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The bioavailability in rats orally treated with silver nanoparticles is studied through the analysis of liver, kidney, feces and urine.
Insight into silver nanoparticles bioavailability in rats

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A comprehensive study of the bioavailability of orally administered silver nanoparticles (AgNPs) was carried out using a rat model. The silver uptake was monitored in liver and kidney tissues, as well as in urine and in feces. A significant accumulation of silver was found in both organs, the liver being the principal target of AgNPs. A significant (~50%) fraction of silver was found in feces whether the fraction excreted via urine was negligible (<0.01%). Intact silver nanoparticles were found in feces by asymmetric flow field-flow fractionation (AsFFF) coupled to UV-Vis. Laser ablation – ICP MS imaging showed that AgNPs were able to penetrate into the liver, in contrast to kidney where they were retained in the cortex. Silver speciation analysis in cytosols from kidney showed metallothionein complex as the major species whereas in liver the majority of silver was bound to high-molecular (70-25 kDa) proteins. These findings demonstrate the presence of Ag(I) released by the oxidation of AgNPs in the biological environment.

1. Introduction

Due to their antimicrobial properties, silver nanoparticles (AgNPs) are used in many consumer and medical products. However, an increasing number of reports suggest that AgNPs may be potentially toxic which spurs research on the absorption, distribution, metabolism and excretion (ADME) behavior of metal-based nanomaterials in biological systems, recently referred to as “nanometallomics”. Several in vitro studies indicated that AgNPs are toxic to mammalian cells derived from skin, liver, lung, retina, nervous system and reproductive organs. On the other hand, in-vivo studies, especially of the bioavailability by oral exposure, are rather scarce. A study of the biodistribution of 60 and 56 nm AgNPs in rats demonstrated a dose-dependent accumulation of silver in liver, lungs, kidney, stomach, testes and brain. A significant accumulation of small-size (22, 42 and 71 nm) AgNPs in virtually all the above mentioned tissues was also observed in mice. However, silver was not detected in tissues from mice treated with large-sized (323 nm) AgNPs, proving that small-sized silver nanoparticles could be much easier absorbed in gastrointestinal tract and translocated in the organs. Loescher et al. showed that silver administered orally to rats in the form of AgNPs was less bioavailable that if administered as silver acetate. Van der Zande et al. found no differences in the tissue distribution pattern of two types of AgNPs in rats (<20 nm uncoated and <15 nm PVP coated). All these studies were based on the total silver determination in bulk tissue samples by GF AAS, ICP MS upon microwave-assisted digestion.

A deeper insight into the location, transportation, and functional mechanism of AgNPs can be gained by the plethora of hyphenated techniques typically applied to determine the size-distribution of engineered nanomaterials, offering a potential to discriminate between the intact and metabolized NPs in complex matrices. Molecular targets of nanoparticles can be investigated by the plethora of hyphenated techniques as demonstrated for CdS nanoparticles. The aim of this work was to study the fate of orally administered AgNPs in rats by the determination of their...
accumulation in organs (kidney and liver) and excretion (urine and feces) complemented by the tissue distribution imaging and speciation studies.

2. Experimental

2.1. Instrumentation

Total silver analysis
A CEM microwave digestion system, model MARSXpress, (CEM, Matthews, USA) equipped with XP1500 microwave vessels was used for the digestion of liver and kidney samples. Silver in tissues and urine samples was determined by using an ELAN DRC-e ICP MS (Perkin Elmer, Toronto, Canada). The sample introduction system consisted of a glass concentric slurry nebulizer and cyclonic spray chamber (Glass Expansion, Melbourne, Australia) operated at manufacturer’s recommended standard conditions. Feces were analysed by flame AAS using a Perkin-Elmer Model 2380 (Perkin Elmer) with an air: acetylene (40:20 ratio) flame.

Silver imaging
A NewWave UP-213 laser ablation (LA) system (NewWave Research, Freemont, USA) was coupled to an Agilent model 7500cs (Agilent, Tokyo, Japan) as described elsewhere, however, no hydrogen addition was necessary.32 The laser ablation system was operated in a focused spot mode at the repetition rate of 20 Hz with spot size of 100 µm and scan speed of 100 µm s⁻¹. The ablated matter was transported into the ICP with He gas (500 mL min⁻¹) and mixed in a T-connector with aerosol obtained using a Micromist nebulizer and a double pass Scott spray chamber prior to ICP MS.

Asymmetric Flow Field-Flow Fractionation
An AF2000 system (Postnova Analytics, Landsberg, Germany) was used to identify silver nanoparticles in rat feces. Separation conditions were optimized as described elsewhere.33 The eluent was directed from the channel through an UV-Vis diode-array detector (Shimadzu, Duisburg, Germany) recording the signal between 200 and 650 nm. The plasmon resonance of silver nanoparticles was used for their on-line detection by UV-Vis. A complete spectrum was registered every 2 s.

Size Exclusion Chromatography - ICP MS
A Superdex 75 10/300 GL column (GE Healthcare, Pittsburgh, USA) was coupled to an Agilent model 7500 (Agilent). Chromatographic separations were performed by using a model 1200 series HPLC pump (Agilent) as delivery system. The exit of the column was connected directly to the nebulizer of the ICP MS equipped with a collision/reaction cell as described elsewhere.31

2.2. Samples, reagents and materials

Silver nanoparticles
Collargol, CAS registry number 9007-35-6, available as powder and consisting of silver nanoparticles with an average diameter ca. 15 nm (with total silver content ~70%) in a protein matrix (~30%) (Fig. 1) was obtained from Laboratorios Argenol S.L. (Zaragoza, Spain). Collargol is described in pharmacopeias as a strong antiseptic.

Standards
A commercially available metallothionein (MT-2) isolated from rabbit liver (Enzo Life Sciences, Farmingdale, USA) was used. It was provided by the manufacturer as a solution in 25 mM Tris/HCl and 50 mM NaCl at pH 8, with a concentration of 780 µg mL⁻¹. Dogfish Liver Certified Reference Material DOLT-4 (National Research Council Canada, Ottawa, Canada) with a certified silver content of 0.93 ± 0.07 mg kg⁻¹ was used to check the accuracy of the total silver determination.

Reagents
Analytical reagent grade chemicals purchased from Sigma-Aldrich (St. Louis, USA) were used throughout unless otherwise stated. Ultrapure water (resistivity >18 MΩ cm⁻¹) was obtained from a Milli-Q Advantage system (Millipore, Billerica, USA).

2.3. Procedures

Animals
Weanling male Sprague Dawley rats (Rattus norvegicus) (Janvier, Le Genest-St-Isle, France) weighing 92 ± 3 g were randomly separated into three groups (A, B, C). Group A consisted of 24 rats randomly separated into two subgroups of twelve animals each: each subgroup consisted of 6 control rats and 6 experimental rats treated with Collargol for 81 days. All groups of rats were maintained in plastic cages in temperature-controlled environment (21 ± 1 °C) with a relative humidity 60 ± 4%, and subjected to a 12-h light/dark cycle from 7 p.m. to 7 a.m. and allowed free access to both food and water.

Rats were handled in compliance with European Union rules and according to the guidelines of NIH34 and the Committee on Animal Care at the University of Montpellier (France).
Diets and feeding procedures
Rats were fed on a standard diet supplied by SSNIFF (Spezialdiäten GmbH, Soest, Germany). Vitamins and mineral mixes were formulated according to AIN-93 guidelines.35 Rats and uneaten food were weighed daily. Rats received daily by gavage either tap water (control group) or an aqueous solution of Collargol (experimental group) at a dose of 500 mg kg\(^{-1}\). Body Weight (BW) for the first two groups (A and B) and 820 mg kg\(^{-1}\) BW for the third group (C). Collargol powder was dissolved in ultra-pure water and sonicated for 60 s. The volume of the suspensions fed was adjusted daily to the weight of rats. At the end of each experimental period, rats were deprived of food overnight.

Sampling collection
For each animal and for each experimental period, the liver and the kidney were excised, weighed, sectioned for analyses and stored at -80 °C. For each animal and 30-d period, urine and feces were collected, pooled and frozen each day during the 7 last days, i.e. once the animals were adapted to the treatment.

Acid digestion of rat organs and feces
Lyophilized liver and kidney samples were ground manually to a particle diameter below 1 mm. A ground sample (100-200 mg) was weighed accurately (± 0.1 mg) in a microwave digestion vessel. 7 mL of conc. HNO\(_3\) and 3 mL of conc. HCl were added and the digestion was performed at 200 °C and 800 psi for 30 min. In order to avoid the formation of insoluble AgCl the digestion was performed in the presence of excess of chloride to stabilize silver as the AgCl\(^{2-}\) complex. After digestion the volume was made up to 50 mL with 3% (v/v) HCl. Feces samples were ground with a gridding apparatus and a tank ball to a particle diameter below 1 mm. A 100-mg ground sample was weighed (±0.1 mg) in a vessel. 1 mL of conc. HNO\(_3\) and 3 mL of conc. HCl were added. Samples were digested in a sand bath and allowed to evaporate near to dryness. The remained solid was dissolved in 1.5 mL of conc. HCl and the solution was made up to 15 mL with 10% (v/v) HCl in a conical tube. The solutions were sonicated and centrifuged at 21 °C for 10 min at 3000 rpm.

A white precipitate was often observed at the bottom of the tube. The supernatant was removed and the solid was dissolved in 1.5 mL of conc. HCl. Both volumes were combined and made up to 50 mL with 10% (v/v) HCl. Digestions were made in duplicate.

Laser ablation - ICP MS imaging
Kidney and liver samples were dissected and embedded in Tissue-Tek® OCT (Sakura, Alphen aan den Rijn, The Netherlands), frozen in liquid nitrogen, and stored at -80 °C. Sections were cut into 35 µm thick slices using a Leica CM1950 cryostat (Leica Biosystems, Nussloch, Germany). The cryosections were directly mounted onto glass slides. The tissue section was systematically scanned (line by line) by a focused laser beam. The silver concentration heat map was constructed using Matlab software (Mathworks, Natick, USA).

Solubilization of feces prior to AsFIIFF
A 100-mg ground sample was accurately weighed (± 0.1 mg) in a conical tube. 2 mL of tetramethylammonium hydroxide (TMAH) 25% (w/w) and 400 µL of cysteine 0.5% (w/w) were added and mixed in a turbler for 24 h at 28 rpm. The solution was made up to 10 mL with a solution of 0.1% cysteine and 0.05% (w/w) Triton X-100, sonicated for 1 min and centrifuged at 21 °C for 15 min at 3000 rpm before analysis. Solubilizations were made by duplicate.

Size exclusion chromatography - ICP MS analysis
A 0.7-g sample (kidney or liver) was ground in liquid nitrogen. A volume of 2 mL of 200 mM ammonium acetate (pH 7.5) containing 1 mM diethiothreitol (DTT) and 0.1 mM phenylmethylsulfonylfluoride (PMSF) was added and the solution was sonicated for 1.5 min (power 25%, 3 s stop, 5 s pulse). The mixture was centrifuged at 4 °C for 20 min at 120,000 g; the supernatant was collected and freeze-dried. Prior to analysis, it was diluted in 200 µL of water. An aliquot of 10 µL was diluted with 95 µL of water, injected on the column and eluted at 0.55 mL min\(^{-1}\) with 100 mM ammonium acetate buffer (pH 7.5).

3. Results and discussions
3.1 Total silver content of rat organs
To study the distribution of silver in the different exposure groups, the total silver content of liver and kidney was determined as described above. The analytical performance of the method was tested in terms of recovery, accuracy and quantification limit. Recoveries (tested for 50 µg of Ag) ranged from 92-107%. Accuracy was verified by analyzing a certified reference material, DOLT-4. The result obtained (0.916 ± 0.027 µg g\(^{-1}\)) was in good agreement with the certified value (0.93 ± 0.07 µg g\(^{-1}\)). Instrumental quantification limit, calculated as 10 times the standard deviation of instrumental blank (n=10) divided by the sensitivity, was 0.007 µg g\(^{-1}\). The method quantification limit, calculated as 10 times the standard deviation of method blank (n=10) divided by the sensitivity, was 0.012 µg g\(^{-1}\).

The silver contents of liver and kidney were determined for control and experimental rats at 45-d exposure (group A). Results showed a significant accumulation of silver in both organs from the experimental group, well above the silver content found in liver and kidney from the control group (Table 1). These results identify liver and kidney as two target organs for orally administered AgNPs in rats.

The higher content observed in liver could be explained by the higher presence of sulfur in this organ and the consequent formation of silver-thiol complexes.36 Despite the significant accumulation of silver in both organs, no toxic effects were observed in rats after 45-d exposure. Rats after 81-d exposure were reported elsewhere to show signs of oxidative stress and inflammation suggesting a long-term effect.37 In order to check this hypothesis, the total silver content in liver and kidney from experiments at 30, 45 (group A) and 81 days (group C) was determined and compared. Results obtained (Table 2) showed that the total amount of silver in liver and kidney increased significantly after 81-d exposure, which could explain the toxic effects found by Ebabe et al.37 Moreover, in the same study liver was reported as a target organ, which correlates with the high amount of silver found in liver after 81-d exposure. However, the high silver content after 81-d exposure could not only be a consequence of the feeding time but also of the amount of silver.
Concerning the silver excretion, it can be supposed that silver is absorbed, led to liver through the portal vein and excreted into the bile. In a similar way it can be argued that the silver accumulated in kidneys is eliminated in urine. Indeed Kim et al.\textsuperscript{13} showed that silver is excreted in feces. The silver concentration found in liver after 30 + 30\textsuperscript{Vd} experiment was higher than for 30 and 45\textsuperscript{Vd} exposure. Therefore, the results here were normalized for the Collargol exposure dose defined as the ratio between the silver content determined in each organ (µg Ag/kg organ) and the daily Collargol dose (µg silver/kg body weight) (Fig. 2) showing that the long-term accumulation is produced regardless of the exposure dose.

### 3.2. Total silver content of feces and urine

Concerning the silver excretion, it can be supposed that silver is

**Table 1** Silver content (µg g\textsuperscript{-1}) of lyophilized kidney and liver from control (T) and AgNPs fed (C) rats for 45-d exposure. Average ± standard deviation (n=6)

<table>
<thead>
<tr>
<th>Rat</th>
<th>Liver</th>
<th>Kidney</th>
<th>Rat</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>T 1</td>
<td>0.067 ± 0.002</td>
<td>0.042 ± 0.003</td>
<td>C 1</td>
<td>4.655 ± 0.179</td>
<td>1.986 ± 0.062</td>
</tr>
<tr>
<td>T 2</td>
<td>0.077 ± 0.002</td>
<td>0.060 ± 0.002</td>
<td>C 2</td>
<td>1.903 ± 0.069</td>
<td>1.344 ± 0.050</td>
</tr>
<tr>
<td>T 3</td>
<td>1.493 ± 0.041</td>
<td>0.235 ± 0.032</td>
<td>C 3</td>
<td>3.315 ± 0.059</td>
<td>1.336 ± 0.051</td>
</tr>
<tr>
<td>T 4</td>
<td>0.917 ± 0.045</td>
<td>0.020 ± 0.001</td>
<td>C 4</td>
<td>5.566 ± 0.116</td>
<td>1.142 ± 0.035</td>
</tr>
<tr>
<td>T 5</td>
<td>0.266 ± 0.006</td>
<td>0.109 ± 0.011</td>
<td>C 5</td>
<td>1.487 ± 0.026</td>
<td>1.608 ± 0.065</td>
</tr>
<tr>
<td>T 6</td>
<td>0.029 ± 0.002</td>
<td>0.110 ± 0.003</td>
<td>C 6</td>
<td>4.004 ± 0.059</td>
<td>4.714 ± 0.085</td>
</tr>
<tr>
<td>Control</td>
<td>0.638 ± 0.696</td>
<td>0.109 ± 0.111</td>
<td>AgNPs fed</td>
<td>3.488 ± 1.581\textsuperscript{a}</td>
<td>2.022 ± 1.351\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} significantly different from control group, P<0.05.

**Table 2** Silver content (µg g\textsuperscript{-1} dry mass) of livers and kidneys from AgNPs fed rats for 30, 45 and 81-d exposure. Average ± standard deviation (n = 6 for 30 and 45 days; n=14 for 81 days)

<table>
<thead>
<tr>
<th>Organ</th>
<th>Days</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>30</td>
<td>5.09 ± 2.41</td>
<td>3.49 ± 1.59</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td></td>
<td>2.02 ± 1.35</td>
</tr>
<tr>
<td>Kidney</td>
<td>81</td>
<td>0.89 ± 0.47</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 2** Comparison of total silver content determined in liver and kidney between different exposure groups. Results are normalized for the silver exposure dose and presented as the ratio between the silver content determined in each organ (µg Ag/kg organ) and the daily Collargol dose (mg Ag/kg body weight) administered to rats daily, as the Collargol dose for 81-d exposure was higher than for 30 and 45-d exposure. Therefore, the results here were normalized for the Collargol exposure dose defined as the ratio between the silver content determined in each organ (µg silver/kg organ) and the daily Collargol dose (µg silver/kg body weight) (Fig. 2) showing that the long-term accumulation is produced regardless of the exposure dose.

**Table 3** Silver content of rat feces and urine from AgNPs fed rats for 30 and 30 + 30-d exposure. Average ± standard deviation (n=6)

<table>
<thead>
<tr>
<th>Days</th>
<th>Feces / g kg\textsuperscript{-1}</th>
<th>Urine / µL L\textsuperscript{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>16.13 ± 1.33</td>
<td>40.88 ± 4.11</td>
</tr>
<tr>
<td>30 + 30</td>
<td>&lt; 0.01</td>
<td>0.35 ± 0.04</td>
</tr>
</tbody>
</table>

To study the elimination of silver, the total silver content in feces and urine from experimental rats of group B (rats treated with Collargol for 30 days and another 30 days with tap water only) was analyzed. Results showed an almost complete elimination of silver after 30-d post-exposure (Table 3). Silver found in urine was a 0.8% of the silver found after 30-d exposure, whereas in feces no silver (<0.01 g kg\textsuperscript{-1}) was detected. Furthermore, the silver content of liver was determined and compared with that in feces from the same experimental group B. The silver concentration found in liver after 30 + 30-d experiment (0.05 µg g\textsuperscript{-1}) was well below the silver content found in liver after 30-d exposure (5.09 µg g\textsuperscript{-1}). This finding was in good agreement with the analysis obtained for feces, as in both cases no silver accumulation was found after exposure.

### 3.3 Silver distribution in liver and kidney

The analysis of total silver showed the accumulation in liver and kidney, and the analysis of urine and feces confirmed its elimination via the digestive tract. Besides, the different
3.4 Identification of silver nanoparticles in feces

Once in the gastrointestinal system, AgNPs can behave in two different ways: (i) they could be absorbed as intact nanoparticles, and/or (ii) be dissolved by oxidation releasing of Ag (I). According to the literature, the first scenario can occur in some extent. Van der Zande et al. detected intact AgNPs in liver, spleen and lungs upon exposure to rats. Loeschnet al. also detected nanosized silver granules in the ileum of animals.
exposed to silver nanoparticles. Interestingly, in both studies nanoparticulate silver was also detected for animals exposed to Ag(I) salts. This indicates that a fraction of nanoparticles might be bioavailable. Besides, nanoparticles detected in tissues can be formed \textit{in vivo} from Ag(I) and they are probably composed of silver salts.\textsuperscript{16}

Neither the total silver measurements nor LA-ICP MS imaging can provide information on whether the silver is present as dissolved or particulate forms. Therefore, in order to identify the form of Ag in rat feces, a procedure using AsFIFFF was developed. Feces were solubilized with TMAH in order to avoid the use of strongly oxidizing HNO\textsubscript{3}. The use of TMAH was recently reported to preserve AgNPs in cells\textsuperscript{27} and tissues.\textsuperscript{38}

Fig. 5 shows the fractogram obtained for feces from rats after 30-d exposure (group A). It shows the presence of Ag nanoparticles with the size distribution resembling that of AgNPs in Collargol in the same medium which confirms the presence of intact AgNPs in the feces after the solubilization by TMAH.

3.5. Insight into molecular targets of silver in liver and kidney

In order to get an insight into the speciation of silver accumulated in liver and kidney, the relevant cytosols from 30, 45 (group A) and 81-d (group C) experiments were analyzed by SEC-ICP MS. Fig. 6 shows that silver is complexed by a number of biomolecules in the wide range with standing out fractions at >70, 66, 29 and 7 kDa.

In the case of liver, the morphology of the chromatograms is practically independent of the exposure time and similar to that for sulphophilic elements such as Cu or Cd.\textsuperscript{39} The overall intensity increases (as this is the case for the total Ag concentration). This suggests the existence of an abundant pool of proteins able to absorb the incoming silver by complexation once the MT pool is saturated. These are very likely to be cysteine-rich residues responsible for potent silver-binding capability.\textsuperscript{40}

In the case of kidney, the ratio of the complexes with high molecular mass ligands (>70-33 kDa) to that with low molecular weight ligands is increasing as a function of the exposure time. The low molecular weight fraction was identified by a comparison with the relevant standard as the complex of Ag with metallothionein. Kurasaki et al.\textsuperscript{41} reported an induction of Ag-induced metallothionein in kidneys by injecting silver lactate. The localization of Ag-MT in the kidney cortex was confirmed using autofluorescent signals and immunohistochemistry.\textsuperscript{41}

These results confirm the presence of Ag in the form of complexes with biomolecules as a result of the oxidation of silver nanoparticles during digestion, as it has been confirmed by Liu et al.\textsuperscript{42}
Fig. 6 $^{107}$Ag signal obtained for cytosols extracted from liver and kidney from experiments at 30, 45 and 81 days compared with MT standard spiked with silver.
Conclusions

An in vivo study based on oral administration of AgNPs to rats indicates a significant accumulation of silver in kidney and liver, the latter being the principal target of AgNPs. The silver is relatively labile, being eliminated after 30 days. Silver is homogeneously distributed in liver whereas in kidney is preferentially located in cortex. Silver speciation demonstrated the oxidation of AgNPs in the biological environment producing Ag(I) complexed by a number of proteins, the major of which in kidney is metallothionein. The presence of intact AgNPs in rat feces was detected for the first time.

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