A mechanistic study of TiO₂ nanoparticle toxicity on *Shewanella oneidensis* MR-1 with UVA illumination: bacterial growth, riboflavin secretion, and gene expression

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Abstract

Toxicity of nanomaterials to ecological systems has recently emerged as an important field of research, and thus, many researchers are exploring the mechanisms of how nanoparticles impact organisms. Herein, we probe the mechanisms of bacteria-nanoparticle interaction by investigating how TiO₂ nanoparticles impact a model organism, the metal-reducing bacterium *Shewanella oneidensis* MR-1. In addition to examining the effect of TiO₂ exposure, the effect of synergistic UVA illumination was explored in this study, as TiO₂ nanoparticles are known photocatalysts. The data reveal that TiO₂ nanoparticles cause an inhibition of *S. oneidensis* growth at high dosage without compromising cell viability, yet co-exposure of nanoparticles and UVA illumination does not increase the adverse effects on bacterial growth relative to TiO₂ alone. Measurements of intracellular reactive oxygen species and riboflavin secretion, on the same nanoparticle-exposed bacteria, reveal that TiO₂ nanoparticles have no effect on these cell functions, but application of UVA illumination with TiO₂ nanoparticles has an impact on the level of riboflavin outside bacterial cells. Finally, gene expression studies were employed to explore how cells respond to TiO₂ nanoparticles and UVA illumination, and these results were correlated with cell growth and cell function assessment. Together these data suggest a minimal impact of TiO₂ NPs and UVA illumination on *S. oneidensis* MR-1, and the minimal impact could be accounted for by the nutrient-rich medium used in this work. These measurements demonstrate a comprehensive scheme combining various analytical tools to enable a mechanistic understanding of nanoparticle-cell interactions and to evaluate the potential adverse effects of nanoparticles beyond viability/growth considerations.

Keywords

Titanium nanoparticles, *Shewanella oneidensis*, UV illumination, riboflavin, gene expression

1. Introduction

The biocidal effect of titanium dioxide (TiO₂) nanoparticles (NPs) has resulted in extensive development of TiO₂-NP-based anti-microbial materials (Fu et al., 2005; Wang et al., 2005; Perelshtein et al., 2012), but also generated rising concern about the adverse effects of TiO₂ NPs on non-target species in ecological systems. Previous studies have revealed toxicity of TiO₂ NPs to ecologically relevant organisms, including algae, zebrafish, water fleas, and fish (Sharma, 2009; Bar-Ilan et al., 2012; Ma et al., 2012; Manzo et al., 2015). The intended biocidal effects of TiO₂ NPs, combined with the potential release of TiO₂ NPs into the environment (Kaegi et al., 2008; Kiser et al., 2009) with anticipated increase in the usage of TiO₂ NPs (Robichaud et al., 2009; Menard et al., 2011), generates an emerging need to assess the ecological risk of TiO₂ NPs on relevant organisms (Wiesner et al., 2006; Adam et al., 2015; Semenzin et al., 2015).

Although released NPs can potentially affect all organisms within the food web, low trophic level organisms like bacteria are most likely to interact with released NPs due to their wide distribution and their localization in microenvironments where nanoparticles may accumulate. Quite a few studies have addressed the influence of TiO₂ NPs on bacteria species, but most of these studies focus on *E. coli* and other pathogenic bacteria (Kuhn et al., 2003; Kim and An, 2012; Li et al., 2012; Tong et al., 2013). *Shewanella oneidensis* MR-1, an environmentally beneficial gram-negative bacterium of genus *Shewanellae*, has a unique dissimilatory metal-reducing capability to respire heavy metals, including Mn(III), Mn(IV), Fe(III), and Cr(VI) (Heidelberg et al., 2002; Hau and Gralnick, 2007). With this capability, *S. oneidensis* MR-1 plays an important role in solubilizing metal salts for species throughout the food web as well as having a potential role in bioremediation of toxic elements in the ecosystem. Because of these environmental implications, *S. oneidensis* MR-1 has been employed to study toxicological response to environmental factors like pH, heavy metals and electron acceptors (Beliaev et al., 2002; Bencheikh-Latmani et al., 2005; Chourey et al., 2006; Leaphart et al., 2006). Though to a

very limited extent, it also has been employed as an environmentally relevant bacterial model in nanotoxicity studies (Wu et al., 2010; Wu et al., 2011; Maurer-Jones et al., 2013) as is done here.

Mechanistic studies of the adverse effects of toxic agents can provide important implications for the assessment of ecological risk and the prediction of toxicity. One of the most studied mechanisms of TiO₂ NP impact on biological systems is the generation of reactive oxygen species (ROS), which could potentially be a molecular initiating event in damage to cells by nanoparticles (Reeves et al., 2008; Sharma, 2009; Xue et al., 2010; Li et al., 2012; Maurer-Jones et al., 2012). As a photocatalyst, TiO₂ NPs absorb photons and generate electron-hole pairs, and these charge carriers move to the NP surface; ROS can be generated during the reaction between the TiO₂ NP surface and the surrounding aqueous medium (Chen and Mao, 2007). Previous work has shown that UV-illuminated TiO₂ NPs exhibited higher toxicity across species than TiO₂ NPs alone, and ROS generated by TiO₂ NPs are believed to be the means by which TiO₂ NPs kill bacteria (Kuhn et al., 2003; Kim and An, 2012; Tong et al., 2013). Li et al. built up a linear correlation between the total ROS generation by metal oxide NPs, including TiO₂ NPs, and the toxicity of these NPs to *E. coli* (Li et al., 2012). The production of intracellular ROS could result in subsequent cellular response including lipid peroxidation or DNA damage, either of which could be measured as endpoints (Cabiscol et al., 2000). In *S. oneidensis* MR-1, the secretion of riboflavin is one important cellular endpoint that can be monitored. Riboflavin is a redox molecule that acts as an electron shuttle between the cell surface and external acceptors like metal oxide surfaces and plays a critical role in cell-mediated metal reduction (Marsili et al., 2008; Brutinel and Gralnick, 2012). The presence of riboflavin is also important in its role as a photosensitizer as it was found to generate ROS in a light-dependent manner (Grzelak et al., 2001). In fact, previous research has demonstrated that the amount of *S. oneidensis*-secreted riboflavin increased as a cellular response upon TiO₂ NP exposure (Maurer-Jones et al., 2013).

Efforts have been made to elucidate the molecular responses upon TiO₂ NP exposure by monitoring changes in gene expression levels (Park and Yeo, 2013; Kubacka et al., 2014; Rocheleau et al., 2015; Sohm et al., 2015). Nanoparticles have been shown to cause changes in RNA expression levels in organisms varying from human cells to bacteria. Using gene expression levels to assess nanotoxicity can provide direct information on mechanisms of nanotoxicity and help identify biomarkers for future high-throughput nanotoxicity assessments (Klaper et al., 2009; Higashisaka et al., 2011). Although oxidative stress has been identified as one of the major toxicity mechanisms in other bacterial models with various nanoparticles (Choi and Hu, 2008; von Moos and Slaveykova, 2014), other modes of action have been revealed via gene expression studies (Petkovic et al., 2011; Cui et al., 2012; Lee et al., 2012; Kubacka et al., 2014; Qiu et al., 2015). Kubacka et al. revealed changes in the level of gene expression related to various pathways other than oxidative stress, including the activation of detoxification and repair pathways and a decrease in a pathway related to cell-cell communication, in a pathogenic bacterium upon TiO₂-UV treatment (Kubacka et al., 2014). Ideally, gene expression data, measured and correlated with other cellular responses and changes in cell population growth, could enable the construction of a sequential series of biological responses that can be used in the identification of adverse outcome pathways for nanoparticles and assist in risk assessment for TiO₂ NPs.

Herein, we establish an integrated toxicology approach, from a molecular initiating event to cell population change, to comprehensively explore the mechanisms of nanoparticle interaction with bacterial cells, using *Shewanella oneidensis* MR-1 as model organism exposed to TiO₂ NPs and concurrent UVA illumination. The impact of TiO₂ NPs and UVA illumination on bacterial cell viability and population growth were monitored, followed by assessments of intracellular ROS generation and riboflavin secretion. Gene expression changes upon continuous nanoparticle and UVA exposure were also monitored at different time points. By combining several analytical methodologies to probe impacts from cell viability to gene expression, this work

enables a mechanistic understanding of nanoparticle-bacterial cell interaction and brings a new integrative scheme to assess the risk of nanoparticle exposure to the environment.

2. Materials and Methods

2.1. Bacteria preparation

LB broth (BD Difco, BD Diagnostics, Franklin Lakes, NJ), made by dissolving 25 grams of pre-mixed LB broth powder (consisting of 10 g tryptone, 5 g yeast extract and 10 g NaCl) in 1 liter of Milli-Q water followed by autoclaving, was used in this work as the bacteria cell culture medium. *S. oneidensis* MR-1 was a kind gift from Professor Jeffery Gralnick, University of Minnesota Department of Microbiology and was stored at -80°C before use. Bacteria were inoculated onto a LB broth agar plate and incubated at 30°C for 24 hours or until visible colonies formed. Colonies were inoculated into 5-15 mL of autoclaved LB broth in sterile culture tubes and grown overnight.

2.2. Monitoring cell growth over time

Bacterial growth curves were monitored by measuring the optical density (OD) of a bacterial suspension over time with different nanoparticle doses. After the bacterial suspension reached stationary phase, 200 μ L of suspension was diluted with fresh autoclaved LB broth to a total volume of 5 mL to reach a diluted cell density of approximately 10^7 cells/mL. The diluted bacteria suspension was grown in a 30-32°C orbital shaker at 300 rpm for 6 hours either in the dark or under UV illumination with continuous exposure to 0, 1, 5, 10, 25, or 100 mg/L TiO₂ nanoparticles. 200 μ L aliquots were transferred periodically to a Costar® 96-well assay plate (Corning Incorporated, NY), and measurements of OD at $\lambda = 600$ nm (OD₆₀₀) were recorded with a Synergy 2 Multi-Mode Microplate Reader (BioTek, VT). Background due to nanoparticle

extinction and assay plate wall absorbance was subtracted to generate normalized data, which was plotted and analyzed by Prism (GraphPad Software, CA).

2.3. Monitoring cell respiration

Cell growth was also characterized by measuring oxygen uptake by the cell suspension over time. A PF-8000 aerobic/anaerobic respirometer system (Respirometer Systems and Applications, LLC, Arkansas) was used to monitor oxygen uptake. This instrument consists of biological reaction vessels, a stirring base for mixing samples, and a control module that contains the pneumatic and electronic controls for oxygen or gas flow measurement. Bacteria were grown in LB broth to stationary phase, and diluted 1:10 into fresh autoclaved LB broth in reaction vessels to reach a total volume of 100 mL. TiO₂ nanoparticles were added into the reaction vessels to a final concentration of 25 mg/L or 100 mg/L. A tube filled with 1 mL 30% potassium hydroxide solution was inserted into each reaction vessel to absorb carbon dioxide released by bacteria aerobic respiration. The negative pressure created by oxygen consumption of bacteria was compensated by oxygen injection continuously to keep a constant pressure in the headspace of reaction vessel. Oxygen uptake was recorded automatically by the instrument, and each measurement was done in duplicate.

2.4. Glucose 6-phosphate dehydrogenase cytotoxicity assay for cell viability

Cell viability was measured using a commercial cytotoxicity assay, the Vybrant™ Cytotoxicity Assay Kit (Molecular Probes, OR), which uses an enzymatic fluorescence reaction to measure the glucose 6-phosphate dehydrogenase (G6PD) released from dead cells. Bacteria were first grown in LB broth overnight to stationary phase ($OD_{600} = 1.5-1.7$) and then exposed to 100 mg/L TiO₂ nanoparticles for 30 minutes, either in the dark or under UV illumination. The TiO₂ NPs were sonicated for 30-45 minutes prior to exposure (same for all exposures in this study). Following exposure, 1 mL of bacterial suspension was centrifuged at $17,000 \times g$ for 2 minutes, and the supernatant was collected for measurement. A resazurin/reaction 2X mixture was prepared

following the kit's protocol, and 50 μ L of the mixture was added into 50 μ L supernatant on a 96 well plate. The microplate was incubated at 37°C for 30 minutes in the dark before fluorescence readings were taken with excitation at 530 nm and emission at 590 nm. Cell death was proportional to the intensity of fluorescence signal. The background fluorescence signal was obtained from a control group that only contained LB broth and the resazurin/reaction mixture, and this signal was subtracted from experimental fluorescence signals.

2.5. Intracellular ROS generation measurement

Dichlorodihydrofluorescein diacetate (DCFDA), which can be deacetylated by cellular esterases to a non-fluorescent intermediate that is later oxidized to fluorescent 2', 7'-dichlorofluorescein (DCF) upon reaction with ROS, was used as an indicator of ROS. DCFDA was diluted to a 10 μ M working solution using fresh LB medium. Then, 100 mL of bacterial suspension was grown to the stationary phase ($OD_{600} = 1.5-1.7$) followed by centrifugation for 10 minutes at 1,500 $\times g$. After decanting the supernatant, the bacteria cell pellet was resuspended with 40 mL DCFDA 10 μ M working solution. The resulting suspension was then incubated at 30°C for 1 hour in the dark to avoid photo-oxidization of the ROS indicator. The bacteria suspension was centrifuged again at 1,500 $\times g$ for 10 minutes, and the supernatant was removed. 8 mL of fresh LB broth was applied to wash the pellet three times and the cell pellet was diluted with 60 mL of fresh LB broth.

TiO₂ NP suspension was added to 2.94 mL of the final bacteria suspension to reach a NP concentration of 100 mg/L. 360 μ L of bleach was used as a positive control as it can generate an excessive amount of ROS. The bacterial suspension was incubated in the dark for 15 minutes after TiO₂ nanoparticle exposure, transferred to a 96 well flat bottom polystyrene reading plate (Corning Incorporated, NY) and then either exposed to UV illumination or kept in the dark for another 15 minutes. The fluorescent signal from the ROS indicator was excited at 488 nm and measured at 515 nm by a Synergy 2 Multi-Mode Microplate Reader (BioTek, VT).

A control experiment was done to explore the interference of TiO₂ NPs on the detection of fluorescence of DCF, the fluorescent molecule converted from DCFDA in cells. 92.5 nM and 481 nM of DCF was diluted in fresh LB broth to generate fluorescence signals that are comparable to what were detected in bacterial suspension in the dark and under UV illumination, respectively. 100 mg/L TiO₂ NPs were added to DCF solution and mixed well, and the fluorescence signals were read using the same method as stated above. Five material replicates were done in this control experiment.

2.6. HPLC measurement of riboflavin secretion

Flavin secretion, an important function of *S. oneidensis*, was measured by HPLC analysis of bacterial suspension supernatant. First, bacterial suspensions were exposed to 100 mg/L TiO₂ nanoparticles in the dark or under UV illumination. Then, 1 mL of bacterial suspension was collected from same tube after 30 min, 1 hour, or 6 hours, and centrifuged for 15 min at 5000 ×g. Exposure was done in triplicate. 200 µL of the supernatant was transferred to an amber HPLC vial with a 250 µL glass insert, and placed on the autosampler of an Agilent 1200 HPLC (Agilent Technologies, CA). A Zorbax Eclipse XDB-C₁₈ 4.6 × 150 mm, 5 µm analytical column was washed with acetonitrile and equilibrated using mobile phase, a 70:30 mixture of 20 mM citric acid buffer (pH 3.3)/methanol. The injection volume was 30 µL, and isocratic elution was performed with a flow rate of 1 mL/min. Riboflavin was detected by a fluorescence detector after flowing through an Eclipse XDB-C₁₈ 4.6 × 12.5 mm, 5 µm analytical guard column, with excitation at 450 nm and emission at 530 nm. Riboflavin elution was achieved after approximately 6.7 min.

2.7. RNA extraction

RNA was extracted from bacteria cells exposed 25 mg/L of TiO₂ nanoparticles and sent to the University of Minnesota Genomics Center (UMGC) at the University of Minnesota for quantitative real-time PCR analysis. A Direct-zol™ RNA MiniPrep kit (Zymo Research, CA),

allowing quick and high-quality DNA-free RNA extraction and purification using spin columns, was used according to the manufacturer's recommendations for total RNA extraction. Bacteria were grown in LB broth to stationary phase and exposed to 25 mg/L of TiO₂ nanoparticles in the dark or under UV illumination in triplicates. At 0, 0.5, 1, and 6 hours after nanoparticle exposure and/or UV illumination, 1 mL of bacteria suspension was centrifuged at 1500 ×g for 10 minutes. The supernatant was removed and the cell pellet was sufficiently resuspended in 800 µL of RNeasy® (Qiagen, Crawfordsville, IN). The extraction kit protocol was followed using a centrifugation speed of 12,000 ×g with an on-column DNase I treatment at 30°C for 15 minutes. RNA was finally eluted from the column using 25 µL of DNase/RNase-free water at 16,000 ×g for 1 minute. The total RNA solution was normalized to 200 ng/µL and submitted for quantitative real-time PCR analysis.

2.8. qPCR and data analysis

Quantitative real-time PCR (qPCR) was done by the University of Minnesota Genomics Center using the same protocol and instrument described earlier (Maurer-Jones et al., 2013). For each gene of interest, three sets of primers were designed using Universal ProbeLibrary ProbeFinder software, followed by validation of amplification efficiency. The primer pairs with the highest amplification efficiency for each gene were chosen for subsequent qPCR experiments. Table 1 shows a full list of genes investigated along with their corresponding functions and their primers used in qPCR experiments. The raw data was processed using the qPCR data analysis program, Miner (Zhao and Fernald, 2005), which applies an objective method to determine efficiency and the fractional cycle number (Ct) at threshold of individual PCR reactions. Ct values were determined using Miner program, and relative fold change was calculated based on the $\Delta\Delta C_t$ method (Schmittgen and Livak, 2008). Among two candidates, *gyrB* and *recA*, *recA* was chosen as the housekeeping gene due to a lower standard deviation in expression level.

Table 1 Target genes, corresponding functions, and their primers for qPCR experiment.

Target gene	Forward primer (5' - 3')	Reverse primer (5' - 3')	Probe number	Function	Accession number
Fe/Mn superoxide dismutase (<i>sodB</i>)	GCA ATG TTC GCC CTG ACT AC	CCT GCG AAG TTT TGG TTC AC	#95	Superoxide dismutase activity	NP_718453.1
Diguanylate cyclase-like protein (<i>mxrA</i>)	CAT TTC GCA AGT TGA ACA CC	CCA CAC CAA TAT GAC GCA AA	#44	Indirectly modulate cyclic di-GMP	NP_719709.1
Phage shock protein B (<i>pspB</i>)	TTG ATT GCG AAA GCC GAT A	ATC AAG AAT CGC CTC TAA GGT TT	#46	Phage shock response	NP_717416.1
Double-stranded DNA translocase (<i>ftsK</i>)	GAT AGT CGA TGG CAT TGT GGT	TAG GCT TAG TCG GCA CAG GT	#142	Cytokinesis	NP_717901.1
Catalase HPII (<i>katB</i>)	GGC ATT GAT CCT GAT TCT TCT C	TCC AAC GAG GGA AGT TAC CA	#157	Catalase activity, oxidative stress	NP_716697.1
Extracellular iron oxide respiratory system surface decaheme cytochrome c component (<i>omcA</i>)	TCG ATT ATG GCA AAG TGC AT	CGT GAC AGG CTG CAC AAT	#28	Electron carrier activity	NP_717388.1
DNA gyrase B subunit (<i>gyrB</i>)	AGA ATG GAT CGA CGC CTT TA	ATA CAG TAC GCC GCT GTT TTC	#31	DNA replication	NP_715653.1
Recombinase A (<i>recA</i>)	GGT GAC TCA CAC ATG GGC TTA	CCG CTA ACT TAC GCA TTG CT	#136	DNA-dependent ATPase activity	NP_718983.1

3. Results

3.1. TiO_2 nanoparticle characterization

Synthesis of acid-catalyzed TiO_2 NPs followed by a 48-hour hydrothermal treatment was performed using a sol-gel method developed by Isley and Penn (Isley and Penn, 2008). Details can be found in the Supporting Information. The dynamic light scattering (DLS)- based size and ζ -potential of synthesized nanoparticles were measured in both Milli-Q water and LB broth before toxicity tests, and the results are summarized in Figure S1. It was noted that upon re-suspension in LB broth, the hydrodynamic diameter increased drastically, with a significant peak shift in size distribution, indicating that TiO_2 NPs aggregated in LB broth. The large aggregation was also confirmed by hyperspectral microscopic images (Figure S2; more information on hyperspectral imaging can be found in Supporting Information). Meanwhile, the absolute size of the primary

TiO₂ NPs, determined from TEM images, did not change. This aggregation is expected based on the high concentration of salts and nutrients in LB broth (French et al., 2009). Changes in ζ -potential from positive to almost neutral in LB broth may result from both pH change (from 6.05 of Milli-Q water to 7.30 of LB broth) and peptide adsorption onto the TiO₂ NP surface, as LB broth contains high concentration of peptides. Stability results show that, once re-dispersed in LB broth, the aggregation status of TiO₂ remains quite stable for 6 hours, the experimental time frame in this study.

3.2. Effect of TiO₂ NPs and UV illumination on cell growth

t-test, $p=0.0314$). (f) Cytotoxicity assays following TiO₂ and UV exposure on *S. oneidensis* showed TiO₂ nanoparticles, even at 100 mg/L, did not kill cells compared to control groups.

Bacterial cell density (OD_{600}) was monitored hourly upon 1, 5, 10, 25, or 100 mg/L TiO_2 NP exposure, either in the dark or under continuous UV illumination. TiO_2 NP extinction and LB broth background at 600 nm were subtracted from the original readings to obtain background-subtracted OD_{600} values, as plotted in Figure 1(a)(b). Data from 1 and 10 mg/L exposure followed the same trend but are not shown in the figure for clarity. Data from 3 hour time point were identified as outliers (ROUT method, $Q=1.0\%$, GraphPad Prism) (Motulsky and Brown, 2006) and excluded from further statistical analyses. In both cell growth curves in the dark and under UV illumination in Figure 1(a) and 1(b), there appeared to be a dose-dependent negative effect of TiO_2 NP treatment, a trend that was also supported upon analysis of the final cell density after 6 hours; this finding is consistent with precedent work (Maurer-Jones et al., 2013). However, it should be noted that the optical density change, even with the highest nanoparticle dose (100 mg/L), was less than 10%. Thus, the effect of TiO_2 exposure under these conditions can be considered minor. For the most part, no additional effect of UV illumination and TiO_2 nanoparticle treatment was observed, with the exception of a few trials where the interaction of TiO_2 and UV illumination was determined to be significant when testing final OD_{600} values using a two-way ANOVA. Given the fact that (1) the significance was not repeated for all trials and (2) the difference in OD_{600} change was at most 14%, it is concluded that, overall, UV illumination and TiO_2 nanoparticle treatment shows no interactive effect.

The first 4-hour time period of bacterial growth was identified as the log/exponential growth phase by fitting the logarithmic transformed cell number values using linear regression, and the growth rate K was calculated from the following equations:

$$N = N_0 e^{Kt}$$

$$\log N = (K \log e)t + \log N_0$$

$$K = \ln 10 \times slope = 2.303 \times slope$$

where N is the cell number, calculated from optical density (1 Abs = 10^9 cells/mL), and N_0 is the cell number at the beginning (0 hour). K ($hour^{-1}$) refers to the growth rate in the log phase, and e is

the natural logarithm. Two-way ANOVA revealed a statistically significant difference in the growth rate, K , between dark and UV illumination ($F(1,24)=20.87$, $p=0.0001$); a Tukey post-hoc test showed that at 100 mg/L TiO_2 dose, the growth rate K at log phase under UV illumination is statistically significantly lower than that in the dark (Tukey's multiple comparison, $p=0.0087$). TiO_2 exposure did not have any effect on bacterial growth rate in the log phase.

After 4 hours, bacterial growth slowed as it entered the stationary phase; thus, the growth rate cannot be determined by the equations above. To explore the bacteria growth at this transition stage, the differences in OD_{600} values between the 4- and 6-hour time points were calculated and used to represent the bacteria growth in this period (Figure 1(d)). Two-way ANOVA revealed that both UV illumination ($F(1,24)=107.9$, $p<0.0001$) and TiO_2 concentration ($F(5,24)=4.471$, $p=0.0051$) have statistically significant effects on the bacterial growth at 4 and 6 hours. The positive effect of UV illumination on cell growth during 4-6 hours compensated for the slowed growth during the log phase induced by UV exposure, resulting in no significant effect of UV illumination on the final OD at 6-hour (Figure 1(c), two-way ANOVA, $F(1,24)=0.2968$, $p=0.5909$). Meanwhile, TiO_2 treatment has a significant effect on the final OD reading at 6-hour (two-way ANOVA, $F(5,24)=18.13$, $p<0.0001$), and a Tukey post-hoc test showed that 100 mg/L TiO_2 NP treatment statistically significantly decreased the OD reading at 6-hours compared to control and other lower concentrations (Tukey's multiple comparison, $p<0.05$).

Oxygen uptake by the bacterial populations was also measured over time by monitoring oxygen uptake with either 25 mg/L (data not shown) or 100 mg/L TiO_2 nanoparticle exposure. It should be noted that the respiration measurement was only performed in the dark due to the limitations of the instrument. Using the first derivative of the oxygen uptake curve, the maximum oxygen uptake rate ($k_{\max}$) and the time point when the maximum rate was reached ($t_{\max}$) were calculated. The quotient of $k_{\max}$ divided by $t_{\max}$ was calculated to represent bacterial oxygen uptake, as shown in the inset of Figure 1(e). A decrease in the value indicates inhibition of bacterial oxygen uptake.

A two-tailed, unpaired student t-test showed that upon 100 mg/L TiO₂ NP exposure, bacterial respiration was inhibited ($p=0.0314$), consistent with the growth curve results.

3.3. Effect of TiO₂ NPs and UV illumination on cell death

A cytotoxicity assay was also applied to test bacterial cell viability upon TiO₂ NP exposure and/or UV illumination for a short time period (30 min). Cells are known to release the cytosolic enzyme glucose 6-phosphate dehydrogenase (G6PD) upon cell damage. In the Vybrant cytotoxicity assay, G6PD is measured by a two-step enzymatic reaction that generates a red-fluorescent probe, resorufin. The results show that TiO₂ NP treatment, even at the highest dose considered herein (100 mg/L), did not increase cell death. Interestingly, two-way ANOVA indicated a significant effect of UV illumination on increasing cell viability ($F(1,8)=14.70$, $p=0.005$) after the 30-minute exposure. Considering the short time frame, this effect can be explained by an increased growth rate upon UV illumination in the first hour of bacterial growth (Two-way ANOVA, $F(1,24)=7.698$, $p=0.01$, data not shown). No additional effect between nanoparticles and UV illumination was observed on cell viability in the cytotoxicity assay.

3.4. ROS generation affected by TiO₂ NPs and UV illumination

Figure 2. (a) A minor decrease in fluorescence intensity was detected upon TiO₂ exposure both in dark and under UV illumination (Tukey's multiple comparison test, $p<0.05$). (b) The presence of TiO₂ NPs resulted in a minor, but statistically significant, decrease in the fluorescence of DCF (two-way ANOVA,

$F(1,16)=11.99$, $p=0.0032$). Star denotes the statistical significance between groups (Tukey's multiple comparison test, $p<0.05$).

Bacterial cells were loaded with dichlorodihydrofluorescein diacetate (DCFDA), a probe which can be converted to fluorescent 2', 7'-dichlorofluorescein (DCF) upon reaction with cellular esterases and ROS, to indicate the amount of intracellular ROS, followed by exposure of 100 mg/L TiO_2 NPs and/or UV illumination. It was hypothesized that with the presence of TiO_2 NPs, an increase of intracellular ROS level would be observed due to the photocatalytic properties of TiO_2 NPs. Surprisingly, the results (Figure 2(a)) showed that TiO_2 NPs decreased the fluorescent signals, indicating that the amount of intracellular ROS had a minor, but statistically significant, decrease. To explore the source of this minor decrease in fluorescent signals, a control experiment was done to see if TiO_2 NPs interfered with the DCF fluorescence. Results showed that it was very likely that the decrease in fluorescent signals was attributed to the optical interference of TiO_2 NPs in suspension (Figure 2(b)). Thus, it was concluded that the treatment of TiO_2 NPs, even at the highest dose here, did not induce changes in intracellular ROS level. The dramatic increase in ROS levels upon UV illumination was probably due to the photo-induced transformation of DCFDA to fluorescent DCF (Bilski et al., 2002), as their magnitude was comparable to the positive control (data not shown).

3.5. Riboflavin secretion affected by TiO_2 NPs and UV illumination

Figure 3. Riboflavin secretion was affected by UV illumination at all time points (statistical significance not shown) and further decreased in the presence of TiO₂ NPs after 0.5- and 1-hour exposure (two-way ANOVA and post-hoc Tukey's test within one time point, $p < 0.05$). Asterisks denote statistical significance.

Riboflavin secretion upon TiO₂ NPs and/or UV light exposure was examined at different time points (30 min, 1 hour and 6 hour) using HPLC. For all three time points, no change was observed in riboflavin secretion upon TiO₂ NP exposure in the dark, even with the highest TiO₂ nanoparticle dose in this study (100 mg/L). UV illumination significantly decreased the amount of riboflavin detected in the supernatant, mostly attributed to the photodegradation of riboflavin under UV light; it was confirmed by a control experiment that no riboflavin peaks were observed for a 4 μ M solution of riboflavin after a 6-hour UV illumination (data not shown). Interestingly, there was a statistically significant interaction between the effects of TiO₂ and UV on riboflavin secretion upon 0.5- and 6-hour exposure (two-way ANOVA, $F(1,8)=9.694$ and 8.633 , $p=0.0144$ and 0.0188 , respectively), indicating that the presence of TiO₂ NPs further reduced the amount of riboflavin in the supernatant of the bacterial suspension.

3.6. Effect of TiO₂ NPs and UV illumination of gene expression

Figure 4. Gene expression pattern of 6 different genes in *S. oneidensis* MR-1 upon TiO₂ nanoparticle (25 mg/L) and/or UV exposure for different time periods. TiO₂ NPs did not affect gene expression at this dose, and UV illumination induced minor effects. Asterisks denote statistical significance in post-hoc Tukey's test ($p < 0.05$).

In the gene expression studies, 25 mg/L was used as the TiO₂ NP dose as it was found to be the highest dose that did not induce inhibition in bacterial growth. Two-way ANOVA and a post-hoc Tukey's multiple comparison test was applied to log₂-transformed relative fold change values. *gyrB* and *recA* were included as candidates for housekeeping genes, and *recA* was finally chosen as the housekeeping gene after comparing the variation of the expression levels of these two genes. Results showed that TiO₂ nanoparticle (25 mg/L) and UV exposure induced almost no effect on *S. oneidensis* MR-1 gene expression. Two-way ANOVA showed statistically significant effects of UV illumination on *sodB* expression at 0.5 hour ($F(1,8)=5.367$, $p=0.0492$) and 1 hour ($F(1,8)=5.630$, $p=0.0451$), *mxdA* expression at 0.5 hour ($F(1,8)=10.05$, $p=0.0132$), and *pspB* expression at 1 hour ($F(1,8)=24.12$, $p=0.0012$). In a post-hoc Tukey's test ($p < 0.05$), there was a statistically significant decrease in the expression level of the *pspB* gene in UV-illuminated

samples when compared to either control or TiO₂-treated samples in the dark. Overall, TiO₂ NPs did not affect gene expression at the dose of 25 mg/L.

4. Discussion

*4.1. Minimal inhibition of TiO₂ NPs and UV illumination on *S. oneidensis* growth*

Measurements of cell growth, oxygen uptake, cell death, and intracellular ROS generation collectively indicated that UV illumination, even at high intensity, does not enhance the toxicity of TiO₂ nanoparticles; this result forces us to reject our initial hypothesis that UV illumination would enhance the toxicity of TiO₂ NPs by generating excess ROS. This finding is also not consistent with previous research on other gram-negative bacteria species (Tong et al., 2013). Considering the fact that a very nutrient-rich medium, LB broth, was used in this study, it is possible that the ROS generated from TiO₂ NPs are readily quenched by components in LB medium, such as peptides, making the external ROS level too low to damage the cell membrane and further impact intracellular ROS; this was further proved by the gene expression results (see below). In addition, as TiO₂ NPs tend to aggregate in LB medium, resulting in loss of sub-100 nm population and the gain of a population of effectively microscale particles (Figure S1(d)), the TiO₂ particles likely generated less ROS than expected for well-dispersed nanoparticles due to the reduced surface area (Li et al., 2012). Finally, as TiO₂ NPs are not internalized by but are in close proximity to the bacterial cells (Figure S2), TiO₂ NPs simply have no chance to directly generate ROS within the cell plasma. While TiO₂ NPs, either in the dark or under UV illumination, still induced inhibition of bacterial growth at high dosage, we postulate that the inhibition of growth is mostly caused by adsorption of nutrients to TiO₂ NP surfaces rather than ROS generation from TiO₂ NPs, due to the fact that (1) UV illumination did not enhance the inhibition; (2) the inhibition began after log phase, when the growth started to be limited by the availability of nutrients in media; and (3) cell viability after short exposure was not decreased by the

combination of TiO₂ exposure and UV illumination. Thus, in future research, a minimal medium will be more desirable than LB broth and might reveal toxicity in viability/growth that was not observed in this study. However, it is still possible that TiO₂ NPs and UV illumination caused subtle effects that cannot be observed by only viability/growth measurements; this possibility was addressed by cellular function assessment and gene expression studies subsequently.

4.2. Riboflavin level change upon TiO₂ NP and UV exposure

Riboflavin, a redox molecule secreted by *S. oneidensis* MR-1 cells, acts as an electron shuttle between the cell surface and external acceptors, like metal oxide surfaces (Marsili et al., 2008; Brutinel and Gralnick, 2012). However, the redox potential of TiO₂ conduction band at pH 7 (-0.7 V vs. SHE (Dias et al., 2012)) is considered too low for reduction by flavins (with a midpoint potential of -0.2 to -0.25 V (Marsili et al., 2008)). Thus, we were not expecting riboflavin level to increase upon TiO₂ exposure; on the contrary, considering that fact that bacterial growth was inhibited by TiO₂ NPs, the hypothesis was that riboflavin secretion would be somewhat inhibited, correlating with growth results. However, for all three time points, no change was observed in riboflavin secretion upon only TiO₂ NP exposure, even with the highest TiO₂ nanoparticle dose in this study (100 mg/L). This indicated that riboflavin secretion does not correlate with bacterial growth. A previous study showed that riboflavin secretion increased upon 24-hour TiO₂ NP exposure in a dose-dependent manner (Maurer-Jones et al., 2013); this was not observed in this study, possibly due to the different time frame of these two studies.

As expected, UV illumination induced a decrease in riboflavin amount in cell suspension drastically, probably due to the photodegradation of riboflavin upon UV exposure, as shown in control experiments and previous work (de la Rochette et al., 2000; Ahmad et al., 2006); control experiments showed that no riboflavin peaks were observed after 6 hours under UV illumination for a 4 µM solution of riboflavin (data not shown). The degradation was not likely due to ROS as adding antioxidants, including N-acetylcysteine or glutathione, did not recover the riboflavin level

in cell suspension (Figure S3). Considering the fact that all riboflavin was degraded after a 6-hour exposure in control experiments, it seems that bacterial cells in suspension “reestablish” riboflavin levels to counteract depletion of riboflavin from UV illumination. In a previous study, electrochemical activities were observed in a mediator-less microbial fuel cell using *Shewanella putrefaciens* cells, indicating that cells established a level of mediators for electron shuttling (Kim et al., 2002). Interestingly, TiO₂ NPs further reduced the riboflavin level in addition to UV exposure, indicating some additive effect between UV illumination and TiO₂ NPs on the amount of riboflavin outside cells. This additional effect may be due to changes in either cell riboflavin secretion or photodegradation of riboflavin outside bacterial cells. Studies have shown that riboflavin can be adsorbed on TiO₂ surfaces, likely via the phenolic group, and photo-excited flavin can transfer electrons into the conduction band of TiO₂ (Shinohara et al., 1991; Kathiravan and Renganathan, 2008). Thus, it is possible that TiO₂ NPs act as a mediator/catalyst in riboflavin photodegradation.

4.3. Gene expression change upon TiO₂ NP and UV exposure

To further investigate subtle effects that cannot be observed by only viability/growth measurements, 6 genes related to oxidative stress, exopolysaccharide (EPS) synthesis, extracytoplasmic stress, cell division and metal-reducing pathways were included in this gene expression study to test the hypothesized mechanisms of how bacterial cells respond to TiO₂ NPs. Different mechanisms of bacterial stress response were addressed by these 6 genes. TiO₂ NPs were hypothesized to kill bacteria via generating ROS under UV illumination (Kuhn et al., 2003), while previous transcriptional analysis displayed that a series of ROS-scavenging related genes were up-regulated upon UVA illumination (Qiu et al., 2005). Thus, the *katB* gene, one of the peroxide response-inducible genes controlled by the redox-sensitive transcriptional activator OxyR (Jiang et al., 2014), and the *sodB* gene, a gene coding for Fe-containing superoxide dismutase (Farr and Kogoma, 1991), were chosen to represent the cell responses to oxidative

stress from excessive ROS potentially generated by TiO₂ NPs and UV illumination. Analysis of the results using two-way ANOVA showed that the *katB* gene was not influenced by TiO₂ NP and/or UV light exposure while UV illumination had a statistically significant effect on the expression level of the *sodB* gene after 30-min and 1-hour exposure, but TiO₂ NPs did not show any effect. This correlated with the observation from the intracellular ROS assay, where TiO₂ NPs did not induce any increase in ROS level within bacterial cells. However, though the expression level of the *katB* gene did not change, it does not necessarily indicate a lack of oxidative stress, as other ROS-scavenging related genes may respond instead of the *katB* gene based on observations from previous work (Qiu et al., 2005). Interestingly, the expression level of superoxide dismutase decreased instead of the up-regulation that we expected; this may indicate a decrease of oxidative stress from superoxide ions upon UV illumination, but the expression level change was very minor. Overall, the response from the *katB* and *sodB* genes did not implicate a role for oxidative stress as a result of TiO₂ NP exposure.

Cyclic di-GMP is an intracellular signaling molecule in a lot of bacterial species (Thormann et al., 2006). Biofilm formation, an important function of *S. oneidensis* that is related with bacterial interaction with mineral surface and electron transfer (Thormann et al., 2004; Godeke et al., 2011), was postulated to be controlled by cyclic di-GMP via production of EPS (Thormann et al., 2006). In the same piece of work, a $\Delta mxdA$ mutant exhibited strong defect in biofilm formation, followed by a further discovery that the intracellular cyclic di-GMP level was indirectly modulated by MxdA in *S. oneidensis* MR-1 (Rakshe et al., 2011). Based on these precedent studies, the *mxdA* gene, coding for a diguanylate cyclase-like protein, was chosen to indicate potential changes in biofilm formation. Two-way ANOVA analysis showed that after a 30-minute exposure, UV illumination had a statistically significant effect on the expression level of the *mxdA* gene, while no impact was observed upon TiO₂ NP exposure. No effect was observed at longer time points. These results indicate the potential modulation of cyclic di-GMP within the cells, but this effect vanished as the exposure time lengthened with a time frame of 6-hour. Previous work

indicated that the TiO₂-exposed bacteria had slower biofilm growth with no change in the amount of EPS observed, and the *mxrA* gene showed no difference in expression level upon 1-hour and 6-hour TiO₂ exposure (Maurer-Jones et al., 2013).

Filamentous phage infection, osmotic shock, and ethanol treatment have been shown to induce the response of phage-shock-protein (Psp) genes in bacterial cells (Weiner et al., 1991; Darwin, 2005; Maxson and Darwin, 2006). It was postulated that extracytoplasmic stress disrupts the membrane potential by dissipating the proton-motive force on cell membranes (Darwin, 2005). As precedent work (Maurer-Jones et al., 2013) and microscopic images (Figure S2) showed that TiO₂ NPs were not internalized by but in proximity to the bacterial cells, it was reasonable to hypothesize that TiO₂ NPs exert membrane stress to bacterial cells by associating with the bacterial membrane; thus, as part of the Psp response system, the *pspB* gene was chosen to reveal potential extracytoplasmic stress (Darwin, 2005). Results show that *pspB* gene expression was down-regulated after 1 hour upon UV illumination, but no change was observed at other time points or upon TiO₂ exposure. Considering the role of PspB protein in Psp response, acting largely as a signal detector/transducer that detects extracytoplasmic signals and initiates the Psp response by binding to PspA protein, *pspB* gene expression might not be directly regulated upon membrane stress. Further experiments can be done using *pspA* or *pspG* as genes of interest, as they are the two genes that are regulated by PspF regulon following PspA protein binding to the PspB/C complex during the Psp response (Maxson and Darwin, 2006). The down-regulation of *pspB* expression upon UV illumination is hard to interpret without additional information; no precedent work has reported change of *pspB* expression level upon UV illumination. Further experiments might include a membrane potential measurement upon TiO₂ NP exposure and UV illumination to obtain more information on membrane stress.

The other two genes of interest, *ftsK* and *omcA*, did not show any statistically significant changes upon TiO₂ NP or UVA exposure. The FtsK protein actively pushes DNA through the closing division septum during bacterial chromosome segregation in cell division (Aussel et al.,

2002), so we expected the *ftsK* gene expression level to be a biomarker for bacterial growth. However, though *S. oneidensis* MR-1 displayed a growth inhibition upon TiO₂ NP exposure, the expression level of *ftsK* gene did not change. The OmcA protein is a part of the metal-reducing pathway of *S. oneidensis* MR-1, which is important for the bacterial respiration of oxidized metals like Fe(III) and Mn (IV). It is located on the outer membrane, and previous research shows that this protein is required for electron transfer from the inner membrane to the electron terminal acceptors via electron shuttles like flavins (Coursolle et al., 2010). Based on this information, we expected the expression level of the *omcA* gene to be indicative of riboflavin secretion. However, *omcA* gene expression level did not change upon exposure, and was not correlated with riboflavin secretion measured by HPLC. A better candidate for riboflavin biomarker might be a gene directly related with riboflavin biosynthesis (e.g. *ribA/B*, *ushA*) (Covington et al., 2010; Brutinel and Gralnick, 2012).

It should also be noted that though there were some statistically significant changes in gene expression levels, the degree of change was relatively small in a biological context (smaller than 2). With a higher dose of 100 mg/L, one might see more change, but in a previous study, 100 mg/L of TiO₂ nanoparticles also did not show any relative fold change larger than 2 (Maurer-Jones et al., 2013). Based on the result of this study, none of the genes can be considered as biomarkers for the inhibition of growth. For future work, the *nqrF* gene, coding for Na-translocating NADH-quinone reductase, might be a candidate as a biomarker of cell respiration and growth (Steuber, 2001; Beliaev et al., 2005).

5. Conclusions

In this study, we showed that TiO₂ nanoparticles inhibited the growth on *Shewanella oneidensis* MR-1 at high dosage but had no effect on cell viability, while concomitant UVA illumination did not induce extra inhibition in growth. It is likely that the minimal impacts are due to the nutrient-rich medium used in this study. Because environmental systems are not likely to be nutrient rich,

future work will examine effects in minimal medium that only contains minimal amount of salts (Horst et al., 2012). Further assessments on reactive oxygen species and riboflavin secretion indicated that TiO₂ NPs induced neither intracellular ROS accumulation nor riboflavin secretion, but had additive effect with UVA illumination to further reduce the amount of riboflavin outside cells. Finally, we demonstrated that gene expression is a tool to explore NP toxicity mechanisms in combination with cell function, thus providing more information on how cells respond to NPs and understanding the subtle influences of NPs to the ecological systems.

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Notes

The authors declare no competing financial interest.

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Supporting Information Available: The supporting information contains methods on (1) TiO₂ nanoparticle synthesis and characterization, (2) actinometry of UVA illumination, and (3) hyperspectral imaging of TiO₂ NP-exposed bacterial cells. It also contains figures on (1) TiO₂ nanoparticle

characterization using TEM, DLS and ζ -potential, (2) hyperspectral image of TiO₂ NPs with bacterial cells, and (3) riboflavin secretion upon TiO₂ NP exposure and/or UV illumination with or without N-acetylcysteine or glutathione.

Abbreviations

UV, ultraviolet; TiO₂, titanium dioxide; NPs, nanoparticles; *S. oneidensis* MR-1, *Shewanella oneidensis* MR-1; ROS, reactive oxygen species; OD, optical density; DCFDA, dichlorodihydrofluorescein diacetate; DCF, 2', 7'-dichlorofluorescein; RNA, ribonucleic acid; qPCR, quantitative real-time PCR; Ct, fractional cycle number; DLS, dynamic light scattering; TEM, transmission electron microscopy; HPLC, high-performance liquid chromatography; ANOVA, analysis of variance; EPS, exopolysaccharide; Psp, phage-shock-protein.

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