IMPACT OF VARIOUS METALLIC NANOPARTICLES ON METAL HOMEOSTASIS

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we postulated that metallic NP should interfere with metal homeostasis, which is closely related to oxidative stress
Interferences between Nanoparticles and metal homeostasis

Labile metallic nanoparticles: CuO, ZnO, Ag..

cellular model: hepatocytes (HepG2) because metals end up in liver

1) Characterization of the NP in the medium
2) Dissolution studies
3) Cell viability
4) Q-PCR on genes involved in redox and metal homeostasis in subtoxic conditions
5) Visualization, localization, quantification (TEM; µXRF; XAS; ICP-MS)

- **Goal**
  Decipher the mechanisms of disruptions at the cellular and molecular levels

- **Main result:** metallic NP interfere with metal homeostasis even in subtoxic conditions but not always using the same mechanisms
ZnO-NP case study

<table>
<thead>
<tr>
<th></th>
<th>ZnO-NP-SVF</th>
<th>ZnO-NP-silane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size distribution of</td>
<td>Multimodal, not</td>
<td>Multimodal, not</td>
</tr>
<tr>
<td>ZnO-NP in medium</td>
<td>measurable</td>
<td>measurable</td>
</tr>
<tr>
<td>(DLS)</td>
<td></td>
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<tr>
<td>SEM images of ZnO-NP</td>
<td><img src="image1" alt="SEM image" /></td>
<td><img src="image2" alt="SEM image" /></td>
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<tr>
<td>at 900µM after</td>
<td></td>
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<tr>
<td>24h incubation in</td>
<td></td>
<td></td>
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<tr>
<td>complete medium</td>
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Best dispersion Conditions

(Cup-horn sonication)

Dissolution study

ZnO-NP-FBS (○)
ZnO-NP-silane (■)
at 90 µM in complete MEM

HepG2 cells treated with different metal nanoparticles for 24h

- **Ag and Cu → Nano-effect**
- **Zn → Fast dissolution**
- **CuO-NP more toxic than Cu salt → Trojan horse**
- **Ag-NP less toxic than Ag salt → Protective**
- **Salt and NP same effect**
### Cellular responses induced by CuO-NP, ZnO-NP and Ag-NP

#### mRNA fold increase after 6 h exposure

<table>
<thead>
<tr>
<th>sub-toxic conditions</th>
<th>CuO-NP PVP</th>
<th>CuCl₂</th>
<th>ZnO-NP silane</th>
<th>ZnO-NP SVF</th>
<th>ZnAcetate</th>
<th>Ag-NP PVP</th>
<th>Ag-NP citrate</th>
<th>AgNO₃</th>
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<td></td>
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<td>AgNO₃</td>
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<tr>
<td></td>
<td>60 µM</td>
<td>90 µM</td>
<td>60 µM</td>
<td>90 µM</td>
<td>60 µM</td>
<td>100µM</td>
<td>50 µM</td>
<td>25 µM</td>
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<tr>
<td>HSPA6</td>
<td>6.0</td>
<td>1.2</td>
<td>4.6</td>
<td>2.1</td>
<td>1.9</td>
<td>435</td>
<td>994</td>
<td>553</td>
</tr>
<tr>
<td>HMOX</td>
<td>13.8</td>
<td>1.9</td>
<td>4.1</td>
<td>2.9</td>
<td>4.6</td>
<td>34</td>
<td>46</td>
<td>34.5</td>
</tr>
<tr>
<td>MET</td>
<td>38.4</td>
<td>11.1</td>
<td>46.9</td>
<td>44.5</td>
<td>56.4</td>
<td>362</td>
<td>539</td>
<td>30.5</td>
</tr>
<tr>
<td>GCLM</td>
<td>1.6</td>
<td>2.1</td>
<td>4.4</td>
<td>3.7</td>
<td>4.1</td>
<td>12</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>ZnT1</td>
<td>2.2</td>
<td>1.6</td>
<td>4</td>
<td>3.8</td>
<td>3.4</td>
<td>6</td>
<td>9</td>
<td>-</td>
</tr>
</tbody>
</table>

**In all cases**
- Met, GCLM, ZnT1 overexpression
- Metal homeostasis control
- Weak oxidative stress response
- HMOX only (not SOD or CAT)

**Specificities:**
- ZnO-NP same result than Zn salt
- CuO-NP more effect than Cu salt
- Ag stronger expression for all targets
  - Very high overexpression for HSPA6---> protein folding problem

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HSP6: heat shock protein
HMOX: heme oxygenase
MET: Metallothionein
GCLM: in GSH synthesis
ZnT1: Zn exporter
CuO-NP trigger disruption of Cu and Zn homeostasis under sub-toxic conditions

NP-CuO-NP act as a Trojan horse to bring copper into vesicles where acidic conditions may favor release into cells.

HepG2 cells after 24h-incubation with CuO-NP 250 µM

after 6 h, 24 h and 48 h incubation with 90 μM Zn compounds for 24 h followed by a 24 h recovery.

Cellular zinc content measured by ICP-AES

protection by EDTA against Zn toxicity → Zn dissolution in the medium

TEM observation of ZnO-NP treated HepG2 sections

Control

ZnO-NP-FBS
90 μM, 6 h

ZnO-NP-silane
90 μM, 6 h


ZnT2 upregulated and decrease mitochondrial transmembrane potential

6 h incubation with Zn compounds at **90 µM (sub toxic dose)**

Suggesting a storage of zinc in mitochondria, mitochondria alterations

after 24 h incubation with 90 µM Zn compounds
Sub-toxic doses of both ionic and nanoparticulate forms of zinc induce zinc homeostasis disruption, mitochondria alterations and increased autophagy.
**Ag-NP Conclusion** (as described in G. Veronesi talk)

- Ag-NP dissolve intracellularly in acidic vesicles

  ![Single cell analysis](image)

  - redox and metal homeostasis disruptions

  - Ag(I) forms complexes with thiol-containing biomolecules as AgS₂ (GSH) and AgS₃ (Met) complexes

  ![Diagram of cellular processes](image)

General conclusion: mechanisms of disruptions by labile MeNPs in hepatocytes

Met : Metallothionein
ZnT, Zip : Zinc transporter
MTF : Metal regulatory transcription factor
MRE : Metal response element
Predictive toxicology
- Interferences between metal homeostasis and metallic NP
- Nano-effect due to endocytosis and dissolution (NP-CuO)
- no general correlation between NP and cytotoxicity

Metallothionein biomarker of metal ion exposure  AgNP>ZnNp & CuONP
release of Zn(II) → MTF activation/translocation → Zn(II) exporter Znt1
GSH major player
late induction of a moderate oxidative stress

Labile NP → higher inflammatory responses than other NP (literature)
  → Redox and metal homeostasis disruption
- Biological chelators assisted-dissolution of metallic NP (AgNP; I. Worms talk)

Perspectives: Safer-by-design approach
- Control of the dissolution by the coating
- Bio-inspiration for eco-conception

(postsers Marchioni S3.2-P2 & Laisney S3.2-P4)
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7th International IMBG meeting on Metallic Nanoparticles: Health, Environment, and Safer-by-Design

September 11 – 15th 2017
Villars de Lans, France

Advanced courses
Sept 11 – 12th
Practical methodologies and analyses:
- Analytical chemistry, imaging, spectroscopy & molecular biology techniques
- NPs in complex media: tricks and pitfalls
- Safer-by-design approach

Conference
Sept 13 – 15th
Behavior, fate and impacts:
- in the environment
- on health
Safer-by-design, coating and surface reactivity
Applications (health, agriculture, textile, ..)

Scientific committee:
A. Baeza-Squiban; M. Carrière; L. Charlet; S. Lanone; I. Michaud-Soret; J. Rose; G. Sarret; G. Veronesi; M. Wiesner

Organizers:
Géraldine Sarret, Marie Carrière & Isabelle Michaud-Soret

website: http://imbg-grenoble.fr/
Study of ZnO-NP toxicity

Viability of HepG2 cells after a 24h incubation

Sub-toxic dose
90 μM
(7.3 μg mL$^{-1}$)
gene expression studies

ZnO-NP Toxicity is equivalent to Zinc salt in Hepatocytes
mRNA expression of Zn- and redox-stress genes

after a 6 h incubation with Zn compounds at 90 µM (sub toxic dose)

Potential ZnO-NP specific effect (*)

Globally similar results between NP and salt

(*) Moos et al (2011) Metallomics 3(11), 1199-211
ZnO-NP Conclusions

ZnO-NP toxicity seems to a great extent a direct consequence of zinc dissolution and subsequent increase in intracellular and mitochondria zinc concentrations.
Increased exposure of environment & humans to NP

→ mechanistic studies at the molecular and cellular levels were essential for predictive toxicology

Weir et al., Env.science & Tech., 2012
Wilson Center (USA) (2013)
www.nanotechproject.org/cpi

>442 products containing Ag-NP

1000 to 5000 tonnes/year (2015)
Woodrow Wilson Institut
Decrease mitochondrial transmembrane potential after 24 h incubation with 90 μM Zn compounds.

Amount of rhodamine 123 internalized in the cells expressed as a percentage of the mean fluorescence of control cells (three independent experiments). ***: p < 0.001.

AgNP intracellular dissolution (as described in G. Veronesi talk)

X-ray fluorescence microscopy on ID16B on whole cells coupled with cell section observed by TEM

1. Control

2. AgNO₃

High sensitivity → allows Ag(I) detection

Ag hot spots → Ag-NP as well as diffuse signal → Ag(I) species
Larger hot spots with citrate → vesicles with agglomerated Ag-NP

mRNA expression in HepG2 by quantitative PCR analysis after a 6 h incubation with Zn compounds at 90 μM.
Protection by EDTA or Ca2+ against Zn toxicity

HepG2 pretreated for 15 min with 1 mM EDTA or 2 mM CaCl2 before adding Zn compounds at the toxic dose of 250 μM for 24 h. EDTA or CaCl2 treatment alone had no effect on viability of HepG2 cells.

Comparison with CuO-NP

CuO-NP more toxic than salt
Influence of copper chelating proteins on the dissolution of silver nanoparticles and their toxicity
Effect of bafilomycin A on Zn toxicity

HepG2 cells were pretreated 1 h with 100 nM Baf A before adding Zn compounds for 24 h at subtoxic doses of 90 and 150 μM
Dissolution of Ag-NP: silver release quantified by ICP-AES

Ag-NP coated citrate

Supernatant
pellet

silver (µg)

% silver
days

with GSH
no ligand

1 3 6
1 3 6

Dissolution of Ag-NP: silver release quantified by ICP-AES

Ag-NP coated citrate

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Main results

1) Correlation coordination number and \( d(\text{Ag-S}) \) for Ag-complex with bioinspired ligands and Ag-biomolecules containing 1 to 20 thiols (GSH, Atox1, metallothioneins (MeT))

2) Simultaneous measurements \textit{in cellulo} of dissolved Ag(I) and remaining NP-Ag part
Ag(I) is mainly bound to intracellular GSH in macrophages and also to MeT in hepatocytes (HepG2)

3) Detection of dissolved Ag(I) in a unique cell thanks to the \( \mu \text{XRF} \) on ID16B (exceptional sensitivity of \textbf{few attograms /pixel of 70x70nm} \(^2\)) as a function of the coating

Veronesi et al. \textit{Nanoscale} \textbf{(2015)}, 7,7323
Veronesi , Deniaud et al, \textbf{(2016)} \textit{Nanoscale}
X Ray Fluorescence elemental images highlighting the distribution of (A) Zn and (B) Ag, and (C) their co-localization in a single hepatocyte (HepG2) exposed for 24 h to citrate-coated Ag-NP.

Images were acquired in the X-ray nanoprobe ID16B-NA of ESRF. Interestingly, both particulate (intense Ag hot spots) and ionic forms (diffuse signal) of silver could be visualized with XRF.
Does ZnO-NP interfere with redox equilibrium in Hepatocytes (HepG2) ?

**Superoxide dismutases (Cu-Zn or Mn SOD)**

- \( \text{O}_2 \xrightarrow{e^-} \text{O}_2^{2-} \xrightarrow{e^-} \text{H}_2\text{O}_2 \xrightarrow{e^-} \text{OH}^- + \text{OH}^- \xrightarrow{e^-} 2 \text{OH}^- \)

**Peroxidases**

**Peroxiredoxines**

**Oxidative Stress**

- Oxidative stress leads to the activation of Nrf2, which binds to Keap1 and is degraded, allowing Nrf2 to enter the nucleus and bind to ARE to activate Phase II antioxidant enzymes.

**GSH production and regeneration**

- NADPH production
- GSH utilization
- Quinone detoxification

**GSH production and regeneration**

- G6PD
- PGD
- ME1
- IDH1
- TXN1
- PRDX1
- TXNRD1

**Iron sequestration**

- FTL
- FTH
- HMOX

**GSH utilization**

- GPX2
- GSTA1 to GSTA3, GSTA5
- GSTM1 to GSTM3
- GSTP1

**Quinone detoxification**

- NOO1

**GCLM**

- GCLC
- GSR
- XCT

**NADPH production and regeneration**

- NADPH production

**Phase II Antioxidant Enzymes**

- Keap1
- Nrf2
- Maf
- ARE
Measurement of the amount of Zn in HepG2 after incubation with 90 µM ZnO-NP or Zn acetate for 6 h and 24 h (ICP-OES)

Very similar zinc accumulation between NPs and salt
The intra-cellular concentration of Zinc increased from 6h to 24h
No evidence of zinc release

≠

More copper is found in HepG2 exposed for 24h to CuO-NP than to CuCl₂
Studies of ZnO-NP, CuO-NP and Ag-NP

These particles can dissolve in water-based media and release ionic species

Cu ions release from CuO-NP

% dissolution

time, h

Cell culture media at pH 6.5

Cell culture media at pH 8

Water

pH decrease favors ion release from NP → similar mechanism in endo- and lysosomal vesicles