Investigation of the uptake and cytotoxic mechanisms of amine-modified silver nanoparticles

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INTRODUCTION

the impact of NP on biological system, especially their toxic effects and mechanism is still limited
### Table 1. Applications of commonly used nanoparticles

<table>
<thead>
<tr>
<th>Nanoparticle Type</th>
<th>Abbreviation</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon nanotube (single or multiwalled)</td>
<td>CNT (SWNT, MWNT)</td>
<td>Cell delivery, biosensors</td>
</tr>
<tr>
<td>Silver</td>
<td>Ag</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>Quantum dot</td>
<td>QD</td>
<td>Fluorescent imaging</td>
</tr>
<tr>
<td>Aluminum</td>
<td>Al</td>
<td>Fuel additive</td>
</tr>
<tr>
<td>Iron</td>
<td>Fe</td>
<td>Magnetic imaging</td>
</tr>
<tr>
<td>Titanium dioxide</td>
<td>TiO$_2$</td>
<td>Paint, water treatment, food, cosmetics</td>
</tr>
<tr>
<td>Zinc oxide</td>
<td>ZnO</td>
<td>Transparent sunscreens</td>
</tr>
<tr>
<td>Manganese oxide</td>
<td>MnO</td>
<td>Catalysis, batteries</td>
</tr>
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</table>
Numerous commercial applications of AgNPs lead to an increased risk of human exposure

Due to the effective antimicrobial activities and surface plasmon resonance properties, AgNPs have gained particular interest for various commercial applications (e.g. wound dressings and photovoltaics).

the increase of human exposure and potential adverse health effect has led to increasing public concern
Gaps in the available data set, both in relation to exposures and hazard, do not allow reaching any definite conclusions that could be used for regulatory decision making.

Results show that repeated inhalation in the workplace and possibly consumer inhalation may cause risks. Also (uncontrolled) nano-silver drug intake and burn treatment of large parts of the body with wound dressings may cause risks.

Main future work should focus on generating occupational and consumer exposure data, as well as toxicity data on absorption (are particles or only ions absorbed?).

information on genotoxicity, and further information on the toxicity following inhalation exposure to sizes and agglomeration states as uncounted in the workplace.
Silver as an antimicrobial, meanwhile may trigger cytotoxicity
Trojan-horse mechanism in the cellular uptake of silver nanoparticles verified by direct intra- and extracellular silver speciation analysis.

Hsiao IL¹, Hsieh YK¹, Wang CF¹, Chen IC¹, Huang YJ¹.
Schematic of the known pathways for intracellular uptake of nanoparticles

This process play key roles in executing their biomedical functions and in toxicity
Mechanisms of AgNP cytotoxicity in the mammalian cell
Toxicity testing methods suggested by NCL

U.S. Environmental Protection Agency (EPA) 2009

National Research Council (NRC) 2007

Toxicity testing on the cellular response pathways

As the preferred toxicity testing strategy in the 21st century

**Toxicity**

**Oxidative Stress**
- Hep G2 Hepatocyte Glutathione Assay
- Hep G2 Hepatocyte Lipid Peroxidation Assay
- Hepatocyte Primary ROS Assay

**Cytotoxicity (necrosis)**
- LLC-PK1 Kidney Cytotoxicity Assay (MTT and LDH Release)
- Hep G2 Hepatocarcinoma Cytotoxicity Assay (MTT and LDH Release)

**Cytotoxicity (apoptosis)**
- LLC-PK1 Kidney Apoptosis Assay (Caspase 3 Activation)
- Hep G2 Hepatocarcinoma Apoptosis Assay (Caspase 3 Activation)
- Hep G2 Hepatocarcinoma Homogeneous Apoptosis Assay (Caspase 3/7 Activation)

**Autophagy**
- Autophagic Dysfunction Assay: Qualitative Analysis of MAP LC3I to LC3-II Conversion by Western Blot
- Autophagic Dysfunction in LLC-PK1 Cells
Figure 1: Autophagy produces metabolic fuel through the degradation of biomolecules.

Notes: Damaged proteins, organelles, and other biomolecules are sequestered into double-membrane vesicles called autophagosomes. LC3 is essential for autophagosome maturation. The mature autophagosomes fuse with the lysosome, and biomolecules are degraded by hydrolytic enzymes into metabolic fuel.

Abbreviation: LC3, lipiddated cytosolic-associated protein light chain.
Review

Autophagy as a Possible Underlying Mechanism of Nanomaterial Toxicity

Vanessa Cohignac $^{1,2,*}$, Marion Julie Landry $^{1,2,*}$, Jorge Boczkowski $^{1,2}$ and Sophie Lanone $^{1,2,*}$

Figure 2. Hypothetic relationship between the autophagy and the biological responses to nanomaterial.


2007, ATO-induced autophagic cell death in U118 human glioma cells...
2009, Combination treatment with ATO and IR enhances autophagic effects...
2010, ATO and IR enhances cell-killing effects in human fibrosarcoma

2010, Pterostilbene-induced Apoptosis and Autophagy in Human Bladder Cancer Cells
2011, Synergistic effects of arsenic trioxide and radiation in osteosarcoma cells through autophagy and apoptosis
2011, ATO induces autophagy and apoptosis through down-regulation of survivin

2012, MP enhances the radiation sensitivity through the stimulation of ER stress and induction of autophagy
2012, Monascuspiloin Induces Apoptosis and Autophagic Cell Death in Human Prostate Cancer Cells
2012, Chemopreventive Effects of Pterostilbene on Urethane Induced Lung Carcinogenesis via the Induction of Apoptosis and Autophagy.
2013, SAHA enhance radiosensitivity and suppresses lung metastasis in breast cancer in vitro and in vivo

2014, Cytotoxicity, oxidative stress, apoptosis and the autophagic effects of AgNPs in NIH3T3
2014, ATO induces programmed cell death through stimulation of ER stress
2015, Cationic polystyrene nanospheres induce autophagic cell death
Experimental considerations for performing in vitro nanoparticle toxicity studies

(1) Analysis the physicochemical parameters of AgNPs.
(2) Cellular uptake and mechanisms of AgNPs.
(3) Cytotoxicity of AgNPs.
(4) The toxic mechanisms of AgNPs.
### Table 1 Characterization of AgNPs

<table>
<thead>
<tr>
<th>Characterization</th>
<th>Method</th>
<th>Condition</th>
<th>Results</th>
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</thead>
<tbody>
<tr>
<td>Size</td>
<td>TEM</td>
<td>Dry</td>
<td>26.2 ± 7.6 nm</td>
</tr>
<tr>
<td>Morphology</td>
<td>TEM</td>
<td>Dry</td>
<td>Spherical</td>
</tr>
<tr>
<td>Composition and purity</td>
<td>EDX</td>
<td>Dry</td>
<td>Ag (99.6%)</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>PALS</td>
<td>Water</td>
<td>-28.4 mV</td>
</tr>
<tr>
<td>Hydrodynamic size</td>
<td>DLS</td>
<td>Water</td>
<td>40.1 ± 1.7 nm</td>
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<tr>
<td>Polydispersity index (PDI)</td>
<td>DLS</td>
<td>Water</td>
<td>0.204</td>
</tr>
<tr>
<td>(\lambda_{\text{max}})</td>
<td>UV-Vis</td>
<td>Water</td>
<td>389 nm</td>
</tr>
<tr>
<td>Hydrodynamic size</td>
<td>DLS</td>
<td>DMEM</td>
<td>92.9 ± 2.1 nm</td>
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<tr>
<td>Polydispersity index (PDI)</td>
<td>DLS</td>
<td>DMEM</td>
<td>0.289</td>
</tr>
<tr>
<td>(\lambda_{\text{max}})</td>
<td>UV-Vis</td>
<td>DMEM</td>
<td>398 nm</td>
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Fig. 1. Characterization of AgNPs. (a) UV-vis spectra of AgNPs in water or DMEM plus 1% FBS. (b) Transmission electron microscope (TEM) documentation of NP morphology: AgNPs were mainly spherical in shape with a mean diameter of 26 ± 7.6 nm (mean± SD) (scale bar representing 20 nm). (c) An X-ray diffraction (XRD) pattern of AgNPs.
Fig. 2. Cellular uptake of AgNPs by NIH 3T3 cells. TEM images demonstrated the interaction of AgNPs with cells. Scale bars: 2 μm (a, b, c and e); 0.5 μm (d and f) The results of side-scatter(ed) light of flow cytometry demonstrated that AgNPs were apparently engulfed by NIH 3T3 cells.
Cellular uptake pathway of AgNPs

Fig. 8. (C) and (D) Cellular uptake pathway for AgNPs internalization in NIH 3T3 cells. Cells were pre-incubated with different pharmacological inhibitors for 30 min (Nystatin 7.5 μM; Chlorpromazine 10 μM; Wortmannin 200 nM) and particle uptake was analyzed by flow cytometric side scatter. Combine group means that cells were treated with AgNPs (5 μg/ml), Chlorpromazine and Wortmannin. (n=3, Δ, *, #, p<0.05).

Fig. 3. Cytotoxicity of AgNP-treated NIH 3T3 cells. Cells were grown for 1 day in 6-well plates and exposed to AgNPs at the different concentrations for 24 hrs.
Effects of AgNPs on cellular reactive oxygen species (ROS), glutathione (GSH) and the expression of heme oxygenase 1 (HO-1)

Fig. 4. Effects of AgNPs on cellular reactive oxygen species (ROS), glutathione (GSH) and the expression of Heme oxygenase 1 (HO-1).
Measurements of apoptosis and necrosis in NIH 3T3 cells treated with AgNPs

Fig. 5. Measurement of apoptosis in NIH 3T3 cells that received various treatments. Scale bars: 50 μm.

(a) DAPI staining: apoptotic bodies

(b) Annexin V/PI: Early apoptosis; Necrosis

(c) 0 2 5 10 15 (μg/ml)

<table>
<thead>
<tr>
<th>Pro-caspase 3</th>
<th>Cleaved-caspase 3</th>
<th>PARP</th>
<th>Cleaved-PARP</th>
<th>GAPDH</th>
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Fig. 5. Measurement of apoptosis in NIH 3T3 cells that received various treatments. Scale bars: 50 μm.
AgNPs induced morphological and biochemical markers of autophagy in NIH 3T3 cells

Fig. 6. AgNPs induced morphological and biochemical markers of autophagy in NIH 3T3 cells.
(a) Microphotograph of AVOs in NIH 3T3 cells. (b) Quantification of AVOs with AO-stained cells. (c) The immunofluorescence detection of LC3. Scale bars: 20 μm (up); 10 μm (down). (d) Western blotting.
Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity
Effect of SAS and LAS on uptake and localization of AgNPs

Fig. 7. Localization of AgNPs in NIH 3T3 cells. NIH 3T3 cells were treated for 3 hrs with 15 μg/ml R6G-SAS and R6G-LAS then incubated with lysosensor DND-189 for lysosome staining. Scale bars are 200 μm.

Fig. 8.1 Nanoparticle-cell membrane surface adhesion is dependent on the material and bio-molecular surface identity of the nanoparticle.
Autophagosome formation and lysosomal dilatation by AgNPs treatment

Fig. 8. Double-immunocytochemical staining of NIH 3T3 cells with anti–LC3 and anti–LAMP-1 antibodies. Cells were sham washed or treated for 12 hrs with SAS or LAS. A nearly complete separation between autophagosomes and dilated lysosomes was evident in SAS-treated cells.

lysosome associated membrane protein-1 (LAMP-1)

The vacuoles that formed after SAS treatment corresponded closely to dilated lysosomes

LysoSensor green DND-189

The vacuoles that formed after SAS treatment corresponded closely to dilated lysosomes

Fig. 8. Double-immunocytochemical staining of NIH 3T3 cells with anti–LC3 and anti–LAMP-1 antibodies. Cells were sham washed or treated for 12 hrs with SAS or LAS. A nearly complete separation between autophagosomes and dilated lysosomes was evident in SAS-treated cells.
ER stress signaling

ER stress is an important cellular self-protection mechanism, allowing cells to adapt to stress by initiating autophagy or inducing apoptosis.
Fig. 9. Effect of AgNPs on ROS production and ER stress. Cells were treated with several doses (2, 5, 7.5, 10 and 15 μg/ml) of AgNPs for 6 hrs and 12 hrs.
AgNPs induced autophagy via ER stress in NIH 3T3 cells. Cells were treated with 15 μg/ml AgNPs with or without 500 μM TUDCA for 12 h. Detection of green and red fluorescence in AO-stained cells using flow cytometry. (A)Control; (B)SAS; (C)LAS; (D)TUDCA; (E)SAS+TUDCA; (F)LAS+TUDCA. (n=3, * p<0.05.)
Oxidative stress
Danger signals production
Lysosome
Ag
H+
ROS
ER stress
Uptake
Autophagy
Enlarged lysosomes
Lysosome dysfunction
Apoptosis
Apoptotic bodies
Fragmentation
Condensation
Autophagy dysfunction
Lysosome dysfunction
Autophagosome accumulation
TUDCA
IRE1
Ag
p62
LC3
Clathrin-mediated
cytosis
Macropinocytosis
Summary
Conclusion

The induction of ER stress and autophagy were the critical toxic effects associated with AgNPs.

The smaller-sized AgNPs resulted in more significant cytotoxicity than the larger-sized AgNPs through the induction of more extensive dysfunction of autophagy.

This study illustrates the influence of AgNPs on biological systems and may provide insights to guide the development of safe products for biomedical applications of AgNPs.

References:


## Conclusion

### Table 3. Summary of cellular and molecular mechanisms underlying AgNPs toxicity

1. Induction of oxidative stress
   - Lipid peroxidation (e.g. damage to the plasma membrane, disruption of the lysosomal membrane integrity)
   - DNA damage, protein misfolding and aggregation, organellar injury, etc.
2. Activation of intracellular signaling pathways
   - Stimulation of ER stress, apoptosis, autophagy, and inflammasome pathways
3. Interference with mitochondrial electron transport and aerobic respiration
   - Mitochondrial impairment
4. Defective autophagy
   - Blockade of autophagosome-lysosome fusion
5. Release of toxic Ag cations
   - Interference with copper homeostasis

# unique for AgNPs and other like metallic NPs; * AgNPs-specific

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