Supplementary Information for

Sorption of silver nanoparticles to laboratory plastic during (eco)toxicological testing

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Materials and Methods

Nanoparticles and chemicals

AgNO₃ was from Sigma-Aldrich and was dissolved in DI water at 17.5 mg Ag/mL. Silver nanoparticles (AgNPs) were from Nanocomposix. 30 and 70-nm sized (manufacturer information) citrate (product # AGCB30 and AGCB70) and branched polyethylene imine (bPEI) (product # AGBB30 and AGBB70) coated Biopure AgNPs were selected due to their widespread use in various toxicological studies (Fabrega et al., 2011) (Tejamaya et al., 2012) (Mwilu et al., 2013) (Ivask et al., 2014) (Hunt et al., 2014) and were received as 1 mg/mL aqueous suspensions. The as-received particles were diluted with water to 100 µg/mL, placed on 300 mesh Cu grids and visualised under transmission electron microscope (JEOL JEM2100F). Particles' primary size was measured from TEM images (25 -30 particles were counted) using Gatan software. Hydrodynamic diameter (d_h) and surface charge (ζ potential) of the as-received nanoparticles dispersions were measured using dynamic light scattering (DLS) method in PSS Nicomp 380 particle sizer. For d_h , 50 µg AgNP/mL samples either in water or in test media were used, data was collected for 5 min and d_h was determined from intensity-based Nicomp distribution of the particles. d_h and ζ potential were measured twice or trice. The Ag concentration in AgNPs dispersions was constantly monitored by digesting the AgNPs suspension with conc. HNO₃ and subsequent analysis by ICP-MS.

Ecotoxicological and toxicological test media

Dispersions of AgNPs and AgNO₃ solutions were prepared in a medium which is generally used for algal growth inhibition assay (OECD 201 medium, further designated ecotoxicological test medium; for composition, see Table S1) and in a standard toxicological test medium which is routinely used to grow various cell lines (RPMI medium, Sigma-Aldrich product # R0883, supplemented with 10% foetal bovine serum (Hyclone), GlutaMAX, Na-pyruvate and penicillin-streptomycin (Life Technologies); for composition, see Table S1). AgNPs were characterised in MilliQ water and in each test medium by DLS (see above) and by ICP-MS in time resolved (TR) mode (single particle ICP-MS). Before hydrodynamic size (DLS) measurements of 1-10 µg/mL AgNPs in test media, the NPs suspensions were incubated for 24 h at 37 °C (NPs in cell culture medium) or 72 h at room temperature (NPs in algal medium), to imitate the respective assay conditions. Single particle ICP-MS (sp-ICP-MS) was performed on suspensions prepared from stock AgNP suspensions (1 mg Ag/mL), after bath sonication for 3 min, diluted to final concentrations of 50 pg/mL with the test media. The measurements were performed by Agilent 8800 triple quadrupole ICP-MS using 3 ms dwell time. Transport efficiency of the sample introduction system was determined by analysing standard 60 nm Au NPs (NIST, USA). For calibration of the system, dissolved ionic Ag (0-1 ng/mL), in both 2% HNO₃ and matrices used for NP characterisation (ecotoxicological medium and cell culture medium, Table S1) was used and the obtained curves were used for particle size calculations. Raw data were collected and plotted in frequency histograms, where the background threshold was determined by separating low counts with the highest frequencies, following the separation method described earlier (Mitrano et al., 2014). All the pulse intensities over the threshold were considered as NP events, converted to Ag mass by standard calibration curves and then to AgNP diameter, assuming spherical shape.

AgNPs sorption assay

Sorption assay with AgNPs was carried out using four different clear polystyrene microplates from NUNC. 96-well untreated (NUNC, product # 167008) and Nunclon Delta surface treated (NUNC,

product # 138396) plates and 12-well untreated (NUNC, product # 150200) and Nunclon Delta surface treated (NUNC, product # 150628) were used. AgNPs and AgNO₃ in ecotoxicological test medium at concentrations 10, 50 and 250 ng Ag/mL and in cell culture medium at concentrations 50, 250 and 1000 ng Ag/mL were prepared in 50 mL polypropylene tubes (Greiner, product # 227261). To determine the concentration of Ag added to the sorption test, an aliquot was removed from AgNPs suspensions/AgNO₃ dilution in 50 mL polypropylene tubes, acidified (see below) and analysed in ICP-MS. For sorption test, 200 µL and 2 mL AgNPs suspensions/AgNO₃ dilutions was pipetted onto 96- and 12-well plates, respectively. The plates with cell culture medium were incubated for 24 h at 37°C and 5% CO₂ in the dark and the plates with ecotoxicological test medium (algal growth medium) were incubated for 72 h at 25°C on an illuminated shaker. These incubation conditions were selected according to standard conditions for toxicological assays with mammalian cell lines and algal cells, respectively. After incubation 190 µL and 1.8 mL was collected from 96 and 12-well microplates, respectively. HNO₃ until the final concentration of 1-5% was added to samples in ecotoxicological test medium. For samples in cell culture medium, 17% HNO₃ and 4% HCl was added to the samples, the samples were transferred to sealable Teflon tubes and digested for 10 min at 170 °C and 1800 W using a MARS6 microwave reaction system (CEM). Three parallel samples were prepared for ICP-MS measurements, to obtain standard deviation. Blank samples (respective test media or water) was always included for control and the concentration of Ag in those samples was < 0.05 ng/mL. Statistical analysis on the data was performed using Data analysis package of MS Excel. Significant differences between samples were analysed using the t-test.

Adsorption of AgNPs to microplates (96-well microplates were used as examples) in cell culture medium and in algal growth medium was fitted using Langmuir and Freundlich isotherms. Fitting of the data with Langmuir isotherm was carried out using linearised Langmuir model:

$$\frac{C}{q} = \frac{1}{K_L q_{\max}} + \frac{1}{q_{\max}} \times C$$

where C is concentration (g/L) of Ag that was removed from the plate after 24 h or 72 h of exposure in cell culture medium or algal medium, respectively (recovery), K_L is Langmuir constant (L/m² in our case), q is adsorption of Ag and q_{max} is the maximal adsorption of Ag. q is calculated essentially as in Kafshgari et al. (2013)

$$q = \frac{V(C_0 - C)}{A}$$

where V is the volume of AgNPs added to the test (L), C₀ is the starting concentration of Ag and A is area of the microplate well that was in contact with AgNPs (0.000214m² in case of 96-well plates).

q_{max} and K_L were found from function $\frac{C}{q} = f(C)$.

Fitting of the adsorption data with linearised Freundlich model was carried out according to the following formula:

$$\log(q) = \log K_F + \frac{1}{n} \log C$$

where K_F (g/m²) is the Freundlich constant and n is the Freundlich component. K_F and n were found from function $\log(q) = f(\log(C))$.

Characterisation of microplate surfaces

The surface properties of untreated and Nunclon Delta surface treated microplates were characterised by contact angle measurements, X-ray photoelectron spectrometry (XPS) and rotating disk method. Before measurements, the untreated and Nunclon Delta surface treated 12-well microplates were broken to 0.4-1 cm pieces.

Static water contact angles were determined using a sessile drop apparatus consisting of a sample holder, a 10 μ L syringe (Hamilton, Reno, USA) and an Olympus SZ-PT microscope and lens system connected with a Sony CCD camera. Approximately 1 μ L droplets of 18.2 M Ω .cm MilliQ water were dispensed onto plate surface, a photo was taken and angle of the drop was calculated using ImageJ software and drop analysis plug-in (<http://rsb.info.nih.gov/ij/>).

XPS analysis of microplate surfaces was performed using Kratos Axis Ultra instrument with DLD equipped with monochromatic aluminium x-ray running at 225W with a characteristic energy of 1486.6eV; analysis was performed at 4×10^{-9} Torr and binding energy in the range of 10 and 1110 eV was registered. The area of analysis was a 0.3 mm x 0.7 mm and analysis depth was approximately 15 nm. A quick scan from 5 to 1000 eV and a detailed scans at 275-305 eV, 525-545 eV and 390-410 eV were performed. To analyse the sorption of components of ecotoxicological and cell culture medium onto the plate surfaces, the pieces of microplates were exposed to algal growth medium at room temperature for 72 h and to cell culture medium at 37 °C for 24 h. After exposure, the plate microplate pieces were rinsed several times with DI water, dried and the analysis was performed as described above. XPS data processing was performed using CasaXPS processing software version 2.3.15 (Casa Software Ltd., Teignmouth, UK).

The Rotating Disk method was used to measure surface ζ -potential. For that, 25 mm diameter disks cut from the microplate surfaces were spun at 3000 rpm in DI water at 1.5 mm distance from a reference electrode using Zeta Metrix Zeta Spin Model 1.2 as essentially described in (Sides et al., 2006). To analyse the effect of media components attached onto the microplate surfaces, the 25 mm disks were exposed to algal growth medium for 72 h at room temperature or to cell culture medium for 24 h at 37 °C. After exposure, the disks were dried and analysed as above. Surface zeta potential was calculated as in (Sides et al., 2006).

Table S1. Composition of the test media used in this study. All the ingredients of the OECD 201 medium were of analytical grade. Components of cell culture medium – RPMI 1640 (# R0883), GlutaMAX, Na-pyruvate and Penicillin-Streptomycin were from Sigma. Fetal bovine serum was from Hyclone.

Medium	Composition	pH
Algal growth medium (OECD 201 medium)^a (OECD, 2002)	15mg/L NH ₄ Cl 12mg/L MgCl ₂ • 6H ₂ O 18mg/L CaCl ₂ • 2H ₂ O 15mg/L MgSO ₄ • 7H ₂ O 1.6mg/L KH ₂ PO ₄ 64µg/L FeCl ₃ • 6H ₂ O 100µg/L Na ₂ EDTA • 2H ₂ O 185µg/L H ₃ BO ₃ 415µg/L MnCl ₂ • 4H ₂ O 3µg/L ZnCl ₂ 1.5µg/L CoCl ₂ • 6H ₂ O 10ng/L CuCl ₂ • 3H ₂ O 7µg/L Na ₂ MoO ₄ • 2H ₂ O 50mg/L NaHCO ₃ 50mg/L Na ₂ SiO ₃ • 9H ₂ O	7.5
Cell culture medium^b	0.1 g/L Ca(NO ₃) ₂ • 4H ₂ O 0.049 g/L MgSO ₄ 0.4 g/L KCl 2 g/L NaHCO ₃ 6 g/L NaCl 0.8 g/L Na ₂ HPO ₄ 0.2 g/L L-arginine • HCl 0.05 g/L L-Asparagine 0.02 g/L L-Aspartic acid 0.065 g/L L-Cystine • 2HCl • H ₂ O 0.02 g/L L-Glutamic acid 0.01g/L Glycine 0.015 g/L L-Histidine• HCl •H ₂ O 0.02 g/L Hydroxy-L-Proline 0.05 g/L Isoleucine 0.05 g/L L-Leucine 0.04 g/L L-Lysine• HCl 0.015 g/L L-Methionine 0.015 g/L L-Phenylalanine 0.02 g/L L-Proline 0.03 g/L L-Serine 0.02 g/L L-Threonine 0.005 g/L L-Tryptophan 0.022 g/L L-Tyrosine 0.02 g/L L-Valine 0.3 g/L L-Glutamine 0.3 mg/L D-Biotin 3 mg/L Choline chloride 1 mg/L Folic acid 3.5 mg/L Myo-inositol 1 mg/L Niacinamide 1 mg/L p-Aminobenzoic Acid 0.25 mg/L D-Pantothenic Acid • ½Ca 1 mg/L Pyridoxine • HCl 0.2 mg/L Riboflavin	(depends on air CO ₂ content)

1 mg/L Thiamine • HCl
0.005 mg/L Vitamin B12
4.5 g/L D-Glucose
0.001 g/L reduced Glutathione
0.0053 g/L Phenol red • Na
1 mM Na-Pyruvate
100 U/L Penicillin
100 mg/L Streptomycin
10% Fetal Bovine Serum

^a All the components were of analytical grade

^b Medium without fetal bovine serum, streptomycin, penicillin, Na-pyruvate was purchased from Sigma-Aldrich (product # R0883)

Figure S1. Transmission electron microscopy images of 30 and 70 nm branched polyethylene imine (bPEI) and citrate coated AgNPs. A): 30 nm bPEI-coated AgNPs; B): 30 nm citrate-coated AgNPs; C): 70 nm bPEI-coated AgNPs; D): 70 nm citrate-coated AgNPs.

Figure S2. AgNPs diameter in stock suspension and in different test media as measured by time-resolved, i.e., single-particle ICP-MS method. A): 30 nm bPEI coated AgNPs; B): 30 nm citrate coated AgNPs; C): 70 nm bPEI coated AgNPs; D): 70 nm citrate coated AgNPs.

Figure S3. Sorption of nanoparticles suspended algal growth medium onto laboratory plastic. Sorption of 30 and 70 nm citrate and bPEI coated AgNPs onto 96 and 12-well microplates with different surface properties. A), B): sorption of 10 ng/mL AgNPs suspension; C), D): sorption of 50 ng/mL AgNPs suspension; E), F): sorption of 250 ng/mL AgNPs suspension. Filled columns: untreated microplates, open columns: Nunc Delta surface treated microplates. Error bars indicate standard deviations of three replicate experiments. a - indicates significant ($p < 0.05$) differences between before incubation condition and after incubation on microplates. b - indicates significant ($p < 0.05$) differences between untreated and Nunc Delta surface treated plates.

Figure S4. Sorption of nanoparticles suspended in cell culture medium onto laboratory plastic. Sorption of 30 and 70 nm citrate and bPEI coated AgNPs onto 96 and 12-well microplates with different surface properties. A), B): sorption of 50 ng/mL AgNPs suspension; C), D): sorption of 250 ng/mL AgNPs suspension; E), F): sorption of 1000 ng/mL AgNPs suspension. Filled columns: untreated microplates, open columns: Nunc Delta surface treated microplates. Error bars indicate standard deviations of three replicate experiments. a - indicates significant ($p < 0.05$) differences between before incubation condition and after incubation on microplates. b - indicates significant ($p < 0.05$) differences between untreated and Nunc Delta surface treated plates.

Figure S5. Recovery of citrate coated 70 nm AgNPs from different microplates. A) recovery in algal growth medium, B) recovery in cell culture medium at different initial AgNPs concentrations (concentration of added AgNPs). Recovery increases with increasing AgNPs concentration. This indicates saturation of AgNPs binding sites on microplate surfaces with increasing AgNPs concentration.

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