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SILVER NANOPARTICLES: SUMMARY OF THE DOSSIER

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Series on the Safety of Manufactured Nanomaterials

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SILVER NANOPARTICLES: SUMMARY OF THE DOSSIER

IOMC
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Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris, 2017

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Nos. 44-54, These items are the dossiers derived from the Testing Programme on Manufactured Nanomaterials which are located at:
http://www.oecd.org/chemicalsafety/nanosafety/testing-programme-manufactured-nanomaterials.htm

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ABOUT THE OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 35 industrialised countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in eleven different series: **Testing and Assessment**; **Good Laboratory Practice and Compliance Monitoring; Pesticides; Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel . Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents;** and **Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (www.oecd.org/chemicalsafety/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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FOREWORD

The OECD Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (the Joint Meeting) held a Special Session on the Potential Implications of Manufactured Nanomaterials for Human Health and Environmental Safety in June 2005. This was the first opportunity for OECD member countries, together with observers and invited experts, to begin to identify human health and environmental safety related aspects of manufactured nanomaterials. The scope of this session was intended to address the chemicals sector.

As a follow-up, the OECD Workshop on the Safety of Manufactured Nanomaterials was organised (December 2005), in Washington, D.C. to determine the "state of the art" for the safety assessment of manufactured nanomaterials with a particular focus on identifying future needs for risk assessment within a regulatory context.

Following the conclusions and recommendations of the Workshop [ENV/JM/MONO(2006)19], the OECD Council established the OECD Working Party on Manufactured Nanomaterials (WPMN) as a subsidiary body of the OECD Chemicals Committee in September 2006. This programme concentrates on human health and environmental safety implications of manufactured nanomaterials (limited mainly to the chemicals sector), and aims to ensure that the approach to hazard, exposure and risk assessment is of a high, science-based, and internationally harmonised standard. It promotes international co-operation on the human health and environmental safety of manufactured nanomaterials, and involves the safety testing and risk assessment of manufactured nanomaterials.

This document is the summary of the dossier on silver nanoparticles prepared by Korea, as the lead of the silver nanoparticle project. The dossier on silver nanoparticles is publicly available as ENV/JM/MONO(2015)16 Part 1-6.

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1. PHYSICAL AND CHEMICAL PROPERTIES

1.1. Identification of the substance

CAS Number	:	7440-22-4
IUPAC Name	:	Silver
Molecular Formula	:	Ag
Structural Formula	:	Ag
Molecular Weight	:	107.87
Synonyms	:	Silver

The Ag nanomaterials used in this document are listed below. Reference Ag nanomaterial is Citrate-stabilized AgNPs and NM300 nano-silver.

Name	Remarks
cAgNPs	Citrate-stabilized AgNPs 10nm, -39mV
AgNPs	Silver powder, 6-55 nm
PVP-AgNPs	12 nm, -10mV
H2-AgNPs	13 nm, 22 mV
NanoComposix uncapped nanoscale silver	10, 20, 30, 50 nm
SARPU 200KW	
BPEI-AgNPs	10 nm, +40mV
NM300 silver	< 20nm

1.2. Purity/Impurities/Additives

No information is available.

1.3. Physical and chemical properties

1.3.1. Appearance/physical state/colour

The appearance of the nanomaterials was investigated in the study by transmission electron microscope (TEM) (Sung *et al.*, 2008). AgNPs were generated from silver wire by evaporation/condensation method by International Organization for Standardization (ISO) 10801. AgNPs in the inhalation chamber were sampled on filter which coated with carbon, mounted on an electron microscope grid(200 mesh, Veco, Eerbeek, the Netherlands), and visualized under a transmission electron microscope(TEM, Hitachi 7100, Japan). The diameters of 800 randomly selected particles were measured at a magnification of 100,000, and the silver particles were analyzed using an energy-dispersive x-ray analyzer(EDX-200, Horiba, Japan) at an accelerating voltage of 75kV. AgNPs were spherical in shape and none aggregated/ agglomerated forms with diameters under 55 nm. The diameters were log normally distributed from 6 to 55 nm and the CM4.5D and geometric standard deviation (GSD) were 18.84 nm and 1.45, respectively, with a good correspondence to the mobility diameters.

1.3.2. Particle size distribution(Granulometry)

Particle size distribution of the nanomaterials was investigated in the study by TEM(Klein, 2011). The experiments were conducted using NM300 nano-Silver < 20 nm reference nanomaterial. The material was a nano-Silver colloids dispersed with a nominal silver content of 10 w/w%. In dark at 4 ºC, the mean particle size of three distributions was determined to be >15 ~ <17 nm and D90 was be >3 ~ <20 nm for NM300

The particle size distribution (granulometry) of the nanomaterials was investigated in studies by ISO 10801/10808(Sung *et al.*, 2008). Geometric Mean particle sizes of the different aerosolized nanoparticles were measured by scanning mobility particle sizer (SMPS). The median diameter and GSD were 18.48 nm and 1.45, respectively.

1.3.3. Dissolution/Dispersibility

The dissolution/dispersibility study was conducted to determine the water solubility/dispersibility of AgNPs using electrophoretic light scattering (ELS) spectrophotometer and TEM. Hydrodynamic diameter (HDD, short-term, DI water with pH 2 and PBS) of cAgNPs in pH 2 steadily increased and reached above 1 μm in 60 min. In addition, the particle size of PVP-AgNPs was also slightly increased from 65 to 85 nm. This difference of two AgNPs is due to the additional stability mechanism: i.e., steric hindrance of PVP-AgNPs; electrostatic repulsion by carboxyl groups of citrate-AgNPs. No change of HDD in pH 7 was observed for both AgNPs. In pH 9 DW, AgNPs were negatively charged and caused the greatest electrostatic repulsion, thus providing excellent particle dispersion. In long-term test (24 hrs), citrate-AgNPs exposed in pH 2 and PBS media showed steadily increasing particle size, and finally it settled at the bottom of the beaker due to the formation of bulk particles. Due to the effect of protein corona in FBS, citrate-AgNPs maintained its slightly increased particle size (66 nm). This feature was also observed for PVP-AgNPs (75 nm). The light exposure of citrate-AgNPs leads to photo-oxidation of adsorbed citrate, destroying the strong citrate double-layer kinetic stabilization, which may subsequently change the size and shape of AgNPs (Redmond et al., 2007). UV-vis spectra of PVP-AgNPs were not changed, but those of citrate-AgNPs were greatly decreased for irradiation of 455 nm. While PVP-AgNPs maintained their initial size regardless of photo irradiation, citrate-AgNPs were markedly increased and reached 3.6μm. Citrate undergoes photochemical oxidation on AgNPs through the following reaction (Wu et al., 2008): citrate+1/2O_2→acetone-1,3-dicarboxylate+CO_2+OH^-. That is, Ag plasmon-induced photooxidation of adsorbed citrate injects electrons into the silver nanocrystal, and then these electrons accumulate on the Ag

particle, leading to particle growth (Xue et al., 2008). The charge transfer photooxidation is irreversible due to fast decarboxylation of oxidized citrate, which thus destroys the strong citrate double layer kinetic stabilization of the initial colloid (Redmond et al., 2007). Under the test conditions, PVP-AgNPs stabilized by steric hindrance have good dispersion stability in biological media, compared to citrate-AgNPs stabilized by electrostatic repulsion.

1.3.4. Aggregation/agglomeration

The agglomeration/aggregation state of silver nanoparticles (cAgNPs) was investigated using dynamic light scattering (DLS) spectrophotometer and TEM (NIER, 2010). The cAgNPs showed a distinct aggregation state in each dispersion medium. In water and MEM media, aggregate of cAgNPs was observed little due to electrostatic and electrosteric effects (steric hindrance combined with electrostatic repulsion). In distilled and deionized water phase, particle size of cAgNPs was virtually unchanged at low temperature, 4℃. At room temperature, particles grew to form aggregates and their size was slightly increased with time. In MEM containing 10% FBS, the size of cAgNPs was slightly increased in the initial condition due to the reaction between protein in MEM and citrate in cAgNPs resulting in protein corona. However, aggregation rate of cAgNPs in MEM was nearly zero, presumably because of the steric hindrance of protein corona. Salts in PBS solution induced aggregation of cAgNPs and their size was increased to several hundred nanometres. In this condition, the aggregation rate was about 2.8 - 3.4 x 10^{-23} m^3/s. In addition, aggregation and agglomeration were affected by pH of solution; at pH 2, sedimentation of cAgNPs was observed while they were stable in a high pH condition, such as pH 9, regardless of temperature. Under the test conditions, agglomeration and aggregation of cAgNPs were observed in various solutions such as water and MEM (little aggregation) and PBS solution (more aggregation).

The hydrodynamic diameter (HDD) of silver nanoparticles was examined as a function of pH, ionic strength, and background electrolyte (Badawy *et al*., 2010). The surface charging properties of the AgNPs were also examined under the same test conditions. The results indicated that electrosterically stabilized PVP-AgNPs may have a great potential for mobility and transport in the environment as compared to electrosterically stabilized AgNPs. The study also showed that the type of coating and the stabilization mechanism have a profound impact on the aggregation potential of AgNPs. Thus, the potential fate and transport of AgNPs are more closely associated with the chemistry of the capping agent. This information may provide an insight into the potential toxicity and mobility of AgNPs in natural and engineered environments.

1.3.5. Crystalline phase

The crystalline phase of silver nanoparticles (cAgNPs) was determined using TEM (NIER, 2011). 1% (wt %) of AgNPs solution was drooped to liquid Cu coated grid. Water was removed and the grid was dried for 24 hours in dust free place. To take TEM image, brightness and focus of the environment was controlled. When measuring AgNPs size, both high and low concentration resolution images were obtained for representability. The crystalline size was easily determined with TEM image analysis and was 7.00 ± 2.25 nm for a total of 433 nanoparticles. Although AgNPs showed a wide range of particle distribution, there were few or no particles over 10 nm in size. The crystalline size of cAgNPs determined with TEM showed uniform size.

The crystalline phase of silver nanoparticles (cAgNPs) was determined using X-ray diffraction method (XRD) (NIER, 2011). To analyze crystalline phase of cAgNPs, cAgNPs particles in solution were dried, on the assumption that crystalline phase of colloidal cAgNPs should not change during the drying process. XRD spectra showed typical silver metal peaks for(JCPDS#01-071-3762) Ag(111) and Ag(200) at around 38° and 45°, and Ag(220) and Ag(311) at 65° and 78°, respectively. To confirm the assumption of drying method, mixing method was performed as follows: Very small quantities of 100 ppm cAgNPs

solution were dropped on the amorphous silica powder, which showed a broad peak at 20° in XRD data. The positions of typical peaks in XRD spectra were consistent with those of drying method. Crystalline sizes were analyzed with the Scherrer equation. When full width at half maximum was measured for the NM111 peak, the primary particle size, namely, crystalline size was 18 nm. Although cAgNPs were aggregated during the drying and mixing process, its primary size was maintained. cAgNPs showed typical XRD peaks for metal silver (JCPDS #01-071-3762). The drying and the mixing process did not affect the XRD data. The crystalline size calculated by Scherrer equation was about 18 nm, which was maintained during aggregation.

1.3.6. Crystallite and grain size

A crystallite and grain size study was conducted according to OECD Guideline 110 to determine the crystalline size of silver nanoparticles (cAgNPs) by UV analysis and DLS analysis (NIER, 2011). In UV analysis, cAgNPs showed a characteristic peak at 400 nm. When particles were grown by surrounding environments such as pH, temperature or amount of salts, the characteristic peak in UV spectrum was shifted into red wave. In water at pH 2 and PBS, the UV peak was decreased and even disappeared by sedimentation. Due to the proportional relationship between the cAgNPs size and the agglomeration or aggregation state, cAgNPs size was quite naturally increased with aggregation state. Above pH 7, size and size distribution of cAgNPs were not changed with solution temperature in distilled and deionised water while particle sizes were decreased with solution temperature in MEM. Size distribution in water solution became broader at low temperatures than that at high temperatures. Also in MEM, it showed a weaker correlation with solution pH. Size and size distribution of cAgNPs could be measured by DLS and were strongly correlated with agglomeration/aggregation state. However, particle size in TEM analysis was not matched with that of in DLS analysis. Namely, TEM data showed the size of primary particle of AgNPs, while DLS results showed the secondary particle size. HDD in DLS analysis was several times larger than primary particle size in TEM analysis. Additionally, DLS usually requires high concentrations of NPs that likely result in some NP aggregation potentially leading to larger diameters. (Domingos et al. 2009).

1.3.7. Zeta potential

A zeta potential study was conducted to determine the surface charge of silver nanoparticles (cAgNPs) by measuring the zeta potential (NIER, 2011). cAgNPs were stabilized via electrostatic repulsion. Therefore, solution pH was a critical factor which determined the surface charge of cAgNPs. Raw solution of cAgNPs showed-60 mV of zeta potential, indicating a good stability which ranged in a good stability (zeta potential of colloids in water and waste water, ASTM Standard D 4187-82, American Society for Testing and Materials, 1985). When cAgNPs were dispersed in high pH solution (above pH 6), their zeta potential was slightly changed to -50 ~ -30 mV, which ranged in a moderate stability. When pH was decreased to 2, zeta potential approached neutral and the repulsive electrostatic force between cAgNPs particles was minimized. Therefore, zero potential zone (low pH, less than 3) was very unstable and aggregates were readily formed. Hydrodynamic sizes at low pH were also larger than those at high pH. Effect of dispersion medium on zeta potential was as follows: In water phase, the ratio of cAgNPs to Ag^+ ion was maintained at 9:1 for 5 days and their zeta potential was maintained at -60 mV, which exhibited a good stability. In PBS, cAgNPs were aggregated aggressively and therefore zeta potential was not measurable. Although cAgNPs in MEM showed zeta potential of -20 mV (incipient instability by ASTM D4187-82), they were very stable in MEM. Proteins in MEM compete with the nanoparticle surface to form a protein corona that largely defines the biological identity of the particle. This protein corona increased additional stability due to steric hindrance effect. Therefore, the protein on cAgNPs prevented nanoparticles from aggregating in MEM. Under the test conditions, pH and salts in solution were found to significantly affect cAgNPs stabilization via electrostatic repulsion. In low pH, zeta potential converged to zero. This suggests particle growth via aggregation/agglomeration

1.3.8. Additional physico-chemical information

A study was conducted to determine the grids storage container for silver nanoparticles in different medium, high pH, under irradiation with room light with different wavelengths using TEM and UV-visible spectroscopy in accordance with the OECD's sponsorship programme, ENV/JM/MONO(2009)20/REV (Roh *et al.*, 2010, Roh *et al.*, 2012). After 10 ppm of AgNPs was irradiated with normal light with different wavelengths, the peak changes of UV-vis spectra were observed. AgP in dark conditions had three extinction bands at ca. 350, 400, and 650 nm. On the other hand, AgS in UV-vis spectra showed a single characteristic peak at ca. 400 nm. Therefore, this peak at 400 nm revealed a particle size of AgP and AgS to be in the range of 37 ~ 40 nm. In comparison to dark conditions, the third peaks at 455 and 530 nm wavelengths were blue-shifted, while that for 630 nm wavelength was red-shifted. Even though AgS after light irradiation maintained its peak position, its absorbance was decreased. As the wavelength of light decreased, the absorbance peak also decreased. A decrease in the intensity of their SPR band indicated particle dissolution as well as a possible increase of Ag+ concentration in solution. AgP in DW was 40 nm in TEM with 46 nm of HDD, but after irradiation, it was 33 nm with 45 nm of HDD. In addition, its zeta potential decreased from -45 to -25 mV, which is a slightly more stable value. It is noteworthy that the dissolution of Ag+ from AgP seemed to decrease in size and photo-oxidation of citrate on the AgP surface caused a decrease in the surface charge. As a result, we can conclude that the photo-oxidation of AgP occurs toward lower the surface energies by truncating the edges and tip sides. However, in pH 9, the size and HDD of AgP were maintained at 39 nm and 46 nm without any change of zeta potential even though the particle was under light irradiation. This means that Ag+ dissolution and citrate oxidation were fully repressed by treatment of additional OH-. The diameters in TEM and HDD in DLS AgS in DW were 37 nm and 43 nm, respectively. After irradiation, the size of the primary particle was decreased to 33 nm, but its HDD was increased to 248 nm. While the size of individual AgS particles decreased, the secondary particle showed a collective behavior. Because the surface energy of AgS was evenly distributed on the spherical shape, Ag+ release was not sufficient to lower the surface energy during irradiation. Therefore, the lowering of surface energy via photo-oxidation of AgP occurred by the aggregation of particles. The zeta potential of AgS in DW was decreased after irradiation, but that in the pH 9 solution remained constant. In addition, the diameter and HDD of AgS in pH 9 were slightly changed from 34 and 42 to 33 and 46 nm, respectively. Therefore, we found that additional OH- acted as a buffer to repress the release of Ag+ and oxidation of citrate. From the results, it was recommended that AgNPs can be stored in transparent bottles under room light in high pH conditions (pH 9).

A study was conducted to confirm silver content in vials. Measurement of the silver content of the sample containing NM300 was done by Argentometry acc. to Volhard and GF-ASS using aqua regia. The dissolved silver content of the samples was determined using ultrafiltration membranes (Sartorius Vivaspin 500, MWCO 3000) in accordance with the SOP TUDr UMSICHT SOP for GF-ASS measurements in UMSICHT final report of the partner UFT Bremen (Köser et al., 2013). As a result, argentometry gave only 84.3 ± 2.5% silver content of the nominal silver concentration of 20 mg Ag/mL. Measuring the silver content of NM300K samples in water with nominal concentration on 20 Ag mg/L using GF-AAS gave 15.0 ± 3.0 mg Ag/L. Measuring the total silver content of NM300K samples in test media according to OECD TG 201, OECD TG 202, OECD TG221, DIN 38412-48 modified by Neumann-Hensel and Melbye (2006) with the nominal concentration of 10 mg Ag/L gave 7.49 ± 0.38 mg Ag/L. The dissolved silver content of NM300K samples in test media was determined by GF-ASS of the ultrafiltrated and then digested samples.

Rogers et al., 2012 investigated physical and chemical changes that occur during exposure of AgNPs to synthetic stomach fluid (SSF) system. Manufacturer reported size and zeta potential characteristics for the citrate-stabilized AgNPs (40 nm nominal diameter, Biopure, 1.0 mg/mL) which were obtained from Nanocomposix(San Diago, CA) were confirmed by DLS and TEM. The results reported here indicate a

multistep process occurs after the exposure of AgNPs to SSF. First, AgNPs rapidly aggregate possibly due to a change in zeta potential that occurs in acidic media. Second, Ag ions are released from particles by oxidation in acidic media. Third, in the presence of Cl-, AgCl is formed in proximity to particles and appears to sinter particles together. Hence, ingested AgNPs may be converted to a variety of aggregated and chemically modified particles in the stomach. Given the wide range of coating compounds that vary in chemical properties, surface charge, etc., the herein reported results may not be representative of AgNPs preparations in general. Bioavailability of Ag from these Ag-containing materials will also depend on the interactions between this mixture of Ag-containing species and the absorptive surfaces of the gastrointestinal tract.

The silver nanoparticles were generated from silver wire by evaporation/condensation method following ISO 10801. Silver nanoparticles in the inhalation chamber were sampled on to filter. The filters used were coated with carbon, mounted on an electron microscope grid, and visualized under a transmission electron microscope. The diameters of 800 randomly selected particles were measured at a magnification of 100,000, and the silver particles were analyzed using an energy-dispersive x-ray analyzer at an accelerating voltage of 75 kV. The silver nanoparticles observed by TEM were spherical in shape and none aggregated/agglomerated forms with diameters under 55 nm. The diameters were log normally distributed from 6 to 55 nm and the CMD and GSD were 18.48 nm and 1.45, respectively, with a good correspondence to the mobility diameters.

Table 1.1. Summary of basic physical and chemical properties

Endpoints	Method	Results	Reference
Melting/Freezing point	-	961.9 ℃	Carapella *et al.*, 1979
Boiling point	-	2,212 ℃ (1013.25 hPa)	Weast *et al.*, 1988
Density	-	ca. 10.43 ~ 10.49 g/cm^3 (20 ℃)	Carapella *et al.*, 1979; Butts *et al.*, 1967
Vapour pressure	-	1,013 hPa (1,031 ℃)	Carpella *et al*., 1979

2. GENERAL INFORMATION ON EXPOSURE

2.1. Environmental Fate and Pathways

2.1.1. Photodegradation

No information is available.

2.1.2. Stability in Water

The impact of stabilization mechanism on the aggregation kinetics of silver nanoparticles was tested by U.S. EPA according to EPA QA/QC guidance where standard USEPA or OECD harmonized guidelines with modifications were used. Hydrogen Reduced Silver Nanoparticles (H_2-AgNPs), Citrate coated Silver Nanoparticles (Citrate-AgNPs), Polyvinylpyrrolidone coated Silver Nanoparticles (PVP-AgNPs), and Branched Polyethyleneimine coated Silver Nanoparticles (BPEI-AgNPs) were used for the test. The four AgNPs were synthesised in house according to methods published in the literature with slight modification. The aggregation behavior of the electrostatically stabilized H_2-AgNPs and Citrate-AgNPs was in agreement with the classical Derjagun-Landau-Vewery-Overbeek (DLVO) theory. The H_2-AgNPs and Citrate-AgNPs exhibited both reaction-limited and diffusion-limited regimes. The H_2-AgNPs had critical coagulation concentrations (CCC) of 25, 30 and 3 mM in the presence of $NaNO_3$, NaCl and $Ca(NO_3)_2$ salts, respectively. The Citrate-AgNPs had CCC of 70, 70 and 5 mM in the presence of $NaNO_3$, NaCl and $Ca(NO_3)_2$ salts, respectively. The aggregation behavior of the PVP- AgNPs and BPEI-AgNPs was not in agreement with the classical DLVO theory. Regardless of the ionic strength and the background electrolyte type and valence, the PVP-AgNPs and BPEI-AgNPs did not aggregate and thus, no CCC was found for the PVP-AgNPs and BPEI-AgNPs under the experimental conditions of the study.

Table 2.1.2. Summary of stability in water

Test Material	Method	Results	Reference
H_2-AgNPs	Stabilization mechanism on the aggregation kinetics of silver nanoparticles EPA QA/QC guidance	H_2-AgNPs had critical coagulation concentrations (CCC) of 25, 30 and 3 mM in the presence of $NaNO_3$, NaCl and $Ca(NO_3)_2$ salts, respectively.	U.S. EPA, 2012
Citrate-AgNPs		Citrate-AgNPs had CCC of 70, 70 and 5 mM in the same conditions of H_2-AgNPs.	
PVP-AgNPs		PVP-AgNPs and BPEI-AgNPs did not aggregate and thus, no CCC was found for the PVP-AgNPs and BPEI-AgNPs under the experimental conditions of the study.	
BPEI-AgNPs			

2.1.3. Transport between Environmental Compartments

No information is available.

2.1.4. Biodegradation

A biodegradation test of silver in soil was conducted by using ancient coins to calculate a dissipation time (DT_{50}) of silver. As a consequence of the study, the coins survived with a surface layer of silver sulphide.

2.1.5. Abiotic Degradability and Fate

No information is available.

2.1.6. Bioaccumulation

A bioaccumulation test of silver was conducted using the blue mussel *Mytilus edulis* (Calabrese *et al*.,1984). The bioaccumulation of silver metal was unlikely to occur as it was insoluble in water.

2.1.7. Adsorption to Soil and Sediment

An adsorption and desorption test with cAgNPs (ABC Nanotech Co., Ltd) was conducted according to OECD Test Guideline 106 (NIER, 2010; NIER, 2011). cAgNPs suspension diluted with de-ionized water was used for adsorption and desorption test to soil and exposed to cAgNPs solution (concentrations of 1-83,000 mg/L) 3 mL and 1 g of dried soil for 24 hours at 25 ℃. After 24 hours, adsorption reaction reached the equilibrium state. Desorption test was performed as same with adsorption test, but de-ionized water was used instead of cAgNPs solution. As a result, quartz sand showed lower capacity compared to other test soils samples. Significantly high portion of cAgNPs were desorbed from the field soil samples. Low adsorptive and high desorptive properties of soil means high mobility of cAgNPs in soil environment whereas high adsorptive and low desorptive properties of soil means accumulation. No relationship between characteristics of soil and adsorption/desorption was observed.

A transformation of three commercial nanoparticles (Sigma-Aldrich), two silver and one zinc oxide, was tested by U.S. EPA according to EPA QA/QC guidance where standard USEPA or OECD harmonized guidelines with modifications were used (Schekel *et al*., 2010). One silver nanomaterial, named Ag Organic, was organically coated with a proprietary compound for dispersion in solvents and has a size of 100 nm. The other silver nanomaterial, named Ag Uncoated, had no coating and has a particle size of 148 nm. Kaolin was reacted with the individual nanomaterials for periods of 1 hour to 18 months. An initial solution concentration of 3 mg/L silver, 10 g/L kaolin, and a background electrolyte of 0.01 M $NaNO_3$ or NaCl were used. The pH was maintained at 6 throughout the experiment and the reaction vessels were sealed and continuously agitated in a 25 °C incubation environment. As a result, at pH 6, both the kaolin surface and the silver nanoparticles were negatively charged limiting direct interaction of the materials. However, silver chloride was identified with the metallic silver nanoparticles in sodium chloride suspensions and may be attributed to an in situ silver chloride surface coating.

A soil percolation test with two German soils, a Cambisol (RefeSol 01A) based on sand and a Luvisol (Holtensen) based on silt was conducted according to DIN 19528 (Hoppe *et al*., 2014, Hoppe *et al*., 2015). To test the remobilization of NM300K from soils, long term incubation tests were conducted. The tested soils were spiked NM300K and stored in the dark at room temperature (22.5 °C). Individual water content was adjusted weekly. The soils were percolated with different solutions (artificial rain water (AFR), DOC leachate from litter (DOC)) after 3 and 100 days of incubation. As a result, in RefeSol 01A, the recovery of

Ag total after percolation was 94% (SD = 12%, n = 9) in the DOC variant and 87% (SD = 7%, n = 9) in the AFR variant. In the Holtensen sample, the recovery of Ag total after percolation was 98.5% (SD = 5%, n = 9) in the DOC variant and 99.3% (SD = 6%, n = 9) in the AFR variant. The results of all recovery rates were consistent with the measured released Ag total content in leachates.

A batch test in soil was conducted according to a method developed by Utermann *et al*., 2005 which was similar to DIN 19528 (Hoppe *et al*., 2014, Hoppe *et al*., 2015). The stock dispersion (100 mg/L) was stored in the dark and diluted with dionized water for target contents of 0.2, 0.5, 2, 5, 10, and 20 mg/L. Tests were conducted under standard laboratory temperature (25 °C). All samples were taken from German farmland mostly located in Lower Saxony and used for the test. As a result, previous batch tests showed a high adsorption capacity for silver ions (Ag+). Adsorption capacity enhanced with increasing C content, clay content, and pH values. The retention of NM300K followed a linear distribution between retained and colloidal stable fraction. Hence, the slope of the linear distribution was defined as retention coefficient (Kr). The retention was low for 18 soils (Kr = 0.6–12.6 L/kg, group one) and high for six (Kr = 308–2,391 L/kg, group two) of the investigated soils.

An adsorption and desorption test with cAgNPs (ABC Nanotech Co., Ltd) was conducted using a batch equilibrium method according to OECD Test Guideline 106 (Park *et al*., 2012). cAgNPs diluted with de-ionized water was used for 24 hours at 25 ± 1 ℃. The partitioning of AgNPs to soil can be categorized in two types: adsorption-first and deposition-first process. The dispersion stability in each soil micro-environment is a key parameter that determines whether AgNPs will be adsorbed or deposited. In addition, considering that AgNPs will undergo decomposition to Ag+ ions in the end, it is noteworthy that the accumulation or mobility of AgNPs in soil environment will change over time due to the differences in sorption behavior between AgNPs and Ag+ ions. As a result, nearly all of the AgNPs were translocated to the sediment sample in the pH range of 2～12, and the aggregation, deposition and sedimentation of AgNPs were found to be the main reasons for the transport toward the soil phase.

Table 2.1.7. Summary of adsorption to soil and sediment

Test Material	Method	Results	Reference
cAgNPs (ABC Nanotech Co., Ltd)	3 mL of cAgNPs solution (1-83,000 mg/L) and 1 g of dried soil for 24 hrs OECD TG106	Quartz sand showed lower capacity of adsorption compared to other test soils samples and high portion of cAgNPs were desorbed from the field soil samples.	NIER, 2010; NIER, 2011
Ag Organic (100 nm), Ag Uncoated (148 nm), ZnO (Sigma-Aldrich)	3 mg/L of silver, 10 g/L of kaolin, and 0.01 M $NaNO_3$ or NaCl EPA QA/QC guidance	At pH 6, both the kaolin surface and the silver nanoparticles are negatively charged limiting direct interaction of the materials.	Schekel *et al*., 2010
Cambisol (RefeSol 01A), Luvisol (Holtensen)	Soils were spiked AgNM300K and stored in the dark at 22.5 °C DIN 19528	The recovery of Ag total in RefeSol 01 and Holtensen sample was 94% (87%) and 98.5% (99.3%) in the DOC (AFR) variant, respectively.	Hoppe *et al*., 2014, Hoppe *et al*., 2015
Soils (German farmland)	0.2, 0.5, 2, 5, 10, and 20 mg/L of test solution method developed by Utermann *et al*., 2005 (DIN 19528)	Adsorption capacity enhanced with increasing C content, clay content, and pH values. The retention is low for 18 soils and high for six of the investigated soils.	Hoppe *et al*., 2014, Hoppe *et al*., 2015
cAgNPs (ABC Nanotech Co., Ltd)	cAgNPs diluted with de-ionized water for 24 hrs OECD TG 106	In the pH range of 2~12, nearly all of the AgNPs were translocated to the sediment sample due to the aggregation, deposition and sedimentation of AgNPs.	Park *et al*., 2012

2.1.8. Additional Environmental Fate and Behavior Information

An activated sludge process and adsorption test with cAgNPs (ABC Nanotech Co., Ltd) were conducted according to OECD Test Guideline 303 and 106, respectively (NIER, 2010; NIER, 2011). An activated sludge used was obtained from Gimpo sewage treatment plant in Korea. cAgNPs suspension was diluted with de-ionized water and exposed to activated sludge with concentrations of 1 and 10 mg/L for 24 hours at 25 ℃.

Effect of exposure concentration and time on fate of cAgNPs in activated sludge process

Considerable amount of cAgNPs was not absorbed in the activated sludge when high concentration (10 mg/L) was treated compared to the low concentration (1 mg/L). 68% of treated cAgNPs was absorbed in the sludge at 10 mg/L concentration while 94% was absorbed at 1 mg/L concentration. In addition, the effluent concentration and % of cAgNPs were progressively decreased with time. The removal percent was increased from 8 hrs to 24 hrs exposure by ca. 5% and 22% in the case of 1 mg/L and 10 mg/L cAgNPs, respectively.

Effect of synthetic sewage on cAgNPs concentration in effluent

cAgNPs were exposed to activated sludge in the presence or absence of synthetic sewage, and the concentration in the effluents was compared. In the presence of synthetic sewage, more cAgNPs (ca. 3.7% and 24% of 1 and 10 mg/L cAgNP exposure, respectively) were adsorbed to the

activated sludge whereas the low concentration of cAgNPs was measured in the effluent without synthetic sewage (De-ionized water).

Effect of cAgNPs in Effluent water quality

The effluent COD, NH_4-N, NO_2-N, and NO_3-N concentrations of the activated sludge process were monitored after the cAgNP exposure, and the values were compared with control. There was a significant difference between the COD concentrations of control and cAgNP exposure, whereas nitrogen concentrations were not significantly affected by cAgNP exposure.

As a result, the fate of cAgNPs was affected by the exposure concentration, time and synthetic sewage, and the effluent COD was affected by cAgNPs exposure, whereas the nitrogen concentrations were not affected by the exposure.

The removal of engineered NPs (AgNPs, TiO_2NPs, and SiO_2NPs) by activated sludge was investigated in DI water (Park *et al*., 2013). The removal rate is expressed as the remaining concentration and the % removal when the initial concentration of the three NPs employed was 10 mg/L (in the case of AgNPs, the NPs concentration was 1 mg /L). As a result, considerable removal of all three NPs by the activated sludge was observed in a time-dependent manner. More than 90% of the three engineered NPs were removed within 24 h. In addition, the presence of both a synthetic wastewater component and EPS significantly enhanced the NP removal efficiency.

Cooper CF and Jolly WC (1969) and Ward *et al* (1979) observed silver uptake by *Lolium perenne L.* and *Trifolium repens L.* Silver uptake by the plants appeared to be directly related to the soil concentration. As a result, silver in leaves was derived from airborne deposition; silver in roots was derived from soil uptake.

Table 2.1.8. Summary of additional environmental fate and behavior information

Test Material	Method	Results	Reference
cAgNPs (ABC Nanotech Co., Ltd)	Activated sludge of 1 and 10 mg/L for 24 hrs OECD TG 303 OECD TG 106	The fate of cAgNPs was affected by the exposure concentration, time and synthetic sewage.	NIER, 2010; NIER, 2011
AgNPs TiO_2NPs SiO_2NPs	10 mg/L of AgNPs, Activated sludge of 1 and 10 mg/L for 24 hrs OECD TG 303 OECD TG 106	The removal of all three NPs by the activated sludge occurred in a time-dependent manner.	Park *et al*., 2013
Silver	silver uptake by *Lolium perenne L.* and *Trifolium repens L.*	Silver in leaves of the plants was derived from airborne deposition and silver in roots was derived from soil uptake.	Cooper CF and Jolly WC, 1969; Ward *et al*., 1979

2.1.9. Summary

2.1.9.1. Photodegradation

No information is available.

2.1.9.2. Stability in Water

The impact of stabilization mechanism on the aggregation kinetics of silver nanoparticles was conducted according to EPA QA/QC guidance. The H_2-AgNPs had critical coagulation concentrations (CCC) of 25, 30 and 3 mM in the presence of $NaNO_3$, NaCl and $Ca(NO_3)_2$ salts, respectively. The Citrate-AgNPs had CCC of 70, 70 and 5 mM in the same conditions of H_2-AgNPs. The PVP-AgNPs and BPEI-AgNPs did not aggregate and thus, no CCC was found for the PVP-AgNPs and BPEI-AgNPs under the experimental conditions of the study.

2.1.9.3. Transport between Environment and Compartments

No information is available.

2.1.9.4. Biodegradation

A biodegradation test of silver in soil was conducted by using ancient coins to calculate a dissipation time (DT_{50}) of silver and the coins survived with a surface layer of silver sulphide.

2.1.9.5. Abiotic Degradability and Fate

No information is available.

2.1.9.6. Bioaccumulation

The bioaccumulation of silver metal was unlikely to occur as it was insoluble in water.

2.1.9.7. Adsorption to Soil and Sediment

An adsorption and desorption test with cAgNPs was conducted according to OECD TG 106. 3 mL of cAgNPs solution (1-83,000 mg/L) and 1 g of dried soil were used for 24 hours. Quartz sand showed lower capacity of adsorption compared to other test soil samples and high portion of cAgNPs were desorbed from the field soil samples.

A transformation of Ag Organic (100 nm), Ag Uncoated (148 nm) and one zinc oxide was conducted according to EPA QA/QC guidance. 3 mg/L of silver, 10 g/L of kaolin, and 0.01 M $NaNO_3$ or NaCl were used. At pH 6, both the kaolin surface and the silver nanoparticles were negatively charged limiting direct interaction of the materials.

A soil percolation test with a Cambisol (RefeSol 01A) and a Luvisol (Holtensen) was conducted according to DIN 19528. The tested soils were spiked NM300K and stored in the dark at 22.5 °C. The recovery of Ag total in RefeSol 01 and Holtensen sample was 94% (87%) and 98.5% (99.3%) in the DOC (AFR) variant, respectively.

A batch test in soil taken from German farmland was conducted according to a method developed by Utermann *et al.*, 2005 similar to DIN 19528. The stock dispersion was diluted for target contents of 0.2,

0.5, 2, 5, 10, and 20 mg/L. Adsorption capacity enhanced with increasing C content, clay content, and pH values. The retention was low for 18 soils and high for six of the investigated soils.

An adsorption and desorption test with cAgNPs was conducted according to OECD Test Guideline 106. cAgNPs diluted with de-ionized water were used for 24 hours. In the pH range of 2～12, nearly all of the AgNPs were translocated to the sediment sample due to the aggregation, deposition and sedimentation of AgNPs.

2.1.9.8. Additional Environmental Fate and Behavior Information

An activated sludge process and adsorption test with cAgNPs was conducted according to OECD TG 303 and 106. The cAgNPs suspensions were exposed to activated sludge with concentrations of 1 and 10 mg/L for 24 hours. The fate of cAgNPs was affected by the exposure concentration, time and synthetic sewage.

The removal of all three NPs (AgNPs, TiO_2NPs, and SiO_2NPs) by the activated sludge was conducted according to OECD TG 303 and 106. 10 mg/L of AgNPs was used as an initial concentration and exposed to activated sludge with concentrations of 1 and 10 mg/L for 24 hours. The removal of all three NPs by the activated sludge occurred in a time-dependent manner.

A silver uptake by *Lolium perenne L.* and *Trifolium repens L* was tested. Silver in leaves of the plants was derived from airborne deposition and silver in roots was derived from soil uptake.

.HAZARDS TO THE ENVIRONMENT

3.1. Aquatic Toxicity

3.1.1. Acute Toxicity

Fish

An acute toxicity test of cAgNP (ABC Nanotech Co., Ltd) with *Cyprinus carpio* was conducted at 25, 50, 100 and 200 μg/L for 48 and 96 hours (24 °C, pH 7.2) according to OECD test guideline 203 (Abei, 1974; Bradford, 1976; Lee *et al*., 2012). cAgNP suspension was mixed with fresh water which was dechlorinated and continuously aerated. The homogeneity and stability in NP freshwater medium were confirmed through TEM measurements, high resolution nano scale microscopic measurement, and ionization measurement using ICP-AES analysis. As a result, enzymatic activities in the brain of the fish exposed to 200 μg/L of cAgNPs were significantly reduced, and varied antioxidant enzyme activity was recorded in the liver and gills. When the concentration of cAgNPs increased to 100 μg/L and 200 μg/L, GST activity was more significantly reduced in the gills than in the liver and brain. Similar to GST activity, CAT activity in the liver changed at different cAgNP concentrations, but it remained relatively stable in the brain and gills. SOD activity differed from that of GST and CAT in which it was relatively stable in all examined tissues. When observing other biochemical indices, there was no significant difference except for NH3 and blood urea nitrogen concentrations in fish exposed to 50 μg/L of cAgNPs.

An acute toxicity test of NM300K and silver ion (Merck) with *Danio rerio* was conducted five times under static conditions for 48 hours according to DIN 38415 T6 (Polleichtner *et al.*, 2013). Test media were prepared freshly and filtered using regenerated cellulose (0.45 μm) before the characterization experiments. A stock dispersion with 2 % silver content was prepared prior to the characterization experiments and ultrasonicated for 15 min. Test samples were then prepared according to UBA test protocols by placing 10 μL of NM300K (2%) in clean glass vials (20 mL) and then adding test medium up to the final sample volume of 20 mL. As a positive control, $AgNO_3$ was used and effects of silver ions were calculated based on the effects of $AgNO_3$. Data were available for five independent tests for NM300K and two independent tests for $AgNO_3$. The lowest EC_{50} value found within the 5 exposure experiments was 0.292 mg/L (NOEC: 0.1 mg/L) (experiment 5). EC_{50} values calculated for the exposure to Ag ions (based on results for $AgNO_3$) were 0.062 and 0.08 mg/L (NOECs: 0.051 mg/L).

Table 3.1. Summary of acute toxicity to fish

Test Material	Species	Method	Result	Reference
cAgNP (ABC Nanotech Co., Ltd)	*Cyprinus carpio*	25, 50, 100 and 200 μg/L, 48 and 96 hours OECD TG 203	Liver was most susceptible and the antioxidant enzyme system was mostly active in the liver. The activities of antioxidant enzymes (GST and CAT) fluctuated with different Ag-NP concentrations, whereas SOD activity remained stable.	Abei, 1974; Bradford, 1976; Lee *et al*., 2012

NM300K	*Danio rerio*	48 hours, static DIN 38415 T6	EC_{50}: 0.292~1.668 mg/L; NOEC: 0.1 mg/L	Polleichtner *et al*, 2013
Silver ion (Merck)			EC_{50}: 0.062~0.8 mg/L; NOEC: 0.051 mg/L	

Invertebrates

An acute toxicity test of cAgNP (ABC Nanotech Co., Ltd) with *Daphnia magna* was conducted at 0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009, and 0.01 mg/L under static conditions for 48 hours according to OECD test guideline 202 (Asghari *et al*., 2012). Test organisms used were neonates (< 24 h old) and exposed at 20 ± 2 ℃ of test temperature. Test solutions were renewed every 24 hours during exposure to avoid significant change in the concentration of AgNPs. EC_{10}, EC_{50}, and EC_{90} values in this acute toxicity test to *Daphnia magna* on mortality were 0.003 mg/L, 0.004 mg/L, and 0.005 mg/L, respectively.

An acute toxicity test of NM300K with *Daphnia magna* was conducted four times under static conditions for 48 hours according to OECD test guideline 202 (Polleichtner *et al*., 2013). As a positive control, $AgNO_3$ was used and the first experiment on NM300K was not valid so that experiments 2-4 were used to assess the acute toxicity on daphnids. EC_{50} and NOEC values to *Daphnia magna* on mortality ranged from 0.024 to 0.046 mg/L and were 0.03 mg/L, respectively. Experiment 4 was prolonged to 96 h of exposure and no significant change in effect concentrations was found.

An acute toxicity test of NM300 with three aquatic invertebrates was conducted at 50, 80, 128, 204.8 and 327.7 μg/L for *Daphnia magna*, 1.5, 3, 6, 12 and 24 μg/L for *Daphnia galeata*, and 1.56, 3.13, 6.25, 12.5, 25 and 50 μg/L for *Daphnia pulex* under static conditions for 48 hours according to OECD test guideline 202 (Völker *et al*., 2013). Test organisms used were neonates (< 24 h old) and exposed at 20 ± 1 ℃ of test temperature and pH 7~9. EC_{10} values of *Daphnia magna*, *Daphnia galeata* and *Daphnia pulex* on immobilization were 60.3 μg/L, 11 μg/L, and 4.37 μg/L, and EC_{50} values were 121 μg/L, 13.9 μg/L, and 8.95 μg/L, respectively.

An acute toxicity test of multiple sizes (10, 20, 30, and 50 nm) of nanosilver (nanoComposix) with *Daphnia magna* was conducted under static conditions for 48 hours according to OECD TG 202 and 203 for the first two tests and a modified OECD test guideline 204 for third test (Hoheisel *et al*., 2012). Test solutions were renewed every 24 hours during exposure. LC_{50} of multiple sizes of commercially prepared nanosilver ranged from 4.31 to 30.36 mg total Ag/L with increasing toxicity associated with decreasing particle size. A strong relationship between estimated specific particle surface area and acute toxicity was observed. Nanosilver suspensions (10 nm) treated with cation exchange resin to reduce the concentration of Ag associated with it were approximately equally toxic to *D. magna* compared to untreated nanosilver (48 h LC_{50} was 2.15 and 2.79 mg total Ag/L, respectively).

Table 3.2. Summary of acute toxicity to invertebrates

Test Material	Species	Method	Result	Reference
cAgNP (ABC Nanotech Co., Ltd)	*Daphnia magna*	0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009, and 0.01 mg/L, 48 h, static OECD TG 202	EC_{10}: 0.003 mg/L (95% CL: 0.003-0.003 mg/L); EC_{50}: 0.004 mg/L (95% CL: 0.004-0.004 mg/L); EC_{90}: 0.005 mg/L (95% CL: 0.005-0.005 mg/L)	Asghari *et al*., 2012
NM300K	*Daphnia magna*	48 h, static OECD TG 202	EC50: 0.024~0.046 mg/L; NOEC: 0.03 mg/L	Christian, 2013
NM300	*Daphnia magna*	50, 80, 128, 204.8 and 327.7 μg/L 48 h, static OECD TG 202	EC_{10}: 60.3 μg/L (95% CL: 39.9-91.0 μg/L); EC_{50}: 121 μg/L (95% CL: 101-146 μg/L)	Völker et al., 2013
	Daphnia galeata	1.5, 3, 6, 12 and 24 μg/L 48 h, static OECD TG 202	EC_{10}: 11 μg/L (95% CL: 8.47-14.3 μg/L); EC_{50}: 13.9 μg/L (95% CL: 9.13-21.4 μg/L)	
	Daphnia pulex	1.56, 3.13, 6.25, 12.5, 25 and 50 μg/L, 48 h, static OECD TG 202	EC_{10}: 4.37 μg/L (95% CL: 3.07-6.22 μg/L); EC_{50}: 8.95 μg/L (95% CL: 7.54-10.6 μg/L)	
Nanosilver (10, 20, 30, and 50 nm) (nanoComposix)	*Daphnia magna*	48 h, static OECD TG 202 OECD TG 203 OECD TG 204 (modified)	LC_{50}: 4.31~30.36 mg total Ag/L, Increasing toxicity associated with decreasing particle size	Hoheisel *et al*., 2012

3.1.2. Chronic Toxicity

Fish

A chronic toxicity test of silver powder (bare AgNPs, Sigma-Aldrich) was conducted with *Oryzias latipes* under semi-static conditions at 25 ℃ of test temperature according to OECD TG 210, 212 and OECD draft proposal for a new guideline (2006) of Fish embryo toxicity test (Adams *et al*., 2006; Powers *et al*., 2006; Fanklin *et al*., 2007; Bae, 2012). Fish embryos were exposed to the concentrations of AgNPs (50, 100 and 150 mg/L) and Ag^+ ion ratios (2, 9, 5, 14, 23, 40, 52, 76 and 100 %) at 25 μg/L of AgNPs. For heart beat test (3, 5 and 7 days), morphological changes with a digital camera were observed at 48, 72 and 120 h. 96-well microtiter plate was used as a test vessel and twelve individual embryos were treated per exposure concentration with three independent replicates of a microtiter plate. Test solution was changed every 48 hours and eggs were exposed to an AgNPs suspension in a 96-well microtiter plate. The percentage of surviving eggs was calculated using Abbott's formula, which was corrected for the mortality

in the controls. The toxicity of AgNPs did not increase linearly with ion ratio, but reached a maximum at an ion ratio of 23%. Otherwise the agglomeration rate reached a maximum at an Ag^+ ion ratio of 50%. AgNP suspensions were detected to accelerate agglomeration in an aqueous phase. Both the agglomeration rate and toxicity of AgNPs solution were related to the Ag^+ ionic ratio in the solution. Ag^+ ions not only increase the toxicity, but also accelerate suspension instability of AgNPs. The AgNPs were able to penetrate through the chorion and distributed widely in the body structures of hatched *O. latipes*.

A chronic toxicity test of cAgNP (ABC Nanotech Co., Ltd) was conducted with *Oryzias latipes* for 14 days at 25 ℃ of test temperature according to OECD TG 212 (Tollefsen *et al*., 2002; Boudreau *et al*., 2005 and 2004; Osaki *et al*., 2006; Cho *et al*., 2013). Working solutions (0.1, 0.25, 0.5, 0.75 and 1.0 mg/L of cAgNP) were obtained by diluting the stock solution with respective amounts of DI water. Thirty fertilized eggs were randomly divided equally into a 6-well polystyrene plate (i.e., 10 eggs per well) containing 3 mL of AgNP solution for each treatment. Test solutions were renewed every second day. Survival, phenotypic deformities, and hatchability were monitored daily with microscopy (Stemi SV11; Zeiss Co., Germany) until day 14. Embryos that did not hatch within 14 days were defined as dead and hatchability was calculated from the sum of dead and unhatched individuals. Larvae were reported as dead if the heart stopped beating and/or did not respond when gently touched. The LC_{50} of the embryo test after 24 to 96 h exposure decreased from 1.46 mg/L (CL: 95% 0.81-2.10) to 0.84 mg/L (95% CL: 0.67-1.00). In comparison, the 24 h LC_{50} value in the acute adult toxicity test was not calculated (> 1 mg/L), with no change in LC_{50} over the 48 h exposure period (0.8 mg/L; 95% CL: 0.68-0.96).

A chronic toxicity test of NM300K was conducted with *Danio rerio* for 35 days at 25.0-27.3 ℃ of test temperature and pH 8.2-8.7 according to OECD TG 210 (Schaefers and Weil, 2013). Nanoparticulate silver was investigated in two fish early life stage toxicity tests in a large static system (250 L), in which the test suspension was permanently mixed by pumps (WP 300, Tetra GmbH, Melle, Germany). The nominal test concentrations were 12.5, 25, 50, 100, and 200 µg Ag/L water in the first test and 12.5, 50, and 100 µg Ag/L water in the second test. The test vessels were filled with test water and temperated before beginning of exposure. 2 mL containing bottles of NM300K were filled up to 10 mL with aqua distilled water, ultra-sonificated for 15 min and directly applied to the test vessels. The test organisms were introduced into the test vessels on the same day. In the first test, one aquarium per treatment was used, containing four fish cages (pseudo-replicates). In the second test, two aquaria per treatment contained six fish cages each. Hatch was not affected up to 136 µg/L (mean measured). Post-hatch survival was significantly reduced at concentrations ≥ 47 µg/L, and the NOEC was determined to be 23 µg/L. The most sensitive endpoint was growth, measured as total individual length and wet weight with a NOEC of 5.9 µg/L. The test setup was demonstrated to be suited for the testing of nanomaterials, proven by sensitive results and high statistical power.

Table 3.3. Summary of chronic toxicity to fish

Test Material	Species	Method	Result	Reference
Silver powder (Bare AgNPs) (Sigma-Aldrich)	*Oryzias latipes*	48, 72, and 120 h, semi-static OECD TG 210 OECD TG 212 OECD draft proposal for a new guideline (2006) of Fish embryo toxicity test	The maximum toxicity of AgNPs and agglomeration rate reached at an Ag^+ ion ratio of 23% and 50%, respectively.	Adams *et al*., 2006; Powers *et al*., 2006; Fanklin *et al*., 2007; Bae, 2012
cAgNP (ABC Nanotech Co., Ltd)	*Oryzias latipes*	0.1, 0.25, 0.5, 0.75 and 1.0 mg/L, 14 days OECD TG 212	LC_{50} of the embryo test after 24 to 96 h exposure decreased from 1.46 mg/L to 0.84 mg/L. LC_{50} of the acute adult toxicity test after 24 h was not calculated (> 1 mg/L), with no change in LC_{50} (0.8 mg/L) over the 48 h exposure period.	Tollefsen *et al*., 2002; Boudreau *et al*., 2005 and 2004; Osaki *et al*., 2006; Cho *et al*., 2013
NM300K	*Danio rerio*	35 days OECD TG 210	NOEC: 136 mg/L (number hatched); NOEC: 23 mg/L (post-hatch success); Growth: NOEC: 5.9 mg/L (length); NOEC: 5.9 mg/L (weight)	Schaefers and Weil, 2013

Invertebrates

A chronic toxicity test of NM300K with *Daphnia magna* was conducted at 10, 20, 51, 65, and 216.8 μg/L under semi-static conditions for 21 days according to OECD test guideline 211 (Polleichtner *et al*., 2013). EC_{10}, NOEC, and LOEC values in this chronic toxicity test to *Daphnia magna* on reproduction were 0.6 μg/L, 0.2 μg/L, and 0.5 μg/L, respectively. In addition, EC10, NOEC, and LOEC values on mobility were 2.6 μg/L, ≥ 16.8 μg/L and > 16.8 μg/L, and NOEC and LOEC values on age at first reproduction were ≥ 16.8 μg/L and > 16.8 μg/L, respectively.

A chronic toxicity test of NM300 with three aquatic invertebrates was conducted at 2.5, 5 and 10 μg/L for *Daphnia magna*, 1.25, 2.5, 5 and 10 μg/L for *Daphnia galeata*, and 1.25, 2.5 and 5 μg/L for *Daphnia pulex* for 21 days according to OECD test guideline 211 (Völker *et al*, 2013). Test organisms used were neonates (< 24 h old) and exposed at 19~21 ℃ of test temperature and pH 7~9. EC_{10} values of *Daphnia magna* and *Daphnia galeata* on reproduction were 0.92 μg/L and 3.45 μg/L, respectively and there was no effect on reproduction in parental generation on *Daphnia pulex*.

Table 3.4. Summary of chronic toxicity to invertebrates

Test Material	Species	Method	Result	Reference
NM300K	*Daphnia magna*	10, 20, 51, 65, and 216.8 µg/L, 21 days, semi-static OECD TG 211	Reproduction: EC_{10}: 0.6 µg/L (95% CL: 0-2 µg/L); NOEC: 0.2 µg/L; LOEC: 0.5 µg/L	Polleichtner *et al.*, 2013
			Mobility: EC_{10}: 2.6 µg/L; NOEC ≥ 16.8 µg/L; LOEC > 16.8 µg/L	
			Age at first reproduction: NOEC ≥ 16.8 µg/L; LOEC > 16.8 µg/L	
NM300	*Daphnia magna*	2.5, 5 and 10 µg/L, 21 days OECD TG 211	EC_{10}: 0.92 µg/L (95% CL: 0.33-2.53 µg/L)	Völker *et al*, 2013
	Daphnia galeata	1.25, 2.5, 5 and 10 µg/L, 21 days OECD TG 211	EC_{10}: 3.45 µg/L (95% CL: 1.07-11.2 µg/L)	
	Daphnia pulex	1.25, 2.5 and 5 µg/L, 21 days OECD TG 211	No effect on reproduction	

Algae and cyanobacteria

A growth inhibition test of NM300K with *Desmodesmus subspicatus* was conducted four times under static conditions for 72 hours according to OECD TG 201 (Polleichtner *et al.*, 2013). Determined Endpoints were growth rate and yield. Effects were recorded upon 72 h of exposure and some experiment effects were also recorded upon 24 h and 48 h of exposure. No significant differences based on exposure concentrations were found for the different recording times. EC_{50} values on growth for NM300K within the 4 experiments exposed at 0.005, 0.007, 0.010, 0.025, 0.050, 0.075, 0.100 and 0.250 mg/L varied from 0.057 to 0.1 mg/L (NOECs 0.01 - 0.048 mg/L). Calculated EC_{50} values for Ag ions were one order of magnitude below the EC_{50} values of NM300K. No ECx values were derived for the dispersant.

A growth inhibition test of NM300K with *Anabaena flos-aquae* was conducted two times under static conditions for 72 hours according to OECD TG 201 (Polleichtner *et al.*, 2013). Nominal concentrations were 0.002, 0.004, 0.02, 0.1 and 0.4 mg/L in test 1 and 0.0005, 0.0013, 0.0078, 0.0195 and 0.0488 mg/L in test 2. Determined Endpoints were yield, biomass and section-by-section growth rate. EC_{10}, EC_{20}, EC_{50}, LOEC and NOEC were calculated and the data were obtained for NM300K in 2 experiments and $AgNO_3$ in 1 experiment. Effect concentrations of Ag ions were determined based on calculations from experiment with $AgNO_3$. As a result, EC_{50} values for NM300K were 0.005 and 0.0011 mg/L (yield) and 0.007 and 0.0017 mg/L (growth rate), and NOECs were 0.004 mg/L (yield) and 0.004 mg/L (growth rate) (exp. 1). NOECs could not be determined in exp. 2. EC_{50} values for Ag ions were 0.000024 mg/L (yield) and 0.000027 mg/L (growth rate). Analysis of the silver content was performed at test start and test termination. For calculation of EC values based on calculated concentrations, the geometrical mean of silver concentration originating from test start and test termination was taken to reveal EC_{50} values for NM300K of 0.00078 mg/L (yield) and 0.00108 mg/L (growth rate), and NOECs could not be determined in experiment 2.

A growth inhibition test of NM300K with *Pseudokirchnerella subcapitata* (Algensammlung Pringsheim) was conducted at 1-10,000 µg Ag/L (nominal), 21 °C and pH 8.1 under static conditions for 72 hours according to ISO 8692 (Engelke, 2012). The green algae were exposed to 7 concentrations with control and 4 independent test runs (2 replicates/run). Percent biomass/growth rate inhibition per concentration in control response was satisfactory. The doubling time of the biomass parameter did not always fulfill the validity criterion according to DIN 8692, but all tests fulfilled the validity criterion of the coefficient of variation < 5%. EC_{50} values on growth varied from 423 to 1,167 µg/L.

Table 3.5. Summary of toxicity to algae and cyanobacteria

Test Material	Species	Method	Result	Reference
NM 300K	*Desmodesmus subspicatus*	0.005, 0.007, 0.010, 0.025, 0.050, 0.075, 0.100 and 0.250 mg/L, 72 hours, static OECD TG 201	Growth rate: EC_{50}: 0.057~0.1 mg/L; NOEC: 0.001~0.048 mg/L	Polleichtner *et al.*, 2013
NM 300K	*Anabaena flos-aquae*	0.0005, 0.0013, 0.0078, 0.0195 and 0.0488 mg/L, 72 hours, static OECD TG 201	EC_{50}: 0.005~0.0011 mg/L (yield); 0.007~0.0017 mg/L (growth rate) NOEC: 0.004 mg/L (yield); 0.004 mg/L (growth rate)	Polleichtner *et al.*, 2013
Silver ion ($AgNO_3$)		0.002, 0.004, 0.02, 0.1 and 0.4 mg/L, 72 hours, static OECD TG 201	EC_{50}: 0.000024 mg/L (yield); 0.000027 mg/L (growth rate)	
NM 300K	*Pseudokirchnerella subcapitata*	1-10,000 µg/L, 72 hours ISO 8692	Growth rate: EC_{50}: 423~1,167 µg/L	Engelke, 2012

Aquatic Plants

A growth inhibition test of NM300K with *Lemna minor* was conducted under static conditions for 7 days according to OECD test guideline 221 (Polleichtner *et al.*, 2013). 3 independent tests for NM300K, 1 test for NM300K DIS and independent tests for $AgNO_3$ were performed. Results of effects of silver ions were calculated based on the effects of $AgNO_3$. The species were exposed to 0.001-7.8 mg/L (experiment 1) or 0.006-7.8 mg/L (experiment 2 and 3) of NM300K and 0.001-0.78 mg/L of $AgNO_3$. Endpoints based on frond number as well as biomass were determined as yield, mean growth rate and section-by-section growth rate. EC_{10}, EC_{20}, EC_{50}, LOEC and NOEC values were determined. EC_{50} values for growth rate (frond no.) upon exposure to NM300K were from 2.372 to 3.052 mg/L, while calculated EC_{50} value for silver ions upon exposure to silver nitrate was 0.164 mg/L.

A growth inhibition test of NM300K with *Lemna minor* was conducted at 300-10,000 μg Ag/L (nominal), 25 °C and pH ≤ 5.5 under static conditions for 7 days according to OECD test guideline 221 (modified EN ISO 20079) (Engelke, 2012). 3 independent tests (3 replicates/run) with 6 concentration runs and 6-well polystyrene plates were used as exposure vessel due to the advantage of requiring lower volumes for the test (10 ml). As a result, EC_{50} values on frond number varied from 744 to 1,697 μg/L.

A growth inhibition test of NM300K with *Myriophyllum spicatum* was conducted at 23 ± 2 °C under static conditions for 14 days according to Maletzki *et al*., 2010 using the Andrews medium (Polleichtner *et al.*, 2013). 2 experiments with NM300K and 1 experiment with $AgNO_3$ were performed. Effect concentrations of Ag ions were calculated based on the results upon exposure to $AgNO_3$. The species were exposed to 0.001, 0.01, 0.1, 1 and 10 mg/L (experiment 1) and 0.01, 0.02, 0.1, 0.25, 1 and 2.5 mg/L (experiment 2) of NM300K as nominal concentrations. As a result, EC_{50} values on main shoot length in both tests were 1.2122 mg/L and 4.8712 mg/L, respectively.

Table 3.6. Summary of toxicity to aquatic plants

Test Material	Species	Method	Result	Reference
NM300K	*Lemna minor*	0.001-7.8 mg/L (exp. 1), 0.006-7.8 mg/L (exp. 2 and 3), 7 days, static OECD TG 221	Frond number: EC_{50}: 2.372~3.052 mg/L	Polleichtner *et al.*, 2013
Silver ion ($AgNO_3$)		0.001-0.78 mg/L, 7 days, static OECD TG 221	Frond number: EC_{50}: 0.164 mg/L	
NM300K	*Lemna minor*	300-10,000 μg/L 7 days, static OECD TG 221 (modified EN ISO 20079)	Frond number: EC_{50}: 744~1,697 μg/L	Engelke, 2012
NM300K	*Myriophyllum spicatum*	0.001, 0.01, 0.1, 1 and 10 mg/L (exp. 1), 0.01, 0.02, 0.1, 0.25, 1 and 2.5 mg/L (exp. 2), 14 days, static Method according to Maletzki *et al*., 2010	Main shoot length: EC_{50}: 1.2122 mg/L (exp. 1); EC_{50}: 4.8712 mg/L (exp. 2)	Polleichtner *et al.*, 2013

Microorganisms

A SOS chromo test of cAgNPs (ABC NANOTECH Co., LTD) with *Escherichia coli PQ37* was conducted at 0, 0.03, 0.32, 3.23, 16.13 and 32.26 mg/L of cAgNP and 0, 0.03, 0.32, 3.23, 16.13 and 32.26 mg/L of Ag+ for 2 hours (Ames *et al*., 1975; Hofnung, 1982; Huisman *et al*., 1984; Quillardet and Hofnung, 1985; Quillardet *et al*., 1985; Dimpfl and Echols, 1989; Quillardet and Hofnung, 1993; Ishioka *et al*., 1997; Lantzsch and Gebel, 1997; Anderson and Kowalczykowski, 1998). The SOS chromo test was performed to assess the genotoxicity of cAgNPs with 4NQO as positive control. To confirm the characterization, homogeneity and (short term) stability of prepared test items, DLS size measurement, Zeta potential measurement and SEM measurements were used for estimating the size and aggregation level of NP suspension in test media. The SOS response was induced in *E. coli PQ37* exposed to 4NQO as β-galactosidase activity increases. However the activities of β-galactosidase and alkaline phosphatase of *E. coli PQ37* exposed to cAgNPs and Ag+ were nearly maintained. As a result, cAgNPs and Ag+ was not genotoxic based on the result of the SOS chromo test.

An antimicrobial activity and biofilm inactivation test of cAgNPs (ABC NANOTECH Co., LTD) with *Pseudomonas aeruginosa PA01* was conducted at 10 mg/L of AgNPs, and 1 and 10 mg/L of AgNO3 (Ag ions) for 150 minutes (25 °C, 100 rpm) (Lee *et al*., 1998; Hong *et al*., 2008; Park *et al*., 2013). To confirm the characterization, homogeneity and (short term) stability of prepared test items, DLS size measurement, Zeta potential measurement and SEM measurements were used for estimating the size and aggregation level of NP suspension in test media. Silver ion showed a higher antimicrobial activity than cAgNPs. cAgNPs at 10 mg/L showed a similar antimicrobial activity to a 1.0 mg/L solution of silver ions, indicating a 10 x greater antimicrobial activity of the ions compared to the nanoparticles under the test conditions used. Silver ion also demonstrated a higher biofilm inactivation rather than cAgNPs. The Ag ions induced over a 2 log inactivation after 150 min, whereas a similar exposure to AgNPs only resulted in a 0.3 log inactivation of biofilm cells. As a result, Silver ion had a higher antimicrobial activity and biofilm inactivation than cAgNPs.

A survival rate test of AgNPs (Sigma-Aldrich) with *Escherichia coli* was conducted at 0.4 and 0.8 mg/L and 25 °C according to EPA (2007) Nanotechnology White Paper (Ministry of Environment, 2010). Bare AgNPs suspensions were prepared using physical methods, i.e., sonication, stirring, and nanofilteration from commercial Ag nanopowder and AgNPs were stirred for 1 day. Polycationic materials were used extensively to disrupt cell membranes and transport materials into cells. The results showed that when the AgNPs were coated with cationic organic materials (CTAB and PAH), the surface charge of the AgNPs became positive, resulting in the lysis of *E. coli* cells. Cationic charged AgNPs showed the highest lethal effects, probably because the cell membrane is negatively charged and may enter into electrostatic interactions with the positively charged AgNPs. Both cationic stabilizers had a strong inherent toxicity and inactivated *E. coli*. In addition, the cationic surfactant (CTAB) showed a lethal bactericidal action, even at low concentrations (0.03 mM). The toxicity of PAH-coated AgNPs (at 0.06 mM PAH) was accelerated compared to the toxicity of bare AgNPs and PAH. In conclusion, the inactivation of *E. coli* was not correlated with the properties of neutral stabilizers (TW80, PVP, and CMC), but rather with ionic type and hydro/lipophilicity. The findings demonstrate that the toxicity of AgNPs was accelerated in cases of stabilizers with cationic properties and hydrophobicity, but was not reduced by biocompatible compounds or polymers.

A growth inhibition, colony forming unit and liquid-to-plate assay of cAgNPs (ABC NANOTECH Co., LTD) with *Escherichia coli* and *Bacillus subtilis* was conducted for 12 hours (Dunnett, 1955; Hamilton *et al*., 1977; US EPA, 1999; An and Kim, 2009; Lee *et al*., 2009; Kim *et al*., 2010). Nominal concentrations were 0, 10, 30 and 50 mg/L for growth inhibition assay, 0, 1, 5, and 10 mg/L for CFU assay, and 0, 0.025, 0.05, and 0.25 mg/L for LTP assay. To confirm the characterization, homogeneity and (short term) stability of prepared test items, DLS size measurement, Zeta potential measurement and SEM

measurements were used estimating the size and aggregation level of NP suspension in test media. In growth inhibition test, growth of *E. coli* was inhibited on early stage at 30 mg/L and 50 mg/L. In CFU assay, the colony formation of *E. coli* was inhibited by cAgNP. However, the negative effect was not significant for *B. subtilis*. The EC_{50} with confidence limit of *E. coli* and *B. subtilis* were 3.6 (95% CL: 3.2-4.2) and >10 mg/L, respectively. In LTP assay, the colony areas were reduced by 9%, 33%, 70% and 91% on the *E. coli* plates and by 48%, 89%, 98% and 96% on *B. subtilis* plates at 0.05 mg AgNP/L, respectively. The EC_{50} on *E. coli* were > 0.25 mg/L, 0.07 (95% CL: 0.06-0.09) mg/L, < 0.025 mg/L, and < 0.025 mg/L after 0, 1, 2, and 3 h, respectively. The EC_{50} on *B. subtilis* under each exposure time were calculated as 0.1 (95% CL: 0.01-1.41) mg/L, 0.07 (95% CL: 0.06-0.09) mg/L, < 0.025 mg/L, and < 0.025 mg/L, respectively.

A growth inhibition and NP LB agar test of cAgNPs (ABC NANOTECH Co., LTD) with *Escherichia coli* and *Bacillus subtilis* was conducted at 0 to 50 mg/L (growth inhibition test) and 0, 1, 5 and 10 mg/L (NP LB agar test) and for 12 to 24 hours under static condition according to Lee *et al*., 2009 for LTP test (Sondi and Salopek-Sondi, 2004; Kim *et al*., 2007; Pal *et al*., 2007; Tong *et al*., 2007; Yoon *et al*., 2007; Maneerung *et al*., 2008; An and Kim, 2009; Dror-Ehre *et al*., 2009; Gurunathan *et al*., 2009; Lee *et al*., 2009). To confirm the characterization, homogeneity and (short term) stability of prepared test items, DLS size measurement, Zeta potential measurement and SEM measurements were used for estimating the size and aggregation level of NP suspension in test media. NPs extended inhibited cell proliferation. The inhibition of *E. coli* growth depends on the NP concentrations. Growth of *B. subtilis* shows a typical S-shape curve under lower NP concentrations. Initial growth inhibition was clear for *E. coli* at 50 mg/L. TEM observation showed that NPs was present in the microbial cells, and they caused to destroy the cell membranes. TEM-EDS confirmed that the particles inside the cells were NPs aggregates. LB agar test showed that colony formation of *E. coli* was clearly prohibited by NPs, however, the effect was not significant for *B. subtilis*. Therefore, *E. coli* seems to be more sensitive to NPs compared to *B. subtilis*. This result has agreement with the growth inhibition result. The EC_{50} was 3.64 mg/L for growth inhibition test and 3.16 to 4.19 mg/L for NP LB agar test in *E. coli* after 24 hours. In *B. subtilis*, the EC_{50} was > 10 mg/L for growth inhibition test after 24 hours.

A solid-contact test of NM300K with *Arthrobacter globiformis* (German collection of Microorganisms, DSMZ) was conducted at 30 °C for 2 hours according to DIN 38412-48 (Engelke, 2012). After the uncontaminated soil was sieved and autoclaved at 120 °C, it was thoroughly mixed with the highest concentration of the test material which was prior diluted in deionized water. To obtain a range of Ag-concentrations (10-100 mg/kg dw for NM300K), the spiked soil was mixed with an appropriate amount of uncontaminated RefeSol 01-A. All soil samples were thoroughly mixed and tests were always conducted 20 hours after spiking. As a result, the EC_{50} was 34 (95% CL: 30.24-38.65) mg/kg for dehydrogenase inhibition.

A luminescent bacteria test of NM300K with *Vibrio fisheri* was conducted for 30 minutes under static condition according to DIN EN ISO 11348-3 (Polleichtner *et al.*, 2013). Silver nitrate ($AgNO_3$) was used as a positive control. Data were available for three independent tests for NM300K (one was analyzed 2 times with differing color correction), one test for NM300K DIS and one independent test for silver nitrate. EC_{10}, EC_{20} and EC_{50} values were determined. As a result, the EC_{50} of NM300K and silver nitrate was 10.56-13 mg/kg and 1.76 mg/L (one independent test), respectively.

Table 3.7. Summary of toxicity to microorganisms

Test Material	Species	Method	Result	Reference
cAgNPs (ABC NANOTECH Co., LTD)	*Escherichia coli PQ37*	0, 0.03, 0.32, 3.23, 16.13 and 32.26 mg/L (cAgNPs), 0, 0.03, 0.32, 3.23, 16.13 and 32.26 mg/L (Ag ion), 2 hours	cAgNPs and Ag+ was not genotoxic based on the result of the SOS chromo test.	Ames *et al*., 1975; Hofnung, 1982; Huisman *et al*., 1984; Quillardet and Hofnung, 1985; Quillardet *et al*., 1985; Dimpfl and Echols, 1989; Quillardet and Hofnung, 1993; Ishioka *et al*., 1997; Lantzsch and Gebel, 1997; Anderson and Kowalczykowski, 1998
cAgNPs (ABC NANOTECH Co., LTD)	*Pseudomonas aeruginosa PA01*	10 mg/L (AgNPs), 1 and 10 mg/L (Ag ion), 150 min	Silver ion had higher antimicrobial activity and biofilm inactivation than cAgNPs.	Lee *et al*., 1998; Hong *et al*., 2008; Park *et al*., 2013
AgNPs (Sigma-Aldrich)	*Escherichia coli*	0.4 and 0.8 mg/L EPA (2007) Nanotechnology White Paper	The toxicity of AgNPs was accelerated in cases of stabilizers with cationic properties and hydrophobicity, but was not reduced by biocompatible compounds or polymers.	Ministry of Environment, 2010
cAgNPs (ABC NANOTECH Co., LTD)	*Escherichia coli*	0, 10, 30 and 50 mg/L (growth inhibition), 0, 1, 5, and 10 mg/L (CFU assay), 0, 0.025, 0.05, and 0.25 mg/L (LTP assay) 12 hours	Growth inhibition: growth of *E. coli* was inhibited on early stage at 30 mg/L and 50 mg/L. CFU assay: EC_{50} : 3.6 mg/L (95% CL: 3.2-4.2 mg/L); LTP assay: <0.025 mg/L (3h)	Dunnett, 1955; Hamilton *et al*., 1977; US EPA, 1999; An and Kim, 2009; Lee *et al*., 2009; Kim *et al*., 2010
	Bacillus subtilis		CFU assay: EC_{50} > 10 mg/L; LTP assay: <0.025 mg/L (3h)	
cAgNPs (ABC NANOTECH Co., LTD)	*Escherichia coli*	0 to 50 mg/L (growth inhibition), 0, 1, 5 and 10 mg/L (NP LB agar test), 12-24 hours, static	Growth inhibition: EC_{50}: 3.64 mg/L; NP LB agar test: EC_{50}: 3.16-4.19 mg/L (24 h)	Sondi and Salopek-Sondi, 2004; Kim *et al*., 2007; Pal *et al*., 2007; Tong *et al*.,

	Bacillus subtilis	Lee *et al*., 2009 for LTP test	Growth inhibition: EC_{50} > 10 mg/L (24 h)	2007; Yoon *et al*., 2007; Maneerung *et al*., 2008; An and Kim, 2009; Dror-Ehre *et al*., 2009; Gurunathan *et al*., 2009; Lee *et al*., 2009
NM300K	*Arthrobacter globiformis*	30 °C, 2 hours DIN 38412-48	Dehydrogenase inhibition: EC_{50}: 34 mg/kg (95% CL: 30.24-38.65 mg/kg)	Engelke, 2012
NM300K	*Vibrio fisheri*	30 min, Static DIN EN ISO 11348-3	EC_{50}-NM300K: 10.56-13 mg/kg EC_{50}-Silver nitrate: 1.76 mg/L	Polleichtner *et al.*, 2013

Other organisms

A toxicity test of NM300 with *Potamopyrgus antipodarum* (New Zealand mud snail) was conducted at 0.1, 10, 31.25, 62.5, 125, 250, 500 and 1,000 µg/L and 16 ± 1°C under semi-static conditions for 28 days (Völker *et al*., 2014). For all exposure experiments, female *P. antipodarum* (shell height 3.6-4.2 mm) were used. Prior to the experiments, the reproductive status of twenty snails was assessed. In each experiment, exposure groups consisted of four replicates with ten individuals each. NM300 was diluted in demineralized water to obtain a stem dispersion. Precise volumes of this dispersion were pipetted into test media and stirred for 30 sec. to achieve final test concentrations. Tests were conducted in 250 mL glass beakers filled with 200 mL fully reconstituted water with an identical composition for the cultivation medium. Exposure media were exchanged twice a week and the following water parameters were maintained: pH 8.0 ± 0.5, oxygen saturation > 60%, conductivity 770 ± 100 µS/cm. After renewal of the test media, animals were fed with 0.2 mg TetraPhyll® (Tetrawerke, Melle, Germany) per animal and day. As a result, effect values were 5.57 µg/L (EC_{10}) and 15.0 µg/L (EC_{50}) with 95% confidence intervals of 2.97 to 10.5 µg/L and 10.6 to 21.2 µg/L, respectively.

A toxicity test of NM300 with *Sphaerium corneum* (European fingernail clam) was conducted at 5, 25, 50, 100 and 500 µg/L and 16 ± 1°C under semi-static conditions for 28 days (Völker *et al*., 2015). For the exposure experiments, only healthy and mature individuals of *S. corneum* (size 5-10 mm) that reacted to an external stimulus were used. Prior to the experiments, the reproductive status of ten clams was assessed. Exposure groups consisted of four replicates with five individuals each. NM300 was diluted in demineralized water to obtain a stem dispersion. Precise volumes of this dispersion were pipetted into test media and stirred for 30 sec. to achieve final test concentrations. The animals were exposed in 600 mL glass beakers filled with 400 mL ISO medium. The test water was renewed every other day and the following water parameters were maintained: pH 7.5 ± 0.5, oxygen saturation >80%, conductivity 770 ± 100 µS/cm. Animals were fed with a suspension of green algae (*Scenedesmus acutus*). As a result, the NOEC and LOEC values were 100 and 500 µg/L, respectively.

Table 3.8. Summary of toxicity to other organisms

Test Material	Species	Method	Result	Reference
NM300	*Potamopyrgus antipodarum*	0.1, 10, 31.25, 62.5, 125, 250, 500 and 1,000 µg/L, 28 days, semi-static	EC_{10}: 5.57 µg/L (95% CL: 2.97-10.5 µg/L); EC_{50}: 15.0 µg/L (95% CL: 10.6-21.2 µg/L)	Völker *et al*., 2014
NM300	*Sphaerium corneum*	5, 25, 50, 100 and 500 µg/L, 28 days, semi-static	Behavior (reduced activity): NOEC: 100 µg/L; LOEC: 500 µg/L	Völker *et al*., 2015

3.2. Sediment Toxicity

3.2.1. Sediment-Water Toxicity

Chironomids

A sediment-water toxicity test of NM300K with *Chironomus riparius* (Bayer Crop AG) was conducted at 0.3125, 0.625, 1.25, 2.5, 5.0 and 10.0 mg/L, 20 ± 2 °C and pH 8.0-8.2 under static conditions for 28 days according to OECD test guideline 219 and in compliance with GLP (Hund-Rinke and Klawonn, 2013). Four to five days before adding the test organisms to the test vessels, egg masses were taken from the cultures and placed in small aerated vessels with test water at about 20 °C. First instar larvae (one day post hatching) were used in the test. As the larvae were added one day before spiking, the age of the larvae was about 2 days at day 0 (day 0 = day of spiking the water phase). Four replicates per concentration were conducted. A stock dispersion of 200 mg/L was prepared by mixing the respective amount of NM300K with tap water. 50 mL of the stock dispersion was used for the 10 mg/L concentration. Appropriate amounts of the stock dispersion were diluted to obtain further concentrations. Each vessel was spiked with 50 mL of suitable dispersion. At several points of time aqueous samples (5 mL) were taken at four depths (about 1.5 cm, 3.0 cm, 4.0 cm, and 5.5 cm). The samples were combined and used for analysis. Chironomid emergence was measured as the endpoint. Emergence rate, development time and rate, and sensitivity were analyzed for statistically significant difference using computer software ToxRat Professional version 2.10.4.1 by ToxRat® Solutions GmbH. In summary, the EC_{50} values for emergence rate were 2.201 mg/L (95% CL: 2.041-2.357 mg/L) for midges, 2.415 mg/L for males and 1.835 mg/L (95% CL: 1.610-2.091 mg/L) for females, respectively and NOEC was 1.25 mg/L for all three parameters. The EC_{50} values for development rate were 7.508 mg/L for midges, 5.828 mg/L for males and 15.369 mg/L for females, respectively and NOEC was 0.625 mg/L for all three parameters. The LOEC value for growth rate was 2.5 mg/L.

3.3. Terrestrial Toxicity

3.3.1. Acute Toxicity

Soil invertebrates except arthropods

A soil toxicity test of cAgNPs (ABC NANOTECH Co., LTD) with *Caenorhabditis elegans* was conducted at 0.05, 0.5 and 5 mg/L for 24 hours (Williams and Dusenvery, 1990; Roh *et al*., 2006; Rho *et al*., 2007). The stock of silver nanoparticles was black colloidal suspension, and included degree of purity in 20%. Citrate (0.5-1.5%) was used as a stabilizer of the colloidal particles. To estimate the size and

aggregation level of NP suspension, homogeneity and (short term) stability of prepared test items such as the measurement of DLS, SEM, and Zeta potential were used. Stress-related gene expression test was performed as test system and metallothionein (*mt-1*), metallothionein (*mt-2*), heat shock protein (*hsp-16.2*), heat shock protein (*hsp-16.41*), glutathione S-transferase (*gst-4*), and cytochrome P450 family protein 35A2 (*cyp-35a2*) were observed as target genes. The sequences of primers were determined from the *C. elegans* database (http://www.wormbase.org). The expression of *mt-1*, *mt-2*, *hsp-16.2*, *hsp-16.41*, *gst-4*, and *cyp35a2* did not show any significance with increasing cAgNP concentrations. In conclusion, there were no the lethal effects of cAgNPs at the level of the whole organism, and it showed that cAgNPs do not induce the stress of *C. elegans* in the genetic levels.

A soil toxicity test of cAgNPs (ABC NANOTECH Co., LTD) with *Caenorhabditis elegans* (wild type N2 strain) was conducted up to 100 mg/L for 24 hours (Kim *et al*., 2008; Ma *et al*., 2009; Roh *et al*., 2009; Wang *et al*., 2009; Roh *et al*., 2010; Kim *et al*., 2012). The stock of silver nanoparticles was black colloidal suspension, and included degree of purity in 20 %. Citrate (0.5-1.5 %) was used as a stabilizer of the colloidal particles. To estimate the size and aggregation level of NP suspension, homogeneity and (short term) stability of prepared test items such as the measurement of DLS, SEM, and Zeta potential were used. *C. elegans* was exposed to NP NGM agar medium for homogeneous distribution of NPs. The effect of NPs on survival of the test organism showed a dose-dependent decrease up to 100 mg/L. There was no significant effect on *C. elegans*, at the concentrations greater than 100 mg/L of cAgNP. This phenomenon was due to the nanoparticle aggregation. Similar trend was observed in the reproduction test. The number of larva born during the exposure period had a trend of being reduced up to 10 mg/L in comparison with control. Therefore, cAgNPs adversely induced the reproductive effect on adult of *C. elegans*.

A soil toxicity test of cAgNPs (ABC NANOTECH Co., LTD) with *Eisenia andrei* (red tiger worm) and *Perionyx excavatus* (blue worm) was conducted up to 2,000 mg/kg and at 25 °C for 7 days according to OECD test guideline 207 (Kurek and Plytycz, 2003; Hendawi *et al*., 2004; An and Lee, 2008; Wijnhoven *et al*., 2009). The stock of silver nanoparticles was black colloidal suspension, and included degree of purity in 20 %. Citrate (0.5-1.5 %) was used as a stabilizer of the colloidal particles. To estimate the size and aggregation level of NP suspension, homogeneity and (short term) stability of prepared test items such as the measurement of DLS, SEM, and Zeta potential were used. NRR (Neutral Red Retention) assay according to Hendawi *et al*., 2004 and Kurek and Plytycz, 2003 was used to confirm coelomocytes effect of NPs exposed to earthworm and BAFs were measured to determine uptake of NPs and its accumulation in plant tissue after 2 d. In case of *E. andrei*, 7d-NOEC values (pinocytosis) were 300 mg/kg dry soil as a result of NRR assay and pinocytosis effect was reduced approximately 4% at the maximum concentration of 2,000 mg/kg. However, in the case of *P. excavatus*, coelomocyte density (NOEC > 2,000 mg/kg) and pinocytosis effect (NOEC > 2,000 mg/kg) were not changed with increasing cAgNP concentrations.

An acute toxicity test of AgNPs (Sigma-Aldrich) with *Caenorhabdities elegans* was conducted at 0.1, 0.2, 1.5, 1, 2 and 4 mg/L and 20 °C for 24 and 72 hours according to an accepted scientific principles (Ministry of Environment, 2010). Short-term survival and growth experiments (24 h) were compared with the sensitivity of physiological level responses of *C. elegans* to AgNPs. Short-term testing only provided a snapshot of the physiological status, thus longer term testing (72 h) was conducted to evaluate the effects on reproductive potential. Although AgNPs exposure did not affect the survival and growth of the wild type, reproduction was seriously affected, with the number of offspring per individual dramatically decreased (70% of the controls in 0.1 and 0.5 mg/L AgNPs). The mutant strains' survival and growth response were not different from the wild type, but the reproductive responses of the mtl-2 (*gk125*) and sod-3 (*gk235*) mutants were less sensitive (40-60% less at 0.1 mg/L and 10% at 0.5 mg/L) to AgNPs exposure than the wild type, while the response of the daf-12 (*rh286*) mutant was similar to the wild type.

An acute toxicity test of cAgNPs (ABC NANOTECH Co., LTD) with *Eisenia andrei* (red tiger worm) and *Perionyx excavatus* (blue worm) was conducted for 7 days according to accepted scientific principles (Chemosphere, 2008; Nanotechnology, 2009; Ecotoxicol Environ Safety, 2004; Pedologia, 2003). Each test unit contained 5 g of dried NP-amended soil, and DI water was added to give an overall moisture content of about 35% of the dry weight. 15 replicates for *E. andrei* and 10 replicates for *P. excavatus* in each treatment were applied during 7 days. NRR (Neutral Red Retention) assay according to Hendawi *et al*., 2004 and Kurek and Plytycz, 2003 was used to confirm coelomocytes effect of earthworm exposed NPs and BAFs was measured to determine uptake of NPs and its accumulation in plant tissue after 2 d. 7d-NOEC values (pinocytosis) for *E. andrei* were 300 mg/kg dry soil as a result of NRR assay. Pinocytosis effect was reduced approximately 4% at the maximum concentration of 2,000 mg/kg compare to control based on NRR assay. In the case of *P. excavatus*, coelomocyte density and pinocytosis effect were not changed with increasing cAgNP concentrations. The bioaccumulation factors of cAgNP were calculated to be 0.001 and 0.004 for *E. andrei* and *P. excavatus*, respectively. The 7d-EC_{50} (survival) and NOEC (survival) for *E. andrei* were estimated to be 530(397-705) mg/kg and 300 mg/kg, respectively. As a result, there is no significant effect on the coelomocyte viability and pinocytosis activity.

An acute test of cAgNPs (ABC NANOTECH Co., LTD) with *Caenorhabditis elegans* was conducted for 24 hours according to an accepted scientific principles (ASTM, 2008; Mechanism of Ageing and Development, 2008; Environmental Toxicology and Chemistry, 2009; Environmental Science Technology, 2009; Environmental Toxicology and Pharmacology, 2009; Environmental Pollution, 2009). To confirm homogeneous distribution in test medium, SEM measurements were used. And high resolution nano scale microscopic measurement was used for confirmation of distribution as a screening. And the NP-dissolution on test medium was measured using ICP-AES. The effect of NPs on survival of *C. elegans* showed a dose-dependent decrease up to 100 mg/L. There was no significant effect on *C. elegans*, at the concentrations greater than 100 mg/L of cAgNP. Similar trend was observed in the reproduction test. The number of larva born during the exposure period had a trend of being reduced up to 10 mg/L in comparison with control. This indicated that cAgNPs adversely induced the reproductive effect on adult of *C. elegans*. In NP NGM agar test, as a result, the LC_{50} (EC_{50}) and NOEC were 55.3 mg/L, < 10 mg/L for survival and > 100 mg/L, 1 mg/L for reproduction, respectively. In Ag ion toxicity test, the LC_{50} (EC_{50}) and NOEC were 22.31 mg/L, > 0.5 mg/L for survival and > 10 mg/L, 1 mg/L for reproduction, respectively.

An acute toxicity test of cAgNPs (ABC NANOTECH Co., LTD) with *Caenorhabditis elegans* was conducted at 0.05, 0.5 and 5 mg/L for 24 hours according to an accepted scientific principle (Environmental Toxicology and Chemistry, 2006; Toxicology, 2007; Environmental Toxicology and Chemistry, 1990). To confirm homogeneous distribution in test medium, SEM measurements were used. And high resolution nano scale microscopic measurement was used for confirmation of distribution as a screening. And the NP-dissolution on test medium was measured using ICP-AES. As *in vitro* test, stress-related gene expression test was performed and metallothionein (*mt-1*), metallothionein (*mt-2*), heat shock protein (*hsp-16.2*), heat shock protein (*hsp-16.41*), glutathione S-transferase (*gst-4*) and cytochrome P450 family protein 35A2 (*cyp-35a2*) were observed for target genes. In conclusion, the expression of *mt-1*, *mt-2*, *hsp-162*, *hsp-16.41*, *gst-4* and *cyp35a2* did not show any significance with increasing cAgNP concentration. Therefore were no the lethal effects of cAgNPs at the level of the whole organism, and it showed that cAgNPs did not induce the stress of *C. elegans* in the genetic levels.

Table 3.9. Summary of acute toxicity to soil invertebrates except arthropods

Test Material	Species	Method	Result	Reference
cAgNPs (ABC NANOTECH Co., LTD)	*Caenorhabditis elegans*	0.05, 0.5 and 5 mg/L, 24 hours	cAgNPs don't induce the stress of *C. elegans* in the genetic levels.	Williams and Dusenvery, 1990; Roh *et al*., 2006; Rho *et al*., 2007
cAgNPs (ABC NANOTECH Co., LTD)	*Caenorhabditis elegans*	~100 mg/L, 24 hours	cAgNPs induced adverse effect on *C. elegans* by decreasing survival rate and the number of larva in a dose dependent manner. Mortality: LC_{50}: 53.03 mg/L; (95% CL: 28.51-105.14 mg/L) NOEC < 10 mg/L Reproduction: EC_{50} > 100 mg/L; NOEC: 1 mg/L	Kim *et al*., 2008; Ma *et al*., 2009; Roh *et al*., 2009; Wang *et al*., 2009; Roh *et al*., 2010; Kim *et al*., 2012
cAgNPs (ABC NANOTECH Co., LTD)	*Eisenia andrei*	~2,000 mg/kg 7 days OECD TG 207	Pinocytosis: NOEC: 300 mg/kg Coelomocytes viability: NOEC > 2,000 mg/kg	Kurek and Plytycz, 2003; Hendawi *et al*., 2004; An and Lee, 2008; Wijnhoven *et al*., 2009
	Perionyx excavatus		Pinocytosis: NOEC > 2,000 mg/kg Coelomocytes viability: NOEC > 2,000 mg/kg	
AgNPs (Sigma-Aldrich)	*Caenorhabditi es elegans*	0.1, 0.2, 1.5, 1, 2 and 4 mg/L, 24 and 72 hours	AgNPs exposure did not affect the survival and growth of the wild type and mutant strains' while reproduction was seriously affected with decreased number of offspring. The responses of the mtl-2 (*gk125*) and sod-3 (*gk235*) mutants were less sensitive to AgNPs exposure than the wild type, while the response of the daf-12 (*rh286*) mutant was similar to the wild type.	Ministry of Environment, 2010
cAgNPs (ABC NANOTECH Co., LTD)	*Eisenia andrei*	7 days, NRR assay, BAF aasay, Coelomocytes viability, Survival	Pinocytosis: NOEC: 300 mg/kg Coelomocytes viability: NOEC > 2,000 mg/kg BAF: 0.0006 kg/kg worm Survival: EC_{50}: 530 mg/kg (95% CL: 397-705 mg/kg);	Chemosphere, 2008; Nanotechnology, 2009; Ecotoxicol Environ Safety, 2004; Pedologia, 2003

			NOEC: 300 mg/kg	
	Perionyx excavatus		Pinocytosis: NOEC > 2,000 mg/kg Coelomocytes viability: NOEC > 2,000 mg/kg BAF: 0.0039 kg/kg worm	
cAgNPs (ABC NANOTECH Co., LTD)	*Caenorhabditis elegans*	24 hours,	NP NGM agar test Survival: LC_{50}: 55.03 mg/L (95% CL: 28.51-105.14 mg/L); NOEC <10 mg/L Reproduction: EC_{50} >100 mg/L; NOEC: 1 mg/L	ASTM, 2008; Mechanism of Ageing and Development, 2008; Environmental Toxicology and Chemistry, 2009; Environmental Science Technology, 2009; Environmental Toxicology and Pharmacology, 2009; Environmental Pollution, 2009
			Ag ion toxicity test Survival: LC_{50}: 22.31 mg/L (95% CL: 10.88-45.72 mg/L); NOEC > 0.5 mg/L Reproduction: EC_{50} >10 mg/L; NOEC: 1 mg/L	
cAgNPs (ABC NANOTECH Co., LTD)	*Caenorhabditis elegans*	0.05, 0.5 and 5 mg/L, 24 hours, Stress-related gene expression test	The expression of *mt-1*, *mt-2*, *hsp-162*, *hsp-16.41*, *gst-4* and *cyp35a2* were not showed any significance with increasing cAgNP concentration.	Environmental Toxicology and Chemistry, 2006; Toxicology, 2007; Environmental Toxicology and Chemistry, 1990

Terrestrial plants

An acute toxicity test of cAgNPs (ABC NANOTECH Co., LTD) with *Phaseolus radiatus* (mung bean) and *Sorghum bicolor* (sorghum) was conducted at 0, 5, 10, 20 and 40 mg/L (agar medium) and 0, 100, 300, 500, 1,000 and 2,000 mg/kg (soil medium) for 48 hours according to Lee *et al*., 2008 for the DAT test (Lee *et al*., 2012; Lee *et al*., 2008; Ma *et al*., 2010; Environmental Toxicology and Chemistry, 2008; Chemosphere, 2010). Plant agar media were used for easy dispersion of NPs without precipitation. The dual agar test (DAT) was performed and bioaccumulation factor (BAF) was calculated. In the DAT test, it was found that the growth of *P. radiatus* and *S. bicolor* was decreased with increasing cAgNP concentrations. The EC_{50} of *P. radiatus* and *S. bicolor* were 13 (10-17) and 26 (5-139) mg/L, respectively. The 48hr-EC_{50} values of root and shoot of *S. bicolor* were < 5 and > 40 mg/L, respectively. The brown tip and necrosis were found in exposed roots of *P. radiatus* and *S. bicolor*. The NP accumulated in *P. radiatus* and *S. bicolor* in DAT was increased dependently on the exposure concentration. The bioaccumulation in the DATs of *P. radiatus* and *S. bicolor* were 5.47 ± 0.36 and 3.29 ± 2.66 mg/kg, respectively, in the maximum exposure concentration of 40 mg/L. The bioaccumulation factors of *P. radiatus* and *S. bicolor* were calculated to be 0.14 and 0.08 L/kg, respectively. In case of the soil test, the cAgNPs accumulations in roots of *P. radiatus* and *S. bicolor* were measured to be 15.38 ± 4.03 and 12.46 ± 6.74 mg/kg plant

respectively in the highest concentration of 2,000 mg/kg dry soil. On the other hand, the cAgNPs accumulations in the shoot were measured to be 1.91 ± 1.07 and 1.47 ± 1.14 mg/kg plant for *P. radiatus* and *S. bicolor*, respectively. The bioaccumulation factors of root of *P. radiatus* and *S. bicolor* were calculated to be 0.008 and 0.006 kg /kg, respectively. On the other hand, the bioaccumulation factors of shoot of *P. radiatus* and *S. bicolor* were calculated to be 0.001 and 0.001 kg/kg, respectively. Therefore, the growth rates of the both plants were inhibited as a result of exposure to NPs.

A seedling emergence and seedling growth test of NM300K with *Phaseolus aureus* was conducted at 1.5, 4.5, 13.5, 40.5 and 121.5 mg/kg dry soil and 22 ± 10 °C for 14 days according to OECD TG 208 (Schlich and Hund-Rinke, 2013). Silver nanoparticles were applied by mixing the test material and air-dried carrier soil with the same physicochemical properties of the test soil. Enough Ag-NPs were added to the carrier to achieve the final test concentration when 5% carrier soil and 95% test soil were mixed to homogeneity. The soil was mixed with a spoon instead of a pestle to avoid modifying the Ag-NPs. Uncontaminated soil (at 20–30% WHCmax) was spread on a plate, and the spiked carrier soil was evenly distributed over the test soil before mixing. The mixed soil was adjusted to 55% WHCmax using deionized water. This procedure was performed for every test concentration. As a result, the EC_{25} value for 14 days was 33.5 mg/kg soil dw.

Table 3.10. Summary of acute toxicity to terrestrial plants

Test Material	Species	Method	Result	Reference
cAgNPs (ABC NANOTECH Co., LTD	*Phaseolus radiatus*	0, 5, 10, 20 and 40 mg/L (agar medium), 0, 100, 300, 500, 1,000 and 2,000 mg/kg (soil medium), 48 hours Lee *et al*., 2008 for the DAT test	Growth: EC_{50}: 13 mg/L (95% CL: 10-17 mg/kg);	Lee *et al*., 2012; Lee *et al*., 2008; Ma *et al*., 2010; Environmental Toxicology and Chemistry, 2008; Chemosphere, 2010
	Sorghum bicolor		Growth: EC_{50}: 26 mg/L (95% CL: 5-139 mg/kg); Root: EC_{50} < 5 mg/L; Shoot: EC_{50} > 40 mg/L;	
NM300K	*Phaseolus aureus*	1.5, 4.5, 13.5, 40.5 and 121.5 mg/kg, 14 days OECD TG 208	EC_{25}: 33.5 mg/kg	Schlich and Hund-Rinke, 2013

3.3.2. Chronic Toxicity

Soil invertebrates except arthropods

An earthworm reproduction test of NM300K with *Eisenia andrei* was conducted for 56 days according to OECD test guideline 222 (Schlich and Hund-Rinke, 2013). The STP simulations confirmed that at environmentally relevant concentrations > 90% of AgNPs remain bound to sewage sludge. Effects of AgNPs bound to sewage sludge and added to soil were similar to those of pristine NM300K after degradation of the sludge. The predicted no-effect concentration for NM300K in soil of 0.05 mg/kg dry soil was determined a maximum threshold of 300 mg/kg dry sludge per application, considering the maximum addition of sewage sludge in Germany (5 tons per hectare every 3 years).

An earthworm reproduction test of NM300K with *Eisenia andrei* (Regenwurmfarm Tacke) was conducted at 15, 30, 60, 120 and 200 mg/kg soil (dry mass) and 19~21 °C for 56 days according to OECD test guideline 222 and in compliance with GLP (Hund-Rinke and Klawonn, 2013). The test organisms were synchronized adult earthworms, which were 2-12 months old with a clitellum, and a wet mass

between 250 mg and 600 mg. The worms were conditioned in the artificial soil for 7 days before use, and the same feed as used in the test was given in a sufficient amount. The soil used in the test was a natural sandy soil (certified RefeSol 01-A, batch IME 01: sand 71%, silt: 24%, clay: 5%, Org C: 0.93%, pH 5.7, clay: 5%). NM300 K was stabilized in a dispersant and the stock dispersion contains 10% of silver. The concentration of silver ions in the test soil was measured by incubation of DGTs in the vessels. Two DGTs per test vessel were incubated for two days. The incubation started on day 0, day 26 and day 54. The total content of Ag was determined in the soil and in the earthworms. Earthworms were incubated for 24 h on wet filter paper to purge their gut and then frozen at -20 °C until analysis. As a result, the effect values of EC_{10}, EC_{20}, EC_{50}, LOEC and NOEC were 1.147 mg/kg (95% CL: 1.118-1.172 mg/kg), 1.309 mg/kg (95% CL: 1.289-1.328 mg/kg), 1.688 mg/kg (95% CL: 1.670-1.709 mg/kg), 1.5 mg/kg and 0.75 mg/kg, respectively for reproduction.

Table 3.11. Summary of chronic toxicity to soil invertebrates except arthropods

Test Material	Species	Method	Result	Reference
NM300K	*Eisenia andrei*	56 days OECD TG 222	Reproduction: PNEC: 0.05 mg/kg dry soil	Schlich and Hund-Rinke, 2013
NM300K	*Eisenia andrei*	15, 30, 60, 120 and 200 mg/kg, 56 days OECD TG 222	Reproduction: EC_{10}: 1.147 mg/kg (95% CL: 1.118-1.172 mg/kg); EC_{20}: 1.309 mg/kg (95% CL: 1.289-1.328 mg/kg); EC_{50}: 1.688 mg/kg (95% CL: 1.670-1.709 mg/kg); LOEC: 1.5 mg/kg; NOEC: 0.75 mg/kg	Hund-Rinke and Klawonn, 2013

Terrestrial plants

A chronic toxicity test of cAgNPs (NAMATECH Co. Ltd) with *Lycopersicon esculentum* (tomato) was conducted at concentrations from 50 to 5,000 mg/L and 24 °C for 14 days and 6 weeks (Song *et al*., 2013). Seed germination and root elongation were observed and 100 mm/15 mm petri dish, one piece of filter paper for each petri dish, 5 mL of deionized water (germination) or test solution (root length) were used as materials. As a result, every treated plant showed almost full germination after 12 days. AgNPs did not fully penetrate the seed coat and endosperm and thus had limited effects on the embryos. Those treated with AgNP showed significant decreases in root growth even at the lowest concentration (50 mg/L) while those exposed to the highest concentration (5,000 mg/L) failed to show significant increase in root growth throughout the experimental period. After 14 days, AgNPs showed a 2 to 3 fold increase diameter in 100mg/L treatment. In conclusion, AgNPs resulted in significantly decreased root elongation at every concentration. In greenhouse experiments, mature plants showed evidence of phytotoxicity due to AgNPs by exhibiting low chlorophyll contents, higher SOD, and less fruit production.

Soil microorganisms

A nitrogen transformation test of NM300K with dry soil was conducted at 15, 45, 100 and 200 mg/kg dry soil and 20 ± 2 °C for 28 days according to OECD test guideline 216 and in compliance with GLP (2012Schlich and Hund-Rinke, 2013). RefeSol soils were selected as reference soils on behalf of the German Federal Environment Agency (Umweltbundesamt UBA) and they are known to be suitable for

testing the influence of substances on the habitat function of soils (bioavailability, effects on organisms). RefeSol 01A matches the properties stated in various OECD terrestrial ecotoxicological guidelines (e.g. tests with plants and soil microflora). During all experiments, red clover was sown on the stored soils and no pesticides were used. Appropriate amounts of soil were sampled 1–4 weeks before the test. If the soil was too wet for sieving, it was dried at room temperature to 20-30% of the maximum water holding capacity (WHCmax) with periodic turning to avoid surface drying. If the tests did not start immediately after sieving, the soil was stored in dark at 4 °C under aerobic conditions. Silver nanoparticles were applied by mixing the test material and air-dried carrier soil with the same physicochemical properties of the test soil. Enough Ag-NPs were added to the carrier to achieve the final test concentration when 5% carrier soil and 95% test soil were mixed to homogeneity. The soil was mixed with a spoon instead of a pestle to avoid modifying the Ag-NPs. Uncontaminated soil (at 20–30% WHCmax) was spread on a plate, and the spiked carrier soil was evenly distributed over the test soil before mixing. The mixed soil was adjusted to 55% WHCmax using deionized water. This procedure was performed for every test concentration. Test soil contained 1.02% of organic carbon content and 0.78 mg/kg of nitrogen content and 100 g of dry soil were sampled after 3 hours, 7 days, 21 days and 28 days. In conclusion, NM-300K inhibited the nitrogen transformation of the soil micro-organism. However, the nitrogen transformation was strongly stimulated by the dispersant (NM-300K DIS) in both tested concentrations. Therefore, depending on the test concentration, the potential ammonium oxidation should be observed instead of the N-Transformation test. As a result, AgNP absorption to sludge and aging in soil caused toxic effects on soil microorganisms of the terrestrial ecosystem at environmentally relevant concentrations. After 28 days, the NOEC was < 0.56 mg/kg dry soil and the EC10was 0.5 mg/kg dry soil.

A carbon transformation test of NM300K with dry soil was conducted at 1.67, 5, 15, 45 and 100 mg/kg dry soil and 20 ± 2 °C for 28 days according to OECD test guideline 217 and in compliance with GLP (Schlich and Hund-Rinke, 2013). RefeSol soils were selected as reference soils on behalf of the German Federal Environment Agency (Umweltbundesamt UBA) and they are known to be suitable for testing the influence of substances on the habitat function of soils (bioavailability, effects on organisms). RefeSol 01A matches the properties stated in various OECD terrestrial ecotoxicological guidelines (e.g. tests with plants and soil microflora). During all experiments, red clover was sown on the stored soils and no pesticides were used. Appropriate amounts of soil were sampled 1–4 weeks before the test. If the soil was too wet for sieving, it was dried at room temperature to 20-30% of the maximum water holding capacity (WHCmax) with periodic turning to avoid surface drying. If the tests did not start immediately after sieving, the soil was stored in dark at 4 °C under aerobic conditions. Silver nanoparticles were applied by mixing the test material and air-dried carrier soil with the same physicochemical properties of the test soil. Enough Ag-NPs were added to the carrier to achieve the final test concentration when 5% carrier soil and 95% test soil were mixed to homogeneity. The soil was mixed with a spoon instead of a pestle to avoid modifying the Ag-NPs. Uncontaminated soil (at 20–30% WHCmax) was spread on a plate, and the spiked carrier soil was evenly distributed over the test soil before mixing. The mixed soil was adjusted to 55% WHCmax using deionized water. This procedure was performed for every test concentration. Test soil containing 1.02% of organic carbon content and 0.78 mg/kg of nitrogen content and 100 g of dry soil were sampled after 3 hours, 7 days, 21 days and 28 days. In conclusion, the EC_{10} and EC_{50} were 0.9 mg/kg and 13 mg/kg dry soil, respectively.

A soil enzyme activity test of cAgNPs (ABC NANOTECH Co., LTD) with nature soil was conducted for 7 days according to Kandeler and Gerber, 1988; Gong, 1997; Brohon *et al*., 2001; Tabatabai, 1994 and Adam and Duncan, 2001. Soil was surface sample (0-5 cm) collected from the campus of Konkuk University (Seoul, Republic of Korea). Samples were air dried for 3 days, sieved (< 2 mm), and stored in plastic bags at 4 °C until test. Amended soil with cAgNPs in the absence of substrate was used as the method blank to correct the absorbance due to cAgNPs themselves and prepared for each concentration of cAgNPs used in the study. The moisture content was adjusted to about 60 % of the water holding capacity and then the soils were incubated at 25 °C for in dark. Urease, dehydrogenase (DHA), acid phosphatase

(APA), arylsulfatase (ASA), ß-glucosidase (BGA) and hydrolysis of fluorescein diacetate (FDA) were evaluated to observe soil enzyme activities. There was an adverse effect of cAgNPs on the activities of five representative enzymes and the hydrolysis of FDA in soil environment. Activity of DHA, ASA, and urease immediately declined after cAgNP amendment with increasing concentration. The urease and DHA exhibited more sensitiveness to cAgNPs than other four enzymes. No significant effect of silver ions, which can be dissolved from cAgNPs under experimental condition, was observed on the activities of soil enzymes. The EC_{50} values of DHA, FDA, urease, APA, ASA and BGA were 107.98 (95% CL: 62.82-185.61), > 1,000, 14.2 (95% CL: 8.78-22.97), > 1,000, > 1,000 and > 1,000 mg/kg dry soil for each enzyme for 7 days. The NOECs for same enzyme activities were 10, 100, < 1, > 1,000, 10 and 100 mg/kg dry soil for each.

Table 3.12. Summary of toxicity to soil microorganisms

Test Material	Media	Method	Result	Reference
NM300K	RefeSol soil (German Federal Environment Agency)	15, 45, 100 and 200 mg/kg, 28 days OECD TG 216	N-transformation: NOEC < 0.56 mg/kg; EC10: 0.5 mg/kg (95% CL: 0-1.0 mg/kg)	Schlich and Hund-Rinke, 2013
NM300K	RefeSol soil (German Federal Environment Agency)	1.67, 5, 15, 45 and 100 mg/kg, 28 days OECD TG 217	C-transformation: EC_{10}: 0.9 mg/kg; EC_{50}: 13 mg/kg	Schlich and Hund-Rinke, 2013
cAgNPs (ABC NANOTECH Co., LTD)	Nature soil (Konkuk University, Korea)	7 days Enzyme activity test	EC_{50}-DHA: 107.98 mg/kg (95% CL: 62.82-185.61 mg/kg); EC_{50}-FDA > 1,000 mg/kg; EC_{50}-Urease: 14.2 mg/kg (95% CL: 8.78-22.97 mg/kg); EC_{50}-APA > 1,000 mg/kg; EC_{50}-ASA > 1,000 mg/kg; EC_{50}-BGA > 1,000 mg/kg NOEC-DHA: 10 mg/kg; NOEC-FDA: 100 mg/kg; NOEC-Urease < 1 mg/kg; NOEC-APA > 1,000 mg/kg; NOEC-ASA: 10 mg/kg; NOEC-BGA: 100 mg/kg	Kandeler and Gerber, 1988; Tabatabai, 1997; Gong, 1997; Brzezinska *et al*., 1998; Adam and Duncan, 2001; Brohon *et al*., 2001; An and Kim, 2009

Additional information

A respiration inhibition test of NM300K with sludge (Schmallenberg, Germany) was conducted at 1, 3, 9, 27, 50, 81 and 100 mg/L and 20 ± 2 °C according to OECD test guideline 209 (Schlich and Hund-Rinke,2013). Primary particle size of NM300K was 15nm and 2 mL of the substance containing 200 mg of silver nanoparticles. Dry content of sludge was 4 g/L ± 10% and resulting dry content of sludge in the test vessel was 1.6 g/L. Test was performed in 1 L glass flask washed with dishwashing detergent. For validity of the test, 3,5-dichlorphenole was used as reference. The EC_{50} has to be between 5 and 30 mg/L. The variation between replicate control samples should be less than ± 15%. In conclusion, an EC_{10} of 27.9 mg/L and an EC_{50} of 43.0 mg/L were determined for NM300K.

A surface charge-department toxicity test of AgNPs with bacillus species was conducted according to EPA QA/QC guidance where standard US EPA or OECD harmonized guidelines with modifications were

used (Badawy *et al*., 2011). The H_2-AgNPs (13 nm, -22 mV) have uncoated surfaces. The Citrate-AgNPs (10 nm, -38 mV) were electrostatically stabilized through the ionization of the carboxyl groups of the citrate molecule adsorbed on the surface of the AgNPs. The PVP-AgNPs (12 nm, -10 mV) were stabilized through the steric repulsion caused by the adsorption of PVP on the surface of the AgNP. The BPEI-AgNPs (10 nm, + 40 mV) were electrostatically stabilized due to the adsorption of the BPEI molecules on the nanoparticle surface. The four investigated silver nanoparticles were purified to remove residual impurities from the synthesized silver nanoparticles suspensions using 10 KD polyethersulfone ultrafiltration membranes. The results clearly demonstrated that AgNPs exhibited surface charge-dependent toxicity and completely inhibited the bacterial activity at concentrations greater than 3 ppb, 10 ppb, 100 ppb and 800 ppb, respectively. The toxicity followed the order of BPEI-AgNPs > PVP-AgNPs > H_2-AgNPs > Citrate-AgNPs. The more negative Citrate-AgNPs were the least toxic, whereas the positively charged BPEI-AgNPs were the most toxic NPs. The results demonstrated the importance of the physical interactions, between the AgNP and the bacterium, on the toxicity of AgNPs. These physical interactions were highly governed by the surface charge of not only the AgNPs but also the cellular membranes of the bacteria examined. As a result, surface charge was one of the most important factors that has to be taken into consideration when evaluating the toxicity of AgNPs in the environment.

Table 3.13. Summary of additional information

Test Material	Media or species	Method	Result	Reference
NM300K	Sludge (Schmallenberg, Germany)	1, 3, 9, 27, 50, 81 and 100 mg/L OECD TG 209	EC_{10}: 27.9 mg/L; EC_{50}: 43 mg/L	Schlich and Hund-Rinke, 2013
H_2-AgNPs, Citrate-AgNPs, PVP-AgNPs, BPEI-AgN	Bacillus species	EPA QA/QC guidance	The AgNPs exhibited surface charge-dependent toxicity and inhibited the bacterial activity at concentrations greater than 3 ppb, 10 ppb, 100 ppb and 800 ppb, respectively. The toxicity followed the order of BPEI-AgNPs > PVP-AgNPs > H_2-AgNPs > Citrate-AgNPs.	Badawy *et al*., 2011

3.4. Summary

3.4.1. Aquatic Toxicity

Acute toxicity to fish

An acute toxicity test of cAgNP with *Cyprinus carpio* was conducted at 25, 50, 100 and 200 µg/L for 48 and 96 hours according to OECD TG 203. Liver was the most susceptible and the antioxidant enzyme system was most active in the liver. The activities of antioxidant enzymes (GST and CAT) fluctuated with different Ag-NP concentrations, whereas SOD activity remained stable.

An acute toxicity test of NM300K and silver ion with *Danio rerio* was conducted under static conditions for 48 hours according to DIN 38415 T6. The EC_{50} values for the exposure to NM300K were 0.292 and 1.668 mg/L (NOEC: 0.1 mg/L). The EC_{50} values for the exposure to Ag+ ions were 0.062 and 0.08 mg/L (NOECs: 0.051 mg/L).

An acute toxicity test of Silver powder (AgNPs) with *Oryzias latipes* was conducted at 50, 100 and 150 mg/L under semi-static conditions for 48, 72 and 120 hours according to OECD TG 210, 212 and OECD draft proposal for a new guideline (2006) of Fish embryo toxicity test. The maximum toxicity of AgNPs and agglomeration rate reached at an Ag+ ion ratio of 23% and 50%, respectively.

An acute toxicity test of Silver powder (AgNPs) with *Oryzias latipes* was conducted at 0.1, 0.25, 0.5, 0.75 and 1.0 mg/L of cAgNP for 14 days according to OECD TG 212. The LC_{50} values of the embryo test after 24 to 96 h exposure decreased from 1.46 mg/L to 0.84 mg/L. The LC_{50} values of the acute adult toxicity test after 24 h were not calculated (> 1 mg/L), with no change in LC_{50} value (0.8 mg/L) over the 48 h exposure period.

Acute toxicity to invertebrates

An acute toxicity test of cAgNP with *Daphnia magna* was conducted at 0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009, and 0.01 mg/L under static conditions for 48 hours according to OECD TG 202. The EC_{10}, EC_{50}, and EC_{90} values were 0.003 mg/L, 0.004 mg/L, and 0.005 mg/L, respectively.

An acute toxicity test of NM300K with *Daphnia magna* was conducted under static conditions for 48 hours according to OECD TG 202. The EC_{50} was ranged from 0.024 to 0.046 mg/L and NOEC values on mortality were 0.03 mg/L.

An acute toxicity test of NM300 with three aquatic invertebrates was conducted at 50, 80, 128, 204.8 and 327.7 μg/L for *Daphnia magna*, 1.5, 3, 6, 12 and 24 μg/L for *Daphnia galeata*, and 1.56, 3.13, 6.25, 12.5, 25 and 50 μg/L for *Daphnia pulex* under static conditions for 48 hours according to OECD TG 202. The EC_{10} values of *Daphnia magna*, *Daphnia galeata* and *Daphnia pulex* on immobilization were 60.3 μg/L, 11 μg/L, and 4.37 μg/L, and EC_{50} values were 121 μg/L, 13.9 μg/L, and 8.95 μg/L, respectively.

An acute toxicity test of multiple sizes (10, 20, 30, and 50 nm) of nanosilver with *Daphnia magna* was conducted under static conditions for 48 hours according to OECD TG 202, 203 and a modified OECD TG 204. The LC_{50} value ranged from 4.31 to 30.36 mg in total Ag/L with increasing toxicity associated with decreasing particle size

Chronic toxicity to fish

A chronic toxicity test of silver powder (bare AgNPs, Sigma-Aldrich) with *Oryzias latipes* was conducted under semi-static conditions according to OECD TG 210, 212 and OECD draft proposal for a new guideline (2006) of Fish embryo toxicity test. The maximum toxicity of AgNPs and agglomeration rate reached at an Ag^+ ion ratio of 23% and 50%, respectively.

A chronic toxicity test of cAgNP with *Oryzias latipes* was conducted at 0.1, 0.25, 0.5, 0.75 and 1.0 mg/L for 14 days according to OECD TG 212. The LC_{50} of the embryo test after 24 to 96 h exposure decreased from 1.46 mg/L to 0.84 mg/L. LC_{50} of the acute adult toxicity test after 24 h was not calculated (> 1 mg/L), with no change in LC_{50} (0.8 mg/L) over the 48 h exposure period.

A chronic toxicity test of NM300K with *Danio rerio* was conducted for 35 days according to OECD TG 210. Hatch was not affected up to 136 μg/L and the NOEC was determined to be 23 μg/L. The most sensitive endpoint was growth, measured as total individual length and wet weight with a NOEC of 5.9 μg/L.

Chronic toxicity to invertebrates

A chronic toxicity test of NM300K with *Daphnia magna* was conducted at 10, 20, 51, 65, and 216.8 µg/L under semi-static conditions for 21 days according to OECD TG 211. EC_{10}, NOEC, and LOEC values on reproduction were 0.6 µg/L, 0.2 µg/L, and 0.5 µg/L, respectively. EC_{10}, NOEC, and LOEC values on mobility were 2.6 µg/L, ≥ 16.8 µg/L, and > 16.8 µg/L, and NOEC and LOEC values on age at first reproduction were ≥ 16.8 µg/L and > 16.8 µg/L, respectively.

A chronic toxicity test of NM300 with three aquatic invertebrates was conducted at 2.5, 5 and 10 µg/L for *Daphnia magna*, 1.25, 2.5, 5 and 10 µg/L for *Daphnia galeata*, and 1.25, 2.5 and 5 µg/L for *Daphnia pulex* for 21 days according to OECD TG 211. EC_{10} values of *Daphnia magna* and *Daphnia galeata* on reproduction were 0.92 µg/L and 3.45 µg/L, respectively and there was no effect on reproduction in parental generation on *Daphnia pulex*.

Toxicity to algae and cyanobacteria

A growth inhibition test of NM300K with *Desmodesmus subspicatus* was conducted at 0.005, 0.007, 0.010, 0.025, 0.050, 0.075, 0.100 and 0.250 mg/L under static conditions for 72 hours according to OECD TG 201. EC_{50} values on growth rate were from 0.057 to 0.1 mg/L and NOECs were from 0.01 to 0.048 mg/L.

A growth inhibition test of NM300K with *Anabaena flos-aquae* was conducted under static conditions for 72 hours according to OECD TG 201. Nominal concentrations were 0.002, 0.004, 0.02, 0.1 and 0.4 mg/L for Ag ions and 0.0005, 0.0013, 0.0078, 0.0195 and 0.0488 mg/L for NM300K. EC_{50} values for NM300K were 0.005 and 0.0011 mg/L (yield), 0.007 and 0.0017 mg/L (growth rate) and NOECs were 0.004 mg/L (yield, growth rate). EC_{50} values for Ag ions were 0.000024 mg/L (yield) and 0.000027 mg/L (growth rate).

A growth inhibition test of NM300K with *Pseudokirchnerella subcapitata* was conducted at 1-10,000 µg/L under static conditions for 72 hours according to ISO 8692. EC_{50} values on growth rate were from 423 to 1,167 µg/L.

Toxicity to aquatic plants

A growth inhibition test of NM300K with *Lemna minor* was conducted under static conditions for 7 days according to OECD TG 221. The species were exposed to 0.001-7.8 mg/L (experiment 1) or 0.006-7.8 mg/L (experiment 2 and 3) of NM300K and 0.001-0.78 mg/L of $AgNO_3$. EC_{50} values on frond number varied from 2.372 to 3.052 mg/L for NM300K and 0.164 mg/L for silver ion.

A growth inhibition test of NM300K with *Lemna minor* was conducted at 300-10,000 µg/L under static conditions for 7 days according to OECD TG 221 (modified EN ISO 20079). EC_{50} values on frond number varied from 744 to 1,697 µg/L.

A growth inhibition test of NM300K with *Myriophyllum spicatum* was conducted under static conditions for 14 days according to Maletzki *et al*., 2010. The species were exposed to 0.001, 0.01, 0.1, 1 and 10 mg/L for experiment 1 and 0.01, 0.02, 0.1, 0.25, 1 and 2.5 mg/L for experiment 2. EC_{50} values on main shoot length in both tests were 1.2122 mg/L and 4.8712 mg/L, respectively.

Toxicity to microorganisms

A SOS chromo test of cAgNPs with *Escherichia coli PQ37* was conducted at 0, 0.03, 0.32, 3.23, 16.13 and 32.26 mg/L of cAgNP and 0, 0.03, 0.32, 3.23, 16.13 and 32.26 mg/L of Ag ion for 2 hours. cAgNPs and Ag ion were not genotoxic based on the result of the SOS chromo test.

An antimicrobial activity and biofilm inactivation test of cAgNPs with *Pseudomonas aeruginosa PA01* was conducted at 10 mg/L of AgNPs, and 1 and 10 mg/L of Ag ions for 150 minutes. Silver ion had higher antimicrobial activity and biofilm inactivation than cAgNPs.

A survival rate test of AgNPs with *Escherichia coli* was conducted at 0.4 and 0.8 mg/L according to EPA (2007) Nanotechnology White Paper. The toxicity of AgNPs was accelerated in cases of stabilizers with cationic properties and hydrophobicity, but was not reduced by biocompatible compounds or polymers.

A growth inhibition, colony forming unit and liquid-to-plate assay of cAgNPs with *Escherichia coli* and *Bacillus subtilis* was conducted at 0, 10, 30 and 50 mg/L for growth inhibition assay, 0, 1, 5, and 10 mg/L for CFU assay, and 0, 0.025, 0.05, and 0.25 mg/L for LTP assay for 12 hours. In growth inhibition test, growth of *E. coli* was inhibited on early stage at 30 mg/L and 50 mg/L. In CFU assay, the EC_{50} values on *E. coli* and *B. subtilis* were 3.6 and >10 mg/L, respectively. In LTP assay, the EC_{50} on *E. coli* and *B. subtilis* were < 0.025 mg/L and < 0.025 mg/L after 3 h, respectively.

A growth inhibition and NP LB agar test of cAgNPs with *Escherichia coli* and *Bacillus subtilis* was conducted at 0 to 50 mg/L for growth inhibition and 0, 1, 5 and 10 mg/L for NP LB agar test and for 12 to 24 hours under static condition according to Lee *et al*., 2009 for LTP test. The EC_{50} was 3.64 mg/L for growth inhibition test and 3.16 to 4.19 mg/L for NP LB agar test in *E. coli* after 24 hours. In *B. subtilis*, the EC_{50} was > 10 mg/L for growth inhibition test after 24 hours.

A solid-contact test of NM300K with *Arthrobacter globiformis* was conducted at 30 °C for 2 hours according to DIN 38412-48. The EC_{50} was 34 mg/kg for dehydrogenase inhibition.

A luminescent bacteria test of NM300K with *Vibrio fisheri* was conducted for 30 minutes under static condition according to DIN EN ISO 11348-3. The EC_{50} of NM300K and silver nitrate was 10.56-13 mg/kg and 1.76 mg/L, respectively.

Toxicity to other organisms

A toxicity test of NM300 with *Potamopyrgus antipodarum* was conducted at 0.1, 10, 31.25, 62.5, 125, 250, 500 and 1,000 µg/L under semi-static conditions for 28 days. The EC_{10} and EC_{50} values were 5.57 µg/L and 15.0 µg/L, respectively.

A toxicity test of NM300 with *Sphaerium corneum* was conducted at 5, 25, 50, 100 and 500 µg/L under semi-static conditions for 28 days. The NOEC and LOEC values were 100 and 500 µg/L, respectively.

3.4.2. Sediment Toxicity

Sediment-water toxicity to chironomids

A sediment-water toxicity test of NM300K with *Chironomus riparius* was conducted at 0.3125, 0.625, 1.25, 2.5, 5.0 and 10.0 mg/L under static conditions for 28 days according to OECD TG 219 and in compliance with GLP. The EC_{50} values for emergence rate were 2.201 mg/L (midges), 2.415 mg/L (males)

and 1.835 mg/L (females), and NOEC was 1.25 mg/L for all three parameters. The EC_{50} values for development rate were 7.508 mg/L (midges), 5.828 mg/L (males) and 15.369 mg/L (females), and NOEC was 0.625 mg/L for all three parameters. The LOEC value for growth rate was 2.5 mg/L.

3.4.3. Terrestrial Toxicity

Acute toxicity to soil invertebrates except arthropods

A soil toxicity test of cAgNPs with *Caenorhabditis elegans* was conducted at 0.05, 0.5 and 5 mg/L for 24 hours. cAgNPs did not induce the stress of *C. elegans* in the genetic levels.

A soil toxicity test of cAgNPs with *Caenorhabditis elegans* was conducted up to 100 mg/L for 24 hours. cAgNPs induced adverse effects on *C. elegans* by decreasing survival rate (LC_{50}: 53.03 mg/L; NOEC: < 10 mg/L) and the number of larva (EC_{50}: > 100 mg/L; NOEC: 1 mg/L) in a dose dependent manner.

A soil toxicity test of cAgNPs with *Eisenia andrei* and *Perionyx excavatus* was conducted up to 2,000 mg/kg for 7 days according to OECD TG 207. In case of *E. andrei*, NOEC value (pinocytosis) was 300 mg/kg and pinocytosis effect was reduced at 2,000 mg/kg. In the case of *P. excavatus*, coelomocyte density (NOEC > 2,000 mg/kg) and pinocytosis effect (NOEC > 2,000 mg/kg) were not changed with increasing cAgNP concentrations.

Chronic Toxicity to soil invertebrates except arthropods

An earthworm reproduction test of NM300K with *Eisenia andrei* was conducted for 56 days according to OECD TG 222. After 28 days, the PNEC was 0.05mg/kg dry soil.

An earthworm reproduction test of NM300K with *Eisenia andrei* was conducted at 15, 30, 60, 120 and 200 mg/kg soil for 56 days according to OECD TG 222 and in compliance with GLP. The values of EC_{10}, EC_{20}, EC_{50}, LOEC and NOEC were 1.147 mg/kg, 1.309 mg/kg, 1.688 mg/kg, 1.5 mg/kg and 0.75 mg/kg, for reproduction.

An acute toxicity test of AgNPs with *Caenorhabdities elegans* was conducted at 0.1, 0.2, 1.5, 1, 2 and 4 mg/L for 24 and 72 hours. AgNPs exposure did not affect the survival and growth of the wild type and mutant strains' while reproduction was seriously affected with decreased number of offspring. The responses of the mtl-2 (*gk125*) and sod-3 (*gk235*) mutants were less sensitive to AgNPs exposure than the wild type, while the response of the daf-12 (*rh286*) mutant was similar to the wild type.

An acute toxicity test of cAgNPs with *Eisenia andrei* and *Perionyx excavatus* was conducted for 7 days. The NOEC values for pinocytosis and coelomocyte density with *E. andrei* were 300 mg/kg and > 2,000 mg/kg, respectively. In the case of *P. excavatus*, coelomocyte density (NOEC > 2,000 mg/kg) and pinocytosis effect (NOEC > 2,000 mg/kg) were not changed. The bioaccumulation factors were 0.001 (*E. andrei*) and 0.004 (*P. excavatus*). The 7d-EC_{50} and NOEC for survival with *E. andrei* were 530 mg/kg and 300 mg/kg, respectively.

An acute test of cAgNPs with *Caenorhabditis elegans* was conducted for 24. In NP NGM agar test, the LC_{50} (EC_{50}) and NOEC were 55.3 mg/L, < 10 mg/L for survival and > 100 mg/L, 1 mg/L for reproduction, respectively. In Ag ion toxicity test, the LC_{50} (EC_{50}) and NOEC were 22.31 mg/L, > 0.5 mg/L for survival and > 10 mg/L, 1 mg/L for reproduction, respectively.

An acute toxicity test of cAgNPs with *Caenorhabditis elegans* was conducted at 0.05, 0.5 and 5 mg/L for 24 days. The expression of *mt-1*, *mt-2*, *hsp-162*, *hsp-16.41*, *gst-4* and *cyp35a2* did not show any significance with increasing cAgNP concentration.

Acute toxicity to terrestrial plants

An acute toxicity test of cAgNPs with *Phaseolus radiatus* and *Sorghum bicolor* was conducted at 0, 5, 10, 20 and 40 mg/L (agar medium) and 0, 100, 300, 500, 1,000 and 2,000 mg/kg (soil medium) for 48 hours according to Lee *et al*., 2008 for the DAT test. The EC_{50} values of *P. radiatus* and *S. bicolor* for growth were 13 mg/L and 26 mg/L, respectively. The EC_{50} values of *S. bicolor* for root and shoot were < 5 and > 40 mg/L, respectively.

A seedling emergence and seedling growth test of NM300K with *Phaseolus aureus* was conducted at 1.5, 4.5, 13.5, 40.5 and 121.5 mg/kg for 14 days according to OECD TG 208. The EC_{25} was 33.5 mg/kg.

Chronic toxicity to terrestrial plants

A chronic toxicity test of cAgNPs with *Lycopersicon esculentum* was conducted at from 50 to 5,000 mg/L for 14 days and 6 weeks. Root elongation significantly decreased at every concentration. In greenhouse experiments, mature plants showed evidence of phytotoxicity exhibiting low chlorophyll contents, higher SOD, and less fruit production.

Toxicity to soil microorganisms

A nitrogen transformation test of NM300K with RefeSol soils was conducted at 15, 45, 100 and 200 mg/kg for 28 days according to OECD TG 216 and compliance with GLP. NM300K inhibited the nitrogen transformation of the soil micro-organism. Tests on the potential ammonium oxidation of soil organisms exposed to NM300K resulted in a NOEC of < 0.56 mg/kg and an EC10 of 0.5 mg/kg upon 28 days.

A carbon transformation test of NM300K with RefeSol soils was conducted at 1.67, 5, 15, 45 and 100 mg/kg for 28 days according to OECD TG 217 and in compliance with GLP. The EC_{10} and EC_{50} were 0.9 mg/kg and 13 mg/kg dry soil, respectively.

A soil enzyme activity test of cAgNPs with nature soil was conducted for 7 days. The EC_{50} values of DHA, FDA, urease, APA, ASA and BGA were 107.98, > 1,000, 14.2, > 1,000, > 1,000 and > 1,000 mg/kg for each enzyme. The NOEC values for same enzyme activities were 10, 100, < 1, > 1,000, 10 and 100 mg/kg.

4. TOXICOLOGICAL INFORMATION

4.1. Toxicokinetics, metabolism and distribution

4.1.1. Basic toxicokinetics

The toxicokinetics guideline TG 417 is not suitable for nanomaterials as the kinetics of nanomaterials is governed by other processes than for molecules: Transport of particles across barriers is, unlike for most molecules, not based on partitioning, which is gradient-driven, but on active (energy-consuming) transcellular transport. Particles are not expected to be metabolised. Distribution is through active processes of the mononuclear phagocyte system (MPS) rather than diffusion (OECD document ENV/JM/MONO(2016)24). Nevertheless, two toxicokinetics studies on silver nanomaterials have been proposed.

One toxicokinetics study was conducted according to OECD TG 417 and in compliance with GLP (NIER, 2011; Lee *et al.*, 2013). Citrate capped silver nanoparticles(cAgNPs, ABC Nanotech Co., Ltd., Korea) were administered to New Zealand White rabbits(4 males/dose) at 0.5 and 5 mg/kg bw as single dose via intravenous injection. Blood sampling was performed from ear vein of treated animals at 0, 5, 10, 30 mins, 1, 2, 6, 12 hours, 1 2, 3, 4, 5, 6, 7, 14, 21 and 28 days, respectively. Blood samples were also collected from non-treated control group at 1, 7 and 28 days. Faeces and urine were collected for 24 hours, 1, 2, 3, 4, 5, 6, 7, 14, 21 and 28 days after treatment. Tissues (liver, kidney, spleen, lung, brain, testis, and thymus) from 4 rabbits of a treated group were obtained for AgNPs distribution analysis at 1, 7, and 28 days after treatment. Bioavailability, tissue distribution and excretion ratios were calculated. No mortality was observed. AgNPs were easily accumulated in the body, although a decline in blood concentration was observed. The AUE (last) was 0.90 ± 0.16 and 3.65 ±00.68 μg·day/mL and t1/2 was 16.3 ± 2.9 days and 11.7 ± 1.3 days in 0.5 and 5 mg/kg bw-treated group, respectively. The half-time of release of silver from organs into blood could not be calculated because more than 50% of AgNPs were still retained in all the tested organs until the final day of 28 days. Excretion through feces after injection supports the idea of biliary excretion of AgNPs, which needs further investigation on the detailed mechanisms of AgNPs excretion.

The other toxicokinetics study was conducted according to OECD TG 417 and in compliance with GLP (Park *et al.*, 2011). Citrate capped silver nanoparticles (cAgNPs, ABC Nanotech Co., Ltd., Korea) were administered to Sprague-Dawley rats (4 males/dose) at 1 and 10 mg/kg bw as single dose via oral gavage or intravenous injection. Blood was collected at 10 min, 1, 2, 4, 8, 24, 48 and 96 hours after a single treatment. Liver, lung, and kidney were obtained at 24 and 96 hours after treatment. Excretion of AgNPs via faeces and urine was determined at 24 hours after treatment. Control animals were sacrificed after vehicle treatment, immediately. Bioavailability, tissue distribution and excretion ratios were calculated. Liver was found to be the main organ where absorbed silver nanoparticles were accumulated. Most of orally administered silver nanoparticles were excreted via feces. The absorption rates of cAgNPs through gastrointestinal tract showed very low-bioavailability of 1.2% and 4.2% in 1 and 10 mg/kg bw treated group, respectively. Most of orally administered cAgNPs were found in feces, suggesting a poor absorption rate.

Table 4.1. Summary of basic toxicokinetics studies

Test Material	Species/strain	Method	Results	Reference
cAgNPs (ABC Nanotech Co., Ltd.)	New Zealand rabbits (4 male/dose)	0.5 and 5 mg/kg bw, single intravenous injections, OECD TG 417	No mortality was observed. AgNPs were easily accumulated in the body, although a decline in blood concentration was observed. Excretion through feces after injection supports the idea of biliary excretion of AgNPs, which needs further investigation on the detailed mechanisms of AgNPs excretion.	NIER, 2011; Lee *et al.*, 2013
cAgNPs (ABC Nanotech Co., Ltd.)	Sprague-Dawley rats (4 male/dose)	1 and 10 mg/kg bw, single oral gavage or intravenous injection, OECD TG 417	Liver was found to be the main organ where absorbed silver nanoparticles were accumulated. Most of orally administered silver nanoparticles were excreted via feces. The absorption rates of cAgNPs through gastrointestinal tract showed very low-bioavailability.	Park *et al.*, 2011

4.2. Acute Toxicity

4.2.1. Inhalation

No information is available.

4.2.2. Dermal

Acute dermal toxicity study was conducted according to OECD TG 402 and in compliance with GLP (MKE, 2010; Kim *et al.*, 2013). Citrate capped silver nanoparticles (cAgNPs, ABC Nanotech Co., Ltd., Korea) were applied to five male and female Sprague-Dawley rats of 8 weeks and 11 weeks of age, respectively. No deaths or abnormal findings were observed at the maximum concentration for 14 days. Although the dose tested was specified as 2,000 mg/kg bw, 2,400 mg/kg bw was administered. It is because the specific gravity of 1.2 was not considered in preparation for the test material. LD_{50} of cAgNPs was considered to be higher than 2,000 mg/kg bw in rats.

4.2.3. Oral

Acute oral toxicity study was conducted according to OECD TG 423(MKE, 2010). Citrate capped silver nanoparticles (cAgNPs, ABC Nanotech Co., Ltd., Korea) at 300 and 2,000 mg/kg bw applied six male Sprague-Dawley rats of 7 weeks of age. A starting dose was 300 mg/kg bw, resulting in no observed symptom. Therefore, the dose was increased up to 2,000 mg/kg bw. No deaths or abnormal findings were observed at the maximum concentration for 14 days. LD_{50} of cAgNPs was considered to be higher than 2,000 mg/kg bw in rats.

Table 4.2. Summary of acute toxicity: dermal, oral

Test Material	Species/strain	Method	Results	Reference
Dermal				
cAgNPs (ABC Nanotech Co., Ltd.)	Sprague-Dawley rats (5 males /dose, 5 females/dose)	2,000 mg/kg bw, single intravenous injections, OECD TG 402	LD_{50} of cAgNPs was considered to be higher than 2,000 mg/kg bw in male and female rats.	MKE, 2010 ; Kim *et al.*, 2013
Oral				
cAgNPs (ABC Nanotech Co., Ltd.)	Sprague-Dawley rats (6 males/dose)	300 and 2,000 mg/kg bw, single oral gavage injection, OECD TG 423	No deaths or abnormal finding were observed at the maximum concentration for 14 days. LD_{50} of cAgNPs was considered to be higher than 2,000 mg/kg bw in male rats.	MKE, 2010

4.3. Irritation/corrosion

4.3.1. Skin irritation/corrosion

One skin irritation study was conducted according to OECD TG 404 and in compliance with GLP (MKE, 2010; Kim *et al.*, 2013). Citrate capped silver nanoparticles (cAgNPs, ABC Nanotech Co., Ltd., Korea) at 0 or 0.5 mL/6 cm^2 were exposed to three female New Zealand White Rabbits for 4 hours. Erythema and eschar formation and oedema formation were assessed 1, 24, 48 and 72 hours after application. Erythema, eschar formation or oedema formation was not observed in all the treated animals. The primary irritation index was 0.0. As a result, cAgNPs is not a skin irritant on female rabbits.

The other skin irritation study was conducted according to OECD TG 404 and in compliance with GLP (NIER, 2011). Three male New Zealand White Rabbits were dermally exposed to 0.5 mL/6 cm^2 skin of citrate capped silver nanoparticles (cAgNPs, ABC Nanotech Co., Ltd., Korea) for 4 hours. 1.0% citrate solution was used for negative control. Erythema and eschar formation and oedema formation were assessed 1, 24, 48 and 72 hours after application. Erythema, eschar formation or oedema formation was not observed in all the treated animals. The primary irritation index was 0.0. As a result, cAgNPs is not a skin irritant on female rabbits.

4.3.2. Eye irritation

Eye irritation study was conducted according to OECD TG 405 and in compliance with GLP (MKE, 2010; Kim *et al.*, 2013). Three male New Zealand White rabbits were exposed to 0.1 mL/eye (approximately, 0.02 g/eye) of citrate capped silver nanoparticles (cAgNPs, ABC Nanotech Co., Ltd., Korea) in one eye while another eye remained untreated and served as control. The eyes were examined and the changes were observed at 1, 24, 48 and 72 hours after application, and ocular reactions of conjunctivae, cornea and iris were scored according to the another eye remained untreated and served as control. The eyes were examined and the changes were observed at 1, 24, or oedema fea, iris, conjunctivae and chemosis were 0. Therefore, the index of acute ocular irritation was 0.0. As a result, cAgNPs is not an eye irritant on male rabbits.

Table 4.3. Summary of skin irritation and eye irritation

Test Material	Species/strain	Method	Results	Reference
Skin				
cAgNPs (ABC Nanotech Co., Ltd.)	New Zealand White rabbits (3 male /dose)	Male : 0.5 mL/6 cm^2 Applied gauze patch for 4 hours, OECD TG 404	Erythema, eschar formation or oedema formation was not observed in all the treated animals. The primary irritation index was 0.0. As a result, cAgNPs is not a skin irritant on female rabbits.	MKE, 2010 ; Kim *et al.*, 2013
cAgNPs (ABC Nanotech Co., Ltd.)	New Zealand White rabbits (3 female /dose)	Female : 0 or 0.5 mL/6 cm^2 Applied gauze patch for 4 hours, OECD TG 404	Erythema, eschar formation or oedema formation was not observed in all the treated animals. The primary irritation index was 0.0. As a result, cAgNPs is not a skin irritant on female rabbits.	NIER, 2011
Eye				
cAgNPs (ABC Nanotech Co., Ltd.)	New Zealand White rabbits (3 male /dose)	0.1 mL(0.02 g), OECD TG 405	The index of acute ocular irritation was 0.0. cAgNPs is not an eye irritant on male rabbits.	MKE, 2010 ; Kim *et al.*, 2013

4.4. Sensitization

4.4.1. Skin sensitization

One skin sensitization study was conducted according to OECD TG 406 and in compliance with GLP (NIER, 2011). CrlOri:HA guinea pigs(20 males/dose) were induced dermally with 0.4 mL of Citrate capped silver nanoparticles(cAgNPs, ABC Nanotech Co., Ltd., Korea) in a 2.5 x 2.5 cm2 occlusive patch. This induction process was performed on the 27th day with a 0.1% (w/v) 1-chloro-2,4-dinitrobenzene(DNCB) and the test substance. For negative control, however, distilled water was applied while for the positive control, 1% (w/v) DNCB was applied instead of the test substance. 10 males for positive control group and 10 males for negative control group were used. Skin reaction was graded according to "Magnusson and Kligman grading scale for the evaluation of challenge patch test reactions" in OECD TG No. 406. No skin reaction was observed in any of the treated groups at 24 and 48 hours after the challenge. 1%(w/v) DNCB induced skin sensitization. cAgNPs were a weak sensitizer in Guinea pig

The other skin sensitization study was conducted according to OECD TG 406 and in compliance with GLP(Kim *et al.*, 2013). Hartley guinea pigs (20 males) were induced with 3 pairs of intradermal injection. Citrate capped silver nanoparticles(cAgNPs, ABC Nanotech Co., Ltd., Korea) of 0.1 mL volume(20.48%) were given in the shoulder region at 1st induction. The volume of the test substance used for a 2 x 4 cm^2 occlusive patch was 0.5 mL at 2nd induction and challenge phase. The patch was lasted for 48 hours at 2nd induction phase and lasted for 24 hours at challenge phase. For negative control (10 males), 1.0% citrate solution was applied instead of the test material. Interval between the first and second induction was 1 week and, after 2 weeks later, challenge was performed. Skin reaction was graded according to "Magnusson and Kligman grading scale for the evaluation of challenge patch test reactions" in OECD TG 406. One out of 20 test animals (5%) exhibited grade 1 erythema at 24 or 48 hours after challenge, but no other skin reaction was observed in other animals. In this study, cAgNPs were a weak sensitizer in Guinea pig

Table 4.4. Summary of skin sensitization

Test Material	Species/strain	Method	Results	Reference
cAgNPs (ABC Nanotech Co., Ltd.)	CrlOri:HA Guinea pigs (20 male /dose)	0.4 mL, 2.5 x 2.5 cm^2 occlusive patch for 6 hours, OECD TG 406	No skin reaction was observed in any of the treated groups at 24 and 48 hours after the challenge. cAgNPs were a weak sensitizer in Guinea pig.	NIER, 2011
cAgNPs (ABC Nanotech Co., Ltd.)	Hartley Guinea pigs (20 male /dose)	3 pairs of intradermal injection of 0.1 mLvol.(20.48%), 2 x 4 cm^2 occlusive patch, OECD TG 406	One out of 20 test animals(5%) exhibited grade 1 erythema at 24 or 48 hours after challenge, but no other skin reaction was observed in other animals. cAgNPs were a weak sensitizer in Guinea pig.	MKE, 2010 ; Kim *et al.*, 2013

4.5. Repeated dose toxicity

4.5.1. Repeated dose toxicity: oral

Repeated dose oral toxicity studies were conducted according to OECD 422 and in compliance with GLP (Hong *et al.*, 2014). Citrate capped silver nanoparticles (cAgNPs, ABC Nanotech Co., Ltd., Korea) were administered by oral route to Sprague-Dawley rats (50/sex/dose) at 62.5, 125 and 250 mg/kg bw/day, once daily for 42 days(Male: 14 days before mating, 14 days during the mating and 14 days of post-mating, Female: 14 days before mating, during the mating and gestation and 4 days of lactation). A daily application volume (10 ml/kg) was adjusted according to the most recent body weight and the vehicle control rats were treated with an equivalent volume of distilled water. Examinations during the study included clinical observations, mortality, bodyweight, food and water consumption, haematology, blood chemistry, organ weight, gross and histopathological examinations. No signification toxicity or mortality was observed. Alopecia was observed in the vehicle control and treatment groups of both sexes. Salivation was observed in 1 female of the 250 mg/kg bw/day group on Day 1 of gestation. No significant differences in body weight, food and water consumption were observed in any of the dose groups. No statistically significant changes in haematological analysis were observed in any of the treatment groups, including

recovery group. In the serum biochemical analysis and urinalysis, no treatment related changes were observed. In both sexes, decreases in IP and AST and an increase in CI were also considered to be spontaneous because these were neither dose-related nor within the normal ranges. In recovery groups, a statistically significant increase in absolute and relative weights of liver was observed in males, and an increase in absolute weights of kidneys and adrenal glands were observed in females. No gross or histopathological findings were observed at necropsy. Under the test conditions, the NOAEL of AgNPs was considered to be higher than 250 mg/kg bw/day in Sprague-Dawley rats.

Repeated dose oral toxicity studies were conducted according to the OECD Guideline 407 and in compliance with GLP (Jeong *et al.*, 2010). Silver nanoparticles (cAgNPs, Namatech Co., Ltd., Korea) were administered by oral route to Sprague-Dawley rats (10/sex/dose) at 0, 30, 100 and 1,000 mg/kg bw/day (dosing volumes were 10 ml/kg), once daily for 28 days. Examination during the study included mortality, toxic effects, body weight, macroscopic and microscopic examination. No significant difference in body weight was observed in any of the dose groups. The treated samples showed luminal and surface particles and the tissue also contained silver nanoparticles. A dose-dependently increased accumulation of silver nanoparticles was observed in the lamina propria of both the small and large intestine, and also in the tip of the upper villi in the ileum and protruding surface of the fold in the colon. The cAgNPs treated rats exhibited a higher number of goblet cells that had released their mucus granules than the controls, resulting in more mucus materials in the crypt lumen and ileal lumen. Moreover, cell shedding at the tip of the villi was frequent. Lower amounts of neutral and acidic mucins were found in the goblet cells in the cAgNPs treated rats. Plus the amount of sialomucins was increased, while the amount of sulfomucins was decreased. In particular, in the colon of the sample treated rats, sialyated mucins were detected in the lamina propria, the connective tissue under the epithelia. cAgNPs are a powerful intestinal secretagogue and induce an abnormal mucin composition in the intestinal mucosa.

Repeated dose oral toxicity studies were conducted according to the OECD Guideline 408 and in compliance with GLP (MKE, 2008; Kim *et al.*, 2010) Silver powder (Namatech Co., Ltd., Korea) was administered by oral gavage to Fischer 344 rats(10/sex/dose) at 0, 30, 125 and 500 mg/kg bw/day for 90 days. Examinations during the study included mortality, clinical signs, body weight, food and water consumption, haematology, clinical chemistry, ophthalmoscope, organ weights, gross and histopathological examinations. No mortality or clinical signs were observed. Decrease in body weight gain was observed in male rats treated with 500 mg/kg bw/day after 4, 5 week?? and 7 ~ 13 weeks. No significant differences in food and water consumption were found between treated and control groups. Increase of monocyte number and decrease of reticulocytes were detected in female rats treated with 500 mg/kg bw/day and in female rats treated with 30 mg/kg bw/day, respectively. No difference in prothrombin time and activated partial thromboplastin time was observed among groups. Total cholesterol and total bilirubin were elevated in male rats treated with 125 mg/kg bw/day of silver powder, and total cholesterol was increased in male rats treated with 500 mg/kg bw/day. The decreases in magnesium, total protein, and inorganic phosphorus were detected in female rats treated with 125 mg/kg bw/day. The increases in total cholesterol and alkaline phosphatase and the decreases in magnesium, total protein, and inorganic phosphate were observed in female rats treated with 500 mg/kg bw/day of silver powder. No significant organ-weight changes were observed in either the male and female rats except for an increase in the weight of the left testis in the 500 mg/kg bw/day male rats, and for decrease in the weight of right kidney in the 30 and 125 mg/kg bw/day female rats. No significant difference in gross pathology was observed between treated and control groups. Bile duct hyperplasia and focal inflammation in liver were prominent, although dose-response relationship was not detected. The LOAEL and NOAEL of silver powder were 125 mg/kg bw/day and < 30 mg/kg bw/day, respectively in Fischer 344 rats.

Furthermore, non-GLP conform studies was conducted according to OECD 407 and 408 .In a repeated dose oral toxicity study conducted according to OECD 407(NIER No. 2010-49-1224), citrate capped silver nanoparticle (cAgNPs, ABC Nanotech Ce., Ltd., Korea) were administered by oral via

drinking water to Sprague-Dawley rats (5/sex/dose) at 0, 25, 100 and 400 mg/kg bw/day for 28 days. Examinations during the study included mortality, toxic effects, body weight, clinical biochemistry and macroscopic examination. No significant toxicity or mortality was observed. No significant difference in body weight was observed in any of the dose groups. Serum GPT was increased while blood urea nitrogen (BUN) was decreased in female rats, which showed dose-dependency. cAgNPs tested did not induce any toxicity in a repeated dose 28-day oral toxicity study in rodents.

In a repeated dose oral toxicity study conducted according to OECD 408(NIER No. 2010-49-1224), citrate capped silver nanoparticles(cAgNPs, ABC Nanotech Ce., Ltd., Korea) were administered by oral via drinking water to Sprague-Dawley rats(10/sex/dose) at 0, 25, 100 and 400 mg/kg bw/day for 90 days. Examination during the study included mortality, toxic effects, body weight, clinical biochemistry and macroscopic examination. No significant toxicity or mortality was observed. No significant difference in body weight was observed in any of the dose groups. Serum triglyceride (TG) was decreased while total bilirubin levels were increased in treated groups. However, there was no dose-dependent tendency and statistical significance was not tested. cAgNPs tested did not exhibit any toxicity in a repeated dose 90-day oral toxicity study in rodents.

4.5.2. Repeated dose toxicity: inhalation

Studies were performed according to 412 und 413 not yet adapted to accommodate nanomaterials. Nevertheless, two repeated inhalation toxcity studies on silver nanomaterials have been proposed.

A repeated dose inhalation toxicity study was conducted according to the OECD 412 Guideline and in compliance with GLP (Ji *et al.*, 2007) Silver powder (Daedeok Science, Korea, Purity: 99.98%) was administered by inhalation to Sprague-Dawley rats (10/sex/dose) at low (target dose, $1.2x10^4$ particles/cm^3, $1.2x10^6$ nm^2/cm^2), middle (target dose, $1.2x10^5$ particles/cm^3, $8.5x10^7$ nm^2/cm^2), and high(target dose, $1.2x10^6$ particles/cm^3, $1.8x10^9$ nm^2/cm^2)doses for 6 h/day, 5 days/week, for 90 days. Fresh air was used as control. Examination during the study included mortality, clinical observation of animals, body weight change, monitoring of food and water consumption, haematology, blood clinical chemistry, gross pathology, measurement of organ weights and histopathology. No mortality or clinical signs were observed. No significant difference in body weight and food/water consumption was observed in any of the dose groups. There were no significant dose related changes in the haematology values for the male rats. The percentage of neutrophils and eosinophils increased significantly ($p < 0.05$) in female rats in the low-dose group when compared with the control. The MCH in the female rats in the middle-dose group increased significantly ($p < 0.05$) when compared with the female rats in the high-dose group. The high-dose group revealed significantly increased ($p < 0.05$) calcium in both the male and female rats when compared with the control, and increased total protein ($p < 0.05$) in the male rats when compared with the control. Meanwhile, the low-dose group of male rats showed increased gamma-GT ($p < 0.05$) when compared with the control group. The exact meaning of these differences was impossible to clarify. No significant gross pathological or organ weight changes were observed. Histopathological examination of the male rat livers revealed one case of cytoplasmic vacuolization in the control, four cases in the low-dose group, and one case each in the middle- and high-dose groups, respectively. For the female rats, two cases of cytoplasmic vacuolization were detected in the control and low-dose group, respectively, six cases in the middle dose group, and seven cases in the high-dose group. Two cases of hepatic focal necrosis were detected in the male rats of the high-dose group and one case in the female rats of the high-dose group. The other organs, including the kidneys, spleen, lungs, adrenals, heart, reproductive organs, brain, and nasal cavity were also examined histopathologically, with no distinct findings. The silver concentration in the lung tissue from the groups exposed to silver nanoparticles for 28 days revealed a statistically significant ($p < 0.01$) dose-dependent increase. Although no clear silver concentrations were detected in the blood for any of the dose groups, a clear increase ($p < 0.05$) in silver concentration was observed in the liver for the high-dose group, along with a statistically significant ($p < 0.01$) increase in silver concentration in the brain. The olfactory bulb, which showed higher silver-concentration levels than the brain, also revealed a dose-dependent increase ($p < 0.01$) in both the male and female rats. The LOAEL of silver nanoparticles was considered to be 1.2 to ^{4} particles/cm^3 (= $1.22le^6$ nm^2/cm^2) in Sprague-Dawley rats.

A repeated dose inhalation toxicity study was conducted according to the OECD 413 Guideline and in compliance with GLP (KCL, 2008; Sung *et al.*, 2009). Silver nanoparticles (generated from solid silver wire by ISO/10801) were administered by inhalation particulate to groups of Sprague-Dawley rats (10/sex/dose) at the concentrations of 0, $0.6x10^6$ particles/cm^3 (= 49 μg/m^3), $1.4x10^6$ particles/cm^3 (= 133 μg/m^3) and $3.0x10^6$ particles/cm^3 (= 515 μg/m^3) by continuous exposure, 6 h/day, 5 days/week, for 90 days. HEPA filtered clean air was supplied to negative control group. Examinations during the study included mortality, clinical observation of animals, body weight change, monitoring of food and water consumption, haematology, blood clinical chemistry, ophthalmological examination, urinalysis, gross pathology, measurement of organ weights and histopathology. No mortality or clinical signs were observed. Decrease in body weight was detected in females exposed to $1.4x10^6$ particles/cm^3 from 3-week exposure. No significant difference in food consumption was observed in any of the dose groups. No significant differences in haematology and urinalysis were observed between treated and control groups. Increases of creatinine and total protein and decreases of chloride were detected in males exposed to $0.6x10^6$

particles/cm^3. However, all the values were within normal range and dose response was not detected. No abnormalities were observed in ophthalmoscopic examination. In male rats, cervical lymph node congestive spot, spleen hypertrophy, bladder congestive spot, intestinal nodule, and brain retraction were detected. In female rats, cyst in ovary, spleen hypertrophy, black spot in liver and adrenal gland, and intestinal nodule were observed. No significant difference was observed in organ weights in any of the dose groups. Liver and lung were appeared to be the target organs of inhaled silver nanoparticles. BAL analysis revealed concentration dependent increase in albumin, lactate dehydrogenase and total protein were found in female rats. Decreases in tidal volume were observed in the male rats, which was concentration-dependent. In case of female rats, such decrease was detected in the group exposed to $1.4x10^6$ particles/cm^3. No change was detected in prothrombin time and activated partial thromboplastin time. Significant increase in erythrocyte clotting was found in female rats exposed to $3.0x10^6$ particles/cm^3. Distribution in lung, kidney, liver, blood, brain and olfactory nerve was prominent and concentration dependent. The NOAEL of silver nanoparticles was $1.0x10^6$ particles/cm^3 or 100 μg/m^3 in Sprague-Dawley rats.

4.5.3. Repeated dose toxicity: dermal

No information is available.

4.5.4. Repeated dose toxicity: other routes

No information is available.

Table 4.5. Summary of Repeated dose toxicity studies: inhalation, oral

Test Material	Species/strain	Method	Results	Reference
Oral				
cAgNPs (ABC Nanotech Co., Ltd.)	Sprague-Dawley rats (50/sex/dose)	62.5, 125 and 250 mg/kg bw/day, Oral injections, Once daily for 42 days, OECD TG 422	The NOAEL of AgNPs was considered to be higher than 250 mg/kg bw/day in Sprague-Dawley rats. In recovery groups, a statistically significant increase in absolute and relative weights of liver was observed in males, and an increase in absolute weights of kidneys and adrenal glands were observed in females.	NIER, 2011
cAgNPs (ABC Nanotech Co., Ltd.)	Sprague-Dawley rats (10/sex/dose)	0, 30, 100 and 1,000 mg/kg bw/day, Oral injections, Once daily for 28 days, OECD TG 407	Silver nanoparticles are a powerful intestinal secretagogue and induce an abnormal mucin composition in the intestinal mucosa.	Jeong *et al.*, 2010
Silver powders (Namatech Co.,	Fischer 344 rats (10/sex/dose)	0, 30, 125 and 500 mg/kg bw/day,	The LOAEL and NOAEL of Silver	MKE, 2008 ; Kim *et al.*,

Ltd.)		Oral injections, Once daily for 90 days, OECD TG 408	powder was 125 mg/kg bw/day and < 30 mg/kg bw/day, respectively in Fischer 344 rats.	2010
cAgNPs (ABC Nanotech Co., Ltd.)	Sprague-Dawley rats (5/sex/dose)	0, 25, 100 and 400 mg/kg bw/day, Oral injections, Once daily for 28 days, OECD TG 407	cAgNPs tested did not induce any toxicity in the repeated dose for a 28-day oral toxicity study in rodents.	NIER No. 2010-49-1224
cAgNPs (ABC Nanotech Co., Ltd.)	Sprague-Dawley rats (10/sex/dose)	0, 25, 100 and 400 mg/kg bw/day, Oral injections, Once daily for 90 days, OECD TG 408	cAgNPs tested did not exhibit any toxicity in the repeated dose for a 90-day oral toxicity study in rodents.	NIER No. 2010-49-1224
Inhalation				
Silver powder (Daedeok Sci.)	Sprague-Dawley rats (10/sex/dose)	$1.2x10^4$ particles/cm^3 ($1.2x10^6$ nm^2/cm^2), $1.2x10^5$ particles/cm^3, ($8.5x10^7$ nm^2/cm^2), $1.2x10^6$ particles/cm^3, ($1.8x10^9$ nm^2/cm^2), Inhalation route, Once daily for 90 days, OECD TG 412	The current 28-day study of silver nanoparticle inhalation in Sprague-Dawley rats indicated that the silver nanoparticle doses used did not cause any significant health effects. However, the lung silver concentration exhibited a dose dependent increase following silver nanoparticle inhalation exposure.	Ji *et al.*, 2007
AgNPs (generated from solid silver wire by ISO/10801)	Sprague-Dawley rats (10/sex/dose)	0, $0.6x10^6$ particles/cm^3 (49 μg/m^3), $1.4x10^6$ particles/cm^3 (133 μg/m^3), $3.0x10^6$ particles/cm^3 (515 μg/m^3), Inhalation route, Once daily for 90 days, OECD TG 413	The NOAEL of silver nanoparticles was $1.0x10^6$ particles/cm^3 (= 100 μg/m^3) in Sprague-Dawley rats.	MKE, 2008 ; Sung *et al.*, 2009

4.6. Genetic toxicity

4.6.1. Genetic toxicity: in vitro

The bacterial reverse mutation assay is not adequate for nanomaterials. Nevertheless, one study on silver nanomaterials has been proposed.

A bacterial reverse mutation assay was conducted according to the OECD Guideline 471 and in compliance with GLP (KCL, 2011; Kim *et al.*, 2013), strains of *S.typhimurium* (TA 1535, TA 1537, TA 98 and TA 100) and *E.coli* (WP2uvrA) were exposed to citrate capped silver nanoparticles (cAgNPs, ABC

Nanotech Co., Ltd., Korea). Based on a range-finding test, cytotoxic concentrations were as follows: with metabolic activation: > 0.63 μL/plate (TA98 and TA1537); 1.25 μL/plate (TA100, TA1535 and WP2uvrA); without metabolic activation: > 0.16 μL/plate (TA100); 0.31 μL/plate (TA98, TA1535, TA1537 and WP2uvrA). Metabolic activation system used in this test was S9 mix 0.1 mL (=0.01 mL S9)/plate; S9 fraction prepared from liver homogenates of Sprague-Dawley male rat induced with Aroclor 1254. Vehicle and positive control groups were also included in mutagenicity tests. No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to test item at any concentrations in either the presence or absence of S9 mix. The positive and vehicle controls induced the appropriate responses in the corresponding strains indicating the validity of the study. The cAgNPs are not considered as mutagenic in *S. typhimurium* (TA 1535, TA 1537, TA 98 and TA 100) and *E. coli* (WP2uvrA) strains.

An *in vitro* chromosome aberration test was conducted according to the OECD Guideline 473 and in compliance with GLP (KCL, 2011; Kim *et al.*, 2013), Chinese hamster ovary fibroblast, CHO-K1 cells were exposed to citrate capped silver nanoparticles (cAgNPs, ABC Nanotech Co., Ltd., Korea). Based on a range-finding test, cytotoxic concentration were as follows: with metabolic activation: 0.156 μL/mL; without metabolic activation: 0.078 μL/mL (6 h) and 0.078 μL/mL (24 h). In test without S9 mix, frequencies of aberrant metaphase in 24 h treated cells were 0.5, 0.5, 0.0, 0.5 for 0, 0.001, 0.003, 0.005 μL/mL treated cells, respectively. Cells treated for 6 h and recovered for 18 h exhibited 0.5, 2.0, 0.5 and 1.5 of frequency in 0, 0.005, 0.010 and 0.019 μL/mL treated groups, respectively. In test with S9 mix, frequency of aberrant metaphase was 0.5 in all groups. No significant increases in chromosomal aberrations were observed at any concentrations in either the presence or absence of S9 mix. The cAgNPs are not considered as clastogenic in Chinese hamster ovary fibroblast, CHO-K1 cells with and without metabolic activation.

4.6.2. Genetic toxicity: in vivo

An *in vivo* bone marrow micronucleus assay was conducted according to the OECD Guideline 474 and in compliance with GLP (KCL, 2007; Kim *et al.*, 2008), groups of Sprague-Dawley rats (10/sex/dose) were oral gavage administered with Silver nanoparticles (Namatech Co., Ltd., Korea) at the dose levels of 0, 30, 300 and 1,000 mg/kg bw/day for 28 days. Polychromatic (PCE) and normochromatic (NCE) erythrocyte ratio was established to determine the toxicity and incidence of micronucleated immature erythrocytes (MNPCE) from 2,000 immature erythrocytes was scored. Treatment of test substance did not show any cytotoxic effects to bone marrow cells and did not induce significant increase in micronuclei formation in all treated groups. No clinical signs or mortality were observed. No significant changes in body weight, food/water consumption were observed. The LOAEL and NOAEL was 300 and 30 mg/kg bw/day, respectively. Oral administration of silver nano did not increase the incidence of micronuclei formation, suggesting it is not genotoxic under the test conditions.

An *in vivo* bone marrow micronucleus assay was conducted according to the OECD Guideline 474 and in compliance with GLP (KCL, 2011; Kim *et al.*, 2011), 4 groups of Sprague-Dawley rats (10/sex/dose) were administered via inhalation with Silver nanoparticles (ABC Nanotech Co., Ltd., Korea) at the dose levels of fresh-air control, 0.7×10^6 particles/cm^3, 1.4×10^6 particles/cm^3, and 2.9×10^6 particles/cm^3 for 6 h/day, 5 days/week, for 13 weeks. Polychromatic (PCE) and PCE/normochromatic (NCE) erythrocyte ratio was established to determine the toxicity and incidence of micronucleated immature erythrocytes (MNPCE) from immature erythrocytes was scored. There were no statistically significant differences in the micronucleated polychromatic erythrocytes or in the ratio of polychromatic erythrocytes among the total erythrocytes after silver nanoparticle exposure when compared with the control. No clinical signs or mortality were observed. No significant changes in body weight, food/water consumption were observed. The LOAEL and NOAEL was 125 and 30 mg/kg bw/day, respectively. The

present results suggested that a 90 day exposure to silver nanoparticles by inhalation did not induce genetic toxicity in male or female rat bone marrow *in vivo*.

However, as concerns the in vivo bone marrow micronucleus test: as long as it cannot be demonstrated that the test compound has reached the target tissue, a negative outcome of the test does not guarantee the absence of genotoxicity

Table 4.6. Summary of Genetic toxicity studies: *in vitro*, *in vivo*

Test Material	Species/strain	Method	Results	Reference
In vitro				
cAgNPs (ABC Nanotech Co., Ltd.)	*S.typhimurium* (TA 1535, TA 1537, TA 98 and TA 100), *E.coli* (WP2uvrA)	*S.typhimurium* TA98, TA100, TA1535, TA1537, *E.coli* WP2uvr - Without S9 mix: 0, 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, 0.31 μL/plate (= 0, 1, 2, 4, 8, 16, 32, 62 μg/plate) - With S9 mix: 0, 0.04, 0.08, 0.16, 0.31, 0.63, 1.25, 2.5 μL/plate (= 0, 8, 16, 32, 62, 126, 250, 500 μg/plate). *S.typhimurium* TA100: - With S9 mix: 0, 0.02, 0.04, 0.08, 0.16, 0.31, 0.63, 1.25 μL/plate (= 0, 4, 8, 16, 32, 62, 126, 250 μg/plate), A bacterial reverse mutation assay, OECD TG 471	The cAgNPs are not considered as mutagenic in *S. typhimurium*(TA 1535, TA 1537, TA 98 and TA 100) and *E. coli*(WP2uvrA) strains.	KCL, 2011; Kim *et al.*, 2013
cAgNPs (ABC Nanotech Co., Ltd.)	Chinese hamster ovary fibroblast (CHO-K1 cell)	- without S9 mix: 0, 0.001, 0.003, 0.005 μL/mL(24 h) 0, 0.005, 0.010, 0.019 μL/mL(6 h) 18 h recovery period. - with S9 mix: 0, 0.039, 0.078 and 0.156 μL/mL *in vitro* chromosome aberration test, OECD TG 473	The cAgNPs are not considered as clastogenic in Chinese hamster ovary fibroblast, CHO-K1 cells with and without metabolic activation.	KCL, 2011; Kim *et al.*, 2013
In vivo				
Silver nanoparticles	Sprague-Dawley rats	0, 30, 300, 1,000 mg/kg bw/day,	Oral administration of silver nano did not	Ji *et al.*, 2007

(Namatech Co., Ltd.)	(10/sex/dose)	Once daily for 28 days, Oral gavage injection, *in vivo* bone marrow micronucleus assay, OECD TG 474	increase the incidence of micronuclei formation, suggesting it is not genotoxic under the test conditions. The LOAEL and NOAEL was 300 and 30 mg/kg bw/day, respectively.	
Silver powders (Namatech Co., Ltd.)	Fischer 344 rats (10/sex/dose)	0.7×10^6 particles/cm^3, 1.4×10^6 particles/cm^3, 2.9×10^6 particles/cm^3 Once daily for 90 days, Inhalation route, *in vivo* bone marrow micronucleus assay, OECD TG 474	The present results suggested that a 90 day exposure to silver nanoparticles by inhalation did not induce genetic toxicity in male or female rat bone marrow *in vivo*. The LOAEL and NOAEL was 125 and 30 mg/kg bw/day, respectively.	Kim *et al.*, 2009

4.7. Carcinogenicity

No information is available.

4.8. Toxicity to reproduction

4.8.1. Toxicity to reproduction

A Combined Repeated Dose Toxicity Study with the Reproduction / Developmental Toxicity Screening Test was conducted according to OECD Guideline 422 and in compliance with GLP (KTR, 2011; Hong et al., 2013), Citrate capped silver nanoparticle (cAgNPs, ABC Nanotech Co., Ltd., Korea) was orally administered to groups of Sprague-Dawley rats (50/sex/dose) at 0, 62.5, 125 and 250 mg/kg bw/day (dosing volumes were 10 ml/kg) for 42 days (Male: 14 days before mating, 14 days during the mating, and 14 days of post-mating; Female: 14 days before mating, during the mating and gestation, and 4 days of lactation). During the study, data was recorded on mortality, clinical signs, estrus cycle, mating evaluation, functional observation, gestation period, the number of corpora lutea and implantation, delivery rate, the number of live and dead pups, the percentage of live and dead pups to implantations, pre-implantation loss, post-implantation loss, sex ratio, survival rate, number of neonates with external anomalies and body weights of pups. No treatment-related changes in detailed functional observation were observed in any of the treatment groups. No statistically significant differences were observed in the following parameters examined: gestation period, the number of corpora lutea and implantation, delivery rate, the number of live and dead pups, the percentage of live and dead pups to implantations, pre-implantation loss, post-implantation loss, sex ratio, survival rate, number of neonates with external anomalies, and body weights of pups on postnatal day 0 and day 4. NOAEL of cAgNPs is considered to be more than 250mg/kg bw/day for general toxicity in parent animals.

4.8.2. Developmental toxicity

A Combined Repeated Dose Toxicity Study with the Reproduction / Developmental Toxicity Screening Test was conducted according to OECD Guideline 422 and in compliance with GLP(KTR, 2011; Hong *et al.*, 2014), Citrate capped silver nanoparticle (cAgNPs, ABC Nanotech Co., Ltd., Korea)

was orally administered to groups of Sprague-Dawley rats(50/sex/dose) at 0, 62.5, 125 and 250 mg/kg bw/day (dosing volumes were 10 ml/kg) for 42 days (Male: 14 days before mating, 14 days during the mating, and 14 days of post mating; Female: 14 days before mating, during the mating and gestation, and 4 days of lactation). During the study, data was recorded on clinical observations, gestation period, the number of corpora lutea and implantation, delivery rate, the number of live and dead pups, the percentage of live and dead pups to implantations, pre-implantation loss, post-implantation loss, sex ratio, survival rate, number of neonates with external anomalies and body weights of pups. No statistically significant differences were observed in the following parameters examined: the number of live and dead pups, the percentage of live and dead pups to implantations, sex ratio, survival rate, number of neonates with external anomalies, and body weights of pups on post-natal day 0 and day 4. NOAEL of the test article are considered to be more than 250 mg/kg bw/day for general toxicity in F1 pups.

Table 4.7. Summary of Reproduction toxicity studies: *in vitro, in vivo*

Test Material	Species/strain	Method	Results	Reference
Reproduction toxicity				
cAgNPs (ABC Nanotech Co., Ltd.)	Sprague-Dawley rats(50/sex/dose)	0, 62.5, 125 and 250 mg/kg bw/day, Oral injection for 42 days(Male: 14 days before mating, 14 days during the mating, and 14 days of post mating; Female: 14 days before mating, during the mating and gestation, and 4 days of lactation), OECD TG 422	NOAEL of cAgNPs is considered to be more than 250mg/kg bw/day for general toxicity in parent animals.	KTR, 2011; Hong *et al.*, 2013
Developmental toxicity/teratogenicity				
cAgNPs (ABC Nanotech Co., Ltd.)	Sprague-Dawley rats(50/sex/dose)	0, 62.5, 125 and 250 mg/kg bw/day, Oral injection for 42 days(Male: 14 days before mating, 14 days during the mating, and 14 days of post mating; Female: 14 days before mating, during the mating and gestation, and 4 days of lactation), OECD TG 422	NOAEL of the test article are considered to be more than 250 mg/kg bw/day for general toxicity in F1 pups.	KTR, 2011; Hong *et al.*, 2014

4.9. Specific investigations

4.9.1. Neurotoxicity

No information is available.

4.9.2. Immunotoxicity

No information is available.

4.9.3. Specific investigations: other studies

No information is available.

4.10. Exposure related observations in humans

4.10.1. Health surveillance data

A health surveillance study was conducted in a workplace manufacturing silver nanoparticles. A total of 5 workers were normally involved in the silver nanomaterial manufacturing (Lee *et al.*, 2011), only 2 workers participated in the voluntary biomonitoring program. The workers were 37 and 42 years of age, male, and had worked for 7 years in the silver nanoparticles manufacturing industry. The air samples were taken by drawing air through mixed cellulose ester filters in sampling cassettes (37 mm diameter, 0.8 mm nominal pore-size, and 2 in. cowl) obtained from Pall Corp.(P/N 64678; Ann Arbor, MI). The filter samples for personal sampling were collected in the breathing zone using MSA (Escort Elf pump)-operated sampling pumps at a flow rate of 1.5 ~ 2.0 L/min and SKC (Leland Legacy pump) operated sampling pumps at a flow rate of 6.9 ~ 7.3 L/min when the work duration was short. The sampling with personal samplers was performed during the normal work period from 9:30 to 16:00 and typically lasted 159 ~ 350 min. The total suspended particulate concentration was determined gravimetrically based on the NIOSH manual of analytical methods (NMAM) 0500(1994). Silver concentrations on the filter were analysed using an ICP (Perkin Elmer optima 5300DV) based on the NMAM 7300-ICP method (NIOSH, 2003) after wet digestion using the ICP-OES Plasma Spectrometer method. The concentration of silver in blood and urine was analysed with a flameless method using an atomic absorption spectrophotometer equipped with a Zeeman graphite furnace (Perkin Elmer 5100ZL, Zeeman Furnace Module, USA) based on the NIOSH 7300 method. Two male workers who had worked for 7 years in the business of manufacturing silver nanomaterial were being exposed to 0.15777 and 0.10869 mg/m^3 of total suspended particulate, however the silver concentrations were 0.35 and 1.35 mg/m^3, which are lower than the current occupational exposure limit for both silver dust and soluble silver compounds. The health surveillance data indicated that the nanomaterial manufacturing workers were exposed to much less than the concentrations of silver dust (100 mg/m^3) or soluble silver (10 mg/m^3) threshold limit values for the workplace air or silver nanoparticle 90-day inhalation toxicity NOAEL of 100 mg/m^3 (Sung *et al.*, 2009). Furthermore, their blood and urine concentrations of silver were within the normal reported range, and their health status estimated by their blood biochemistry and haematology showed no significant findings.

4.10.2. Epidemiological data

No information is available.

4.10.3. Direct observations: clinical cases, poisoning incidents and other

No information is available.

4.10.4. Sensitization data (humans)

No information is available.

4.10.5. Exposure related observations in humans: other data

A study was conducted at workplaces where the workers handle nanomaterials, personal sampling, area monitoring, and real-time monitoring using a scanning mobility particle sizer (SMPS) and dust monitor were performed to estimate the potential exposure of workers. The air samples were taken by drawing air through mixed cellulose ester filters in sampling cassettes(37 mm diameter, 0.8 mm nominal pore-size, and 2 in. cowl) obtained from Pall Corp.(P/N 64678; Ann Arbor, MI). The filter samples for personal sampling were collected in the breathing zone using MSA (Escort Elf pump) operated sampling pumps at a flow rate of 1.5 ~ 2.0 L/min and SKC (Leland Legacy pump) operated sampling pumps at a flow rate of 6.9 ~ 7.3 L/min when the work duration was short. The sampling with personal samplers was performed during the normal work period from 09:30 to 16:00 and typically lasted 159 ~ 350 min. An scanning mobility particle sizer (SMPS) combining a differential mobility analyser (DMA, 4220, HCT Co., Ltd., Korea) and condensation particle counter(CPC, 4312, HCT Co., Ltd., 0 ~ 108 particles/cm^3 detection range) was used to monitor the particle size distribution with the electrical mobility diameter ranging from 15 to 710.5 nm. A dust monitor (Model 1.108, Grimm) was used to observe the particle size distribution with the diameter ranging from 0.3 to 20 μm. After wet digestion using the inductive coupled plasma (ICP)-OES Plasma Spectrometer method, silver concentrations on the filter were analysed using an ICP(Perkin Elmer optima 5300DV) based on the NIOSH Manual 7300-ICP method (NIOSH, 2003a). AgNPs were morphologically identified using a scanning transmission electron microscope. The silver metal concentrations ranged from 0.00002 to 0.00118 mg/m^3, which were also lower than the silver dust 0.1 mg/m^3 and silver soluble compound 0.01 mg/m^3 occupational exposure limits set by the ACGIH. Similarly, the particle concentrations at the silver nanoparticle manufacturing workplaces increased when the sodium citrates were weighed or reacted with the silver nitrates, and during the cleaning of the workplace. The number of silver nanoparticles in the samples obtained from the workplace manufacturing silver nanoparticles using induced coupled plasma ranged from 57,789 to 2,373,309 particles/cm^3 inside the reactor with an average size of 20 ~ 30 nm and 535 ~ 25,022 particles/cm^3 with a wide range of particle sizes due to agglomeration or aggregation after the release of nanoparticles into the workplace air. In contrast, the silver nanoparticles manufactured by the wet method ranged from 393 to 3526 particle/cm^3 with an average size of 50 nm. Thus, when taken together silver nanoparticle concentrations were relatively lower than existing occupational exposure limits.

4.11. Toxic effects on livestock and pets

No information is available.

4.12. Additional toxicological information

No information is available.

4.13. Summary

4.13.1. Toxicokinetics, metabolism and distribution

The toxicokinetics studies were conducted according to OECD TG 417 and in compliance with GLP. cAgNPs were administered to rabbits (4 males/dose) at 0.5 and 5 mg/kg bw as single dose via intravenous injection. No mortality was observed. AgNPs were easily accumulated in the body, although a decline in blood concentration was observed. cAgNPs were administered to rabbits (4 males/dose) at 0.5 and 5 mg/kg bw and rats(4 males/dose) at 1 and 10 mg/kg bw as single dose via oral gavage or intravenous injection

according to OECD TG 417 and in compliance with GLP. Liver was found to be the main organ where absorbed silver nanoparticles were accumulated. Most of orally administered silver nanoparticles were excreted via feces. The absorption rates of cAgNPs through gastrointestinal tract showedvery low-bioavailability.

4.13.2. Acute toxicity

Dermal

Acute dermal toxicity studies were conducted according to OECD TG 402. LD_{50} of cAgNPs was considered to be higher than 2,000 mg/kg bw in male and female rats.

Oral

Acute oral toxicity studies were conducted according to OECD TG 423. No deaths or abnormal findings were observed at the maximum concentration for 14 days. LD_{50} of cAgNPs was considered to be higher than 2,000 mg/kg bw in male rats.

4.13.3. Irritation/corrosion

Skin

Skin irritation studies were conducted according to OECD TG 404 and in compliance with GLP. cAgNPs were exposed to three female rabbits at 0 or 0.5 mL/6 cm^2 and three male rabbits at 0.5 mL/6 cm^2 for 4 hours. cAgNPs are not a skin irritant on female and male rabbits.

Eye

Eye irritation study was conducted according to OECD TG 405 and in compliance with GLP. cAgNPs were exposed to three male rabbits at 0.1 mL/eye in one eye while another eye remained untreated and served as control. cAgNPs are not an eye irritant male rabbits.

4.13.4. Sensitization

Skin sensitization studies were conducted according to OECD TG 406 and in compliance with GLP. Guinea pigs (20 males/dose) were induced dermally with 0.4 mL of cAgNPs in a 2.5 x 2.5 cm^2 occlusive patch. No skin reaction was observed in any of the treated groups at 24 and 48 hours after the challenge. cAgNPs were a weak sensitizer in Guinea pig.

In another OECD TG 406 test, guinea pigs (20 males/dose) were induced with 3 pairs of intradermal injection of cAgNPs (0.1 mL volume (20.48%)) in a 2 x 4 cm^2 occlusive patch. One out of 20 test animals (5%) exhibited grade 1 erythema at 24 or 48 hours after challenge, but no other skin reaction was observed in other animals. cAgNPs were a weak sensitizer in Guinea pig.

4.13.5. Repeated dose toxicity

Oral

Repeated dose oral toxicity studies were conducted according to OECD TG 422 and in compliance with GLP. cAgNPs was administered by oral route to groups of Sprague-Dawley rats (50/sex/dose) at 0, 62.5, 125 and 250 mg/kg bw/day, once daily for 42 days (Male: 14 days before mating, 14 days during the mating and 14 days of post-mating, Female: 14 days before mating, during the mating and gestation and 4

days of lactation). NOAEL of AgNPs was considered to be higher than 250 mg/kg bw/day in Sprague-Dawely rats.

Repeated dose oral toxicity studies were conducted according to OECD TG 407 and in compliance with GLP. cAgNPs was administered by oral route to groups of Sprague-Dawley rats (10/sex/dose) at 0, 30, 100 and 1,000 mg/kg bw/day, once daily for 28 days.cAgNPs are a powerful intestinal secretagogue and induce an abnormal mucin composition in the intestinal mucosa.

Repeated dose oral toxicity study was conducted according to OECD TG 408 and in compliance with GLP. cAgNPs was administered by oral route to groups of Fischer 344 rats (10/sex/dose) at the dose levels of 0, 30, 125 and 500 mg/kg bw/day for 90 days. The LOAEL and NOAEL of Silver powder was 125 mg/kg bw/day and < 30 mg/kg bw/day, respectively in Fischer 344 rats.

Furthermore, a non-GLP conform studies were conducted according to OECD 407 and 408.

A repeated dose oral toxicity study was conducted according to OECD 407(NIER No. 2010-49-1224), cAgNPs was administered by oral via drinking water to groups of Sprague-Dawley rats(5/sex/dose) at the dose levels of 0, 25, 100 and 400 mg/kg bw/day for 28 days. cAgNPs tested did not induce any toxicity in the repeated dose for a 28-day oral toxicity study in rodents.

A repeated dose oral toxicity study was conducted according to OECD 408(NIER No. 2010-49-1224), cAgNPs was administered by oral via drinking water to groups of Sprague-Dawley rats(10/sex/dose) at the dose levels of 0, 25, 100 and 400 mg/kg bw/day for 90 days. cAgNPs tested did not induce any toxicity in the repeated dose for a 90-day oral toxicity study in rodents.

Inhalation

A repeated dose inhalation toxicity study was conducted according to the OECD TG 412 and in compliance with GLP. Silver powder was administered by inhalation to groups of Sprague-Dawley rats (10/sex/dose) at the concentrations of $1.2x10^4$ particles/cm^3 ($1.2x10^6$ nm^2/cm^2), $1.2x10^5$ particles/cm^3 ($8.5x10^7$ nm^2/cm^2), and $1.2x10^6$ particles/cm^3 ($1.8x10^9$ nm^2/cm^2) for 6 h/day, 5 days/week, for 90 days. Fresh air used as control. The current 28-day study of silver nanoparticle inhalation in Sprague-Dawley rats indicated that the silver nanoparticle doses used did not cause any significant health effects. However, the lung silver concentration exhibited a dose dependent increase following silver nanoparticle inhalation exposure. The LOAEL of Silver nanoparticles was considered to be 1.2×10^4 particles/cm^3 (= 1.2×10^6 nm^2/cm^2) in Sprague-Dawley rats.

A repeated dose inhalation toxicity study was conducted according to the OECD TG 413 and in compliance with GLP. Silver nanoparticles was administered by inhalation particulate to groups of Sprague-Dawley rats (10/sex/dose) at the concentrations of 0, $0.6x10^6$ particles/cm^3 (49 μg/m^3), $1.4x10^6$ particles/cm^3 (133 μg/m^3) and $3.0x10^6$ particles/cm^3 (515 μg/m^3) by continuous exposure, 6 h/day, 5 days/week, for 90 days. HEPA filtered clean air was supplied to negative control group. The NOAEL of silver nanoparticles was $1.0x10^6$ particles/cm^3 (100 μg/m^3) in Sprague-Dawley rats.

Dermal

No information is available.

4.13.6. Genetic toxicity

In vitro

A bacterial reverse mutation assay was conducted according to the OECD Guideline 471 and in compliance with GLP. Strains of *S.typhimurium* (TA 1535, TA 1537, TA 98 and TA 100) and *E.coli* (WP2uvrA) were exposed to cAgNPs. Based on a range-finding test, cytotoxic concentrations were as follows: with metabolic activation: > 0.63 μL/plate (TA98 and TA1537); 1.25 μL/plate (TA100, TA1535 and WP2uvrA); without metabolic activation: > 0.16 μL/plate (TA100); 0.31 μL/plate (TA98, TA1535, TA1537 and WP2uvrA). The cAgNPs are not considered as mutagenic in *S. typhimurium* (TA 1535, TA 1537, TA 98 and TA 100) and *E. coli* (WP2uvrA) strains.

An *in vitro* chromosome aberration test was conducted according to the OECD Guideline 473 and in compliance with GLP. Chinese hamster ovary fibroblast, CHO-K1 cells were exposed to cAgNPs. Based on a range-finding test, cytotoxic concentration were as follows: with metabolic activation: 0.156 μL/mL; without metabolic activation: 0.078 μL/mL (6 h) and 0.078 μL/mL (24 h). In test without S9 mix, frequencies of aberrant metaphase in 24 h treated cells were 0.5, 0.5, 0.0, 0.5 for 0, 0.001, 0.003, 0.005 μL/mL treated cells, respectively. Cells treated for 6 h and recovered for 18 h exhibited 0.5, 2.0, 0.5 and 1.5 of frequency in 0, 0.005, 0.010 and 0.019 μL/mL treated groups, respectively. In test with S9 mix, frequency of aberrant metaphase was 0.5 in all groups. The cAgNPs are not considered as clastogenic in Chinese hamster ovary fibroblast, CHO-K1 cells with and without metabolic activation.

In vivo

An *in vivo* bone marrow micronucleus assay was conducted according to the OECD Guideline 474 and in compliance with GLP, groups of Sprague-Dawley rats (10/sex/dose) were oral gavage administered with Silver nanoparticles at 0, 30, 300 and 1,000 mg/kg bw/day for 28 days. The LOAEL and NOAEL was 300 and 30 mg/kg bw/day, respectively. Oral administration of silver nano did not increase the incidence of micronuclei formation, suggesting it is not genotoxic under the test conditions.

An *in vivo* bone marrow micronucleus assay was conducted according to the OECD Guideline 474 and in compliance with GLP, 4 groups of Sprague-Dawley rats (10/sex/dose) were administered via inhalation with Silver nanoparticles at the fresh-air control, 0.7×10^6 particles/cm^3, 1.4×10^6 particles/cm^3, and 2.9×10^6 particles/cm^3 for 6 h/day, 5 days/week, for 13 weeks. The LOAEL and NOAEL was 125 and 30 mg/kg bw/day, respectively. The present results suggested that a 90 day exposure to silver nanoparticles by inhalation did not induce genetic toxicity in male or female rat bone marrow *in vivo*.

4.13.7. Carcinogenicity

No information is available.

4.13.8. Toxicity to reproduction

A Combined Repeated Dose Toxicity Study with the Reproduction / Developmental Toxicity Screening Test was conducted according to OECD Guideline 422 and in compliance with GLP. cAgNPs was orally administered to groups of Sprague-Dawley rats (50/sex/dose) at 0, 62.5, 125 and 250 mg/kg bw/day for 42 days (Male: 14 days before mating, 14 days during the mating, and 14 days of post-mating; Female: 14 days before mating, during the mating and gestation, and 4 days of lactation). The NOAEL of cAgNPs is considered to be more than 250mg/kg bw/day for general toxicity in parent animals.

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