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Effect and mechanism of TiO_2 nanoparticles on the photosynthesis of *Chlorella pyrenoidosa*

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ABSTRACT

Titanium dioxide nanoparticles (n-TiO_2) have been used in numerous applications, which results in their release into aquatic ecosystems and impact algal populations. A possible toxic mechanism of n-TiO_2 on algae is via the disruption of the photosynthetic biochemical pathways, which yet remains to be demonstrated. In this study, *Chlorella pyrenoidosa* was exposed to different concentrations (0, 0.1, 1, 5, 10, and 20 mg/L) of a type of anatase n-TiO_2, and the physiological, biochemical, and molecular responses involved in photosynthesis were investigated. The 96 h half growth inhibition concentration (IC_{50}) of the n-TiO_2 to algae was determined to be 9.1 mg/L. A variety of cellular and sub-cellular damages were observed, especially the blurry lamellar structure of thylakoids, indicating the n-TiO_2 impaired the photosynthetic function of chloroplasts. Malondialdehyde (MDA) and glutathione disulfide (GSSG) significantly increased while the glutathione (GSH) content decreased. This implies the increased consumption of GSH by the increased intracellular oxidative stress upon n-TiO_2 was insufficient to eliminate the lipid peroxidation. The contents of photosynthetic pigments, including chlorophyll *a* (Chl *a*) and phycobiliproteins (PBPs) in the exposed algal cells increased along with the up-regulation of genes encoding Chl *a* and photosystem II (PS II), which could be explained by a compensatory effect to overcome the toxicity induced by the n-TiO_2. On the other hand, the photosynthetic activity was significantly inhibited, indicating the impairment on the photosynthesis via damaging the reaction center of PS II. In addition, lower productions of adenosine triphosphate (ATP) and glucose, together with the change of gene expressions suggested that the n-TiO_2 disrupted the material and energy metabolisms in the photosynthesis. These findings support a paradigm shift of the toxic mechanism of n-TiO_2 from physical and oxidative damages to metabolic disturbances, and emphasize the threat to the photosynthesis of algae in contaminated areas.

1. Introduction

Nanomaterials are increasingly used in industrial production as well as in electronic, biological, and medical research (Patra et al., 2012). Titanium dioxide nanoparticles (n-TiO_2) are among the most commonly produced nanomaterials with wide applications in a variety of industries including but not limited to solar cells, cosmetics, food, and environmental remediation due to their excellent photocatalytic activity (Chorianopoulos et al., 2011; Tong et al., 2012; Jang et al., 2016; Bendjabeur et al., 2018). As a result, n-TiO_2 are increasingly released in aquatic environments, which results in inevitable contamination (Amde et al., 2017). The estimated n-TiO_2 concentration in sediments in Switzerland was up to 2.4 mg/kg, which might be further increased with the increasing use of n-TiO_2 (Gottschalk et al., 2009). The possible effects of n-TiO_2 on aquatic life, especially algal sources of primary production, have become a large concern (Kulacki and Cardinale, 2012).

Many studies have investigated the effects of n-TiO_2 on algae, and growth inhibition have been extensively observed (Hong and Otaki, 2006; Kulacki and Cardinale, 2012). Very diverse half growth inhibition values (IC_{50}) have been reported, which are related to physical and chemical properties of n-TiO_2 and culture media (Ji et al., 2011; Menard et al., 2011; Metzler et al., 2011; Lin et al., 2012; Magdolenova et al., 2014; Gao et al., 2018). Evidence showed that nanoparticles (NPs) on the surface of algal cells might reduce light availability for the photosynthesis, resulting in a shading effect (Schwab et al., 2011; Long et al., 2012; Wang et al., 2016). The cell surface binding and/or internalization of NPs may elevate intracellular reactive oxygen species (ROS) level, which would thus induce consumption of the antioxidants (e.g., glutathione, GSH) and affect the activity of related enzymes (such

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as SOD, CAT, etc.) (Li et al., 2015). Previous studies have demonstrated that the toxicity of n-TiO_2 to algae is related to oxidative stress (Morelli et al., 2018; Liu et al., 2018). Li et al. (2015) reported that algae were damaged by the n-TiO_2-induced ROS accumulation and the chloroplast was the site of ROS production. Therefore, the damage caused by n-TiO_2 in the chloroplast could possibly interfere with the light harvest, electron transfer, and energy metabolism in the photosystem (PS), which however remains to be investigated. Moreover, to the best of our knowledge, no research has investigated the effect of n-TiO_2 on the gene expression of algae.

Photosynthesis is a complex series of biochemical reactions that plants and algae use to convert solar energy, water, and carbon dioxide into ATP and glucose, which plays an important role in algal growth (Singh and Singh, 2015). The process can be divided into two parts: the light-dependent reactions and the light-independent reactions (also referred as the dark reactions) (Lu et al., 2015). In the light-dependent reactions, the ATP synthesis is catalyzed by F_0F_1 ATP synthase (Elston et al., 1998). Direct evidence has been found that the proton gradient generated by electron transfer complexes and light-harvesting proteins provides the energy for the synthesis of ATP (Simpson and Knoetzel, 1996), which is used to fix carbon from carbon dioxide into the energy storing carbon compounds in the dark reactions (Lu et al., 2015). In green algae, the photosynthetic process occurs in the thylakoid membrane, which composed of four major protein complexes including PS II, cytochrome b_6f, PS I, and ATP synthase (Choquet and Vallon, 2000). The light harvesting pigments of the PS, including Chl *a* and PBPs, are driven to a higher energy state with the help of light energy, which reflects the photosynthetic efficiency and provides information on the structure and function of PS II (Renger, 2013). In addition, the photosynthetic activity including electron transport rate (ETRmax) and maximum photosynthetic yield (Fv/Fm) have been widely used in detection and evaluation of various stresses on the algal photo-system (Kumar et al., 2014; Chae and An, 2016).

C. pyrenoidosa is a green unicellular alga, which has been used as a classical ecotoxicological model for aquatic ecosystems because of its extra-sensitivity to contaminants (Li et al., 2013; Shao et al., 2015). In the present study, the toxic effects of a type of anatase n-TiO_2 on the photosynthetic system of *C. pyrenoidosa* were evaluated from cellular, biochemical, and molecular levels. The deleterious impacts were characterized as growth inhibition, ultrastructure damage, and lipid peroxidation. GSH and glutathione disulfide (GSSG) were measured to evaluate the antioxidative responses. Moreover, contents of photosynthetic pigments (Chl *a* and PBPs), the photosynthetic activity (ETRmax and Fv/Fm), adenosine triphosphatase (ATPase), ATP, glucose, and the expression of photosynthesis-related genes were investigated to gain a systematical understanding of the toxicity of n-TiO_2 on the photosynthetic process in algae. The results will provide insightful and theoretical guidance for the risk assessment and safe use of nanomaterials.

2. Materials and methods

2.1. Algal cultivation and n-TiO_2 exposure

C. pyrenoidosa was purchased from the Institute of Wuhan Hydrobiology of Chinese Academy of Sciences, China. The algal cells were cultured in 250-mL Erlenmeyer flasks containing 100 mL of the culture medium recommended by the guideline (No. 201) of Organization for Economic Co-operation and Development (OECD) with or without test materials and were kept in an incubation shaker (120 rpm, 25 °C) with illumination by white incandescent lights (100 ± 5 μE/m^2/s, light: dark of 14:10 h) (OECD, 2011). The chemical composition of the culture medium is also given in Table S1 in the Supporting information and the detailed test conditions are summarized in Table S2.

The anatase n-TiO_2 were purchased from Zhejiang Hongsheng Material Technology Co., China, which were characterized in detail in our previous studies (Zhang et al., 2017; Gao et al., 2018). The measured particle size was 12.0 ± 3.5 nm, and the properties and transmission electron microscope (TEM) image are shown in Table S3 and Fig. S1 in the Supporting information, respectively. Different concentrations (0, 0.1, 1, 5, 10 and 20 mg/L) of the n-TiO_2 were suspended with algal culture in 250-mL Erlenmeyer flasks. Before the inoculation of algal cells, media were sonicated (600 W, 40 kHz, 25 °C) in a bath for 30 min to fully disperse the NPs.

2.2. Algal growth assay

The algal cells in each sample were counted at least three times using a counting chamber under a light microscope (LM, Olympus, CX21, Japan), and the density in each treatment group was calculated. The initial algal density was 3.66×10^5 cells/mL and the exposure was maintained for 96 h (Long et al., 2012; Zhang et al., 2017; Gao et al., 2018). The control algae exponentially grew in 96 h (Fig. S2). After the 96 h exposure, the growth inhibition ratio (IR) was calculated as follows: IR (%) = (Xc − Xt)/Xc × 100%, where Xc and Xt are the average cell densities (cells/mL) in the control and treatment groups, respectively. The IC_{50} values, which represent the concentrations of the test substances leading to 50% reduction in the algal growth compared to the control, were calculated from the dose-response curves with SPSS 20.0 for Windows (SPSS, Inc., Chicago, IL, USA).

2.3. Morphology and ultrastructure observations

After the 96 h exposure, the algal cell morphologies were observed using a scanning electron microscope (SEM) (Hitachi S4800, Japan) and a TEM (JEM-1230, JEOL, Japan) following the method described in our previous papers (Long et al., 2012; Zhang et al., 2017; Gao et al., 2018). Briefly, the algal cells were fixed in 2.5% glutaraldehyde, dehydrated in gradient concentrations of ethanol, coated with a layer of gold, and then followed by the SEM observation. For the TEM imaging, the algal cells were fixed in glutaraldehyde overnight followed by staining with osmium tetroxide, dehydration, embedding, and ultrathin section.

2.4. Determination of MDA, GSH, and GSSG

In each group, algal cells from 200 mL of culture were collected by centrifugation at 8000 *g* for 10 min after the 96 h exposure. The samples were homogenized as described by Gao et al. (2018). After the centrifugation at 3000*g* for 10 min at 4 °C, the supernatant was collected and divided for subsequent experiments. Malondialdehyde (MDA) is an oxidation product of lipid peroxidation and an indicator of cell membrane peroxidation. The level of MDA was determined using the thiobarbituric acid reactive substance (TBARS) assay with the MDA assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) with procedures detailed in our previous study (Zhang et al., 2017). GSH and GSSG have been used for evaluating the antioxidative and detoxification states in algae. The contained GSH and GSSG levels were measured using the GSH and GSSG assay kit (Beyotime Institute of Biotechnology, Shanghai, China), which were determined using 5,5-dithiobis-(2-nitrobenzoic)-acid (DTNB) as substrate to measure the absorbance at 412 nm according to Griffith (1980) method. All values were normalized by cell density and were reported in nmol/10^5 cells.

2.5. Contents of photosynthetic pigments and photosynthetic activity

The contents of photosynthetic pigments (Chl *a* and PBPs) in each culture were analyzed according to the method of Johnson et al. (2014) and Deng et al. (2017). After the 96 h exposure, Chl *a* and PBPs were extracted from the algae in 90% ethanol in the dark for 24 h, followed by 15 min of centrifugation at 3000*g*, and diluted to 10 mL with 90%