The role of PVP in the bioavailability of Ag from the PVP-stabilized Ag nanoparticle suspension

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ABSTRACT

We assessed the bioavailability of Ag from Ag nanoparticles (NPs), stabilized with polyvinylpyrrolidone (PVP), to terrestrial isopods which were exposed to 10, 100 and 1000 µg Ag NPs/g of dry food. Different Ag species were determined in the NP suspension that was fed to isopods: (i) total Ag by atomic absorption spectroscopy, (ii) the sum of Ag-PVP complexes and free Ag^+ by anodic stripping voltammetry at the bismuth-film electrode, and (iii) free Ag^+ by ion-selective potentiometry. The amounts of Ag species in the consumed food were compared to the masses of Ag accumulated in the isopod digestive glands. Our results show that all three Ag species (Ag NPs, Ag-PVP complexes and free Ag^+) could be the source of bioaccumulated Ag, but to various degrees depending on the exposure concentration and transformations in the digestive system. We provide a proof that (i) Ag NPs dissolve and Ag-PVP complexes dissociate in the isopod digestive tract; (ii) the concentration of free Ag^+ in the suspension offered to the test organisms is not the only measure of bioavailable Ag. The type of NP stabilizer along with the NP transformations in the digestive system needs to be considered in the creation of new computational models of the nanomaterial fate.

KEYWORDS: Ag ions, Ag complexes, voltammetry, ion-selective electrode, terrestrial isopod

HIGHLIGHTS:

- Ag from Ag NPs, Ag-PVP complexes and free Ag^+ accumulates in isopod hepatopancreas.
- Ag NPs dissolve in the digestive tract of terrestrial isopods.
- Ag-PVP complexes dissociate in the digestive tract of terrestrial isopods.
- NP stabilizer affects the bioavailability of metals from metal NPs.

CAPSULE: The polyvinylpyrrolidone stabilizer for Ag NPs forms labile complexes with Ag^+, which are bioavailable to the test terrestrial isopod.

INTRODUCTION

In the recent years, it has been recognized that nanoparticles (NPs) can undergo a number of transformations such as agglomeration/aggregation, surface association with organic molecules, dissolution, and the formation of corona on their surface (Lowry et al., 2012). These processes govern the bioavailability, cellular internalization and toxicity of NPs. At present, it is believed that ions dissolved from NPs are among the main mediators of their biological effects on organisms (Ivask et al., 2014). Organisms can either increase the dissolution of metal/metal oxide NPs (for example Ag NPs, Cu NPs, $CoFe_2O_4$ NPs and rare-earth oxide NPs; Golobič et al., 2012; Bondarenko et al., 2013; Novak et al., 2013; Huynh et al., 2014; Leclerc and Wilkinson, 2014; Diez-Ortiz et al., 2015; He et al., 2015; Romih et al., 2015) or decrease their dissolution (for example aminosilane-capped ZnO quantum dots; Bellanger et al., 2015). In the case of bacteria and unicellular algae, the NP dissolution takes place outside the organism at the point of contact with NPs (Bondarenko et al., 2013; Huynh et al., 2014; Leclerc and Wilkinson, 2014; Bellanger et al., 2015; He et al., 2015) while in the case of isopods and earthworms, the NP dissolution is suggested to occur inside the digestive tract in contact with digestive juices (Golobič et al., 2012; Novak et al., 2013; Diez-Ortiz et al., 2015; Romih et al., 2015).

Terrestrial isopods (e.g. *Porcellio scaber*, *Porcellionides pruinosus*) have become established model organisms for metal kinetics and metal nanoparticle transformations (Romih et al., 2015; Tourinho et al., 2015, 2016). Focus is set in particular on digestive gland (hepatopancreas), which is the main digestive and metal storage organ (Hames and Hopkin, 1989). The advantage of this test model is an almost exclusive uptake of NPs through oral

route and negligible surface uptake (Vijver et al., 2005), which enables the determination of exact metal mass balances. In our previous studies with isopods *P. scaber* (Golobič et al., 2012; Novak et al., 2013; Romih et al., 2015), we have provided the first evidence that digestive juices enhance the dissolution of selected metal/metal oxide NPs inside the organism. In the study with Cu NPs (Golobič et al., 2012), no particle stabilizers were present and the net bioaccumulation of dissolved ions showed an enhanced dissolution of NPs in the digestive system. In the studies with either bare or citric acid-adsorbed $CoFe_2O_4$ NPs (Novak et al., 2013; Romih et al., 2015), we additionally showed that citric acid enhances the dissolution of NPs in the suspension, but decreases the bioavailability of dissolved metal ions due to chelation at the near-neutral pH of the *P. scaber* digestive system.

To advance our understanding of NP transformations within the digestive system of isopods and the role of NP stabilizer in the bioavailability of dissolved metals, we performed a study with Ag NPs stabilized with polyvinylpyrrolidone (PVP). Silver NPs are most commonly stabilized with PVP, which is known to form weak coordinative bonds with Ag^+ (Zhang et al., 1996; Wang et al., 2005). Suspensions of PVP-stabilized Ag NPs therefore contain at least three different Ag species: Ag^0 (Ag NPs), Ag-PVP complexes and free Ag^+. To the best of our knowledge, the stability constant of Ag-PVP complexes has not been determined yet and the behavior of these complexes in relation to, for example, pH, ionic strength and temperature, is unknown. It is also unknown how Ag-PVP complexes behave in the digestive system of organisms. The bioavailability (as defined by Riding et al., 2013) of Ag from PVP-stabilized Ag NPs therefore cannot be predicted in advance; both biological and chemical means are needed to elucidate the dynamic processes taking place inside the isopod digestive system.

In our previous studies (Golobič et al., 2012; Novak et al., 2013), the amount of free ions in NP suspensions used to spike the food was quantified by the ultracentrifugation

approach coupled with spectrometry. This approach has been one of the most commonly applied in metal/metal oxide NP dissolution analyses, but it has been criticized in terms of incomplete discrimination between ions and particulate matter (Misra et al., 2012; Tantra et al. 2015; Jemec et al., 2016). Namely, spectroscopic techniques may overestimate the amount of free metal ions if the separation between ions and particulate matter is not efficient (Jemec et al., 2016). The novelty of the study presented herein is that electrochemical techniques, such as potentiometry using ion-selective electrodes (ISEs), and anodic stripping voltammetry (ASV), were used to determine the share of free ions and their complexes in the suspension of Ag NPs added to food. These two techniques have been previously recommended (Tantra et al. 2015; Romih et al., 2015, 2016). Anodic stripping voltammetry and potentiometry differ in the metal species they detect: while potentiometry is used for detecting only free ions, the ASV can be exploited for measuring both free ions and labile ion-ligand complexes. Anodic stripping voltammetry offers an advantage over the potentiometric approach as it enables the measurements of a much wider selection of elements and is significantly more sensitive, i.e. enables lower detection limits (Pesavento et al., 2009). The complementary data from potentiometric measurements (for Ag, Cu and Pb) and ASV measurements provide valuable information on metal speciation in the suspensions of the corresponding NPs.

The aim of this study was to assess the bioavailability of Ag for *P. scaber* when offered as a composite Ag NP suspension stabilized with PVP as a surfactant, which was not chemically attached to the NP surface as a coating, but was added to suspension as a surfactant creating sterical hindrance among NPs. The suspension consisted of Ag NPs, Ag-PVP complexes and free Ag^{+}. For this purpose, we first quantified the three Ag species in the suspension applied to the food that was offered to isopods, and compared them to the amount of Ag accumulated in the digestive glands. Due to transformations of Ag in the digestive system and the presence of PVP, which could additionally affect the Ag bioavailability, the

question was whether more free ions as ingested are available for the bioaccumulation or less. If Ag^+ form weak complexes with PVP, which dissociate under physiological conditions, we expect Ag from Ag-PVP complexes to be bioavailable. On the other hand, if Ag-PVP complexes dissociate to a limited extent, we do not expect them to provide a source for Ag accumulation. We discuss the role of PVP as a stabilizing agent for Ag NPs and its effect on the bioavailability of Ag for isopods.

EXPERIMENTAL

Silver nanoparticle sample preparation and characterization

Silver NPs (designated as NNV 003; batch number Parnasos_IG010305_Ag NAMA39_1202_Ag) were supplied by the Colorobbia S.p.A. (Firenze, Italy) within the framework of the EU FP7 NanoValid project. The Ag NPs were supplied as an aqueous suspension with nominal particle concentration 40 g/L, stabilized with PVP as a surfactant which was added into the suspension (not chemically attached to the NP surface). The data on particle size, shape, and ζ-potential was provided by the partners of the EU FP7 NanoValid project. The exact concentration of the PVP stabilizer is considered an intellectual property of the supplier and could not be revealed.

The stock suspension of Ag NPs was diluted with deionized water (MilliQ, Millipore, Bedford, Massachusetts, USA [ρ = 18.5 MΩ·cm]), prior to the use in experiments. For the dissolution measurements we chose the concentrations 12.5, 25 and 50 mg/L of Ag, which are lower than those applied on the leaves (10, 100 and 1000 mg/L). The reason was that PVP in the very same Ag NP suspension had been shown to interfere with the silver ion-selective electrode (Ag-ISE) already at the concentration 25 mg/L (Romih et al., 2016). The total Ag contents in the diluted Ag NP suspensions were checked with flame AAS after overnight digestion in 1 M HNO_3 (suspension/acid ratio 1:1 vol/vol).

Silver nanoparticle dissolution measurements

Eight milliliters of diluted suspensions at concentrations 12.5, 25 and 50 mg/L of Ag were ultracentrifuged in duplicate at 100 000 *g* for 30 min at 20 °C (Beckman Coulter L8-70M class H preparative ultracentrifuge with Type 70.1 Ti rotor and 10 mL thickwall polyallomer tubes). The supernatants were then divided into two aliquots for Ag quantification. The first aliquot was acidified with 65 % HNO_3 (analytical grade purity; Fischer Scientific, Leicester, UK) and the total Ag content was quantified by flame AAS. The second aliquot was left unacidified and was used for the determination of the Ag species (the sum of Ag-PVP complexes and free Ag^+) by ASV and free Ag^+ by potentiometry with Ag-ISE. The stock suspension was also measured by ASV without prior ultracentrifugation or acidification; the measurements were carried out in the same way as for the supernatants of the diluted suspensions. The unacidified supernatants were checked for the presence of unsedimented NPs by the dynamic light scattering (DLS) measurements using Analysette 12 DynaSizer (Fritsch GmbH, Idar-Oberstein, Germany). All the characterization steps were performed on freshly-prepared suspensions.

The ASV approach followed the newly-developed protocol, which was described in Romih et al. (2016). The measurements were performed using the PalmSens potentiostat/galvanostat (PalmSens BV, Utrecht, Netherlands) in combination with the PSTrace 4.4 software (PalmSens BV). The usual three-electrode configuration was employed with the bismuth electrode prepared *in-situ* on a glassy carbon (GCE) disk (d = 2 mm) as the working electrode, a platinum wire as the counter electrode, and a double-junction saturated Ag/AgCl/KCl reference electrode containing 0.1 M HNO_3 as the outer electrolyte to prevent chloride leakage into the sample solution. A computer-controlled magnetic stirrer, rotating at approximately 300 rpm, was employed during the electrochemical accumulation step. All experiments were carried out at room temperature of 23±1 °C in a 20 mL one-compartment

voltammetric cell. The standard solutions of bismuth(III) and silver(I) were provided by Merck (Darmstadt, Germany) and further diluted as required. 0.1 M acetate buffer solution with pH 4.5 was used as a supporting electrolyte. Water used throughout the work was first deionized and then further purified using Elix 10/Milli-Q Gradient unit (Millipore, Bedford, Massachusetts, USA). Following an electrochemical accumulation step of 60 s at −0.8 V, and a subsequent equilibration period of 15 s, an anodic stripping voltammogram was recorded in a quiescent solution by scanning the potential towards more positive values using a square-wave potential scan from −0.6 V to +0.6 V *vs*. the reference electrode, with a frequency of 25 Hz, a potential step of 4 mV, and an amplitude of 50 mV. Before each measurement, a cleaning step was carried out by applying the potential of +0.4 V for 30 s.

The potentiometric measurements were performed using the perfectION™ Ag/S_2 combination ion-selective electrode (Mettler Toledo, Greifensee, Switzerland). The experiments were carried out at room temperature of 23±1 °C in a 20 mL beaker with the use of a magnetic stirrer rotating at 300 rpm. The ionic strength adjustment solution (perfectION™ Ion Electrolyte B, Mettler Toledo) was added to the samples following the protocol of the supplier (ionic strength adjustment solution : sample ratio = 1:50 vol/vol). The Ag-ISE was submerged in the sample after adding the ion strength adjustment solution. The electrode potential was recorded after equilibration for at least two minutes.

The concentrations of free Ag^+ and Ag-PVP complexes in the supernatants of the Ag NP suspensions were determined by the method of three standard additions (Harris, 2010).

Feeding experiment with terrestrial isopods Porcellio scaber

Terrestrial isopods *P. scaber* were collected in September of 2013 from a compost heap in a non-polluted garden in Podutik, Ljubljana, Slovenia. Animals were kept in a climate chamber at 22±1 °C with a 16/8 h light/dark period (120 and 16 lx, respectively; measured

using LI-1000 Data Logger, LI–COR, Nebraska, USA), caged in glass containers with moist loamy sand and peat at the bottom. They were fed dry fallen common hazel leaves (*Corylus avellana*) for three weeks before the Ag exposure. *P. scaber* adults of both sexes, including those at the intermoult and early premoult stages (Zidar et al., 1998) and weighing between 30 and 70 mg of fresh body mass, were selected for the experiments. Moulting individuals (Zidar et al., 1998) and gravid females were excluded.

The experimental setup was performed as described previously (Romih et al., 2015). During the experiment, the animals were fed dry common hazel leaves that were spiked with deionized water (MilliQ, Millipore, Bedford, Massachusetts, USA) as the negative control, $AgNO_3$ (≥99.5 % trace metals basis, Sigma Aldrich, Steinheim, Germany) as the dissolved ion control, and Ag NPs as the experimental compound. All of the test chemicals were prepared freshly prior to the experiment at concentrations of 10, 100, and 1000 mg Ag/L. 100 µL of a test chemical per 100 mg of leaf was applied onto abaxial leaf surfaces with a pipette and spread evenly with a paintbrush (Giotto, F.I.L.A. S.p.A., Pero, Italy), which resulted in the nominal exposure concentrations of 10, 100, and 1000 µg Ag/g of dry leaf mass. The concentrations were chosen on the basis of the work of Pipan-Tkalec et al. (2011), where no adverse effects of Ag NPs on *P. scaber* in terms of feeding rate or body mass change were found in the range of exposure concentrations at 0.1–5000 µg/g of dry food. The leaves were left to dry at room temperature and were re-weighed prior to the experiment. The animals were weighed and placed individually into plastic Petri dishes (∅ = 9 cm) along with the prepared leaves with the contaminated side facing up. No soil or other substrate was added. Each exposure group contained 15 animals; there were no significant differences in the distributions of fresh body masses among the animals divided into different test groups (p>0.05, Mann-Whitney U-test, results not shown). The experiment was maintained for 14 days in controlled and stable conditions at 22±1 °C, 80 % relative humidity (TFA Dostmann

GmbH & Co.KG, Wertheim, Germany), with a 16/8 h light/dark period (120 and 16 lx, respectively) and monitored on a daily basis. The humidity in the Petri dishes was maintained by periodical spraying of deionized water on the internal sides of the lids. At the end of the experiment, the animals were switched to uncontaminated hazel leaves for 1 day to depurate the Ag-spiked food from their digestive systems. The uneaten spiked leaves were collected, air-dried and weighed. Fecal pellets were also weighed after drying in a desiccator for 24 h. After the depuration period, animal mortality was recorded and the surviving animals were weighed. The animals were decapitated and their digestive glands were isolated. Individual digestive glands were placed on small pieces of filter paper (approximately 4 mm × 7 mm in size), stored in plastic tubes, and frozen at -20 °C until further analyses.

A sample of fecal pellets was attached to aluminum holders with a double-sided adhesive carbon tape and inspected for the presence of NPs with a thermal field-emission scanning electron microscope (JEOL JSM-7600F, Tokyo, Japan) under the accelerating voltage of 15 kV (**Supplementary Figures S8–S11**).

Measurements of metal content in food and in the animal tissues

The total Ag concentration in the digestive glands of isopods and in the uneaten leaf remnants after the experiments was determined by flame AAS. Prior to the analysis, the samples were acid digested in concentrated nitric acid (65 % HNO_3, analytical grade purity, Fisher Scientific, Loughborough, UK) in the Milestone Ethos E microwave lab station (Milestone, Bergamo, Italy) equipped with a SK-10 high pressure segmented rotor and 3 mL quartz microsampling inserts. Digestion was conducted at 180°C and 600 W power, with step 1 (heating) lasting 15 min, step 2 (constant temperature) lasting 10 min, and 45 min cooling to 60 °C.

Data analyses of feeding parameters and metal concentrations

The animals that died during moulting and gravid females were excluded from further data processing (n = 19 in total). The numbers of analyzed animals are presented in the figures as part of the x-axis labels. The data are presented as mean values, and uncertainties are expressed as standard deviations (SD). All the data shown in the figures describe nominal concentrations of Ag (10, 100, and 1000 μg/g of dry leaf). The statistical significances of the differences between the control and exposed groups of animals were assessed by the Mann-Whitney *U*-test (*p<0.05, **p<0.01, ***p<0.001) using the OriginPro 8.0 software (OriginLab Corp., Northampton, MA, USA). The formulae for all the calculations used in the present study (the shares of Ag-PVP complexes and free Ag^+ that dissolved from the nanoparticles, the masses of Ag consumed by the isopods and the shares of Ag accumulated into their digestive glands upon exposure to Ag) are provided in the **Supplementary Material** as **Equations (S1) to (S4)**.

RESULTS

Silver nanoparticle characteristics

Silver NPs were supplied as an aqueous suspension with a nominal particle concentration of 40 g/L (the mean measured total Ag concentration was 41.14 g/L; Jemec et al., 2016). The same batch of Ag NPs has been previously characterized and described in Zou et al. (2015), Böhme et al. (2015) and Jemec et al. (2016). In summary, the mean primary particle size of the NPs was 20±7 nm (transmission electron microscopy), and the hydrodynamic diameter in deionized water was 116±7 nm (dynamic light scattering). The ζ-potential of Ag NPs was -19±9 mV.

Concentrations of total Ag in suspensions applied onto leaves and concentrations of Ag on the leaves after the experiment

The actual measured concentration of total Ag in the suspensions used for the dissolution measurements (10.6±0.1, 20.9±0.9 and 44.9±1.1 mg/L; **Table 1**) differed from their corresponding nominal values (12.5, 25 and 50 mg/L, respectively) within a 20 % range. The actual measured concentrations of total Ag on the $AgNO_3$-spiked and Ag NP-spiked leaves (6.5±0.6, 79±33 and 914±38 µg/g of dry leaf mass in the case of $AgNO_3$; 6.1±2.9, 65±7 and 956±68 µg/g of dry leaf mass in the case of Ag NPs; **Table S1, Supplementary Material**) were slightly lower than their corresponding nominal values (10, 100 and 1000 µg/g of dry leaf mass, respectively, for both compounds). Low recovery is a consequence of losses during pipetting due to high viscosity of the Ag NP suspension. Only actual measured values were used in all the calculations.

Concentrations of Ag-PVP complexes and free Ag^+ in the Ag NP suspensions

After the ultracentrifugation of the Ag NP suspensions at nominal concentrations of 12.5, 25 and 50 mg/L of total Ag, the supernatants were measured by flame AAS, ASV using bismuth-film electrode, and potentiometry using Ag-ISE. The flame AAS measurements denote the concentration of total Ag (free Ag ions, Ag-PVP complexes, unsedimented Ag NPs), ASV values denote the sum of free Ag^+ and Ag-PVP complexes, and potentiometric measurements denote free Ag^+ only.

The flame AAS yielded higher values than both other methods, i.e. ASV and potentiometry (**Table 1**), denoting uncomplete sedimentation of NPs during ultracentrifugation. This was corroborated by the dynamic light scattering analysis, which revealed the presence of particles with hydrodynamic diameters of ≥70 nm in the supernatants (**Figures S1–S3, Supplementary Material**).

The ASV measurements (**Table 1)** showed that the supernatants contained 42–47 % of the free Ag^+ and Ag-PVP species in total (depending on the nominal concentration of Ag NPs). We also measured the non-centrifuged stock suspension, and the share of Ag-PVP complexes and free Ag^+ in comparison to the total Ag was 44±3 % (n = 3).

The free Ag^+ content values yielded by the potentiometric measurements were lower than those yielded by ASV, showing the presence of 27, 12 and 12 % of free Ag^+ in the case of 12.5, 25 and 50 mg/L nominal concentrations of Ag NPs, respectively (**Table 1**). The potentiometry, i.e. Ag-ISE, showed unexpectedly deviant behavior at the highest concentration (50 mg/L), as evident from its non-linear response (**Figures S4 and S5, Supplementary Material).** The value obtained at this concentration therefore represents an approximation. The values obtained at the lower two concentrations (12.5 and 25 mg/L) are considered accurate, because the electrode response was linear and the slope remained at approximately 80 % of the ideal value (**Figures S4 and S5, Supplementary Material**).

Please insert Table 1 here.

Concentrations of Ag in isopod digestive glands

To evaluate the potential of terrestrial isopods to accumulate Ag from an Ag NP suspension containing various Ag species (Ag NPs, Ag-PVP complexes and free Ag^+), a feeding experiment with *P. scaber* was performed. After feeding for 14 days on Ag-spiked leaves, the animals from all the exposure groups contained significantly more Ag in their digestive glands than the animals from the control group ($p<0.05$ and $p<0.001$, **Figure 1**). However, there were no statistical differences between the animals exposed to different sources of Ag ($AgNO_3$ or Ag NPs) at same exposure concentrations ($p>0.05$, not marked on **Figure 1**).

Please insert Figure 1 here.

Comparison of the consumed Ag species (Ag NPs, Ag-PVP complexes and free Ag^+) and the bioaccumulated Ag in isopod digestive glands

Data for the consumed amounts of different Ag species and the amount of bioaccumulated Ag in *P. scaber* digestive glands is presented in **Table S1 (Supplementary Material)**. Based on this data we compared the masses of Ag found in the hepatopancreas after the experiment to the masses of each ingested Ag species following the approach of Romih et al. (2015) in order to elucidate which ingested Ag species may be potential sources of bioaccumulated Ag (**Figures 2a-c**). At the exposure concentration of 10 µg Ag/g of dry food, the amount of bioaccumulated Ag was much higher than the amount of consumed free Ag^+ (**Figure 2a**). At the exposure concentration of 100 µg Ag/g of dry food, the amount of bioaccumulated Ag was also higher than the consumed amount of free Ag^+, although this difference was not as pronounced as in the case of 10 µg Ag/g of dry food (**Figure 2b**). At the exposure concentration of 1000 µg Ag/g of dry food, the amount of bioaccumulated Ag was lower than the ingested amounts of any of the Ag species (**Figure 2c**).

Please insert Figure 2 here.

Effects of $AgNO_3$ and Ag NPs on isopod food consumption

The feeding rate of isopods exposed to 10 µg Ag/g of dry food either in the form of $AgNO_3$ or Ag NPs, as well as the feeding rate of isopods exposed to 100 µg Ag/g of dry food in the form of Ag NPs did not significantly differ from the control group (**Figure S6**; $p>0.05$, Mann-Whitney U-test). The feeding rates of isopods exposed to $AgNO_3$ at the exposure concentrations 100 and 1000 µg Ag/g of dry food, and to the Ag NPs at the exposure

concentration 1000 µg Ag/g of dry food were significantly lower than the feeding rate in the control group (**Figure S6;** Mann-Whitney U-test, *p<0.05, **p<0.01 and *p<0.05, respectively).

DISCUSSION

In the present study, we used two analytical methods, i.e. ASV and potentiometry with Ag-ISE, to measure the Ag speciation in suspensions of commercial PVP-stabilized Ag NPs applied to food, and the data on Ag bioaccumulation in digestive gland with the aim to elucidate which Ag species (Ag NPs, Ag-PVP complexes, free Ag^+) in the Ag NP suspension could be the potential source of Ag that was accumulated by the isopods. We provide an indirect proof of the dissolution of Ag NPs in the presence of PVP and the dissociation of Ag-PVP complexes in the digestive system of terrestrial isopods *P. scaber*, which will be discussed below.

Analytical approaches to discriminate among different Ag species in the PVP-stabilized Ag NP suspension

We applied three different approaches to identify different Ag species in the Ag NP suspension: the commonly employed flame AAS coupled with ultracentrifugation, ASV measurements of Ag at the *in-situ* prepared bismuth film electrode following the recently developed protocol (Romih et al., 2016) and potentiometry using the commercial Ag-ISE.

Flame AAS yielded higher values of Ag than both other methods (**Table 1**). This was corroborated by the dynamic light scattering analysis of the supernatants, which revealed the presence of unsedimented particles (**Figures S1–S3, Supplementary Material**). Our results are in line with other reports, which state that ultracentrifugation coupled with spectroscopic determination cannot distinguish among free ions, ion-ligand complexes and unsedimented

NPs (Misra et al., 2012; Tantra et al. 2015), unless ultracentrifugation is performed at very high speeds and long durations (for example 362 769 *g* for 1 h) where complete NP sedimentation is possible (Jemec et al., 2016). However, maximum ultracentrifugation speeds and centrifugal forces depend on the type of centrifuge, rotor and tubes (Beckman Coulter, 2007). The centrifugal force reported in Jemec et al. (2016) is not universally attainable and alternative approaches to the ultracentrifugation coupled with spectroscopy are preferable.

We were able to discriminate between Ag NPs and the sum of Ag-PVP complexes and free Ag^+ in the supernatants after ultracentrifugation as well as in the non-centrifuged stock suspension by the ASV at the *in-situ* prepared bismuth film electrode. The values (in the range of 44–48 %; **Table 1**) were consistent with the previously reported values for the same Ag NP stock (46 %; Jemec et al., 2016).

As expected, the contents of free Ag^+ only, measured by the Ag-ISE, were lower (27 %, 12 % and 12 %) than the values recorded by the ASV (**Table 1**). However, we observed atypical behavior of the Ag-ISE, which was presumably caused by PVP that became adsorbed on the electrode and obstructed the access of free Ag^+ to the sensor surface. The deviations from linearity and/or the decrease of the electrode slope were more pronounced with increasing concentrations of Ag NPs (**Figures S4** and **S5**, **Supplementary Material**). Similar interferences from organic molecules when measuring samples with complex matrices are well-known in ion-selective potentiometry (De Marco et al., 2007; Dimeski et al., 2010). Although ISEs might be attractive for the purpose of measuring free ion content in NP suspensions due to low cost, fast response, preservation of the sample and no requirements for extensive operator training (Koch et al., 2012; Tantra et al., 2015), the interferences due to organic molecules demonstrate that potentiometry is not the method of choice with complex samples. In this case, a better option is the voltammetric counterpart of ion-selective

potentiometry, the Absence of Gradients and Nernstian Equilibrium Stripping (AGNES) technique (Domingos et al., 2013, 2015; Mu et al., 2014; Merdzan et al., 2014).

Transformations of Ag NPs in the isopod digestive system and the bioavailability of Ag from the PVP-stabilized Ag NP suspension

In this study we assumed that only free Ag^+, and not Ag NPs, are accumulated in the isopod digestive glands. This assumption was based on the previously published work by Pipan-Tkalec et al. (2011), Novak et al. (2013), and Tourinho et al. (2016). Namely, when the digestive glands of the NP-fed isopods were inspected by the elemental composition imaging techniques, such as proton-induced X-ray emission (PIXE) or synchrotron X-ray fluorescence microscopy (μXRF), the NP-originating metals (i.e. Ag and Co) were found associated exclusively with the S-cells of the hepatopancreas (Pipan-Tkalec et al., 2011; Novak et al., 2013; Tourinho et al., 2016). The S-cells are a bioaccumulation site for metal ions with the affinity for sulphur-bearing ligands (Hopkin, 1990). Moreover, only Co and not Fe was accumulated into hepatopancreatic cells from the composite $CoFe_2O_4$ NPs, which indicates that NPs were not internalized intact (Novak et al., 2013).

Our results show that the amount of accumulated Ag in digestive glands at the two lower exposure concentrations (10 and 100 μg Ag/g of dry food), was higher than the amount of consumed free Ag^+, which means that only free Ag^+ were not sufficient to explain the amount of Ag accumulated by the isopods **(Figure 2a,b**). We therefore suggest that the remaining Ag must have both dissolved from Ag NPs and dissociated from Ag-PVP complexes. We think that neither of these two Ag species alone (Ag NPs or Ag-PVP complexes) could be a sufficient source of Ag, because it would mean that Ag assimilation efficiency from any of the two Ag sources had to be very high (>80 %). This, however, is physiologically unrealistic (Zidar et al., 2003). For example, the metal assimilation efficiency of *P. scaber* did not exceed 5 % for Co from $Co(NO_3)_2$ (Drobne and Hopkin, 1994) and ~60

% for Cd from $CdCl_2$ (Zidar et al., 2003) at similar exposure concentrations in a similar exposure setup as used in the current study. Moreover, Ag NPs could not provide the only source of remaining Ag, because undissolved NPs were found in the fecal pellets after the feeding experiment (albeit in very small quantities; **Figures S8–S11**, **Supplementary Material**).

Our results therefore show that Ag-PVP complexes were able to dissociate under physiological conditions, although the near-neutral pH in the digestive system of *P. scaber* (Zimmer & Topp, 1997; Zimmer & Brune, 2005) is generally unfavorable for the dissociation of metal-ligand complexes. The facile release of Ag^+ from PVP was most likely permitted by weak coordinative bonds between the C–N and C=O functional groups of PVP and the *sp* orbitals of Ag^+ (Zhang et al., 1996; Wang et al., 2005). The complexation of PVP with Ag^+ and the bioaccessibility of Ag^+ from Ag-PVP complexes could be further supported with additional Ag accumulation experiments where isopods would be exposed to Ag-salt and selected concentrations of PVP. However, in this particular study, we were unable to elucidate the concentration and molecular weight of the PVP added to the Ag NP suspension, because the producer considers it intellectual property. Another way would be the characterization of PVP in the Ag NP suspension, but this exceeds the scope of this study. We suggest that such studies are carried out in the future using different NP stabilizing agents.

At the highest exposure concentration (1000 µg Ag/g of dry food), the amount of ingested free Ag^+ was higher than the amount of accumulated Ag, which means that free Ag^+ only could be a sufficient source of accumulated Ag (**Figure 2c**). However, this does not exclude the possibility that also at this concentration Ag NPs and Ag-PVP complexes dissolved or dissociated in the gut, respectively. We speculate that the tendency of Ag NPs to dissolve at this concentration was less pronounced than at the lower two exposure concentrations, because the amount of accumulated Ag in the case of Ag NP exposure was

significantly lower (p<0.01, Mann-Whitney U-test) than in the case of the same Ag concentration of $AgNO_3$ (**Table S1**, **Supplementary Material**). The isopods exposed to Ag NPs could potentially accumulate as much Ag as the case of $AgNO_3$ (which is considered the physiological storage capacity of *P. scaber* for Ag), but for some reason this did not occur. Most probably, this was due to limited bioavailability of Ag from Ag NPs under given experimental conditions. The lower dissolution of Ag NPs at higher concentration is in line with the data which indicate that Ag NP dissolution in aqueous suspension is inversely related to the NP concentration (Jemec et al., 2016).

Transformations of the Ag NP suspension after drying on the test leaves

After drying of the Ag NP suspension on the leaf surface, the share of each Ag species probably does not exactly equal those measured in the aqueous suspension before application. We assume that possible transformations of the Ag NP suspension on the leaves during drying are: (i) the complexation of Ag^+ to either PVP or Ag NPs, (ii) adsorption/binding of all Ag species to the leaf surface, and (iii) the aggregation/agglomeration of Ag NPs, which may in turn affect the dissolution (Coutris et al., 2012; Schaumann et al., 2015; Tourinho et al. 2015). All these transformations result in less free Ag^+ potentially available for assimilation than those measured in the suspensions applied onto leaves, which further supports the main conclusion of this work. Namely, the dissolution of Ag NPs is lower in the case of larger NP aggregates (Schaumann et al., 2015). Also, AgNO3 and Ag NPs have previously been shown to bind to soil particles (Coutris et al., 2012), which may also be the case for leaves. We measured the amount of total Ag on the leaves after drying. Measured Ag concentrations in the case of Ag NPs were lower than the nominal value as applied with the suspension. However, this was the case also for $AgNO_3$, which means that the difference between nominal and measured concentrations was due to spiking accuracy and not due to NPs falling off the

leaves. PVP is not volatile, therefore we presume that the amount of PVP in the suspension before and after spiking the leaves was similar.

Another potential transformation of Ag NP suspension on leaves may be due to aging of Ag NPs during the 14 days of exposure. It has been previously shown that the tested Ag NP suspension is stable in terms of dissolution and hydrodynamic size (up to a year in stock suspension at 8 °C, and up to 96 h in aqueous suspension at room temperature; Jemec et al., 2016). Shoults-Wilson et al. (2011) studied the speciation of PVP-stabilized Ag NPs in soil during 28 days. They reported that most of the silver remained as Ag^0, and only partial oxidation of Ag NPs has been found. However, it is unclear how the light exposure may transform the Ag NPs on the leaves.

Nevertheless, since the characterization of different Ag species on leaves requires complex techniques and is commonly not feasible, similarly as in the case of soil (Coutris et al., 2012; Schaumann et al., 2015), the characterization in the suspension prior to addition on leaves may currently be the best approximation.

Effects of $AgNO_3$ and Ag NPs on isopod food consumption

The feeding rates of isopods exposed to Ag NPs (1000 µg Ag/g of dry food) were significantly lower than the feeding rate in the control group. This was also reported for terrestrial isopods *Porcellionides pruinosus* fed on Ag NP-contaminated soil for 14 days (EC50 value 127 [56.4–200] µg Ag/g of dry soil; Tourinho et al., 2015). However, no effect on *P. scaber* fed on Ag NPs (up to 5000 µg Ag/g) for 14 days was noted by Pipan-Tkalec et al. (2011). This was most probably due to different Ag NPs tested (commercial Ag NP powder from Sigma-Aldrich).

Environmental implications

Silver NPs are expected to enter the soil environment mainly via land application of sewage sludge or biosolids, and also through direct application of products treated with Ag NPs on compost heaps in gardens. Isopods can be found under stones and dead wood, particularly in gardens, marginal grassland and open woodland. The concentrations of Ag NPs tested in this work (10, 100, and 1000 mg Ag/kg of dry leaf mass) are higher than predicted environmental values reported for soils treated with sludge (0.01–0.1 mg/kg; Gottschalk et al., 2013), but the continuous input of biosolids to agricultural land may lead to increasing Ag NP concentrations over time (Tourinho et al., 2015).

It is of particular interest that labile Ag-PVP complexes proved to be a source of bioaccessible Ag to isopods. From an environmental safety perspective the PVP may not be considered as the most suitable stabilizer of Ag NP suspensions. Although PVP does prevent the aggregation of Ag NPs, the PVP-stabilized Ag NP suspensions have been shown to contain high amounts of Ag-PVP complexes and free Ag^+ content in total, i.e. 36.5 % (Bondarenko et al., 2013), >40 % (Cunningham et al., 2013) or 46 % (Jemec et al 2016) for suspensions prepared by different protocols. On the other hand, suspensions of uncoated Ag NPs (Bondarenko et al., 2013) or Ag NPs coated with casein (Bondarenko et al., 2013) or citrate (Ivask et al., 2014b) in deionized water were shown to contain much lower shares of free Ag^+ and/or Ag-ligand complexes, in the range of 0.82–7.6 %. The dissolution of various Ag NPs (uncoated or coated with citrate, PVP, gelatin, 6-mercaptohexacanoic acid or an unspecified hydrophilic polymer) in the zebrafish hatching medium was in the range of 15–48 % after 120 h; the highest values were again reached by the PVP-stabilized Ag NPs (Cunningham et al., 2013). The differences in the dissolution rates of variously-coated Ag NPs might depend on the potential of the stabilizers to cause NP dissolution (Bondarenko et al., 2013). Another reason for high Ag^+ content in these suspensions might be the fact that Ag NPs are often synthesized by the reduction of Ag^+ to Ag^0, so Ag^+ might be the remnants from

the synthesis step if the NP suspension was not sufficiently purified (Koch et al., 2012). In the latter case, the amount of free Ag^+ in the suspension depends on the stability of complexes between Ag^+ and the NP-stabilizing compounds and on the amount of the stabilizer. We can speculate that PVP in this way acts as a carrier for labile Ag^+ by forming weak coordinative bonds with Ag^+ (Zhang et al., 1996; Wang et al., 2005).

In conclusion, we showed that terrestrial isopods are able to accumulate Ag from three different Ag-species present in a composite Ag NP suspension: free Ag^+, Ag-PVP complexes and Ag NPs. The concentration of free Ag^+ in the suspension offered to the test organism is not the only measure of bioavailable Ag. The data show that also the type of NP stabilizer along with transformations in the digestive system need to be considered when the bioavailability of metals from metal NPs is in question. This knowledge should be taken into consideration in the creation of new computational models in nanomaterial fate evaluations.

SUPPLEMENTARY MATERIAL

The DLS analysis of supernatants of the Ag NP suspensions after ultracentrifugation. The response of the Ag-ISE to the additions of Ag^+ standard to deionized water or to the supernatants of the Ag NP suspensions. Scanning electron micrographs of Ag NP-spiked hazelnut leaves before the experiment and the isopod fecal pellets after the experiment. The feeding rate of the isopods fed with $AgNO_3$-spiked or Ag NP-spiked food for 14 days. The bioaccumulation of different Ag species from $AgNO_3$-spiked or Ag NP-spiked food. Equations used. Supplementary references.

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TABLES WITH CAPTIONS

Table 1. The total concentration of Ag in Ag NP suspensions, measured by atomic absorption spectrometry (AAS); the concentrations of Ag-PVP complexes and free Ag^+, measured by anodic stripping voltammetry (ASV); and the concentrations of free Ag^+ only, measured by potentiometry with the silver ion-selective electrode (Ag-ISE). The values measured by AAS correspond to acidified samples, whereas the ASV and potentiometric measurements were performed without prior acidification.

Nominal Ag concentration in the Ag NP suspensions (mg/L)	**AAS-measured Ag concentration in the Ag NP suspensions (mg/L), mean ± SD (n = 3)**	**Concentration of Ag in the supernatants (mg/L; mean ± SD)**			**The average share of Ag in the supernatants (%)[a]**		
		Total Ag (free Ag^+, Ag-PVP complexes and Ag NPs), AAS (n = 3)	**Free Ag^+ and Ag-PVP complexes, ASV (n = 2)**	**Free Ag^+, potentiometry (n = 2)**	**Total Ag (free Ag^+, Ag-PVP complexes and Ag NPs), AAS**	**Free Ag^+ and Ag-PVP complexes, ASV**	**Free Ag^+, potentiometry**
12.5	10.6 ± 0.1	5.25 ± 0.03	4.5 ± 0.2	2.86 ± 0.07	50	42	27
25	20.9 ± 0.9	11.0 ± 0.1	9.9 ± 0.5	2.5 ± 0.3	53	47	12
50	44.9 ± 1.1	23.6 ± 0.1	19.9 ± 0.2	5.4 ± 0.2	53	44	12[b]

[a], calculated in comparison to the AAS-quantified total Ag content of the suspensions from the corresponding average values.

[b], the value may not be entirely accurate due to suboptimal electrode operation (details are provided in the **Supplementary Material, Figures S4 and S5**).

Ag NPs, silver nanoparticles; Ag-PVP complexes, complexes of silver with polyvinylpyrrolidone.; AAS, atomic absorption spectrometry; ASV, anodic stripping voltammetry; n = number of replicates; SD = standard deviation

FIGURES WITH CAPTIONS

Figure 1

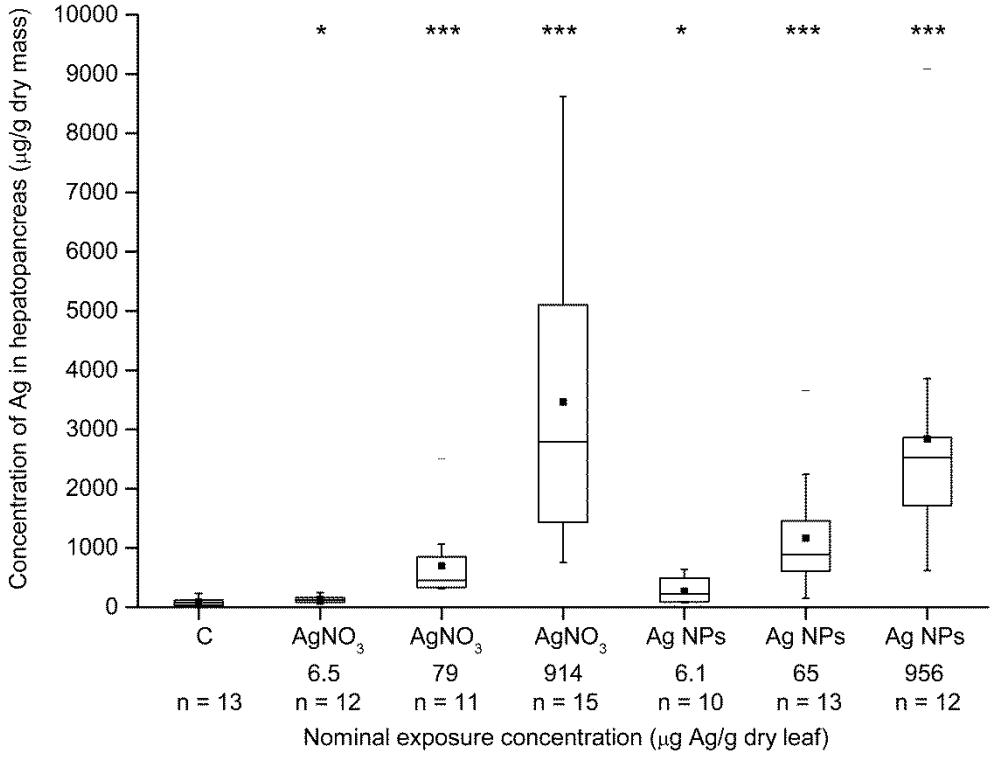
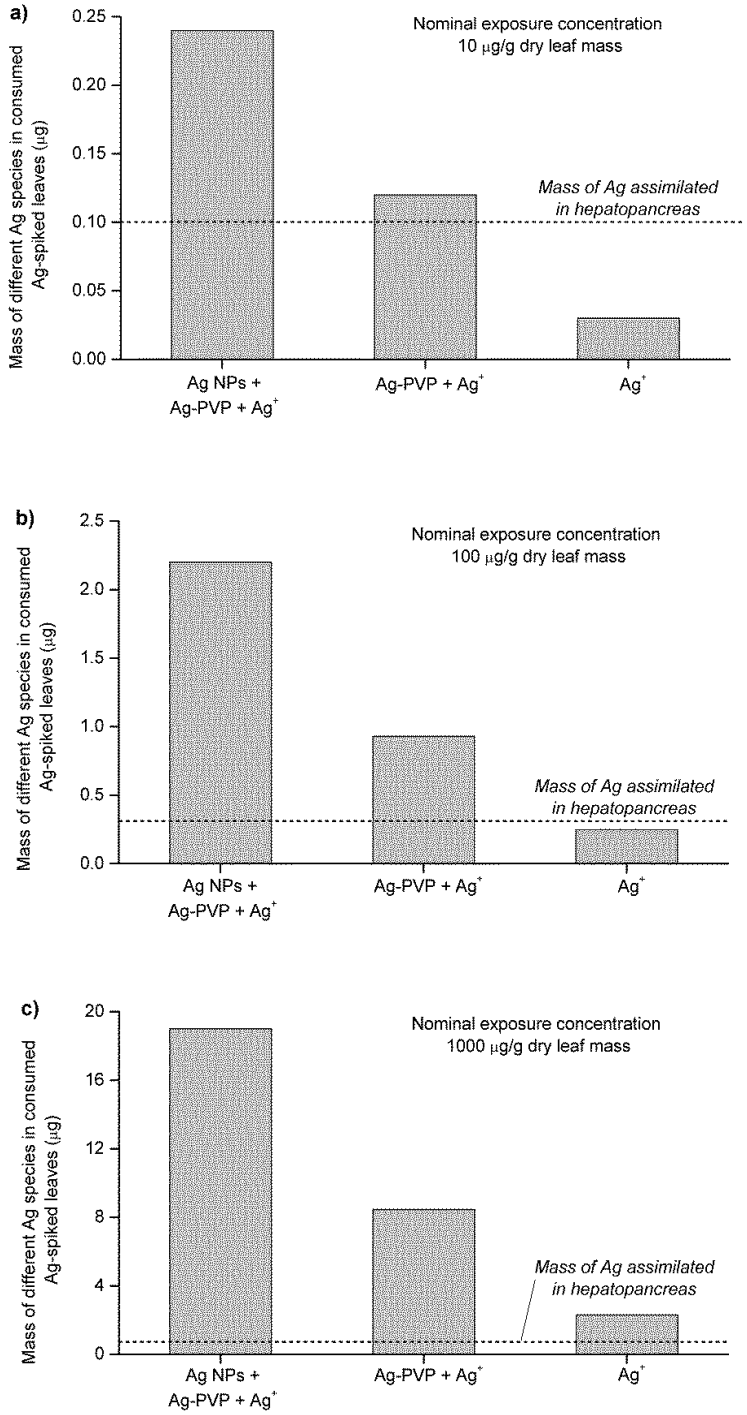


Figure 1. Concentrations of Ag in the digestive glands of isopods *P. scaber* after feeding for 14 days on Ag-spiked food. The animals were fed non-spiked food (control, C) or food that was spiked with $AgNO_3$ (nominally 10, 100, and 1000 µg Ag/g of dry leaf mass) or Ag nanoparticles (Ag NPs; nominally 10, 100, and 1000 µg Ag/g of dry leaf mass). Actual measured exposure concentrations of Ag are provided on the x-axis. Symbols on the box plot represent maximum and minimum values (whiskers: ⊥), mean values (■), outliers (—), statistical differences between the control and the test groups with $p < 0.05$ (*) and $p < 0.001$ (***); n = number of specimens in each test group.

721 **Figure 2**



722

723 **Figure 2.** Comparison of the Ag mass found in the hepatopancreas (dashed line) after the experiment to the
724 masses of each ingested Ag species as the potential source of bioaccumulated Ag from Ag NP suspension at
725 exposure concentrations of **a)** 10, **b)** 100, **c)** 1000 μg Ag/g of dry food. The data represent the averages extracted
726 from **Table S1, Supplementary Material**. Ag NPs, silver nanoparticles; Ag-PVP, complexes of silver with
727 polyvinylpyrrolidone; Ag^+, free silver ions.