Original Article

Size-Dependent Toxicity of Silver Nanoparticles to *Glyptotendipes tokunagai*

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* Short running head:

Size-dependent toxicity of Ag NPs

Abstract

Objectives

This study aims to evaluate the size-dependent toxicity of spherical silver nanoparticles (Ag NPs) to an endemic benthic organism, *Glyptotendipes tokunagai*.

Methods

Ag nanoparticles of three nominal sizes (50, 100, and 150 nm) capped with polyvinyl pyrrolidone (PVP-Ag NPs) were used. Their physicochemical properties, acute toxicity (48 h), and bioaccumulation were measured using third instar larvae of *G. tokunagai*.

Results

The aggregation and dissolution of PVP-Ag NPs increased with exposure time and concentration, respectively, particularly for 50 nm PVP-Ag NPs. However, the dissolved concentration of Ag ions was not significant compared with the median lethal concentration (LC_{50}) value for $AgNO_3$ (3.51 mg/L). The acute toxicity of PVP-Ag NPs was highest for the smallest particles (50 nm), whereas bioaccumulation was greatest for the largest particles (150 nm). However, larger PVP-Ag NPs were absorbed and excreted rapidly, resulting in shorter stays in *G. tokunagai* than the smaller ones.

Conclusions

The size of PVP-Ag NPs significantly affects their acute toxicity to *G. tokunagai*. In particular, smaller PVP-Ag NPs have a higher solubility and stay longer in the body of *G. tokunagai*, resulting in higher toxicity than larger PVP-Ag NPs.

Keywords

Bioacummulation, *Chironomus*, Nanotoxicity, Nanoparticle, Particles size

1. Introduction

The use of nanomaterials in various commercial products has greatly increased recently, as a consequence of rapid developments in nanotechnology [1], [2]. In particular, silver nanoparticles (Ag NPs) that have antibacterial activity are widely used in medical products, mobile devices, cleaning processes, baby care, and textile applications [3]–[5]. Thus, Ag NPs are likely to enter water bodies and cause adverse effects on aquatic organisms. For example, Ag NPs are known to induce high toxicity to *Pseudokirchneriella subcapitata* (algae), *Daphnia magna* (water flea), and *Danio rerio* (zebrafish) [6], [7]. In addition, the impact of NPs in sediment receives more attention, and recent studies have investigated various benthic organisms such as snails and chironomid larvae [8]–[11]. For instance, Ag NPs have a greater impact on the oxidative stress and detoxification of *Chironomus riparius* than Ag ions [10].

The toxicity of NPs is largely dependent on their physical and chemical properties, including surface charge and particle size, which affect the dissolution and aggregation of NPs [12]. Positively charged Ag NPs were found to be more toxic to *bacillus* cells with a negative charge [13], and smaller Ag NPs showed greater influx rates and bioaccumulation in *D. magna* [14]. The bioaccumulation of Ag and CuO NPs in a macrobenthic species, *Macoma balthica*, was also found to depend on the particle size [15]. In general, smaller Ag NPs are dissolved as Ag ions more easily, resulting in greater toxicity [16], [17]. However, a previous assessment of the size-dependent uptake or toxicity of Ag NPs to benthic organisms was very limited.

In the present study, the size-dependent toxicity of Ag NPs to *Glyptotendipes tokunagai* was investigated. Ag NPs were capped with polyvinyl pyrrolidone (PVP) to reduce aggregation. *G. tokunagai* is a dominant species in urban rivers of Korea that has a short life cycle, a high fecundity, and is easy to culture [18].

2. Materials and Methods

Chemicals and test organisms

PVP-Ag NPs of three nominal sizes (50, 100, and 150 nm) were obtained from Nanotech and Beyond (Korea). The PVP-Ag NPs were in a water-based colloid containing 500,000 mg/L Ag and about 12% (w/w) PVP as the coating agent. In addition, silver nitrate ($AgNO_3$, 99.9%) was purchased from Kojima Chemicals (Japan) and used as the control for Ag ions.

G. tokunagai was collected from Jungrang stream (a branch of Han River in Seoul of Korea) in 2007, and cultured over 30 generations in the laboratory of Prof. Yeon Jae Bae, Korea University, Seoul (Korea). *G. tokunagai* was reared in aerated tap water at 20 ± 1°C with a photoperiod of 16 h/8 h (light/dark), and Tetramin (TetraWerke, Germany) was provided as food.

Characterization of PVP-Ag NPs

The morphology of the PVP-Ag NPs was analyzed by transmission electron microscopy (TEM; Tecnai TF20, USA). The hydrodynamic size and surface charge (zeta potential) were measured using dynamic light scattering (DLS) and electrophoretic mobility methods, respectively, by a NanoBrook 90Plus Particle Size Analyzer (Brookhaven Instruments, USA). In addition, the dispersion stability of the PVP-Ag NPs was evaluated by measuring surface plasmon resonance (SPR) absorption using a UV/Vis Spectrophotometer (Optizen POP, Mecasys, Korea).

Ag ions released from the PVP-Ag NPs were analyzed using centrifugal ultrafilters with three replicates per treatment [19]. The PVP-Ag NP solution (10 mL) was centrifuged with 10 kDa centrifugal filters (Amicon Ultra-15 centrifugal filter, Milipore, USA) at 5000 *g* for 20 min. The Ag concentrations in the supernatant were analyzed using an inductively coupled plasma–optical emission spectrophotometer (ICP-OES; Varian Vista PRO, USA).

Toxicity and bioaccumulation testing of PVP-Ag NPs

The test medium used in this study was prepared following the USEPA standard method using moderately hard water (MHW; $NaHCO_3$ = 96 mg/L, $CaSO_4 \cdot H_2O$ = 60 mg/L, $MgSO_4$ = 60 mg/L, KCl = 4 mg/L) at pH 7.5 with a hardness of 100 mg/L as $CaCO_3$ [20]. The PVP-Ag NP solution was prepared in deionized water (18.2 MΩ cm^{-1}, Esse-UP Water System, Mirae St Co., Korea). Acute toxicity tests using *G. tokunagai* under water-only conditions were conducted according to the OECD standard procedures [20]. Six concentrations of PVP-Ag NPs, ranging from 31.25 to 1000 mg/L, and the control (MHW medium) were prepared. One third instar larva (15 days old) was added to the test solution (10 mL) with two replicates, and each replicate consisted of six individuals. Toxicity tests were conducted at 20 ± 1°C with a 16 h light and 8 h dark photoperiod for 48 h. After 48 h of exposure, the mortality of the test organisms was evaluated, and the results are presented in terms of the median lethal concentration (LC_{50}), using the trimmed Spearman-Karber method [21]. *G. tokunagai* mortality was defined as a lack of response when touched using a fine brush.

Bioaccumulation of PVP-Ag NPs (100 mg/L) in MHW medium with *G. tokunaga*i was observed under the same conditions as the acute toxicity testing. Live individuals were separated at a specific exposure time (1, 2, 4, 8, 12, 24, and 48 h) and transferred to clean MHW media for 1 h to remove particles attached to the body and to clear the contents of the gut. The clean larvae were transferred to a 1.5 mL tube, dried at 80°C, and then weighed (dry wt.). The dried larvae were then added to 68% nitric acid (HNO_3, Aristar grade), allowed to stand to dissolve the cellular tissue of the organisms, and digested at 110°C until the acid solution was volatilized. The digestion tube was washed with 2% HNO_3, and the washing solutions were transferred to a 15 mL conical tube (SPL Life Science, Korea). The Ag concentrations in the solution were analyzed using an ICP-OES.

Statistical analysis

All statistical analyses were carried out using SAS Version 9.3 software (SAS Institute Inc., Cary, NC, USA). A one-way analysis of variance (ANOVA) followed by Tukey's test was used to identify significant differences between treatments ($p < 0.05$).

3. Result

Physicochemical properties of PVP-Ag NPs

TEM images of PVP-Ag NPs with different particle sizes are shown in Figure 1. The PVP-Ag NPs were spherical and the primary particle sizes were 56.57 ± 10.13, 100.06 ± 23.25, and 151.00 ± 39.38 nm for 50, 100, and 150 nm PVP-Ag NPs, respectively. The shape of PVP-Ag NPs, as measured by TEM, was spherical in all cases. The zeta potential and hydrodynamic size of the PVP-Ag NPs in MHW medium are given in Table 1. All PVP-Ag NP samples showed a negative charge, with values larger than –30 mV, which may result in the aggregation of PVP-Ag NPs [22].

In fact, hydrodynamic sizes measured by DLS method for 48 h were larger than the corresponding nominal sizes (101.1 ± 19.3, 147.8 ± 6.94, and 174.2 ± 7.85 nm for 50, 100, and 150 nm PVP-Ag NPs, respectively). In addition, the hydrodynamic sizes increased as the exposure time increased, particularly for 50 nm PVP-Ag NPs. The concentration of Ag ions released from PVP-Ag NPs in MHW medium is shown in Figure 2. The smaller PVP-Ag NPs, particularly for the 50 nm samples, gave dissolved Ag concentrations that were higher than those for the larger particles. Moreover, the solubility increased with increasing exposure concentration.

Acute toxicity of PVP-Ag NPs to *G. tokunagai*

The mortality of *G. tokunagai* exposed to PVP-Ag NPs with different particle sizes is shown

149 in Figure 3. In general, the acute toxicity (48 h) of PVP-Ag NPs decreased with increasing
150 particle size, so that the LC_{50} values for 50 and 150 nm PVP-Ag NPs were 297.36 and 820.34
151 mg/L, respectively. No LC_{50} value was calculated for the 100 nm PVP-Ag NPs, and no acute
152 toxicity was observed for the coating material (PVP).

153 Uptake of PVP-Ag NPs by *G. tokunagai* during a 48 h exposure period is shown in Figure 4.
154 Contrary to the results of the acute toxicity tests, the uptake was greater for larger PVP-Ag
155 NPs. In particular, 150 nm Ag NPs were accumulated in a concentration-dependent manner,
156 which was significantly different from those for 50 and 100 nm PVP-Ag NPs ($p < 0.05$)

157

158 **4. Discussion**

159 **Dispersion stability of PVP-Ag NPs**

160 As revealed by DLS measurements (Table 1), the 50 and 100 nm PVP-Ag NPs became
161 larger in MHW medium when compared with the primary particle sizes (Fig. 1). In general,
162 ionic strength and pH have no effect on the aggregation of sterically stabilized PVP-Ag NPs
163 [23]. However, electrostatic repulsion may play a role in controlling the stability of PVP-Ag
164 NPs when Ag NPs were partially coated with PVP [24], likely resulting in the aggregation of
165 PVP-Ag NPs in the MHW medium with a higher ionic strength. In addition, the
166 hydrodynamic size of 50 nm PVP-Ag NPs was significantly different from those for 100 and
167 150 nm PVP-Ag NPs (Table 1; $p < 0.05$). This indicates that 100 and 150 nm PVP-Ag NPs
168 may show similar behaviors in acute toxicity and bioaccumulation.

169 UV/Vis absorption spectra were recorded to evaluate the dispersion stability of PVP-Ag NPs
170 in MHW medium (Fig. 5). All of the PVP-Ag NPs showed an absorption peak at about 440
171 nm, and the intensity of the peak was reduced with increasing primary particle size. The
172 strong absorption peak is a result of the collective oscillations of the metal valence electrons
173 of Ag NPs, known as surface plasmon resonance (SPR) [25]. In general, smaller Ag NPs give

a sharp peak with higher intensity [26]. The SPR peak decreased significantly with increasing exposure time, particularly for the 50 and 100 nm PVP-Ag NPs, possibly because of the aggregation or sedimentation of PVP-Ag NPs [27]. In general, aggregation increases with increasing collision frequency, which is proportional to the number of particles in a given volume [28]. Considering the same concentration based on the mass of NPs, the number of smaller PVP-Ag NPs should be greater than that of larger PVP-Ag NPs, resulting in a higher possibility of aggregation. These findings suggest that the smaller PVP-Ag NPs were not stable in MHW medium for the exposure period of 48 h.

Toxicity of PVP-Ag NPs to *G. tokunagai*

The solubility of the 50 nm PVP-Ag NPs was much higher compared with the solubility of the 100 and 150 nm PVP-Ag NPs, possibly owing to the larger surface area of the smaller 50 nm PVP-Ag NPs [29]. In addition, 50 nm PVP-Ag NPs showed significantly higher acute toxicity to *G. tokunagai* compared with the 100 and 150 nm particles, which is in line with the general fact that smaller particles are more toxic than larger ones [30]. As indicated in Figure 2, the dissolved concentration of Ag released from PVP-Ag NPs was far below the LC_{50} values for $AgNO_3$ (3.51 mg/L) determined in this study. This suggests that the acute toxicity of PVP-Ag NPs to *G. tokunagai* is probably not attributable to Ag ions, but to Ag NPs. However, the dissolution of PVP-Ag NPs in the gut of *G. tokunagai* cannot be ruled out and these Ag ions may contribute to the toxicity observed in this study. Considering that Ag ions can inhibit Na^+/K^+-ATPase activity in biological membranes, whereas Ag NPs may induce membrane deformation and DNA damage [31], the toxicity mechanism should be further studied in order to identify their relative contributions to the observed toxicity of PVP-Ag NPs.

The uptake of PVP-Ag NPs in *G. tokunagai* showed the opposite pattern to the acute toxicity

results (Fig. 3), in which bioaccumulation in *G. tokunagai* was greater for the 150 nm PVP-Ag NPs (Fig. 4). This is also contrary to the result that smaller Ag NPs were accumulated more in *D. magna* [14]. *G. tokunagai*, a deposit feeder in sediment, ingests nutrients from particles suspended in sediment, whereas *D. magna*, a filter feeder, obtains nutrients from water. Thus, theses different feeding habits may be related to their different uptake results. The uptake of PVP-Ag NPs by *G. tokunagai* as a function of time is shown in Figure 6. The larger PVP-Ag NPs were absorbed and excreted rapidly, resulting in a shorter stay in *G. tokunagai*. These findings suggest that the higher toxicity of smaller PVP-Ag NPs could be attributed to the longer retention time. In addition, the higher solubility of smaller PVP-Ag NPs may also lead to the observed toxicity difference.

In summary, the toxicity of PVP-Ag NPs was very dependent on the particle size. Particularly, smaller PVP-Ag NPs were more toxic to *G. tokunagai* compared to larger particle, possibly owing to their prolonged stay and higher dissolution in the body. However, the toxicity mechanism of PVP-Ag NPs should be further studied in order to identify the role of Ag ions and NPs more clearly.

Acknowledgments

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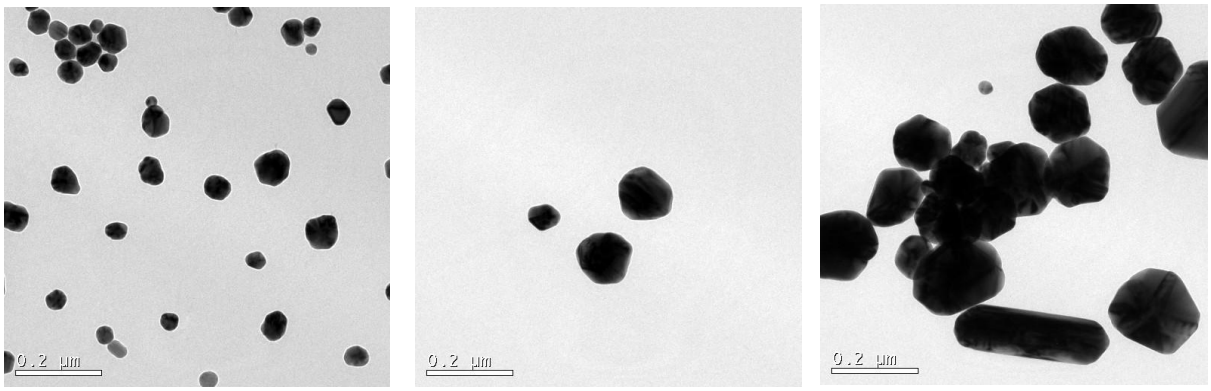
310 31. Zhao CM, Wang WX. Comparison of acute and chronic toxicity of silver nanoparticles
311 and silver nitrate to *Daphnia magna*. Environ Toxicol Chem. 2011;30(4):885–892.

312 Table 1. Hydrodynamic size and zeta potential of PVP-Ag NPs (100 mg/L) in MHW medium as a function of exposure time.

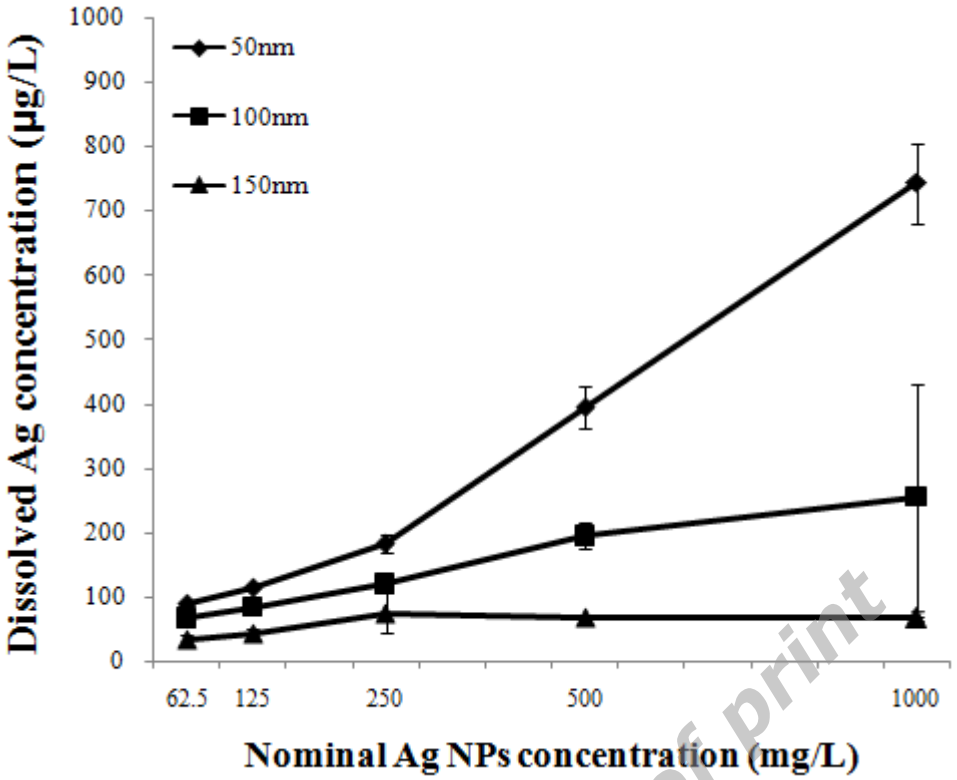
313

Nominal size (nm)	50			100			150		
Exposure time (h)	0	24	48	0	24	48	0	24	48
Hydrodynamic size (nm)	83.07	98.66	121.5	147.62	140.99	154.86	178.91	165.18	178.63
		101.1 ± 19.3 [a]			147.8 ± 6.94 [b]			174.2 ± 7.85 [b]	
Zeta potential (mV)	–2.63	–4.34	–4.47	–7.99	–5.43	–6.03	–7.09	–10.12	–5.72



50nm **100nm** **150nm**

Fig. 1. TEM images of PVP-Ag NPs with nominal particle sizes of 50, 100, and 150 nm.



317

318 Fig. 2. Dissolved concentration of Ag ions released from PVP-Ag NPs in MHW medium

319 after 48 h exposure.

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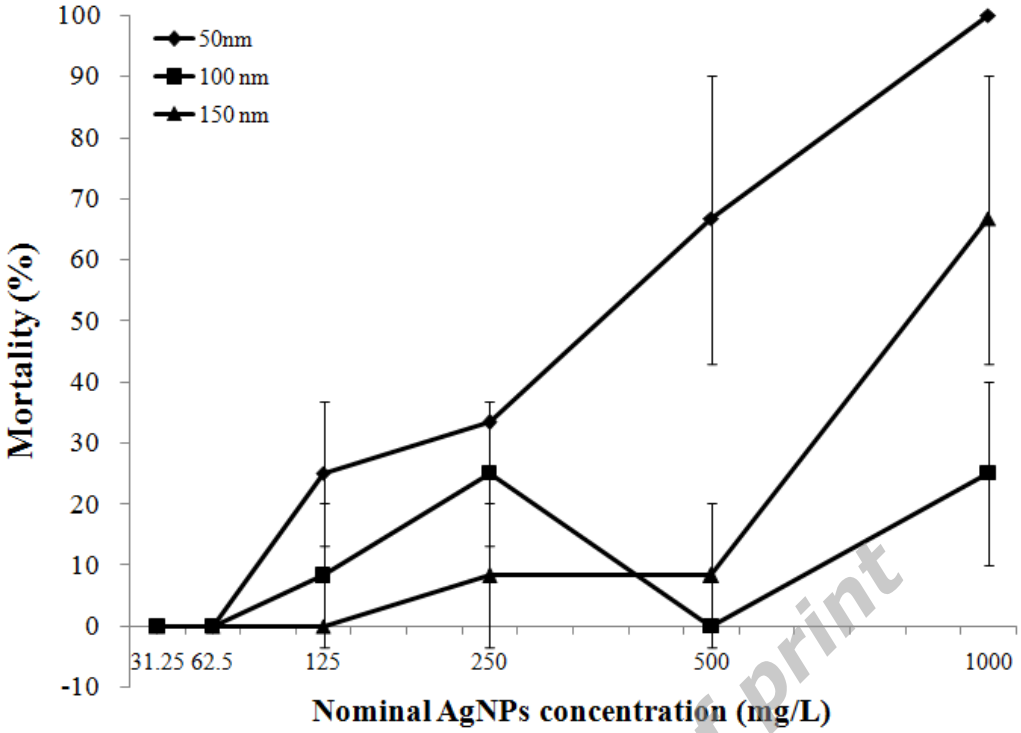

322

323 Fig. 3. Mortality (48 h) of *G. tokunagai* exposed to PVP-Ag NPs as a function of

324 concentration.

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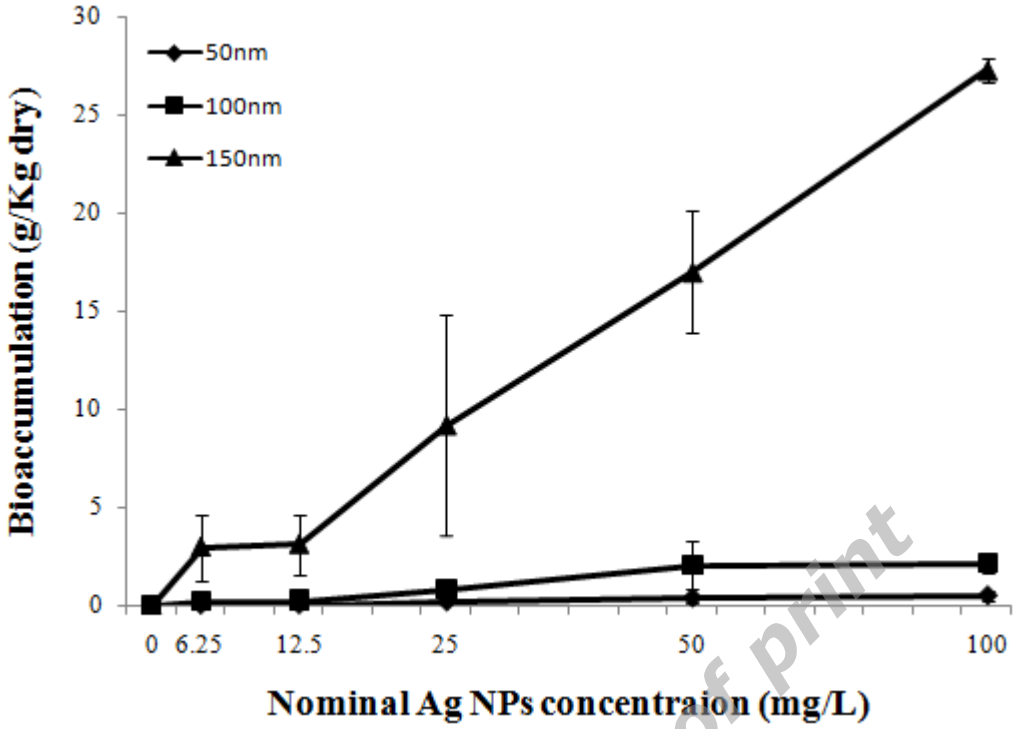
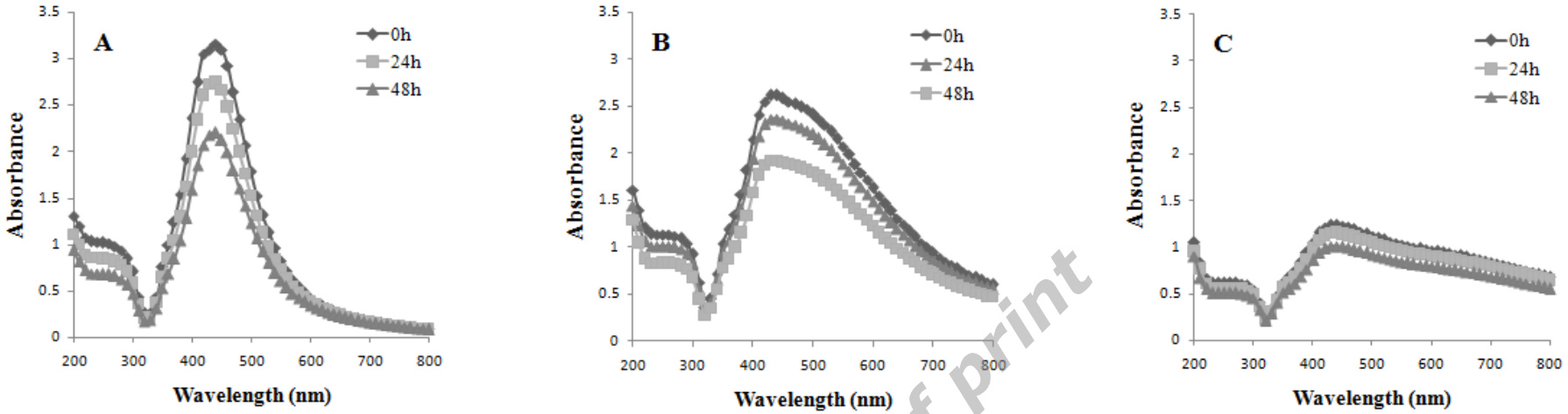


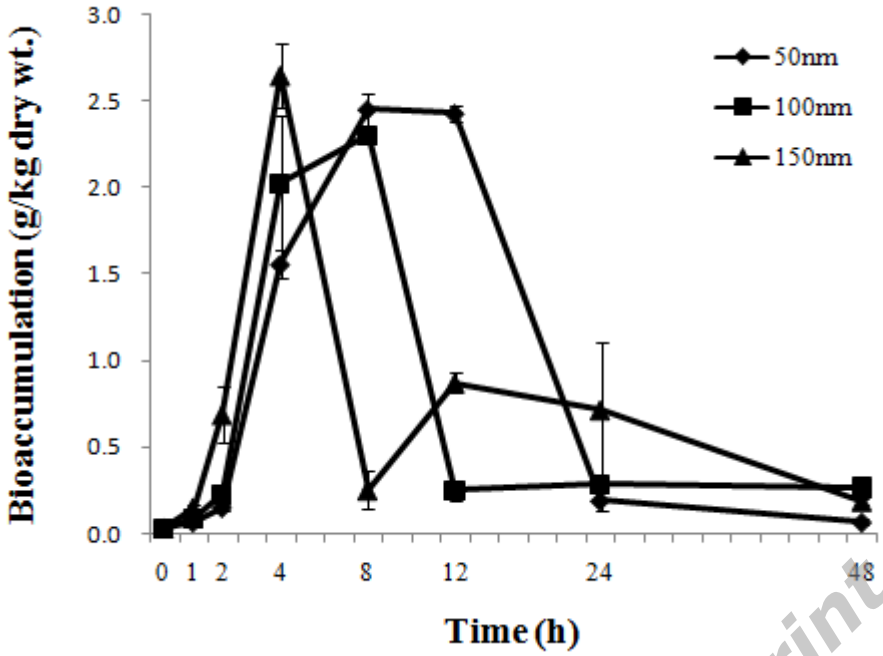
326

327 Fig. 4. Uptake of PVP-Ag NPs in MHW medium by *G. tokunagai* after 48 h exposure.



328

329 Fig. 5. UV/Vis absorption spectra of (A) 50 nm, (B) 100 nm, and (C) 150 nm PVP-Ag NPs in MHW medium as a function of exposure time.



330

331 Fig. 6. Uptake of PVP-Ag NPs (100 mg/L) by *G. tokunagai* in MHW medium as a function

332 of exposure time.