Impact of TiO_2 Nanoparticles on Growth, Biofilm Formation, and Flavin Secretion in *Shewanella oneidensis*

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Abstract:
Supporting information contains nanoparticle characterization, drift observed with quartz crystal microbalance (QCM), Live/Dead BacLight viability assay method and data, extracellular polymeric substance isolation and characterization method and results, detailed sample preparation for TEM uptake analysis, reactive oxygen species results, and relative fold changes for all genes explored in quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Experimental Section

LIVE/DEAD BacLight Viability Assay

Viability of *S. oneidensis* after exposure to TiO_2 was assessed using the fluorescent staining kit LIVE/DEAD® Baclight™ (Life Technologies, Grand Island, NY) where live cells are stained with SYTO-9 fluorescent tag and dead cells are stained with propidium iodide. Cells were diluted to a density of 10^8 cells/mL with LB broth and exposed to varying concentrations (1-100 μg/mL) of as-syn or 25 μg/mL P25/T-Eco TiO_2 nanoparticles for 24 h (at 30 °C and shaking at 200 RPM), with each condition cultured in triplicate and periodic assessment of viability over the 24 h exposure. To perform the viability assay, the staining solution was prepared as specified by the manufacturer (kit L7012). Periodically, 0.5 mL aliquots of the samples were centrifuged at 500g for 10 minutes and pelleted cells were resuspended in a HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer solution with mineral additives to avoid extracellular nucleic acids in the LB broth that bind the fluorescent stain molecules. HEPES buffer, modeled after *Shewanella sp.* basal medium described in Balch *et al.*, consisted of 100 mM HEPES (Sigma Aldrich, St. Louis, MO), 7.8 mM $NaCH_3CO_2$ (EMD Chemicals Inc, Gibbstown, NJ), 1.3 mM $K_2HPO_4{\cdot}3H_2O$ (Mallinckrodt, Phillipsburg, NJ), 1.7 mM KH_2PO_4(JT Baker, Phillipsburg, NJ), 1.7 mM $(NH_4)_2SO_4$ (Fisher Chemical, Fairlawn, NJ), 1 mM $MgSO_4$ (Sigma Aldrich, St. Louis, MO), 9.5 μM $ZnCl_2$ (Sigma Aldrich, St. Louis, MO), 1.9 μM $NiCl_2$ (Sigma Aldrich, St. Louis, MO), 0.5 μM $Na_2MoO_4{\cdot}2H_2O$ (Sigma Aldrich, St. Louis, MO), 0.4 μM $Na_2WO_4{\cdot}2H_2O$ (Alfa Aesar, Ward Hill, MA), 0.2 μM $AlK(SO_4)_2{\cdot}12H_2O$ (Mallinckrodt, Phillipsburg, NJ), 1.8 μM $FeSO_4{\cdot}7H_2O$ (Fisher Chemical, Fairlawn, NJ), 13.5 μM $MnSO_4{\cdot}H_2O$ (Mallinckrodt, Phillipsburg, NJ), 0.2 μM $CuSO_4{\cdot}5H_2O$ (Spectrum Chemical, Redondo Beach, CA), and 3.25 μM $CoSO_4{\cdot}7H_2O$ (Fisher Chemical, Fairlawn, NJ), adjusted to

pH 7.2 with NaOH (Mallinckrodt, Phillipsburg, NJ).[4] 100 µL of the resuspended bacteria were plated on a 96 well plate, mixed with 100 µL of the staining solution and incubated for 15 minutes in the dark. After incubation with the reporter molecules, fluorescence intensity was measured on a multi-well plate reader (Synergy 2, Biotek, Winooski, VT) with excitation wavelength for both molecules centered at 485 nm and the fluorescence intensity of live cell stain, SYTO-9, measured at a wavelength of 530 nm and dead cell stain, propidium iodide, measured at a wavelength of 630 nm. The ratio of live cell to dead cell fluorescence intensity was compared between the control and nanoparticle-exposed bacteria. The viability measurement was repeated after 0, 1, 2, 6, and 24 h of nanoparticle exposure.

Extracellular Polymeric Substance Isolation and Characterization

EPS extraction and characterization was performed as described by Gong *et al.*[5] Samples were prepared in quadruplicate by diluting the bacterial suspension to 10^8 cell/mL and exposing cells to 1-100 µg/mL as-syn or 25 µg/mL P25 of T-Eco TiO_2 nanoparticles for 24 h (shaking at 200 RPM at 30 °C) followed by centrifugation of 3 mL of the samples at 1500g for 10 min. Pelleted cells were resuspended in 10 mL of an aqueous solution of 8.5% (w/w) NaCl and 0.22% (w/w) formaldehyde (JT Baker, Phillipsburg, NJ) by vortexing and placed in the refrigerator for 2 h. Cells were centrifuged at 3700g for 15 min at 4 °C, resuspended in a MQ water wash, and centrifuged again. The supernatant was removed and the EPS weight was taken. MQ water was added in the ratio of 50 mL for every 1 g EPS, samples were sonicated for 3 min and again centrifuged (3700g for 15 min at 4 °C). Cells were resuspended in 5 mL of 10 mM KCl and 10 mL of cold, 200 proof ethanol. The suspension was placed in the refrigerator overnight to allow

EPS precipitation. Then, EPS was pelleted (3700g for 20 min at 4 °C) and resuspended in 10 mL MQ water.

Phenol-sulfuric acid (PSA) was used to quantify sugar content of the EPS, where 2 mL of EPS suspension was mixed with 50 µL of 80% (w/w) phenol solution (Sigma Aldrich, St. Louis, MO) and 5 mL concentrated H_2SO_4 (BDH Aristar, Radnor, PA). The mixture was allowed to incubate for 20 min in a 35 °C water bath followed by stabilization at room temperature for 4 h. The absorbance of each sample was measured at 480 nm in a 96-well plate and compared to a calibration curve made from glucose standards.

Protein quantification was performed via Lowry's method where 0.3 mL of the EPS solution was mixed with 1.5 mL alkaline copper reagent in a glass vial. The alkaline copper reagent was made by combining 2% w/w $Na_2C_4H_4O_6$, 1 mL 1% w/w $CuSO_4 \cdot 5H_2O$ and 98 mL of 2% w/w $NaCO_3$ in 10 mM NaOH. Following the copper solution addition, 75 µL Folin and Ciocalteu's phenol reagent (Sigma Aldrich, St. Louis, MO) was added, incubated for 30 min, and absorbance of the samples was measured at 500 nm. Absorbance values were compared to a calibration curve generated from bovine serum albumin (BSA) standards.

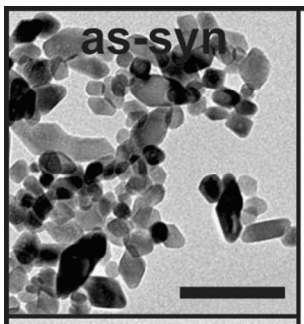
Sample Preparation for TEM Uptake Analysis

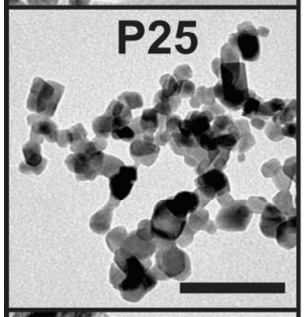
Bacteria were cultured with 25 µg/mL as-syn TiO_2 at varying lengths of exposure then the cells were pelleted by centrifugation (555 g for 10 min) and triple rinsed with 0.1 M sodium cacodylate buffer (Sigma Aldrich, St. Louis, MO) with centrifugation at 89 g for 5 min between each rinse. Final rinse buffer was removed and replaced with a solution of 2.5% glutaraldehyde fixative (Sigma Aldrich, St. Louis, MO) in 0.1 M sodium cacodylate buffer. After a 1 h, the pellet was rinsed three times with sodium cacodylate buffer followed by post-fixing for 1 h in

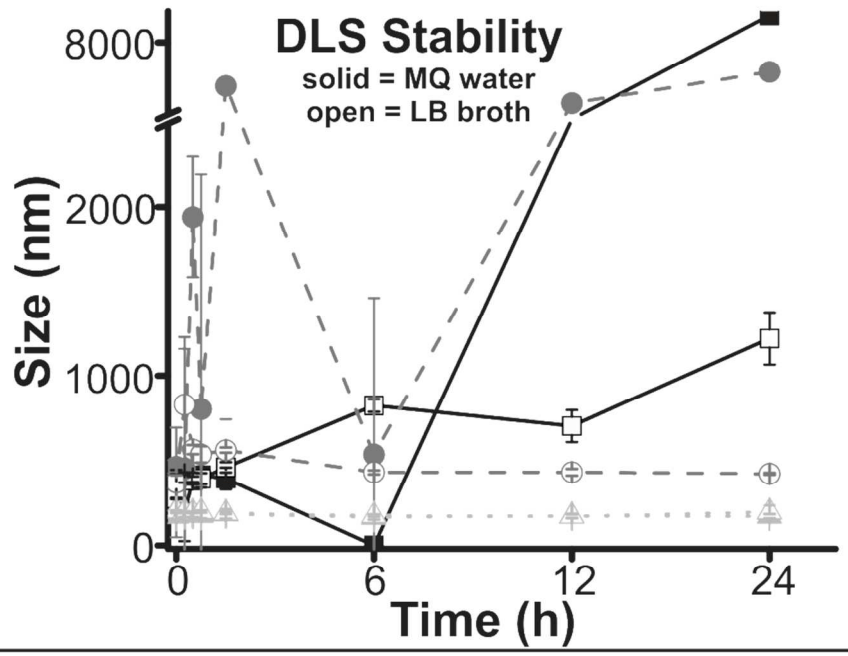
1% osmium tetraoxide (Sigma Aldrich, St. Louis, MO) in 0.2 M sodium cacodylate buffer. The cells were dehydrated in a series of solutions with increasing ethanol in water followed by propylene oxide (Sigma Aldrich, St. Louis, MO). Propylene oxide was replaced with a 1:1 propylene oxide:Epon resin and incubated for 2 h. Finally, samples were infiltrated with 100% resin for 48 h, refreshing the resin 5 times within the 48 h period. Resin was cured for 24 h at 45 °C then 24h at 60 °C. Samples were sectioned into 60-nm-thick sections using a diamond knife on an ultramicrotome, and the sections were collected on Formvar®-coated copper TEM grids (Ted Pella Inc, Redding, CA). Sample grids were stained with uranyl acetate and lead citrate (Sigma-Aldrich, St. Louis, MO) and imaged on JEOL 1200 EXII TEM at 60 kV.

Results

Figure S1. Nanoparticle characteristics. * results taken from previously published work.[1-3]
†measurements taken in bacterial growth broth (LB broth). (ζ-pot = ζ-potential).







TiO_2	Symbol	Size* (nm)	Hydrodynamic Diameter† (nm)	Crystallinity*	ζ-pot† (mV)
as-syn	—■—	11	275±4	93.5% anatase 6.5% brookite	-10±10
P25	--●--	25	371±323	75% anatase 25% rutile	-18±45
T-Eco	···▲···	25 x 7	201±5	100% anatase	-11±28

Figure S2. *S. oneidensis* viability after exposure to TiO_2 nanoparticles as measured with the Live/Dead Baclight™ assay. (A) Viability after 6 h exposure to varied concentrations (0-100 µg/mL) of as-syn TiO_2 nanoparticles. (B) Viability of *S. oneidensis* exposed to 25 µg/mL as-syn TiO_2 nanoparticles for varied times. *$p<0.05$, where each time point is significantly different than all other times of exposure. (C) Viability of *S. oneidensis* after exposure to 25 µg/mL of as-syn, P25, and T-Eco for 6 h.

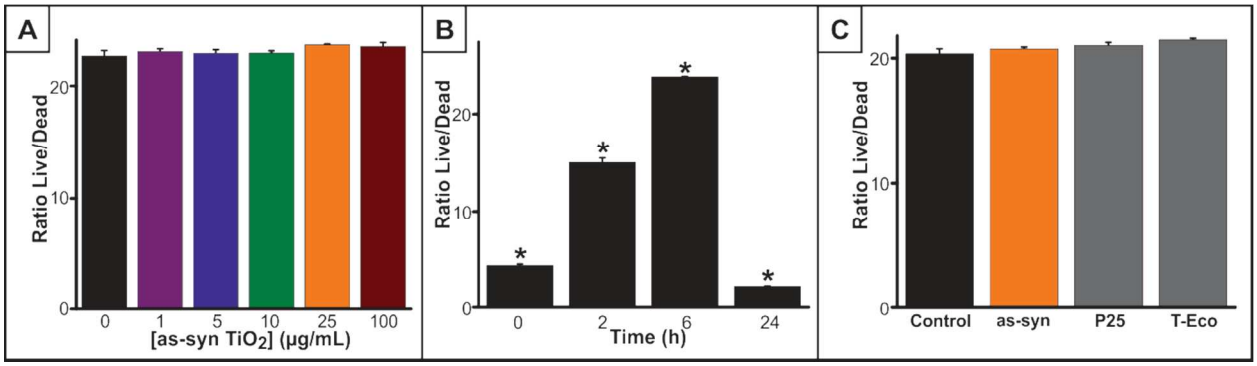


Figure S3. QCM drift without (black) and with (red) the presence of 25 µg/mL as-syn TiO_2 nanoparticles over the time course of a typical QCM experiment. Arrow head indicates addition of TiO_2 to LB broth with (A) showing the frequency drift and (B) indicating resistance drift.

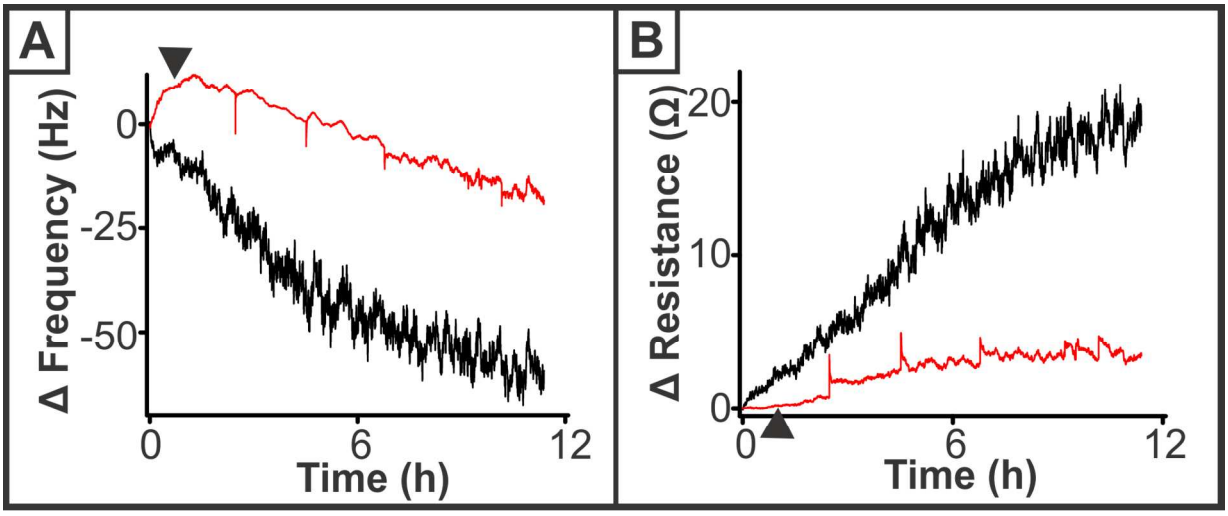


Figure S4. Characterization of EPS after 24 h exposure to varying concentrations and types of TiO_2 nanoparticles.

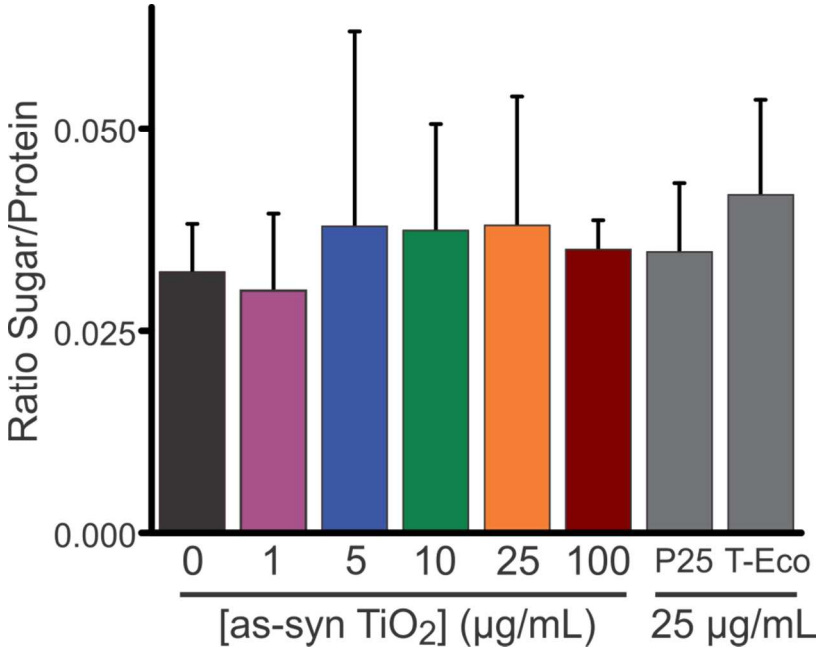


Figure S5. ROS produced in *S. oneidensis* culture over time as measured with the DCFDA assay upon exposure to varied concentrations of as-syn TiO_2 nanoparticles. Early time points (0-6 h) are on a magnified y-axis in the inset.

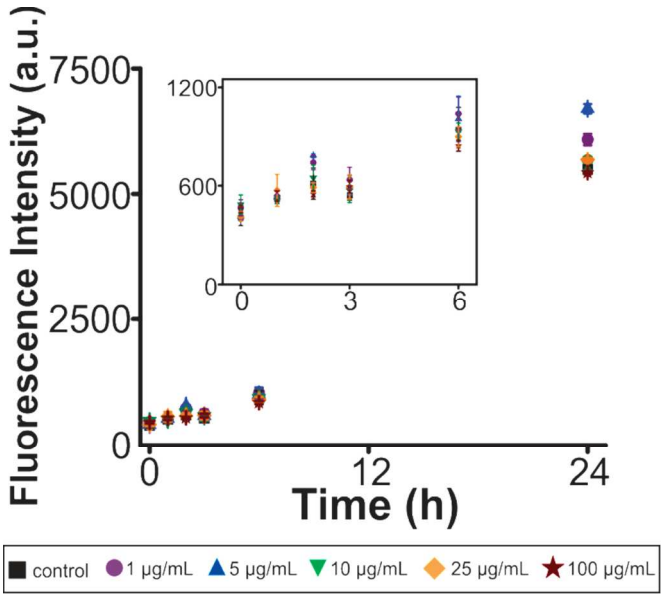


Table S1. Relative fold change of other genes related to flavin secretion, biofilms/EPS, and cell stress investigated with qRT-PCR. Red values with a negative sign indicate a decrease in gene expression as compared to the control, and black positive values are increased gene expression as compared to the control. Filled gray cells indicate significant ($p<0.05$) difference in expression as compared to the control.

		[as-syn TiO_2] (µg/mL)				
Function	**Gene**	**1**	**5**	**10**	**25**	**100**
Flavin secretion	*mtrA*	-1.01	1.10	1.24	1.11	1.01
	cymA	1.10	1.11	1.04	1.25	1.28
	ushA	-1.13	-1.06	-1.14	1.07	1.07
Biofilms and EPS	*mxdB*	-1.37	-1.57	-1.70	-1.73	-1.56
	mxdC	-1.11	1.17	-1.14	1.20	-1.06
	mxdD	-1.16	-1.06	-1.16	-1.03	-1.10
Stress	*gst*	-1.12	1.01	1.18	1.13	1.06
	pspB	-1.04	1.18	1.07	1.62	1.34
	radA	-1.28	-1.04	-1.09	1.01	-1.14
	dnaN	-1.21	-1.01	-1.15	1.11	-1.00

References

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4. W. E. Balch, G. E. Fox, L. J. Magrum, C. R. Woese, R. S. Wolfe, *Microbiol. Rev.* 1979, *43*. 260-96.
5. A. S. Gong, C. H. Bolster, M. Benavides, S. L. Walker, *Environ. Eng. Sci.* 2009, *26*. 1523-1532, DOI: 10.1089/ees.2008.0398.