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Quantitative imaging of translocated silver following nanoparticle exposure by laser ablation-inductively coupled plasma-mass spectrometry

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The likelihood of exposure to antimicrobial silver nanoparticles continues to grow with increasing ubiquity in various medical and consumer products. While translocation of silver nanoparticles to major organs has been examined, the *in situ* location and concentration in the organs is not well characterised. Here we have used laser ablation-inductively coupled plasma-mass spectrometry to quantitatively image serial sections to construct a three-dimensional representation of the distribution of silver in rat spleen following respiratory tract exposure *via* intratracheal instillation of silver nanoparticles. Silver was distributed predominantly in the white pulp of the spleen at concentrations greater than 300 ng/g. Imaging tissue sections via laser-ablation-inductively coupled plasma-mass spectrometry is an excellent tool for the visualisation and quantification of metals attributed to nanoparticles in organs allowing investigation of silver nanoparticle exposure *in vivo*.

Introduction

The antimicrobial properties of silver nanoparticles[1] (Ag-NPs) have led to their widespread use in the manufacture of medical devices, wound dressings and consumer products that range from washing machines to clothing and toys[2-4]. Estimating the number of products currently containing silver nanoparticles is difficult as the composition of the majority of nanostructured surfaces is not reported[5, 6]. However, the number of products containing nanoparticles has increased approximately 20-fold from 2005 to 2010 according to the Nanoparticle Consumer Product Inventory[5]; and AG-NPs were estimated to be included in approximately a quarter of commercial nanomaterial products[6].

Despite increasing ubiquity there have been limited *in vivo* studies addressing the effects of exposure to Ag-NPs. Factors that are known to contribute to their toxicity include concentration, size, shape, surface coating, surface charge and release of ions[4, 7-11]. Regulatory frameworks in Australia[12], Germany[13] and the USA[14] rely on the exposure limits for the bulk material (e.g., silver); however these do not account for the high surface area per mass of nanoparticles and perhaps more importantly that their size and surface properties facilitate their uptake by tissues in the body[15].

In vitro investigations have shown that Ag-NPs are able to induce oxidative stress[3, 16], apoptosis[7], cytotoxicity[17] and genotoxicity[7, 17] in cell lines, and oxidative stress and cytotoxicity in alveolar macrophages[9]. Interpretation and comparison of such studies is complicated by the lack of standards for the assessment of nanoparticles and poorly or differently characterised nanoparticles between studies[18].

There are limited published studies of silver nanoparticle toxicity *in vivo* and these vary by route of exposure, concentration and analysis of the Ag-NPs. Oral exposure of *Drosophila*[19] and intraperitoneal exposure of mice to Ag-NPs confirmed that oxidative stress, genotoxicity, and apoptosis also occur *in vivo*[3, 11]. Dermal and ocular exposure studies show insignificant irritation[20], whilst oral exposure has shown changes in kidney histopathology and liver function markers at high doses in rodent models[21]. Translocation to and accumulation of silver occurs in blood, brain, heart, liver, spleen, lungs, testes and kidneys when Ag-NPs are administered *via* oral or inhalation routes with females experiencing a 2-fold accumulation in the kidneys compared to male rats[11, 22-24]. Inhalation studies demonstrate persistence of silver in the lungs, liver and kidneys of rats at 7 days after inhalation exposure[25]. Regardless of the route of Ag-NP exposure, there is increasing evidence that translocation to and accumulation in secondary organs may be problematic, and this has been recently reviewed[26, 27].

While there is increasing evidence that the unique physico-chemical properties of NPs which make nanomaterials more

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efficient in industrial applications also make these materials more harmful to living organisms. Regulatory guidelines describing the methods by which their toxicity should be assessed are yet to be finally determined and an improved understanding of their distribution and bio-accumulation within tissues will further inform our understanding of their effects. Many of these studies rely on the quantification of Ag-NPs in individual tissues using whole organ digests analysed by elemental spectroscopy[11, 22, 23, 25, 28-30]. It is becoming increasingly recognised that accumulation of metal species in highly specialised tissues such as the brain for example can have deleterious effects depending on the location[31]. Localisation of Ag-NPs within organs has been reported using TEM[11, 22, 25] and microscopy[23, 30], however these methods do not allow *in situ* quantification.

Elemental bio-imaging (EBI) is a quantitative imaging technique that may be used to determine the *in situ* concentration of trace elements in thin sections of biological tissue. EBI typically performed by laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) has been reported for imaging of murine brain[31-34], liver biopsies of Wilson's disease[35], and metal-tagged cancer biomarkers[36, 37]. Ag was previously imaged with EBI following Ag-NP exposure in single cell line toxicological assays[38, 39] and for the non-quantitative localisation in rat liver and kidneys after ingestion[40]. Here, we demonstrate the utility of EBI for determination of location and concentration of Ag within specific structures of organs in a rat intratracheal instillation model of Ag-NP exposure by examination of translocation of silver to the spleen.

Experimental

Instrumentation

All LA-ICP-MS analyses were conducted on an Agilent 7500ce series ICP-MS (Agilent Technologies, Waldbronn, Germany) coupled to a CETAC LSX 213 laser ablation unit (Teledyne CETAC Technologies, Omaha, NE, USA) with a Nd:YAG laser emitting at a wavelength of 213 nm. Laser ablation and ICP-MS conditions were optimised on a gelatine standard containing 28 µg/g Ag. The optimised conditions were a spot size of 50 µm with a scan speed of 100 µm/s and a frequency of 20Hz, the laser cell gas was 0.8 L/min of He with 1.55 L/min Ar as a makeup gas. Prior to ablation, images of the sections were collected using a BZ-9000 inverted fluorescence/bright field microscope (Keyence, Osaka, Japan). Calibration curves and construction of images were performed in ISIDAS, an in-house developed imaging software, with Paraview used for image visualisation[41]. The resulting calibration equations were used to convert the signal intensities of every pixel in each image to concentrations (ng/g). The three-dimensional image was constructed by importing the calibrated two-dimensional vtk files into ImageJ (Rasband, W.S., ImageJ, U. S. NIH, Bethesda, Maryland, USA) as a sequential stack, with StackReg[42] used in rigid mode to register the sections. A three-dimensional vtk file was then created with MATLAB code written in house for image visualisation with Paraview.

For solution analyses, an Agilent Technologies 7500cx ICP-MS (Agilent Technologies, Mulgrave, Australia) was used with sample introduction via a micromist concentric nebuliser (Glass Expansion, West Melbourne, Australia) and a Scott type double pass spray chamber cooled to 2°C. The sample solution and the spray chamber waste were carried with the aid of a peristaltic pump. ICP-MS extraction lens conditions were selected to maximise the sensitivity of a 1% HNO_3:HCl solution containing 1 ng/mL of Li, Co, Y, Ce and Tl. Helium was added into the octopole reaction cell to reduce interferences. Calibration curves were constructed and the results analysed using Agilent Technologies Masshunter software.

Reagents

Ultrapure HNO_3 and the Ag standard were supplied by Choice Analytical (Thornleigh, New South Wales, Australia). Gelatine was purchased from Grüssing GmbH (Filsum, Germany). . Silver nanoparticles (30 – 50nm) coated with 0.2wt% polyvinylpyrolidone (Ag50PVP; CAS 7440-22-4) were prepared and characterised by the NanoGem consortium[43].

Standards

Matrix matched gelatine standards were prepared as previously described[44]. Briefly, standards were made in 10% gelatine with Ag concentrations ranging from 0.01-28 µg/g. The mixtures were homogenised and sliced on a cryotome in 25 µm thick sections. To determine the concentration of the standards, 100 µg of each standard was dissolved in 1 mL of HNO_3 and diluted to 5 mL before analysis by solution ICP-MS. The limit of quantification (LOQ) was calculated as;

$$LOQ=10s_0$$

Where s_0 is the standard deviation of the gas blank signal.

Correlation coefficients >0.99 were obtained in all experiments and LOQs were <30 ng/g.

Rat model

All animal experiments were carried out in the animal facility at the University Clinics of Essen, Germany, and protocols were approved by the institutional ethics authorities (LANUV, Dortmund, Germany). Young female rats weighing 200-250 g (Wistar strain WU) were purchased from Charles River Laboratories (Sulzfeld, Germany) and maintained on a 12 h light/dark cycle. Food and water were provided ad libitum. Toxicological testing was carried out with Ag50PVP nanoparticles (0.6 mg/mL) suspended in 0.9% pyrogen-free NaCl (Fresenius Kabi GmbH, Bad Homburg, Germany). The animal was anaesthetized with 5% isoflurane in air and 500µl of freshly prepared Ag50PVP nanoparticle solution was intratracheally instilled with a Penn Century Microsprayer under visual control. 21 days post-instillation, rats were anaesthetized with ketamine/xylazine. The spleen was

removed, snap frozen in liquid nitrogen and stored at -80°C until required.

Preparation of tissue sections for analysis

The frozen spleen was embedded in Tissue-Tek™OCT (Sakura, USA) and transverse serial sections cut at 25 µm onto glass slides with a cryomicrotome (Microm HM 500), air dried and stored at -80°C until required for analysis.
Prior to analysis frozen sections were post-fixed in 10% neutral buffered formalin and washed 3 x 5 minutes (1 x PBS followed by 2x MilliQ water) and air dried. Photomicrographs were obtained at 10x magnification under bright field illumination for structural differentiation of the red and white pulp.

Results and discussion

The ability of LA-ICP-MS to detect Ag-NPs in biologically relevant complex mammalian organ samples was examined in splenic tissue from a rat model of Ag-NP inhalation. Uptake via the respiratory tract is the most relevant route for occupational Ag-NP exposure; *In vivo* experiments have shown that Ag-NPs instilled into the lungs enter the blood stream[25], and intraperitoneal and intravenous injection that they lead to accumulation in the spleen[23, 24]. The spleen is composed of two functionally and morphologically distinct compartments, the red pulp and the white pulp. The red pulp filters the blood while the white pulp is the largest secondary lymphoid organ that contains approximately 25% of the body's lymphocytes[45]. The spleen is structured so that most of the blood passes along the marginal zone of the white pulp which scavenges pathogens and antigens by phagocytosis by resident macrophages that are then induced to migrate into the white pulp[46]. The efficacy of this process is dependent on characteristics of the nanoparticles and the time elapsed post-exposure[47]. The resulting inhomogenous distribution and accumulation of nanoparticles in the spleen and other organs requires sophisticated imaging methods to determine the spatial distribution and measure localised concentrations of nanoparticles.
The distribution of intravenously administered I^{125}-labelled Ag-NPs have been imaged in mice using single photon emission computerised tomography of the entire animal and showed accumulation in the spleen and the liver[48]. This approach was not able to provide information on the localised distribution and concentration of the nanoparticles and the potential effects of incorporation of iodine into the nanoparticle on charge and surface characteristics remain unknown.
Here we have shown that analysis of splenic sections from rat exposed to Ag-NPs via intratracheal instillation using LA-ICP-MS was able to identify and quantify areas of high concentrations of silver within the organ. The silver may be present as both silver ions and Ag-NPs, however in comparison to other organs the spleen is known to retain Ag-NPs[24]. The Ag were foci distributed through the spleen (Figure 1A). Comparison of the location of these foci with light micrographs of the same sections (Figure 1B) indicates that the silver was concentrated in small, discrete areas of the spleen (Figure 1A) most likely formed by phagocytic cells which are predominantly located within the white pulp of the spleen or within the borderzone[45]. This is consistent with reported findings in that the white pulp is the primary location of these cells in this organ at least 21 days post-exposure[49]. Interestingly there was a weak silver signal also detected in the peripheral red pulp, which may reflect silver ions and/or low concentrations of nanoparticles bound to red blood cells consistent with the reported transport of nanoparticles via circulation[22]. For example carbon nanotubes introduced into the respiratory tract were 90% cleared from the lungs in 90 days but the concentrations measured in organs including the spleen continued to increase over the 360 day duration of the experiment[49].
By analysing consecutive slices of the tissue it was possible to reconstruct a three dimensional image of the mid-section of the spleen showing the inhomogeneous distribution of silver (Figure 2). These accumulations were localised within the white pulp across the five consecutive sections with diameters of approximately 200 µm at concentrations greater than 300 ng/g. These images show that the silver forms large collections in the spleen and demonstrate that LA-ICP-MS has the spatial resolution to play a vital role in determining the bio-distribution and persistence of NPs following exposure.
With the increasing use of nanoparticles there have been recent reports of workplace exposure levels ranging from 0.12-1.35 µg/m^3 [50-52]. In 2016, Weldon *et al.*[2] published an occupational exposure limit (OEL) derived from toxicity and exposure data presented in the literature of 0.19 µg/m^3 suggested to prevent liver and lung damage, as well as argyria, in workers exposed to Ag-NPs. However, while *in vitro* toxicity testing may show Ag-NPs have no cytotoxic effects at a given silver concentration, the data presented here using LA-ICP-MS show that the translocated silver accumulated in high localised concentrations in the spleen and presumably other secondary organs[22, 23, 30, 48]. The silver ions released from these nanoparticles may cause local toxic effects within these organs and these aspects may need to be considered when evaluating the toxicity of these species.

Conclusions

We have demonstrated the ability of LA-ICP-MS to quantify Ag in challenging tissue matrices and provide both spatial localisation and quantitative information, it may now be applied to larger studies to better understand the pharmacodynamics of Ag-NP exposure by monitoring the changes in distribution and local concentrations of Ag in major organs over time. Our findings also indicate that consideration of the toxicity of local concentrations of nanoparticles and their resulting metal ions in secondary organs needs to be considered when developing exposure guidelines.

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Figure 1. A representative transverse section of a rat spleen taken from the central portion of the organ. The magnification of the section is indicated by the scale bar in image B. A) is an image of a splenic section acquired using LA-ICP-MS for ^{107}Ag. Silver ions have been quantified and represented using a colour scale where blue equals 0 ng/g of Ag, and red represents areas equal to or greater than 300 ng/g of Ag. Note the highly concentrated silver foci and the elevated Ag signal in the peripheral red pulp regions. B) Low power photomicrograph of the section prior to ablation.

Figure 2. 3-dimensional image of the mid-section of the spleen generated from 5 consecutive 25 µm thick sections. A large collection of Ag spanning the volume of the 5 sections is indicated by an arrow. Scale is in ng/g with blue equal to 0 and red equal to 300.