase signaling pathways were activated during exposure to silver nanoparticles at lower concentrations which do not induce cytotoxicity.
Conclusion

- The toxicity and inflammatory effects of nanoparticles are dependent on their size. In silver nanoparticles smaller than 20 nm induce cytotoxicity significantly \textit{in vitro}.

- Nanoparticles induce inflammatory immune responses at lower concentrations and chemokines are the major cytokines induced at early stages of exposure to silver nanoparticles.