Physiological Homeostasis Alteration and Cellular Structure Damage of *Chlorella Vulgaris* Exposed to Silver Nanoparticles with Various Microstructure Morphologies

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Abstract

The toxicity of silver nanoparticles (AgNPs) with single morphology exposed to aquatic organisms had been well revealed in the past decade, but few studies have been carried out to evaluate the toxicity differences between AgNPs with various microstructure morphologies, especially to algae. In this work, *Chlorella vulgaris* was used as the tested organism to illustrate the differences of toxic effects between silver nanospheres (AgNSs), silver nanocubes (AgNCs) and silver nanoplates (AgPLs) with concentration of 0.5, 1.0, 2.0, 5.0 mg·L^{-1}, based on the algae's growth (72h), chlorophyll-a content, antioxidant enzyme activity, lipid peroxidation and cell apoptosis (48h). The results showed that the toxicity level exposed to *Chlorella vulgaris* was in the order of AgPLs> AgNCs> AgNSs. The difference shown indicated that the potential toxicity of AgNPs is primarily depended on their microstructure morphologies. This current study initially revealed the structure-effects of AgNPs on *Chlorella vulgaris*, provided a scientific basis for aquatic environmental risk assessment.

1 Introduction

Silver nanoparticles (AgNPs) produced, transported and applicated by humans are ultimately released into the environment and are thus potentially toxic to environmental organisms (Dale et al., 2015). Besides, in natural environment, Ag^{+} can be reduced into nano silver by dehydrogenase or reducing sugar in microorganisms or plants (Mo et al., 2020). AgNPs is highly chemically active and can easily interact with environmental medium (e.g., physical, chemical and biological reaction), which leads to the migration and transformation of AgNPs, eventually affecting their toxic effects (Wang et al. 2020). Although much work was gone into focusing on the biological effects of AgNPs, which systematically revealed action mechanisms and dose-effect relationship of AgNPs with single morphology (e.g., spherical, rodlike, and hexagonal) to various organisms, the shape-dependent toxicity was less explored (Ji et al. 2010; Oukarroum et al. 2012). Noticeably, there is an internal relationship between the structure of pollution and their toxic effect, that is, the structure-activity relationship. Different morphologies of nanoparticles possess different crystal planes and quantum structures, leading to the unique physical and chemical properties (Shen et al. 2015). Several studies have evaluated the ecotoxicity of AgNPs with various morphologies to bacterial, algae and fish. Mayer et al. demonstrated that AgNPs morphologies had no significant effect on the cytotoxicity (2016), while Babak et al. compared the toxicities of silver nanoplates (AgPLs) and silver nanospheres (AgNSs) and found that AgNSs were highly toxic to *Staphylococcus* aureus and *Escherichia coli* (*E. coli*), yet less toxic than AgPLs (2012). In contrast, some studies reported that the toxicities of AgPLs and AgNSs in *P. aeruginosa* and *E. coli* were opposite (Muhammad et al. 2016). Therefore, there is a dispute related to shape-dependent toxicity of AgNPs. Thus, it is of great importance to explore the toxic differences of AgNPs with various morphologies on organisms in ecosystem, providing direct evidence for the risk assessment of AgNPs with different structure.

As a main primary producer in aquatic systems, algae play a crucial role in the environmental homeostasis of water body. The toxicity of AgNPs to microalgae are known to be related to

photosynthetic efficiency inhibition, reactive oxygen species (ROS) generation, metabolism interference, and organelles damage (He et al. 2017; Dorobantu et al. 2015). Whereas, the research concerning the toxic effects of AgNPs with multi-morphologies on algae were still limited. The investigation of structure-activity relationship of between AgNPs and algae is of great scientific value to the comprehensive and in-depth understanding of AgNPs biological toxicity in aquatic environment and the evaluation of safety of water body.

Herein, we compared the toxicities of three AgNPs of various morphologies (AgNSs, AgNCs, and AgPLs) on an alga, *Chlorella vulgaris.* We determined the growth condition, the chlorophyll-a content, antioxidant enzyme activity, lipid peroxidation degree, and cell apoptosis. The experimental results of study could provide valuable information about the toxicity of AgNPs with various microstructure morphologies to aquatic organisms, which might be useful for assessing ecological risk of AgNPs.

2 Materials And Methods

2.1 Tested algae

Experiments were carried out with cultures of the unicellular green algae *C. vulgaris* FACHB-8, purchased from Freshwater Algae Culture Collection at the Institute of Hydrobiology (Wuhan, China).

2.2 Algae growth inhibition test

Pre-cultures of *C. vulgaris* at exponential phase were inoculated into BG11 medium (Table S1) with an initial cell density of 10×10^6 cells/ml for algae growth and chlorophyll-a content assay. Before the inoculation of algae cells, different concentrations of AgNPs with various morphologies were added into the growth media. The nominal concentrations for AgNPs for the test in the medium were 0.5 mg·L^{-1}, 1.0 mg·L^{-1}, 2.0 mg·L^{-1}, 5.0 mg·L^{-1} (AgNPs concentrations verified by inductively coupled plasma mass spectrometry (ICP-MS) showed relatively small deviation). In the exposure test, cultures were grown in 150ml Erlenmeyer flasks containing 30ml of BG11 medium containing different concentrations of AgNPs.

Algae cells were grown in medium containing various AgNPs morphologies and concentration for 72h and algae growth was monitored after every 12h. Cell density was measured by cell counting using an optical microscope (Nikon, China). The method proposed by Sartory (1984) was used to determine the content of photosynthetic pigment.

2.2 Analysis of antioxidant enzyme activity and lipid peroxidation

Cells at 48h were harvested and analyzed for antioxidant enzyme activity and lipid peroxidation analysis. Superoxide dismutase (SOD) activity was assayed by monitoring the inhibition of reduction of Nitroblue Tetrazolium chloride (NBT) photochemically. The determination of Malondialdehyde (MDA) content was

accomplished by the color reaction of Thiobarbituric Acid (TBA) in acid condition (Heath et al. 1968). The ROS level of the algae cells were measured using the ROS assay kit (Beyotime Institute of Biotechnology, Haimen, China). The activity of Peroxidase (POD) and Catalase (CAT) were determined by guaiacol method and UV absorption method respectively.

2.3 Analysis of cells apoptosis

After exposure of 48h, centrifuged at 4000rmp for 10min at room temperature and cultured with 195μL Annexin V-FITC binding solution and 5μL Annexin V-FITC for 10min at 24°C. Next, cells were assayed using an Annexin V-FITC apoptosis detection kit (Beyotime Institute of Biotechnology, Haimen, China), the cell apoptosis and cell size were analyzed on a FACS JAZZ flow cytometer (Becton Dickinson, Sanjose, CA, USA) equipped with an argon laser (excitation at 488 nm).

3 Result And Discussion

3.1 Growth inhibition test

During the experimental process, AgNPs with different concentrations (0.5, 1, 2, 5 mg·L^{-1}) had different morphology response to the tested algae. Compared to the control, *C. vulgaris* was highly sensitive to AgPLs than the others, which showed significantly disruption and aggregation (Fig. 1A). After 48h exposure in AgNPs, evident whitening and stunting appeared on algae cells. The result shows that the presence of AgNPs could lead to the aggregation and even rupture of algae cells, thus affecting their normal physiological and biochemical functions. The 48h inhibition rate of *C. vulgaris* exposed to AgNSs, AgNCs and AgPLs increased with the concentration increasing in a dose related manner (Fig. 1B) ($P < 0.5$). However, tested concentrations of AgNSs and AgNCs did not induce substantial inhibition on cell growth, with the maximum inhibition effect of 43% and 38% at 5 mg·L^{-1}. On the contrary, low concentration of AgPLs (0.5 mg·L^{-1}) could repress cell growth significantly by 60%, indicating a higher toxic effect on cell growth and vitality than AgNSs and AgNCs in this study.

The inhibitory effects of AgNSs, AgNCs and AgPLs on the growth and photosynthetic of *C. vulgaris* are shown in Fig. 1C-H. The cell density of microalgae is the most intuitive parameter to measure the cell growth, and the chlorophyll-a content can be used as one of the indicators to measure the physiological effects of algae cells (Metzler et al. 2012). It can be seen from the figure that the presence of AgNPs inhibited the cells growth and chlorophyll-a synthesis, the inhibition efficiency had a positive relationship with the concentration of AgNPs. Incubation of algae cells in medium containing AgPLs resulted in significant repression on cell growth even at lower concentration of 0.5 mg·L^{-1} at 72h. Increase of the AgNPs concentration to 5 mg·L^{-1} impeded *C. vulgaris* propagation completely. A similar decrease in chlorophyll-a was observed in AgNPs treatment at 0.5, 1, 2 and 5 mg·L^{-1}, which was in agreement with the cell density histogram. The content of chlorophyll-a was only 26.92%, compared with the control group, as the concentration of AgPLs was 5 mg·L^{-1}. The chlorophyll-a content and cell density of AgNSs decreased the least, that is, the effect of AgNSs on photosynthetic pigments activity and growth of algae

was the weakest. Substantial decreased in chlorophyll-a contents of algae exposed to AgNPs indicated that the photochemical activity was repressed and occurrence of photoinhibition was inevitable. For AgNPs with different morphologies, flake nano silver has a stronger biocidal effect than spherical nanoparticles (Pal et al. 2007), the experimental results confirm this viewpoint to some extent.

3.2 Physiological and biochemical test

ROS and MDA are direct indicators of lipid peroxidation and oxidative damage in algae system (Gao et al. 2020). Figure 2 shows the intracellular ROS level (Fig. 2A) and MDA contents (Fig. 2B) of C. vulgaris as a function of the AgNPs concentration. The intracellular ROS level was significantly promoted by increasing AgNPs concentration, whereas relative MDA activity gradually decreased with increasing AgNPs concentrations, resulting a minimum value at 5 of AgNPs. After exposure to 5 mg·L^{-1} AgPLs for 48h, level of the ROS was the highest, and was 2.7-times those of the control, accordingly. As shown in Fig. 2B, the content of MDA was higher than that of the control group, while MDA content exposed to AgPLs increased with the increase of concentration. MDA is one of the products of lipid peroxidation (Gaschler et al. 2017), the content of MDA will be lower than that of the control group when the cells were damaged and ruptured. The results showed that *C. vulgaris* was more sensitive to the AgPLs in terms of MDA and ROS.

In order to determine the effects of AgNPs with different morphologies on enantioselective oxidative stress of *C. vulgaris*, AgNSs, AgNCs and AgPLs exposure group were examined. As seen in Fig. 3, the SOD (Fig. 3A), CAT (Fig. 3B) and POD (Fig. 3C) exhibited similar trend upon exposure to AgNPs after 48h exposure. AgNPs with different morphologies tested differently affected the antioxidant enzyme activities in the algal cells. When *C. vulgaris* cells exposed to different concentrations of AgNPs (0.5, 1, 2 and 5 mg·L^{-1}), SOD activities were enhanced compared with those untreated cells. Besides, the increase of SOD and CAT were most obvious in the AgPLs treated group. SOD enzyme can decompose O_2^-, CAT and POD enzymes are involved in the decomposition of hydrogen peroxide, thus eliminating the influence of ROS. As expected, under the stimulation of AgNPs, the activity of antioxidant enzymes and the antioxidant capacity increased respectively. In this study, the enhancement on CAT, SOD and POD of cultures exposed to AgNPs signify the involvement of antioxidant enzyme in the antioxidant defense against the ROS. Oxidative stress could enhance the antioxidant capacity of cells, and they couldn't maintain internal stability when the content of reactive oxygen species increased to a certain amount.

3.3 Effect of AgNPs on cells membrane damage

The effects of AgNPs on apoptosis of *C. vulgaris* were evaluated by Annexin V-FITC/PI staining. Cell membrane damage was the primary manifestation of apoptosis (Kundrát et al. 2016), when the cell membrane damaged, Annexin V could bind to phosphatidylserine on the surface of cell membrane, and the fluorescence intensity could be detected by FITC and PI dyes. In flow cytometry image, upper left and upper right, lower right quadrants show percentage of early apoptotic cells, advanced stage of apoptotic cells and dead cells, lower left quadrant shows the percentage of live cells respectively. As shown in Fig. 4A, B and C, after exposure to AgNPs with various morphologies for 48 hours, the cell size,

complexity and apoptosis of the experimental group were higher than those of the control group. The percentage of apoptotic cells increased to 80% under the AgPLs treatment, while that in AgNSs treated group was only about 50%, and in AgNCs treated group was about 70%. The result shows that 5 mg·L^{-1} AgNPs could promote the apoptosis of *C. vulgaris* cells, and the degree of promotion was related to the morphology of AgNPs, AgPLs had the greatest damage to the cell membrane compared with AgNSs and AgNCs. Although AgNSs had the smallest nanoscale, it showed the lowest toxicity to *C. vulgaris*, this experimental phenomenon was markedly different from the published literature (Nam et al. 2019).

4. Conclusion

In this study, the toxicity differences of AgNSs, AgNCs and AgPLs in *C. vulgaris* were compared based on the growth inhibition, photosynthetic pigment content, antioxidant enzyme activity, lipid peroxidation, morphology and apoptosis of the cells. The growth and photosynthetic pigment content of *C. vulgaris* were significantly inhibited by all three AgNPs. The results of antioxidant enzymes activities and cell apoptosis indicated that AgPLs possessed the highest potent toxicity to *C. vulgaris*, followed by AgNCs (moderate) and AgNSs (lowest). The experimental findings indicated that the toxic effects of AgNPs on aquatic organisms may primarily depend on its microstructure morphology rather than on its nano size in some certain cases, especially upon exposure to *C. vulgaris.* This work pointed out that the microstructure morphology effects and nanoscale effects are of equal importance for understanding the toxicities of AgNPs in aquatic primary producers, whereas the former may results in more potential impact than the latter when it is exposed to some certain algae, e.g., *C. vulgaris.*

Declarations

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Authors contributions

Conceived and designed the experiments: NCD, HBL and YHL. Performed the experiments: NCD, HBL and YHL. Analyzed the data and prepared the figures: NCD, HBL, YHL, FM, MSW, ZL and XC. Wrote the paper: NCD, HBL and YHL. Reviewed and commented on the paper: NCD, HBL, JNX, RC and HXW. All authors read and approved the manuscript

Data availability Not applicable.

Compliance with ethical standards.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent to publish Not applicable.

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Figures

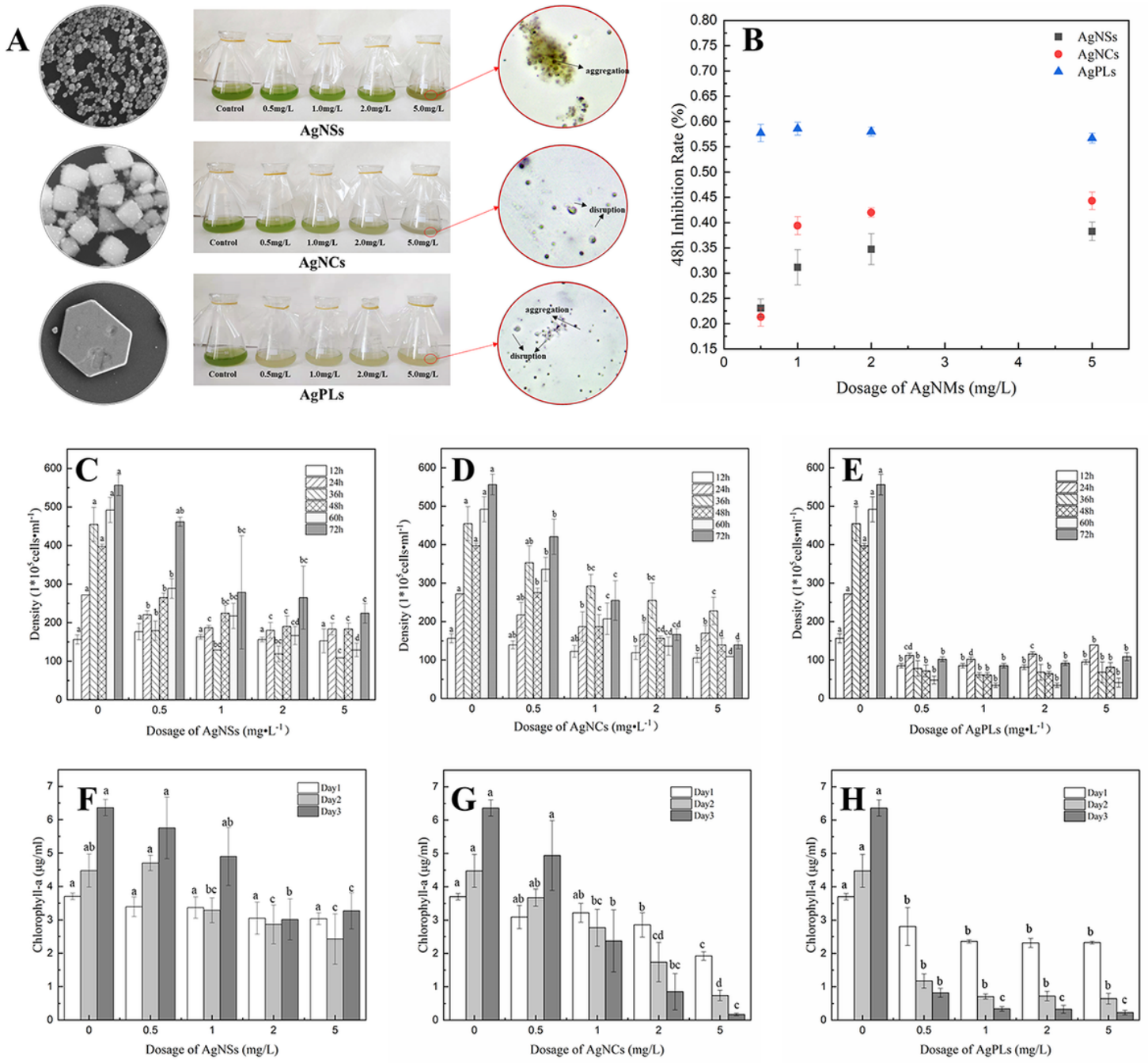

Figure 1

Determination of AgNPs SEM and cell morphology observation, 48h inhibition rates, cell density and chlorophyll-a contents of C. vulgaris exposed to AgNPs with various morphologies for 3 days. AgNPs SEM images and cell morphology (A). 48h inhibition rates (B). Cell density (C, D, E). Chlorophyll-a content (F, G, H). Lower-case letters demonstrate averages comparisons between treatments by LSD test ($p < 0.05$).

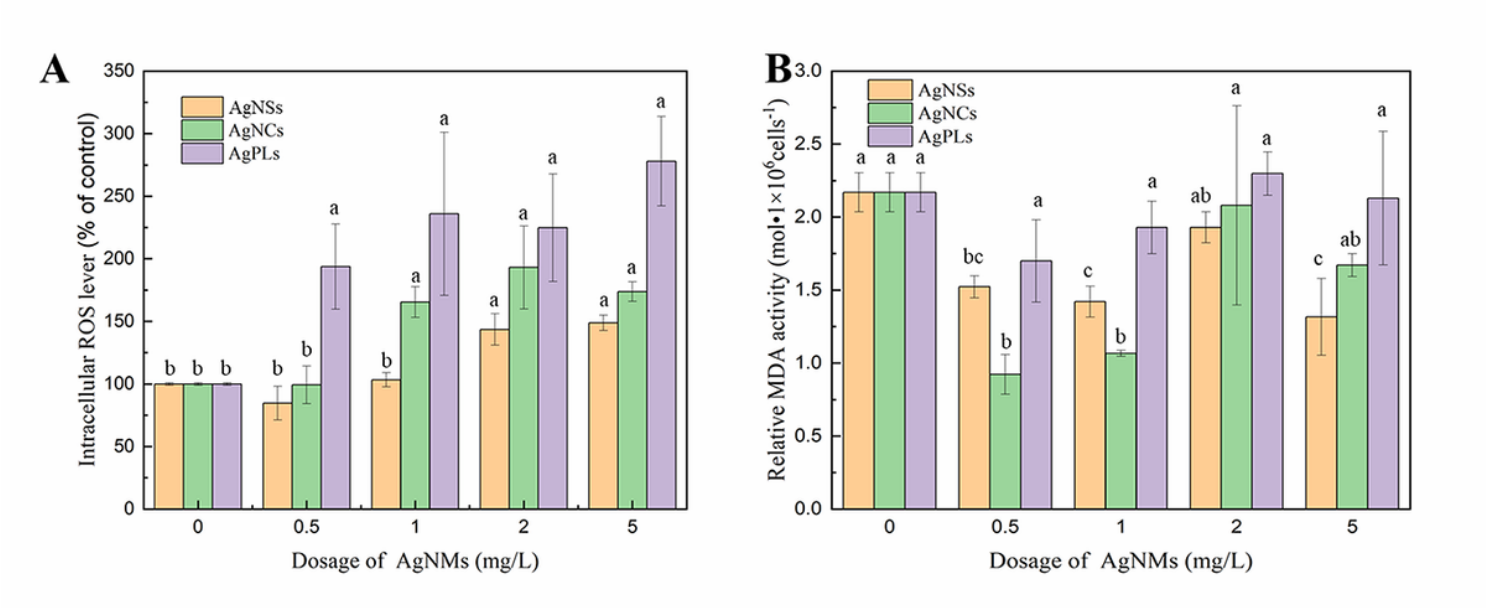

Figure 2

Changes of intracellular ROS level (A), MDA content (B) in C. vulgaris exposed to AgNPs with different concentrations after 48h. Lower-case letters demonstrate averages comparisons between treatments by LSD test ($p < 0.05$).

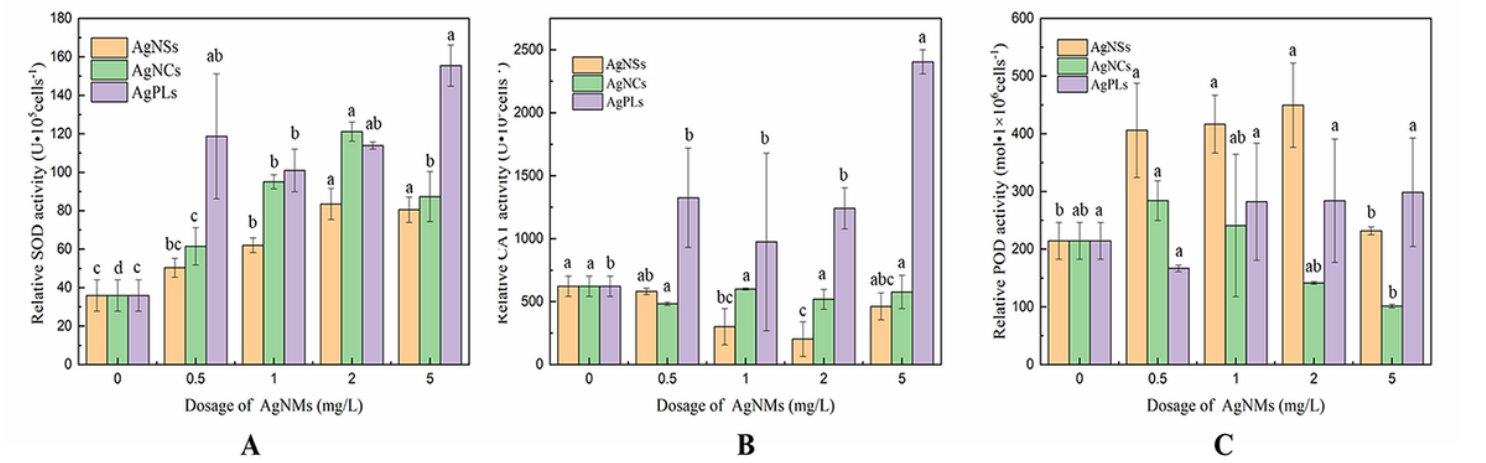

Figure 3

Changes of SOD (A), CAT (B) and POD (C) in C. vulgaris at different concentrations exposed to AgNPs with different concentrations after 48h. Lower-case letters demonstrate averages comparisons between treatments by LSD test ($p < 0.05$).

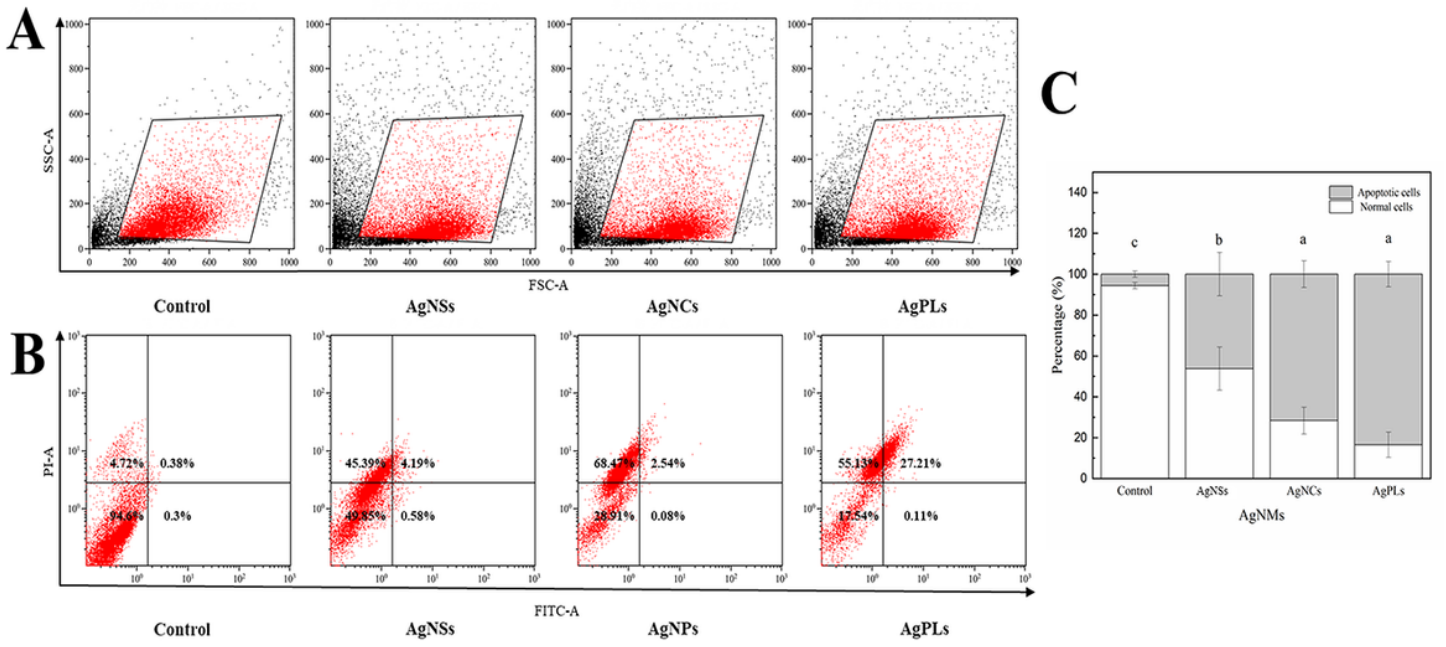

Figure 4

The effect of AgNPs on cell apoptosis in C. vulgaris. The cells were treated with different morphologies of AgNPs in the concentration of 5 mg·L-1 for 48 h, then cells were collected for Annexin V-FITC/PI staining followed by cytometry analysis. (A) Morphology and size distribution of cells in different AgNPs treatment groups. (B) Apoptosis of cells in different AgNPs treatment groups. (C) Percentage of apoptotic and normal cells. Lower-case letters demonstrate averages comparisons between treatments by LSD test ($p < 0.05$).

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