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The synergic impacts of TiO_2 nanoparticles and 17β-estradiol (E2) on the immune responses, E2 accumulation, and expression of immune-related genes of the blood clam, *Tegillarca granosa*

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ABSTRACT

The extensive use of TiO_2 nanoparticles ($nTiO_2$) in industrial products has led to their release into the marine environment, thereby posing a potential risk to marine organisms. However, in addition to affecting marine organisms through its inherent properties, $nTiO_2$ can also act as a vehicle for other toxic pollutants due to their strong adsorption ability through the "Trojan horse" effect. Due to their potential hazard, the endocrine disrupting chemicals (EDCs) such as 17β-estradiol (E2), have been considered as one of the most serious anthropogenic threats to biodiversity and ecosystem health. However, there is still a lack of knowledge regarding the possible synergistic effects of $nTiO_2$ and endocrine disrupting chemicals (EDCs) on marine organisms to date. Therefore, the combined effects of $nTiO_2$ and 17β-estradiol (E2) on the immune responses of the blood clam, *Tegillarca granosa*, were investigated in this study. After 10 days of treatment, the total number, phagocytic activity, red granulocytes ratio, and the phagocytosis of hemocytes were significantly reduced in almost all treatment groups. Furthermore, expressions of genes from NFκβ and Toll-like receptor signaling pathways were significantly altered after exposure to $nTiO_2$ and/or E2, indicating a reduced sensitivity to pathogen challenges. In addition, compared to exposure to E2 alone, co-exposure to E2 and $nTiO_2$ led to a significant increase in the content of alkali-labile phosphate (ALP) in hemolymph, suggesting an enhanced E2 bioconcentration in the presence of $nTiO_2$. In general, the present study demonstrated that $nTiO_2$ enhanced the immunotoxicity of E2 to the blood clam, which may be due to the increased E2 uptake in the presence of $nTiO_2$.

1. Introduction

TiO_2 nanoparticles ($nTiO_2$), which possess unique properties in catalytic activity and ultraviolet (UV) absorption, can be synthesized easily and therefore widely used in a variety of products, such as sunscreens, light-emitting diodes, photovoltaic cells, sporting goods, and water treatment agents [1,2]. In recent decades, this widespread application has boosted the global production of $nTiO_2$, which has been predicted to be more than half a million tons per year by 2020 (www.nanoproject.org). Inevitably, large amounts of $nTiO_2$ will be released into the environment, probably ending in the marine environment through the production, usage, handling, and disposal of products containing $nTiO_2$ [3-5]. Consequently, the effects of $nTiO_2$ on marine organisms and ecosystems represent a major concern in recent years [5]. Several studies have reported that $nTiO_2$ can exert negative effects on a wide range of marine organisms by disrupting their biological processes and physiological functions, including biofilm formation [6], larval development [7,8], growth [9], and immune responses [10,11].

However, $nTiO_2$ is not the only substance in the marine environment that marine organisms could be exposed to in their life cycle. In the past few decades, the extensive use of organic chemicals, specifically in industry and agriculture, have led to a mass release of endocrine disrupting chemicals (EDCs) into the aquatic environment [12], posing a potential threat to the environment and human health. Due to their potential hazard, EDCs have been considered as one of the most serious anthropogenic threats to biodiversity and ecosystem health [13]. Various types of EDCs have been ubiquitously detected in water collected from the ambient environment, ranging from ng/L to μg/L [14-16], which has drawn worldwide concern over their potential impacts on marine organisms and ecosystems. The presence of EDCs has been demonstrated to induce a series of reproductive abnormalities [17] and alter immune function [18] of marine animals even in trace

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amounts. However, current knowledge of the biological effects and the mechanisms of action of EDCs on marine organisms are mostly from vertebrates, while further studies on their effects on marine invertebrates, which represent an important component of marine ecosystems, are still needed.

In addition to its inherent properties, $nTiO_2$ can also affect marine organisms indirectly as a vehicle for other toxic pollutants due to the strong adsorption ability [19,20]. After binding to $nTiO_2$, toxic pollutants can be transported to sites where they would not normally go, and this 'Trojan horse' effect may result in increased uptake and toxicity of other pollutants [3]. Recently, increasing attention has focused on the interaction between $nTiO_2$ and other pollutants, including heavy metals [21], 2,3,7,8–tetrachlorodibenzo -p-dioxin (TCDD) [22], and phenanthrene [23]. However, to the best of our knowledge, the synergistic effects of $nTiO_2$ and EDCs on marine organisms still remain unknown. Moreover, although the immune system in marine organisms represents a significant target for both $nTiO_2$ [24,5]; and EDCs [18], their combined effects on immune responses of marine species remain unknown.

The benthic filter feeders, such as bivalves, are some of the most effective sinks for pollutants in the estuarine and marine food web [25]. Moreover, many marine bivalves are also sensitive to waterborne pollutants and therefore are excellent species in the monitoring of contaminants in the sea [26]. However, the lack of knowledge regarding the impacts of $nTiO_2$ and EDCs severely constrains the use of marine bivalves in practical monitoring. The blood clam, *Tegillarca granosa*, is a traditional aquaculture marine bivalve species widely distributed throughout the Indo-Pacific region [27]. Since living in the intertidal mudflat, where pollutants are often concentrated, blood clams can be exposed to $nTiO_2$ and EDCs pollution simultaneously [28].

In order to improve our present understanding of the combined effects of $nTiO_2$ and EDCs on marine bivalve species, this study was conducted to: 1) examine the synergistic impacts of $nTiO_2$ and 17β-estradiol (E2), as a model of EDCs, on the immune responses in terms of total counts, cell type composition, and phagocytosis activity of hemocyte in *T. granosa*; 2) investigate whether $nTiO_2$ facilitates the uptake of E2 and thus increases the vitellogenin (Vg) level (indicated by the content of ALP); 3) verify whether the expressions of immune-related genes, including TNF receptor associated factors (TRAFs), nuclear factor κβ1 (NFκβ1) were altered after $nTiO_2$ and E2 co-exposure.

2. Materials and methods

2.1. Chemicals

Commercial $nTiO_2$ with particle sizes ≤50 nm were purchased from Klamar (Shanghai, China). The characterization of $nTiO_2$ was performed following the method of our previous study (Table 1) [11]. Particle morphology and size were determined by transmission electron microscopy (Fig. 1) (TEM, JEM-1230, JEOL, Tokyo, Japan). The stock solution (1 g/L) was prepared daily by dispersing the NPs in 1.0 μm-filtered seawater, followed by sonication for 15 min. Test solutions of $nTiO_2$ were prepared immediately prior to use by diluting the stock solution with filtered seawater. The E2 (≥98% purity) used in the present study was obtained from Sigma-Aldrich (St. Louis, USA). Stock solution of E2 (1 g/L) was prepared freshly every day by dissolving the drug in ethanol and diluting to the different required concentrations with filtered seawater.

Table 1
Physicochemical properties of $nTiO_2$ used in this study.

Property	$nTiO_2$
Average diameter	40 ± 8 nm
BET surface area	$60.70\ m^2\ g^{-1}$
Crystal structure	irregular
Purity	99.8%

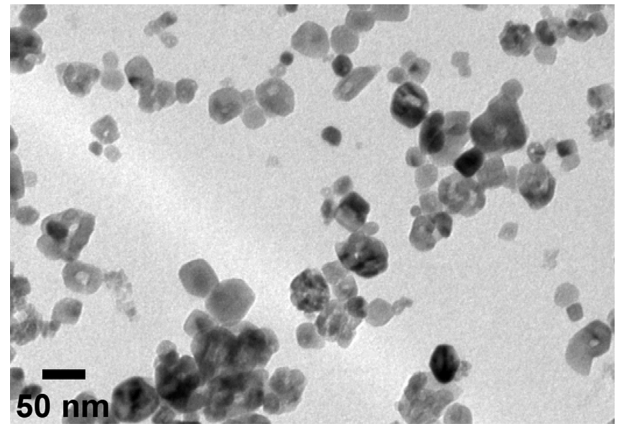

Fig. 1. TEM micrograp of the $nTiO_2$ samples.

2.2. Collection and acclimation of bivalves

Samples of blood clams (8.50 ± 0.62 g) were collected from Yueqing Bay (28° 280′ N and 121°110′ E) in Wenzhou, China in August 2017. To obtain the background concentration of TiO_2, seawater was sampled and analyzed in triplicate. The background concentrations of Ti and E2 were found to be under the detection limits and as low as 20 ng/L, respectively. In order to determine the TiO_2 and E2 concentrations in the tested bivalve species, the $nTiO_2$ and E2 contents in 10 blood clam individuals collected from the sampling sites were analyzed following the methods of the National Standard of China (GB5009.246–2016; Bulletin of the China's Ministry of Agriculture 958-10-2007). TiO_2 concentrations were found under the detection limits (0.3 ng/mg) and E2 concentrations were less than 5 ng/g in all tested samples. Prior to the performance of the experiment, clam samples were acclimatized in a 2000-L indoor tank containing filtered and UV-radiated natural seawater (temperature at 28.0 ± 0.5 °C, pH at 8.10 ± 0.05, and salinity at 21.0 ± 0.5‰) with continuous aeration for 7 days. During the process of acclimation, clams were fed once daily with microalgae *Tetraselmis chuii* at a rate of 5% dry tissue weight [29].

2.3. Exposure experiments

In order to investigate the synergistic effects of $nTiO_2$ and E2 on the immune responses of *T. granosa*, six treatment groups and one control group were set up as follows: (1) control without adding $nTiO_2$ or E2, (2) E2 exposure treatments with 100 or 200 ng/L E2, (3) co-exposure treatments with 0.05 mg/L $nTiO_2$ and 100 ng/L E2 or 0.1 mg/L $nTiO_2$ and 100 ng/L E2, and (4) $nTiO_2$ exposure treatments with 0.05 or 0.1 mg/L $nTiO_2$. According to previous studies, the exposure concentrations of E2 (100 ng/L) and $nTiO_2$ (0.05 and 0.1 mg/L) were chosen to simulate realistic environmental concentrations of these two pollutants [30,31]. In addition, 200 ng/L E2 was also used in the exposure group to compare the impacts exerted on the immune responses between co-exposure groups and those exposed to E2 alone at a higher concentration. For the co-exposure groups, $nTiO_2$ and E2 were mixed together shortly before the start of exposure. Three parallel tanks were used for each treatment as triplicates. After acclimation, 630 clams were randomly assigned to 21 individual tanks (7 treatments × 3 replicates) at a total seawater volume of 50 L with slight aeration. Concentrations of $nTiO_2$ and E2 in each treatment were measured every 2 days during the experiment (Table 2). The clams were fed with *T. chuii* as descried previously and seawater was changed daily with newly added $nTiO_2$ and/or E2 at corresponding designed concentrations after feeding. The exposure lasted for 10 days, and no individual mortality was observed throughout the experimental period (acclimation