

Effects of titanium dioxide nanoparticles on intestinal commensal bacteria

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Abstract Nanomaterials and nanotechnology have great potential in the biological and biomedical field. Recent studies reveal that many nanomaterials possess antibacterial activities. While most of these studies focus on the ability of nanomaterials to inhibit the growth of pathogenic bacteria in vitro, few of them test the effects of nanomaterials on intestinal commensal bacteria. Here, we report that TiO₂ nanoparticles (10, 50 and 100 nm in size) can inhibit the growth of *Drosophila* intestinal commensal bacteria in vitro. This activity depends on the dosage or size, but is independent of the photocatalytic activity of TiO₂ nanoparticles. Surprisingly, dietary TiO₂ nanoparticles of the same dosage fail to display similar effects in *Drosophila* larvae or adults. These flies show a normal amount of intestinal commensal bacteria, as well as a normal developmental cycle, energy store, and locomotor activity. These results imply that the antibacterial effect of TiO₂ nanoparticles differs in vitro and in vivo.

Keywords TiO₂ nanoparticles · Antibacterial activity · Commensal bacteria

1 Introduction

Nanomaterials have wide applications in the biological and biomedical field. Nanoparticles (NPs), carbon nanosheets or nanotubes, and DNA-based nanostructures can serve as platforms for the quantitative detection of protein, DNA, and small molecules [1–5]. Alternatively, they can also serve as vehicles to deliver biological molecules into cells and animals [6–9]. In addition, some nanomaterials possess antibacterial activities due to their distinct physical or chemical properties [10–14]. For example, graphene and graphene oxide may extract phospholipids and destroy bacterial membrane integrity [10]; silver NPs may inhibit bacterial growth via producing positively charged Ag ions [12]; ZnO and TiO₂ NPs show photocatalytic activity, which produces reactive oxygen species (ROS) and hydrogen peroxide (H₂O₂) [13]. However, the precise molecular mechanisms underlying the antibacterial activities of these nanomaterials remain unclear.

Earlier works on antibacterial activities of nanomaterials tend to focus on pathogenic species (harmful bacteria), while the effects of nanomaterials on commensal bacteria (symbiotic bacteria, or beneficial bacteria) have not been explored. Dialogs between commensal bacteria and intestinal epithelial have been shown to affect host development, metabolic homeostasis, immune status, and aging [15–21]. Recent studies suggest a strong link between dysregulation of symbiotic bacteria and metabolic diseases, such as type 2 diabetes and obesity [22, 23]. Given that nanomaterials will be more and more widely applied in the medical and food industries, investigating the effects of nanomaterials on symbiotic bacteria is of great significance. In addition, previous studies mainly

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concern the antibacterial activities of nanomaterials *in vitro*, whether they possess similar activities in the host is intestine has not been addressed.

In this work, we investigated the effects of TiO₂ NPs (10, 50, and 100 nm in size) on *Drosophila* intestinal commensal bacteria. We showed that TiO₂ NPs could inhibit the growth of *Drosophila* intestinal commensal bacteria *in vitro*, in a dosage or particle size-dependent manner. However, the photocatalytic activity of TiO₂ NPs was not required to inhibit symbiotic bacterial growth, suggesting that an alternative mechanism could mediate the antibacterial activity of TiO₂ NPs. Unexpectedly, we did not observe the antibacterial effect of TiO₂ NPs in the *Drosophila* gut. Dietary TiO₂ NPs did not alter the amount of intestinal symbiotic bacteria in *Drosophila* larva or adults, nor did they affect *Drosophila* development, energy store, and locomotor activity. Taken together, these results suggest that the effect of TiO₂ NPs on commensal bacteria differs *in vitro* and *in vivo*.

2 Experimental

2.1 Reagents and fly culture

10, 50, and 100-nm TiO₂ NPs were purchased from Beijing Dk Nano Technology Company. They were dissolved in double distilled water and strongly sonicated with a BILON-R1200 sonicator before mixing with agar media or fly food. w1118 flies were raised at 25 °C and 60 % relative humidity on cornmeal-yeast food. Each fly tube contained fifteen newly laid embryos or newly eclosed adult flies.

2.2 Selective media for bacteria culture

To test the antibacterial activities of TiO₂ NPs *in vitro*, commensal bacteria cultured in liquid selective media were plated onto selective plates with or without premixed TiO₂ NPs. To test the antibacterial activities of TiO₂ NPs *in vivo*, fly larvae or adults were raised on food with or without premixed TiO₂ NPs. Dissected midguts were homogenized in PBS and plated onto selective plates. Selective media were generated as below [24]: *Acetobacteria*: 25 g/l D-mannitol, 5 g/l yeast extract, 3 g/l peptone, and 15 g/l agar (for plates); *Enterobacteria*: 10 g/l Tryptone, 1.5 g/l yeast extract, 10 g/l glucose, 5 g/l sodium chloride, and 12 g/l agar (for plates); *Lactobacilli*: 70 g/l BD Difco Lactobacilli MRS media and 12 g/l agar (for plates). Plates were incubated at 30 °C for 48 h, and bacterial colonies were counted. Colony-forming units (CFU) represented the average number from four plates.

2.3 Measurement of *Drosophila* weight, lipid, and locomotor activity

Drosophila weight was measured with a Mettler Toledo XP6 analytic balance. Triacylglyceride levels were measured as previously described [25]. Briefly, eight third instar larvae or adults were suspended in 400 µl chilled lysis buffer (0.01 mM KH₂PO₄, 1 mM EDTA, protease inhibitors cocktail, 0.05 % Tween 20), lysed by TissueLyser II (Qiagen). The lysate was extracted with 1 ml chloroform:methanol (2:1). The organic phase was transferred to a new tube and air-dried over night at room temperature. The dried lipid was re-dissolved in 300 µl alcohol with 1 % Triton-X100 and assayed with Free Glycerol Reagent (Sigma F6428) and Triglyceride Reagent (Sigma T2449). Lipid levels were normalized to total protein levels. Locomotor activity was measured according to the previous report [26, 27]. Forty-five adult flies were dispersed into three plastic vials and gently tapped to the bottom of the vials. The climbing distance of each fly was measured after 5 s. The experiment was repeated three times.

3 Results and discussion

3.1 Effects of TiO₂ NPs on *Drosophila* commensal bacteria *in vitro*

Previous studies propose that TiO₂ NPs inhibit bacterial growth via their photocatalytic activity, which creates ROS and hydrogen peroxide through UV radiation [13]. However, whether they possess other activities that affect bacterial growth is unclear. In addition, whether they can affect commensal bacteria and pathogenic bacteria in a similar way is largely unknown. To test these possibilities, we cultured *Drosophila* commensal bacteria on plates containing TiO₂ NPs. These bacteria were plated in a darkroom (with red light) and incubated in darkness to avoid UV radiation. To quantify each symbiotic bacterial species, we measured colony-forming units (CFU) using selective plates to identify *Acetobacteria*, *Enterobacteria*, and *Lactobacilli*, three major forms of *Drosophila* commensal bacteria.

Here, we tested TiO₂ NPs with three different sizes (referred to as TiO₂-10 nm, TiO₂-50 nm, and TiO₂-100 nm). The surface of these TiO₂ NPs was modified with hydroxyl groups. Dynamic light scattering showed that they possessed similar zeta potential: -27.3 mV for TiO₂-10 nm, -26.6 mV for TiO₂-50 nm, and -31.6 mV for TiO₂-100 nm. On selective plates containing 200 µg/ml TiO₂ NPs, growth of *Acetobacteria* was not affected by any type of TiO₂ NPs. When the concentration of TiO₂ NPs was increased to 2 mg/ml, all types of TiO₂ NPs could inhibit the growth of *Acetobacteria* to a similar extent (Fig. 1A, D). *Enterobacteria* seemed to be

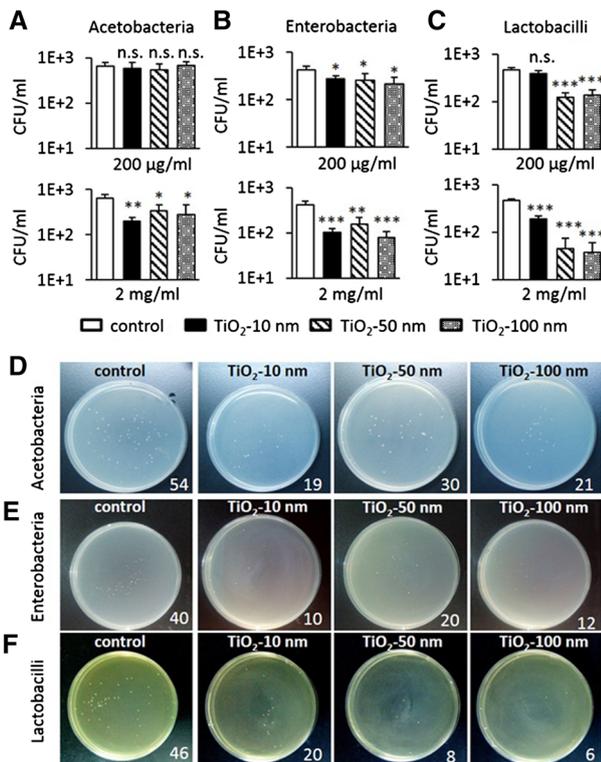


Fig. 1 (Color online) Effects of TiO₂ NPs on *Drosophila* commensal bacteria in vitro. Statistics of colony-forming units (CFU) of in vitro cultured Acetobacteria (A), Enterobacteria (B), and Lactobacilli (C) on selective plates containing 200 µg/ml or 2 mg/ml TiO₂-10 nm, TiO₂-50 nm, or TiO₂-100 nm. n.s. not significant. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Representative results of the effect of 2 mg/ml TiO₂-10 nm, TiO₂-50 nm, or TiO₂-100 nm on the growth of Acetobacteria (D), Enterobacteria (E), and Lactobacilli (F). 100 µl medium was plated on selective plates. The number of colonies on the plates was indicated on the lower right of each panel

more sensitive to TiO₂ NPs than Acetobacteria. The growth of Enterobacteria was significantly inhibited by 200 µg/ml TiO₂ NPs and was further inhibited by 2 mg/ml TiO₂ NPs. Similar to the effects on Acetobacteria, the different sizes of TiO₂ NPs did not cause different antibacterial activities on Enterobacteria (Fig. 1B, E). In contrast, the size of the TiO₂ NPs affected Lactobacilli. At concentrations of both 200 µg/ml and 2 mg/ml, TiO₂-50 nm and TiO₂-100 nm inhibited Lactobacilli growth more severely than TiO₂-10 nm (Fig. 1C, F). These results suggest that TiO₂ NPs can inhibit the growth of commensal bacteria in vitro, in a photocatalytic activity-independent manner. Different genera of commensal bacteria had different sensitivities to the dosage or the size of the TiO₂ NPs.

3.2 Effects of TiO₂ NPs on *Drosophila* commensal bacteria in vivo

Next, we examined the effects of TiO₂ NPs on commensal bacteria in vivo. We dispersed TiO₂ NPs into fly food and fed them to *Drosophila* larvae or adults. As male larvae or male

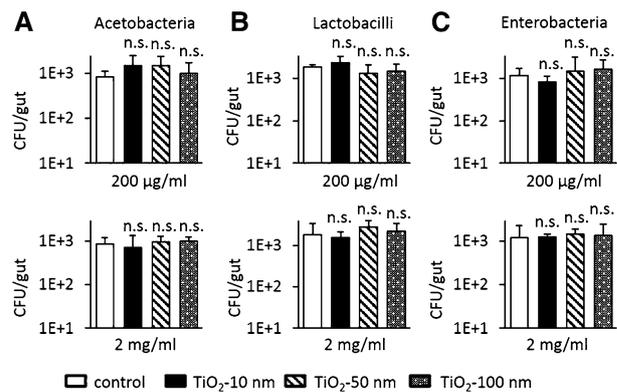


Fig. 2 Effects of TiO₂ NPs on commensal bacteria in *Drosophila* female larvae. Statistics of colony-forming units (CFU) of Acetobacteria (A), Lactobacilli (B), and Enterobacteria (C) from homogenized *Drosophila* larval guts. Larvae were raised on food containing 200 µg/ml or 2 mg/ml TiO₂-10 nm, TiO₂-50 nm, or TiO₂-100 nm until they reached the stop-wondering stage. Female larvae were picked for dissection. n.s. not significant

adults showed a large variance in the number of commensal bacteria among individuals, we decided to focus on female flies. Female larvae were collected before pupation, and female adults were collected after 5 days for gut dissection and quantification of symbiotic bacteria. Under this condition, we found that the growth of Acetobacteria, Enterobacteria, or Lactobacilli in larvae was not affected by three types of TiO₂ NPs, even though the dosage was the same as that used in vitro (Fig. 2). Similarly, either 1 mg/ml or 2 mg/ml TiO₂ NPs of three different sizes failed to significantly inhibit the growth of Acetobacteria, Enterobacteria, or Lactobacilli in adult flies (Fig. 3).

3.3 Effects of TiO₂ NPs on *Drosophila* development and metabolism

The results above were not consistent with those from in vitro. To confirm that our observation indeed reflected a discrepancy between in vitro and in vivo, we further analyzed commensal bacterial-associated developmental and metabolic functions in flies fed with TiO₂ NPs. Gut commensal bacteria play crucial roles in host development, metabolic homeostasis, immune status, and aging [15–21]. Therefore, we would expect to observe defects in developmental cycle, energy store, or locomotor activity in *Drosophila* if their gut commensal bacteria were inhibited by dietary TiO₂ NPs.

We first examined the effect of dietary TiO₂ NPs on *Drosophila* larval development. In the control group, about 90 % of female larvae formed pupae 120 h after egg laying. Either 200 µg/ml or 2 mg/ml dietary TiO₂ NPs of three different sizes did not affect the pupation cycle of the *Drosophila* larvae (Fig. 4A). In addition, the weight and

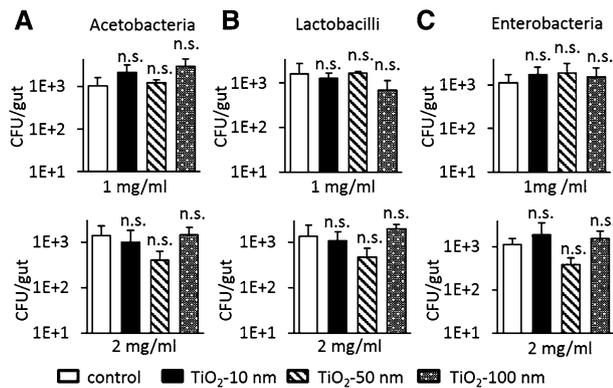


Fig. 3 Effects of TiO₂ NPs on commensal bacteria in *Drosophila* female adults. Statistics of colony-forming units (CFU) of Acetobacteria (A), Lactobacilli (B), and Enterobacteria (C) from homogenized *Drosophila* adult guts. Newly enclosed female flies were raised on food containing 200 µg/ml or 2 mg/ml TiO₂-10 nm, TiO₂-50 nm, or TiO₂-100 nm for 5 days before dissection. *n.s.* not significant

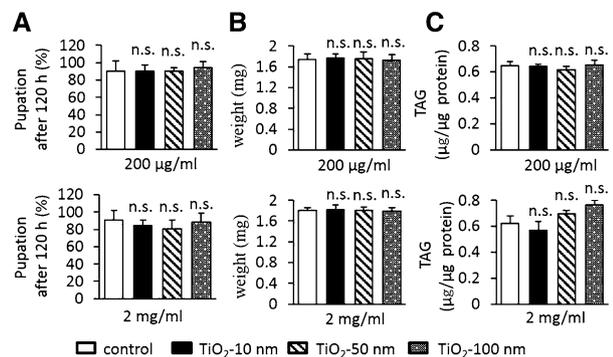


Fig. 4 Effects of TiO₂ NPs on *Drosophila* larval development and metabolism. The effects of 200 µg/ml or 2 mg/ml dietary TiO₂-10 nm, TiO₂-50 nm, or TiO₂-100 nm on pupation rate (A), weight (B), and lipid level (C) of female larvae were shown. *n.s.* not significant

lipid level of the female larvae were not altered by any type of TiO₂ NPs (Fig. 4B, C).

We then examined 5-day-old female adult flies raised on food containing 1 or 2 mg/ml TiO₂ NPs. Similar to our observation in larvae, the weight and lipid level in female adults were comparable to those in the control group (Fig. 5A, B). We also measured the climbing index (i.e., average climbing speed of flies on the wall of the fly tubes) of these flies and observed no significant changes with dietary TiO₂ NPs (Fig. 5C). Together, these results imply that the *in vivo* antibacterial activity of TiO₂ NPs on *Drosophila* gut commensal bacteria differs from *in vitro* activity.

4 Conclusion

Our results show that TiO₂ NPs can inhibit the growth of symbiotic bacteria *in vitro*, in a mechanism that is different from their photocatalytic activity. Enterobacteria are more

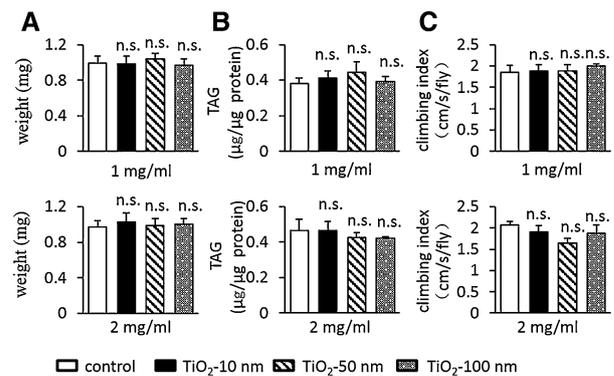


Fig. 5 Effects of TiO₂ NPs on metabolism and locomotor activity in *Drosophila* female adults. The effects of 200 µg/ml or 2 mg/ml dietary TiO₂-10 nm, TiO₂-50 nm, or TiO₂-100 nm on weight (A), lipid level (B), and climbing index (C) of 5-day-old female adults were shown. *n.s.* not significant

sensitive to TiO₂ NPs than Acetobacteria. For these two types of bacteria, the size of the TiO₂ NPs does not affect their antibacterial activities, only dosage matters. In contrast, TiO₂ NPs inhibit the growth of Lactobacilli in both size and dosage-dependent manner.

The results from the *in vivo* experiments do not agree with those from *in vitro*. TiO₂ NPs do not inhibit the growth of symbiotic bacteria in the gut of *Drosophila* larva or adults. The interaction between commensal bacteria and host is mutually beneficial, and host gut can provide a completely different environment from *in vitro* culture media. Therefore, it is not surprising that the host gut can protect commensal bacteria from TiO₂ NPs better than the *in vitro* environment.

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