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RESEARCH ARTICLE

Assessment of silver nanoparticle toxicity for common carp (*Cyprinus carpio*) fish embryos using a novel method controlling the agglomeration in the aquatic media

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Abstract Formation of agglomerates and their rapid sedimentation during aquatic ecotoxicity testing of nanoparticles is a major issue with a crucial influence on the risk assessment of nanomaterials. The present work is aimed at developing and testing a new approach based on the periodic replacement of liquid media during an ecotoxicological experiment which enabled the efficient monitoring of exposure conditions. A verified mathematical model predicted the frequencies of media exchanges which checked for formation of agglomerates from silver nanoparticles AgNP with 50 nm average size of the original colloid. In the model experiments, embryos of common carp *Cyprinus carpio* were exposed repeatedly for 6 h to AgNPs (5–50 μm Ag L^{-1}) either under semistatic conditions (exchange of media after 6 h) or in variants with frequent media exchanges (varying from 20 to 300 min depending on the AgNP colloid concentration and the desired maximum agglomerate size of 200 or 400 nm). In contrast to other studies, where dissolved free metals are usually responsible for toxic effects, our 144-h experiments demonstrated the importance of AgNP agglomerates in the adverse effects of nanosilver. Direct adsorption of agglomerates on fish embryos locally increased Ag concentrations which resulted in pronounced toxicity particularly in variants with larger 400 nm agglomerates. The present study demonstrates the suitability of the novel methodology in controlling the conditions during aquatic nanomaterial toxicity testing. It further provided insights into the mechanisms underlying the effects of AgNP, which rank on a global scale among the most widely used nanomaterials.

Keywords Nanosilver · Agglomeration · Fish embryo · *Cyprinus carpio* · Particle size distribution

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Introduction

An increasing number of practical applications for manufactured nanomaterials (NMs), in nearly all areas of human activity, have led to the increasing risk of release of such materials into the environment. The annual production of NMs is estimated at 1000 t and has a growing trend (Navarro et al. 2008). The development of methods for determination of the actual impact of NMs on the environment is still in the initial stage (Lapresta-Fernández et al. 2012). This is because detection, extraction, or analysis of NMs are challenging due to their small size, unique structure, physical and chemical characteristics, surface coatings, and interactions in the environment, including agglomeration and sequestration (EPA 2007).

Water is a component of the environment which is potentially the most likely to be contaminated by various types of NMs. However, information on the fate of NMs in the aquatic environment or their negative impact on aquatic organisms cannot be considered comprehensive (Karn et al. 2011). A

number of works have investigated the behavior of nanomaterials in aqueous media (Hartmann et al. 2012), standardized media for ecotoxicological testing (Liu et al. 2013), the effect of surface structure (Mudunkotuwa and Grassian 2011), or other parameters which affect the speed and rate of agglomeration (Badawy et al. 2010; Liu et al. 2013). The vast majority of studies, however, have suggested the testing of nanomaterials using the same experimental protocols as exist for common chemicals. Unfortunately, the interpretations and conclusions obtained from simple ecotoxicity experiments are often affected by a number of factors characteristic for NMs, namely the formation of agglomerates. Agglomerates tend to sediment much faster than original NPs which lead to rapid exclusion from the aquatic phase. Consequently, the exposure of testing organisms to the original nanomaterials is limited, and the results of such assays tend to inform more about the toxic effects of the agglomerates (or bulk material) than about the toxicity of nanomaterial.

Nanosilver (AgNP) has a number of applications. Colloidal silver has been used for more than 120 years (Nowack et al. 2011) because of its antibacterial properties. The number of commercially available products containing AgNPs is annually increasing (Ribeiro et al. 2014) and is leading to an increased risk of release into the environment. For this reason, a great deal of research work has focused on determination of the impact of AgNPs on components of the environment. Toxicity tests of AgNPs have been carried out on a number of organisms including unicellular green algae (Bian et al. 2013; Dash et al. 2012), microorganisms (Levard et al. 2013), in vitro cell cultures (Govender et al. 2013), and vertebrates (Massarsky et al. 2013). The environmental fate and ecotoxicity of AgNPs are influenced by a wide range of factors such as particle size, surface area, the composition and valence of the solvating ions, their coating, pH, ionic strength, the presence of chelating substances, humin, humic and fulvic acids, and many others (Reidy et al. 2013). Consequently, AgNPs in the majority of the most frequently used testing media tend to form agglomerates and sediment from the water column.

The present work is aimed at developing and testing a new approach based on the periodic replacement of the liquid media during the ecotoxicological experiment, which enables efficient controlling of the exposure conditions. In our previous work (Oprsal et al. 2013a), we described the combined influence of the ionic strength of a liquid medium and the AgNP concentration on the rate and degree of agglomeration. This allowed for the development of a verified mathematical model predicting the size of agglomerates at a particular time (Oprsal et al. 2013b). In the present study, we applied the model to calculate the frequency of media exchange which ensures the appropriate maximum average size of the agglomerates for a given AgNP concentration. The different frequencies of the media exchange for different nanoparticle concentrations allows for a comparison of the toxicity of the same concentrations of nanoparticles assembled into agglomerates with a variable maximum average size. We further demonstrate utilization of the approach by assessing toxic effects of two silver colloidal systems differing in maximum average agglomerate sizes (200 and 400 nm) on embryos of a common carp (*Cyprinus carpio*), a freshwater fish of a high commercial importance in the fishing industry in Europe and in Asia.

Materials and methods

AgNP preparation

AgNPs were synthesized via reduction of silver nitrate ($AgNO_3$) by glucose ($C_6H_{12}O_6$) (Badawy et al. 2010). The preparation was carried out in an Erlenmeyer flask where 10 ml distilled water, 10 ml of 5 mM solution of silver nitrate, 10 ml of 25 mM ammonia solution, 10 ml of 50 mM sodium hydroxide solution, and 10 ml of 50 mM glucose were mixed. The formed mixture was subsequently stirred at room temperature for 15 min and then poured into a dark storage bottle. The resulting parameters, full width at half maxima (FWHM) and height obtained by atomic force microscopy (AFM), are shown in Fig. 1. These results are in solid agreement with data obtained by SEM (Fig. 2) and dynamic light scattering (DLS, data not shown). The concentration of total Ag in the prepared solution was around 1 mM, and the exact concentration of Ag was verified by optical emission spectrometry with inductively coupled plasma (ICP-OES). Solutions with lower nominal concentrations were prepared by the dilution of the stock solution by a liquid medium for ecotoxicity test.

Characterization of colloidal systems

Hydrodynamic diameter, zeta potential

The mean hydrodynamic diameter D_H and zeta potential were measured with an analyzer (ZetaPALS Potential Analyzer, Brookhaven Instruments Corp., USA) using DLS. The laser in this device operated at 660 nm with a light output of 35 mW. D_H values were measured for 30 s at 10 replications, and these data were statistically processed (mean, median, and standard deviation).

Atomic force microscopy

AFM analysis was carried out on the device SolverProM (NT-MDT, Russia) with a probe HA_NC (resonance frequency 185 kHz, k=4.6 N m^{-1}). The measurements were carried out in the semicontact mode set at 40 % of free oscillations. The samples were prepared by "spin coating" at 90× g on a freshly

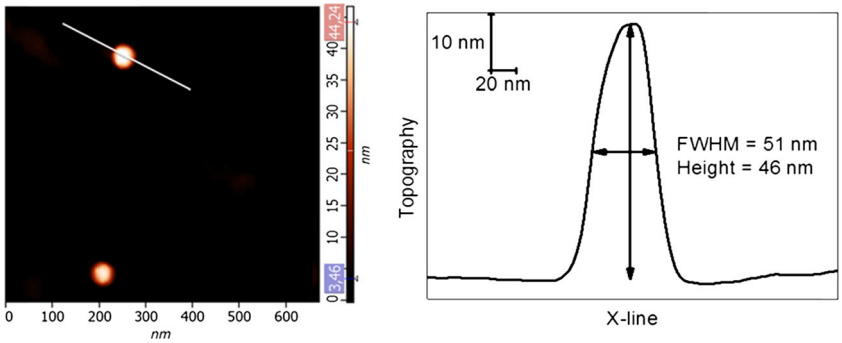

Fig. 1 Atomic force microscopy (AFM) image and profile of tested silver nanoparticles

prepared highly oriented pyrolytic graphite (HOPG) and dried under a vacuum (Bílková et al. 2011).

Scanning electron microscopy

Scanning electron microscopy analysis was carried out on the device JEOL JSM-7500F. The samples were prepared by spin coating at 90×*g* on a freshly prepared HOPG and dried under a vacuum. Samples were sputtered by thin layer of gold.

Analysis of free Ag^+ in tested colloids

The prepared AgNP colloids with total concentrations of Ag 5–250 μM were left for 6 h to agglomerate in the liquid medium under test conditions, and then, 15 ml of the studied colloidal solution were centrifuged at 10,000×*g* for 30 min, and the supernatant was taken for the measurements of the concentration of dissolved silver ions. Ten milliliters of supernatant were subsequently taken from each container, and the Ag concentrations in the samples were measured with an ICP-OES (IntegraXL2, GBC, Dandenong, Australia).

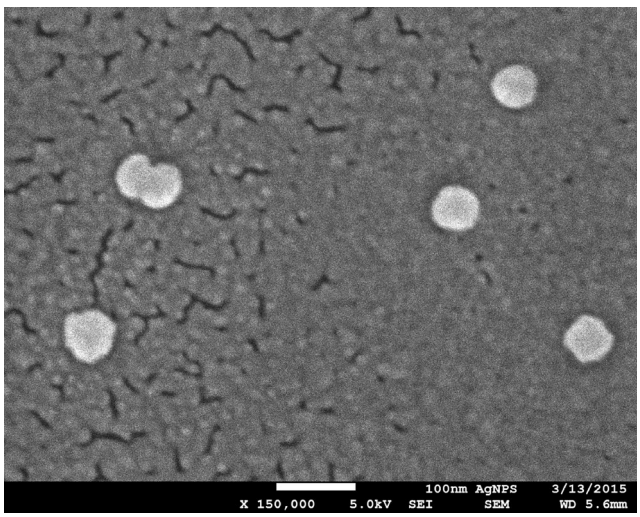

Fig. 2 Scanning electron microscope (SEM) image of tested silver nanoparticles. Scale (100 nm) is shown in the bottom of the picture

Liquid medium for ecotoxicity tests

Medium 203 prepared according to the OECD 203 guideline (OECD 1992) was selected as the default solution for our tests with *C. carpio* embryos. This medium contains 2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.77 mM $NaHCO_3$, and 0.075 mM KCl. The conductivity of medium 203 measured by a conductometer (inoLab cond. 730 with a Tetracon 325 measuring cell, WTW, Germany) was 680 μS cm^{-1}. The ionic strength of the solution affects the thickness of the diffusion layer reducing the stability of the nanoparticles and increasing the agglomeration rate (Baalousha et al. 2013). The influence of the medium 203 dilutions on carp embryos was investigated. The maximum dilution of the medium which did not affect the health status of the embryos was tested. The lowest concentration which still did not cause changes in exposed common carp embryos was found to be 75 % of the original media 203, and this concentration was used for all of the other following experiments.

Test organism

Fertilized eggs of *C. carpio* were obtained from commercial fisheries (Fisheries Třeboň, a.s., Czech Republic). The eggs were transferred to the laboratory immediately after fertilization, washed with a liquid medium, and inspected. The unfertilized or necrotic eggs were removed, and the test was only performed with the healthy specimens. According OECD 203 (OECD 1992) guideline, the fish are inspected at least every day. Fish are considered dead if there is no visible movement and if touching of the caudal peduncle of hatched embryo produces no reaction. Mortality is only endpoint in our tests we used. For sorting the eggs or inspection of test organism, stereo microscope (XTL-101, GX Optical, UK) was used.

Toxicity tests

Experiment 1 lasted 144 h, and the design included repeated periods of exposure to AgNP colloids for 6 h on days 0, 1, 2, 3, 4, and 5 followed by 18 h (overnight) periods when the carp embryos were transferred into fresh media without

nanoparticles. In experiment 2, the embryos were exposed on days 0, 1, 2, 3, 4, and 5, but the colloid media were frequently exchanged during the 6-h exposure. Frequent media exchanges were performed with the aim of checking for the formation of agglomerates. The standard design corresponding to OECD 212 (OECD 1998) protocol for testing of chemicals (i.e., continuous 144-h exposure with the exchange of colloid media every 24 h) was not conducted with respect to the observations in 6-h exposure experiments which resulted in complete agglomeration and sedimentation of NPs during the 6-h period.

Experiment 1—fish, short-term toxicity test with the embryo and Sac-Fry stages, repeated exposures for 6 h

Fish embryotoxicity tests (FETs) were carried out in a semistatic configuration with control and experimental groups of 50 carp embryos. The primary AgNP colloid (concentration of total Ag 1 mM) was diluted in a liquid medium (as prescribed in the OECD 203 guideline) to achieve the desired concentration ranging 5, 10, 25, 50, 100, and 250 μM of total Ag in the solution. The colloids were then characterized and subsequently used for exposure of fertilized eggs and embryos. The experiment was performed in glass beakers. A group of 10 embryos was placed into each beaker, and the dosed volume of AgNP colloid was always 10 ml (one embryo per 1 ml of exposure media). The total experimental time of test was 144 h. We start with 1-day-old healthy fertilized embryo. Experiment with nanoparticles lasted 5 days including 6-h exposure periods followed by 18 h in fresh media without nanoparticle agglomerates every day of experiment.

Experiment 2—the modified FET test with the controlled maximum average size of agglomerates

Further experiments were conducted using a modified protocol with variable exchange media periods. Experiment 2 that lasted same time like in experiment 1 with the different 6-h exposure periods was divided by freshly prepared media exchange. Length of every part of each period depends on selected size of agglomerates at specific media concentration. The periods were calculated as follows.

First, the DLS was used for evaluation of AgNP agglomeration in the particular colloid during the time period sufficient to achieve the steady state. The obtained experimental data were then used for determination of the rate constant k in Eq. (1), which describes the growth of agglomerates (more precisely the increase in D_H) over time (Lee and Ranville 2012).

$$D_H^t = D_H^{\inf} + \left(D_H^0 - D_H^{\inf}\right) \times e^{-kt} \quad (1)$$

In this equation, $D_H{}^0$ represents the hydrodynamic diameter of particles in the tested colloid immediately after its preparation (after the dilution of the primary colloid by the diluted liquid medium 203), $D_H{}^{inf}$ is the hydrodynamic diameter in the steady state, and t is time. The rate constant k strongly depends on the initial AgNP concentration and thus had to be determined for every concentration level separately. $D_H{}^t$ represents the average hydrodynamic diameter in time t. Consequently, when the desired value of this parameter is selected, Eq. (1) can be used for calculation of the time t when this value will be achieved. In other words, if the exchange of the tested colloid is performed in time t, the size of agglomerates does not exceed the value $D_H{}^t$ calculated using Eq. (1). Variable exchange frequency is the way to influence the size of agglomerates occurring in the tested colloid.

FET experiments with controlled agglomerate size were performed using the same experimental setup as in experiment 1. Five beakers were used, and each one contained 10 embryos in 10-ml media. The experiments were designed to check for two sizes of agglomerates (200 and 400 nm) using Ag concentrations ranging from 5 to 50 μM. The experiments lasted for 144 h including 6-h exposure periods on days 0, 1, 2, 3, 4, and 5 during which the colloid media were frequently exchanged (exchange periods ranged between 20 and 120 min depending on the colloid concentration and the desired maximum size of agglomerates—see the "Results"). After the 6-h exposure, the embryos were kept for 18 h overnight in fresh media without nanoparticles.

Statistics

Differences in mortalities between exposure groups were compared by nonparametric Mann–Whitney test; p values less than 0.05 were considered statistically significant. Data were collected in Microsoft Excel and statistically processed with nonparametric module within the Statistica 12 software (StatSoft, Inc., Tulsa, OK, USA).

Results and discussion

The knowledge of the behavior of nanoparticles in liquid media typically used for ecotoxicity tests suggests that reducing ionic strength leads to a reduction in the rate and the extent of agglomeration (Baalousha 2009; Levard et al. 2012; Radomski et al. 2005). That is the point why we tested dilution rate of medium 203 which caused no effect to embryo, and we found most suitable point in 75 % medium 203. During the experiments, concentrations of dissolved Ag^+ in media were tested with ICP-OES. To ensure the accuracy of the results, the amount of dissolved silver was measured 30 min after the preparation of the solution and after 6 h of the exposure time. The measurement of quantities of dissolved silver was carried

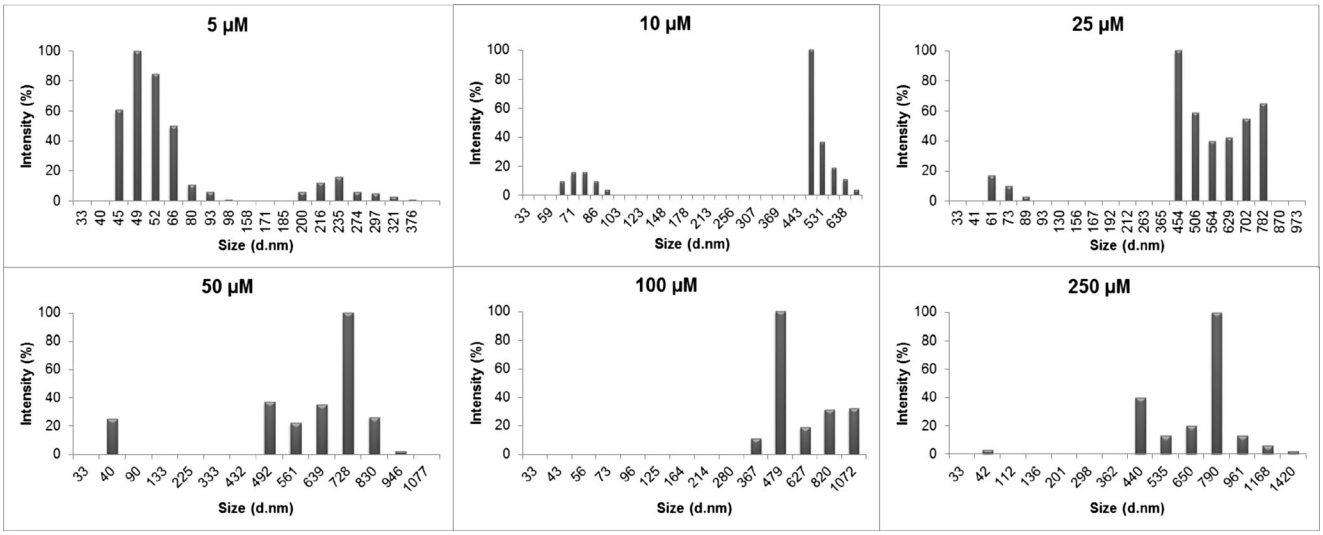

Fig. 3 Aggregate size distribution measured by DLS after mixing with the test media at different AgNP concentration. The original colloid (50-nm average size of particles) was maintained for 6 h in 75 % *v*/*v* medium 203 (prepared according to OECD 203 guideline). The values in the upper part of the figure represent the concentration of AgNPs

out for wide range of concentrations (5–250 μM), but the concentrations of released dissolved silver ion were below the detection limit of the instrument in all variants, i.e., 10 ppb in case of Ag (10 μg L^{-1}; 0.1 μM L^{-1}). No observable dissolution of silver ion from nanoparticle agglomerates was related to the chemistry of the system. Medium 203 contains high concentrations of chlorides that precipitate Ag^{+} to practically insoluble AgCl ($K_S = 1.77 \times 10^{-10}$). During the semistatic experiment, analyses of particle and agglomerate size distributions were analyzed by DLS at varying concentrations after 6 h of incubation (Fig. 3). Figure 3 clearly shows the formation of variable agglomerates where the increasing concentration of AgNPs resulted in the rapid disappearance of small particles around 50 nm and a visible increase in the number of particles around 400 nm. Note that the histograms in Fig. 3 do not have uniform scaling and increasing concentration of the AgNPs leads not only to the disappearance of initial small NPs but also to a shift of the agglomerates to larger sizes. The results thus clearly demonstrate the formation of large agglomerates, whose physicochemical properties are likely to be fundamentally different from the properties of the original colloid.

The formed agglomerates no longer hold together in the solution and necessarily settle to the bottom of the

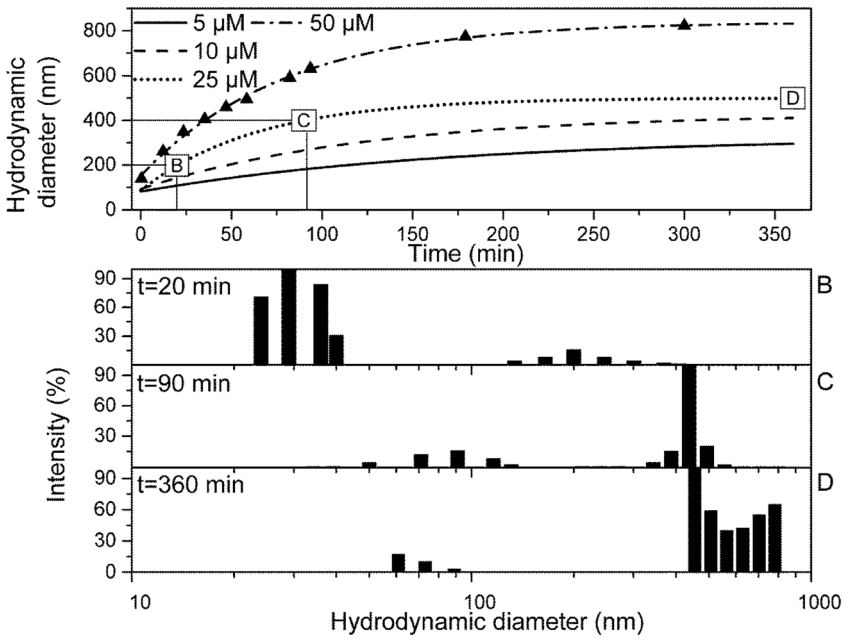

Fig. 4 Agglomeration behavior of AgNPs measured in time. The *upper panel* shows measured points in time for concentration ranges 5–50 μM. Measured points in time are presented like *triangles*. Kinetics of aggregate formation calculated according Eq. (1) at concentrations 5, 10, 25, and 50 μM are shown as *curves*. *Lower panel* shows actually measured particle size distribution of 25 μM AgNP colloid after 20 (*graph B*—corresponding also to point *B* in the upper panel), 90 (*C*) and 360 min (*D*) of the primary colloid dilution in 75 % medium 203 for better illustration of agglomerate size growing in time

experimental beaker which substantially affects the exposure conditions. The formation of agglomerates is determined by the interactions between particles and the total energy of the system. This approach is described in traditional DLVO theory (Derjaguin and Landau 1941). Correspondingly, the rate and the speed of agglomeration are strongly dependent on the concentration and valence of ions in the liquid media traditionally used for toxicity tests. The addition of NPs into the solution leads to a temporary increase in the speed of agglomeration and formation of larger initial agglomerates which sediments faster in a medium with a higher initial concentration and vice versa—smaller agglomerates are formed in solutions with lower concentrations (Quik et al. 2014). This has also been confirmed in the present study (Fig. 3).

As described in the "Materials and methods", the observed formation of agglomerates can be modeled using Eq. (1). The time-dependent decrease in initial particles, i.e., the agglomeration rate, was expressed by the first-order kinetics (Lee and Ranville 2012), and the kinetics of the agglomeration formation for 5, 10, 25, and 50 μM of the initial AgNPs are shown in Fig. 4. The experimental values of agglomerate distributions are also shown for 25 μM as an example (bottom panel in Fig. 4).

Using the models described, we have calculated times of liquid media exchange which would control the formation of agglomerates of the sizes of 200 and 400 nm. These valued (D_H times) are presented in Table 1 and cover the technically feasible range of intervals between 9 and 120 min (for AgNP concentration range 5–50 μM, controlling formation of smaller 200 nm agglomerates) or 34–300 min (for AgNP concentration range 10–50 μM, controlling for 400 nm agglomerates). The following FET experiments were then conducted using the calculated periods when the medium with agglomerates was completely removed and replaced with the fresh AgNP colloid containing media.

The characteristic of NPs to form agglomerates is a major problem for systematic studies of NP toxicity in aqueous media. As shown in Figs. 3 and 4, the 6-h exposure treatments differed not only in NP concentrations (5–250 μM) but also in other characteristics such as size of particles or agglomerates, which makes the interpretation of the toxicity observations extremely difficult. Similarly, it is complicated to compare the results of different studies because of the variable compositions of the exposure media used in different assays (e.g., algae vs Daphnia vs fish) which substantially differ among one other (Seo et al. 2014). Kaewamatawong group attributed the largest part of toxicity in zebrafish FET to dissolved silver ion, and they publish value of EC_{50} in the range 2–126 μg L^{-1} (Kaewamatawong et al. 2012). In study with tilapia (*Oreochromis niloticus*), LC_{50} value was set at 53 μg L^{-1}

Table 1 Rate constants and corresponding times of liquid media exchange for checking the average maximum size of agglomerates on the level of 200 and 400 nm

c AgNPs (μM)	k (h^{-1})	$D_{H\ 200\ nm}$ (min)	$D_{H\ 400\ nm}$ (min)
5	0.84	120	–
10	0.58	45	300
25	0.94	20	90
50	0.99	9	34

c AgNPs concentration of Ag in colloid, *k* rate constant in Eq. (1), $D_{H\ 200}$ *and* $D_{H\ 400\ nm}$ resp. time when the hydrodynamic diameter measured in the particular colloid reaches the value 200 or 400 nm

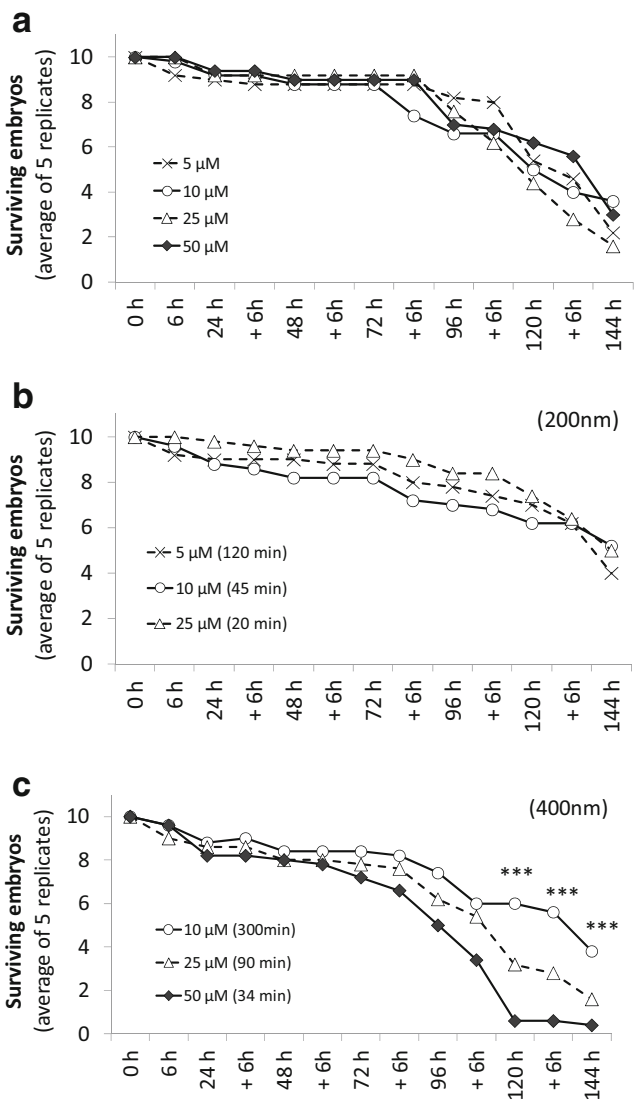

Fig. 5 Toxicity testing of silver nanoparticles (AgNPs): average surviving embryos of five replicate beakers (*error bars* not shown for clarity). **a** Semistatic exposures to four concentrations for 6 h followed by 18 h in fresh media without AgNP, **b** exposure controlling for maximum 200 nm agglomerates, and **c** exposure controlling for maximum 400 nm agglomerates. The values in parentheses in **b** and **c** indicate the periods of frequent media exchanges for the given concentrations; ***Statistically significant differences among all three concentrations 10 vs 25 vs 50 μM (Mann–Whitney test, $p<0.05$)

(Srinonate et al. 2015), for Eurasian perch (*Perca fluviatilis*), EC_{50} value is lower than 30 μg L^{-1} (Bilberg et al. 2010), and for fathead minnow (*Pimephales promelas*), EC_{50} values were 94 and 106 μg L^{-1} for stirred and 125 and 136 μg L^{-1} for sonicated NPs, respectively (Laban et al. 2010). With this respect, comparisons of "sensitivity" of different organisms to NPs (expressed, e.g., in ICx concentrations) is meaningless because the exposures are broadly different in multiple parameters, namely the size and distribution of particles or agglomerates.

In our study with the continuous 6-h exposures (experiment 1), mortalities were observed in all AgNP concentrations—between one and four embryos out of 10 survived after 144 h in individual experimental beakers (mortalities 60–90 %). However, there were no statistical differences among concentrations (Mann–Whitney test, $p>0.05$), and no apparent dose–response relationship (Fig. 5a). The following experiment 2 (Fig. 4b, c) provided variable results. The variant where the frequent exchanges of media controlled for a maximum 200 nm agglomerates (Fig. 5b) provided a similar pattern to experiment 1 with no differences between the doses and a slightly lower toxicity (30–50 % mortalities in individual beakers in all AgNP concentrations tested). In contrast, the experiment checking for 400 nm agglomerates resulted in a dose–response (statistically different effects among the three tested concentrations, Mann–Whitney $p<0.05$, Fig. 5c). The highest colloid concentration tested (50 μM) led to pronounced 90–100 % mortalities (first observed after 120 h of the experiment). In other words, more pronounced AgNP toxicity was caused by higher concentrations of larger agglomerates in the water column. The "continuous" presence of the large 400 nm agglomerates (assured by frequent exchanges of media, Fig. 5c) was the most toxic for common carp embryos, while the same concentrations of AgNP colloid without media exchanges led to fast sedimentation of agglomerates and lower toxicity (compare, e.g., effects of 25 and 50 μM AgNPs in Fig. 5a, c).

The size-specific effects of particles were also confirmed by the observations of individual embryos in the study with apparent sorption of larger Ag agglomerates into the exposed embryos (Fig. 6). This could dramatically increase the actual Ag concentrations locally at the biological surface and lead to mechanical disruption and blocking of biological functions. The latter phenomenon has also been suggested, e.g., by (Chen et al. 2013) which found significantly elevated bioaccumulation of iron in medaka fish in exposed to agglomerating nFe_3O_4. In contrast, lower Fe bioaccumulation was recorded at stable colloid of Fe0 (Chen et al. 2013). Similarly, (Zhu et al. 2012) demonstrated that agglomerates of iron oxide nanoparticles had more pronounced effects on developing embryos of zebrafish *Danio rerio* by inducing both higher mortalities and higher rates of morphological malformations.

The importance of the particulate form of Ag was recently demonstrated in another study with *Daphnia magna* (Seo et al. 2014), where both particulate and dissolved fractions were responsible for the toxicity of AgNPs. In contrast, toxicities of NPs of other metals compared in the same study (CuO, ZnO) were primarily caused by the dissolved fractions exclusively (Seo et al. 2014). Correspondingly, (Stensberg et al. 2014) revealed that silver nanoparticles had greater impacts on mitochondrial functions in *D. magna* (in comparison with the effects of dissolved Ag^+), which could mechanistically explain the observed higher toxicities of Ag nanoparticles.

The importance of careful monitoring of nanoparticle agglomeration in ecotoxicological assays was also recently highlighted by (Auffan et al. 2013). In their study of CeO_2 particle toxicity to *Daphnia*, the authors identified that the actual aquatic exposure duration was only 2 h. After this period, there was no relevant exposure of daphnids to particulate material which was removed from the water column by rapid agglomeration and sedimentation (Auffan et al. 2013). Agglomeration of metal nanoparticles may also have other effects such as changes in the bioavailability of other toxicants (Hartmann et al. 2012) or modifications of the conditions during chronic exposure conditions. For example, (Campos et al.

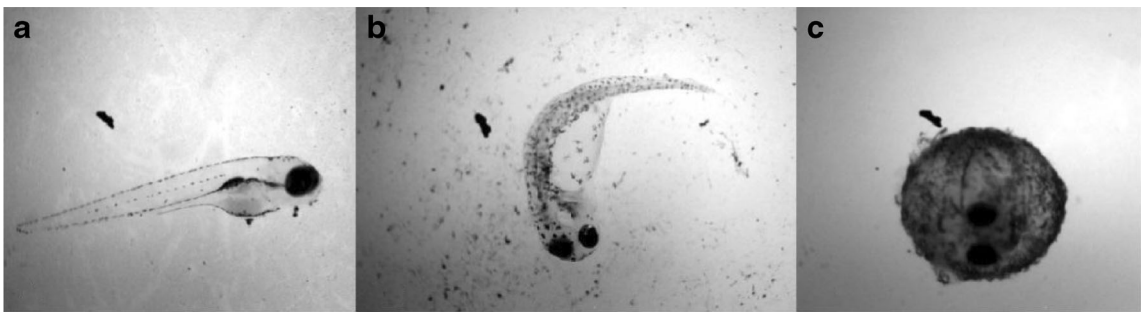

Fig. 6 Example of the influence of AgNPs on the development of carp embryos after 144 h of the experiments. **a** Control embryo, **b** embryo with apparent spine curvature and pericardial edema (exposure to media with AgNP concentration 5 μM) in the experiment controlling for 200 nm agglomerates), and **c** embryo with a chorion covered by AgNP agglomerates (exposure to 25 μM AgNP in the experiment controlling for 400 nm agglomerates)

2013) demonstrated the rapid depletion of food from aquatic media along with the nanoparticle sedimentation which was then manifested as decreased growth and wellness of Daphnia.

The tested concentrations of AgNPs were used to investigate hazard of AgNPs and are naturally much higher in comparison to the environmental concentrations of silver, which is only rarely reflected in regulatory documents (e.g., Scotish EPA WAT-SG-53). However, the goal of the present study was not direct evaluation of risk assessment but methodological development allowing for controlled ecotoxicity testing of metal nanoparticles and agglomerates. In addition—and as we also discuss elsewhere—the effects observed are unlikely to be related to freely dissolved silver ions because none of the samples had Ag in concentrations higher than analytical LOD (10 $\mu g\ L^{-1}$). Also, existing studies on toxicity of silver to fish revealed much higher effective (LOEC) concentrations of 50 and 500 $\mu g\ L^{-1}$ for Japanese medaka (Kashiwada et al. 2012) and zebrafish (Bar-Ilan et al. 2009), respectively.

In conclusion, the novel methodological approach has been developed and tested in the experiments with AgNP toxicity to common carp embryos. In contrast to other studies, we have observed lower toxicological importance of dissolved metal ions, and the effects were apparently related to the formation of AgNP agglomerates. The direct adsorption of agglomerates on the test organisms would seem to be a major contributing factor. Further studies should clarify the mechanisms beyond the observed effects by, e.g., comparing various metal NPs and/or focusing on specific modes of action (Stensberg et al. 2014).

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