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[원 저]

Effects of Size, Impurities, and Citrate Capping on the Toxicity of Manufactured Silver Nano-particles to Larval Zebrafish (*Danio rerio*)

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ABSTRACT

Objectives: This study was conducted to identify factors determining the toxicity of manufactured silver nano-particles (AgNPs) on aquatic organisms.

Methods: For this purpose, we prepared several AgNPs with varied characteristics, including hydrodynamic size (nano-$^{ABC}Ag^{Cit}$ vs -sized-$^{ABC}Ag^{Cit}$), impurities (^{ABC}Ag stock vs ^{ABC}Ag), and citrate capping ($^{ABC}Ag^{Cit}$), using a commercially available manufactured AgNP (^{ABC}Ag stock). Acute tests were conducted using larval zebrafish (*Danio rerioI*). In addition, in order to determine the ecotoxicological potentials of various capping agents, toxicity tests were conducted with microbes, waterfleas, and fish for eight different capping agents that are used for NPs.

Results: The toxicity of AgNPs in terms of 96 h fish LC_{50} increased in the following order: ^{ABC}Ag stock < ^{ABC}Ag = $^{ABC}Ag^{Cit}$ = nano-$^{ABC}Ag^{Cit}$ < μ-sized-$^{ABC}Ag^{Cit}$ < $AgNO_3$. After removing impurities by dialysis, 96 h LC_{50} value decreased significantly from 126.6 μg/L (95% confidence intervals [CI]: 107.0-146.2) (^{ABC}Ag stock) to 78.6 μg/L (CI: 72.7-84.8) (^{ABC}Ag). For μ-sized-$^{ABC}Ag^{Cit}$ (ranging between 3.9 and 40.6 nm) and $^{ABC}Ag^{Cit}$ (40.6 nm and 9.1 μm), the 96 h LC_{50} of the former (43.9 μg/L, CI: 36.0-51.7) was approximately two-fold lower than that of the latter (87.0 μg/L, CI: 73.5-100.3).

Conclusions: In this study, we found that for acute lethality, the contribution of impurities and particle size was significant, but that of citrate was negligible.

Keywords: *Danio rerio*, gill, nano hazard, particle size, silver nanoparticles

I. Introduction

Manufactured silver nanoparticles (AgNPs) have been extensively used in various industrial and healthcare applications because of their antimicrobial effects.[1,2)] While ionic silver (Ag) is considered to have more toxicity than AgNP,[1)] toxicological potentials of AgNPs are expected because of their large surface area-to-volume ratios. However, due to the complexity of the factors including size, shape, chemistry, crystallinity, surface properties, and agglomeration state, little is known about the physic-chemical parameters that may affect the toxicity of AgNPs.

Among several physico-chemical characteristics of NPs that were known to influence the toxicity, the size of NPs is one of the most important determinants of toxicity, since the number of atoms exposed on the surface may increase as particle size decreases.[3,4)] Other properties such as surface characteristics and impurities of NPs may also influence the toxicity of NPs. This is especially the case with transition metals.[5)] Various organic compounds used as a

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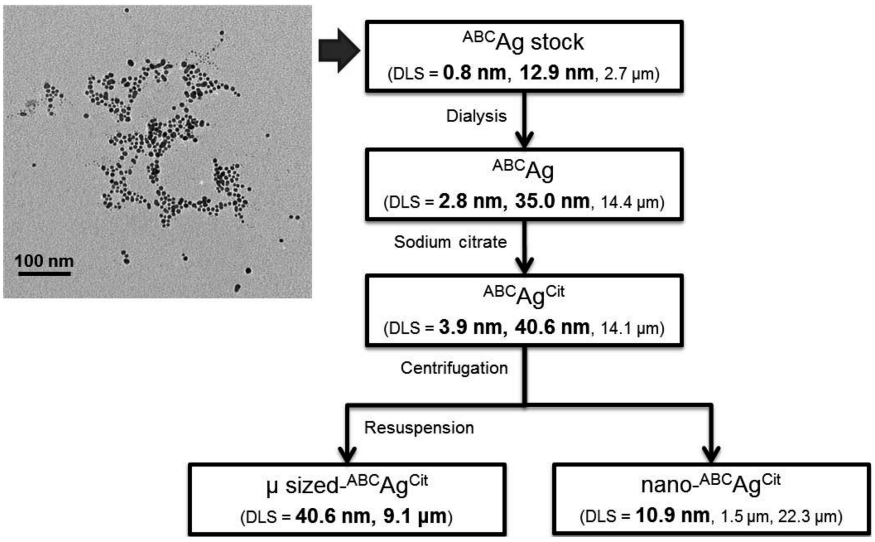

Fig. 1. TEM image of the AgNP colloid purchased and hydrodynamic size of AgNPs derived from modification procedure.

capping agent for NPs may exert toxicity when they are released.

Reported LC_{50}s for AgNPs ranged from 0.0346 to 250 mg/L depending on the test species and exposure duration: With Japanese medaka fish, the 96 h LC_{50} of 0.0346 mg/L were reported for AgNPs with a size of 49.6 nm in suspension.[6] In another study with the medaka fish, Wu *et al*.[2] reported the 48 h LC_{50} of 1.03 mg/L for 20–37 nm AgNPs, suggesting lesser toxicity than the AgNPs of a greater size (49.6 nm). With zebrafish (*Danio Rerio*), the LC_{50} was estimated at 250 mg/L (24 h exposure, undefined),[7] 0.084 mg/L (48 h exposure, 0.2% polyvinyl pyrrolidone coated, 73.55 nm),[8] and 7.07 mg/L (48 h exposure, uncoated, 44.5 and 216 nm).[9] These reports suggest that size or coating characteristics may explain the differences of AgNPs toxicity.

To date, little studies have attempted to determine the influence of the factors such as size, capping agent, or impurities, on the toxicity of AgNPs. This study was conducted to identify the factors determining the toxicity of AgNPs on the aquatic organism. For this purpose, we prepared four types of AgNPs with different characteristics of impurities, capping, or particle size, and evaluated their effects on toxicity using zebrafish (*D. rerio*) as a model animal. In addition, we evaluated the toxicities of commonly used capping agents to aquatic organisms. For this purpose, marine bacterium *Vibrio fischeri* and two model freshwater species including *Daphnia magna* and *D. rerio* were employed.

II. Materials and Methods

1. Preparation of the AgNPs and Instrumental analysis

Manufactured AgNP colloid with a range of 5-25 nm diameter (^{ABC}Ag stock) (SARPU-200KW, lot no. SL-112B4DD01, ABC Nanotech, Korea) was purchased from ABC Nanotech. For the removal of aggregated particles, the ^{ABC}Ag stock solution was filtered through a 0.2 μm syringe filter and stored at 4°C under dark conditions. The AgNPs were prepared by the following procedure (Fig. 1): The colloidal AgNP solution was dialyzed for 24 h in zebrafish culture water (dechlorinated tap water), using a dialysis membrane (molecular weight cut off 3.5 kDa), in order to remove excess ionic Ag and impurities present in the solution, if any (^{ABC}Ag). Citrate capped AgNPs ($^{ABC}Ag^{Cit}$) were generated by capping citrate with molar ratio of Ag:citrate=1:12.5 mM. Nano-sized AgNP fraction (nano-$^{ABC}Ag^{Cit}$) was separated from $^{ABC}Ag^{Cit}$ solution by centrifuging at 14000 rpm for 10 min and subsequently filtering through a 0.22 ìm pore size filter. The faction which was settled down by centrifugation was resuspended by pipetting and was considered as 'micro (μ) sized-$^{ABC}Ag^{Cit}$'. The hydrodynamic size of AgNPs in

Table 1. Molecular weight and formula of capping agents used in acute toxicity tests

Test chemical	MW	Formula
Sodium citrate (citrate)	294.1	$C_6H_8O_7Na_3 \cdot 2H_2O$
Gum arabic (GA)	300,000-800,000	$[C_{26}H_{34}N_2O_{13}]n$
Mercaptoacetic acid (MAA)	92.12	$HSCH_2COOH$
Maleic acid sodium (MAS)	138.05	$C_4H_3O_4Na$
3-Mercaptopropionic acid (MPA)	106.14	$HSCH_2CH_2CO_2H$
Disodium salt oxalic acid (oxalate)	134	NaOCOCOONa
Pyromellitic acid (PMA)	254.16	$C_{10}H_6O_8$
Sodium dodecyl sulfate (SDS)	288.38	$C_{12}H_{25}OSO_3Na$

zebrafish culture water was determined using a dynamic light scattering (DLS) instrument (Qudix, Scatteroscope II, Seoul, Korea). The shape and size of AgNPs were measured using transmission electron microscopy (TEM, Hitachi H-7600, Tokyo, Japan). In order to determine the AgNPs concentration, samples were digested with an acidified solution (4% HNO_3 [v/v]) and analysed by ICP-AES (Optima-4300 DV; PerkinElmer, Waltham, MA, USA).

2. Toxicity Tests

The 96 h acute fish toxicity tests were conducted with $AgNO_3$ and several types of AgNPs based on the OECD 203 Test Guidelines[10] with some modifications. Six days post-fertilization (dpf) larvae were used in the test. Five concentrations and one control group were used, with four replicates for each treatment. Five larval fish were placed in each replicate. Zebrafish were considered dead if there was no visible movement when they were gently touched by the caudal peduncle.[10] Test solutions were renewed at every 48 h, and *Artemia* nauplii were fed just before water renewal. Mortality of the fish was recorded daily until test termination and the median lethal concentration (LC_{50}) values were calculated. Test for $AgNO_3$ was conducted in parallel to compare ionic Ag with AgNPs.

Additionally, to assess toxicity of different capping agents (Table 1), a marine bacterium *V. fischeri* and two model freshwater species including *D. magna* and *D. rerio* were employed. The Microtox® (Strategic Diagnostics Inc., Newark, DE, USA) toxicity assay using *V. fischeri* was conducted to evaluate acute toxicity during the various treatments. Modified 81.9% Basic Test protocol was followed for the toxicity determination, with 5 and 15 min of exposure. For the 48 h acute *Daphnia* toxicity tests, US Environmental Protection Agency (US EPA) guidelines[11] were followed. *D. magna* was cultured and maintained in moderately hard water in Environmental Toxicology Laboratory, Seoul National University following US EPA.[11] Four replicates with five neonates each (<24 h old) were exposed to various concentrations of capping agents. Immobile organisms were considered to be dead and the number of immobile organisms was recorded daily after initiation of exposure. The 96 h acute toxicity tests for zebrafish were conducted with the same method as described above.

For *Daphnia* and fish toxicity tests, water quality parameters, including dissolved oxygen, pH, conductivity and temperature, were monitored daily. Hardness and alkalinity of the culture water were measured and logged whenever new batches of media were prepared, following American Public Health Association.[12] The concentrations of the $AgNO_3$ and AgNPs were expressed in Ag, to make direct comparison between toxicity values possible. All capping agents and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3. Statistical analysis

In the individual toxicity tests of *Daphnia* and zebrafish, the EC_{50} and LC_{50}, and 95% confidence intervals (CI) were calculated by a modified US EPA probit analysis and Spearman–Karber method using ToxStat (version 3.5; West Inc., Cheyenne, WY, USA). The median inhibitory concentrations (IC_{50s}) and CI for each sample were calculated using a vendor-provided software, Microtox Omni (Azur Environmental, Carlsbad, CA, USA).

Table 2. The LC_{50}s of $AgNO_3$ and AgNPs on *D. rerio*

Toxicant	Exposure (h)	LC_{50}	95% confidence interval
$AgNO_3$	24	22.4	(17.0-27.8)
	48	8.9	(7.0-10.7)
	72	5.9	(4.2-7.6)
	96	5.0	(3.4-6.6)
^{ABC}Ag stock	24	174.4	(149.8-198.9)
	48	169.2	(144.6-193.7)
	72	126.6	(107.0-146.2)
	96	126.6	(107.0-146.2)
^{ABC}Ag	24	92.0	(83.6-100.4)
	48	92.0	(83.6-100.4)
	72	78.5	(70.3-86.8)
	96	78.6	(72.7-84.8)
$^{ABC}Ag^{Cit}$	24	113.4	(95.6-131.2)
	48	95.9	(82.9-109.0)
	72	91.1	(77.8-104.5)
	96	87.0	(73.5-100.3)
μ-sized-$^{ABC}Ag^{Cit}$	24	53.5	(44.1-63.0)
	48	49.1	(40.2-57.9)
	72	43.9	(36.0-51.7)
	96	43. 9	(36.0-51.7)
nano-$^{ABC}Ag^{Cit}$	24	104.0	(91.8-117.7)
	48	100.8	(89.0-114.2)
	72	100.8	(89.0-114.2)
	96	93.6	(82.4-106.4)

Units are in ìg/L of Ag ion. Values in parenthesis are 95% confidence interval. Test solution was renewaled after 48 h exposure. LC_{50}: the median lethal concentration.

II. Results

Acute fish toxicities of AgNPs as well as ionic Ag are summarized in Table 2. Values for 96 h LC_{50} of ^{ABC}Ag stock, ^{ABC}Ag, $^{ABC}Ag^{Cit}$, nano-$^{ABC}Ag^{Cit}$, μ-sized-$^{ABC}Ag^{Cit}$, and $AgNO_3$ were 126.6 μg/L (CI: 107.0-146-2), 78.6 μg/L (CI: 72.7-84.8), 87.0 μg/L (CI: 73.5-100.3), 93.6 μg/L (CI: 82.4-106.4), 43.9 μg/L (CI: 36.0-51.7), and 5.0 μg/L (CI: 3.4-6.6), respectively. That is, the toxicity of the different AgNPs and ionic Ag, in terms of 96 h LC_{50}, increased in the following order: ^{ABC}Ag stock < ^{ABC}Ag = $^{ABC}Ag^{Cit}$ = nano-$^{ABC}Ag^{Cit}$ < μ-sized-$^{ABC}Ag^{Cit}$ < $AgNO_3$.

Fig. 2 shows hydrodynamic size distributions of AgNPs. Major sizes of ^{ABC}Ag stock, ^{ABC}Ag, $^{ABC}Ag^{Cit}$, μ-sized-$^{ABC}Ag^{Cit}$, and nano-$^{ABC}Ag^{Cit}$ were 12.9 nm, 35.0 nm, 40.6 nm, 40.6 nm, and 10.9 nm, respectively. AgNPs also, contained particles of 0.8 nm, 35.0 nm, 2.8 nm, 3.9 nm, 9.1 μm, and 22.2 μm, respectively.

The toxicity values and associated CI that were obtained from *V. fischeri*, *D. magna*, and *D. rerio* for each capping agent are presented in Table 3. Citrate showed slight toxicity on *D. magna* (48 h EC_{50}: 371.0 mg/L) but toxicity was not observed in *V. fischeri* (15 min IC_{50}: >409.5 mg/L) and *D. rerio* (96 h LC_{50} = >1000 mg/L). Acute median lethal effects of MAA, MAS, and MPA to *D. magna* were estimated in the mg/L range.

IV. Discussion

The toxicity of the different AgNPs and ionic Ag, in terms of 96 h LC_{50}, increased in the following order: ^{ABC}Ag stock < ^{ABC}Ag = $^{ABC}Ag^{Cit}$ = nano-$^{ABC}Ag^{Cit}$ < μ-sized-$^{ABC}Ag^{Cit}$ < $AgNO_3$. Toxicity contribution of impurities appeared to be important because 96 h LC_{50} significantly decreased from 126.6 μg/L (CI: 107.0-146.2) to 78.6 μg/L (CI: 72.7-84.8) after removing impurities by dialysis. Citrate cappling did not influence the acute toxicity because there was no significant difference in 96 h fish LC_{50} values between ^{ABC}Ag and $^{ABC}Ag^{Cit}$ characterized by presence and absence of citrate capping.

The amount of Ag ion in ^{ABC}Ag stock was estimated at ~0.04% from total Ag concentration of ^{ABC}Ag stock, i.e., 20,444 mg/L, and that of ^{ABC}Ag, i.e., 20,436 mg/L (data not shown), hence the effects of ionic Ag were considered minimal. The 96 h LC_{50} of μ-sized-$^{ABC}Ag^{Cit}$ (43.9 μg/L, CI: 36.0-51.7) was about two fold lower than that of $^{ABC}Ag^{Cit}$ (87.0 μg/L, CI: 73.5-100.3). Considering the size distribution of these two Ag samples (Fig. 2c and d), Ag particles of micrometer size (9.1 μm) of the μ-sized-$^{ABC}Ag^{Cit}$ are responsible for the greater toxicity compared to the $^{ABC}Ag^{Cit}$. This observation is interesting because smaller NPs have greater surface area-to-volume ratios and hence generally show greater toxicity (see review of Luyts, Napierska, Nemery and Hoet[13]). Greater toxicity of the ì-sized-$^{ABC}Ag^{Cit}$ compared to the AgNPs of nano-size range could be in part explained by the characteristics of the fish gill. Bigger particles are more likely to be entrapped in the gill compared to the smaller sized ones:

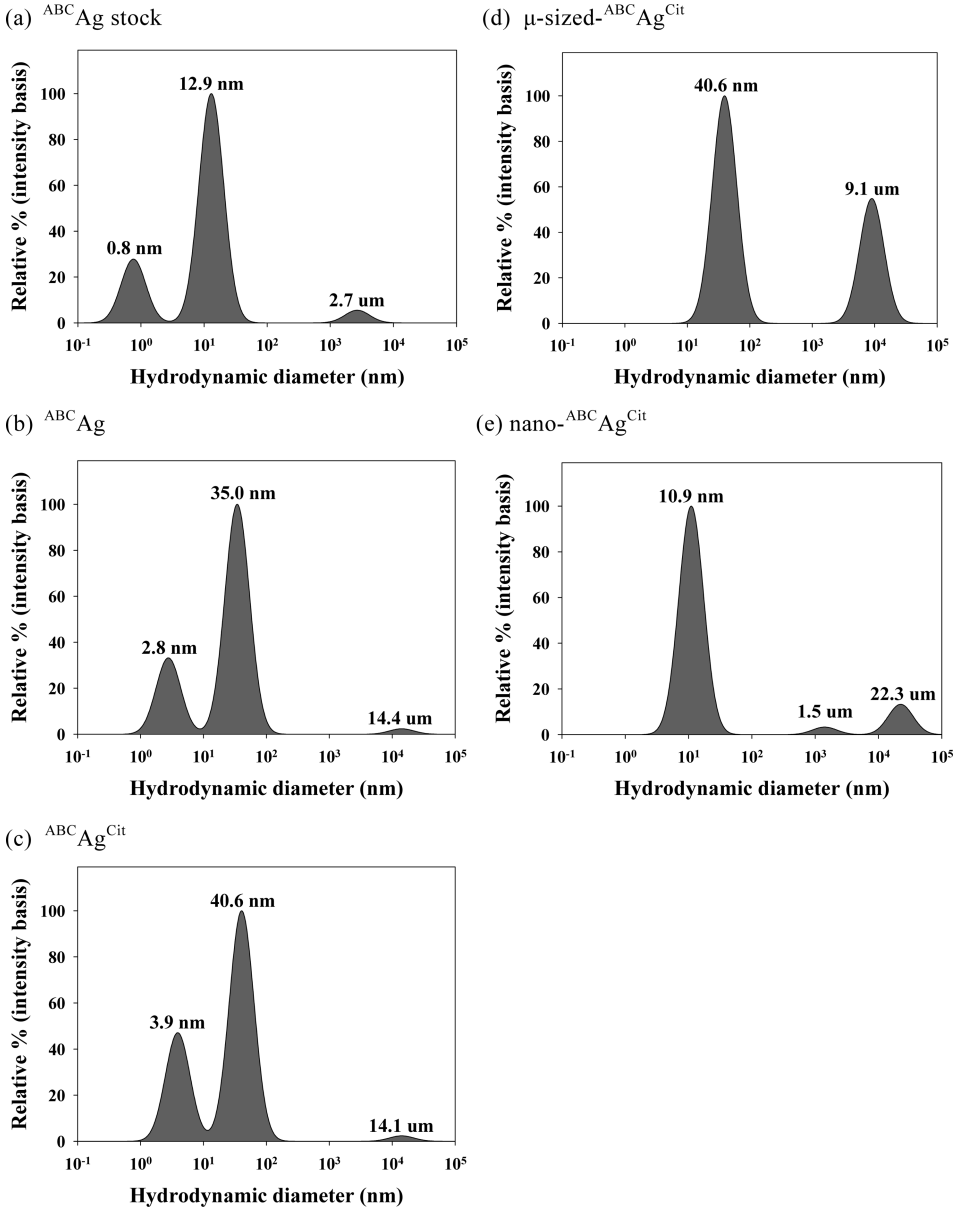

Fig. 2. Hydrodynamic size distributions by intensity measured by dynamic light scattering method for (a) ^{ABC}Ag stock, (b) ^{ABC}Ag, (c) $^{ABC}Ag^{Cit}$, (d) ì-sized-$^{ABC}Ag^{Cit}$, and (e) nano-$^{ABC}Ag^{Cit}$. Measurements were achived using AgNPs dilluted (^{ABC}Ag stock) or suspended (the rest) in zebrafish culture water.

Sanderson et al.[14] reported that finely crushed flakes (0.1–1.0 mm in diameter) stimulated the secretion of mucus sufficiently to form strands and aggregates of mucus-bound particles on the branchial arches. In a previous study using *D. magna*, we have also observed that TiO_2 NPs within a certain size range (>~200 nm to micron) were more likely trapped in the filter apparatus of *Daphnia* and exert greater toxicity that smaller sized particles (manuscript under review). On the other hand, fish gill forms acidic microenvironment which may accelerte the release of ionic Ag from AgNPs entrapped in mucus

Table 3. Toxicity values for capping agents in *Vibrio fischeri*, *Daphnia magna*, and *Danio rerio*

Capping agents	Vibrio fischeri		Daphnia magna		Danio rerio	
	Exposure (min)	IC_{50}	Exposure (h)	EC_{50}	Exposure (h)	LC_{50}
Citrate	5	>409.5	24	683.0 (556.9-809.1)	48	>1000
	15	>409.5	48	371.0 (279.1-462.9)	96	>1000
GA	5	>409.5	24	>1000	48	>1000
	15	>409.5	48	>1000	96	>1000
MAA	5	-	24	3.7 (1.9-5.5)	48	-
	15	-	48	1.5 (1.1-1.9)	96	-
MAS	5	87.18 (78.53-96.8) [3]	24	41.1 (28.4-53.8)	48	>500
	15	65.47 (54.23-79.01)	48	18.3 (19.4-27.1)	96	>500
MPA	5	-	24	6.5 (5.4-7.7)	48	-
	15	-	48	3.6 (2.6-4.5)	96	-
Oxalate	5	>409.5	24	151.6 (106.0 -197.2)	48	>500
	15	>409.5	48	55.8 (38.9-72.7)	96	>500
PMA	5	27.10	24	>500	48	-
	15	10.82	48	70.4 (56.5-84.2)	96	-
SDS	5	1.41 (0.86-2.32)	24	0.24 (0.19-0.29)	48	>12.5
	15	0.53 (0.43-0.66)	48	0.04(0.01-0.07)	96	5.9 (4.8-7.0)

Units are mg/L. Values in parenthesis indicate 95% confidence interval. -: not available; IC_{50}: the median inhibitory concentration; EC_{50}: the median effective concentration; LC_{50}: the median lethal concentration; Citrate: sodium citrate; GA: gum arabic; MAA: mercaptoacetic acid; MAS: maleic acid sodium; MPA: 3-mercaptopropionic acid; Oxalate: disodium salt oxalic acid; PMA: pyromellitic acid; SDS: sodium dodecyl sulfate.

of the fish gill. CO_2 released from the gills can be dissociated to bicarbonate, releasing H^+ ions.[15] Furthermore, fish gills are a primary target of metals from the external environment and serve as a major route for metal uptake.[16-18] Therefore, the toxicities of AgNPs in fish are largely manifested at the gills. However, we could not control perfectly the size of ì-sized-$^{ABC}Ag^{Cit}$ to micron due to technical limitation in removeal of nano-sized particles. In order to elucidate the conclusion that the bigger size of AgNP is the more toxic to fish, preparation method to remove nano particles in the μ-sized-$^{ABC}Ag^{Cit}$ should be deveoped.

Citrate showed slight toxicity on *D. magna* (48 h EC_{50}: 371.0 mg/L) but toxicity was not observed in *V. fischeri* (15 min IC_{50}: >409.5 mg/L) and *D. rerio* (96 h LC_{50} = >1000 mg/L). This result could explain in part why there was no difference in toxicity before/after citrate capping, e.g., between ^{ABC}Ag and $^{ABC}Ag^{Cit}$ (Table 2). Compared to *V. fischeri* and *D. rerio*, *D. magna* was generally more sensitive to the test capping agents except for PMA. However, SDS showed the greatest acute toxic effect on *V. fischeri* (15 min IC_{50}: 0.53 mg/L), *D. magna* (48 h EC_{50}: 0.04 mg/L), and *D. rerio* (96 h LC_{50}: 5.9 mg/L) among the test capping agents. Therefore, the use of SDS as cappling agent should not be encouraged especially when the release of SDS capped NPs into environment is expected.

V. Conclusion

To identify the factors determining the toxicity of manufactured AgNP on the aquatic organism, we prepared four types of AgNPs with different characteristics of impurities, capping, or particle size, and evaluated their effects on toxicity using zebrafish. We found that for acute lethality, contribution of impurities and particle size was significant but that of citrate was negiligible.

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