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ARTICLE

The effect of capping agents on the toxicity of silver nanoparticles to *Danio rerio* embryos

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ABSTRACT

Addition of capping agents like surfactants and polymers during the synthesis of nanoparticles may affect the stability and toxicity of dispersions of nanoparticles. This study revealed the impact of anionic, cationic, and amphoteric surfactants and a cationic polymer on the physical and chemical properties, stability and behavior of silver nanomaterials, as well as on the toxicity of nanosized silver particles with respect to zebrafish embryos. Some of the stabilizers applied were shown to significantly affect embryos of *Danio rerio*. Colloidal dispersions of stabilized silver nanoparticles were demonstrated to induce a complex mechanism of toxicity with respect to embryos of *D. rerio,* which is mainly explained by the toxicity of the organic ligand, while other parameters are somewhat inferior. The newly generated data on the toxicity of nanoparticles and their stabilizers with respect to *D. rerio* embryos reveal the complexity of the toxicity mechanism of nanoparticles impacting living systems.

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Introduction

Nanoparticles (NPs) have already found a wide variety of applications. These applications require high aggregative stability and perfect control over the NPs size and surface functionalities. Recently, the interest in NPs has grown considerably, especially with regard to NPs containing heavy metals and a number of companies already integrated nanomaterials in their products. This can be explained mainly by the unique properties of nanomaterials compared to their bulk counterparts (McCall 2011). Nowadays NPs are amongst others applied in active and selective catalysts, elements of microelectronics, in optical devices (Vance et al. 2015), and even in plant protection products (Kutuzova et al. 2017). In particular, silver NPs are one of the most widely used materials in different fields due to their antibacterial properties: nanosilver is integrated into cleaning and filtering systems and has various medical applications. The increasing interest in nanosilver is mainly explained by the emergence and expansion of pathogenic microorganisms with multiple drug resistance, including resistance to last-generation antibiotics (Krutyakov et al. 2016c).

On the other hand, safety concerns become more and more essential as the number of products containing NPs is constantly increasing. Despite the continued and rapid growth of the field of nanotechnology, the potential harmful effects of nanomaterials on human health or on the environment have not yet been identified in sufficient detail (Gaiser et al. 2011; Gliga et al. 2014).

To understand the toxicity of NPs, the effect of all physical and chemical characteristics needs to be investigated (Oberdörster Stone, and Donaldson 2007; Moore 2006; Rivera Gil et al. 2010). Besides their particle size, the toxic effects of nanoparticles can probably be linked to their intrinsic and extrinsic properties such as the surface area, shape, type, and amount of stabilizer, presence of toxic

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impurities or solvents, etc. (Hua et al. 2014; Golovina and Kustov 2013). Thereupon, these properties are capable of affecting the fate of the NPs as they will have a direct impact on agglomeration and/or aggregation of NPs in environmental and physiological media.

In this study, we assessed the effect of capping agents (stabilizers) on the aggregative stability and toxicity of silver NPs with respect to zebrafish embryos, considering the behavior and stability of NPs in test media as some of the most important characteristics affecting the outcome of biological testing. To obtain specific functional properties of NPs and to stabilize the particles, the NP surface is usually modified by various chemical compounds (Monteiro-Riviere and Tran 2007). The nature of the stabilizer and the stabilizer/silver concentration ratio have a significant effect on the surface charge, the aggregative stability of the colloids, their dispersibility and solubility in various media (Ajitha et al. 2016; Zhu et al. 2013) and the toxicity of NPs.

As stated above, the nature of a stabilizer greatly influences the aggregative stability of NP dispersions in surface waters and in test media. Previously, the spectrum of antibacterial and antifungal activity of Ag NPs stabilized with benzyldimethyl[3-(miristoylamino)-propyl] ammonium chloride was demonstrated. Tests indicate that if such a dispersion influences pathogenic microorganisms (*Escherichia coli ATCC 25922, Staphylococcus aureus FDA 209 P, methicillin-resistant S. aureus INA 00761, vancomycin-resistant L. mesenteroides VKPM B-4177, Saccharomyces cerevisiae RIA 259 and Aspergillus niger INA 00760*), it leads to a synergistic effect of mutual amplification of the activity of silver and the stabilizer (Vertelov et al. 2008). The effect of a stabilizing agent was investigated for gram-negative *E. coli* bacteria by Bae et al. (2011). To modify the surface of the NPs, these authors used anionic and cationic surfactants as well as neutral and charged polymers and found that cationic surfactants increase the antibacterial activity of silver NPs.

In addition, the surface charge of NPs is linked to the uptake of NPs by cells (Nordberg , Fowler, and Nordberg 2014) and due to the negative charge of cells, positively charged NPs, in general, penetrate into cells faster than negatively charged particles. Since most cellular membranes are negatively charged, the zeta potential can affect the NPs ability to permeate through membranes, with cationic particles generally displaying higher toxicity associated with cell wall disruption (Singh et al. 2008; Oh et al. 2010). In turn, the zeta potential of particles depends on the point of zero charges of the particles and the medium composition, with pH being an especially important factor in this respect.

One of the main difficulties in experimental work with NPs and in interpretation of the results obtained is a general lack of information about the properties of the NPs tested and their physical/chemical state under the experimental conditions employed. As the ionic strength of the solution increases, synthetic silver NPs can increasingly form agglomerates (Prathna, Chandrasekaran, and Mukherjee 2011) which in turn affect the toxic properties of NPs. For instance, larger aggregates are more easily removed by macrophage cells than smaller aggregates (Zhu et al. 2009). The mechanisms of aggregation of NPs determine their morphology and the sorption/desorption of ions from dispersions (Gilbert et al. 2009).

In spite of growing attention to the safety of nanomaterials (Wang et al. 2012; Ivask et al. 2014; Wijnhoven et al. 2009), there is no agreement among authors about the mechanisms of possible toxicity of silver NPs and to what extent silver NPs affect the environment and human beings. The data on toxicity of Ag NPs are contradictory and require additional verification. Unfortunately, in most studies the attention paid to the toxic effects of stabilizers on the test objects is insufficient. It is, therefore, difficult to draw firm conclusions about the mechanism of toxicity and the role of a capping agent in process of interaction of NPs with test organisms. The choice of a stabilizing agent significantly influences the results of toxicity tests. Therefore, biological effects of these substances must be evaluated in sufficient detail during experiments.

The purpose of this study was to assess the impact of nanosilver stabilized with anionic, cationic, amphoteric surfactants, and a cationic polymer, on the survival and development of zebrafish embryos (*Danio rerio*). The effect of stabilizing agents is of great interest, since the use of the stabilizer determines the surface properties of the particles, and therefore, the properties of the NPs and

the mechanism of their interaction with the cells and tissues of living organisms.

Materials and methods used

Silver nitrate (99.9+%, Sigma-Aldrich, St. Louis, MO), sodium borohydride (VenPure, 99%, Acros Organics, Morris Plains, NJ) , sodium lauryl ether sulfate (70% aqueous solution, Hansa), benzyldimethyl[3-(myristoylamino)propyl]ammonium chloride monohydrate (99.5%, PharmChem, Donetsk, Ukraine), sodium tallow amphopolycarboxyglycinate (30% aqueous solution, additionally containing up to 10% NaCl, Akzo Nobel), sodium cocaminopropionate (99%, Lakeland, Windermere, UK), polyhexamethylene biguanide hydrochloride (PHMB) (20% aqueous solution, Arch Chemicals, Norwalk, CT) were used in the experiments. All aqueous solutions were prepared with double distilled water. Table 1 presents the information about the capping agents used in this work.

Synthesis of 200 mL colloids of Ag NPs with a concentration of 100 mg/L

Colloidal solutions of AgNPs were obtained using a procedure published elsewhere (Krutyakov et al. 2016a; Krutyakov et al. 2016b; Gusev et al. 2016). Briefly, 50 mL of an aqueous solution of $AgNO_3$ (0.395 g, 0.185 mmol) was added dropwise to a 50 mL aqueous solution of a stabilizer under vigorous stirring. After 15 min, 100 mL of $NaBH_4$ (0.141 g, 0.37 mmol) was added to the mixture dropwise with intensive stirring. This reduction process was carried out at room temperature. The quantity of stabilizer added was adjusted to reach a 100 mg/kg (100 ppm) total concentration in the reaction mixture. The quantity of $AgNO_3$ added was adjusted to reach a 500 mg/kg (500 ppm) total concentration of nanosilver in the reaction mixture.

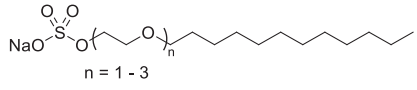
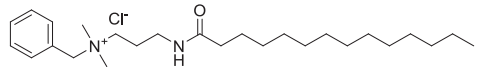
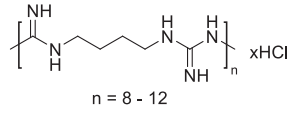
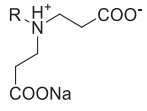
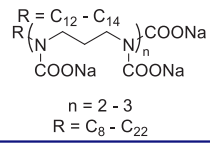
Synthesis of Ag/AgCl composite nanoparticles

Water 96 mL and 4 mL of a 30% H_2O_2 aqueous solution were added to 20 mL of a colloidal Ag dispersion containing 3 g/L silver and 48 g/L sodium tallow amphopolycarboxyglycinate. The mixture was treated with a UV mercury arc lamp (DRT-240) with a power of radiant flux 24.6 W. The irradiation was carried out in a spectral interval of 240–320 nm for 1 h. During this 1 h period, every 15 min the process was interrupted for 10 min under vigorous stirring.

Characterization of nanoparticles

Microphotographs of silver nanoparticles were obtained by means of an electron microscope (Leo 912 AB Omega, Leo Ltd., Germany) with the operating accelerating potential of 100 kV. Samples were prepared by spreading 1–2 μL of a dispersion onto a copper mesh coated with Formvar™ (d = 3.05 mm), which was then dried in open air for

Table 1. List of substances used as stabilizing additives.

Stabilizer	Formula	Symbol
Anionic surfactant sodium lauryl ether sulfate		I
Cationic surfactant benzyldimethyl[3-(myristoylamino)propyl]ammonium chloride		II
Cationic polymer polyhexamethylene biguanide hydrochloride		III
Amphoteric surfactant sodium cocaminopropionate		IV
Amphoteric surfactant sodium tallowamphopolycarboxyglycinate		V

5–10 min. UV–vis absorption spectra were recorded using a Varian Cary 100 spectrophotometer with the cell peltier accessory. The measurements were performed in a quartz cell. Prior to the measurements, the colloid solutions were thermostated at 20 °C. Nanoparticle sizes were determined in the colloidal dispersion by dynamic light scattering (DLS). The measurements were carried out in a quartz cell with a DelsaTMNano C particle analyzer (Beckman Coulter, Beckman Coulter, Brea, CA) at a wavelength of 658 nm using the Delsa Nano Software package. Prior to the measurements, the colloidal dispersions were thermostated at 20 °C. Particle size and concentration measurements were performed with a Nanosight LM10 HS-BF instrument (Nanosight Ltd., Amesbury, UK) based on Nanoparticle Tracking Analysis (NTA). The instrument is equipped with a 405 nm 65 mW laser and a high-sensitivity EMCCD Andor Luca camera. Samples were diluted with particle-free MilliQ water to reach the optimal concentration for NTA according to ASTM E2834–12 (ASTM Standard E2834 2012). Camera settings were optimized for each sample individually. A total of in between 8 and 24 videos of particles Brownian motion were recorded for each sample to reach 2500 tracks in total. All recorded videos were processed with a NTA 2.3 software build 0033. To obtain the joint histogram of the particle size distribution for multiple measurements, single particle diameters and track lengths from each measurement were collected into a global table and binned with weights proportional to track lengths.

Embryo toxicity test

In our experiments, we selected zebrafish embryos as test organisms due to their sensitivity to toxicants and convenience in performing experiments on real-time toxicity assessments. Fertilized zebrafish eggs were obtained from an AB wild-type zebrafish (*Danio rerio*, Hamilton 1822). We carried out the experiments on the hydrobionts according to standard procedures (OECD No. 236, 2013). Adult zebrafishes were maintained at 26 °C in a 12 h light: 12 h dark cycle. An acute exposure regime of 96 h was used, from 0 h post-fertilization (hpf) to 96 hpf, thus including exposure during the major stages of organ development. At the stage of a blastula, zebrafish embryos were distributed, 1 embryo per well, into 24-well plates in 2 mL of freshly prepared solutions containing silver particle dispersions or $AgNO_3$ solution (as a positive control). Zebrafish embryos were exposed in triplicate to either the control or to different concentrations of silver NPs. In addition, we also investigated the impact of a solution of a stabilizer on the development of zebrafish embryos. The embryos were monitored daily during a 96 h period to determine the survival rate (percentage of living embryos), hatching rate (percentage of embryos that had hatched during the exposure period), hatching time (day of hatching) and malformation prevalence (percentage of malformed embryos of surviving embryos). The stages of eggs development were monitored under a binocular microscope ('Carl Zeiss', Stemi 2000C). Throughout all procedures, embryos and solutions were kept at 26 °C. The median lethal concentration (LC50) of zebrafish caused by AgNPs was calculated using GraphPad Prism version 5 according to the dose–response curves obtained.

According to the literature (Asharani et al. 2008; Kannan et al. 2011), the LC50 value for silver NPs can vary considerably. Thus, initially, we carried out a range-finding experiment. For this purpose, the experiments were performed in a wide range of concentrations from 0.001 to 10 mg/L. After the first stage, the experiment was repeated in a narrow range to more accurately assess the LC50 values. Five concentrations and three replicates were used for each assessment. The number of dead embryos, embryos with developmental disorders, and the number of hatched larvae were counted every 24 h. We considered violations such as pericardial edema, scoliosis and a decrease in the body size. The test was carried out during the first 96 h after fertilization. To determine the mechanism of action of the nanoparticle solutions, the harmful effect of stabilizer solutions and their influence on the egg development were analyzed. The data were compared with the toxicity of silver nitrate. At the end of the experiment, the mortality index of the population, the percentage of morphological abnormalities in the embryos, the hatching rate and the morphological variations among survivors were determined. Statistical processing of results was carried out using the program GraphPad Prism version 5.0 (La Jolla, CA).

Results and discussion

The choice of stabilizers was determined by the necessity of stabilization of NPs in dispersion. By using substances of different nature, different stabilization mechanisms can be realized on the one hand, and on the other hand, there is a possibility of varying the size and charge of the produced NPs, thus allowing to control their ecotoxicity. It is known that some surfactants and polymers can induce sufficient aggregative stability of NPs (Kim and Zukoski 1990; Li et al. 2003; Taleb, Petit, and Pileni 1997).

Despite the fact that the same borohydride method of reduction was used, the conditions of stabilization employed for each type of NPs we different (Table 2). Anionic stabilizers electrostatically interact with positively charged silver ions on the surface of NPs. At the same time, the surface of NPs is charged positively as a result of reduction due to excess of silver salt and specific adsorption of silver ions. Negatively charged functional groups in the stabilizing agent may interact with the surface of NPs, which leads to the formation of an inner stabilizing layer of a surfactant (Figure 1).

Due to the strong Ag-N coordination bond, positively charged polymers with a tertiary nitrogen atom in the chain and amphoteric surfactants effectively stabilized silver NPs (Kumar et al. 2003; Beamson and Briggs 1992). Some of the stabilizers have a chloride ion as a counter-ion (polyhexamethylene biguanide hydrochloride) or as an additive (sodium tallowamphopolycarboxyglycinate and sodium cocaminopropionate). Chloride ions can be adsorbed at the surface of a metallic core, and therefore, a positively charged molecule of a polymer is attracted electrostatically to the core of a NP. As a result of this interaction, a coordination bond is formed and the core of the NP becomes negatively charged.

Synthesized Ag/AgCl composite particles containing metallic silver and silver halogenide are reported to have a core-shell structure with an Ag shell on the surface of silver halogenide NPs (Dong, Liang, and Gong 2012; Krutyakov et al. 2016b).

Table 2. Overview of the concentrations of Ag NPs obtained and the (mass) concentration ratio (cr) of stabilizer: NP for each stabilizer.

Nanoparticles	Concentration NPs (mg/L)	Concentration ratio (stabilizer/NPs)
Ag_I	100	10
Ag_II	300	2
Ag_III	500	1 and 0.2
Ag_IV	100	12 and 5
Ag_V	3000	2
Ag/AgCl_V	2500	2

Properties of nanoparticles

Detailed characterization of NPs is of crucial importance for interpretation of biological tests results. The main characteristics of nanoparticles such as the average particle size, surface charge, zeta potential, and the parameter characterizing the stability in test media were investigated. NPs were tested to identify

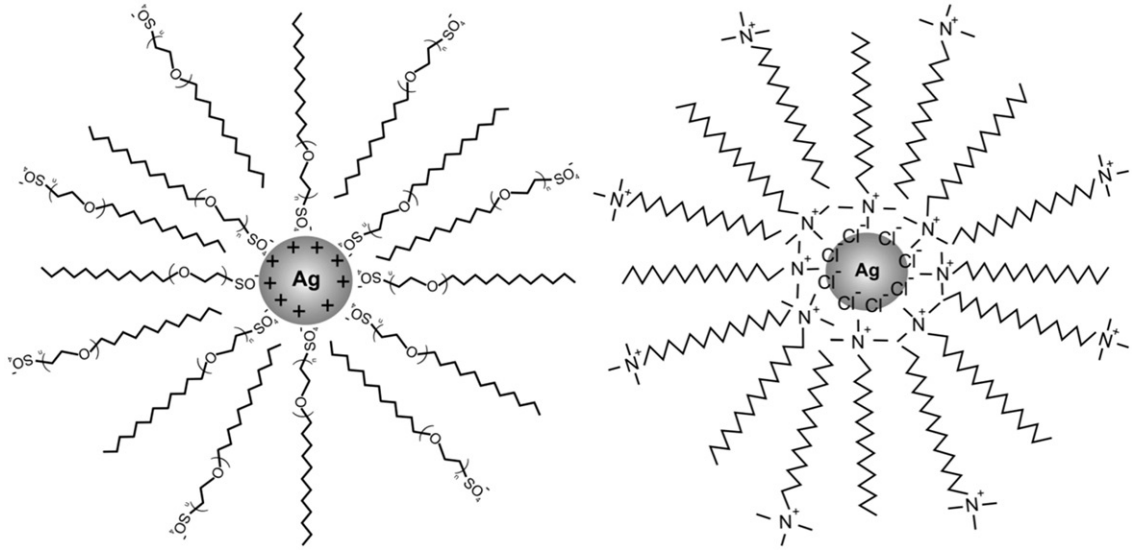


Figure 1. Structure of silver NPs with anionic (left) and cationic (right) surfactants as stabilizers.

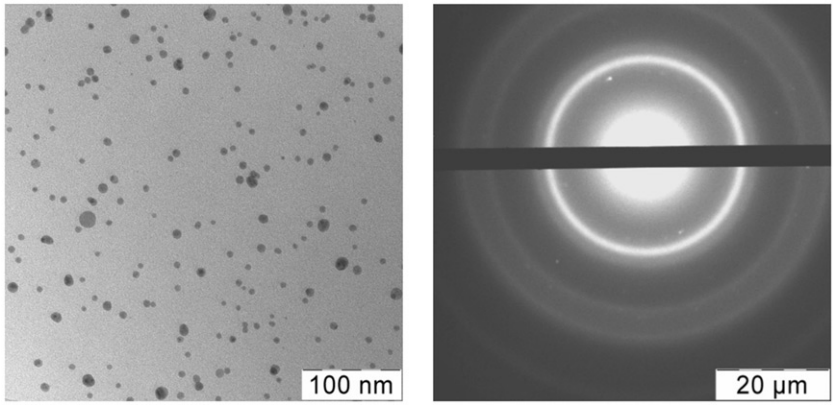


Figure 2. TEM picture of Ag_V NPs with sodium tallowamphopolycarboxyglycinate and diffraction images.

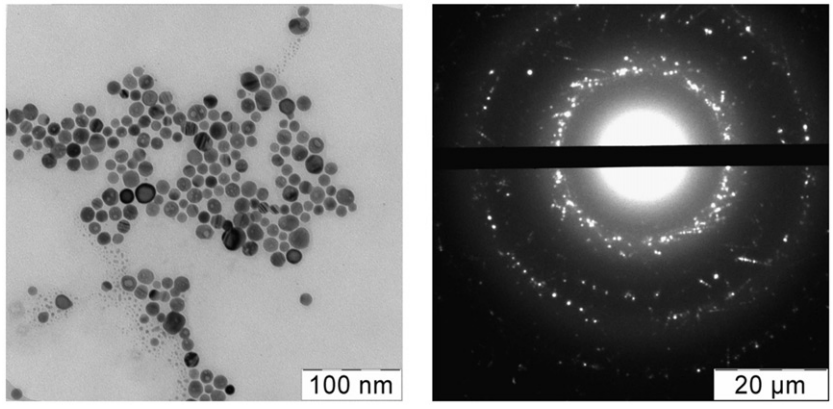


Figure 3. TEM picture of Ag NPs with polyhexamethylene biguanide hydrochloride and diffraction images.

their average size and size distribution, shape and structure. The elemental structure was determined using UV–spectroscopy and X-ray diffraction analysis ('dark field'). The size and shape of Ag NPs were determined by transmission electron microscopy (TEM).

Transmission electron microscopy

All the synthesized silver NPs were spherical shaped and they were single crystals. The diffraction images identified the pure Ag phase (Figures 2 and 3). Figures 2 and 3 depict representative TEM data for Ag_III (cr stabilizer/NPs - 1) and Ag_V samples.

Spectroscopy

NPs were tested in a diluted water solution and in test media (E3 standard (Detrich et al. 1999) – NaCl – 5 mmol; KCl – 0.17 mmol; $CaCl_2$ 0.33 mmol; $MgSO_4$ – 0.33 mmol; pH 7.2 – 7.3). For this purpose, solutions of NPs with a concentration of 10 mg/L in water and 5 mg/L in egg's water for *D. rerio* (EW) were prepared. The UV–Vis spectra of the samples of Ag NPs are shown in Figure 4.

The UV–Vis absorption spectra of the tested samples demonstrate characteristic narrow peaks of surface plasmon resonance of Ag NPs within the range 400–420 nm. Most of the Ag NPs appeared to display sufficient aggregative stability in an aqueous solution and in test media with the exception of Ag_II stabilized with benzyldimethyl[3-(myristoylamino)propyl]-ammonium chloride monohydrate. In the case of the Ag_II NPs, some signs of destabilization were observed such as sedimentation and change of color of Ag NPs dispersions.

DLS and zeta potential

Size characteristics obtained by DLS, NTA, and TEM are in good agreement. The data for Ag_V NPs stabilized by sodium tallowamphopolycarboxyglycinate are shown as an example in Figure 5.

Dynamic light scattering and NTA were performed to determine the hydrodynamic diameter of

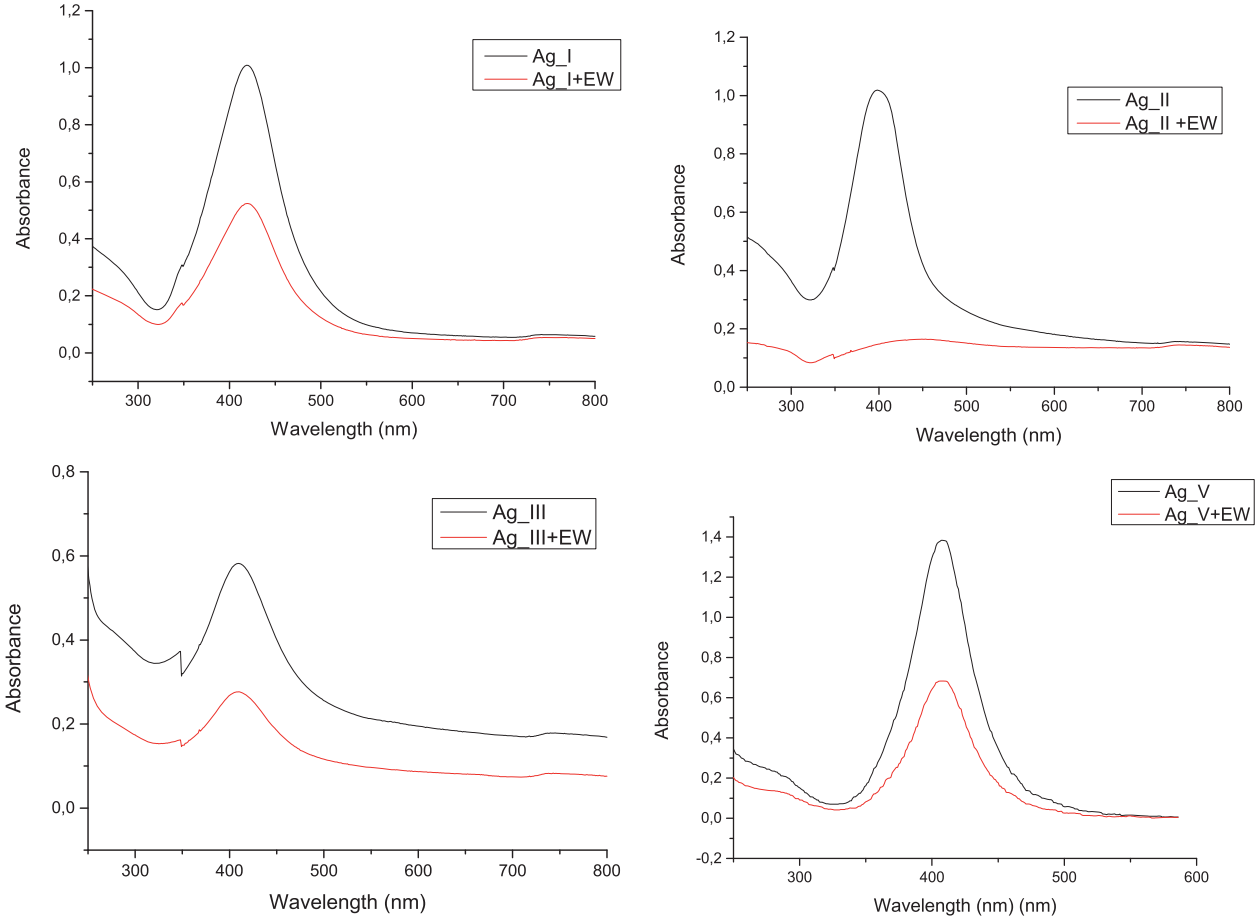


Figure 4. UV-Vis spectra of silver NPs diluted by water (10 mg/L) and test media (5 mg/L).

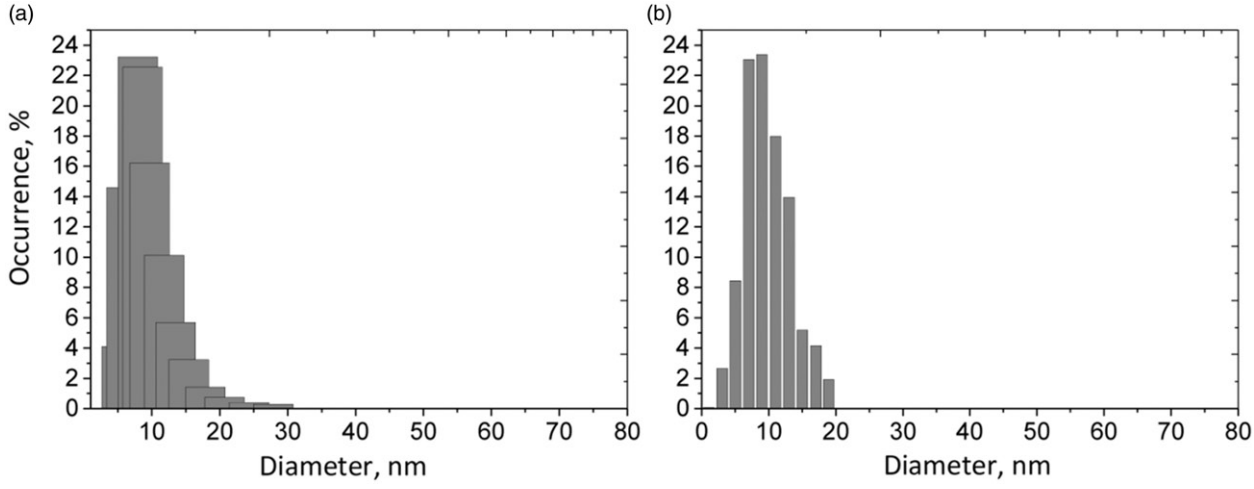


Figure 5. Size distribution of silver NPs (Ag_V) as determined by TEM (a) and DLS (b).

aggregated particles dispersed in water and in test media. Data on size distributions and zeta potentials are given in Table 3.

The zeta-potential depends on the nature of a stabilizing agent in terms of both the charge (negative or positive) and value. NPs with a zeta potential greater than +30 mV or less than −30 mV are considered electrostatically stable, while structural and mechanical barriers can play a certain role for polymers and surfactants. Thus, a cationic stabilizing agent induces a positive zeta-potential, the anionic surfactant results in a negative value, whereas

Table 3. Characteristics and stability of tested Ag NPs.

Nanoparticles	Zeta potential (mV)	Diameter of NPs (nm)	Stability of aqueous dispersion*	ζ-potential in test media (mV)	Diameter of NPs in test media (nm)	Stability of dispersions in test media**
Ag_I	−46.2	7.5	No	−48.9	22.3	Yes
Ag_II	+54.2	25.0	No	−3.1	124.4	No
Ag_III_cr_1	+39.1	7.0	Yes	+42.7	13.5	Yes
Ag_III_cr_0.2	+46.4	3.5	Yes	+6.8	65.3	No
Ag_IV_cr_12	−55.6	9.5	Yes	−50.3	13.8	Yes
Ag_IV_cr_5	−45.3	14.0	Yes	−35.9	13.8	Yes
Ag_V	−17.1	8.5	Yes	−23.8	26.3	Yes
Ag/AgCl _V	−69.2	31.9	Yes	−59.7	54.0	Yes

*concentration 10 mg/L; **concentration 5 mg/L.

amphoteric substances also induce a negative charge and the greatest magnitude of the zeta-potential in absolute terms.

The greater the changes of the zeta-potential for aqueous and test media dispersions, the greater the size of the NPs aggregates. In the test medium, Ag NPs usually demonstrate a higher extent of aggregation which is due to the ionic strength of the medium solution. As can be deduced from Figure 4, some Ag NPs samples (Ag_II and Ag_III with concentration ratio PHMB/Ag – 1) were present as aggregates in egg water. For the other samples, the differences in the size and zeta potential between water and test media were insignificant.

It is obvious that different mechanisms of stabilization take place and this provides a variation in stability and other different properties of NPs solutions in water and test media. The aggregation can change from fast to slow by varying the concentration of a salt or of another additive, as well as pH. According to the Schulze-Hardy rule, the critical coagulation concentration varies as the inverse sixth power of the counterion charge and depends on the type of the ion, even if the ions have the same charge. Thereby divalent cations are far more effective than monovalent cations. Similarly, for coagulation of positively charged Ag NPs, divalent SO_4^{2-} anions are more effective than monovalent Cl^- anions. For negatively charged silver NPs, multivalent cations represent highly effective coagulants.

In the case of multiply charged ions a charge exchange of NPs in the test media for zebrafish embryos is possible, i.e. reversion of the charge and the potential of the colloidal particle. The added ions can be exchanged with counter-ions, replacing them in both the diffuse and the adsorption layers. Therefore, at a sufficiently high concentration of such ions, the charge they induce on the surface can become larger in an absolute sense than the charge of the potential-determining ions. This means a change in the sign of the charge and potential. Now such ions become potential-determining (instead of the former ones) and other counter-ions are arranged around the particle.

As shown by DLS and spectroscopic data, most of the synthesized NPs are stable under the experimental conditions employed in this study. Exceptions were the samples Ag_I and Ag_II stabilized by sodium lauryl ether sulfate and sodium cocaminopropionate, as the color of the dispersions changed and sedimentation started to occur. We observed signs of instability and sedimentation for Ag_III NPs at a low concentration of the stabilizing agent (concentration ration stabilizer/NPs 0.2) during the experiment.

Toxicity of nanoparticles

The nature of a capping agent strongly affects the properties of NPs as well as the toxic behavior and stability in the test media. We obtained and tested NPs dispersions with the same stabilizer at different concentrations. Their effect on *D. rerio* embryos was evaluated. We also analyzed the toxicity of stabilizers themselves with respect to *D. rerio*. Table 4 summarizes the data for solutions of stabilizers and NP dispersions with the same concentration of capping agents.

Toxicity data for Ag NPs stabilized by sodium cocaminopropionate (Ag_IV) in different concentrations are shown in Figure 6. Apparently, the toxic effects of Ag_IV samples with a varying concentration of sodium cocaminopropionate are similar in spite of different concentrations of the latter. Incidentally, we observed 100% mortality of embryos in the experiment with the stabilizer for its

Table 4. Toxicity data for the stabilizers and NPs with the same concentrations.

Nanoparticles	Concentration of the stabilizer in the 1 mg/L dispersion of NPs (mg/L)	Tested concentration of the stabilizer (mg/L)	Mortality for the solution of the stabilizer (%)	Mortality data for NPs with the same concentration of the stabilizer (%)
Ag_I	10	10	100	100
Ag_II	2	2	98.5	67
Ag_III	1	2	100	100
Ag_III	0.2	0.2	12.5	10
Ag_IV	12	12	100	100
Ag_IV	5	5	3.3	58
Ag_V	2	2	62.5	10
Ag_V	2	8	100	100
Ag_xAgCl_V	2	2	62.5	98
Ag/AgCl_V	2	8	100	100

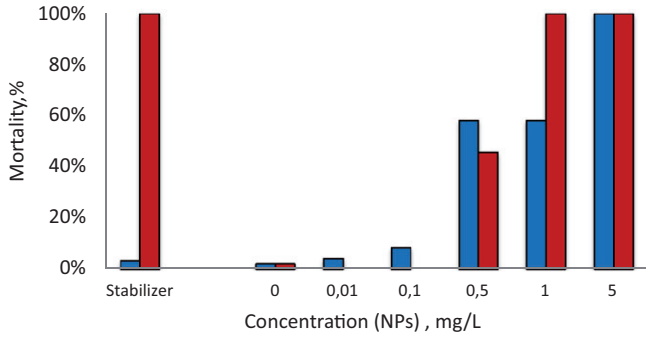


Figure 6. Toxicity data for silver NPs stabilized by sodium cocaminopropionate with concentration ratio 5 (■) and 12 (■) and for the pure stabilizer sodium cocaminopropionate.

concentrated solution (concentration ratio stabilizer/NPs 12) and 3% mortality for the solution with a lower concentration of the stabilizer (concentration ratio stabilizer/NPs 5). Evidently, this stabilizer does not demonstrate any significant toxic behavior with respect to fish embryos.

As shown in Figure 6, the response patterns for the two Ag_IV NPs dispersions are close. The calculated LC50 values for Ag_IV NPs were 0.580 and 0.515 mg/L, respectively, for dispersions with concentrations ratio of the stabilizer of 5 and 12. Thus, the conclusion can be drawn that for the dispersions of Ag_IV the toxic effect with respect to *D. rerio* embryos was determined predominantly by the silver NPs, regardless of the concentration of the stabilizer. Quite opposite results were observed for PHMB-stabilized Ag_III NPs (Figure 7), and it can be concluded that stabilizers considerably modify the toxic effects on *D. rerio* embryos.

When transforming the results into the units of the stabilizer concentration, the curves fall into one concentration range. Thus, it can be concluded that in the case of Ag_III solutions the presence of a stabilizer is a determining factor of toxicity with respect to fish embryos. The experimental data on the toxicity of the pure stabilizer at concentrations of 0.2 and 2 mg/L confirm this assumption (mortality –12.5 and 100% for 0.2 and 2 mg/L, respectively). Consequently, for solutions stabilized by substance III, it is the surfactant that mainly determines the toxicity of colloids.

It should be noted that for some colloidal dispersions, the results obtained were not as clear and unambiguous as in the case of dispersions with III and IV as stabilizers. For instance, data were obtained for silver NPs and Ag/AgCl nanocomposite stabilized by sodium tallowamphopolycarboxyglycinate (V). To determine the contribution of the stabilizer and NPs to the toxicity with respect to zebrafish embryos, the toxic behaviors of Ag_V and silver-based nanocomposite Ag/AgCl_V were analyzed. Figure 8 presents the mortality rates for NPs with sodium tallowamphopolycarboxyglycinate in comparable ranges of the concentration of NPs and stabilizer.

According to the obtained data, the toxic effect of NPs_V dispersions cannot be equivocally explained either by the presence of NPs or by the amount of the stabilizer in the solution. Additional toxicity tests were carried out for pure stabilizers. The mortality scores for zebrafish embryos were 62.5 and 100%, respectively, in 2 and 8 mg/L

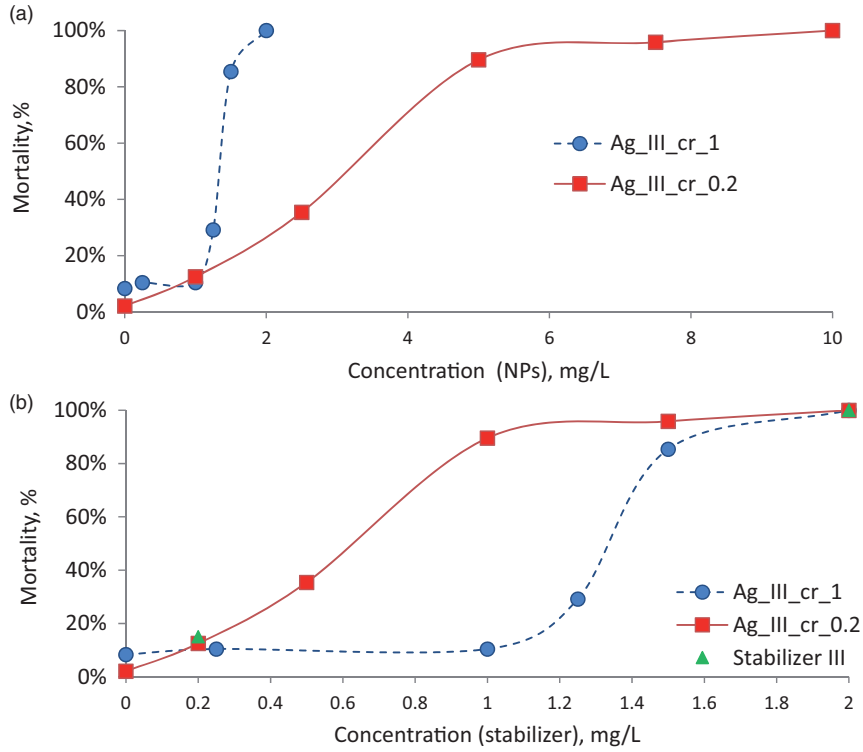


Figure 7. Mortality rate of zebrafish embryos after exposure to the Ag_III NPs dispersions stabilized by PHMB with the concentration ratio (cr) 1 (●) and 0.2 (■) of polyhexamethylene biguanide hydrochloride versus the silver concentration (a) and versus the concentration of the stabilizer (b).

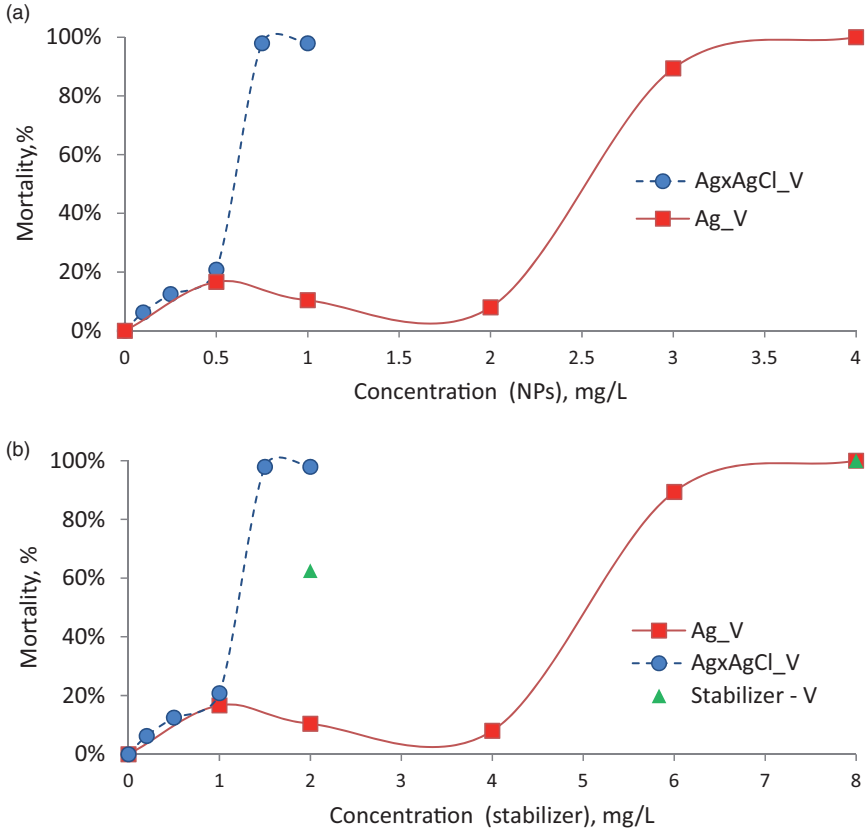


Figure 8. Mortality rate of zebrafish embryos after exposure to silver-based NPs dispersions (Ag_V and Ag/AgCl_V) with sodium tallowamphopolycarboxyglycinate versus the NPs concentration (a) and the concentration of the stabilizer (b).

solutions of sodium tallowamphopolycarboxyglycinate. For the high concentration, there is no disagreement with the experimental results for silver containing NPs, whereas for silver-based NPs with a stabilizer concentration of 2 mg/L the mortality rates of embryos differ from those of the pure stabilizer dispersion (Table 4). The toxicity of the stabilizer is reduced in the presence of NPs and this effect can be explained by the interaction of the stabilizer with silver NPs.

The experimental data for sodium tallowamphopolycarboxyglycinate stabilized NPs can be interpreted in terms of a quite complex mechanism of interaction of silver dispersions with embryos of *D. rerio*. It is obvious that a combined influence of NPs and stabilizer on the development of eggs takes place.

Despite the high toxic effect of some of stabilizing agents with respect to *D. rerio,* there is no reason to neglect the participation of NPs in the mechanism of toxicity of colloid dispersions. It is interesting to observe that NPs may decrease the toxicity of a stabilizer due to adsorption of the stabilizer by the surface of NPs. Such an effect was observed for Ag NPs stabilized by benzyldimethyl[3-(myristoylamino)propyl]ammonium chloride monohydrate and sodium tallowamphopolycarboxyglycinate (Ag_II and Ag_V, respectively). It should be noted that the toxic effect of NPs with different stabilizers is generally lower in comparison with the silver ions toxicity with respect to zebrafish embryos. The summary of the data on LC50 values and the 95% confidence intervals are given in Table 5.

Silver NPs stabilized by sodium cocaminopropionate (LC50 = 0.515 and LC50 = 0.580 mg/L for colloids with concentration ratio of stabilizer/NPs 5 and 12, respectively), and sodium lauryl ether sulfate (LC50 = 0.219 mg/L) were demonstrated to be the most toxic. Similar data were obtained for the silver nanocomposite Ag_xAgCl_V – LC50 = 0.563 mg/L. Ag_III NPs appeared to be less toxic: LC50 = 1.328 and LC50 = 2.917 mg/L for colloids with 0.2 and 1 with concentration ratio of polyhexamethylene biguanide hydrochloride/Ag.

The obtained results on the toxicity of silver NPs and their stabilizing agents allow us to assume the existence of a combined toxic effect of NPs and stabilizers on *D. rerio* embryos. We suggest that the following processes underlie the toxic effect observed depending on the nature of the stabilizer applied:

Table 5. LC50 values for Ag particle dispersions and $AgNO_3$ in zebrafish after 96 h of exposure.

Nanoparticles	LC50 (mg/L)	R^2	95% confidence interval
Ag_I	0.219	0.993	0.216–0.302
Ag_II	0.956	0.998	0.932–0.981
Ag_III_cr_1	1.328	0.973	1.248–1.414
Ag_III_cr_0.2	2.917	0.999	2.763–3.080
Ag_IV_cr_12	0.515	0.999	0.511–0.517
Ag_IV_cr_5	0.580	0.976	0.370–0.909
Ag/AgCl_V	0.563	0.993	0.492–0.644
Ag_V	2.488	0.961	2.028–3.052
$Ag^+(AgNO_3)$	0.049	0.959	0.045–0.051

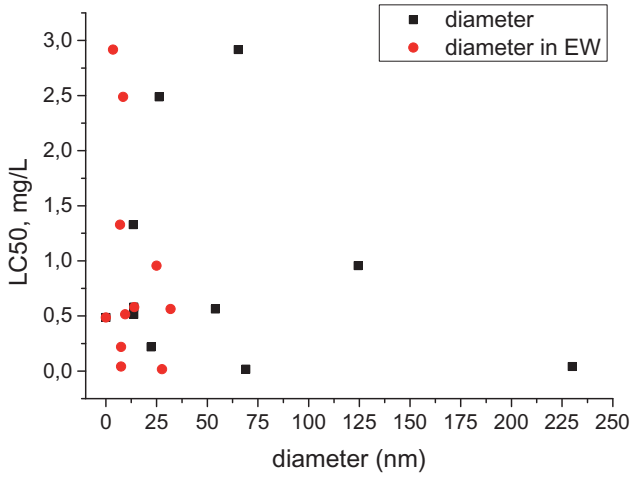


Figure 9. Correlation between LC50 and the initial size of NPs (■) and their diameter in egg's water (■).

(1) toxicity induced by silver NPs in a dispersion with a neutral stabilizer (Ag_IV); (2) stabilizer determines the toxic effect of the solution of NPs (Ag_III) and (3) combined interaction takes place (Ag_V).

All samples tested were found to be toxic to *D. rerio* embryos. The toxic effect of silver NPs is inferior to the toxicity of silver ions (LC50 for Ag^+ ($AgNO_3$) is equal to 0.049 mg/L). For all tested colloidal dispersions, there is a delay in the hatching rate for surviving animals. Typical malformations include: (1) a shortened body length, (2) induced tail malformation, and (3) pericardial edemas in zebrafish embryos.

We found no statistically significant correlation between the toxicity data and the average initial size of NPs or their size in the test media (egg's water). This is illustrated in Figure 9. Thus, the LC50 values do not demonstrate that Ag NPs toxicity is size-dependent. As noticed earlier, size alone cannot explain all of the specific toxic effects of NPs. The lack of correlation between NP toxicity and size might be explained by the complicity of the tested dispersions and strong effects of non-size related properties of NPs.

Conclusions

An important conclusion that can be drawn from this research is that the toxicity of silver NPs depends not only on their size characteristics. The obtained data allow one to conclude that the toxic effect of colloidal suspensions of silver NPs on zebrafish embryos depends mainly on the chemical composition of the NPs itself, the zeta potential, the chemical properties of the stabilizing agent, and the concentration of the stabilizing agent in the colloidal suspension.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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