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Bacterial responses to Cu-doped TiO_2 nanoparticles

Bing Wu, Rick Huang, Manoranjan Sahu, Xueyang Feng, Pratim Biswas *, Yinjie J. Tang *

Department of Energy, Environmental and Chemical Engineering, One Brookings Drive, Campus Box 1180, Washington University in St. Louis, St. Louis, MO 63130, United States

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ABSTRACT

The toxicity of Cu-doped TiO_2 nanoparticles (NPs, 20 nm), synthesized by a flame aerosol reactor, to *Mycobacterium smegmatis* and *Shewanella oneidensis* MR-1, is the primary focus of this study. Both doped and non-doped TiO_2 NPs (20 nm) tended to agglomerate in the medium solution, and therefore did not penetrate into the cell and damage cellular structures. TiO_2 particles (<100 mg/L) did not apparently interfere with the growth of the two species in aqueous cultures. Cu-doped TiO_2 NPs (20 mg/L) significantly reduced the *M. smegmatis* growth rate by three fold, but did not affect *S. oneidensis* MR-1 growth. The toxicity of Cu-doped TiO_2 NPs was driven by the release of Cu^{2+} from the parent NPs. Compared to equivalent amounts of Cu^{2+}, Cu-doped TiO_2 NPs exhibited higher levels of toxicity to *M. smegmatis* (*P*-value<0.1). Addition of EDTA in the culture appeared to significantly decrease the anti-mycobacterium activity of Cu-doped TiO_2 NPs. *S. oneidensis* MR-1 produced a large amount of extracellular polymeric substances (EPS) under NP stress, especially extracellular protein. Therefore, *S. oneidensis* MR-1 was able to tolerate a much higher concentration of Cu^{2+} or Cu-doped TiO_2 NPs. *S. oneidensis* MR-1 also adsorbed NPs on cell surface and enzymatically reduced ionic copper in culture medium with a remediating rate of 61 μg/(liter·OD_{600}·hour) during its early exponential growth phase. Since the metal reducing *Shewanella* species can efficiently "clean" metal-oxide NPs, the activities of such environmentally relevant bacteria may be an important consideration for evaluating the ecological risk of metal-oxide NPs.

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1. Introduction

Due to the significant applications of nanotechnology, the environmental and ecological effects of nanomaterials have to be considered. Evaluation of nanomaterials will not only help ensure the safety of nanotechnological applications, but also help design functional materials that have minimal adverse effects (Biswas and Wu, 2005; Handy et al., 2008; Klaine et al., 2008; Wiesner et al., 2006). Titanium dioxide (TiO_2) has been widely used in many fields (Biswas and Wu, 1998; Hoffmann et al., 1995; Honda and Fujishima, 1972; Sahle-Demessie et al., 2000). To enhance the functional properties and applicability of titanium dioxide, doped versions of TiO_2 are being synthesized to enhance catalytic activity for light harvesting applications (Colón et al., 2006; Namiki et al., 2005; Wang et al., 2008).

Many researchers have conducted studies to evaluate if nano-scale titanium dioxide would have biological impacts (Adams et al., 2006; Jiang et al., 2007, 2009; Oberdörster et al., 2005; Sayes et al., 2006; Warheit et al., 2007). In general, TiO_2 NPs have a relatively low toxicity, although the presence of light can generate reactive oxygen species (ROS) from TiO_2 NPs and thus enhance their antimicrobial activities. On the other hand, copper NPs appear to have higher cytotoxicity than copper ions because they may penetrate the cell membrane and release copper ions inside the cell (Karlsson et al., 2008; Yoon et al., 2007). However, it is still not clear whether there is synergistic effect when TiO_2 NPs are doped with CuO. Also, very few studies have examined the natural remediation of toxic metal NPs from the environment (Lewinski et al., 2008), which can be another important consideration of NPs' ecological impact. This study employed two model bacterial species: *Mycobacterium smegmatis*, a gram-positive bacterium and a model pathogenic strain for the study of *Tuberculosis*; and *Shewanella oneidensis* MR-1, a gram-negative environmentally relevant bacterium. The objectives of this study are: 1) to determine the toxicity of Cu-doped TiO_2 NPs; and 2) to investigate bacterial responses to NPs.

2. Materials and methods

A flame aerosol reactor (FLAR) with a three-port co-flow diffusion burner was used to synthesize Cu-doped TiO_2 NPs (20 nm) and TiO_2 NPs (20 nm) (Jiang et al., 2009). Titanium tetra-isopropoxide (TTIP, 97%, Aldrich-Sigma, USA) and copper (II) ethyl hexanoate (Aldrich-Sigma, USA) were used as the precursors for the synthesis of NPs. Copper (II) ethyl hexanoate was dissolved in xylene and atomized by a stainless steel nebulizer in the high temperature zone. Nitrogen gas at 1.5 liter per minute (lpm) was bubbled through TTIP in a bubbler maintained at 88 °C. Doping percentages were varied by feeding different molar ratios of the precursors into the high temperature

* Corresponding authors. Tang is to be contacted at Tel.: +1 314 935 3441. Biswas, Tel.: +1 314 935 5482.
E-mail addresses: Pratim.biswas@seas.wustl.edu (P. Biswas), yinjie.tang@seas.wustl.edu (Y.J. Tang).

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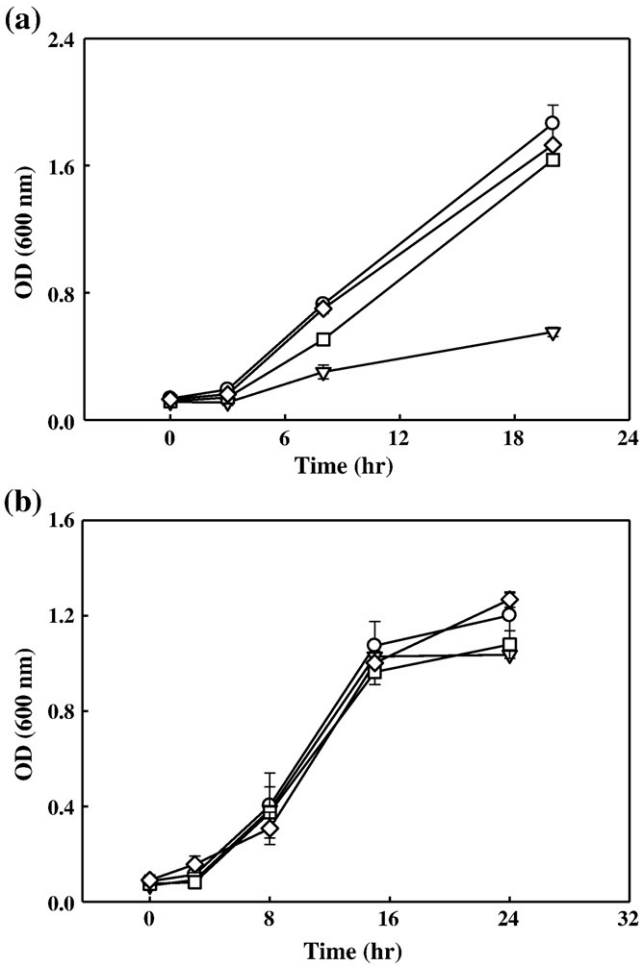

Fig. 1. Responses of *M. smegmatis* (a) and *S. oneidensis* MR-1 (b) to 0.02 g/L NPs ($n=2$): (○) control; (▽) 1.8%-Cu-doped TiO_2 NPs; (□) 0.6%-Cu-doped TiO_2 NPs; (◇) TiO_2 NPs.

combustion zone of the diffusion flame. Oxygen and methane at 7 lpm, and 1.6 lpm, respectively, were introduced into the FLAR system. The temperature and residence time of the NPs in the combustion zone were controlled to obtain the desired size and composition of the NPs. For antibacterial tests, 1.8%-Cu-doped TiO_2 (20 nm, containing 1.8% Cu by weight), 0.6%-Cu-doped TiO_2 (20 nm, containing 0.6% Cu by weight), TiO_2, and CuO NPs (40 nm, Aldrich-Sigma, USA) were suspended in sterilized DI water to make the stock solutions (1 g/L). To reduce agglomeration, the solution was sonicated for 2 min (pulse 20 s on and 20 s off) using a sonicator (Misonix S-4000, USA). The zeta potential of the NPs in water suspensions was measured by a Zeta-sizer (Nanoseries ZS, Malvern, UK).

Two types of bacteria were grown in the presence of NPs: *M. smegmatis* was grown in modified Sauton liquid medium at 37 °C (Tang et al., 2009), and *S. oneidensis* MR-1 was grown in minimal MR-1 medium at 30 °C (Tang et al., 2007). The initial cell density in both cultures was equal to OD_{600} ~ 0.1, and the volume of each culture was 5 mL (placed in a 50 mL falcon tube). All culture tubes were shaken at a speed of 200 rpm in the dark. Cell density was monitored by a UV spectrometer (Genesys, Thermo Scientific, USA) at a wavelength of 600 nm. To monitor microbial remediation of Cu-doped TiO_2 NPs, the cultures with Cu-doped TiO_2 NPs were filtered using a 0.22 μm membrane filter (Nylon, Millipore, USA) after centrifugation at 19,000 × *g* (10 min) to remove cells and large agglomerated NPs. The cell-free supernatant was treated with 2% HNO_3 and 0.5% HCl, and then dissolved copper concentration was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent, USA). The interaction between NPs and cells in the culture was observed by a scanning electron microscope (SEM) and a transmission electron microscope (TEM). All samples for imaging were prepared by the Histology & Microscopy Core Facility of the Washington University School of Medicine.

The effect of NPs on enzyme activity (cell-free) was tested based on NADPH production rates from glucose in the presence of the enzymes hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PD). The two enzymes convert sugar, ATP, and cofactor NADP to gluconate-6-phosphate, ADP, and NADPH (all chemicals were obtained from R-Biopharm, Germany). In the test, samples with known amount of NPs (or $CuCl_2$) were added to the cell-free assay solutions (containing a standard amount of NADP, ATP, the two enzymes, and 0.5 g/L glucose). The reaction was carried out in 2 mL cuvettes. NADPH production rate was calculated based on the change of the UV absorbance at a wavelength of 340 nm. Since NADPH is essential for energy metabolism, measurement of the rate of NADPH production provides a simple approach to test the effect of NPs or ionic copper on general enzyme activities. In addition, the extracellular polymeric substances (EPS) were measured under NP stress. Polysaccharides and proteins were dominant components in the EPS. The polysaccharide concentration was determined according to the phenol-sulfuric acid method using glucose as a standard (Dubois et al., 1956). The protein concentration was determined by Bradford reagent using bovine serum albumin (BSA) as a standard (Bradford, 1976).

3. Results and discussion

Antibacterial properties of the four kinds of NPs (1.8%-Cu-doped TiO_2, 0.6%-Cu-doped TiO_2, TiO_2, and CuO) were tested using *M. smegmatis* or *S. oneidensis* MR-1 culture. When a low concentration of NPs (0.005 g/L) was added, 1.8%-Cu-doped TiO_2, 0.6%-Cu-doped TiO_2 and TiO_2 NPs had no apparent effect on microbial growth (data not shown). With the addition of 0.02 g/L NPs, the growth of *M. smegmatis* was significantly inhibited by 1.8%-Cu-TiO_2 NPs (0.02 g/L NPs contained 0.36 mg/L total copper), and moderately negatively-affected by 0.6%-Cu-doped TiO_2 NPs (0.02 g/L NPs contained 0.12 mg/L total copper) (Fig. 1). *S. oneidensis* MR-1 growth was only slightly affected by 1.8%- and 0.6%-Cu-doped TiO_2 NPs (0.02 g/L), i.e., final cell density was reduced by 10–20%. TiO_2 NPs did not show any effect on

Table 1
Percentage of bacterial growth inhibition after exposure to NPs.

	TiO_2 (20 mg/L)	0.6%-Cu-TiO_2 NPs(20 mg/L)	1.8%-Cu-TiO_2 NPs (20 mg/L)	CuO (20 mg/L)	Cu^{2+} (0.36 mg/L)	TiO_2 (20 mg/L) + Cu^{2+} (0.36 mg/L)
S. oneidensis ($n=4$)	7 ± 6	8 ± 2	15 ± 8	13 ± 4	11 ± 2	7 ± 5
M. smegmatis ($n=4$)	5 ± 7	19 ± 3	55 ± 10	98 ± 5	47 ± 4	51 ± 7
Enzyme[a] ($n=2$)	1 ± 1	22 ± 4	24 ± 5	16 ± 4	4 ± 5	4 ± 5

Inhibition rate (%) = $(1-OD_{600\ (sample)}/OD_{600\ (control)})\times 100\%$; the OD_{600} was measured during the middle exponential growth phase (9–10 h).

[a] Enzymatic assay was conducted in the presence of NPs:

$$Inhibition\ rate\,(\%) = \left[1-\frac{NADPH\ production\ rate\,(\text{with NPs})}{NADPH\ production\ rate\,(\text{control})}\right]\times 100\%.$$

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