SHORT COMMUNICATION

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

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Abstract

Here, we evaluate the extent of sorption of silver nanoparticles (AgNPs) with different primary sizes (30 and 70 nm) and surface properties (branched polyethylene imine, “bPEI” and citrate coating) to laboratory plastic during (eco)toxicological testing. Under conditions of algal growth inhibition assay, up to 97% of the added AgNPs were sorbed onto the test vessels whereas under conditions of in vitro toxicological assay with mammalian cells, the maximum loss of AgNPs was 15%. We propose that the high concentration of proteins and biomolecules in the in vitro toxicological assay originating from serum-containing cell culture medium prevented NP sorption due to steric stabilisation. The sorption of AgNPs to test vessels was clearly concentration dependent. In the conditions of algal growth inhibition assay at 10 ng AgNPs/mL, up to 97% of AgNPs were lost from the test while at higher concentrations (1000 ng AgNPs/mL), the loss of AgNPs was remarkably smaller, up to 64%. Sorption of positively charged bPEI-coated AgNPs was more extensive than the sorption of negatively charged citrate-coated AgNPs and, when calculated on a mass basis, more 70 nm-sized Ag than 30 nm Ag sorbed to plastic surfaces. In summary, this study demonstrates that the loss of AgNPs during (eco)toxicological tests due to sorption on test vessel surfaces is significant, especially in diluted media (e.g. in algal growth medium) and at low NP concentrations. Thus, to ensure the accurate interpretation of (eco)toxicological results, the loss of AgNPs due to adsorption to test vessels should not be overlooked and considered for each specific case.

Although the number of environmental studies as well as human toxicology related studies concerning nanosilver is remarkable, this topic still represents a constant challenge for nanotoxicologists. In April 2015, 447 800 papers were retrieved from ISI Web of Science using keywords ‘Ag OR silver’ AND ‘nano*’ AND ‘*toxic*’. Yet, these papers present often extremely variable toxicity values, even for the same test species and for the same type of toxicological endpoint. In these studies, the half-lethal and half-inhibitory concentrations of silver nanoparticles (AgNPs) to various test organisms varies by 2–3 orders of magnitude (Bondarenko et al., 2013). Although these differences may be attributed to incomparable test conditions and different NPs used, there is strong evidence that certain processes may affect the nanoparticles during or before toxicological testing, as reported by Cohen et al. (2012). Sørensen & Baun (2015) reported poor reproducibility and non-monotonous concentration-response in an algal growth inhibition test when AgNPs were added to growth medium immediately before the test. By contrast, when AgNPs were aged in the test medium for more than 48 h, the assay results were reproducible but AgNPs showed lower toxicity than in freshly prepared medium. This suggests that transformation took place with the NPs during the first hours of toxicological testing. Indeed, recent results suggest that aggregation and dissolution of AgNPs, along with adsorption of silver ions or AgNPs to storage vessels, may occur (Sekine et al., 2015). To date, the phenomena of sorption of NPs during (eco)toxicological testing have not received much attention. Yet this process is likely similar to NP sorption to different surfaces (soils, sediments), occurring in the environment and investigated by several recent studies (Cornelis et al., 2013; Klitzke et al., 2014; Schlich & Hund-Rinke, 2015).

A recent study by Sekine et al. (2015) showed that a remarkable fraction (up to 95%) of AgNPs may attach onto polypropylene, polycarbonate and glass surfaces at concentrations relevant for ecotoxicological assays (50–130 ng/L). However, this earlier study demonstrated the sorption of AgNPs only in one test medium and did not consider the actual assay conditions and surface properties of the test vessels. Thus, the aim of this study was to expand the study of Sekine et al. (2015) by investigating the sorption of AgNPs of different size (70 and 30 nm) and different surface coating (branched polyethylene imine, bPEI and citrate) under conditions of both ecotoxicological and mammalian cell toxicological assays on four different plastic well plates. We mimicked the test conditions of an algal growth inhibition assay originating from serum-containing cell culture medium. This suggests that transformation took place with the NPs during the first hours of toxicological testing. Indeed, recent results suggest that aggregation and dissolution of AgNPs, along with adsorption of silver ions or AgNPs to storage vessels, may occur (Sekine et al., 2015). To date, the phenomena of sorption of NPs during (eco)toxicological testing have not received much attention. Yet this process is likely similar to NP sorption to different surfaces (soils, sediments), occurring in the environment and investigated by several recent studies (Cornelis et al., 2013; Klitzke et al., 2014; Schlich & Hund-Rinke, 2015).

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which was used as a model for an ecotoxicological assay and an
in vitro toxicological assay with mammalian cells, and quantified
the amount that was sorbed onto the walls of the containers during
the assays. Furthermore, we characterised the surface properties
of the microplates and their modification by the different test
media. For the algal growth inhibition assay (recommended by
EU REACH legislation for industrial chemicals), the standard
medium recommended in the OECD 201 guideline was used, and
the test was conducted for 72 h under constant illumination at
room temperature. In an in vitro toxicological assay with
mammalian cell lines, standard cell culture medium (RPMI
supplemented with 10% fetal bovine serum, Glutamax,
Na-pyruvate and penicillin-streptomycin) and 24-hour incubation
in the dark at 37 °C and 5% CO₂ was used. The compositions of
the test media are given in Table S1.

Citrate- and bPEI-coated AgNPs of 30 and 70 nm diameter
were selected due to their widespread use in various toxicological
studies (see SI for references). Aqueous suspensions of AgNPs at
1000 μg/mL were stored in glass vessels (no significant sorption
of the NPs to inner surfaces of the vessels was observed; this was
determined by frequent analysis of Ag content in AgNPs samples).
The primary size of AgNPs determined from transmission
electron microscopy (TEM) images (Figure S1) was in
accordance with the manufacturer specifications. The AgNP size
in algal growth medium and in cell culture medium as determined
with single particle ICP-MS (sp-ICP-MS) was similar to its
primary size (Figure S2). The analysis with sp-ICP-MS provided
simultaneous information on the particle size and the number of
particles in the suspension (Peters et al., 2015) (data not shown),
and confirmed the homogeneous size distribution for all particle
types. Yet, according to dynamic light scattering (DLS), the particles tended to aggregate, especially in the cell culture
medium (Table 1). The differences in particle sizes measured in
test media using the two different methods sp-ICP-MS and DLS,
were likely due to different NP concentrations used. At dilute
concentrations used for sp-ICP-MS measurements (~pg/mL) the particles were likely monodispersed whereas at higher concentrations
used for DLS measurements (~µg/mL), aggregation was
more conspicuous. Citrate- and bPEI-coated AgNPs differed in
their surface charge (ζ-potential): bPEI-coated particles exhibited
high positive and citrate-coated particles high negative ζ-potential
(Table 1).

The sorption assay with AgNPs was carried out using four
different NUNC (Thermo Scientific) microplates: 96- and 12-well
untreated and Nunclon Delta surface-treated clear polystyrene
plates (see Supplementary material). Those plates were selected
due to their common use in environmental and in in vitro toxicity
measurements, and also to allow the comparison between
different surface characteristics and surface-to-volume ratios.
Specifically, Nunclon Delta surface-treated 96-well plates are
often used in experiments with mammalian cells, while plates

| Table 1. Primary diameter (d) determined from TEM images (n = 25), hydrodynamic diameter (dₜ) determined using dynamic light scattering method and surface charge (ζ-potential) of the AgNPs. |
|---------------------------------|----------------|----------------|----------------|----------------|
| Primary d (nm)                  | dₜ of AgNPs in stock (nm) | dₜ of AgNPs in cell culture medium (nm) | dₜ of AgNPs in algal growth medium (nm) | ζ-Potential of AgNPs in stock (mV) |
| 30 nm bPEI-AgNP              | 29.3 ± 4.00 | 58.0 ± 15.3 | 237 ± 147 | 52.0 ± 15.0 | +37 |
| 30 nm citrate-AgNP          | 29.1 ± 2.60 | 40.2 ± 19.0 | 57.3 ± 28.9 | 75.4 ± 49.0 | −40 |
| 70 nm bPEI-AgNP             | 79.7 ± 4.30 | 87.9 ± 27.3 | 343 ± 140 | 78.8 ± 20.9 | +42 |
| 70 nm citrate-AgNP          | 70.5 ± 7.70 | 79.2 ± 24.1 | 113 ± 69.8 | 80.8 ± 44.9 | −44 |

*RPMI medium supplemented with 10% fetal bovine serum, Glutamax, Na-pyruvate and penicillin-streptomycin.

The AgNP concentrations selected for sorption assay in algal
growth medium were 10, 50 and 250 μg/L, while 50, 250, 1000 μg/L were used in mammalian cell culture medium. Those
congestations were chosen on the basis of the half-inhibitory
concentrations of AgNPs for algal growth inhibition and for
in vitro toxicity to mammalian cells. According to the review by
Bondarenko et al. (2013), the half-effective AgNP concentrations
for algal cells range from 5 to 20 000 μg/L while for mammalian
cells in vitro the values range between 600 and 80 000 μg/L. In
order to reproduce the exact conditions of both an algal growth
inhibition assay and a mammalian cells in vitro toxicity assay, the
selected concentration of AgNPs in respective test media were
pipetted onto the microplate and respectively incubated for 72 h at
23 °C on an illuminated shaker or kept for 24 h at 37 °C and 5% CO₂.
After incubation, AgNP suspensions were collected from the
microplates and acidified. When required – i.e. in the case of
mammalian cell culture medium, the samples were also micro-
wave digested before total Ag quantification. All samples were
collected in triplicates and Ag was quantified by ICP-MS. AgNO₃
was also tested to monitor the potential adsorption of ionic silver
that is likely to be released from AgNPs to the surrounding
aquaec acid. The ionic silver concentrations used in the
sorption assays were identical to those used in the case of AgNPs.
All the results are presented as recovery percentages, considering
the total Ag concentration at the beginning of the test as 100%.
The detailed description of all methods is shown in the SI.

Significant sorption of AgNPs onto microplates was observed for
all the tested AgNP samples, plate types and both test media.
On the other hand, no significant amount of ionic Ag was sorbed
to microplates under any condition (Figures 1, S3 and S4). This
result is in agreement with Sekine et al. (2015), who demonstrated
a remarkable adsorption of Ag ions onto glass surfaces but no
significant adsorption onto plastic. The percentage of nanoparticles
sorbed increased with decreasing concentration of the particles (Figure S5). In algal growth medium (average values
for all the particles and plate types), only 25% of AgNPs were
recovered at 10 mg AgNPs/mL, whereas 47% recovery of AgNPs
was achieved at 50 mg AgNPs/mL and 62% at 250 mg AgNPs/mL
(left side of Figure 1 and Figure S3). The concentration-
dependent sorption of AgNPs was observed in mammalian
cell culture medium. At 50, 250 and 1000 ng/mL of AgNPs, 76%,
83% and 88% of AgNPs were recovered, respectively (Figure 1
and Figure S4). In order to determine if AgNP adsorption onto
microplate surfaces followed traditional adsorption isotherms, we
fitted the adsorption data with the Langmuir and Freundlich
adsorption models. Our data were best fitted by the Freundlich
adsorption model (R² values 0.992–0.993), suggesting that AgNPs
adsorbed to microplate surface heterogeneously and attached
preferentially to physically or chemically more favourable sites.
Another clear trend indicated that in algal growth medium, the sorption of AgNPs was remarkably higher than in the cell culture medium (left and right sides of the figure) at 50 and 250 ng/mL on 96- and 12-well microplates with different surface properties. While in algal growth medium (average for 50 and 250 ng AgNPs/mL on 96-well plates in algal and cell culture medium, respectively; (C), (D)): 250 ng AgNPs/mL on 96-well plates in algal growth medium and cell culture medium, respectively. Filled columns: untreated microplates, open columns: Nunclon 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 Nunclon Delta surface-treated plates.

Another clear trend indicated that in algal growth medium, the sorption of AgNPs was remarkably higher than in the cell culture medium (left and right sides of the Figure 1, respectively). While in algal growth medium (average for 50 and 250 ng AgNPs/mL and all plate types) only 54% of AgNPs were recovered, the average recovery of AgNPs in mammalian cell culture medium was 80%. We suggest that in the case of cell culture medium proteins and other biomolecules coated in the microplate surface and thus, sterically prevented the attachment of AgNPs to the surface. On the other hand, biomolecules in the medium also interacted with AgNPs and provided secondary coating which additionally prevented interactions between the particles and the surface of the vessel. Similar observations were reported by Abraham et al. (2013), who showed that adding natural organic matter to AgNP suspensions affected particle sorption to various sorbents (glass, silica gel, sand, and leaf surface). Also, Thio et al. (2012) observed decreased sorption of citrate- and PVP-coated AgNPs in the presence of organic matter natural waters. Another reason for differential sorption of AgNPs to microplates in algal and in cell culture medium could be the specific test conditions.
used for algal assay. Specifically, in algal growth medium, the AgNPs were constantly illuminated for 72 h, whereas the experiments in cell culture medium were conducted in the dark. At this point, we cannot exclude the possibility that the illumination induces AgNP degradation, as has been shown in several papers (George et al., 2014; Gorham et al., 2012)).

In order to investigate the processes taking place on microplate surfaces after their exposure to test media, microplates were incubated with algal growth medium or serum-containing cell culture medium in conditions similar to the toxicity tests and the exposed surfaces were analysed by X-ray photoelectron spectroscopy (XPS) (Figure 2). Before XPS analysis, plates were rinsed several times with DI water and dried. The exposure of microplate surfaces to cell culture medium resulted in significant changes in the surface composition. For both, untreated and Nunclon Delta-treated microplates, the amount of oxygen on the surfaces increased significantly (carbon to oxygen ratio decreased) (Figure 2). Also, nitrogen and sulphur appeared on the plate surfaces. The oxygen/nitrogen ratios in untreated and Nunclon Delta surface-treated microplates that were exposed to cell culture medium were 18.9/10.5 at% (i.e. 1.8) and 22.8/9.8 at% (i.e. 2.5), respectively, being thus similar to O/N ratios of the serum-containing cell culture medium (2.25 according to Kuroda et al. (2003)). The amount of sulfur appearing on microplate surfaces after the treatment with cell culture medium was relatively low (0.3–0.6%). Yet, it allowed us to calculate the oxygen/sulfur ratios on plate surfaces and compare them with O/S ratios reported earlier in the literature. O/S for cell culture medium exposed Nunclon Delta-treated surfaces was 63 (18.9/0.3 at%) and for untreated microplate surface 38 (22.8/0.6 at%) (Figure 2).

Figure 2. Modification of microplate surfaces by algal and cell culture media according to XPS analysis. (A), (B): microplate surfaces after exposition to cell culture medium; (C), (D): microplate surfaces after exposition to algal growth medium. (A), (C): untreated plate surfaces; (B), (D): Nunclon Delta-treated plate surfaces. Surface \( \zeta \)-potential values are indicated on the graphs.

In terms of different surface functionalities of the tested AgNPs, positively charged bPEI-coated particles showed higher sorption compared to negatively charged citrate-coated particles, especially in the algal growth medium (Figure 1) under all tested conditions. This result is consistent with the study by Sekine et al. (2015) describing remarkable affinity of bPEI-coated AgNPs towards plastic and glass surfaces, while reporting minimal adsorption of tannic acid-coated AgNPs. Our findings showed that at 50 and 250 ng AgNPs/mL (in algal growth medium), the amount of bPEI-coated particles sorbed to microplates was 11–19% higher than the amount of surface-sorbed citrate-coated particles. This could be explained by electrostatic interactions between cationic bPEI-coated AgNPs and microplates that exhibited highly negative surface \( \zeta \)-potential (Figure 3).
Among differently sized particles and expressed in mass units (µg/L), the recovery of 70 nm particles was significantly lower than the recovery of 30 nm particles in algal growth medium (Figure 1). Averaging two different coatings and different plate types, the sorption of 70 nm AgNPs was 19% higher than the sorption of 30 nm AgNPs. This difference could be easily explained by the higher content of silver in the larger 70 nm particles (according to the manufacturer’s specifications, one 30 nm AgNP weighs 0.2 fg and one 70 nm AgNP 2.5 fg). Yet, taking into account that the weight difference between 30 and 70 nm AgNPs is about 12.5-fold and that on a per mass basis, only 19% more Ag adsorbed to the microplate surface from the solution containing 70 nm sized AgNPs, the number of 30 nm particles that was retained on microplate surfaces was about 10-fold higher than the number of 70 nm particles.

Interestingly, despite the differences in surface area between the 12- and 96-well microplates (43 mm² and 107 mm² per 100 µL added to the plate, respectively), we did not observe major differences in AgNP sorption to these two different types of microplates. On average, for all NPs and all concentrations, only 8% more particles sorbed to 96-well plates compared to 12-well plates. In contrast, there was a difference in sorption capability of microplates with different surface treatments. This difference was especially pronounced in algal growth medium and in case of bPEI-coated AgNPs (Figure 1). On average, for all the tested concentrations, 17% more bPEI-coated AgNPs was sorbed onto the surface of untreated microplates compared to Nunclon Delta surface-treated plates. The main mechanisms by which AgNPs may adsorb on microplates are van der Waals forces or electrostatic interactions (Abraham et al., 2013). Therefore, we studied the wettability (hydrophobicity), ζ-potential, and chemical attributes of the two different microplate surfaces (Figure 3). Both, untreated and Nunclon Delta surface-treated microplates showed a highly negative surface ζ-potential and thus, this characteristic was excluded as a reason for higher sorption of AgNPs. Moreover, Nunclon Delta surface microplates that sorbed less bPEI-coated AgNPs exhibited even lower surface ζ-potential than untreated plates, which was possibly due to the oxygen plasma treatment applied (Zeiger et al., 2013) (Figure 3E and F). In accordance with our findings, other authors have also suggested that attachment of AgNPs to
solid surfaces could not be completely explained by electrostatic forces (Abraham et al., 2013; Klitzke et al., 2014). Another surface characteristic that has shown to play a role in NP attachment is wettability. Song et al. (2011) analysed the attachment of different AgNPs to hydrophobic and hydrophilic glass beads, and showed that AgNPs with more hydrophobic surface coatings (PVP and gum arabic) attached preferably to hydrophobic glass. On the other hand, we found no significant differences in sorption of relatively hydrophilic citrate-coated AgNPs to different glass surfaces. The results of surface wettability, measured as static water contact angle, showed that in our case, the surface of untreated microplates was significantly more hydrophobic compared to Nunclon Delta-treated microplates (Figure 3A and B). These data indicated that bPEI-coated AgNPs had relatively higher affinity to more hydrophobic surfaces, whereas the adsorption of citrate-coated AgNPs did not significantly depend on wettability of the microplates, as also shown by Song et al. (2011).

In conclusion, although the use of plastic microplates is a common practice in in vitro nanotoxicological experiments as well as in nanocotoxicological studies, this study proves that there may be a significant loss of NPs from the test and hence, caution should be taken when interpreting the test results. Using AgNPs as examples, we showed that up to 90% of AgNPs added to an ecotoxicological or toxicological assay may be retained on the test container surfaces and consequently, be eliminated from the assay. We showed that sorption of AgNPs onto test containers depends on AgNP size, surface characteristics, test conditions and the characteristics of the test containers. As a rule, bPEI-coated AgNPs exhibiting positive surface charge sorbed more strongly to test containers than negatively charged citrate-coated AgNPs. The test medium in which AgNPs were dispersed and exposed also had major influence on nanoparticle sorption onto microplates. Significantly more AgNPs were adsorbed in diluted algal growth medium containing mainly mineral salts compared to cell culture medium containing mainly organic molecules. Slightly more AgNPs were sorbed onto 96-well microplates compared to 12-well microplates, and more hydrophobic microplate surface facilitated the sorption of AgNPs. Thus, various parameters affect the sorption of AgNPs onto the test containers and consequently, the loss of particles from the toxicoological assay. Although this study was conducted using AgNPs, similar loss of NPs during toxicoological assays may also take place for other types of NPs. Thus, these results suggest that the potential loss of NPs should be considered in any eco/toxicological test conducted. As an efficient solution, we suggest that relevant control experiments should be included whenever new types of NPs or test vessels or other test conditions are used.

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**Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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**Supplementary material available online**

Supplementary Table S1 and Figures S1–S5