Metabolites change of *Scenedesmus obliquus* exerted by AgNPs

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**ABSTRACT**

With increasing emission of silver nanoparticles (AgNPs) into the environment, it is important to understand the effects of ambient concentration of AgNPs. The biological effects of AgNPs on *Scenedesmus obliquus*, a ubiquitous freshwater microalga, was evaluated. AgNPs exerted a minor inhibitory effect at low doses. Non-targeted metabolomic studies were conducted to understand and analyze the effect of AgNPs on algal cells from a molecular perspective. During the 48 hr of exposure to AgNPs, 30 metabolites were identified, of which nine had significant changes compared to the control group. These include D-galactose, sucrose, and D-fructose. These carbohydrates are involved in the synthesis and repair of cell walls. Glycine, an important constituent amino acid of glutathione, increased with AgNP exposure concentration increasing, likely to counteract an increased intracellular oxidative stress. These results provide a new understanding of the toxicity effects and mechanism of AgNPs. These metabolites could be useful biomarkers for future research, employed in the early detection of environmental risk from AgNPs.

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**Introduction**

Over the last few years, nanotechnology has been widely used in electronics, energy, materials, medicine, chemicals and clean technologies. Among all nanomaterials, silver nanoparticles (AgNPs) are the most produced (Berube et al., 2010). Due to broad-spectrum antimicrobial property of silver and less negative effect on humans, AgNPs are widely applied in medical devices, cosmetics, clothing, household items, water treatments and detergents etc., which cover nearly all aspects of our daily lives (Marambiojones and Hoek, 2010). Increasingly produced in larger amounts, they are inevitably released into the environment. This has led to growing concerns about the environmental health and safety issues associated with engineered AgNPs (Mathivanan and Ananth, 2012).

The fast growing utilization has increased the release of AgNPs into aquatic environment, which in turn poses potential risks to aquatic organisms and ecosystems (Gottschalk and Nowack, 2011; Klaine et al., 2008). AgNPs also have been reported to have acute toxic effects on human cell lines (Veranobraga et al., 2014), bacteria (Gupta et al., 2014), fish (Garciareyero et al., 2014), and plankton, and AgNP exposure to water also produces bioaccumulation of aquatic organisms (Croteau et al., 2011; Khan et al., 2012; Konnova et al., 2015; Piccapietra et al., 2012; Yooiam et al., 2014). The reported concentration of AgNPs in the environment was as low as 1 μg/L (Holden et al., 2014). Most of the previous studies focused on artificially and unrealistically...
1. Materials and methods

1.1. Materials

AgNPs (20 nm) were purchased from Shanghai Huzheng Nanotechnology Co., Ltd., China. The chemicals required to reconstitute BG11 required reagents are ordered from Sinopharm Chemical Reagent Co., Ltd., China (Analytical grade, Sinopharm Chemical Reagent Co., Ltd., China). N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and FMOC-glycine were procured from Sigma-Aldrich (Chromatographic grade, Sigma-Aldrich, America). HPLC grade acetonitrile and methanol were purchased from Merck (HPLC grade, Merck, Germany). Ultrapure water (18.2 MU/cm) was produced by a Milli-Q water purification system (IQ7000, Milli-Q, America).

1.2. Preparation and characterization of AgNPs

The surface morphology of AgNPs and the hydrodynamic diameters in ultrapure water were characterized by TEM (JEM-2100, Japan Electronics Co., Ltd., Japan). The size of the AgNPs in BG11 culture medium was characterized by the dynamic light scattering (DLS) (DelsaNano C, Beckman Coulter, America).

1.3. S. obliquus culture

S. obliquus (FACHB-417) was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. The culture medium required for S. obliquus growth is BG11. All glassware and culture medium used in the experiment were sterilized in an autoclave (121°C, 30 min). The algae was cultured using aseptic techniques under standard conditions (12 hr:12 hr, light:dark cycle; illumination 2500 lx; 25 ± 1°C). Algal cells agglomerated can be prevented by shaking flask three times a day.

1.4. Exposure of S. obliquus to AgNPs

Sterilized 250 mL flasks containing 100 mL of algae-containing medium were used for all experiments. The algal cells used were in the exponential growth phase, with standardized spectrophotometric densities of 10^4 to 10^6 cell/mL. The algae cells were exposed to AgNP concentrations of 0 (control), 1, 10, 50, 100, 1000 and 2000 μg/L for 7 days.

The experiment was conducted in triplicates for each concentration, shaking each flask three times a day. Algal cell numbers were monitored daily using a spectrophotometer (Cary 60, Agilent, America), measuring absorbance at 680 nm. The growth rate for each sample in each AgNP concentration was calculated using Eq. (1) provided in the OECD guidelines:

\[
\mu_{i,j} = \frac{\ln X_{i,j} - \ln X_{i}}{t_{j} - t_{i}}
\]

where, \(\mu_{i,j}\) (day⁻¹) is the average specific growth rate from time \(t_i\) to \(t_j\), \(X_{i}\) is the biomass at time \(i\), \(X_{j}\) is the biomass at time \(j\).

The percent inhibition was calculated for each exposure concentration growth rate using Eq. (2):

\[
r_i = \frac{\mu_{c} - \mu_{i}}{\mu_{c}} \times 100\%
\]
where, \( r_t \) (%) is the inhibition in average specific growth rate; \( \mu_C \) is the mean value of control group average specific growth rate (\( \mu \)); \( \mu_T \) is the average specific growth rate for the treatment replicate.

To further explore the molecular mechanism of the effects at low doses, we chose 1, 10 to 100 \( \mu \)g/L for the next metabolomic analysis. Because the range of 0–2000 \( \mu \)g/L was chosen to make a dose–response curve in the traditional toxicology experiment. Through the dose–response curve we can get a general understanding of the inhibitory effect of AgNPs on \( S. \) obliquus. Based on the results obtained, we found that the inhibitory effect in the range of 0–100 \( \mu \)g/L is not obvious. Therefore, we selected 0–100 \( \mu \)g/L range of metabolomic analysis in order to further explore the molecular mechanism of change under condition that the morphological damages had not been found. The low dose in the range of 0 to 100 \( \mu \)g/L was also an environmentally relevant concentration of AgNPs.

### 1.5. Metabolomic profiling

#### 1.5.1. Extraction and analysis of metabolites

The procedure for sample pretreatment was adapted from previous publications (Huang et al., 2013; Liang et al., 2013). Prior to metabolomic analysis, 50 mL of the culture medium containing algal cells was stored at −80°C. The FMOG-glycine was dissolved in a mixed solvent of water and methanol (1:4, V/V) at a concentration of 25 g/mL. The mixture was used as the internal standard to extract the metabolites from \( S. \) obliquus samples. The samples were freeze-dried, then weighed and transferred to 1.5 mL Eppendorf tube. Each Eppendorf tube was filled with 300 \( \mu \)L mixture of water: methanol (1:4, V/V), then 6 stainless steel beads (diameter 1.5 mm) were added. The samples were homogenized for 10 min at 25 Hz in Tissue Lyser (QIAGEN, USA) and extracted. In order to further improve the extraction efficiency, the samples were sonicated in ice water for 10 min. Because of the strong pressure pulse produced by ultrasound, the cell structure is disrupted and the cell lysis is accelerated, thereby increasing the extraction efficiency. After the sample was centrifuged (14,000 r/min, 4°C) for 10 min, 10 \( \mu \)L of supernatant was collected and transferred into micro bottles, then dried in CentriVap concentrator (Labconco, USA). After drying, it was derivatized for metabolite analysis. The derivatization was conducted with two agents: 100 \( \mu \)L methoxyamine in pyridine (5 mg/L) at 60°C for 2 hr and 100 \( \mu \)L MSTFA at 37°C for 16 hr.

Then the sample was analyzed with Agilent 7890A Series gas chromatograph coupled system to an Agilent 7200 Q-ToF mass detector. The GC column was HP-5 MS UI (30 m \( \times \) 0.25 mm I.D. \( \times \) 0.25 \( \mu \)m film, Agilent, USA), carrier gas helium, and the flow rate set at 1 mL/min. Injection volume was 0.2 \( \mu \)L in splitless mode. Injection temperature and transfer line temperature were 250 and 280°C, respectively. Under splitless mode, injection volume was 0.2 \( \mu \)L with 250°C. The transfer temperature was 280°C. The oven temperature was set at 90°C for 1 min, then increased to 130°C at rate of 20°C/min and to 280°C at rate of 6°C/min. Finally it was increased to 300°C at a rate of 25°C/min then hold for 6 min. The total run time of the GC was 34.8 min. The solvent cutting time was 4.5 min. The ion source was ran at 230°C in electron ionization (EI) mode. The scan range for ToF was from m/z 50 to 800 (Du et al., 2017).

#### 1.5.2. Statistical analysis

Mass Hunter Workstation Software (Agilent, USA) was used for data acquisition and processing. The data were normalized based on the abundance of the internal standard (FMOC-glycine). Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed using SIMCA 13.0. The heatmap of metabolite ratios was visualized using Multi Experiment Viewer software (MeV). Each set of data after log transformation was compared using the t-test (Microsoft Excel 2013). The features with variable importance in projection (VIP) scores above 1 in OPLS-DA model and \( p \) values lower than 0.05 were considered statistically significant in separating groups.

### 2. Results and discussion

#### 2.1. Growth inhibition of AgNPs on \( S. \) obliquus

TEM imaging shows that the Citrate Coated AgNPs exist in a variety of shapes, but most of them are roughly spherical, with the average diameter estimated to be around 20 nm (Appendix A Fig. S2). The AgNPs aggregated in BG11 culture medium with the average hydrodynamic diameter of AgNPs increasing from 74.3 ± 8.6 nm in the pure water to 82.10 ± 11.2 nm in BG11 culture medium (Appendix A Fig. S3). The presence of electrolytes in the BG11 medium could induce the slight aggregation of AgNPs.

The growth inhibition rate of \( S. \) obliquus was studied by exposing the algae with various concentrations of AgNPs in BG11 medium, each concentration is repeated three times. The dose–response curve of the exposure results is shown in Fig. 1. With increased AgNP concentration, the percentage of growth inhibition of \( S. \) obliquus was observed to increase as well. The percentage of growth inhibition in the low concentration 1 to 10 \( \mu \)g/L was 0.342% to 2.706%. It can be seen that AgNPs at low concentrations had no obvious inhibition effect on \( S. \) obliquus, and even showed positive stimulus at 1 \( \mu \)g/L. In order to give insight to the effect of AgNPs on \( S. \) obliquus at low concentrations, we chose metabolomic technology to analyze the inhibitory effect of AgNPs on \( S. \) obliquus from molecular mechanisms.

#### 2.2. Metabolite identification and function analysis

In this study, metabolomic techniques were used to analyze the changes of metabolites in \( S. \) obliquus exposed to 1, 10 and 100 \( \mu \)g/L of AgNPs. We set up three replicate samples at each concentration. The metabolite was tested using gas chromatography-quadrupole time of flight mass spectrometry (GC-QToF-MS) and the results are shown in Table 1. The study identified 30 metabolites including 12 types of organic acids, 5 types of carbohydrates, 9 types of amino acids and other 4 compounds.

Further analysis of metabolic group data, principal component analysis (PCA) was performed (Fig 2a). In the PCA analysis, the Quality Control (QC) is clustered in the score plot, indicating that the method used in the experiment was stable and the instrument was running well. In order to better compare the changes in metabolites between control group and each concentration groups, we conducted further orthogonal partial
least squares-discriminant analysis (OPLS-DA) (Fig. 2b–d). The analysis was carried out between each exposure concentration group and the control group. After OPLS-DA, Variable Importance in Projection (VIP) values were used. The t-test was also performed between the two groups. Statistical significance between the two groups was defined as VIP > 1 and p value < 0.05.

The abundance of 9 metabolites, D-fructose, D-galactose, fumaric acid, glycine, L-aspartic acid, malic acid, palmitic acid and sucrose, was significantly affected by exposure to AgNPs (Table 2). In addition, the 30 metabolites of the identified results were visualized by Multi Experiment Viewer (MeV) version 4.8 as shown in the heatmap (Fig. 3). Red color means the metabolites with positive significances in discriminating between groups, blue is opposite.

Compared with the control group, the metabolites content of the treatment group (1, 10, 100 μg/L AgNPs) showed different trends. For example, the metabolites of D-fructose, D-galactose, sucrose, fumaric acid, L-aspartic acid and malic acid decreased gradually with the increase of AgNP concentration. These compounds in the 1 μg/L content are higher than those of the control group, showing a stimulating effect, especially D-galactose and sucrose, which were 7.75 and 7.71 times the control group. But at 100 μg/L the content of these compounds was lower than that of the control group, showing inhibition. The content of D-galactose and sucrose, was 0.01 times and 0.06 times of the control group, respectively at 100 μg/L AgNPs) showed a significant reduction in the levels of these metabolites. With the increase in AgNP concentration, the self-repair mechanism of algal cells could have become irreversibly damaged, leading to galactose metabolic pathway derangement and significant changes, which is consistent with the results of our inhibitory studies.

The adsorption of AgNPs on the surface of S. obliquus may have had an effect on the cell wall of the algae. At low dose (1 μg/L) AgNPs, this could have stimulated the cell wall repair mechanism, leading to upregulation of galactose metabolism as the metabolic products were higher than control. With the increase in AgNP concentration, the self-repair mechanism of algal cells could have become irreversibly damaged, leading to galactose metabolic pathway derangement and significant reduction in the levels of these metabolites. A significant increase in sucrose content at 1 μg/L may also be a stress response mechanism of algal cells, improving their own defense capabilities through the accumulation of energy sources (Kajikawa et al., 2015).

At the same time, sucrose hydrolysis produces glucose and fructose which degrade into pyruvate by glycolysis, and is involved in the TCA cycle through decarboxylation to acetyl coenzyme A (Tavan and Ayar, 2015). Malic acid is an intermediate of the TCA cycle and can be present in algal cells as precursors of carbohydrate, fat and amino acid synthesis pathways (Huang et al., 2016). In addition, it is an important intermediate for bio-oxidation defense mechanisms and cellular mechanisms of tolerance induction (Agrawal et al., 2012). Its content increased significantly with adverse environmental changes, which is consistent with the results of our inhibitory studies.

![Fig. 1 – C60 concentration (C) response curve for Scenedesmus obliquus.](image-url)
Carbon metabolism is an important step in the metabolic processes mentioned above, and malic acid is an important intermediate substance in carbon metabolism that plays a role in maintaining ion balance and pH regulation (Iwasaki et al., 1988). Fumaric acid, L-aspartic acid and glycine, which were detected together with malic acid, are also involved in the process of carbon metabolism as shown in Appendix A Fig. S5. This confirmed the inhibitory effect of AgNPs on S. obliquus.

Moreover, glycine is an endogenous antioxidant, constituting reduced glutathione. It is important to counteract increased oxidative stress, which can result from AgNP exposure (Noctor et al., 1999).

2.3. Speculated mechanism

Many studies have suggested that AgNPs can be adsorbed onto the surface of microbial cells by electrostatic attraction, hydrogen bonding, specific coordination, etc. The nanoparticles may enter the cells through the pores in the cell wall. It can also enter cells from the cell wall and breaks into the cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>P-value</th>
<th>Variable importance in projection</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 vs 1</td>
<td>0 vs 10</td>
<td>0 vs 100</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>2.09E-02</td>
<td>6.21E-04</td>
<td>3.70E-03</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>4.88E-02</td>
<td>7.80E-01</td>
<td>4.85E-02</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>4.77E-03</td>
<td>8.69E-01</td>
<td>8.59E-01</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2.47E-02</td>
<td>3.54E-02</td>
<td>6.14E-02</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.78E-01</td>
<td>1.52E-01</td>
<td>4.64E-02</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>2.07E-02</td>
<td>1.17E-02</td>
<td>1.99E-03</td>
</tr>
<tr>
<td>Malic acid</td>
<td>5.46E-01</td>
<td>3.41E-02</td>
<td>3.55E-01</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>2.59E-01</td>
<td>7.37E-01</td>
<td>4.70E-02</td>
</tr>
<tr>
<td>Sucrose</td>
<td>4.82E-02</td>
<td>4.67E-01</td>
<td>4.86E-02</td>
</tr>
</tbody>
</table>

* The 0, 1, 10, 100 in this line means 0 μg/L, 1 μg/L, 10 μg/L and 100 μg/L.
The dissociation of metal ions from AgNPs can be carried into cells by specific channels, but the dissolution of AgNPs is very limited and the dissolution mass ratio within 24 hr is less than 10% (Loza et al., 2014; Miao et al., 2010; Piccapietra et al., 2012). These adsorption and internalization processes can cause a series of bio-toxic effects, such as shading effect, cell physical damage, cell morphological changes, further absorption of nanoparticles and so on (Miao et al., 2009).

Previous studies have found that S. obliquus has significant absorption of AgNPs. In the process of absorption of AgNPs, the cell wall of S. obliquus is damaged, and algal cells have significant oxidative stress effect (Zhang et al., 2016). Combined with the results of metabolomics we can infer the following speculated molecular mechanism.

In the pathways by which AgNPs enter algal cells, the possibility of entry from the areas of cell wall rupture is greatest. As a result, galactose metabolic pathway was significantly affected; the metabolite of this pathway changed from stimulation to inhibition with concentration increasing (Fig. 5a–d). Galactose is an important component of the cell wall. At low doses, increased content indicates that cells are actively repairing damaged cell walls. At higher doses, accumulation of AgNPs inhibits galactose metabolism pathway, leading to irreparable damage of the cell wall, and thus provides the possibility for the large number of AgNPs entering cells. This speculation coincides with the conclusion of Razack et al. (2016). AgNPs that enter the cell cause increased intracellular oxidative stress and may interact with the cell contents to form phytochelatin (PC) (Chen et al., 2012; Domingos et al., 2011; Dwivedi and Ma, 2014; Gekeler et al., 1989). In this process, intracellular glutathione content will be significantly increased to counteract the oxidative stress. The increase in glycine observed further supports our inference as glycine is an important component of glutathione amino acids and glutathione changes are closely linked. The trend of malic acid is similar to that of glycine (Fig. 5f). Malic acid can induce biological tolerance mechanisms and is also closely related to oxidative stress. Moreover, as AgNPs continue to accumulate in algal cells, this hinders the cell from obtaining sufficient essential nutrients. Therefore, algal cells respond by accumulating energy sources like sucrose to counteract the effects of AgNPs (Smith and Stitt, 2007), which was observed in our results (Fig. 5b).

Fig. 3 – Heatmap of metabolite changes of Scenedesmus obliquus exposed to various concentrations of AgNPs.

Fig. 4 – Interaction between nanoparticle and Scenedesmus obliquus.
3. Conclusions

S. obliquus is the primary producer of most aquatic ecosystems. It is therefore important to understand the impact of AgNPs on it. In this study, the effects and the molecular mechanisms of low-dose AgNPs were investigated and described by using metabolomic analysis. It was found that AgNPs may damage the cell wall of S. obliquus, upregulating galactose metabolism (for cell wall repair) and substances to counteract oxidative stress. AgNP might interrupt the TCA cycle and the accumulation of energy sources (sucrose). This study not only showed new insights into the molecular mechanism of AgNPs toxicity, but also provides important data on metabolites for future studies. Ultimately, it provides an early forecast of ecological risks using nanoparticles based on other metals in the aquatic environment.

For future studies, the toxic effects of different species of nanoparticles on single-celled organisms at low doses can be further explored. Life-cycle studies could also be conducted to investigate whether the toxic effects produced by AgNPs are lasting.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2018.05.017.


