**Particle Size Effect and the Mechanism of Hematite Reduction by
the Outer Membrane Cytochrome OmcA of *Shewanella Oneidensis*
MR-1**

JUAN LIU^{a,b,*}, CAROLYN I. PEARCE^{b,c}, LIANG SHI^b, ZHEMING WANG^b, ZHI SHI^b, ELKE
ARENHOLZ^d AND KEVIN M. ROSSO^{b,*}

^aCollege of Environmental Sciences and Engineering, Peking University, Beijing, 100871, China

^bPacific Northwest National Laboratory, Richland, WA 99352, USA

^cSchool of Chemistry, The University of Manchester, Manchester, M13 9PL, UK

^dAdvanced Light Source, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

To be submitted to *Geochimica et Cosmochimica Acta*

*To whom correspondence may be addressed:

E-mail: juan.liu@pku.edu.cn (J. Liu) or kevin.rosso@pnl.gov (K.M. Rosso)

Keywords: hematite · nanoparticle · size effect · microbial iron reduction · aggregation · electron
transfer · cytochrome

ABSTRACT

The cycling of iron at the Earth's near surface is profoundly influenced by dissimilatory metal reducing microorganisms, and many studies have focused on unraveling electron transfer mechanisms between these bacteria and Fe(III)-(oxyhydr)oxides. However, these efforts have been complicated by the fact that these minerals often occur in the micro- to nanosize regime, and in relevant natural environments as well as in the laboratory are subject to aggregation. The nature of the physical interface between the cellular envelope, the outer-membrane cytochromes responsible for facilitating the interfacial electron transfer step, and these complex mineral particulates is thus difficult to probe. Previous studies using whole cells have reported reduction rates that do not correlate with particle size. In the present study we isolate the interaction between the decaheme outer-membrane cytochrome OmcA of *S. oneidensis* and nanoparticulate hematite, examining the reduction rate as a function of particle size and reaction products through detailed characterization of the electron balance and the structure and valence of iron at particle surfaces. By comparison with abiotic reduction via the smaller molecule ascorbic acid, we show that the reduction rate is systematically controlled by the sterically accessible interfacial contact area between OmcA and hematite in particle aggregates; rates increase once pore throat sizes in aggregates become as large as OmcA. Simultaneous measure of OmcA oxidation against Fe(II) release shows a ratio of 1:10, consistent with a cascade OmcA oxidation mechanism heme by heme. X-ray absorption spectroscopies reveal incipient magnetite on the reacted surfaces of the hematite nanoparticles after reaction. The collective findings establish the importance of accessibility of physical contact between the terminal reductases and iron oxide surfaces, and through apparent consistency of observations help reconcile behavior reported at the larger more complex scale of whole cell studies.

1. INTRODUCTION

In the natural environment, reduction of sparingly soluble Fe(III) oxide minerals to the highly soluble Fe(II)_(aq) form by dissimilatory metal-reducing bacteria (DMRB) is a prevalent redox process that controls iron cycling in the subsurface, and can influence the fate and transport of redox-active metals, as well as organic and inorganic contaminants (e.g., Islam et al., 2004; Lovley et al., 1996). To access extracellular oxide mineral electron acceptors, it has been proposed that c-type cytochromes work in concert to comprise a long-range electron transport network across the periplasm and outer membrane of the cell envelope, terminating at decaheme cytochromes whose function is to facilitate the interfacial electron transfer (ET) step (Clarke et al., 2011; Shi et al., 2009). Along with MtrC, OmcA is one of these terminal reductases. It is found on the outer membrane and in the extracellular polymeric substances of *Shewanella oneidensis* MR-1, an extensively studied DMRB for its role in environmental biogeochemistry and its considerable potential influence on the remediation of contaminated environments (Cao et al., 2011; Fredrickson et al., 2008).

The 85 kDa OmcA shows broad redox potentials ranging from -320 to -20 mV vs. SHE (Standard Hydrogen Electrode) (Shi et al., 2006). A variety of laboratory measurements have demonstrated that its 10 heme groups are involved in ET to metal acceptors, along with those in MtrC (Johs et al., 2010; Ross et al., 2009; Shi et al., 2006; Wang et al., 2008; Xiong et al., 2006). Determination of the molecular structure of MtrF, an MtrC homolog, and more recently the structure determination of OmcA itself (Edwards et al., 2014), suggest that these proteins transfer electrons directly to the surface of metal oxides via one or more solvent-exposed hemes (Clarke et al., 2011; Shi et al., 2012a) within a conserved staggered cross heme wire motif. For example, with regards to the widespread Fe(III)-oxide mineral hematite (α -Fe₂O₃), experimental and computational studies have shown that purified OmcA is able to both specifically bind to hematite surfaces and transfer electrons to it (Eggleson et al., 2008; Johs et al., 2010; Kerisit et al., 2007; Lower et al., 2008; Wigginton et al., 2009; Xiong et al., 2006).

Despite recent discoveries relating to the structural, electrochemical, and spectroscopic characteristics for these key outer-membrane cytochromes, resolving their function at a detailed level requires close examination of how properties of the electron accepting metal oxide itself can affect the interfacial ET step. At the whole cell level, in experiments with *S. oneidensis*, metal oxide reduction rates were shown to depend on characteristics of the metal oxide such as particle size, surface area, surface structure, solubility, mineral structure, impurity, and crystallinity (Bonneville et al., 2009; Bose et al., 2009; Ekstrom et al., 2010; Neal et al., 2003; Roden and Zachara, 1996). For small particle sizes, such as for many of the metal oxides in nature (Hochella et al., 2008), additional characteristics such as extent of particle aggregation and size distribution become important, in principle, because they can impact the steric accessibility of solid surface area to cytochromes, which themselves are of nanometer size and situated in a geometrically constrained configuration on the outer membranes of cells .

In spite of its importance for understanding the interfacial ET rate, unraveling the impacts of nanoparticle properties on DMRB activity has been difficult. Attempts using whole cell studies have left the issue unresolved. Roden (2003) suggested that initial rates of microbial Fe(III) oxide reduction are linearly correlated to BET surface area and the reactive surface site density of the solid phase. However, Bose et al. (2009) reported that the microbial reduction rates for 11, 12, 30, 43, and 99 nm hematite nanoparticles are not proportional to particle size, and postulated that metal oxide particles of different sizes may attach to DMRBs in different modes. Yan et al. (2008) suggested that the reduction rates should be directly correlated to the bacteria-hematite contact area, not to the total oxide surface area. Studies to date thus appear to be in conflict, possibly because of the complexities of understanding the manifold of interactions between whole cells and very small particles overall (Bosch et al., 2010; Bose et al., 2009; Roden, 2003; Yan et al., 2008). Novel experiments are needed that minimize the number of modes of physical and chemical interaction between the particles and terminal reductases to understand mechanisms of ET between DMRB and metal oxides in the environment.

The present study investigates the particle size effect directly, making use of purified OmcA as the electron donor and hematite particles with well-defined sizes as electron acceptors. We report reduction kinetics of hematite particles with mean primary particle sizes of 15, 30, 55, and 173 nm, by OmcA, and we compare to the size-dependence of the hematite reduction kinetics by ascorbic acid, a reducing agent analog for biogeochemical organic compounds in nature (Echigo et al., 2012). Characteristics of the hematite particles before and after reaction with OmcA were investigated using a variety of methods, including in situ micro-X-ray diffraction (μ -XRD), transmission electron microscopy (TEM), and Fe $L_{2,3}$ -edge X-ray absorption/magnetic circular dichroism (XAS/XMCD) spectroscopy. The observed size effect is resolved and discussed in terms of separate factors such as primary particle size itself, the molecular size of the reductant, and particle aggregation. This study is the first investigation of the effect of particle size on ET kinetics between hematite and an important c-type cytochrome in the transmembrane electron transfer network of a primary DMRB.

2. MATERIALS AND METHODS

All glassware and plastic bottles for wet chemical experiments were soaked in 1% HNO_3 overnight and then rinsed several times with distilled and deionized water before use. All chemicals, plastic syringes, tubes, vials, pipette tips, and syringe filters were deoxygenated for at least 24 h inside the glovebox prior to use. All spectroscopic kinetic measurements as well as batch reactions (including OmcA and ascorbic acid reduction experiments) were conducted in the glovebox. Degassed and deionized water (DDW) was stored in an anoxic chamber for preparation of all solutions and suspensions. All chemicals were reagent grade or better. All experiments were performed in triplicate. All reduction experiments were performed at least in triplicate in a high-purity dry N_2 glovebox (< 1 ppm O_2).

2.1 Hematite Nanoparticle Synthesis.

Hematite nanoparticles in 15, 30, 55, and 173 nm sizes were synthesized by forced hydrolysis of Fe(III) salt solution according to Schwertmann and Cornell (2000). Briefly, the 15 nm hematite nanoparticles were synthesized by slowly adding 80 mL of 1 M ferric nitrate ($\text{Fe}(\text{NO}_3)_3 \cdot 9 \text{H}_2\text{O}$) solution dropwise at the speed of 0.5 mL/min into 1 L of boiling distilled MilliQ water while stirring vigorously. After the $\text{Fe}(\text{NO}_3)_3 \cdot 9 \text{H}_2\text{O}$ addition was finished, the resulting suspension was removed from the heat and cooled overnight. For synthesizing 30 nm nanoparticles, 500 mL of 0.002 M HCl solution in a screw-cap bottle was heated in an oven at 98 °C overnight. Then, 4.04 g of $\text{Fe}(\text{NO}_3)_3 \cdot 9 \text{H}_2\text{O}$ was added to the solution. The bottle was then closed and vigorously shaken. After that, it was returned to the oven immediately and held at 98 °C for 7 days. These two synthesis suspensions were dialyzed in standard grade Cellulose dialysis membranes (1000 MWCO, Spectra Por) against MilliQ water until the conductivity remained constant. 55 nm and 173 nm hematite particles were synthesized by a similar method. To synthesize 55nm hematite, 0.002 M HCl solution (2L) was heated to 98 °C in a closed polypropylene bottle, and then FeCl_3 was added to it. The final concentration of FeCl_3 is 0.02 M. After the bottle was tightly capped and vigorously shaken, the closed bottle was held at 98 °C in a thermostatted oven for ten days. For 173nm particle synthesis, 0.04 M FeCl_3 and 0.001 M HCl solutions were used. The resulting red suspensions were centrifuged and resuspended by sonication in DDW for 3 repetitions. All hematite stock suspensions were purged with nitrogen gas for one hour before transfer to the glovebox, and then were purged again inside the glovebox using high purity nitrogen gas for one hour.

2.2 Characterization of Hematite Particles.

Particle density in all suspensions was measured by digestion of 0.1 mL of particle suspensions in 9.9 mL of 5 M HCl overnight with shaking. Total Fe concentration was determined using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) by adding 0.02 mL of the digested solutions to 4.98 mL 2% HNO_3 and measuring on an ICP-OES (Perkin-Elmer, Optima 2100DV). Subsamples of the hematite suspensions were rapidly frozen using liquid N_2 and freeze-dried (Labconco freeze dry

system, Kansas City, Missouri) for BET specific surface area (SSA) measurements. Multipoint BET SSA of hematite particles was measured with a quantachrome Autosorb automated gas sorption system (Quantachrome Co.) using nitrogen gas at 77 K.

Particle size and morphology of hematite particles were measured on a JEOL JEM-2100F field-emission transmission electron microscope (TEM) operating at 200 kV. TEM samples were prepared by dipping a 400 mesh copper grid coated with lacey carbon film into diluted nanoparticle suspension and then drying it inside the glovebox. Samples were taken out of the glovebox directly before measurements.

The hydrodynamic diameters of the hematite particles were measured using Dynamic Light Scattering (DLS) under aqueous conditions consistent with those used for reduction experiments by OmcA and for reduction experiments by ascorbic acid (see below). These measurements were performed using a ZetaSizer Nano ZS (Malvern Instrument, UK) equipped with 4 mW He–Ne Laser at 25.0 ± 0.1 °C and at a scattering angle 90°.

The crystalline phases of hematite particle suspensions, before and after reaction with OmcA, were characterized in situ by Micro X-ray diffraction analysis (μ -XRD) using a Rigaku D/Max Rapid II instrument with a MicroMax 007HF generator fitted with a rotating Cr anode ($\lambda = 2.2897\text{\AA}$) and a 2D image plate detector. The suspensions were loaded into boron-rich 0.5 mm O.D. capillary tubes (Charles Supper Company) in the glovebox, and the capillaries were sealed with capillary wax (Charles Supper Company) to keep the suspensions under anoxic conditions during measurements.

High-resolution Fe $L_{2,3}$ edge XMCD for the samples were measured at room temperature on beamline 4.0.2 at the Advanced Light Source (ALS), Berkeley, CA, using the eight-pole resistive magnet endstation (Arenholz and Prestemon, 2005). This beamline has an elliptically polarizing undulator and a monochromator with a resolving power of 6000 at the $L_{2,3}$ edges. Samples were mounted on a copper manipulator using carbon tape in an N₂ glovebox and the XAS signal was monitored in total-electron-yield (TEY) mode with an effective probing depth of ~4.5 nm. The sample was aligned at 90° to the beam

which is parallel to the applied magnetic field. At each photon energy point of the spectrum, the x-ray absorption was measured for two opposite magnetization directions by reversing the applied magnetic field (0.6 Tesla), while keeping the circular polarization of the incident x-rays constant. The XAS signals for each magnetization direction were normalized to the incident beam and subtracted to give the XMCD spectrum (Pattrick et al., 2002; Pearce et al., 2010).

2.3 Purification of OmcA.

The details of protein purification and characterization of OmcA were described in Shi et al (2006). Briefly, an OmcA-encoding gene was cloned and expressed in wild-type *Shewanella oneidensis* MR-1. In order to facilitate subsequent protein purification, a His₆ sequence was included at the C terminus of OmcA. *S. oneidensis* MR-1 containing the recombinant OmcA was cultured and then lysed by using a French press. The unbroken cells and debris were removed by centrifugation, and OmcA in the supernatant was isolated by a column of Ni²⁺-NTA agarose. After purification, all OmcA suspensions were adjusted to 10 μ M. The purified OmcA (10 μ M) in 20 mM HEPES (pH 7.0) with 150 mM NaCl was purged with dry N₂ gas for more than 1 hour in the glovebox, and then stored at 4°C in serum bottles capped with thick rubber stoppers and crimp sealed.

2.4 Reduction of Hematite by Ascorbic Acid.

Batch-dissolution experiments of hematite particles with ascorbic acid in the absence of light and oxygen were conducted according to a modified version of the method reported by Echigo et al (2012). The hematite stock suspensions were vigorously shaken until there were no visible settled particles remaining at the bottom of bottles. Calculated volumes of the hematite suspensions, acetate buffer (100mM, pH = 4), and sodium nitrate were then added into 60 mL glass serum reaction bottles capped with rubber stoppers and wrapped with black aluminum foil. All the suspensions were continuously shaken for more than four hours in advance and allowed to reach equilibrium before reactions with ascorbic acid. The initial concentrations of hematite particles, sodium nitrate, and ascorbic acid were 2.5

mM, 10 mM, and 0.5 mM, respectively, with a total reaction volume of 30 mL. These concentrations of electrolyte and weak carboxylic acid are generally above the critical coagulation conditions (CCC) for hematite particles even at pH = 4, which means that hematite particles are in aggregates in suspension in these experiments (He et al., 2008; Zhang and Matijevic, 1984). At desired time intervals, 2.0 mL aliquots were collected using 5.0 mL syringes, and immediately filtered through 0.2 μ m Nylon syringe filter membranes (Whatman). The [Fe(II)] in the filtrate was analyzed by ferrozine assay (Stookey, 1970) using a Shimadzu UV-2501 PC spectrophotometer. The initial rates were determined by fitting the data from the first 80 min of reactions to a second order polynomial (Echigo et al., 2012), $m_{\text{Fe(II)}} = a + bt + ct^2$.

2.5 Reduction of Hematite by OmcA.

Purified OmcA was in the oxidized form with a characteristic absorption peak at 408 nm (α peak) and a much weaker peak at $\sim$ 530 nm. Reduction of OmcA results in a shift of the α peak and the appearance of absorption peaks at 522 and 552 nm (Figure 1). The absorbance at 552 nm is measured to calculate the concentration of reduced OmcA.

Batch reduction experiments with OmcA were conducted in 3.5 mL screw-cap quartz cuvettes using a diode array UV-Vis spectrometer (HP8452, Agilent). A Peltier cell holder with a stirring system was used to continuously stir suspensions in the cuvettes throughout experiments. Just before every reduction experiment, purified OmcA was fully reduced by gradually adding $\sim$ 5 mM sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) solution until no change in absorbance at 552 nm was observed (Wang et al., 2008). Kinetic studies were initiated by spiking a certain volume of hematite stock suspensions into the reduced protein solution. The absorbance at 552 nm was tracked as a function of time. After baseline correction, the Beer-Lambert Law can be used to calculate the concentration of oxidized OmcA at time t (C_t) from the measured absorbance (A_t) (Wang et al., 2008):

$$C_t = \frac{A_t - C_0 \epsilon_{ox}}{\epsilon_{ox} - \epsilon_{red}} \quad (1)$$

where C_0 is the initial concentration of oxidized OmcA, ϵ_{ox} and ϵ_{red} are the molar absorption coefficients of the oxidized and reduced OmcA, respectively. All reduction experiments with OmcA were conducted in HEPES (pH 7.0, 20 mM with 150 mM NaCl) buffer solution by mixing 0.4 μ M protein and hematite suspensions at a concentration of 20 μ M Fe(III) equivalents. The reduction rates were calculated from the slopes of the initial 15 - 20% portion of the time course curves (more than 300 points) (Shi et al., 2012b). The square of the correlation coefficient, R^2 , was greater than 0.95 in all data fits. Real-time analysis of the Fe(II) concentration was not feasible because of lack of a proper detection method for this small volume of solution.

Electron balance in hematite reduction by OmcA was quantified using batch reactions of 2 μ M of OmcA and hematite suspensions at a concentration of 100 μ M [Fe(III)] equivalents in 20mM HEPES buffer solution at pH 7. For each experiment, 0.5 mL of solution was sealed in a 2 mL cryogenic vial immersed in ice inside a cooler. The cooler was shaken continuously for 24 hours in the glovebox. At the end of experiments, the amount of reduced OmcA was measured using A_{552} in the absorption spectrum. The Fe(II) concentration in the solution was measured using the ferrozine assay. Hematite and OmcA were separated from solution by a 30 kDa centrifuge filter (Amicon Ultra-0.5, Millipore, Billerica, MA, USA) at 13,000x g for 12 minutes, and then added 0.4 mL of the filtrate into 1.6 mL ferrozine (1000 mg/L, pH 7). A control sample, FeCl₂ solution, was prepared as an external standard and analyzed using the same procedure as for reduction experiments, in order to detect any oxidation or adsorption of aqueous Fe(II) during the experiments; none was observed.

3. RESULTS

3.1 Starting Materials

All hematite particles were synthesized similarly via forced hydrolysis of Fe(III) salt solution. The sizes and BET SSAs of the synthetic hematite particles are summarized in Table 1. μ -XRD patterns of all samples exhibit only the characteristic peaks for hematite (Figure 2), thus no secondary phase was present prior to reaction. The change of the full-width at half-maximum (FWHM) indicated the different particle sizes. TEM images (Figure 3) showed that all particles, except the 30 nm ones, exhibit a nearly isotropic shape with similar surface roughness. The 30 nm nanoparticles have a rhombohedral shape, which is in agreement with previous studies (Bose et al., 2009; Echigo et al., 2012; Madden and Hochella, 2005).

3.2 Reduction by Ascorbic Acid

Ascorbic acid was chosen as the reducing agent in the abiotic reactions for the following reasons: ascorbic acid is a widely distributed micronutrient required for multiple biological functions and also an important regulator of iron uptake; the well-established mechanism of hematite reduction by ascorbic acid can be helpful to understand the size-dependent reactivity (Lanzl et al., 2012; Suter et al., 1991). The reactivity of ascorbic acid in hematite reduction obviously decreased with increasing pH in the range of 2 – 6 (Lanzl et al., 2012). To obtain measurable change in the concentration of aqueous Fe^{2+} before obvious changes happening in the surface properties or aggregation state, the abiotic reduction by ascorbic acid was conducted at pH 4. Lanzl et al. (2012) reported that the relative reactivity of 8 and 40 nm hematite particles was unchanged at varying pH in the range of 2 to 6, indicating the different pH would not affect the size effect on reactivity trend.

The concentration of aqueous Fe(II) ions released from hematite particles as a function of time from reductive reaction with ascorbic acid at pH 4 are shown in Figure 4A. In all experiments, the initial mass concentration of hematite particles in the various suspensions was the same. Thus, while the available acceptor Fe(III) concentration was the same, the total surface area of the particles measured by BET was increasingly larger in reactions with decreasing particle size. Measured reduction rates were, therefore,

normalized to the BET SSA. The SSA-normalized initial rates of hematite particles in the four sizes are in the order $15\text{ nm} > 30\text{ nm} \geq 55\text{ nm} > 173\text{ nm}$ (Figure 4B and Table 1). Results from similar experiments reported by Echigo (2012) and by Suter (1991) are also provided in Figure 4B, which shows that our measured rates are consistent with precedent on the same basis. All data indicate that the abiotic reduction rates by ascorbic acid are systematic and inversely proportional to primary particle size.

Aggregation state of particles in suspensions may influence the amount of available surface area and interfacial reactivity of particles, so the hydrodynamic diameter measurements of hematite particles in different sizes at the pH 4 conditions for reduction by ascorbic acid were conducted using DLS. The measured hydrodynamic diameters for all sizes of particles were much larger than their primary particle size, revealing extensive aggregation of all particles under the condition (Figure 5). In addition, the average hydrodynamic diameter of the smallest particles was statistically slightly larger than the largest ones, which is consistent with the results reported by previous studies (Lanzl et al., 2012).

3.3 Reduction by OmcA

Hematite reduction by OmcA was carried out at pH 7, in order to maintain near-physiological conditions and the maximal activity of OmcA (Shi et al., 2006). Despite this pH was higher than that of the reactions with ascorbic acid, the size-dependent reactivity trend in reduction experiments with ascorbic acid at pH = 4 and pH = 7 would be same, as mentioned above. Thus, the different pH values in the abiotic and biotic reactions would not affect the comparison of the size-dependent reactivity trends. In order to ensure the stability of hematite particle suspensions in contact with OmcA, as well as to understand if there were any effects of adsorption on the spectroscopic absorbance of OmcA, control experiments with purified OmcA in the oxidized form and hematite particles of different sizes were conducted. The data (Figure 6) show that the absorbance at 552 nm (A_{552}) in all cases was essentially constant except for a slight increase when run over a long period of time, likely due to increased light scattering by slowly aggregating hematite particles; this effect is minor and independent of particle size.

For subsequent experiments with reduced OmcA, a dependence of A_{552} on protein adsorption on nanoparticle surfaces or other processes unrelated to ET can thus be assumed negligible. Moreover, the stability of OmcA reduced by $\text{Na}_2\text{S}_2\text{O}_4$ in HEPES buffer solution without hematite was also studied. Although A_{552} decreased by about 8% by the end of this experiment (Figure 6), the change in the first ~100 minutes was subtle. Because only the initial 15 - 20% portion of the time course was to be used to calculate reduction rates, the decrease in the long term experiment is inconsequential. Therefore, the change of A_{552} yielding kinetic curves for hematite reduction by OmcA primarily indicates the progressive oxidation of OmcA as it reacts with the nanoparticles.

Representative kinetics for OmcA reduction of hematite particles with different sizes are compared in Figure 7A. The change of reduced OmcA concentration with time was calculated according to the absorbance change at 552 nm using equation 1. In all reduction reactions, the concentration of reduced OmcA decreased in a similar way; the reduction rates were initially fast and decayed monotonically. Again on a basis of constant initial mass concentration of particles, the fraction of OmcA oxidized by hematite at the end of each reaction was dependent on particle size in the order $15\text{ nm} > 30\text{ nm} > 55\text{ nm} \geq 173\text{ nm}$. Considering that accumulation of surface bound Fe(II) could pose kinetic constraints on the long-term extent of hematite reduction with OmcA, it is only possible to accurately assess the impact of oxide properties on reduction kinetics during the initial stages of reduction (Roden, 2003). The initial rates were derived from the slopes of the initial portion of the time course and compared in Figure 7B. Once surface area normalized, the initial rates occur in the order $173\text{ nm} \gg 15\text{ nm} > 30\text{ nm} > 55\text{ nm}$ (Table 1, Figure 7B).

The aggregation state of each size of hematite at the pH 7 conditions for reduction by OmcA was also studied using DLS. The measured hydrodynamic diameters revealed extensive aggregation of all hematite particles in the 20 mM HEPES buffer solution with 150 mM NaCl at pH 7 (Figure 8). Similar to the trend observed at the conditions for reduction by ascorbic acid, the average hydrodynamic diameter of

15 nm particles was slightly larger than that of 173 nm particles, indicating the smaller particles aggregated to a greater extent. The high salt concentration in the buffer solutions significantly influenced the aggregation state of particles. The effect of ascorbic acid or OmcA at much lower concentration can be negligible.

3.4 Reduction Products

To gain further mechanistic insight, batch experiments were performed under conditions identical to those used in the kinetic studies, at a scale which allowed us to obtain measurable concentrations of Fe(II) ions ([Fe(II)]) simultaneously while measuring OmcA concentrations, along with sufficient product material for characterization by μ -XRD, TEM, and Fe $L_{2,3}$ -edge XAS/XMCD. At the end of 24 h, around 60% of reduced OmcA was reacted (Figure 9). No obvious increase in the reaction extent was observed by prolonging reaction time beyond 24 h, even though [Fe(III)] in hematite was much more than 10 times the concentration of reduced OmcA ([OmcA_{red}]). Similar incomplete enzymatic oxidation was observed in homogeneous reactions with Fe(II)-complexes (Liu et al., 2012a) and also in heterogeneous reactions with iron oxide nanoparticles (Liu et al., 2013). Importantly, although not all OmcA was reacted with hematite in these batch experiments, the ratios of produced [Fe(II)] to the decrease of [OmcA_{red}] at the end of the 24 h period was close to 10 electron equivalents in all cases (Figure9). On the other hand, only ~ 0.1% Fe(III) of hematite was reduced by OmcA at the end of reaction, so the reaction products may not be able to completely passivate hematite surface or determine the reaction extent.

μ -XRD results of hematite particles after reaction with ascorbic acid and OmcA show that no secondary phase was detectable under the measured conditions (Figure 2) (The patterns of 15 nm hematite after reactions are shown in Figures 2. Others are not shown). TEM images of 15 nm and 30 nm nanoparticles after reaction with OmcA did not show any secondary phase. μ -XRD and TEM analyses also showed no indication of a particle size or morphology change upon reaction.

However, Fe $L_{2,3}$ -edge XAS/XMCD spectroscopy yielded insights into changes in the structure and valence of Fe at particle surfaces after reaction. This *ex situ* technique provides information on the total relative amounts of Fe(III)/Fe(II) present in the upper 5 nm of the oxide surface, deduced from Fe site coordination indicated by XMCD spectral features and the degree of magnetic ordering indicated by the XMCD intensity (Liu et al., 2013; Pearce et al., 2010; Pearce et al., 2012). Figure 10 shows XAS/XMCD spectra for 15 nm and 30 nm hematite particles before reaction, after reaction with ascorbic acid, and after reaction with OmcA. The XAS spectra in Figure 10A and 10B show that in terms of total Fe(III)/Fe(II) content the hematite particles are essentially unchanged upon reaction with ascorbic acid; no residual Fe(II) is found and the spectral features are consistent with initial hematite. After reaction with OmcA, however, residual Fe(II) at the surface is evident by the increased intensity of the low energy shoulder on the L_3 peak. In the corresponding XMCD spectra (Figure 10C and 10D) we again see no evidence of a magnetic surface phase present after reaction with ascorbic acid. In contrast, after reaction with OmcA a measurable XMCD signal is observed with the three main peaks characteristic of magnetite (Pearce et al., 2006) indicating that magnetically ordered species of Fe are present as octahedrally coordinated Fe(II), tetrahedrally coordinated Fe(III), and octahedrally coordinated Fe(III).

4. DISCUSSION

4.1 Abiotic versus biotic reduction: particle size effect

Figure 4 shows that, in the reactions with ascorbic acid, our hematite particles behave classically with respect to the reductive dissolution rate as a function of particle size. The reduction of hematite by ascorbic acid nominally consists of three steps: (1) adsorption of ascorbate through formation of a surface Fe(III)-ascorbate complex; (2) Fe(II) formation on the surface through transfer of one electron within the surface complex; (3) the release of Fe(II) ions from the hematite surface into solution, usually considered the rate-limiting step (Suter et al., 1991). The release rate of Fe(II) is directly related to the solubility of

the iron oxide solid which, in turn, can be expected to exponentially increase as the grain size decreases according to the modified Kelvin equation (Adamson and Gast, 1997). Particularly when particle size reaches the nanoscale, solubility is predicted to dramatically increase. Higher dissolution rates for smaller nanoparticles have been widely observed in previous studies (e.g. Echigo et al., 2012; Liu et al., 2009; Madden and Hochella, 2005). Although in practice, additional size related effects can influence the kinetics, such as particle morphology (Liu et al., 2008), surface defects, phase transformation (Navrotsky et al., 2008), structural disorder, aggregation state (Echigo et al., 2012), and so on, in this study the systematically higher reduction rates of the smaller hematite nanoparticles are consistent with size-dependent solubility playing a dominant role in the reaction rate with ascorbic acid.

The trend of the reduction rates of hematite particles with the bacterial outer-membrane cytochrome OmcA is rather different from that observed in reduction experiments with ascorbic acid, and also inconsistent with the theoretical size-dependent solubility argument mentioned above, because of an unexpectedly fast reduction rate per SSA for the largest particle size. Importantly, similar behavior has been observed in the whole cell DMRB iron oxide reduction studies examining particle size dependence, despite disagreement among them as to the origin of this effect (Bose et al., 2009; Yan et al., 2008). In the study by Bose et al. (2009), reduction of hematite nanoparticles with *S. oneidensis* MR-1 yielded surface-area-normalized-bacterial reduction rates in the order 99 nm >> 12 nm > 30 nm > 43 nm. The Fe(III) reduction rate for the 99 nm nanoparticles was one order of magnitude higher than the rate observed for the smaller nanoparticles. They proposed that different cell contact and cell coverage on larger and smaller nanoparticles could produce this result (Bose et al., 2009). Yan et al. (2008) studied reduction of 10, 30, and 50 nm hematite nanoparticles by *Geobacter sulfurreducens*. The results showed that the surface area-normalized rate was highest for the 30 nm particles and lowest for the 10 nm particles. They suggested that the relative decrease in the bacteria-hematite contact area caused by aggregation was likely greater for the 10 nm particles than for the 30 nm particles, which yields a higher surface area-normalized rate for 30 nm particles (Yan et al., 2008). The suggestion of a difference between effective surface area

and total surface area attributable to particle aggregation effects is thus not unprecedented, but the underlying cause was not resolved from these whole cell studies.

In our study, comparison of size-dependence of the reductive dissolution by ascorbic acid versus that by OmcA clearly reveals a steric effect of surface area access by the OmcA molecule itself, and demonstrates its function as an interfacial ET enzyme that requires direct contact with the oxide surface in the absence of an electron shuttle. The BET surface area normalized rates are proportional to primary particle size in reactions with ascorbic acid, but not uniformly in reactions with OmcA. In both kinds of experiments, the hematite particles are not homodisperse sols but instead comprised of particle aggregates that physically restrict access to SSA for all but sufficiently small electron donating molecules. Therefore, the size, morphology, and structure of aggregates may significantly influence the BET surface area normalized rates.

OmcA, which is hypothesized to function as a terminal reductase, nominally by direct contact with metal oxide minerals, is physically too large to access the SSA in the interior of the smaller particle aggregates, for all but the 173 nm hematite particles. The recently resolved structure of OmcA (Edwards et al., 2014) shows that this protein consists of four domains with ten hemes organized into a ‘staggered cross’ motif similar to MtrF, with dimensions of $\sim 9.5 \text{ nm} \times 6.0 \text{ nm} \times 5.0 \text{ nm}$. Separately, the maximum dimension of the molecular envelope of OmcA was measured to be 9.6 nm for its oxidized form, and 8.9 nm for $\text{Na}_2\text{S}_2\text{O}_4$ reduced OmcA (Johs et al., 2010). Generally, OmcA is thus at least an order of magnitude larger than the molecular sizes of N_2 (0.36 nm in diameter) used in BET measurements (Yang et al., 2001), and also ascorbic acid ($0.60 \text{ nm} \times 0.49 \text{ nm} \times 0.36 \text{ nm}$) (Revuelta et al., 2011). Approximating the 173 nm nanoparticle aggregates as consisting of close-packed spheres, the maximum radius of an electron-donating molecule that can pass through aggregate interstices is 13.4 nm, consistent with access by OmcA and the relatively fast observed reduction rate for these particles. But the maximum is 4.2 nm for the 55 nm hematite particles, and smaller still for the 30 nm and 15 nm particles, consistent with occlusion of OmcA from contact with intra-aggregate spaces and lower observed reduction rates per unit

SSA. Beyond simple geometric approximations, it is well known from light scattering studies that smaller primary particle sizes form more compact aggregates (Chowdhury et al., 2013). Intra-aggregate pores with an average size less than 2 nm have been observed in the aggregates of ~6 nm ferric iron oxyhydroxide nanoparticles using small-angle X-ray scattering (Gilbert et al., 2007). Moreover, in stable aggregates with a similar hydrodynamic diameter in aqueous suspensions, the number of 173 nm nanoparticles is much less than that of the smaller nanoparticles. This means a larger proportion of particles are exposed to bulk solution in the suspension of 173 nm hematite particles, which also facilitates the reaction with OmcA. The slightly larger aggregates of 15 nm nanoparticles (Figure 4 and 8) imply that even a larger proportion of nanoparticles are inside aggregates, so the inaccessibility of some intra-aggregate pores could be more prominent in the case of the smallest nanoparticles. Our recent liquid cell transmission electron microscopy also showed that the aggregates of the smaller hematite nanoparticles were statistically more compact and slightly larger than those of the larger hematite nanoparticles (Liu et al., 2016). BET surface area can be used to estimate the effective surface area in the reaction with ascorbic acid, because of the similar size of N₂ and ascorbic acid. However, BET surface area could overestimate the effective surface area in the case of OmcA due to inclusion of the inaccessible surface area in aggregates.

Our observations of the reactive behavior of cytochrome isolates help reconcile, with respect to nanoparticle metal oxides, the reactive behavior of whole cells in previous work. That a steric effect is found for both an outer-membrane protein isolate and analogous whole cell studies suggests that in the whole cell studies the observed particle size-dependent effect pertains to accessibility to intra-aggregate spaces by membrane-bound terminal reductases. The conceptual model for reduction by whole cells via direct contact of the outer-membrane cytochromes with the oxide surface to facilitate ET would thus seem to be substantially reinforced by the present findings. The effect of endogenous electron shuttles, such as flavins, on aggregation state and reduction rates of nanoparticle aggregates needs to be investigated further.

4.2 Mass balance and product characteristics

A possible explanation for the incomplete reaction between OmcA and hematite (Figure 9A) is that the cell potential difference between the hematite particles and OmcA decreases to zero before all the OmcA reacts, as the concentration of Fe(III) in the nanoparticles decreases and the concentration of oxidized OmcA increases over the course of the reaction. Another explanation is that product oxidized OmcA or Fe(II) ions or both built up an armoring surface phase that reduced the reactive surface area on hematite particles over time, inhibiting further reaction.

Figure 9B indicates that the molar ratio of oxidized OmcA to Fe(II) release is close to 1:10. This observation is intriguing because every OmcA molecule consists of ten hemes, and each of them likely has a mildly different redox potential (Breuer et al., 2015). The measured electron balance of 10 suggests that the hematite particles did not react with a subgroup of the most reducing hemes in all OmcA molecules, but rather a subgroup of OmcA molecules oxidized to completion, so as to dispense electrons in the observed ratio of 1 OmcA oxidized to 10 Fe(II) ions produced. A similar 1 to 10 ratio for magnetite oxidation with a decaheme c-type cytochrome, MtoA, has been reported (Liu et al., 2012b). The OmcA molecules that reacted with hematite particles were themselves fully oxidized, and incomplete oxidation of total OmcA as indicated by the A₅₅₂ band is accounted for by what would then have to have been a fully reduced OmcA residual. This electron balance of 10 was observed in experiments with hematite in all sizes. It suggests that at the molecular level the electron transfer between reduced OmcA and hematite is a dynamically cascading process, such that for an OmcA molecule that reacts with the oxide the first ET triggers the remaining nine. If true, this behavior would not necessarily be evident from knowledge of the initial redox potentials of individual hemes in OmcA. Once electron transfer begins, conformational changes may shift the redox potential of the remaining hemes to more reducing potentials, sequentially, until oxidation of that molecule completes (Breuer et al., 2015). The corresponding *in vivo* condition may thus entail a balance of electron input and output to this outer membrane cytochrome that dynamically sustains continuous ET.

XMCD spectra (Figure 10) reveal the different reduction mechanisms on product formation of reactions with OmcA and ascorbic acid, which was not shown by TEM and μ -XRD. The XMCD signals indicate the presence of magnetite on the particle surface after reactions with OmcA. Although residual OmcA bearing Fe in cytochromes is physically present on the surface of particles after reactions, it is unlikely that the Fe signal observed in Figure 10 is attributable to OmcA due to lack of expected magnetic ordering among Fe in a sorbed OmcA surface layer and due to a lack of detectable Fe in separate XAS measurement of the OmcA without hematite. The observed XMCD spectra in Figure 10C and 10D thus likely represent nanoparticle surface Fe(III) and residual Fe(II) species that result from the reduction reaction by OmcA. On the contrary, the lack of an XMCD signal for hematite reacted with ascorbic acid demonstrates that no magnetite formed on product surfaces under the condition studied. The reductive dissolution of hematite by ascorbic acid was carried out at pH 4, the release of Fe(II) from the hematite surface into solution is accelerated by protonation of surface sites neighboring the Fe(II) leaving group, limiting the accumulation of Fe(II) at the hematite surface.

On the other hand, hematite reduction by OmcA was carried out at pH 7, in order to maximize OmcA activity and maintain near-physiological conditions (Shi et al., 2006). The formation of magnetite at the surface (topmost several angstroms) of the hematite particles results from the interaction of OmcA with hematite surface and the specific mechanism of electron transfer across the interface, which leads to a *localized* build-up of Fe(II) to high enough concentrations at these conditions (Hansel et al., 2003) for transformation to trace magnetite on hematite surface, even under ambient conditions (Lu and Tsai, 2015). The intensity of the XMCD becomes significantly larger on going from the 15 nm particles to the 30 nm particles, consistent with the presence of larger single crystalline domains on which to template and grow structurally and magnetically ordered mineral phases as ET products upon reduction by OmcA.

4.3 Biogeochemical Implications

OmcA is an important multi-heme c-type cytochrome in the electron transfer network for direct enzymatic reduction of oxide surfaces by DMRB such as *S. oneidensis*. Its ability to specifically absorb on metal oxide mineral surfaces and its special terminal reductase role in *S. oneidensis* MR-1 make this study highly relevant to whole-cell studies. Technically, further investigation on the role of endogenous electron shuttles and other redox-active cofactors along the extracellular electron transport chain in *S. oneidensis* MR-1 is needed to correlate the results of this study with the findings of previous *in vivo* experiments. Nevertheless, our results are in general agreement with, and explain, previous particle-size dependence findings observed in whole-cell experiments of *S. oneidensis* MR-1 with hematite, suggesting an important mechanistic link based on the requirement for close interaction between outer-membrane cytochromes and metal oxides to facilitate interfacial ET. Our findings reaffirm the importance of systematically investigating the effect of mineral properties on microbial redox reactions. Moreover, it is helpful to determine the overall role of nanoparticles in microbial redox processes and to understand the fate of nanoparticles in the environment.

The difference observed between reactions with ascorbic acid and OmcA imply that the influence of particle size might be quite different in abiotic and biotic reactions more generally. Our findings suggest that the influence of aggregation on reactivity of nanoparticles would generally be more substantial in biotic reactions that are mediated by larger and physically more complex biomolecules. Aggregation is inevitable for most nanoparticles in the environment under most aqueous conditions. However, the impact of aggregation on reactivity of nanoparticles in environmentally relevant systems is still difficult to be quantitatively described. BET surface area should not be used to estimate the reactive surface area in aggregated particle suspensions, when the size of the reactant molecules, such as OmcA, is much bigger than the gas molecules used for BET measurements. To study microbial reactivity of nanoparticles with bacteria or macromolecules, new methods are needed to realistically quantify the amount of surface area accessible for sorption and heterogeneous reaction (Cwierny et al., 2008). Furthermore, the formation of incipient Fe(II)-bearing mineral phases on the surface of Fe(III)-oxides at

neutral pH values as a result of microbial activity has important implications for understanding basic mechanistic processes in the biogeochemical cycling of iron in the environment.

ACKNOWLEDGEMENTS

This material is primarily based upon work supported by the PNNL Science Focus Area project funded by the U.S. Department of Energy (DOE) Office of Biological and Environmental Research (OBER), Subsurface Biogeochemical Research program. Nanoparticle characterization and expertise provided by KMR was through the Geosciences program at PNNL funded by the DOE Office of Basic Energy Sciences, Chemical Sciences, Geosciences, and Biosciences Division. JL acknowledges support from the National Natural Science Foundation of China (No. 41472306). A portion of the experiments were performed at the Environmental Molecular Sciences Laboratory (EMSL), a national user facility supported by the DOE OBER and located at PNNL. XA and XMCD measurements were performed at BL4.0.2 at the Advanced Light Source supported by the DOE Office of Science, Office of Basic Energy Sciences under contract No. DE-AC02-05CH11231. We gratefully acknowledge helpful suggestions from Prof. Michael F. Hochella, Jr. and Dr. Harish Veeramani.

518 REFERENCES

- 519 Adamson, A.W., Gast, A.P., 1997. Physical Chemistry of Surfaces, 6th ed. Wiley-Interscience, New York.
- 520 Arenholz, E., Prestemon, S.O., 2005. Design and performance of an eight-pole resistive magnet for soft
521 x-ray magnetic dichroism measurements. *Rev. Sci. Instrum.* 76, 8.
- 522 Bonneville, S., Behrends, T., Van Cappellen, P., 2009. Solubility and dissimilatory reduction kinetics of
523 iron(III) oxyhydroxides: A linear free energy relationship. *Geochimica Et Cosmochimica Acta* 73,
524 5273-5282.
- 525 Bosch, J., Heister, K., Hofmann, T., Meckenstock, R.U., 2010. Nanosized Iron Oxide Colloids Strongly
526 Enhance Microbial Iron Reduction. *Applied and Environmental Microbiology* 76, 184-189.
- 527 Bose, S., Hochella, M.F., Gorby, Y.A., Kennedy, D.W., McCready, D.E., Madden, A.S., Lower, B.H., 2009.
528 Bioreduction of hematite nanoparticles by the dissimilatory iron reducing bacterium *Shewanella*
529 *oneidensis* MR-1. *Geochim Cosmochim Ac* 73, 962-976.
- 530 Breuer, M., Rosso, K.M., Blumberger, J., Butt, J.N., 2015. Multi-haem cytochromes in *Shewanella*
531 *oneidensis* MR-1: structures, functions and opportunities. *J. R. Soc. Interface* 12, 27.
- 532 Cao, B., Shi, L.A., Brown, R.N., Xiong, Y.J., Fredrickson, J.K., Romine, M.F., Marshall, M.J., Lipton, M.S.,
533 Beyenal, H., 2011. Extracellular polymeric substances from *Shewanella* sp HRCR-1 biofilms:
534 characterization by infrared spectroscopy and proteomics. *Environ Microbiol* 13, 1018-1031.
- 535 Chowdhury, I., Walker, S.L., Mylon, S.E., 2013. Aggregate morphology of nano-TiO₂: role of primary
536 particle size, solution chemistry, and organic matter. *Environ Sci-Proc Imp* 15, 275-282.
- 537 Clarke, T.A., Edwards, M.J., Gates, A.J., Hall, A., White, G.F., Bradley, J., Reardon, C.L., Shi, L., Beliaev, A.S.,
538 Marshall, M.J., Wang, Z.M., Watmough, N.J., Fredrickson, J.K., Zachara, J.M., Butt, J.N., Richardson,
539 D.J., 2011. Structure of a bacterial cell surface decaheme electron conduit. *P Natl Acad Sci USA* 108,
540 9384-9389.
- 541 Cwiertny, D.M., Handler, R.M., Schaefer, M.V., Grassian, V.H., Scherer, M.M., 2008. Interpreting
542 nanoscale size-effects in aggregated Fe-oxide suspensions: reaction of Fe(II) with goethite.
543 *Geochimica Et Cosmochimica Acta* 72, 1365-1380.
- 544 Echigo, T., Aruguete, D.M., Murayama, M., Hochella, M.F., 2012. Influence of size, morphology, surface
545 structure, and aggregation state on reductive dissolution of hematite nanoparticles with ascorbic
546 acid. *Geochimica Et Cosmochimica Acta* 90, 149-162.
- 547 Edwards, M.J., Baiden, N.A., Johs, A., Tomanicek, S.J., Liang, L.Y., Shi, L., Fredrickson, J.K., Zachara, J.M.,
548 Gates, A.J., Butt, J.N., Richardson, D.J., Clarke, T.A., 2014. The X-ray crystal structure of *Shewanella*
549 *oneidensis* OmcA reveals new insight at the microbe-mineral interface. *Febs Lett* 588, 1886-1890.
- 550 Eggleston, C.M., Voros, J., Shi, L., Lower, B.H., Droubay, T.C., Colberg, P.J.S., 2008. Binding and direct
551 electrochemistry of OmcA, an outer-membrane cytochrome from an iron reducing bacterium, with
552 oxide electrodes: A candidate biofuel cell system. *Inorg Chim Acta* 361, 769-777.
- 553 Ekstrom, E.B., Learman, D.R., Madden, A.S., Hansel, C.M., 2010. Contrasting effects of Al substitution on
554 microbial reduction of Fe(III) (hydr)oxides. *Geochimica Et Cosmochimica Acta* 74, 7086-7099.
- 555 Fredrickson, J.K., Romine, M.F., Beliaev, A.S., Auchtung, J.M., Driscoll, M.E., Gardner, T.S., Nealson, K.H.,
556 Osterman, A.L., Pinchuk, G., Reed, J.L., Rodionov, D.A., Rodrigues, J.L.M., Saffarini, D.A., Serres,
557 M.H., Spormann, A.M., Zhulin, I.B., Tiedje, J.M., 2008. Towards environmental systems biology of
558 *Shewanella*. *Nat Rev Microbiol* 6, 592-603.
- 559 Gilbert, B., Lu, G.P., Kim, C.S., 2007. Stable cluster formation in aqueous suspensions of iron
560 oxyhydroxide nanoparticles. *J Colloid Interf Sci* 313, 152-159.
- 561 Hansel, C.M., Benner, S.G., Neiss, J., Dohnalkova, A., Kukkadapu, R.K., Fendorf, S., 2003. Secondary
562 mineralization pathways induced by dissimilatory iron reduction of ferrihydrite under advective
563 flow. *Geochim Cosmochim Ac* 67, 2977-2992.

564 He, Y.T., Wan, J.M., Tokunaga, T., 2008. Kinetic stability of hematite nanoparticles: the effect of particle
 565 sizes. *J Nanopart Res* 10, 321-332.
 566 Hochella, M.F.J., Lower, S.K., Maurice, P.A., Penn, R.L., Sahai, N., Sparks, D.L., Twining, B.S., 2008.
 567 Nanominerals, mineral nanoparticles, and Earth systems. *Science* 319, 1631-1635.
 568 Islam, F.S., Gault, A.G., Boothman, C., Polya, D.A., Charnock, J.M., Chatterjee, D., Lloyd, J.R., 2004. Role
 569 of metal-reducing bacteria in arsenic release from Bengal delta sediments. *Nature* 430, 68-71.
 570 Johs, A., Shi, L., Droubay, T., Ankner, J.F., Liang, L., 2010. Characterization of the Decaheme c-Type
 571 Cytochrome OmcA in Solution and on Hematite Surfaces by Small Angle X-Ray Scattering and
 572 Neutron Reflectometry. *Biophys J* 98, 3035-3043.
 573 Kerisit, S., Rosso, K.M., Dupuis, M., Valiev, M., 2007. Molecular computational investigation of electron-
 574 transfer kinetics across cytochrome-iron oxide interfaces. *Journal of Physical Chemistry C* 111,
 575 11363-11375.
 576 Lanzl, C.A., Baltrusaitis, J., Cwiertny, D.M., 2012. Dissolution of Hematite Nanoparticle Aggregates:
 577 Influence of Primary Particle Size, Dissolution Mechanism, and Solution pH. *Langmuir* 28, 15797-
 578 15808.
 579 Liu, J., Aruguete, D.A., Jinschek, J.R., Rimstidt, J.D., Hochella, M.F., 2008. The non-oxidative dissolution of
 580 galena nanocrystals: Insights into mineral dissolution rates as a function of grain size, shape, and
 581 aggregation state. *Geochim Cosmochim Acta* 72, 5984-5996.
 582 Liu, J., Aruguete, D.M., Murayama, M., Hochella, M.F., 2009. Influence of Size and Aggregation on the
 583 Reactivity of an Environmentally and Industrially Relevant Nanomaterial (PbS). *Environ Sci Technol*
 584 43, 8178-8183.
 585 Liu, J., Pearce, C.I., Liu, C.X., Wang, Z.M., Shi, L., Arenholz, E., Rosso, K.M., 2013. Fe₃-xTi_xO₄
 586 Nanoparticles as Tunable Probes of Microbial Metal Oxidation. *J. Am. Chem. Soc.* 135, 8896-8907.
 587 Liu, J., Wang, Z., Belchik, S.M., Edwards, M.J., Liu, C., Kennedy, D.W., Merkley, E.D., Lipton, M.S., Butt,
 588 J.N., Richardson, D.J., Zachara, J.M., Fredrickson, J.K., Rosso, K.M., Shi, L., 2012a. Identification and
 589 characterization of MtoA: a decaheme c-type cytochrome of the neutrophilic Fe(II)-oxidizing
 590 bacterium *Sideroxydans lithotrophicus* ES-1. *Frontiers in Microbiology* 3.
 591 Liu, J., Wang, Z., Sheng, A., Liu, F., Qin, F., Wang, Z., 2016. In Situ Observation of Hematite Nanoparticle
 592 Aggregates Using Liquid Cell Transmission Electron Microscopy. *Environ Sci Technol* 50, 5606-5613.
 593 Liu, J., Wang, Z.M., Belchik, S.M., Edwards, M.J., Liu, C.X., Kennedy, D.W., Merkley, E.D., Lipton, M.S.,
 594 Butt, J.N., Richardson, D.J., Zachara, J.M., Fredrickson, J.K., Rosso, K.M., Shi, L., 2012b. Identification
 595 and characterization of MtoA: a decaheme c-type cytochrome of the neutrophilic Fe(II)-oxidizing
 596 bacterium *Sideroxydans lithotrophicus* ES-1. *Front. Microbiol.* 3, 37.
 597 Lovley, D.R., Coates, J.D., BluntHarris, E.L., Phillips, E.J.P., Woodward, J.C., 1996. Humic substances as
 598 electron acceptors for microbial respiration. *Nature* 382, 445-448.
 599 Lower, B.H., Lins, R.D., Oestreicher, Z., Straatsma, T.P., Hochella, M.F., Jr., Shi, L., Lower, S.K., 2008. In
 600 vitro evolution of a peptide with a hematite binding motif that may constitute a natural metal-oxide
 601 binding archetype. *Environmental Science & Technology* 42, 3821-3827.
 602 Lu, J.F., Tsai, C.J., 2015. Reduction kinetics of hematite to magnetite under hydrothermal treatments.
 603 *Rsc Adv* 5, 17236-17244.
 604 Madden, A.S., Hochella, M.F., 2005. A test of geochemical reactivity as a function of mineral size:
 605 Manganese oxidation promoted by hematite nanoparticles. *Geochimica Et Cosmochimica Acta* 69,
 606 389-398.
 607 Navrotsky, A., Mazeina, L., Majzlan, J., 2008. Size-driven structural and thermodynamic complexity in
 608 iron oxides. *Science* 319, 1635-1638.

- Neal, A.L., Rosso, K.M., Geesey, G.G., Gorby, Y.A., Little, B.J., 2003. Surface structure effects on direct reduction of iron oxides by *Shewanella oneidensis*. *Geochimica Et Cosmochimica Acta* 67, 4489-4503.
- Patrick, R.A.D., Van der Laan, G., Henderson, C.M.B., Kuiper, P., Dudzik, E., Vaughan, D.J., 2002. Cation site occupancy in spinel ferrites studied by X-ray magnetic circular dichroism: developing a method for mineralogists. *Eur J Mineral* 14, 1095-1102.
- Pearce, C.I., Henderson, C.M.B., Patrick, R.A.D., Van der Laan, G., Vaughan, D.J., 2006. Direct determination of cation site occupancies in natural ferrite spinels by L-2,L-3 X-ray absorption spectroscopy and X-ray magnetic circular dichroism. *Am Mineral* 91, 880-893.
- Pearce, C.I., Henderson, C.M.B., Telling, N.D., Patrick, R.A.D., Charnock, J.M., Coker, V.S., Arenholz, E., Tuna, F., van der Laan, G., 2010. Fe site occupancy in magnetite-ulvospinel solid solutions: A new approach using X-ray magnetic circular dichroism. *Am Mineral* 95, 425-439.
- Pearce, C.I., Qafoku, O., Liu, J., Arenholz, E., Heald, S.M., Kukkadapu, R.K., Gorski, C.A., Henderson, C.M.B., Rosso, K.M., 2012. Synthesis and properties of titanomagnetite ($\text{Fe}_{3-x}\text{Ti}_x\text{O}_4$) nanoparticles: A tunable solid-state Fe(II/III) redox system. *J Colloid Interf Sci* 387, 24-38.
- Revuelta, M.V., van Raap, M.B.F., Zelis, P.M., Sanchez, F.H., Castro, G.R., 2011. Ascorbic Acid Encapsulation in Hydrophobic Silica Xerogel. *Food Technol Biotech* 49, 347-351.
- Roden, E.E., 2003. Fe(III) oxide reactivity toward biological versus chemical reduction. *Environ Sci Technol* 37, 1319-1324.
- Roden, E.E., Zachara, J.M., 1996. Microbial reduction of crystalline iron(III) oxides: Influence of oxide surface area and potential for cell growth. *Environmental Science & Technology* 30, 1618-1628.
- Ross, D.E., Brantley, S.L., Tien, M., 2009. Kinetic Characterization of OmcA and MtrC, Terminal Reductases Involved in Respiratory Electron Transfer for Dissimilatory Iron Reduction in *Shewanella oneidensis* MR-1. *Applied and Environmental Microbiology* 75, 5218-5226.
- Schwertmann, U., Cornell, R.M., 2000. *Iron Oxides in the Laboratory*. Wiley-VCH, Weinheim.
- Shi, L., Chen, B.W., Wang, Z.M., Elias, D.A., Mayer, M.U., Gorby, Y.A., Ni, S., Lower, B.H., Kennedy, D.W., Wunschel, D.S., Mottaz, H.M., Marshall, M.J., Hill, E.A., Beliaev, A.S., Zachara, J.M., Fredrickson, J.K., Squier, T.C., 2006. Isolation of a high-affinity functional protein complex between OmcA and MtrC: Two outer membrane decaheme c-type cytochromes of *Shewanella oneidensis* MR-1. *J Bacteriol* 188, 4705-4714.
- Shi, L., Rosso, K.M., Clarke, T.A., Richardson, D.J., Zachara, J.M., Fredrickson, J.K., 2012a. Molecular Underpinnings of Fe(III) Oxide Reduction by *Shewanella Oneidensis* MR-1. *Frontiers in microbiology* 3, 50.
- Shi, L.A., Richardson, D.J., Wang, Z.M., Kerisit, S.N., Rosso, K.M., Zachara, J.M., Fredrickson, J.K., 2009. The roles of outer membrane cytochromes of *Shewanella* and *Geobacter* in extracellular electron transfer. *Env Microbiol Rep* 1, 220-227.
- Shi, Z., Zachara, J.M., Shi, L., Wang, Z.M., Moore, D.A., Kennedy, D.W., Fredrickson, J.K., 2012b. Redox Reactions of Reduced Flavin Mononucleotide (FMN), Riboflavin (RBF), and Anthraquinone-2,6-disulfonate (AQDS) with Ferrihydrite and Lepidocrocite. *Environmental Science & Technology* 46, 11644-11652.
- Stookey, L.L., 1970. Ferrozine - a New Spectrophotometric Reagent for Iron. *Analytical Chemistry* 42, 779.
- Suter, D., Banwart, S., Stumm, W., 1991. Dissolution of Hydrous Iron(III) Oxides by Reductive Mechanisms. *Langmuir* 7, 809-813.
- Wang, Z.M., Liu, C.X., Wang, X.L., Marshall, M.J., Zachara, J.M., Rosso, K.M., Dupuis, M., Fredrickson, J.K., Heald, S., Shi, L., 2008. Kinetics of Reduction of Fe(III) Complexes by Outer Membrane Cytochromes MtrC and OmcA of *Shewanella oneidensis* MR-1. *Appl. Environ. Microbiol.* 74, 6746-6755.

655 Wigginton, N.S., Rosso, K.M., Stack, A.G., Hochella, M.F., Jr., 2009. Long-Range Electron Transfer across
 656 Cytochrome-Hematite (α -Fe₂O₃) Interfaces. *Journal of Physical Chemistry C* 113, 2096-2103.
 657 Xiong, Y.J., Shi, L., Chen, B.W., Mayer, M.U., Lower, B.H., Londer, Y., Bose, S., Hochella, M.F., Fredrickson,
 658 J.K., Squier, T.C., 2006. High-affinity binding and direct electron transfer to solid metals by the
 659 *Shewanella oneidensis* MR-1 outer membrane c-type cytochrome OmcA. *J Am Chem Soc* 128,
 660 13978-13979.
 661 Yan, B., Wrenn, B.A., Basak, S., Biswas, P., Giammar, D.E., 2008. Microbial reduction of Fe(III) in hematite
 662 nanoparticles by *Geobacter sulfurreducens*. *Environmental Science & Technology* 42, 6526-6531.
 663 Yang, Q.H., Hou, P.X., Bai, S., Wang, M.Z., Cheng, H.M., 2001. Adsorption and capillarity of nitrogen in
 664 aggregated multi-walled carbon nanotubes. *Chem Phys Lett* 345, 18-24.
 665 Zhang, Y., Matijevic, E., 1984. Stability of Monodispersed Hematite Sols in the Presence of Citric-Acid.
 666 *Colloid Polym Sci* 262, 723-726.

667

668 **Table 1. Hematite Nanoparticles Investigated and Their Reduction Rates**

Particle size (nm)	BET SSA (m^2/g)	SSA normalized rates in reaction with ascorbic acid ($10^{-7} \text{ M hr}^{-1} \text{ m}^{-2}$)	SSA normalized rates in reactions with OmcA ($10^{-4} \text{ M hr}^{-1} \text{ m}^{-2}$)
15 ± 4	59.6	1.928 ± 0.364	2.252 ± 0.313
30 ± 6	39.1	0.994 ± 0.294	1.280 ± 0.862
55 ± 7	34.0	0.919 ± 0.327	0.580 ± 0.115
173 ± 63	3.5	0.153 ± 0.134	14.970 ± 4.013

669 Error bars represent one standard deviation of mean from three replicates.

670

671

672 Figure 1. Absorption spectra of OmcA (2 μ M) in 50 mM NaCl and 100 mM HEPES buffer (pH = 7) in
 673 the oxidized (black line) and the reduced form (red line). The appearance of characteristic peak at 552 nm
 674 indicates the reduction of OmcA.

Figure 2. μ -XRD patterns of synthetic hematite (A) 15, 30, 55, and 173nm hematite particles before reactions; (B) patterns of 15 nm hematite nanoparticles before reaction (black, bottom), after reaction with OmcA (red, middle), and after reaction with ascorbic acid (blue, top); The patterns were matched with the reference pattern of hematite (PDF No. 33-0664), shown at the bottom (C). No secondary phase was observed.

Figure 3. TEM images of 15 nm (A), 30 nm (B), 55 nm (C) and 173 nm (D) hematite particles.

Figure 4. Reduction of hematite particles (0.4 g L^{-1}) with ascorbic acid (0.5 mM) at pH 4 in absence of oxygen and light: (A) Concentrations of Fe(II) produced from 15, 30, 55, and 173 nm hematite particles vs. time; (B) BET surface area normalized initial rates of hematite particles reported in this study (black), by Echigo (2012) (red, the original rates measured at pH 3.35 were converted to the presented data at pH 4 using the equation (Lanzl et al., 2012): $r = k [\text{H}^+]^n$, $n = 1.0$), and by Suter (1991) (blue). Error bars represent one standard deviation of mean from three replicates. The reduction rate of 30 nm in this study was very similar to that reported by Echigo (2012), leading to the overlap of the two spots in the figure.

695

696 Figure 5. The hydrodynamic diameter distribution of 173 nm (A), 30 nm (B), and 15 nm (C) hematite
 697 particles in 100 mM acetate buffer (pH = 4, 10 mM NaNO₃). The concentration of hematite particles was
 698 0.1 mM for all experiments. The hydrodynamic diameters of 15 nm, 30 nm, and 173 nm hematite
 699 particles were 1096.0 ± 132.7 nm, 812.8 ± 93.3 nm, and 776.6 ± 679.0 nm (standard deviation),
 700 respectively.

Figure 6. Change of absorbance at 552 nm vs time in the control experiments: fully reduced OmcA without hematite in 20 mM HEPES buffer solution (magenta, top); purified OmcA in oxidized form with 15 nm (black), 30 nm (red), 55 nm (blue), and 173 nm (green) hematite particles. No obvious change in the absorbance at 552 nm was observed over time in this study for all hematite particles with oxidized OmcA.

Figure 7. Size-dependent reduction of hematite particles with OmcA. (A) variation of the concentration of reduced OmcA as a function of time in the reduction experiments with 15 nm (black), 30 nm (red), 55 nm (blue), and 173 nm (purple) hematite particles. (B) BET SA-normalized initial rates as a function of primary particle size. Error bars represent one standard deviation of mean from three replicates.

714

715 Figure 8. The hydrodynamic diameter distribution of 173 nm (A), 30 nm (B), and 15 nm (C) hematite
716 particles in 20 mM HEPES buffer (pH 7, 150mM NaCl). The concentration of hematite particles was 0.01
717 mM for all experiments. The hydrodynamic diameters of 15 nm, 30 nm, and 173 nm hematite particles
718 were 406.1 ± 61.0 nm, 280.3 ± 52.3 nm, and 309.5 ± 46.0 nm (standard deviation), respectively.

Figure 9. Electron balance experiments: The percentage of reduced OmcA was oxidized at the end of one day experiment (above). OmcA was not totally oxidized, even there is excess Fe(III) in hematite compared to initial concentration of reduced OmcA ($[OmcA_{red}]$); The ratio of produced $[Fe(II)]$ to the decrease in $[OmcA_{red}]$ at the end of one day experiment (below). In reactions of hematite with different sizes, the ratios are all close to 10. Error bars represent one standard deviation of mean from three replicates.

Figure 10. XAS (top) and XMCD spectra (bottom) for (A, C) 15 nm and (B, D) 30 nm hematite particles before and after reaction with ascorbic acid (AA) and OmcA.