Interfacial and Long-Range Electron Transfer at the Mineral-Microbe Interface

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Abstract

The electron transfer mechanisms of multiheme cytochromes were examined with scanning tunneling microscopy (STM). To simulate bacterial metal reduction mediated by proteins in direct contact with mineral surfaces, monolayers of purified decaheme cytochromes from the metal-reducing bacterium *Shewanella oneidensis* were prepared on Au(111) surfaces. Recombinant tetracysteine sequences were added to two outer-membrane decaheme cytochromes (OmcA and MtrC) from *S. oneidensis* MR-1 to ensure chemical immobilization on Au(111). STM images of the cytochrome monolayers showed good coverage and their shapes/sizes matched that predicted by their respective molecular masses. Current-voltage ($I-V$) tunneling spectroscopy revealed that OmcA and MtrC exhibit characteristic tunneling spectra. Theoretical modeling of the single-molecule tunneling spectra revealed a distinct tunneling mechanism for each cytochrome: OmcA mediates tunneling current coherently whereas MtrC temporarily traps electrons via orbital-mediated tunneling. These mechanisms suggest a superexchange electron transfer mechanism for OmcA and a redox-specific (i.e. heme-mediated) electron transfer mechanism for MtrC at mineral surfaces during bacterial metal reduction.

Additionally, a novel electrochemical STM configuration was designed to measure tunneling current from multiheme cytochromes to hematite (001) surfaces in various electrolyte solutions. Current-distance ($I-s$) profiles on hematite (001) reveal predictable electric double layer structure that changes with ionic strength. The addition of the small tetraheme cytochrome *c* (STC) from *S. oneidensis* on insulated Au tips resulted in modified tunneling profiles that suggest STC significantly modulates the double layer. This observation is relevant to understanding metal reduction in cases where terminal metal-reducing enzymes are unable to come in direct contact with reducible mineral surfaces. Electronic coupling to the mineral surface might therefore be mediated by a localized ion swarm specific to the mineral surface.
Acknowledgements

"We are each so atomically numerous and so vigorously recycled at death that a significant number of our atoms—up to a billion for each of us, it has been suggested—probably once belonged to Shakespeare. A billion more came from Buddha and Genghis Kahn and Beethoven, and any other historical figure you care to name." —Bill Bryson, *A Short History of Nearly Everything*

This quote, among several memorable ones, stuck with me the most after reading the book from which it came during my second year at Virginia Tech. To me, it is a brilliantly illustrated example of how we are all interconnected; not only as humans, but as occupants of this amazing planet we call Earth. But the quote could be extended a bit—not only are we atomically connected to every historical figure in human history, but also to every microbe, every tree, and actually a good majority of the geologic material as well. Hence, I believe the study of our natural world is an extension of finding out who we are as people. As verification of this interconnectivity, I would not have been able to finish my degree—studying a small part of nature—without the generous help of so many people.

I’d like to start off my acknowledging all of the support and wisdom that my advisor Mike Hochella has offered me over past five years. It would take far too many pages to describe how much he has done for me, so instead I’ll borrow a typical phrase he would use and turn it around on him: Mike, you are the best! You have far surpassed all of my expectations as to of what an advisor is capable. I hope in some way I can come close to teaching, engaging, and communicating science the way that you do so well. Thank you for all that you have taught me.

The rest of my committee, Randy Heflin, Kevin Rosso, and Brian Lower, have been equally involved in my development as a scientist and as a person. The last two, in particular, essentially took me on as their own student while out at PNNL in Richland, WA. I very much admire their dedication and enthusiasm towards science, but more than that, I consider both of them dear friends. I will fondly remember the cumulative year I spent at PNNL away from Blacksburg because of the two of you.

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Dedication

For two students, Matthew Gwaltney and Juan Ortiz, who were killed on April 16, 2007 along with 30 other students and faculty. Although I only knew them from a few weekend basketball games we played together during the weeks preceding their deaths, they will remain in my memory forever. Matt and Juan were pursuing their Master’s degrees in Civil and Environmental Engineering and were close to finishing. I dedicate the completion of my degree in their honor just over one year later.
Attributions

Several colleagues and coworkers aided in the writing and research behind several of the chapters of this dissertation. A brief description of their background and their contributions are included here.

Prof. Michael F. Hochella (Department of Geosciences, Virginia Tech) is the primary research advisor and Committee Chair. He provided general scientific guidance and support with the writing of all Chapters. Furthermore, Prof. Hochella provided the majority of financial support through several research grants.

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Dr. Brian Lower (Chemical Sciences Division, Pacific Northwest National Laboratory) is a committee member and provided assistance with cytochrome purification and experimental design. He also helped write Chapter 2.

Dr. Liang Shi (Biological Sciences Division, Pacific Northwest National Laboratory) supplied all of the cytochrome material for all of the experimental work throughout the dissertation. He also helped write Chapter 2.

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Kelly L. Haus (Department of Geosciences, Virginia Tech) is a PhD candidate in the Hochella research group and helped with the writing of Appendix D.
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1) Introduction

The mineral-microbe interface is one of the most dynamic interfaces in nature. From the millions of bacterial species on Earth—the vast majority of which we haven’t cultured yet—to the just over 4,500 known mineral species, the sheer abundance of the two components is enough to suggest that they’re bound to interact. But it’s not merely a coincidence that microorganisms interact with minerals; nature has selected for it. Some bacteria evolved to ‘eat’ minerals and, conversely, some minerals would not exist without the actions of bacteria. Moreover, the nature of the interactions is in a constant state of flux. Bacteria in particular are very responsive to slight changes in environmental conditions. For example, some bacteria prefer to ‘breathe’ oxygen if it’s available, but can switch to a multitude of other chemical compounds in anoxic environments, including minerals. The same can also be said about the minerals: depending on a number of environmental factors (e.g. water chemistry, presence of certain gases, even the size of the mineral), a mineral’s reactivity can change remarkably. So not only is there an incredible diversity in the abundance of microbe and mineral species on the planet, but they are constantly responding to the environment. In effect, this is overwhelming evidence to suggest that the permutations of this interface are essentially infinite.

But why do microbes need to even interact with minerals? And why is this important for deepening our understanding of how nature works? Furthermore, if this interface is ‘infinite,’ how do we even approach studying something so complex? The answers, as we shall see, are both elegantly simple and excruciatingly complex.

Microorganisms comprise the majority of Earth’s biomass and exist everywhere—from the deepest ocean to high in the atmosphere (Whitman et al., 1998). They play important roles in many Earth processes including controlling climate and shaping the landforms that we see. In nearly every niche in which they inhabit, they are interacting with minerals. In these ecological niches, microorganisms and their metabolic byproducts can induce mineral growth or dissolution, sometimes even simultaneously. My dissertation focuses on understanding how one particular bacterial species of metal-reducing bacteria, *Shewanella oneidensis*, respires on certain minerals (e.g. Fe- and Mn-oxides). My approach combines elements of biochemistry, physics, materials science,
Bacterial metal reduction

Dissimilatory metal reduction is a process by which certain species of bacteria couple the oxidation of an energy source (e.g. formate, lactate, H₂) with the reduction of redox-active polyvalent metals to generate energy. Metals, in general, present a unique respiratory challenge for bacteria because the phase of the metal is variable depending on environmental conditions. For example, Fe(III) exists predominately as a dissolved aqueous species at low pH, but readily precipitates as a solid-phase mineral in circumneutral pH waters. Moreover, metal speciation is dramatically dependent upon its redox-state. Bacterial metal reduction is one of the most common biogeochemical reactions in nature because it occurs in soils, sediments, surface waters, and oceans (LOVLEY et al., 2004). In fact, it is one of the first metabolisms thought to have evolved in bacteria several billion years ago, resulting in significant sedimentary deposits throughout geologic time (NEALSON and MYERS, 1990; VARGAS et al., 1998).

*S. oneidensis* has proven to be an ideal organism to study in hopes of understanding the mechanisms of bacterial metal reduction because it can utilize abundant metals in the environment, such as Fe(III) and Mn(IV), as well as a wide range of anthropogenic contaminants including Cr(VI), Se(VI), U(VI), Tc(VII), V(V), and As(V). Aided by its recent genome sequencing, researchers have recently learned a great deal about the biochemical metal-reduction pathways in *S. oneidensis*. For instance, reduction is thought to be mediated by the transfer of electrons from a number of c-type cytochromes that are constituents of the electron transport pathway (DiCHRISTINA et al., 2005; SHI et al., 2007). Cytochromes are redox-active proteins that contain prosthetic heme cofactors bound within an organic protein matrix. Cytochromes function as electron carriers by taking advantage of a reversible change in the oxidation state of covalently bound Fe(II/III) within the heme center. *S. oneidensis* has as many as 14 multiheme (≥4 hemes) cytochromes, many encoded by genes in a single operon, which are thought to have specific cellular roles during metal reduction.
Electron transfer from a heme group within a cytochrome to reducible Fe(III) sites on the surface of a Fe(III)-oxide depends on many factors. The first-order rate constant ($k_{ET}$) of a single electron transfer step is strongly dependent on the reaction driving force ($-\Delta G^{\circ}$) and the reorganization energy ($\lambda$) (MARCUS and SUTIN, 1985). Factors that control these quantities include heme redox potentials, heme abundance, and proper conformation and folding (GRAY and WINKLER, 2003). When cytochromes are presumed to reduce Fe(III)-oxides, direct contact with the mineral surface is thought to increase the electronic coupling between the cytochrome’s heme cofactor(s) and Fe(III) sites at the mineral surface, thus increasing $k_{ET}$ and the efficiency of bacterial respiration. Recent examples demonstrating how certain cytochromes bind more tightly to mineral surfaces than other similar cytochromes (LOWER et al., 2007) illustrate the intimate link between mineral contact and bacterial metal reduction. Despite a great deal of interest (and funding) directed towards research on this organism, many knowledge gaps still remain in terms of describing the mechanisms of how *S. oneidensis* is able to reduce mineral surfaces. For example, which cytochromes can serve as a terminal metal-reducing enzyme? Are certain cytochromes designed to reduce specific metals/minerals? What happens if these cytochromes are not able to come in direct contact with the mineral surface?

My dissertation focuses on understanding at a molecular level how a few target cytochromes from *S. oneidensis* transfer electrons when in contact (or near-contact) with a solid surface or mineral. As stated earlier, the complexity of this interface requires novel approaches for understanding the many variables that control bacterial metal reduction. For my dissertation, I used an instrument not typically associated with mineral-microbe interactions: the scanning tunneling microscope (STM). Most work with cytochrome proteins is done in bulk systems using electrochemical techniques, but the STM allows for single cytochrome molecules to be studied with respect to its ability to transfer electrons.

**Scanning tunneling microscopy**

The basic design of STM includes an ultra-fine metallic tip wired into a circuit with a conductor (or semi-conductor) held at variable bias to maintain a constant
tunneling current through a feedback loop (Binnig and Rohrer, 1982). Tunneling current results when the (semi)conductive sample and tip are very close—often within a few angstroms of each other—until the electron wavefunctions in the tip overlap the electronic wavefunctions in the sample. If a bias voltage is applied across the junction, an electrical tunneling current is induced and electrons flow from tip to sample, or vice-versa, depending on the bias direction. In contrast to electron hopping, tunneling allows the passage of electrons through energy barriers that would otherwise not be allowed. Tunneling is directly proportional to tip-sample distance; by maintaining a constant current, the tip can essentially trace the topography of the surface in the $z$ direction. In essence, STM operates based on the ability of the sample, molecular adsorbate, or tip to conduct electrons (i.e. electronic structure) (Tersoff and Hamann, 1985). The realization that this electronic structure could be probed using STM lead to the development of tunneling spectroscopy (TS), which is the primary technique I used to study multiheme cytochromes from $S$. oneidensis. Interpretation of TS data is not inherently straight-forward, but an extensive theoretical framework developed over the years allows for the relation of single-molecule tunneling current measurements to more conventional electron transfer quantities.

**Dissertation outline**

The three primary chapters of my dissertation describe my research efforts using STM/TS to understand electron transfer between multiheme cytochromes from $S$. oneidensis and mineral surfaces. I studied primarily three proteins that have various roles in bacterial metal reduction. Two of the proteins I studied were the outer-membrane decaheme cytochromes OmcA and MtrC. These proteins putatively serve as terminal metal-reducing enzymes on the exterior of the cell and are therefore the most likely candidates for a terminal metal-reducing enzyme. Unfortunately, neither OmcA nor MtrC have a known protein crystal structure, which are indispensable in terms of the information they contain regarding both structure and inferred chemistry. For example, the crystal structure for the small periplasmic tetraheme cytochrome (STC) from $S$. oneidensis has revealed complex electron transfer pathways not seen before (Harada et
al., 2002; LEYS et al., 2002). For that reason, I also studied STC in hopes of linking experimental data to molecular modeling.

Chapter 2 describes our first efforts in characterizing OmcA and MtrC with STM. We demonstrate an efficient way to chemically attach these cytochromes to Au(111)—a proxy for a mineral surface—and describe a model for how molecular monolayers of the cytochromes are formed on the metal surface. Moreover, we show that using TS, the cytochromes appear to have very specific electronic structures when adsorbed to Au(111). It was published in Geochimica et Cosmochimica Acta in early 2007 and has already been cited multiple times.

Chapter 3 goes one step further by actually interpreting electron transfer mechanisms for OmcA and MtrC based on modeling of tunneling spectra. This is an effort to ultimately place tunneling spectra within an appropriate theoretical framework connecting tunneling measurements to electron transfer quantities. The results suggest that these two cytochromes have different roles for metal reduction when adsorbed to a solid surface. This chapter was published in Journal of Physical Chemistry B in late 2007.

Chapter 4 describes a slightly different approach to addressing a related question, namely, how do cytochromes reduce minerals if not in direct contact with the surface? This becomes important in a variety of situations in which the bacterium (or cytochrome) cannot electronically couple to reducible sites on a mineral surface (i.e. surface Fe(III) atoms on Fe-oxides). Using a novel experimental design with STC proteins attached to homemade Au STM tips, current-distance profiles were measured in various solutions at hematite (001). We were able to show that tunneling from STC to hematite in solution is controlled at least in part by the electrical double layer. This effect may allow for bacterial metal reduction to remain efficient despite few, if any, cytochromes in direct contact with hematite. This chapter is currently in preparation to be submitted to a special issue of Journal of Physical Chemistry C as an invited article.

During my time at Virginia Tech, I also participated in a number of collaborations that either did not contribute directly to my dissertation research, or were smaller experiments that as of yet do not have a place in a potential publication. These works are split into four appendices. Appendix A describes the results of synchrotron-based X-ray
reflectivity measurements on films of OmcA, MtrC, and STC in hopes of resolving the molecular structure of protein monolayers. Appendix B is a collection of STM images of molecular monolayers of the periplasmic decaheme cytochrome MtrA, mixed monolayers of MtrC and OmcA, and the observation of submolecular resolution on OmcA molecules. Appendix C presents STM/TS measurements on STC that will eventually be coupled with bulk protein electrochemistry measurements and molecular modeling simulations performed by researchers at Pacific Northwest National Laboratory as part of the Biogeochemistry Grand Challenge project. Appendix D is a review paper I wrote that ended up appearing on the cover of *Journal of Environmental Monitoring*. The manuscript, which is a bit outside my area of expertise, highlights recent advances in understanding environmental nanoparticles, from their origin to their transport, and the environmental processes that they impact.

REFERENCES


2) Electron tunneling properties of outer-membrane decaheme cytochromes from *Shewanella oneidensis*

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**ABSTRACT**

We have characterized the outer-membrane decaheme cytochromes OmcA and MtrC from *Shewanella oneidensis* MR-1 at the single molecule level using scanning tunneling microscopy (STM) and tunneling spectroscopy (TS). These cytochrome proteins are of great interest because they are thought to mediate bacterial electron transfer reactions in anoxic waters that control the reductive dissolution of oxide minerals. In our study, to characterize the electron transfer properties of these proteins on a model surface, the purified cytochromes were chemically immobilized as molecular monolayers on Au(111) substrates via a recombinant tetra-cysteine sequence as verified by X-ray photoelectron spectroscopy. Atomic force microscopy images confirm the monolayer films were ~5–8 nm thick which is consistent with the apparent lateral dimensions of individual cytochrome molecules obtained with STM. Current-voltage TS of single cytochrome molecules revealed that OmcA and MtrC have different abilities to mediate tunneling current despite having otherwise very similar molecular and biochemical properties. These observations suggest that, based on their electron tunneling properties, the two cytochromes could have specific roles during bacterial metal reduction. Additionally, this study establishes single-molecule STM/TS as an effective means for revealing insights into biogeochemical redox processes in the environment.
2.1 INTRODUCTION

Since life’s inception on the planet, microorganisms have been interacting with Earth materials and influencing global geochemical processes (see reviews by Nealson and Stahl, 1997; Newman and Banfield, 2002). In fact, dissimilatory metal-reducing bacteria (DMRB) are thought to be similar to some of the first organisms that evolved on Earth, and hence may represent the first true biogeochemical agents to have existed on our planet (Vargas et al., 1998). Geomicrobiological cycling of metals like iron, the most abundant redox-active metal in the Earth’s crust, and manganese by DMRB continues to be a prevalent redox process in modern environments and influences the fate and transport of not only these metals, but of organic and inorganic contaminants as well (Nealson et al., 2002; Lovley et al., 2004; Kappler and Straub, 2005). Given the low solubility, and hence solid-phase, of Fe(III) and Mn(IV) in natural waters, the molecular and nanoscale interactions between oxide surfaces and microorganisms (see reviews by Hochella, 2002; Gilbert and Banfield, 2005) become significant controls on this ubiquitous and dynamic redox couple.

One specific DMRB under widespread study is *Shewanella oneidensis* MR-1, a gram-negative facultative anaerobe that inhabits a wide variety of marine and surface waters as well as soils and sediments in the subsurface critical zone (Myers and Nealson, 1988; Venkateswaran et al., 1999). Recent studies have provided a glimpse into the remarkable electron transport chain of *S. oneidensis*, noting the importance of abundant multiheme cytochromes—particularly OmcA and MtrC—in bacterial iron and manganese respiration (see reviews by Tiedje, 2002; Schroder et al., 2003; Dichristina et al., 2005).

OmcA (SO1779) and MtrC (SO1778; also referred to as OmcB) are decaheme c-type cytochromes with calculated molecular weights of 83 and 79 kDa, respectively (Myers and Myers, 1997; Myers and Myers, 1998; Beliaev et al., 2001). Each contains lipoprotein consensus sequences for anchoring to the bacterial outer-membrane (Myers and Myers, 2004). It has been suggested that they have overlapping roles in Mn(IV)-oxide reduction implying that they could both act as terminal metal-reducing enzymes (Myers and Myers, 2003). Further support of this notion has been verified by the demonstration that purified OmcA and MtrC form a stable protein complex that has a higher reduction activity relative to either of the cytochromes alone (Shi et al., 2006).
Moreover, the midpoint potentials of OmcA and MtrC have been estimated with electrochemical redox titrations (Richardson et al., personal communication) and suggest that the complex may be tuned to a high redox potential. Despite having a broad biochemical and electrochemical knowledge of the function of these cytochromes, the molecular-scale properties controlling the electron transfer behavior of these cytochromes, particularly in an adsorbed state, remain poorly understood. Moreover, the mechanism(s) by which DMRB utilize these cytochromes to shuttle electrons to iron and manganese-bearing mineral surfaces remains elusive.

One hypothesis under investigation is that the terminal reduction pathway is mediated enzymatically by outer-membrane multiheme cytochromes (e.g. OmcA, MtrC) that are in direct contact with the oxide surface. Evidence supporting this hypothesis has been provided by studies of cell adhesion and direct electron transfer. For example, nanoscale adhesive forces between *S. oneidensis* and goethite (α-FeOOH) may be due to bonding interactions with outer-membrane multiheme cytochromes exposed at the cell surface (Lower et al., 2001; Lower et al., 2005). It has also been proposed that *S. oneidensis* has the ability to distinguish between polyvalent and unreactive metal-oxide surfaces (Lower et al., 2001) and even between different crystallographic faces on one iron-oxide mineral (Neal et al., 2005). Adhesion propensity corresponds quite well to the calculated electron transfer behavior and reactivity at these same faces (Neal et al., 2003), supporting the possibility that interfacial electron transfer and cytochrome-mediated adhesion may be directly related to metal reduction. Presumably this direct contact is necessary to increase the electronic coupling of the cell’s electron transfer moieties (e.g., redox-active heme cofactors within membrane-associated cytochromes) to the iron oxide surface allowing for efficient electron transfer (i.e., *tunneling*).

Alternatively, there appears to be substantial evidence supporting indirect reduction pathways that do not require direct contact between the cell and the oxide surface. In these cases, reduction is thought to be mediated either by small redox-active molecules acting as electron shuttles or by the production of extracellular pillin-like appendages recently-termed ‘nanowires.’ In the case of shuttles, it is possible that outer-membrane cytochromes donate electrons to biosynthetic shuttle molecules such as quinones, which then transfer electrons to specific sites on the oxide surface (Newman
and Kolter, 2000; Lies et al., 2005). Reduction of hematite ($\alpha$-Fe$_2$O$_3$) surfaces by hydroquinone molecules appears to be a relatively facile process (Stack et al., 2004; Anschutz and Penn, 2005) despite the dramatic influence of adsorption, pH, and quinone structure on the thermodynamics of such reactions (Uchimiya and Stone, 2006). Reductive dissolution features on hematite surfaces observed at locations not associated with specific cell attachment sites of *S. putrefaciens* CN32 (Rosso et al., 2003) may also support this pathway. However, calculated electron transfer rates suggest that the relatively fast diffusion of electrons at hematite surfaces may instead account for nonlocal dissolution features (Kerisit and Rosso, 2006). Moreover, it has recently been shown that enzyme-containing membrane fractions can directly reduce Fe(III) and Mn(IV)-oxides without the presence of chelators or small electron shuttles (Ruebush et al., 2006).

Secretion of nanowires, so-called because of their apparent ability to conduct an electrical current, was recently proposed to be an alternative pathway for long-range oxide reduction (Reguera et al., 2005; Gorby et al., 2006). Although there are two contradicting reports regarding the nanowires produced by *S. oneidensis*, a recent study shows that not only does *S. oneidensis* produce functional nanowires, but also that its nanowires are composed in part of outer-membrane multiheme cytochromes—the same cytochromes thought to be responsible for direct enzymatic reduction (Gorby et al., 2006).

As is often the case with such dynamic interfaces in microbial systems, the predominating whole-cell mechanism for bacterial metal reduction may in fact be an amalgamation of the three proposed pathways above and may even involve presently unknown mechanisms. Nonetheless, a common component of all direct and indirect solid-phase oxide reduction models is the involvement of outer-membrane multiheme cytochromes, particularly the cytochromes OmcA and MtrC. In this study, we attempt to address an important aspect of the direct enzymatic reduction model by interrogating the ability of OmcA and MtrC to mediate interfacial tunneling current when adsorbed to a solid surface.

To address the need of an analytical technique capable of probing the electron tunneling properties of adsorbed cytochrome molecules, various scanning probe microscopy techniques have historically been employed. To date, scanning tunneling
microscopy (STM) and current-sensing atomic force microscopy (CS-AFM), coupled with tunneling spectroscopy (TS) on immobilized single-center metalloproteins, have revealed the nature of interfacial tunneling at a single-molecule level (see reviews by Davis and Hill, 2002; Zhang et al., 2002; Davis et al., 2005). The development of these techniques has arrived to the point where single-molecule measurements on multicenter metalloproteins are achievable. Here we have employed these techniques to study the decaheme cytochromes OmcA and MtrC adsorbed to Au(111) substrates with the objective of attempting to directly probe their mechanisms of electron transfer (see section 2.1 for justification).

In this study, we: 1) establish a protocol for chemical immobilization of OmcA and MtrC in the form of monolayer films on Au(111) surfaces, 2) characterize these cytochrome films using STM, AFM, and XPS, and 3) demonstrate, through current-voltage TS, clear differences in the molecular conductance of the two cytochromes. These tunneling differences may eventually be representative of their intrinsic interfacial electron transfer properties.

2.2 MATERIALS AND METHODS

2.2.1 Cytochrome purification and film preparation

The most common substrate used for making single-molecule STM measurements on adsorbed metalloproteins is Au(111). This substrate provides several advantages over oxide surfaces. Au(111) is a stable surface in air and water on the time scale of STM measurements and will not spontaneously oxidize these cytochromes as an iron oxide surface could (e.g., hematite). When properly prepared, an Au(111) substrate is comprised primarily of large atomically-flat terraces hundreds of nanometers wide suitable for high-resolution imaging of cytochrome monolayers. Au(111) is an excellent conducting substrate for STM compared to semiconducting oxides. Furthermore, the electronic structure near the Fermi energy for a metal is in general comparatively simple and better understood relative to metal oxides. For example, relatively few features appear in the TS conductance curves for Au(111), and those that do are very reproducible and well understood (Davis et al., 1991; Avouris et al., 1995; Kowalczyk et al., 2006) whereas the same cannot be said for oxide surfaces such as hematite (e.g. Becker et al.,
We viewed these Au(111) characteristics to be important for maximizing our ability to interpret the TS data.

The use of Au(111) substrates also provides a key feature in that methods for chemical immobilization of proteins on the surface are already well established. Cysteine residues exposed on many metalloprotein surfaces are able to form covalent thiol bonds with Au(111) which stabilize cytochrome films for imaging with STM. Chemical binding employing a thiol-gold bridge improves chances for efficient electronic coupling between the metalloprotein and the electrode surface (Vondrak et al., 1999). Proteins, and in particular, cytochromes, have been documented to have electrostatic affinities for oxide surfaces (Khare et al., 2005; Eggleston et al., 2006) but protocols for immobilizing cytochromes using chemical binding to oxide surfaces are not well developed at this time. Historically, recombinant proteins with surface-exposed cysteine residues have been constructed to achieve chemical binding (Lo et al., 1999; Andolfi et al., 2002), and that is the strategy used in this study. X-ray photoelectron spectroscopy (XPS) has widely been used to detect such Au-S thiol bonds in organo-sulfur films including single-center metalloproteins (e.g. Chi et al., 2000; Hansen et al., 2003).

Recombinant OmcA and MtrC contained a 4×Cys/5/6×His tag (AACCPCGCCKGKIPQPLLGLDSTRTGHHHHHHH) at their C-termini for efficient protein detection, purification, and attachment to the Au(111) surface. The recombinant proteins were expressed in *S. oneidensis*. OmcA was purified as described in a previously established protocol (Shi et al., 2005; Shi et al., 2006) and STM imaging showed OmcA was well bound to the Au surface (see Results section for further explanation). In contrast to OmcA, initial STM images of our previously purified MtrC suggested that it failed to bind to Au(111). Because this MtrC was also refractory to the purification by immobilized metal ion chromatography (IMAC) despite the presence of 6×His tag at its C-terminus, we believed its tag was buried inside the protein molecule. To make its tag surface-exposed, we added a linker sequence (DDDDK) between MtrC and the tetra-cysteine sequence. The newly constructed MtrC bonded well to the Ni-NTA column and was purified by IMAC. Indeed, the newly purified MtrC was firmly bound to the Au(111) surface, and formed a stable film.
The purified cytochromes were stored at –20°C in buffer solution with the following solutes: 20 mM HEPES, 5 mM β-mercaptoethanol, protease inhibitor, 250 mM NaCl, 1% octyl β-D-glucopyranoside (OGP), and 10% glycerol at pH 7.6. Additional 2 mM β-mercaptoethanol was added to eliminate the formation of disulfide bonds between thiol groups in the tetra-cysteine tag before depositing onto the gold substrate. Due to the presence of lipoprotein consensus sequences for anchoring to the outer-membrane, solubilization in water of the isolated cytochromes requires detergent (OGP) to saturate the exposed hydrophobic regions of the protein surface. The original buffer solution was exchanged twice with 20 mM OGP detergent, 50 mM HEPES buffer, pH 7.6, using Microcon centrifuge filters (10,000 MWCO) at 14,000 rpm and 10°C (Davis et al., 2000). The concentration of OGP was kept below its critical micelle concentration of 25 mM.

The Au(111) layer was ~200 nm thick, supported on muscovite mica, and freshly annealed in a hydrogen flame (Molecular Imaging, Inc.). A hydrophobic seal was made at the contact between gold and mica using Tempfix adhesive (SPI Inc.) to ensure the protein solution did not contact disrupt the Au(111) surface from underneath. Statically charged particles were then removed with a Zerostat 3 piezoelectric pistol before finally depositing approximately 50 µL of the protein solution (final concentration <20 mM) onto the Au(111) surface. The solution was left in contact with the Au(111) surface for 12–16 hours at 4°C in a glass Petri dish. Afterwards, the protein solution was carefully removed from one corner of the film using a pipette (an area that was not used for imaging) and the remaining solution wicked away using the edge of a Kimwipe until no evidence of solution on the substrate was visibly present. The film was then left to equilibrate at room temperature and ambient humidity for 1–2 hours before STM imaging. Extensive rinsing of the films was not necessary as it interferes with the hydrophobic nature of the OGP overlayer (see section 3.2.1). Films were used for experiments no longer than ten days after preparation. Control films of 20 mM OGP detergent and 50 mM HEPES buffer were prepared using the same protocol except without protein in solution. Bare Au(111) control samples were only treated with the piezoelectric pistol prior to imaging.

2.2.2 STM/TS and AFM operation
Using a Molecular Imaging, Inc. Pico SPM controller and 300S-type scanner, STM and AFM imaging was performed in air at ambient humidity. STM imaging was conducted under constant-current mode using electrochemically-etched 80:20 Pt-Ir tips. Images were always obtained by using positive bias voltages (~0.5–1 V) for engaging the tip to the sample surface. In this system, the tip is kept at ground potential while the bias voltage is applied to the substrate thus all bias voltages reported reflect the sample bias relative to the tip. AFM images were collected in contact mode using Pt-Ir coated silicon-nitride cantilevers with a force-constant of 0.3 N/m (MikroMasch Inc.).

During TS operation, the magnitude of the tunneling current ($I$) passing through an individual protein molecule as the bias voltage ($V$) is changed can be viewed as carrying information regarding the propensity of the cytochromes to specifically mediate electron tunneling between the STM tip and the Au substrate. For the collection of $I(V)$ spectra, the tip was quickly centered over a chosen cytochrome immediately after its appearance in an STM image. The feedback loop was automatically turned off, and the tunneling current was recorded as a function of applied bias voltage. The bias was swept through a 3 V range from $+1.5$ to $-1.5$ V at 10 milliseconds sweep duration. Each spectrum consisted of 200 sampling points. Tunneling spectra were always collected starting from the same imaging bias voltage and set point current. The bias voltage sweep was always in the same direction. The representative cytochrome $I(V)$ spectra presented below are averages of several thousand individual spectra (3050 for OmcA, ~8000 for MtrC) collected on several different protein films using many different Pt-Ir tips. Raw $I(V)$ spectra were initially analyzed using Igor Pro 4.0 (WaveMetrics, Inc). Extreme outliers with obvious differences in $I(V)$ signal (<5%) were removed before the remaining spectra were averaged together to construct the representative spectra presented below. Differential conductance ($dI/dV$) spectra were generated using the Savitzky-Golay (1964) moving least-squares method and a 23-point window and removing discontinuities at zero bias voltage. Normalized differential conductance spectra $[(dI/dV)/(I/V)]$ were generated by normalizing to the best-fit exponential ‘background.’

All spectra were collected at an initial tip-sample separation distance as defined by 1.08 V and 0.5 nA. Additionally, in every case the collection of each $I(V)$ curve as
described above was automatically repeated at five different increasing tip-sample distances (Z) in 1 Å steps from the original separation distance. This allowed for analysis of $I(Z)$ spectra for chosen bias voltages. Exponential decay in the $I(Z)$ spectra was used as a check to ensure that true tunneling was always occurring during the measurements (i.e., currents were attributed to electronic tunneling as opposed to electrochemical or ionic charging).

2.2.3 XPS operation

All XPS spectra were obtained with a Perkin-Elmer 5400 X-ray photoelectron spectrometer (Physical Electronic Industries, Inc.) using an aluminum X-ray source and two-channel collector. The X-ray anode was operated at 12 keV and 250 watts and the vacuum was kept below 5 x $10^{-7}$ Torr. The pass energy for wide-range survey scans was 89.45 eV, and for narrow scans it was 17.9 eV. The S 2p spectra presented are averages of 100 sweeps from 178–158 eV binding energy with 25 milliseconds for every 0.1 eV step. All spectra are referenced to Au 4f$_{7/2}$ at 83.8 eV. Data was analyzed using AugerScan 3.12 (RBD Enterprises Inc.).

2.3 RESULTS AND DISCUSSION

2.3.1 X-ray Photoelectron Spectroscopy

Survey XPS scans of OmcA and MtrC films, as well as a control film comprised of only OGP detergent and HEPES buffer, show the presence of several Au photopeaks, as well as C 1s, N 1s, and O 1s photopeaks. The absence of a Fe 2p$_{3/2}$ photopeak indicated that heme Fe atoms in the cytochromes were below XPS detection limits. Despite a low signal-to-noise ratio, measurable differences were apparent in the narrow scans of S 2p photopeaks between 178–158 eV binding energies for OmcA and MtrC films and the OGP control film (Fig. 2.1). Although its intensity is weak, the representative peak near 162–162.7 eV observed in OmcA and MtrC samples is indicative of thiol bonds with Au(111) (Chi et al., 2000; Duwez, 2004). This peak is absent in the OGP control film, suggesting that the objective of creating a thiol bond between the cytochrome films and the Au substrate was achieved.
We also observed the presence of an additional weak S 2p peak near 168.5 eV in both cytochrome samples. In other organo-sulfur self-assembled monolayers (SAMs), a primary cause of weak unexpected higher energy peaks is oxidation of the sulfur species on a time scale of less than four hours for films exposed to air (Schoenfisch and Pemberton, 1998). Although the additional peak is not indicative of any chemical interaction with the Au(111) surface, its presence suggests that not all sulfur in the film is associated with Au-S bonds. This is not surprising as there are unbound sulfur species associated with β-mercaptoethanol and HEPES buffer, as well as non-binding cysteine residues in the cytochromes. However, in organo-sulfur SAMs, unbound thiols are expected to have peaks slightly above 163 eV (Duwez, 2004); no such peaks were detected. Due to the relatively low sulfur content in these cytochrome films, it is not unexpected to see weak S 2p photopeaks. If oxidation is indeed occurring on the films, it may also be contributing to the loss of intensity arising from thiol sulfur species.

These measurements only qualitatively suggest the presence of such interactions between the tetra-cysteine tag and Au(111). They do not suggest all of the molecules are bound in this fashion nor does it eliminate the possibility of other unreacted thiols present in the film. Nevertheless, when the XPS evidence for the presence of thiol bonds is
combined with the STM observations below, immobilization of the cytochromes into stable films is clearly established. Note also that the presence of Au-S bond implies minimal separation between the cytochrome molecule and the Au(111) surface, increasing the probability for electronic interactions between one or more redox-active heme cofactors and the gold surface.

2.3.2 STM/AFM Observations

Initial STM imaging

We first characterized freshly-annealed clean Au(111) surfaces with STM in ambient conditions at a variety of imaging length scales. The observed topographic features consisted of large terraces separated by monoatomic steps, as expected for this well-studied surface (e.g. Han et al., 1997; Zhang et al., 2004; Morimoto et al., 2005). For the cytochrome films, while the general ‘terraced’ structure of the underlying Au(111) surface was still evident (see Fig. 2.2a), high resolution images of the terraces showed strikingly different features on the terraces themselves. Based on other studies of STM imaging of metalloproteins and the molecular weights of OmcA and MtrC, we expect that individual protein molecules should appear as ‘bumps’ on the order of a few nanometers in diameter (e.g. Friis et al., 1999; Chi et al., 2000; Chi et al., 2001; Davis and Hill, 2002; Andolfi et al., 2003; Bonanni et al., 2003; Hansen et al., 2003; Andolfi et al., 2004b; Zhang et al., 2004). No such features were initially observed. Instead, images initially consisted of large flat terraces each covered with randomly dispersed patches, or ‘pits,’ of low apparent tunneling probability.

The ‘pits’ were ~1–3 nm wide and irregularly spaced (Fig. 2.2a, inset), with an STM apparent depth of ~2–3 Å. Features similar to these have been reported in the literature for other systems, primarily for organo-sulfur self-assembled monolayers (SAMs) (see reviews by Poirier, 1997; Yang and Liu, 2003; Vericat et al., 2005). ‘Pits’ have also been observed in ambient conditions on alkanethiol-functionalized Au(111) substrates with adsorbed metalloprotein monolayers (e.g. Chi et al., 2000; Chi et al., 2001; Zhang et al., 2003a; Zhang et al., 2003b; Zhang et al., 2004). In our experiments, images such as these were routinely obtained initially from film to film regardless of cytochrome type. These image features were stable for indefinite periods of time.
Imaging at very small length scales did not resolve any features that could be assigned as individual cytochromes. For reasons discussed below, we will refer to this initial SAM-like topography as the ‘unactivated’ film topography.

![Series of typical STM topographs of ‘unactivated’ topography (A), a single irreversible ‘activation’ event (B), and ‘activated’ topography of cytochrome films (C). Bias voltage for A was 0.84 V. For B, the bias voltage was lowered from 1.08 V (top of the image) through zero bias until finally stopping near –1.5 V where the abrupt change in image contrast occurs. The bias voltage was then raised through zero bias back to +1.08 V. Bias voltage for C was +2.2 V. 0.5 nA current set-point for all images. Scale bars are 50 nm for A–C and 20 nm for the inset in A.](image)

*Figure 2.2 Series of typical STM topographs of ‘unactivated’ topography (A), a single irreversible ‘activation’ event (B), and ‘activated’ topography of cytochrome films (C). Bias voltage for A was 0.84 V. For B, the bias voltage was lowered from 1.08 V (top of the image) through zero bias until finally stopping near –1.5 V where the abrupt change in image contrast occurs. The bias voltage was then raised through zero bias back to +1.08 V. Bias voltage for C was +2.2 V. 0.5 nA current set-point for all images. Scale bars are 50 nm for A–C and 20 nm for the inset in A.*

‘Activation’ of cytochrome films

Despite this apparent failure to resolve individual cytochromes initially, we discovered that the cytochromes were indeed present, residing as an intact monolayer that could only be resolved by STM after modifying the tunneling conditions. Here we describe this process in detail.

By varying the bias voltage to increasingly more negative values, the SAM-like features on the Au(111) terraces could be immediately changed. Bias voltages more negative than ~ –1.5 V led to the appearance of clearly resolved features consistent with a monolayer of cytochromes. The resulting images, collected after having surpassed the threshold bias voltage, are referred to as the ‘activated’ film topography. The transition between ‘unactivated’ and ‘activated’ film topographies was very rapid, often shorter than the time required to collect a single scan line, yielding a very sharp transition in the images (Fig. 2.2b). The images after activation showed a layer of close-packed, nearly spherical features ranging from 5 to 10 nm in size, consistent with the expected sizes for
OmcA and MtrC (Fig. 2.2c). These features uniformly covered entire Au(111) terraces and did not show any preferential adsorption to specific topographic features on the surface. Step heights consistent with those of the underlying Au(111) surface were maintained. Imaging conditions were stable and data could be collected for hours at variable bias voltages even outside the normal bias range of $\pm 1.5$ V. The new features assigned as cytochromes are acquired at the expense of the old SAM-like features in an irreversible fashion. For instance, following film activation at large negative bias voltages, the apparent ‘pits’ were irretrievable upon readjusting the bias voltage back to the low positive values used for engaging the tip to the sample.

Several lines of evidence lead us to conclude that this activation behavior arises due to bias-induced interactions between the tip and a SAM-like overlayer of residual OGP detergent ‘blanketing’ the cytochrome film. The nominal interpretations of the origin of apparent ‘pits’ in SAMs are that they arise from either gold substrate vacancy islands that form during the self-assembly process, domain boundaries where the SAM is highly disordered, or molecular defects in the SAM (Vericat et al., 2005). We do not expect gold vacancy islands to contribute to the self-assembled OGP overlayer given the fact that there is a considerably large cytochrome monolayer sandwiched between the gold substrate and OGP. Disorder in the OGP overlayer may possibly be causing the ‘pits’, but because of the presence of the underlying cytochrome film it is reasonable to expect that the ‘pits’ could arise from subtle irregularities in the cytochrome distribution.

Bias-dependent reorganization of self-assembled surfactant films has been previously observed (Boussaad and Tao, 1999). Based on this fact and the observations described above, we rationalize the observed behavior as follows. During deposition and self-assembly of the cytochrome film, as cytochromes bind to the Au(111) via thiol bonds, OGP maintains interaction with the hydrophobic domains of the cytochrome. The hydrophilic polar head group of OGP remains exposed to the aqueous solution while the cytochromes themselves have minimal contact with solution. As the packing of cytochromes approaches a full monolayer and the molecules become densely packed on the surface (see Fig. 2.4), the OGP facing the solution phase self-assembles into a largely ordered detergent overlayer protecting the hydrophobic cytochromes from solution.
When the bias voltage is assigned a large magnitude at negative bias voltage, the electric field between the tip and sample is evidently strong enough to disrupt the OGP overlayer while not altering the underlying cytochrome film itself. The metal STM tip is strongly hydrophilic and in principle can interact with hydrophilic head groups of the upper surface of the OGP overlayer if enough energy is supplied. We hypothesize that the electric field induced at high bias voltage induces rearrangement of the self-assembled OGP overlayer under the tip so that the outward-facing hydrophilic head-groups of OGP wet the tip. This removes intervening detergent from between the tip and cytochrome film, creating a ‘window’ through the otherwise intact surfactant overlayer allowing the STM tip to engage the underlying cytochrome film directly (Fig. 2.3). This is what is reflected in the transition from unactivated to activated film topographies in the STM images. Tip wetting phenomena such as this are not uncommon for STM performed in air under ambient conditions (Patel et al., 1997). Further support of this hypothesis is the observation that STM images of activated films could be reverted back to the unactivated film topography only when the tip is fully disengaged from the sample surface. Re-engaging in the same location showed that the OGP overlayer is locally restored and the previously formed ‘window’ was eliminated. This observation suggests that bias-induced transformation of the tip structure is not responsible for the activation effect. Additional evidence against changes in tip structure include the reproducibility of the observations as well as the stable imaging of the activated cytochrome films over a wide range of bias voltages after activation.

*Figure 2.3 Cartoon of bias-induced viewing ‘window’ formed from reorganization of OGP overlayer (film ‘activation’) revealing an intact cytochrome layer underneath.*
The ‘window’ formed between the tip and OGP overlayer is very stable as long as the tip is engaged, traveling perfectly with the tip during scanning with no signs of interference with imaging. The ‘window’ is also stable during the collection of $I(V)$ spectra and we found no evidence that the ‘window’ could be closed by the same electrostatic mechanism by which it is opened, for example by reversal of the bias polarity to large positive values. Therefore, after creation of the activation ‘window’, the collection of $I(V)$ spectra could involve a wide bias range as needed. In contrast, the collection of $I(V)$ spectra on the unactivated films, i.e., on the SAM-like OGP overlayer were limited to a bias range that did not surpass the threshold bias.

**STM of cytochrome molecules**

OmcA molecules were observed to be ~8 nm in diameter laterally on average whereas MtrC molecules appeared to be ~5 nm in diameter on average (Fig. 2.4). The observed sizes of the cytochromes in the activated films are within the expected range for globular proteins of a similar molecular weight, as expected for ambient imaging of biomolecules (Alliata et al., 2004). The apparent sizes observed on the films, including the slight variation between OmcA and MtrC, may be a function of not only their molecular shapes, but whether or not the cytochromes are binding to the Au(111) surface in a preferred orientation.

Because crystal structures for either cytochrome are unavailable, it is unknown, in terms of location on the protein surface and molecular axis, where the cysteine residues that covalently bond to the substrate are exposed. However, there is evidence to indicate binding of both cytochromes to the Au(111) surface is through the tetra-cysteine tag located at the C-termini of the cytochromes. Although recombinant OmcA and MtrC have 27 and 29 cysteines in their polypeptides, respectively, 20 of them are used to covalently bind 10 heme moieties; another serves as the lipid-binding site. Thus, only six and eight cysteine residues are free, respectively, for each polypeptide to bind to Au(111), in which four are from the tetra-cysteine tag. As a control, we attempted to image purified MtrA, a 32 kDa periplasmic (i.e., soluble) decaheme $c$-type cytochrome from S. oneidensis (Pitts et al., 2003). Due to the fact that MtrA contains no tetra-cysteine tag, stable films were not observed. Instead it was found that MtrA molecules
were easily moved about on the Au(111) surface by the STM tip. Furthermore, MtrC with a recessive tetra-cysteine tag (i.e. the recombinant MtrC that lacks a DDDDK linker sequence between its C-terminus and the tetra-cysteine tag; described in section 2.1) did not bind to IMAC columns and no images of cytochrome molecules on gold were obtained using STM on this particular MtrC construct. Therefore there is substantial evidence suggesting an exposed C-terminus tetra-cysteine tag mediates chemical immobilization of OmcA and MtrC on the Au(111) surface.

Figure 2.4 Typical STM topographs of ‘activated’ MtrC (A) and OmcA films (B). Tunneling conditions for OmcA: Bias 2.2 V, current setpoint 0.5 nA. Tunneling conditions for MtrC: Bias voltage 1.08 V, current setpoint 0.5 nA. Scale bars, 20 nm.

AFM of cytochrome films

STM does not provide true height information of the adsorbed cytochromes. In an effort to determine the vertical dimensions of individual cytochromes for comparison with the lateral dimensions observed by STM, contact-mode AFM imaging of the cytochrome films was conducted in air and solution. After repeated failed attempts to achieve molecular resolution in solution, the height of the film itself was assessed by imaging in air. Due to capillary and van der Waals forces from imaging in air, we had poor control on the applied forces, which often led to shearing away portions of the cytochrome film from the surface. This however facilitated measurements of the film
thickness by comparing the height difference between the upper surface of the film and the underlying Au substrate. The contrast of the intact film to that of the previous scan area is clearly evident (Fig. 2.5).

The horizontal cross-section in Fig. 2.5, which bisects the previous scan area indicated by the ~2.7 µm square feature in the center of the image, suggests the cytochrome film thickness is roughly 5–8 nm. This is in excellent agreement with the STM apparent diameters of the cytochromes, and confirms the fact that these films consist of only one monolayer of globular-shaped cytochrome molecules. It should be noted that no activation technique was needed to visualize the films, as expected due to the high loading forces involved with contact mode AFM relative to STM.

2.3.3 Tunneling spectroscopy

Current-voltage tunneling spectra
TS was used to gain insight into the electron tunneling properties of individual OmcA and MtrC molecules. Representative $I(V)$ spectra for clean Au(111), OmcA, and MtrC are shown in Fig. 2.6a. As expected, the most obvious difference is between the clean Au(111) and the cytochrome films. Clean Au(111) has a very steep exponential rise in the current with increasing bias voltage magnitude as expected for a metallic substrate, whereas the cytochrome spectra are consistent with a more resistive tunneling junction. The $I(V)$ behavior for OmcA is near exponential, but the $I(V)$ behavior for MtrC is noticeably different in that it does not follow a smooth functional form in the positive bias region. The general features of the $I(V)$ spectra just described are highly reproducible on average for individual cytochromes in these films.

Figure 2.6 A) Representative $I(V)$ spectra on Au(111) and activated cytochrome films of OmcA, and MtrC. Imaging bias voltage, 1.08 V; current set-point, 0.5 nA. B) Representative $I(Z)$ curves for OmcA and MtrC collected at six different bias voltages, where zero tip-sample separation is defined as the separation distance for imaging at each particular bias voltage and setpoint current conditions.

For each TS location, we collected $I(V)$ spectra at five tip-sample separations starting from the imaging tip-sample separation distance and increasing by 1 Å ‘$Z$’ increments. The entire spectral set at each sampling location is collected in 50 ms by software automation to minimize the effects of drift. This provided us with the ability to assess the decay in the tunneling current as a function of tip-sample separation for any bias voltage in the $I(V)$ range. $I(Z)$ spectra were used to ensure that the current was a true tunneling current as opposed to electrochemical or ion conduction currents. True
tunneling current decays exponentially with increased separation whereas ion conduction has a linear $I(V)$ signature (Hamers, 1993). Fig. 2.6b shows representative spectra for OmcA at selected bias voltages. Exponential dependence on separation distance is demonstrated across the entire bias range. This verifies that the primary contribution to the STM was from true electronic tunneling through the cytochromes.

As a check to ensure we were probing only the tunneling properties of the cytochromes and not other molecules possibly in the tunneling junction (e.g., detergent or buffer molecules), $I(V)$ spectra were taken on the OGP overlayer—on unactivated terraces and within the apparent low-tunneling ‘pits’—and on the OGP-only film that was described above in section 2.1. The $I(V)$ spectra on these three surfaces were highly asymmetric with little increase in current in the positive-bias range (Fig. 2.7). This rectifying behavior is a phenomenon that has been observed by STM elsewhere, for example, on copper-phthalocyanine molecules (Pomerantz et al., 1992) as well as on photosynthetic proteins (Stamouli et al., 2004). The $I(V)$ behavior on the unactivated OGP overlayer is very similar to the $I(V)$ behavior of the OGP-only film. The fact that near-symmetric (i.e., non-rectifying) exponential $I(V)$ curves are always observed for OmcA and MtrC (Fig. 2.6a) but never on the control films suggests that, after activation and the formation of the ‘window’, the OGP overlayer does not affect the $I(V)$ measurements on the cytochromes.

*Normalized differential conductance*

Normalized differential conductance spectra (Fig. 2.8) plotted for OmcA and MtrC enhance the subtle differences in the $I(V)$ spectra. Inflections in slope of the $I(V)$ spectra appear as peaks in the $dI/dV$ form and, when normalized to $I/V$, the bias-dependence of the tunneling transmission probability is removed (Hamers, 1993). This mode of analysis stems from Tersoff-Hamann tunneling theory which provides for the possibility that under ideal conditions at low bias voltage, peaks that appear in the $(dI/dV)/(I/V)$ spectra approximately correspond to a high local density of states of the sample at the location of the tip (Tersoff and Hamann, 1985). Under this assumption, the $(dI/dV)/(I/V)$ spectra for MtrC reflects a large density of states evident in the positive bias
range while OmcA shows no such features. This is the first glimpse into the electronic structure of these two redox-active metalloproteins.

![Graph showing tunneling current vs tip bias for OGP films and MtrC cytochrome films.]

**Figure 2.7** Representative $I(V)$ spectra of OGP films (dotted) as well as two different locations on the 'unactivated' topography of MtrC cytochrome films; SAM-pits (dashed) and 'unactivated' terraces on the OGP overlayer (solid). Imaging bias voltage, +1.08 V; current set-point, 0.5 nA.

![Graph showing normalized differential conductance spectra for OmcA and MtrC cytochrome films.]

**Figure 2.8** Normalized differential conductance $[(dI/dV)/(I/V)]$ spectra for OmcA (open circles) and MtrC (closed circles) 'activated' cytochrome films.

Because the representative spectra shown in Fig. 2.6a for OmcA and MtrC are averages of several thousand individual $I(V)$ spectra, the corresponding peaks in the $(dI/dV)/(I/V)$ spectra may have been slightly broadened. However, based on other STM studies of small redox-active molecules, the effect of averaging still maintains peak
positions within ±0.1 V from individual I(V) measurements (Han et al., 1997). The differences seen in the tunneling spectra are thus statistically significant and correspond to true differences in the tunneling conductance of OmcA and MtrC.

If the cytochrome molecule being probed possesses unoccupied or partially occupied energy levels lying between the Fermi levels of the tip and substrate (which are energetically separated by the bias voltage), then in principle these energy levels can assist in the tunneling process (see reviews by Nitzan and Ratner, 2003; Mccreery, 2004). Ignoring different possible mechanisms for tunneling current modification due to these ‘bridge’ states for the moment, in general normalized differential conductance spectra would show increased conductance in the bias voltage range where this condition is met. The assist mechanism is due to either resonant tunneling (Schmickler, 1993), where electrons tunnel directly between tip and substrate by a resonant transition through the unoccupied levels of the molecule, or it is due to “two-step electron transfer” where electrons transferred into the acceptor level are partially stabilized and even temporarily trapped there by nuclear relaxation (Kuznetsov et al., 1992). The difference lies in the energetic position of the bridge state(s) relative to the tip or substrate Fermi levels, the electronic couplings between the tip, molecule, and substrate, and the vibrational dynamics of the molecule. These details are beyond the scope of the current paper. Using electrochemical STM in electrolyte solution under bipotentiostat control, redox energy levels can sometimes be tuned into or out of tunneling resonance, but these experiments are extremely difficult (e.g. Tao, 1996). In our case the measurements are performed without electrochemical control and therefore the cytochrome energy levels sampled during the bias voltage ramp, if any, are by pure chance. It is noteworthy that for these experiments, TS in air provides an advantage over aqueous solution-based TS (e.g., via electrochemical STM) in that the cytochromes used in this study are not expected to be stable over long time scales in solution as they are primarily hydrophobic.

Due to the natural function of these cytochromes for electron transfer—specifically optimized for efficiency without dissipating painstakingly harvested energy—it is natural to consider peaks in the MtrC spectra (Fig. 2.8) as arising from participation of heme cofactor electronic energy levels in the tunneling process. However, other possible explanations for the observed bias-dependent conductance
changes should be considered first. In general, ET rates depend strongly on the dielectric properties of the surrounding medium, with larger reorganization free energies and therefore slower ET rates occurring in polar media with high dielectric constants, such as water (Marcus and Sutin, 1985). In biological ET, a primary function of the protein is to create a low dielectric environment around the heme cofactors to limit the medium contribution to the reorganization free energy. Proteins accomplish this by folding in such a way so as to exclude solvent water from around the heme groups, creating a compact environment with low polarizability. At equilibrium, the structure adopted entails a complex balance between intraprotein, protein-solvent, and solvent-solvent interactions (Simonson, 2003). In multiheme proteins, another primary function of the protein is to facilitate interheme ET, either by arranging heme cofactors in close proximity to each other (usually between 5–10 Å apart) or by providing intervening functional groups that can act as electron tunneling bridges, allowing interheme ET distances of up to 14 Å (Page et al., 1999).

We now expand this picture by considering factors relevant to interrogating electron tunneling behavior of a protein using tunneling spectroscopy: 1) Many proteins are charged and have a net permanent dipole in their native conformation (Simonson, 2003). In principle, the charge and/or dipole can couple to the electric field in the tip-sample junction leading to protein orientation or conformation changes with changing bias, particularly when the bias polarity is changed; 2) The structure of the protein is usually coupled in a significant way to the oxidation state of its heme cofactors (Gray and Winkler, 2003). Thus the protein conformation is redox-dependent. This means that variable forces applied to the cytochrome by the changing tip-substrate field could in principle concomitantly shift heme electronic energy levels; and 3) Solvent and ion access to heme or chargeable functional groups in the protein depends on protein structure. By affecting the protein structure, the bias voltage ramp could therefore alter dielectric or protein charge characteristics which in turn would affect the bias-dependence of the tunneling process. To illustrate a possible effect of the large electric fields in the tip-sample junction, it is easy to show that the force on a single test charge in a model tunneling junction under conditions used here are on the nN scale (i.e. bond breaking). For example, the electric field magnitude (E) across a 5 nm tip-substrate
tunneling gap filled with protein (dielectric constant $\kappa \sim 10$) (Marcus and Sutin, 1985) is $\sim 3 \times 10^9$ N/C. Assuming the force is proportional to the magnitude of the charge ($q$), a test charge of $1.6 \times 10^{-19}$ C would be subjected to $\sim 0.5$ nN of force towards the negatively-charged electrode. For comparison, previously measured forces attributed to conformational unfolding of outer-membrane proteins in S. oneidensis are on the order of 0.2–1 nN (Lower et al., 2001). The effects of multiple charges and/or dipoles in the tunneling gap may induce even stronger net forces acting on the molecule, but such effects are difficult to predict and are considered outside the scope of this paper.

Therefore, despite the numerous recent examples in the literature that have utilized STM on metalloproteins in air as an effective means of single-molecule analysis (e.g. Davis et al., 2000; Chi et al., 2001; Andolfi et al., 2003; Bonanni et al., 2003; Alliata et al., 2004; Andolfi et al., 2004a; Andolfi et al., 2004b; Davis et al., 2004), there is always a concern regarding effects of the STM measurement itself on the proteins under study. However, it is clear from the high reproducibility of the STM images and TS data in this study that the electric fields associated with STM operation are not irreversibly altering the proteins. Upon film activation, the proteins are very stable under the tip for long periods of repeated scanning and over a wide range of bias voltages. We note also that electric fields of this magnitude are expected to be intrinsically imposed on these kinds of proteins when embedded in a bacterial outer membrane (Lindsay, 1993). In our case, we attribute the stability of the proteins in part to preservation of the local microenvironment (hydration, local saturation of hydrophobic domains, etc.) by the OGP overlayer. Although we cannot claim that the cytochromes are in their native conformation on the surface initially or during STM imaging, reproducible cytochrome dimensions before and after the collection of TS spectra suggest that the molecules are retaining a stable conformation.

Notwithstanding the possible field-induced effects, we conclude in this case that none of them are likely causes for the observed differences between MtrC and OmcA in the normalized differential conductance spectra (Fig. 2.8). Although the structures of OmcA and MtrC are not yet known, there is substantial evidence suggesting these two proteins are structurally and chemically very similar, beyond the simple fact that they are both decaheme cytochromes with similar molecular weight and size. For example, both
MtrC and OmcA are functional Fe(III)-NTA reductases that lack activity toward nitrite or nitrate, with virtually identical rates of Fe(III)-NTA reduction and maximum activity in the same temperature and pH range (Shi et al., 2006). In their oxidized forms, the ferric heme iron ions are low spin species with histidine ligands providing both the distal and proximal ligands in both MtrC and OmcA (Richardson et al, personal communication). Both cytochromes exhibit heme operating potentials within a similar range, with both ranges notably higher than seen for similar proteins from a number of *Shewanella* species. The most significant difference shown so far between MtrC and OmcA is in the ‘clustering’ of the redox potentials of their ten heme groups, with MtrC potentials occurring in the region of −80 to −170 mV in a 3:7 ratio and OmcA potentials occurring at −66 mV, −149 mV, and −212 mV in a 3:3:4 ratio (Richardson et al, personal communication). We therefore expect these two proteins to behave similarly within the tunneling junction except for the energy and density of electronic states associated with heme groups. Returning now to the clearly observable differences in the OmcA and MtrC normalized differential conductance spectra (Fig. 2.8), given the above considerations and given that no structural changes in the cytochromes are evident after collection of tunneling spectra, we conclude that features in the MtrC spectra derive from the participation of heme electronic energy levels in the tunneling process. For OmcA no such features are seen. The tunneling process through OmcA is therefore a so-called ‘direct tunneling’ mechanism between tip and substrate, wherein the OmcA molecule provides only a smooth modification to the tunneling transmission probability over the bias range used (Fig. 2.6a). Interpretation of the features observed in the MtrC spectra in terms of a physical model of electron transfer is left to future work.

**2.4 CONCLUSIONS**

OmcA and MtrC, two decaheme c-type cytochromes from *S. oneidensis* proposed to be responsible for direct enzymatic reduction of oxide surfaces have different abilities to mediate interfacial tunneling current when adsorbed to a solid surface. The differences in the tunneling conductance may reflect variation in the roles of these otherwise very similar cytochromes during enzyme-mediated bacterial metal reduction. These results could also have implications for other metal reduction pathways by possibly providing
insights into the origin of the conductive properties of bacterial nanowires and/or helping describe extracellular electron transfer interactions between multiheme cytochromes and electron shuttles. How this relates to their electrochemical properties and their operation as a protein complex is yet to be determined. Towards this end, analysis of the tunneling spectra in terms of models based on electron transfer theory may allow for new insights into the mechanisms of elementary electron transfer processes mediated by OmcA and MtrC at oxide surfaces. Future experiments employing similar techniques may help constrain the nanoscale redox reactions that occur at the dynamic interface between bacteria, cytochromes, and oxide surfaces.

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3) Mechanisms of electron transfer in two decaheme cytochromes from a metal-reducing bacterium

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ABSTRACT

In this report, we analyze and interpret single-molecule current-voltage ($I$–$V$) tunneling spectra collected for two decaheme $c$-type cytochromes using a scanning tunneling microscope. The cytochromes (OmcA and MtrC) are outer-membrane proteins from the metal-reducing bacterium \textit{Shewanella oneidensis} and function as metal-reducing enzymes. Although the two cytochromes are similar in heme count, charge-carrying amino-acid content, and molecular mass, their $I$–$V$ spectra are significantly different. The $I$–$V$ spectra for OmcA show smoothly varying symmetric exponential behavior. These spectra are well fit by a coherent tunneling model that is based on a simple square barrier description of the tunneling junction. In contrast, the $I$–$V$ spectra for MtrC have significant breaks in slope in the positive tip bias range. Two large peaks in the normalized differential conductance spectra of MtrC were fit to a tunneling model that accounts for the possibility of transient population of empty states stabilized by vibrational relaxation. Reorganization energies deduced for the two features are similar to those normally assigned to metal centers in other metalloproteins. Work function measurements of the cytochrome films were used to convert the energies of these two spectral features to the normal hydrogen electrode (NHE) scale for comparison with the redox potential domain previously measured by protein film voltammetry, which showed good correspondence. We conclude that MtrC mediates tunneling current by discretely-resolved heme orbital participation at -81 mV and -365 mV vs. NHE. The difference in tunneling behavior between OmcA and MtrC suggests distinct physiological functions for
the two cytochromes; in contrast to OmcA, MtrC appears to be tuned to a specific operating potential.

3.1 INTRODUCTION

Electron transfer (ET) enzymatically mediated by redox-active proteins (e.g., cytochromes) is essential machinery for many natural biochemical processes including photosynthesis and respiration. Biochemical ET pathways often involve cytochromes specifically adapted to function as incomparably highly efficient ET channels. Drawing upon an example important to environmental chemistry (BROWN et al., 1999; ROSSO and DUPUIS, 2006), the respiration of metal reducing bacteria on Fe(III)-oxide mineral phases can be controlled by the production and localization of multiheme c-type cytochromes to the outer membrane. For the bacterium *Shewanella oneidensis*, direct contact between the cell and oxide surface has been proposed to often be a prerequisite for efficient metal respiration (DICHristina et al., 2005; Nealson et al., 2002). In this case, the outer-membrane decaheme cytochromes OmcA and MtrC are hypothesized to act as terminal metal-reducing enzymes by mediating interfacial ET when in direct contact or close proximity to the oxide surface (Beliaev et al., 2001; Myers and Myers, 2001; Myers and Myers, 2003; Shi et al., 2006). Presumably, adhesion between the cytochrome and oxide surface leads to electronic coupling between one or more heme centers and crystalline Fe(III) electron acceptors, allowing for efficient ET across the cell-oxide interface (LOWER et al., 2007; Neal et al., 2003; Xiong et al., 2006). Although the crystal structures of these cytochromes are not yet known, recent studies are beginning to shed light into their electrochemical and redox properties (HARTSHORNE et al., 2007). Understanding mechanisms of ET through OmcA and MtrC is of interest for distinguishing their biologic function in the electron transport system of *S. oneidensis*. This is technologically important because the reduction efficiency of *S. oneidensis* is directly related to its use in microbial fuel cells (Chang et al., 2006) and for the bioremediation of heavy metals and radionuclides (Lloyd, 2003; Marshall et al., 2006). Moreover, c-type cytochromes such as OmcA have been considered as the principal components of biofuel cells. More generally, understanding the interfacial ET...
mechanisms of OmcA and MtrC is of interest for deepening our understanding of the fundamental origins of ET efficiency in redox-active proteins.

Protein ET is typically investigated in solution using electrochemical redox-titrations or as adsorbed protein films on electrode surfaces using protein film voltammetry (PFV) (ARMSTRONG and WILSON, 2000; HIRST, 2006). Due to the obscuring, and often rate-limiting nature of protein diffusion in electrochemical redox titrations, PFV is an important complementary technique for directly probing protein electrochemistry. However, both approaches are bulk techniques that access only the average behavior of the collective system. Single-molecule techniques are steadily emerging, motivated by the design and implementation of molecular electronics (NITZAN and RATNER, 2003; XU and TAO, 2003). Single-molecule tunneling junctions probe electronic transmission (i.e., conductance) on the nanoampere scale through leads attached to opposite sides of the molecule. Scanning tunneling microscopy (STM) is a single-molecule technique that provides a variant of a metal-molecule-metal tunneling junction that has a weak electronic interaction across at least one of the two metal-molecule interfaces (i.e., tip-molecule interaction). STM provides the ability to image sub-molecular structure (CUI et al., 2001; TAO et al., 2000) as well as probe molecular conductance by measuring current-voltage ($I-V$) behavior with tunneling spectroscopy (TS).

We recently performed an STM/TS study of the decaheme cytochromes OmcA and MtrC from S. oneidensis (WIGGINTON et al., 2007). Monolayer films were created by attaching purified cytochromes to a Au(111) substrate through a recombinant tetra-cysteine sequence exposed on the protein surface. These protein films entail an overlying detergent self-assembled monolayer that preserves the local environment of the proteins, even in air, while still allowing the STM tip to penetrate and directly probe individual molecules. Images of both cytochrome films showed stable dense molecular monolayers with molecular sizes in the range 5–8 nm in diameter. OmcA and MtrC are similar in their heme count, charge-carrying amino-acid content, molecular mass, apparent STM-size, and cellular location in S. oneidensis. Despite these similarities, the tunneling conductance of MtrC is significantly different than that of OmcA. The OmcA $I-V$
spectra follow a smooth symmetric exponential behavior whereas MtrC $I-V$ spectra show significant breaks in slope in the positive tip bias range (WIGGINTON et al., 2007).

Measurement and interpretation of STM-based tunneling conductance of redox-active proteins in solution and in air continues to become more frequent. Such studies have primarily focused on proteins with defined crystal structures such as azurin, a blue copper protein isolated from *Pseudomonas aeruginosa*, using STM or a similar configuration such as conducting atomic force microscopy (C-AFM) (ALESSANDRINI et al., 2003; CHI et al., 2005; CORNI, 2005; FACCI et al., 2001; FRASCERRA et al., 2005; FRIIS et al., 1999; ZHAO et al., 2004). Additional proteins examined by such techniques include other blue-copper proteins (ANDOLFI et al., 2004), photosynthetic reaction centers (LEE et al., 1995; LUKINS, 2000; STAMOULI et al., 2004), iron-sulfur proteins (ZHANG et al., 2004), and various small cytochromes or heme proteins/peptides (ALBRECHT et al., 2006; DAVIS et al., 2000; HANSEN et al., 2003; KHOMUTOV et al., 2002; MORIMOTO et al., 2005; SATTERLEE and MAZUR, 2006).

Tunneling spectroscopy of proteins in air has both advantages and disadvantages versus other environments such as in vacuum or aqueous solution. Protein conformation is extremely sensitive to the presence of water, requiring either an aqueous solution environment or at least humid air which provides several monolayers of adsorbed water on surfaces at ambient conditions. STM in vacuum allows for high cleanliness and $I-V$ tunneling spectroscopic capabilities, but the possibility of denaturing the proteins is great. The conformation of soluble proteins in aqueous solution should be very stable assuming the pH is near a value of the isoelectric point of the protein. Membrane-bound insoluble proteins such as OmcA and MtrC, however, require detergent molecules to maintain solubility. Even then, tunneling spectroscopy in solution is extremely difficult due to unavoidable Faradaic currents. A form of tunneling spectroscopy is possible in solution using a bipotentiostat to simultaneously adjust the tip-substrate and substrate-solution potentials, but collection of $I-V$ spectra in the conventional sense is generally not feasible. Because of the presence of a protective self-assembled monolayer over the MtrC and OmcA films, our setup is unique. We can establish tunneling contact with an STM operating in air by penetrating the overlayer with the tip, which apparently allows the underlying proteins to retain a hydrated environment and stable conformations.
This also simplifies the tunneling junction as compared to aqueous solution and allows us to collect \( I-V \) spectra in the conventional way. Furthermore, it takes advantage of the insoluble nature of the cytochromes we are studying by preserving hydrophobic interactions with the exterior of the molecule.

The objective of the present study is to analyze the differences in molecular conductance between OmcA and MtrC using mechanistic models appropriate for tunneling through large redox-active molecules, and to establish if any connection exists between the energies of the spectroscopic features and PFV-based midpoint potentials for these protein films. From this analysis, the tunneling mechanisms for OmcA and MtrC are compared and discussed in terms of their different biochemical functions for dissimilatory metal reduction by \textit{S. oneidensis}.

**Theory Background**

Two authoritative reviews on tunneling spectroscopy are presented by Hamers (1993) and Hipps (2006) but for our purposes it is important to review specific tunneling theory concepts within the context of our experimental design (for a definition of symbols and abbreviations, see Table 3.1). When a bias voltage \( (V) \) is applied across the junction between STM tip and substrate separated by a few Ångstroms, a tunneling current \( (I) \) is induced. In the conventional picture for a metal-vacuum-metal junction, electrons passing between the sample and tip (and vice versa) tunnel coherently through a trapezoidal potential barrier, as shown in Figure 3.1a. The tunneling process is an elastic one in that the tunneling electrons lose no energy to the barrier and it is exponentially-dependent on bias voltage and tip-sample separation distance. This relationship breaks down, however, when an electroactive molecule, such as a cytochrome, is present in the tunneling junction and the transmission probability is modified.

An electroactive molecule provides additional energy levels that can participate in the tunneling process (i.e., the potential barrier is no longer a vacuum but is replaced by a set of discrete electronic and vibrational states with which the tunneling electrons may interact). Inelastic and orbital-mediated tunneling are two plausible processes affecting the transmission of electrons through this kind of tunneling junction. Inelastic tunneling occurs when tunneling electrons exchange energy with vibrational or electronic states in
the molecule; the energies of the tunneling electrons are not conserved at the expense of molecular excitations. This effect was described by Sumi as manifesting a phonon-progressional staircase structure in $I-V$ spectra (SUMI, 1998). Very sharp linewidths in differential conductance spectra are obtained at low temperatures but vibrational inelastic tunneling features are typically not experimentally sought above 10K due to thermal broadening of the Fermi levels (HAMERS, 1993; HIPPS, 2006; SUMI, 1997). In our spectra, collected at room temperature, features are much more intense and energetically broader than expected for inelastic tunneling effects at low temperature. Hence we assume they do not arise from inelastic tunneling when interpreting our tunneling spectra. Thus, we now focus our attention on various forms of orbital-mediated incoherent tunneling processes as a possible basis to explain the features in our spectra.

Table 3.1. List of important symbols and abbreviations

<table>
<thead>
<tr>
<th>term</th>
<th>definition</th>
<th>units</th>
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<tbody>
<tr>
<td>STM</td>
<td>Scanning tunneling microscopy</td>
<td>--</td>
</tr>
<tr>
<td>PFV</td>
<td>Protein film voltammetry</td>
<td>--</td>
</tr>
<tr>
<td>ET</td>
<td>Electron transfer</td>
<td>--</td>
</tr>
<tr>
<td>NHE</td>
<td>Normal hydrogen electrode</td>
<td>--</td>
</tr>
<tr>
<td>$V$</td>
<td>Bias voltage</td>
<td>V</td>
</tr>
<tr>
<td>$I$</td>
<td>Tunneling current</td>
<td>nA</td>
</tr>
<tr>
<td>$D_{ox}(\varepsilon)$</td>
<td>Density of oxidized states</td>
<td>--</td>
</tr>
<tr>
<td>$D_{red}(\varepsilon)$</td>
<td>Density of reduced states</td>
<td>--</td>
</tr>
<tr>
<td>$(dI/dV)/(I/V)$</td>
<td>Normalized differential</td>
<td>--</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>Voltage ratio parameter</td>
<td>--</td>
</tr>
<tr>
<td>$\phi$</td>
<td>Tunneling barrier height</td>
<td>V</td>
</tr>
<tr>
<td>$L$</td>
<td>Tunneling barrier width</td>
<td>Å</td>
</tr>
<tr>
<td>$E_m$</td>
<td>Electrochemical midpoint</td>
<td>mV</td>
</tr>
<tr>
<td>$\Phi$</td>
<td>Electronic work function</td>
<td>eV</td>
</tr>
<tr>
<td>$\varepsilon_{ox}$</td>
<td>Oxidized redox center potential</td>
<td>mV</td>
</tr>
<tr>
<td>$E_0$</td>
<td>Formal redox center potential</td>
<td>mV</td>
</tr>
<tr>
<td>$\varepsilon_{red}$</td>
<td>Reduced redox center potential</td>
<td>mV</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Nuclear reorganization energy</td>
<td>eV</td>
</tr>
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</table>

Orbital-mediated tunneling occurs when electronic states of the molecule are energetically situated between the Fermi levels of the tip and substrate and assist in electronic transmission between them. Similar to inelastic tunneling, orbital-mediated tunneling manifests peaks in the differential conductance. But in contrast to inelastic
tunneling, these peaks are associated with elastic processes in the sense that the energy of electrons tunneling via an electronic state on the molecule corresponds to an absolute energy of that state (HIPPS, 2006). In further contrast, orbital-mediated tunneling features have been routinely observed at room temperature and are typically not temperature-dependent around room temperature (HIPPS, 2006; MAZUR and HIPPS, 1995). Schmickler (1993) and Kuznetsov et al. (1992) postulated that discrete states of individual electroactive molecules could be experimentally probed by with STM. Mazur and Hipps (1995) demonstrated that a relationship exists between standard reduction potentials and the energetic position of orbital mediated bands for a variety of small organic and organometallic compounds. Tao demonstrated that unoccupied states of iron protoporphyrin could be tuned electrochemically into tunneling resonance using STM in solution (TAO, 1996). Since that time, a number of studies have reported similar observations using STM and C-AFM in vacuum, air, and solution.

To assist in the tunneling conductance, the energies of the participating molecular orbitals must lie within the bias voltage interval. The energies of these orbitals depend not only on the electronic structure of the molecule itself, but also on the nature of the electrical contact(s) to the molecule (e.g., strong electronic coupling to one or both electrode versus weak electronic coupling) (CUI et al., 2002; SCHMICKLER, 1993), on the propagation of the bias potential drop across the molecule (HILDEBRANDT and MURGIDA, 2002; MUJICA et al., 2000; WACKERBARTH and HILDEBRANDT, 2003), and on fluctuations in the structure of the molecule (GRAY and WINKLER, 2003b; LINDSAY, 1993). In models of the tunneling conductance, the energies of participating orbitals are described as having an energetic distribution of certain width and mean value to account for fluctuations in the local molecular structure and surrounding solvent. For example, Schmickler proposed that unoccupied states participating in tunneling have a Gaussian-like energy distribution (SCHMICKLER, 1993). In this model, the current is proportional to $D_{\text{ox}}(\epsilon)$, defined as the density of oxidized states available at the Fermi level of the negatively biased electrode. Likewise the reduced states, $D_{\text{red}}(\epsilon)$, have a Gaussian-like energetic distribution (Fig. 3.1b).
Further details of the orbital-mediated tunneling mechanism depend on rates of ET through the molecule relative to rates of thermally induced nuclear relaxation. If the residence time of a tunneling electron on the molecule approaches that of the period of the modes that reorganize the nuclear configuration of the oxidized state to that of the reduced state—typically on the order of $10^{-13}$ seconds for hemes in metalloproteins (Marcus and Sutin, 1985)—then it becomes possible to temporarily trap the electron on the molecule. The coherent process of resonance tunneling entails negligible residence time for tunneling electrons on the molecule compared to periods of nuclear motion. The coherence progressively gives way to incoherent electron hopping as ET rates through the molecule decrease, which provides time for spontaneous relaxation of the nuclear configuration to stabilize the system towards the reduced state. In this case, the first ET from one electrode to the molecule must then be followed by a second transfer from the molecule to the other electrode. The residence time on the molecule depends in part on the rate of the second ET step relative to the nuclear relaxation, and in part on the energy of the reduced state relative to the Fermi level of the accepting electrode (Fig. 3.1b). It is therefore possible to envision a relatively slow second step leading to transient accumulation of charge on the molecule. If the second transfer occurs during relaxation but before the reduced state is fully stabilized, this mechanism was termed vibrationally...
coherent ‘two-step’ ET by Kuznetsov and co-workers (KUZNETSOV et al., 1992; ZHANG et al., 2002) (Fig. 3.1c). If the reduced state becomes stabilized, the second step has a thermal activation energy associated with it and the overall metal-molecule-metal transfer consists of a sequence of two equilibrated ET steps (Fig. 3.1c). Therefore, in this second ‘two-step’ scenario, this vibrationally incoherent two-step mechanism of conductance involves transient reduction and oxidation of the molecule, and is strongly dependent on fluctuations in the nuclear configuration.

It should be noted that several additional researchers have been working towards addressing the connection between molecular conductance and ET as well, but these formalisms are not as of yet applicable to our experimental conditions (BERLIN and RATNER, 2005; BIXON and JORTNER, 2005; NITZAN, 2001a; NITZAN, 2001b; NITZAN and RATNER, 2003; SEGAL et al., 2000).

3.2 METHODS

Tunneling spectra were previously collected in air at identical imaging conditions (+1.08 V tip bias and 0.5 nA current set-point) (WIGGINTON et al., 2007) Averages of several thousand $I-V$ spectra were fit to a modified Simmons model as presented in Ref. 25 using the nonlinear regression software NLREG (Phillip H. Sherrod) which follows a nonlinear least-squares algorithm (DENNIS et al., 1981). Three parameters were used to fit the functional form to the data and up to 15 iterations were used to confirm parameter and relative function convergence. Differential conductance $[(dI/dV)/V]$ spectra for MtrC were generated using the Savitzky-Golay moving least-squares method with a 23-point window (SAVITZKY and GOLAY, 1964). Discontinuities at zero bias voltage were removed. MtrC $(dI/dV)/(I/V)$ spectra were generated by normalizing to the best-fit exponential background as defined by the fit to the modified Simmons equation. The $(dI/dV)/(I/V)$ curves were analyzed using a peak fitting routine in Igor Pro 4.0 (Wavemetrics, Inc). The least-squares fit of a linear combination of four Gaussian functions was sought using the peak centers, full-widths at half-maximum, and peak heights for fit optimization. Four was the minimum number of peaks required to mimic the overall $(dI/dV)/(I/V)$ behavior. The Gaussian parameters for the two main features
were then converted into the quantities of the two-step tunneling model according to Han and coworkers (Han et al., 1997).

To acquire the protein film work functions, ultraviolet photoelectron spectroscopy (UPS) was conducted in a turbo-pumped, dual-chamber ultrahigh vacuum system equipped with a Leybold EA-11 hemispherical analyzer. UPS spectra were collected with a 21.2 eV He(I) excitation energy and a pass energy of 10 eV which gives an analyzer resolution ($\Delta E$) of 0.15 eV. All samples were analyzed with a -5.0 V bias to assure good determination of low-KE edge (vacuum level) energies. Cytochrome monolayer films for UPS analysis were prepared according to a protocol previously described (Wigginton et al., 2007). It is unknown whether or not exposure to vacuum affected the protein films, but the protective effect of the overlying detergent layer in air makes it possible that the films remained unaffected for short periods of time in vacuum.

### 3.3 RESULTS AND DISCUSSION

**Coherent elastic tunneling in OmcA**

The representative $I-V$ spectrum for OmcA is smooth and symmetric, following an exponential increase in tunneling current with bias voltage. The $(dI/dV)/(I/V)$ form showed no significant features (Wigginton et al., 2007). These observations suggest that tunneling at both positive and negative tip bias occurs coherently through the molecule, which effectively acts as a simple insulating spacer. One simple treatment of coherent elastic tunneling through a thin insulating film was originally proposed by Simmons (1963). This theoretical framework describes the probability of an electron traversing through a rectangular barrier with a defined width, $L$, and height, $\phi$ (Fig. 3.1a). A modified Simmons model has been applied to protein monolayers by Zhao and coworkers for force-dependent conductance spectra of azurin using C-AFM (Zhao et al., 2004). The modification allows for asymmetry in the $I-V$ spectrum due to rectifying behavior or an asymmetric voltage drop across the tunneling junction. In this case, the current density ($i$) is defined as:

$$i = \frac{e^2}{2\pi \hbar L^2} \left[ (\phi - \alpha V) \exp(-K \sqrt{\phi - \alpha V}) - (\phi + (1 - \alpha)V) \exp(-K \sqrt{\phi + (1 - \alpha)V}) \right]$$

and

\[ (1) \]
\[ K = \frac{4\pi L}{h}\sqrt{2\pi\alpha} \]

(2)

where, \(e\) and \(m\) are the electron charge and mass, \(h\) is the Planck constant, and \(\alpha\) is the voltage drop parameter. Asymmetry in the voltage drop across the gap results in \(\alpha\) deviating from 0.5.

Fig. 3.2a shows the OmcA \(I-V\) curve plotted with the corresponding Simmons model fit and calculated parameters. A good fit was obtained as evidenced by the total sum of deviations being less than 1 (0.99) with an adjusted \(R^2\) value of 0.99. The fit yields \(\alpha = 0.509 \pm 5.3 \times 10^{-4}\) for OmcA, which is nearly perfectly symmetrical. The barrier height (1.75 \(\pm\) 0.1 eV) and barrier width (8.35 \(\pm\) 0.03 Å) values are similar to those previously calculated for azurin at low contact forces (Zhao et al., 2004). Zhao and coworkers concluded from their study of azurin that conduction was primarily through the protein matrix and not through the copper redox center localized within (Davis et al., 2006; Zhao et al., 2004). Similarly, our analysis suggests that tunneling through OmcA is a coherent process involving no apparent participation of discrete electronic states, such as those associated with heme centers, over the chosen bias interval.

![Figure 3.2 I-V spectra from Ref. 22 (solid lines) for OmcA (A) and MtrC (B) fit to Simmons' coherent elastic tunneling model (dashed line) described by Eq. 1.](image)

Fig. 3.2b shows a similar treatment of the MtrC \(I-V\) curve but with considerably different results. The Simmons model does not fit the data as well as OmcA, with a sum
of deviations of 2.9 and an adjusted R\(^2\) value of 0.94. It is even more evident from the figure that significant deviations from the model for MtrC exist especially in the positive bias region. Therefore, these variations are not easily explained by a coherent elastic tunneling model.

**Orbital-mediated tunneling in MtrC**

*Analysis of Tunneling Spectra*

For MtrC, two significant spectral features are readily apparent as Gaussian-like peaks in the normalized differential conductance form \((dI/dV)/(I/V)\) (Wigginton et al., 2007) (Fig. 3.3). Features of this shape are anticipated in the orbital-mediated tunneling models of Schmickler and Kuznetsov. Here we apply the model developed by Kuznetsov for orbital-mediation as a vibrationally incoherent two-step ET process (Kuznetsov et al., 1992; Zhang et al., 2002). The current density is proportional to the following quantity

\[
i \propto \pi \left( \frac{k_B T \lambda}{R_{sm} R_{mt}} \right) \frac{R_{sm} R_{mt}}{R_{sm} + R_{mt}}
\]

where \(k_B\) is the Boltzmann constant, \(T\) is temperature, and \(\lambda\) is the reorganization energy. The molecular adsorbate coupling parameters between the substrate and tip (\(R_{sm}\) and \(R_{mt}\) respectively) are defined as

\[
R_{sm} = \int_{-\infty}^{\infty} \rho_s(\epsilon) V_{sm}^2 f_s(\epsilon) \exp \left[ -\frac{(\epsilon - E_0 - \lambda + \alpha V)^2}{4\lambda k_B T} \right]
\]

and

\[
R_{mt} = \int_{-\infty}^{\infty} \rho_s(\epsilon + eV) V_{mt}^2 (1 - f_t(\epsilon + eV)) \exp \left[ -\frac{(\epsilon - E_0 - \lambda + \alpha V)^2}{4\lambda k_B T} \right]
\]

where \(E_0\) is the formal potential of the molecule, \(\epsilon\) is the energy level of the redox center (either oxidized, \(\epsilon_{ox}\), or reduced, \(\epsilon_{red}\)), \(f(\epsilon)\) is the Fermi function of the sample or tip, \(\rho_s\) is the density of states of the substrate, and \(V_{sm}\) and \(V_{mt}\) are the matrix tunneling elements for ET from the substrate to molecule, and molecule to tip, respectively (Kuznetsov et al., 1992; Zhang et al., 2002).
This model suggests that the full-width at half-height of the orbital-mediated features in the differential conductance curves is directly related to the reorganization energy ($\lambda$). The reorganization energy is the energy required to distort the nuclear configuration of the ET reactants (i.e., a donor-acceptor pair) into that of the products, without having transferred the electron (MARCUS, 1956). In the case of a multi-heme cytochrome, it is possible to envision that $\lambda$ is associated with the energy to distort bonds in the participating heme orbitals in the cytochrome and from the energy to repolarize the surrounding polypeptide and associated solvent to a configuration consistent with temporary occupation of those orbitals. The importance of this quantity for understanding protein ET stems from the fact that thermal ET is a Franck-Condon process controlled by nuclear fluctuations. The reorganization energy enters into most modern expressions of the ET rate (MARCUS and SUTIN, 1985). It is these thermal fluctuations in the cytochrome that extend the energies of the participating electronic states statistically around the most probable value, imparting width to the differential conductance peaks (ALESSANDRINI et al., 2006; GERISCHER, 1969). The reorganization energy is further manifested in the model as the energy difference between the molecular state available to accept an electron and the formal potential, $E_0$, of the redox center.
(GERISCHER, 1969). In other words, \( \varepsilon_{\text{ox}} = \lambda - E_0 \) and \( \varepsilon_{\text{red}} = \lambda + E_0 \) (Fig. 3.1b) (HAN et al., 1997).

To obtain good fits to the MtrC spectrum shown in Figure 3.3, a linear combination of four peaks of the Gaussian form

\[
\frac{dI}{dV} = A \exp \left[ - \frac{(V - V_p)^2}{B^2} \right] + C
\]

were simultaneously fit, where \( A \) is the height of the peak, \( B \) is related to the half-width at half-height of the Gaussian, \( C \) is the background height of the \((dI/dV)/(I/V)\) spectra, and \( V_p \) is the predicted peak voltage (HAN et al., 1997). Two minor peaks at the bias extremes are ignored, focusing instead on the two prominent peaks. The resulting Gaussians for the two prominent peaks were then separately fit to a simplified form of the Kuznetsov model. All densities of states, coupling terms, and Fermi functions were collected in front of the integration signs into one fitting parameter, \( b \), in front of the rate quotient. The reorganization energy and the formal potential, \( E_0 \), were allowed to vary, yielding a three-parameter form of the Kuznetsov model. Resulting best fit quantities for the two prominent peaks are given in Table 3.2. Standard errors for all predicted terms were 0.05 or less. \( \varepsilon_{\text{ox}} \) was taken as the absolute peak position of the Gaussian while \( \varepsilon_{\text{red}} \) was calculated directly from the estimates of \( \lambda \) and \( E_0 \).

**Table 3.2. ET parameters for MtrC estimated from orbital-mediated tunneling model (Eqs 3-5). Quantities in V or eV where appropriate.**

<table>
<thead>
<tr>
<th></th>
<th>Peak 1</th>
<th>Peak 2</th>
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<tbody>
<tr>
<td>( \varepsilon_{\text{ox}} )</td>
<td>0.28</td>
<td>0.55</td>
</tr>
<tr>
<td>( E_0 )</td>
<td>-0.30</td>
<td>0.22</td>
</tr>
<tr>
<td>( \varepsilon_{\text{red}} )</td>
<td>-0.87</td>
<td>-0.11</td>
</tr>
<tr>
<td>( \lambda )</td>
<td>0.57</td>
<td>0.33</td>
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The resulting values for \( \lambda \) can be compared to values for heme groups in cytochromes and related molecules reported elsewhere. For cytochrome self-exchange reactions in solution, \( \lambda \) is typically on the order of 0.5 to 0.8 eV (FEDURCO, 2000; MARCUS and SUTIN, 1985; MOSER et al., 1992; SIMONSON, 2002; SIMONSON, 2003). The blue-copper protein azurin is predicted to exhibit even higher values of \( \lambda \) from 0.7 to 0.8 eV (DiBILIO et al., 1997; WINKLER et al., 1997). However, these values were not
predicted for cytochromes at interfaces in an STM configuration. Very few examples in
the literature exist where $\lambda$ has been estimated from measuring current passing through a
redox-active protein in a tunneling junction. To the best of our knowledge, all attempts
thus far have focused on azurin. Estimates by Alessandrini and coworkers place $\lambda$ in a
range from 0.13 to 0.53 eV based on bias-dependent imaging of sub-monolayers of azurin
(ALESSANDRINI et al., 2003). Related quantum-mechanical calculations by Corni predict
$\lambda$ to be around 0.43 eV (CORN, 2005). Chi and coworkers recently calculated $\lambda$ to be
0.35 to 0.45 eV from bias-dependent image contrast on hydrophobic organic self-
assembled monolayers (CHI et al., 2005), which is in good accordance with Corni’s
calculations but much lower than values predicted for the azurin self-exchange reaction.
Lower values of $\lambda$ are predicted for redox-active proteins in the STM configuration
because the interfacial configuration and the presence of the STM tip reduces the space
for conformational degrees of freedom and surrounding solvent. Possible strong field
effects from high bias voltages may also contribute to the lowering of $\lambda$ but proteins often
retain native-like conformations even in high external electric fields (POMPA et al., 2005).

Given the reasonably good correspondence between the $\lambda$ values deduced for
MtrC from the tunneling spectra and literature estimates of $\lambda$ for cytochrome redox
centers, we conclude that the two prominent features in the MtrC spectra arise from
orbital-mediated tunneling via heme center electronic states. Therefore, ET through the
molecule at positive tip bias likely follows a two-step mechanism: electrons originating
from Au(111) reduce the oxidized redox center, which is subsequently transiently
oxidized by the tip, according to vibrationally incoherent electron hopping (see Fig.
3.1b). We cannot completely rule out other possible contributions to the tunneling
current based on the participation of other energy levels (e.g. amino acid residues or other
aromatic groups within the otherwise insulating polypeptide matrix). The presence of
two additional smaller peaks in $(dI/dV)/(I/V)$ may be evidence for such a case (Fig. 3.3).

The model also predicts energies for two redox potentials in MtrC. To compare
these values with previously measured electrochemical midpoint potentials, however,
these energies must be accurately converted to a standard electrochemical scale.
**Comparison to Electrochemistry**

STM energy levels are referenced to the Fermi energy of the metal on an absolute vacuum scale whereas the electrochemical midpoint potentials are referenced to a standard electrode such as the normal hydrogen electrode (NHE). The conversion of energies from one scale to another is based on the difference in their work functions ($\Phi$). The work function of the NHE is a well-defined quantity ($\Phi_{\text{NHE}} = 4.43$ eV) (Reiss and Heller, 1985) but the shift in the work function for Au(111) after adsorption of a monolayer of molecular adsorbates is a quantity that must be measured. In some cases, $\Phi_{\text{Au}}$ can decrease more than 1.5 eV due to organosulfur adsorbates depending on the coverage, exposure time, and the details of the molecular structure (Alloway et al., 2003; De Renzi et al., 2005). Thin films of other small organic molecules weakly adsorbed to Au(111) have also been shown to lower $\Phi_{\text{Au}}$ by more than 1 eV (Schroeder et al., 2003). Therefore, determining the shift in the work functions ($\Delta \Phi$) between the cytochrome films and the clean Au(111) surface must be done before comparing the electrode potentials. This can be accomplished by measuring the energy shift of the low-KE photoemission edge (i.e., vacuum level) using ultraviolet photoelectron spectroscopy (UPS), as has been previously shown (see Hipps, 2006 for numerous examples).

Through UPS analysis, $\Phi_{\text{Au}}$ for the clean Au(111) substrates used in these experiments was measured to be $6.3 \pm 0.1$ eV by measuring the overall width of the valence band spectrum (Figure 3.4). This value is slightly higher than previously reported literature values of single-crystal Au(111) (Schroeder et al., 2003), but this can be explained by the apparent polycrystalline nature of the Au(111) samples as apparent from previous STM images (Wigginton et al., 2007). The work function of MtrC monolayer films was measured relative to Au(111) and $\Delta \Phi$ was determined to be $1.7 \pm 0.05$ eV (Fig. 3.3). By subtracting $\Delta \Phi$ from $\Phi_{\text{Au}}$, the absolute work function of MtrC films, $\Phi_{\text{MtrC}}$, is $4.6 \pm 0.1$ eV.

Knowledge of $\Phi_{\text{MtrC}}$ allowed us to place the redox potentials determined for the two MtrC differential conductance features on the NHE scale for direct comparison with the midpoint potential domain, $E_{\text{m}}$, measured electrochemically by Richardson and co-workers (Hartshorne et al., 2007). The left side of Fig. 3.5 shows the positive tip bias
range relative to the Fermi energy of Au(111) along with the corresponding peaks in \( (dI/dV)/(I/V) \). The converted potentials of the two peaks observed in MtrC are \(-81\) mV and \(-365\) mV versus NHE. From this figure, it is clear that these values are two well-resolved potentials within the wide electrochemical redox domain previously measured (HARTSHORNE et al., 2007). Given that sub-component potentials of \( E_m \) were not accessible using the traditional ‘bulk’ electrochemical techniques, our study demonstrates that single-molecule tunneling experiments are a highly sensitive technique capable of resolving individual redox potentials of multicenter proteins.

**Figure 3.4** UPS spectra of bare Au(111) (dotted black line) plotted alongside cytochrome films of OmcA (dashed red line), and MtrC (solid blue line) chemically adsorbed to the surface.

**Figure 3.5** Energetic alignment between the two prominent peaks (green dotted lines) corresponding to experimental tunneling spectra (solid blue line) and the broad midpoint potential range measured electrochemically\(^\text{12}\) (\( E_m \) vs. NHE) for MtrC.
Physiological implications regarding OmcA and MtrC

Given the differences in the conductance of OmcA and MtrC despite their similar properties and arrangement on the substrate, our analysis infers that these two cytochromes can be distinguished based on their intrinsic ability to transmit electrons. From a physiological standpoint, this conclusion is intriguing because it suggests the two cytochromes have different roles for mediating interfacial ET at the interface between a metal-reducing bacterium and a reducible metal oxide surface. It is possible that absolute energies of heme center orbitals in OmcA do not fall within the bias interval probed relative to the Fermi energy of Au(111), but the typical range of redox potentials of cytochromes (± 400 mV vs. NHE) (GRAY and WINKLER, 2003b) is completely sampled in our measurements. The lack of spectroscopic features for OmcA and the agreement between the STM and PFV data for MtrC leads us to the conclusion that OmcA is intrinsically less electroactive than MtrC. Unfortunately, as of yet there is no published electrochemistry data available for OmcA to compare with our tunneling spectra.

The fact that coherent elastic tunneling is the dominant tunneling mechanism in OmcA suggests coupling of donor and acceptor by superexchange tunneling through the cytochrome polypeptide matrix (GRAY and WINKLER, 2003b). Superexchange tunneling between redox centers is thought to be a plausible pathway for ET in multicenter proteins (JEUKEN et al., 2002) and has also been proposed to be the tunneling mechanism for azurin in a C-AFM configuration (DAVIS et al., 2006). These observations suggest that OmcA may not be constructed for the function of mediating ET at specific potentials, as is the case for other multicenter proteins (LEGER et al., 2006), and as such, would function much differently on the cell surface than MtrC.

The excellent agreement of the tunneling spectra with PFV, combined with reasonable predictions for $\lambda$ predicted by the orbital-mediated two-step model suggests that MtrC mediates tunneling current through the participation of at least two heme center electronic states. The activity of these two heme centers (or two groups of several heme centers), allows MtrC to be tuned to one or more specific redox potentials. These potentials conceivably could be specifically modified by conformation changes to trigger
ET. Recent ET calculations for heme dimers in other multiheme proteins from *S. oneidensis* and related species provide similar evidence for intraprotein ET between hemes in such a manner (Smith et al., 2006). This is also consistent with the concept of specific ET pathways mediated by two pentaheme domains within MtrC (Hartshorne et al., 2007). The two resolved redox potentials obtained in our study may provide clues as to the unique roles of these pentaheme domains in relation to the oxide surface.

The observed differences in ET mechanism for OmcA and MtrC may reflect the fact that these two cytochromes are known to form an extracellular complex (Shi et al., 2006). Protein complexes in nature often serve the purpose of providing separate pathways to increase ET efficiency, such as in the photosynthetic reaction center (Bixon et al., 1991; Chan et al., 1991; Heller et al., 1995). Considering the primary protein sequences of OmcA and MtrC, it is also plausible that differences in their amino acid content could account for different intramolecular tunneling mechanisms/pathways (Gray and Winkler, 2003a). OmcA and MtrC have close or identical numbers of most amino acids known to contribute to ET processes (e.g., tryptophan, phenylalanine, histidine, cysteine). However, OmcA has more than twice the number of tyrosine residues (31) than MtrC (14). Due to the aromatic nature of its phenol functional group, tyrosine is known to contribute to ET in proteins via a superexchange mechanism (Clay et al., 2002). The increased presence of tyrosine may allow for more efficient superexchange tunneling in OmcA. Future studies using site-specific mutants of OmcA and MtrC are required to fully understand if that is indeed the case. Additionally, the conductivity of bacterial extracellular ‘nanowires’ may depend on specific ET pathways within and between OmcA and MtrC as these cytochromes are hypothesized to be critical structural and charge-carrying components of nanowires (Gorby et al., 2006).

It should be noted that it is possible that differences in tunneling spectra between OmcA and MtrC are unrelated to the inherent protein function/design; structural or orientational differences between the two cytochromes may be present when covalently bound to Au(111). The orientation of the protein molecules on the surface could affect the accessibility of heme centers in the tunneling junction. The C-terminus tetra-cysteine tag, which is responsible for the covalent anchoring of the molecules to Au(111) in this study, is presumed to orient OmcA and MtrC on the surface in a uniform fashion, but
their relative orientations may be different enough to affect interfacial ET. Resolving these questions depends in large part on the future availability of detailed structure information for these two cytochromes.

### 3.4 CONCLUSIONS

Analysis of single-molecule conductance spectra for OmcA and MtrC has differentiated these two similar cytochromes based on their ability to transmit electrons. Conductance through OmcA appears to follow a coherent tunneling mechanism with no evidence for the participation of heme center electronic states. In contrast, two main conductance features are found for MtrC having characteristics consistent with orbital-mediated tunneling via heme centers at discrete redox potentials. Reorganization energies associated with the MtrC features fall within the range typically expected for redox centers in cytochromes. The reasons behind the differences in the conductance mechanisms for these cytochromes is not entirely clear, but our findings suggest that differences in the ET mechanism between OmcA and MtrC exist that could in turn reflect differences in their bulk electrochemistry as well as their physiological role in bacterial metal respiration. In contrast to OmcA, the function of MtrC appears to entail operation at specific potentials, possibly reflecting a purpose of conformation-triggered ET or direct interaction with mineral surfaces. Further progress on this topic will be enabled by the arrival of protein structure information. For example, computational methods such as *ab initio* electronic structure calculations and molecular dynamics simulations could potentially provide valuable links between single-molecule conductance measurements and ET kinetics in multicenter proteins (Kerisit et al., 2007).

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REFERENCES


4) Influence of bacterial multiheme cytochromes on long-range electron tunneling to hematite (001) surfaces

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ABSTRACT

Electrochemical scanning tunneling microscopy (EC-STM) was used to measure tunneling current profiles across the nanoscale space between a multiheme bacterial cytochrome and the hematite (001) basal plane. The small tetraheme cytochrome (STC) was purified from Shewanella oneidensis and chemically adsorbed to Au STM tips to allow for precise control over the protein/hematite separation distance. Current-distance (I-s) curves collected on hematite (001) at constant surface potentials show features consistent with a double layer dependent strongly on ionic strength. With STC in the tunneling junction, the double layer structure was significantly modified to show characteristics of enhanced tunneling through adsorbed water and ion layers at the near surface region. These results suggest that cytochrome-mediated electron transfer during bacterial metal reduction could be extended as much as several nanometers away from the mineral surface. Furthermore, breaks in tunneling current profiles following conformational changes of individual STC molecules imply that cytochrome conformation and heme cofactor orientation control interfacial and long-range electron transfer within the electrical double layer of a semiconducting mineral surface.

4.1 INTRODUCTION

Metal oxide surfaces control a wide range of interfacial chemical processes in nature, such as photocatalysis, trace metal adsorption, and biologically-mediated metal reduction because of their ubiquity and unique surface reactivities (BROWN et al., 1999). Electron transfer reactions are involved in a majority of these processes, which subsequently influences the fate and speciation of elements in the environment (ROSSO and DUPUIS, 2006). Electron transfer reactions are considered the simplest chemical
reactions and in biological systems provide the basic driving force for life (MARCUS and SUTIN, 1985). Dissimilatory metal reducing bacteria survive in anoxic environments by mediating electron transfer to polyvalent metal oxide surfaces to conserve energy; these reactions have important consequences in environmental chemistry (NEALSON et al., 2002).

Proteins containing multiple prosthetic heme cofactors (i.e. multiheme cytochromes) are critically important components of the electron transport pathway between metal-reducing bacteria and mineral surfaces (DiCHRISTINA et al., 2005; GRALNICK and NEWMAN, 2007; SHI et al., 2007) and may influence the way in which bacterial cells associate with minerals. For instance, adhesion forces between Shewanella oneidensis and iron (hydrox)oxides is thought to be a result of cytochrome interactions with the surface (LOWER et al., 2001; LOWER et al., 2005), and indeed cell accumulation and surface attachment is often associated with increased biological reduction (i.e. electron transfer) rates at specific mineral surfaces (NEAL et al., 2003; NEAL et al., 2005). Interestingly, the two outer-membrane multiheme cytochromes from S. oneidensis thought to be most important during metal reduction, OmcA and MtrC, bind specifically with hematite (α-Fe₂O₃) (LOWER et al., 2007). Recent molecular dynamics simulations have shown that multiheme cytochromes change conformation when adsorbed to hematite to allow for effective coupling between heme cofactors and surface-bound Fe(III) required for electron transfer (KERISIT et al., 2007). Experimental documentation of conformational changes for monoheme (KHARE et al., 2006) and multiheme cytochromes (EGGLESTON et al., 2008) confirm this observation. Further evidence of the link between cell attachment and reduction was recently seen in microbial fuel cells (BRETSCHGER et al., 2007). Cytochromes therefore are responsive to mineral surfaces and may indeed interact specifically with them to enhance electron transfer.

In some environments, metal-reducing bacteria may be able to reduce redox-active metal-oxides such as hematite even if outer-membrane cytochromes are not permitted to come in direct contact with the mineral surface. Low molecular weight electron shuttles (NEWMAN and KOLTER, 2000; MARSILI et al., 2008) and extracellular nanowires (GORBY et al., 2006) have been presented as two possible mechanisms that facilitate bacterial reduction in circumstances where cytochrome-metal-oxide contact is
not expected, such as sessile communities or in biofilms (LIES et al., 2005). Although these two extracellular reduction mechanisms seem plausible, they may be energetically expensive in terms of ATP required to employ such reduction strategies.

An alternative possibility that is not mutually exclusive from any reduction strategy is a control of electron transfer by a combination of the mineral surface structure and solution chemistry. In this case, the intrinsic electrical double layer at the mineral surface, which is primarily controlled by surface potential, pH, and ionic strength, interacts specifically with outer-membrane or surface-exposed cytochromes at significant distances beyond the non-adiabatic limit for electron transfer (i.e. less than 2 nm) (GRAY and WINKLER, 2003). This was proposed conceptually by Nealson et al. (2002) wherein microscopic observations of *S. oneidensis* cultures did not necessarily need to maintain contact with Mn-oxides in order to reduce the mineral surface but needed to remain close and/or in brief contact. This observation lead to the speculation that cells could effectively reduce the electrical double layer at the mineral-water interface instead of specific reducible polyvalent metal atoms residing on the mineral surface (i.e. Fe(III) atoms). A more recent report by Lies et al. (2005) considered the low probability that outer-membrane cytochromes could actually maintain close enough proximity to a mineral surface for electron transfer as evidence to support an electron shuttling mechanism, but this reasoning could also support reduction of an extended electrical double layer. Under this scenario, outer-membrane cytochromes would presumably be required to store reducing equivalents (i.e. electrons) within many of their heme cofactors until a mineral and its associated electrical double layer with an appropriate redox potential comes within the narrow spatial range required for electron transfer. Cytochrome-mediated reduction of a more broadly extended electrical double layer could hypothetically extend the distance required for biological reduction and alleviate the need for energy-expensive reduction strategies, increasing the efficiency of anaerobic respiration on solid-phase electron acceptors.

Limited work has investigated the role that the electrical double layer plays on charge transfer at semiconducting mineral surfaces. Experiments on thin iron oxide films suggest tunneling through the liquid gap is rate-limiting as opposed to the space charge region associated with the semiconducting iron oxide surface (DIEZ-PEREZ et al., 2006a).
Indeed, the surface potential, which is intimately related to interfacial water and associated charge-compensating ions, has been shown to be incredibly important in dictating reactivity of specific hematite ($\alpha$-Fe$_2$O$_3$) surfaces (YANINA and ROSSO, 2008). If charge is donated to an oxidizing potential at the surface within the electrical double layer, electron transfer can potentially occur at a surface such as hematite without direct electron tunneling from cytochrome heme cofactors to Fe(III) sites. Surface Fe(III) sites on hematite (001) have been treated as a redox center in the theoretical framework of a resonant tunneling model (EGGLESTON, 1999; EGGLESTON et al., 2003; EGGLESTON et al., 2004) that was originally developed by Schmickler (1992). An additional way of treating electron transfer in hematite surfaces is through electron mobility via a small polaron hopping model (ROSSO et al., 2003a). Small polarons traversing through bulk hematite from the surface has been predicted to be efficient for bacterial respiration (KERISIT and ROSSO, 2006) despite concerns of the inhibitory effect of bulk defects on charge transport (KERISIT and ROSSO, 2007). Experimental confirmation of this concept was recently presented wherein it was documented that charge transport through bulk hematite is a function of a chemically induced surface potential gradient and low electrical resistivity (YANINA and ROSSO, 2008). This observation supports nonlocal dissolution features that have previously been observed on hematite during biological reduction (ROSSO et al., 2003b).

Very little experimental evidence of metal-reducing bacteria or their associated multiheme cytochromes reducing mineral surfaces has been probed at a level in which interfacial electron transfer was specifically measured. As biological reduction in its most basic reaction is simply electron tunneling, these reactions can theoretically be probed in solution for various electrode potentials and solution conditions. One such technique, electrochemical scanning tunneling microscopy (EC-STM), combines the single-molecule sensitivity of scanning probe microscopies (ALESSANDRINI et al., 2006) with in situ electrochemical control of electrode potentials (SCHINDLER et al., 2005). Hematite (001) surfaces have previously been used in EC-STM studies as the working electrode (WE) to examine cytochrome (EGGLESTON et al., 2006) and electron shuttle (STACK et al., 2004a; STACK et al., 2004b) adsorption and/or reduction.
To address the lack of knowledge regarding interfacial electron transfer between bacterial multiheme cytochromes and metal oxide surfaces and their associated electrical double layer, we investigated distance-dependent tunneling from the small tetraheme cytochrome c (STC) from *S. oneidensis* MR-1 to hematite (001) basal planes with EC-STM. We measured thousands of current-distance (*I*-*s*) profiles under a number of ionic strength solutions at fixed surface potentials to test the effect of the electrical double layer on electron tunneling. Although the outer-membrane cytochromes MtrC and OmcA are believed to be the two cytochromes principally involved with solid-phase metal reduction for *S. oneidensis*, the lack of structural information of these two proteins limits the ability of experimental electron transfer measurements, such as an earlier study by our group on OmcA and MtrC (WIGGINTON et al., 2007b), to be combined with theoretical simulations and electron transfer calculations.

STC is periplasmic and not implicated in extracellular metal reduction, but has been suggested to be involved in the reduction of soluble metals able to diffuse through the outer membrane such as U(VI) (FREDRICKSON et al., 2002). STC is similar to outer-membrane metal reductases because of the similar axial ligand binding of their heme cofactors and is therefore a model cytochrome for understanding the fundamental mechanisms of cytochrome-mediated reduction of metal oxide surfaces.

The hematite (001) basal plane is one particular mineral surface that has received abundant attention because of its ubiquity in nature as the predominant crystal face of one of the most stable Fe-oxide minerals in nearly all oxidizing conditions. Hematite is a wide band gap semiconductor and exhibits the hexagonal corundum-type crystal structure. (001) is structurally distinct from other crystallographic faces or edge structures in that it contains not only several different Fe(III) sites (EGGLESTON, 1999), but contains mixed Fe and O terminations as well as adsorbed or adatom Fe apart from bulk terminations (EGGLESTON et al., 2003; EGGLESTON et al., 2004). Spectroscopic investigations on this surface suggest that water is also a primary component of the surface structure whereas hydrated and/or hydroxylated chemical terminations influence reactivity (JUNTA-ROSSO and HOCHELLA, 1996; TRAINOR et al., 2004). Indeed, the specific reactivity at hematite (001) can be described by differences in local electronic structure compared to bulk hematite (BECKER et al., 1996).
4.2 MATERIALS AND METHODS

EC-STM design and STM tip fabrication

A homemade electrochemical (EC) cell was made with hematite (001) as the working electrode mounted on a steel puck. The natural hematite used in this study has been previously shown to be an n-type semiconductor at 25°C with large concentrations of donor impurities such as Ti and Sn, making it conductive enough for STM imaging (EGGLESTON et al., 2003). Before construction of the EC cell, the hematite sample was cleaned extensively with concentrated KOH to remove adventitious material.

Fig. 4.1 shows the arrangement of the hematite EC cell. The Ag/AgCl quasi-reference electrode (RE) was made by oxidizing Ag wire in 1M KCl and was found to vary +118 mV relative to a standard Ag/AgCl reference electrode (223 mV vs NHE). Pt wire was used as the counter electrode (CE). Au STM tips were fabricated by etching 0.25 mm Au wire (99.9+% purity, Sigma Aldrich) in HCl/ethanol mixture solution using a DC power supply. Tips were rinsed with nanopure water and methanol and blown dry with high purity nitrogen gas. The sharpened tips were then coated with Apiezon wax to minimize leakage currents (NAGAHARA et al., 1989). For our STM tips, the leakage current was routinely measured to be <0.01 nA. STC-functionalized tips were prepared similarly to previous protocols for chemically immobilized cytochromes on Au(111) by incubation with reduced and purified STC molecules overnight (WIGGINTON et al., 2007b; ROSSO et al., 2008). Cell volume for all EC-STM experiments was 150–200 µL. The hematite cell was periodically cleaned with methanol and nanopure water between measurements.

Figure 4.1 Cartoon cross-section of electrochemical cell with hematite (001) as the working electrode. Reference electrode (RE) was Ag/AgCl wire, counter electrode (CE) was Pt wire, and Au STM tip was insulated with Apiezon wax.
**Purification of the small tetraheme cytochrome c**

STC is a 12.1 kDa tetrahem erythrocrome that is produced abundantly by *S. oneidensis* within the periplasmic space (TSAPIN et al., 2001). Native STC did not bind preferentially to Au(111) surfaces in previous experiments (ROSSO et al., 2008) so a recombinant form was constructed. Recombinant STC was graciously provided by Liang Shi (Pacific Northwest National Laboratory). This construct contains a 4 x Cys/V5/6 x His tag at its C-terminus to encourage the formation of Au-thiol bonds at the STM tip with the tetracysteine sequence. This construct was previously used for OmcA and MtrC binding to Au(111) (WIGGINTON et al., 2007b). STC was further purified and dialyzed to a final working solution of 50 mM HEPES pH 7.2 and did not require detergent to solubilize the protein.

**Characterization techniques**

Scanning electron microscopy (SEM) of homemade STM tips was performed using a LEO (Zeiss) 1550 field-emission SEM with an in-lens backscattered electron detector. X-ray photoelectron spectroscopy was conducted using a PHI Quantera SXM-03 with a spot size of 100 µm to verify effective binding of STC molecules to the STM tips.

**Collection of current profiles**

*I-s* spectra were collected using a Molecular Imaging PicoPlus SPM. Each suite of measurements was taken with one tip at many locations on the hematite (001) surface and at two different current setpoint values (0.5 and 1.0 nA). Several repeat experiments were conducted to demonstrate reproducible behaviors, but the spectra presented here were only collected with one tip to minimize the effect of tip shape and/or leakage current, unless otherwise noted. All *I-s* spectra are retraction curves collected with the feedback loop temporarily turned off. Each curve was collected individually over 0.1 sec and collected in series, with a 0.1–1 sec pre-sweep delay in between acquisitions. For bare hematite experiments, the tip was engaged with a +350 mV tip bias voltage and 0.5 nA current setpoint and *I-s* spectra were collected only when imaging conditions were favorable (i.e. steps were visible on the hematite surface). For STC-functionalized tips, *I-s*
spectra were collected over several more surface locations to account for the fact that imaging the surface prior to the collection of spectra was not possible without compromising the integrity of the cytochromes attached to the tip.

The hematite surface potential was set near the open circuit potential of the hematite cell, which was near \(-0.21\text{V}\) in KCl solutions equilibrated with ambient atmosphere. KCl solutions (pH \(\sim 5.8\)) were exchanged out carefully as to not disturb the tip by flushing new solution into the cell 3-4 times. Measurements were collected sequentially in solutions of increasing ionic strength (i.e. 1 mM first, followed by 10 mM and then finally 100 mM).

4.3 RESULTS

Characterization of STM tips and EC cell

To verify that sufficiently sharp STM tips were fabricated with adequate Apiezon wax coatings, several STM tips were imaged using SEM. Figure 4.2a shows a backscattered SEM image of a typical tip. As this imaging method is mass-dependent, the light color at the end of the tip corresponds to heavier Au atoms and the dark areas corresponds to the Apiezon wax coating. Although the degree of coating was variable from tip to tip, the low leakage currents measured for most tips indicated good coverage.

Fig. 4.2b shows an XPS spectrum in the S 2p region for a bare Au STM tip and an STC-functionalized tip. The presence of a peak near 162 eV for STC-functionalized tips is indicative of Au-S bonds, presumably with the protein’s tetracysteine sequence. This was previously seen for OmcA and MtrC decaheme cytochromes from \textit{S. oneidensis} on Au(111) (Wigginton et al., 2007b). The presence of another S 2p peak near 168 eV is indicative of minor surface sulfate contamination on the bare Au tips.

STM imaging of the hematite (001) surface, as seen in Fig. 4.2c, shows relatively clean surfaces with clearly resolved atomic steps over a large scan area. Atomic resolution was not possible with these tips because their sharpness was not optimized for imaging atoms on hematite.
Figure 4.2. (A) Scanning electron micrograph of Apiezon wax-coated Au STM tip. Scale bar 10 µm. (B) XPS spectra of bare Au STM tip and STC-functionalized STM tip in the S2p region. See text for details. (C) STM image of hematite (001) used in this study. Scale bar 500 nm.

Bare Au tips on hematite (001)

Current-distance ($I-s$) profiles taken on hematite (001) in the absence of adsorbed STC were collected using a number of tips at several sample locations. Figure 4.3 shows a suite of individual $I-s$ measurements from 0–3 nm retraction spectra at two current setpoints (0.5 and 1 nA) for three different ionic strengths. The average curve is shown in black with error bars corresponding to standard error for each experiment. Approximately 100 curves were collected for each experiment before extreme outliers (e.g. due to electronic noise) were discarded. Distribution histograms of the tunneling limit distance, $s_0$, for each experimental condition ($s_0$ is defined as where the tunneling profile reaches ~0.0 nA tunneling current) are shown in the inset of each plot. For low ionic strength solutions, many of the $s_0$ values were outside the range that the tip was retracted and thus correspond to large bars in the histogram for the maximum value (i.e. 3 nm).
Figure 4.3. (A-C) Current-distance profiles collected with 1.0 nA initial setpoint for bare Au STM tips in 100 mM (A), 10 mM (B), and 1 mM KCl (C). (D-F) Current-distance profiles collected with 0.5 nA initial setpoint current for 100 mM (D), 10 mM (E), and 1 mM (F). All insets correspond to distribution of $s_0$ (defined in text) for each experiment.

Because the $I-s$ profiles have such a wide distribution, particularly at lower ionic strength, averaging the curves masks specific features found on each curve. Fig. 4.4 shows representative curves for each ionic strength collected using a 1.0 nA setpoint current indicating a significant change in slope, often a pre-exponential (i.e. ‘linear’) tunneling regime with ionic strength. This is better illustrated in a semi-logarithmic plot
of tunneling current where the point of significant change in initial slope is contracted with increasing ionic strength (Fig. 4.4b). Note: the discrepancy in the current ‘plateaus’ for the three spectra at the furthest tip-sample separation distance is due to slight changes in leakage current when exchanging solutions. However, the important features are seen at the beginning of the curves and do not appear to be affected by leakage current.

Figure 4.4. (A) Representative current-distance profiles collected with 1.0 nA initial setpoint current for 100 mM, 10 mM, and 1 mM KCl showing distinctive changes of slope. (B) Same information plotted on a semi-logarithmic scale.

STC-functionalized tips on hematite (001)

A similar range of experiments were conducted using STC-functionalized STM tips on hematite (001). Figure 4.5 shows a suite of measurements, along with the average curve with standard error bars, collected using one tip in 0–5 nm retraction curves across a range of experimental conditions. The inset shows $s_0$ distribution histograms for each experimental condition.
Figure 4.5. (A-C) Current-distance profiles collected with 1.0 nA initial setpoint for STC-functionalized Au STM tips in 100 mM (A), 10 mM (B), and 1 mM KCl (C). (D-F) Current-distance profiles collected with 0.5 nA initial setpoint current for 100 mM (D), 10 mM (E), and 1 mM (F). All insets correspond to distribution of $s_0$ (defined in text) for each experiment.

An additional observation for STC functionalized tips not seen with bare Au tips was that many spectra contained additional significant breaks in current in the primary decay region of $I-s$ spectra. These features were observed in roughly 10% of spectra collected with STC. A representative curve is shown in Fig. 4.6. These features were
quantified by measuring the current \( I_w \) and distance \( s_w \) where the exponential tunneling regime resumes as the tip is further withdrawn from the surface (see HAISS et al., 2004). The distributions of \( I_w \) and \( s_w \) values for 1.0 nA setpoints over four different STC-functionalized tips are shown in Fig. 4.6. The corresponding average position in each solution for \( s_w \) are 2.43±1.2 nm (1 mM), 2.72±1.42 nm (10 mM), and 2.91±1.7 nm (100 mM). Average \( I_w \) values for the same conditions are 0.344±0.19 (1mM), 0.532±0.31 (10mM), 0.543±0.1977 (100mM). Similar trends were observed for spectra collected with 0.5 nA setpoint.

\[ \text{Figure 4.6. (Left) Representative single-molecule tunneling profile for STC-functionalized Au STM tips. (Right) Histograms of } I_w \text{ and } s_w \text{ values for many STC-functionalized tips in different ionic strength solutions.} \]

4.4 DISCUSSION

Probing the electrical double layer at hematite (001)

The ionic strength dependence at constant surface potential on tunneling profiles, particularly for \( s_0 \) (Fig. 4.3), demonstrates that the tunneling limit is controlled at least in part by the electrical double layer at the hematite surface. These trends are consistent with a contracted double layer for higher ionic strengths and extended for lower ionic strengths. However, a significant portion of the averaged \( I-s \) curves exhibit an extended non-exponential tunneling region (Fig. 4.4). This behavior could be a function of several factors related to interfacial water and/or ordering of ions at hematite (001). Here we briefly discuss possible interpretations of how the electrical double layer is influencing the shape of these tunneling profiles.
Non-exponential tunneling profiles have been shown at very short distances (<1 nm) on a number of metallic surfaces to be caused by fluctuations in tunneling barrier height as interfacial water layers enter the tunneling junction (Halbritter et al., 1995; Hong et al., 1998; Hugelmann and Schindler, 2003; Nagy and Wandlowski, 2003; Hugelmann and Schindler, 2004; Woo et al., 2007). In these circumstances, tunneling is vacuum-like wherein the barrier height is strictly influenced by ordered ions and/or molecules at the interface. However, extended non-exponential decay profiles—out much further than the first few monolayers of water—are less well understood, particularly for semiconductor electrodes. Such features have previously been observed on semiconducting Fe-oxide films when the tip potential was specifically set within the band gap of the Fe-oxide (Diez-Perez et al., 2006a). In that system, chloride surface states transiently existing within the Fe-oxide band gap allowed for new tunneling pathways between the tip and Fe-oxide (Diez-Perez et al., 2006b). Similar non-exponential tunneling profiles have been observed on other semiconductor surfaces (Hiesgen et al., 2005) which have been attributed to the electrical double layer in the tunneling junction, but $s_0$ values for those spectra never reached beyond ~1.5 nm.

The source of extended non-exponential tunneling profiles at semiconductor surfaces is most likely a convolution of fluctuations in changes in the ordering of charge-compensating ions at the semiconductor surface and faradaic currents. The contributions of these factors is not simple to determine, especially on a wide band gap semiconductor like hematite where the potential of the surface and tip, as well as the bias magnitude, can significantly influence the degree of band bending, surface charge, and accumulation/depletion of charge carriers in the semiconductor (Hiesgen et al., 2001; Hiesgen et al., 2005). Fig. 4.7 shows an illustration of tip-induced band bending on hematite that could be occurring in our system. Here, a space charge layer (or depletion zone) is created because the potential of hematite is held positive of flatband potential.

Slight changes in the degree of band bending are likely occurring in hematite throughout the collection of $I$-$s$ spectra as the distance between hematite and tip is rapidly changed, but they are difficult to approximate. The observed differences in $I$-$s$ spectra for the three electrolyte solutions are consistent for distances set by both 0.5 nA and 1.0 nA setpoint currents (Fig. 4.3) which suggests band bending does not have an effect on the
overall shape of $I-s$ curves. Therefore the observed trends are independent of band bending (although subtle differences in the spectra collected at 0.5 nA and 1.0 nA setpoints could be explained by this phenomenon, particularly the deviation in initial current values relative to setpoint current).

![Figure 4.7](image)

**Figure 4.7.** (A) Band diagram representative of hematite in equilibrium with KCl solution. (B) The effect of the STM tip on the degree on localized band bending at the hematite surface assuming the tip is negatively charged with a positive bias voltage. $E_f$ is the Fermi level, CB is the conduction band, and VB is the valence band for hematite or Au tip, $V_b$ is defined as the bias voltage between tip and hematite.

The extension of $s_0$ with decreasing KCl concentrations (Fig. 4.3) is consistent with predictions based on electrical double layer models (Stumm and Morgan, 1996) where the double layer thickness is compacted as more negatively-charged ions are present in the electrolyte. The electrochemical potential at the hematite surface decays exponentially with $\exp(-\kappa r)$, where $\kappa$ is the inverse Debye length and $r$ is actual tip-substrate separation distance (an unobtainable quantity in this case). Ignoring for the moment surface charge, simple approximations for $\kappa^{-1}$ in 1 mM, 10 mM, and 100 mM KCl solutions are 9.6 nm, 3.04 nm, and 0.96 nm, respectively (Stumm and Morgan, 1996). It is plausible that $s_0$ may represent an approximation of double layer thickness because $\kappa^{-1}$ values for 100 mM and 10 mM are near $s_0$ values, but the large Debye length for 1 mM is certainly far too large to explain the average $s_0$ of ~4 nm. One intriguing alternative possibility that may be related to the double layer is the observation of
significant changes in initial slope observed for different ionic strengths (Fig. 4.4). Indeed, it has been suggested that large changes in slope in $I$-$s$ curves on semiconductors are likely the result of preferential ordering of ions in the tunneling junction (HIESGEN et al., 2005).

Surface charge is also predicted to be influenced by ionic strength, but the hematite surface potential was held constant at -0.21 V vs. Ag/AgCl in the EC cell with the bipotentiostat. Furthermore, the pH in our solutions does not vary enough to affect the surface charge of hematite. Therefore, the only changes in surface charge are due to changing ionic strengths which still supports the concept that the thickness of the double layer has an impact on $s_0$.

**Influence of STC on tunneling profiles**

The $I$-$s$ spectra collected on hematite with STC-functionalized Au STM tips are significantly different than those collected with bare Au STM tips. The trend of increasing $s_0$ with decreasing KCl concentrations is again reflective of changes in the double layer structure with ionic strength; however, the values of $s_0$ for STC-tips are much larger than in the case of bare Au tips (compare Figs. 4.3 and 4.5). For 100 mM KCl solutions, $s_0$ is only extended a few Ångstroms when STC-functionalized tips are used, but in 10 mM KCl, $s_0$ is lengthened by over 2 nm and in 1 mM KCl solutions, $s_0$ increases by over 5 nm.

The question then arises as to how or why does the tunneling limit significantly extend $s_0$ values when STC is present in the tunneling junction? We begin to answer this question by considering the non-adiabatic limit for electron tunneling in solution (~15 Å). The spectra shown in Fig. 4.5 suggest electron transfer much further beyond this typically expected limit. Part of the reason that this has not been observed before, particularly in experiments on adsorbed monolayers of metalloproteins, could be that our substrate is a semiconductor whereas metallic electrodes (e.g. Au, HOPG) have historically been used. The unique electronic structure, perhaps even localized to surface states in hematite, could be responsible for the extension of tunneling distances, but it is most likely related to the fact that for semiconductors, there is expected to be a much thicker double layer than for metal electrodes. When STC is in the presence of this double layer, it apparently
reorganizes the ordering of water molecules and charge compensating ions to the point that electron tunneling occurs at much larger distances.

However, before fully committing to this conclusion, let us consider the geometry of STC molecules on the STM tip. Assuming STC coats the Au STM tips in a similar fashion to how it adsorbs on Au(111) (ROSSO et al., 2008), we can expect monolayer coverage of STC on the tip. STC is a small cytochrome considering its number of heme groups, but is relatively large molecule (~2–4 nm in diameter) for single-molecule tunneling, and as such, only one molecule is likely to be present in the tunneling junction at a time. The presence of one STC molecule will certainly influence the ordering of charge-compensating ions in the tunneling junction however, the tunneling limit doesn't just extend 2–4 nm further based on the molecular size of STC.

It is not predicted that the conformation of STC will change dramatically when going from 100 mM KCl to 1 mM KCl that these differences could be accounted for by changes in the diameter of the molecule. Instead, what is likely happening is that STC influences the double layer by preventing proper ordering of charge-compensating ions at the hematite surface until the molecule has extended far enough away that ions can properly align. In fact, due to the relatively high tunneling current setpoints (0.5 and 1 nA), STC could be coming in physical contact with hematite wherein the tip-sample separation distance for such imaging conditions could be much smaller than the STC molecule itself. One study, however, suggests that at modest imaging conditions, tip-sample separation distances at engagement with the surface can be as high as 3 nm in water (ALLIATA et al., 2004), opening up the possibility that little initial deformation of STC is expected to occur.

Estimating the precise tip-substrate separation distances in our experiments is not straightforward given that methods used to interpret changes in tunneling current, conductance, or barrier height to establish physical contact between tip and substrate are not well established. This is because of the fast that there is no appreciable difference in resistance from solution to semiconductor (HIESGEN et al., 2005). It can reasonably be assumed, however, that there will indeed be differences in tip-substrate separation distance between bare Au STM tips and STC-functionalized tips, but this difference does not explain the significant extension of tunneling profiles. Theoretically, the actual tip-
sample separation distance would be predicted to closer for STC-functionalized tips as compared to bare Au tips because besides its four heme cofactors, the bulk of the STC molecule is a generally less-conductive polypeptide matrix. Moreover, previous tunneling spectroscopy measurements of STC indicate that tunneling current is not likely to be strongly influenced by heme electronic states but instead, controlled by a coherent elastic tunneling process (ROSSO et al., 2008), similar to that observed for the decaheme cytochrome OmcA (WIGGINTON et al., 2007a). This electron transfer mechanism would support shortened $s_0$ values, which is not the behavior that we observed. To the best of our knowledge, there appears to be only one other study in the literature using metalloprotein-functionalized STM tips (ALESSANDRINI et al., 2005), and it did not report any effort to estimate tip-substrate distances or distance-dependent tunneling.

The electronic tunneling barrier ($\phi$), which controls the initial tip-substrate separation distance, is likely to vary when a biomolecule is present in the tunneling gap. As a result, the absolute distances reported for $s_0$ are only qualitatively comparable. Furthermore, proteins adsorbed on semiconducting surfaces have been shown to affect band bending which also influences the energetics of electronic tunneling (CAMPBELL et al., 2007). Although variations in tunneling barrier height between bare STM tips and STC-functionalized tips will undoubtedly influence the tunneling probability for a given experiment, the differences observed are unlikely to be explained simply by changes in barrier height. For example, barrier heights have been shown to stay relatively uniform for metals until a relatively sharp decrease at 3 Å separation distance (i.e. within or the first adsorbed water monolayer) (HAHN et al., 1998). Estimating barrier heights for these $I-s$ curves is difficult due to the significant changes in slope of the $I-s$ curves (Fig. 4.4).

An alternative and much less arbitrary way to quantify the differences in the tunneling profiles besides barrier height and $s_0$ is to estimate the decay parameter $\beta$ which is defined by the exponential form $I = A \exp(-\beta d)$ where $A$ is a constant and $d$ is an approximation for separation distance. Curve fitting was performed on the exponential component of representative spectra for bare Au STM tips (see Fig. 4.4) and STC-functionalized tips. The results are shown in Table 4.1.
Table 4.1: Decay length ($\beta$) estimates for three electrolyte concentrations.

<table>
<thead>
<tr>
<th>[KCl] (mM)</th>
<th>$\beta$ (Å$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare Au</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>4.03</td>
</tr>
<tr>
<td>10</td>
<td>2.38</td>
</tr>
<tr>
<td>1</td>
<td>1.95</td>
</tr>
<tr>
<td>STC</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>4.76</td>
</tr>
<tr>
<td>10</td>
<td>0.78</td>
</tr>
<tr>
<td>1</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Although $\beta$ for 100 mM $I$-$s$ spectra are very similar, it is clear that the decay length is much further extended for STC-functionalized tips for both 10 mM and 1 mM KCl solutions. In previous studies, $\beta$ values on the order of 0.8 Å$^{-1}$ have been observed for other multi-center proteins through a variety of techniques (PAGE et al., 1999; MURGIDA and HILDEBRANDT, 2004; DAVIS et al., 2006; TRAMMELL et al., 2007). It is unknown if this quantity is comparable between these various experiments and our unique, semi-conducting tunneling junction. However, the general trend of an extension of tunneling profiles with STC-functionalized tips is good evidence to suggest some component of the STC molecules are influencing electron transfer. A single STC molecule could provide multiple tunneling channels of variable packing density between the Au tip and hematite which would therefore cause a variation between our measurements and those of previous experiments. This becomes increasingly important if the tip is not sharp enough (CAMPBELL et al., 2007) as is likely the case in our experiments. A clue into this process may reside in the features observed in Fig. 4.6, which we will now describe in detail.

**Single-molecule tunneling through STC**

The significant breaks in slope, or in some cases actual peaks, in $I$-$s$ curves for STC-functionalized tips (Fig. 4.6) and their associated current ($I_w$) and distance ($s_w$) distributions are evidence to suggest individual STC molecules participating in the tunneling process. Recent studies of small organothiols (HAISS et al., 2004; WIERZBINSKI and SLOWINSKI, 2006), redox-active organic molecules (HAISS et al., 2003) and ds-DNA (WIERZBINSKI et al., 2006) have shown distinct breaks in $I$-$s$ curves similar to our results.
which indicate breaks in electrical contact between molecule and substrate or tip when the molecular conformation is changing due to tip-molecule interactions. In these cases, only 1-5% of curves show characteristic breaks in tunneling current (Wierzbinski et al., 2006; Wierzbinski and Slowinski, 2006), which is less frequent than the features we observed in our system. These features are therefore ascribed as single-molecule tunneling spectra dependent upon molecular orientation, electronic coupling, and tip-sample separation distance.

Single molecule conductance in metalloproteins has previously been shown to be linked to compressional forces, which induce conformational changes in the protein (Zhao et al., 2004). Conformational changes in proteins have been shown to trigger electron transfer by rearranging the heme cofactors to more favorable configurations relative to the substrate (or other hemes for the case of intramolecular electron transfer) (Page et al., 1999; Gray and Winkler, 2003). In our system, conformational changes could be occurring either from the strong electric field present in the tunneling junction, or from actual physical contact to the hematite surface. In any case, for the relatively rare instances where these features are observed, the initial conformation of STC in the does not provide the most efficient tunneling pathway. During retraction, however, STC refolds or reorients itself to increase electronic coupling to hematite until finally, normal exponential-like tunneling occurs once the STC molecule is pulled far enough away from the surface.

Considering the structure of STC, specific tunneling pathways may be responsible for the change in electron transfer efficiency. It has been suggested that STC has a chain-like heme architecture which suggests a pathway for directional electron transfer (Harada et al., 2002; Akutsu and Takayama, 2007), but other interpretations of crystal structure suggest each heme can participate in unidirectional electron transfer, or ‘electron-harvesting’ (Leys et al., 2002). This latter behavior would allow STC to be a more universal redox partner because electron transfer could occur when just one heme is energetically aligned with the potential of hematite (or the double layer). Recent molecular modeling simulations have suggested that the relative orientation of hemes strongly influences electronic coupling and thus electron transfer rates (Smith et al., 2006; Kerisit et al., 2007).
The significant number of current break/peak observations in $I$-$s$ profiles taken with STC-functionalized tips could indeed suggest a preferred tunneling pathway through STC, however, the lack of grouped $I_w$ values around many fundamental current values, as observed in other cases for much smaller molecules (e.g. Haiss et al., 2004), was not observed. This disparity is likely due to the fact that the STC molecule is so large that only one molecule is predicted to reside in the tunneling junction (and therefore only one fundamental current value is likely to be a result). What is worth noting, however, is a weak dependence of these values on ionic strength (Fig. 4.6). The overall position of these features, quantified by $s_w$, extends slightly (~5Å) with increasing ionic strength. This trend is opposite of what is observed for $s_0$ values on the average STC spectra and is therefore not related to the changing thickness of the electrical double layer. The slight increase in $I_w$ values with ionic strength could be related to increased ionic conductance in higher ionic strength solutions, but further investigations would be required to verify this.

**Implications for bacterial metal reduction**

The modification of the electrical double layer on hematite (001) by STC has consequences for bacterial metal reduction and cell-mineral interaction forces. One can get an estimate for the general physical interaction between cells (or cytochromes) with a mineral surface by considering previously measured force-distance data. *S. oneidensis* has been shown to exhibit attractive forces to another Fe-oxide, goethite (FeOOH), when the cell and mineral are separated by upwards of ~10 nm or more depending on ionic strength (Lower et al., 2005). Conversely, repulsive forces between hematite AFM probes and monolayers of the outer-membrane cytochromes OmcA and MtrC were measured out to ~50 nm (Lower et al., 2007). These electrostatic and/or steric forces would presumably cause realignment of polar water molecules and rearrangement of ions at both cell and mineral surfaces which would change as the separation distance was decreased up to the point of contact. The fact that these electrostatic forces are experienced at distances beyond the measured tunneling limits in the present study suggest that the electrical double layer might not only play a role in determining the
physical recognition of mineral surfaces by metal-reducing bacteria, but influence the reducing power of multiheme cytochromes at the interface.

It might very well be that certain cytochromes have specific roles at the surface of a mineral such as hematite. *S. oneidensis* cytochromes exhibit a wide range of redox potentials indicating that the electron transport chain is energetically designed to efficiently reduce a host of electron acceptors. The hemes within MtrC alone operate between +100 and -400 mV vs. NHE (HARTSHORNE et al., 2007) which suggest that it is likely flexible in terms of electron-accepting substrate. Furthermore, OmcA and MtrC were recently shown to exhibit different electron transfer mechanisms to Au(111) in which OmcA promotes fast superexchange electron tunneling and MtrC electron transfer is strongly orbital-mediated (WIGGINTON et al., 2007a). By also modifying the electrical double layer at mineral surfaces, the cytochromes also play a passive role in increasing electron transfer efficiency. Supporting evidence is found in which *S. oneidensis* communities have been shown to significantly alter the open circuit potential of anodes in microbial fuel cells (MANOHAR et al., 2008). If bacteria and their electroactive cytochromes are able to alter the surface potential of minerals in nature, this could influence the rate of cytochrome-mediated electron transfer.

In order to fully understand the complex nature of bacterial metal reduction at mineral surfaces, researchers have routinely used a number of experimental and computational techniques from several fields. One underlying component of this interface that until now had remained relatively unexplored is the maximum separation distance between a cytochrome on the outer-membrane of a cell and a mineral such as hematite in order for electron transfer to occur. Our study attempted to use a simple system of purified multiheme cytochromes and hematite as a beginning step towards measuring this quantity. We found that multiheme cytochromes have the ability to modify the ordering of interfacial water and charge-compensating ions in the electrical double layer so that electronic tunneling is probable at extended distances away from the mineral surface. This observation has implications towards understanding electron transfer rates and respiration efficiency whereas cytochromes could presumably reduce a mineral ‘directly’ more frequently outside the typical limit of nonadiabatic electron transfer. It is unknown exactly how electron shuttling molecules, for example, would
interact with the electrical double layer under similar conditions, and we leave the possibility of a similar investigation for future work. Indeed, STM tips have been functionalized with Fe-protoporphyrins (BIZZARRI and CANNISTRARO, 2005) to investigate tunneling mechanisms and could present a fruitful area for future research. Additionally, we observed changes in tunneling efficiency resultant from conformational changes in STC molecules. Combined with electrical double layer experiments, these results suggest that not only do cytochromes have specific roles in mediating interfacial electron transfer, but they may also be intimately related to long-range electron transfer processes occurring at the cell/mineral interface.

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uranium(VI) by the metal-reducing bacterium Shewanella putrefaciens. Geochim Cosmochim Ac 66, 3247-3262.


Appendix A: Monolayer structure of multiheme cytochromes on Au(111) and Si(100) determined by X-ray reflectivity

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ABSTRACT

X-ray reflectivity measurements on monolayers of bacterial multiheme cytochromes on Au(111) and Si(100) surfaces were performed to interrogate vertical heme distribution within the monolayers relative to the surface. The decaheme cytochromes OmcA and MtrC, and the small tetraheme cytochrome (STC) from Shewanella oneidensis have previously been shown to transfer electrons via different mechanisms to Au(111) and it is hypothesized that the electronic coupling of hemes to the surface is responsible for these differences. Here, we attempt to resolve the structure of these cytochrome monolayers on Au(111) and Si(100) surfaces in order to better understand their interfacial electron tunneling behavior. Initial attempts at resolving an ordered heme distribution were unsuccessful but information on the bulk structure of cytochrome films was obtained.

A.1 INTRODUCTION

Multiheme cytochrome proteins are the principal electron-transducing agents in metal reducing bacteria (MRB) (Shi et al., 2007). Solid-phase electron acceptors such as Fe(III)-oxides are principally reduced by outer-membrane cytochromes because the cells must transfer electrons extracellularly, where solid phase redox-active metals predominate in circumneutral waters. In Shewanella oneidensis, a well-characterized MRB, the two primary cytochromes responsible for Fe(III) reduction are the decaheme cytochromes OmcA and MtrC (Beliaev et al., 2001; Myers and Myers, 2001; Myers and Myers, 2003). These cytochromes appear also to be linked to adhesion processes during metal reduction (Lower et al., 2005; Lower et al., 2007; Lower et al., 2001), suggesting an intimate link between electron transfer and contact to solid-phase electron
acceptors. For at least three reasons, little is known about the molecular mechanisms that control cytochrome-mediated reduction of solid-phase electron acceptors: 1) no crystal structure yet exists for an outer-membrane metal reductase, 2) such proteins are difficult to purify due to their inherent hydrophobic nature, and 3) the combination of environmental materials with biological systems has only recently been attempted on a molecular scale. Therefore, in addition to substantial efforts directed towards revealing the molecular structure of these important proteins, novel methods must be developed for interrogating their enzymatic and physiologic properties.

One such method that was recently employed to understand the molecular electron transfer mechanisms of these proteins is scanning tunneling microscopy (STM) (WIGGINTON et al., 2007b). This method, coupled with tunneling spectroscopy (TS), allows for single-molecule measurements of electronic tunneling with the promise of extracting electron transfer mechanisms and parameters from tunneling spectra (ROSSO et al., in prep; WIGGINTON et al., 2007a). In this design, multiheme cytochrome films are formed on Au(111) substrates. The orientation of the molecules is assumed to be in a uniform manner, as is the conformation and activity, however, STM images do not allow for the determination of submolecular structure or bulk film properties. It would be useful to know, for example, how well coupled various heme groups are to the solid surface, and if the structure of the molecule is different on the surface compared to its crystal structure. Efforts to determine protein structure using Molecular Dynamics simulations are only possible with cytochromes that have a known crystal structure, making some soluble cytochromes intriguing candidates (ROSSO et al., in prep) but eliminates the outer-membrane cytochromes. What is truly required is an experimental method that is sensitive to the cytochrome film structure on a nanometer scale.

This report describes efforts to determine the structure of cytochrome films on solid surfaces using specular X-ray reflectivity (XRR). This technique is attractive because submolecular structure of large biomolecules might be possible if the orientation of the molecules within the film is uniform and stable. XRR has been shown to be a sensitive technique for determining protein conformation at interfaces (GIDALEVITZ et al., 1999) as well as metal ion distribution within organic films (GIBAUD and HAZRA, 2000). Such measurements then could hypothetically detect the vertical distribution of heme...
groups relative to the solid surface. XRR also allows precise measurements of film thickness ($d$), roughness ($\sigma$), and scattering length density ($\rho$), which would give estimates of the film coverage and overall structure (GIBAUD and HAZRA, 2000).

Such measurements have been done on other protein films at various interfaces (BOUCHER et al., 2007; GALLANT et al., 1998; GIDALEVITZ et al., 1999; KENT et al., 2004; KENT et al., 2005; LI et al., 2003). We performed high-resolution XRR measurements for two outer-membrane decaheme cytochromes (OmcA and MtrC) and one tetraheme cytochrome (STC) from $S$. oneidensis on Au(111) and Si(100). The spectra were interpreted using refined curve-fitting algorithms based on a multilayer model.

![Figure A.1. Schematic of X-ray reflectivity spectroscopy with definitions of incident angle and Q.](image)

**A.2 METHODS**

### A.2.1 Sample preparation

Cytochrome films were prepared according to previous protocol (WIGGINTON et al., 2007b) with the following exceptions: Au(111) films from Molecular Imaging (Tempe, AZ) were made atomically flat by following a template-stripping procedure (WAGNER et al., 1995) using Si(100) wafers as supporting substrates. Similar Si(100) wafers were also used as cytochrome film substrates because the electron density contrast between the organic protein matrix and Si is much lower than that of organics and Au. Au(111) and Si(100) substrates were cleaned with piranha solution (75% H$_2$SO$_4$, 25%
H$_2$O$_2$) prior to cytochrome incubation. Each substrate was entirely submerged in a dilute cytochrome solution and allowed to incubate overnight at 4°C.

STC films were made without the addition of octyl-β-D-glucopyranoside (OGP), a detergent that is used to solubilize the outer-membrane cytochromes and advantageously forms a stabilizing overlayer on the cytochrome films. Similar to OmcA and MtrC, STC contained a tetra-cysteine (C4) sequence at the C-terminus for efficient purification (Shi et al., 2005) and binding to Au(111) (Ross et al., in prep). Although it was observed with STM that STC without a C4 tag (i.e. STC wild-type) did not form stable monolayers for imaging (Ross et al., in prep), we tested additional films of STC(wt) in an attempt to further explore these results.

A.2.2 Data collection

XRR measurements were collected under ambient conditions on beamline 2-1 at the Stanford Synchrotron Radiation Laboratory. To minimize beam damage to the samples, scans were collected as fast as possible and the shutter was held closed unless collecting spectra. All films were run in triplicate; the first spectra was collected at the aligned height, the second spectra was collected with the stage translated 2.5 μm in the x-direction, and the third taken again at the original location to test for beam damage. Duplicates for each cytochrome/substrate combination were run to measure consistency between samples.

A.2.3 Curve-fitting

Spectra were normalized to background reflectivity and then modeled using Parratt32 v. 1.6. (Christian Braun, HMI Berlin) using a least-squares fitting routine. Spectra for bare substrates were fit initially to get an estimate for the surface of the ‘bulk’ (i.e. substrate) component of the model before modeling the cytochrome films. A 2–4 Å adventitious carbon film was added as a top layer on the substrates to account for surface contamination and resulted in better fits. One representative spectrum for each sample was used for model fitting. Electron density profiles were also calculated using Parratt32.
A.3 RESULTS AND DISCUSSION

A.3.1 Si(100) substrates

The reflectivity data and corresponding modeling results on Si(100) are presented in Fig. A.2 and have been tabulated in Table A.1. Two primary conclusions can be made from these results regarding the structure of cytochrome films on Si(100). First of all, the presence of an adventitious carbon layer was required for accurate model fitting of the data. It is unknown how this layer will interact with the cytochromes during incubation with Si(100), but it should be accounted for. Secondly, the cytochrome film thicknesses are on the order of that seen for individual molecules, suggesting monolayer dispersion on Si(100). For example, MtrC films were shown to be ~4.6 nm thick which correlates very well to our previous measurements of MtrC molecular sizes using STM. However, STC films are slightly thicker than predicted and OmcA films are slightly thinner than predicted.

The reason for this discrepancy is not entirely clear at the moment but we have reason to suspect that there was human error involved with cytochrome sample preparation and that OmcA and STC samples were accidentally switched. Based on previous estimates of OmcA thicknesses from scanning probe microscopies and OWLS (EGGLESTON et al., 2008; WIGGINTON et al., 2007a), OmcA is predicted to be ~6–8 nm in diameter, which corresponds quite well with our measurements of STC. Furthermore, the 2–4 nm diameter of STC is well known because its crystal structure has been solved, and the thickness actually corresponds quite well with our measurements of OmcA. Assuming this error, STC and OmcA films would therefore correspond to monolayer thicknesses as well, but confirmation of this hypothesis has not yet been possible due to the difficulty in arranging time at the synchrotron facility.
Figure A.2. Representative reflectivity and model fits for Si(100) and three cytochrome/Si(100) samples.
Table A.1. Summary of model fitting parameters from XRR measurements for three cytochrome films on Si(100). Units for film thickness (d), scattering length density (ρ), and roughness (σ) are in Å, Å⁻², and Å, respectively.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Bulk Si</th>
<th>Adventitious carbon</th>
<th>Cytochrome film</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>-</td>
<td>4.0</td>
<td>-</td>
</tr>
<tr>
<td>ρ</td>
<td>2e-5</td>
<td>9.4e-6</td>
<td>-</td>
</tr>
<tr>
<td>σ</td>
<td>10</td>
<td>4.1</td>
<td>-</td>
</tr>
<tr>
<td>STC</td>
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<td></td>
</tr>
<tr>
<td>d</td>
<td>-</td>
<td>2</td>
<td>61.5</td>
</tr>
<tr>
<td>ρ</td>
<td>2e-5</td>
<td>5.9e-6</td>
<td>1.2e-5</td>
</tr>
<tr>
<td>σ</td>
<td>3.7</td>
<td>3.0</td>
<td>36.6</td>
</tr>
<tr>
<td>OmcA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>-</td>
<td>3.2</td>
<td>24.4</td>
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<tr>
<td>ρ</td>
<td>2e-5</td>
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<tr>
<td>σ</td>
<td>4.9</td>
<td>3.0</td>
<td>13.5</td>
</tr>
<tr>
<td>MtrC</td>
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</tr>
<tr>
<td>d</td>
<td>-</td>
<td>2.6</td>
<td>46.0</td>
</tr>
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<td>ρ</td>
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</tr>
<tr>
<td>σ</td>
<td>4.2</td>
<td>2.6</td>
<td>35.9</td>
</tr>
</tbody>
</table>

Figure A.3 shows the raw reflectivity data converted into an electron density profile at the Si(100) interface. Each profile shows a smooth profile away from Si(100) indicating relatively homogeneous monolayers in terms of electron density. The lack of an observable concentrations in electron density within the films—perhaps as a result of more dense Fe atoms ordering within low-contrast organic material—can be interpreted in two ways: 1) the sensitivity of the experimental method was not sufficient enough to detect uniformly aligned Fe atom positions within the molecular monolayers, or 2) there was no regular ordering of Fe atoms (i.e. heme groups) within the films. The first interpretation is not likely because previous experiments have been able to resolve monolayers of adsorbed metal atoms within less electron-dense films (GIBAUD and HAZRA, 2000). Therefore, we conclude that although the films are one monolayer thick, they are not uniformly ordered. This result is not surprising because the cytochrome molecules are presumably only electrostatically bound to Si(100) whereas with Au(111) substrates, the cytochromes are chemically attached through Au-S linkages. This
observation demonstrates the fact that molecular orientation on substrates can be strongly influenced by the adsorption mechanism.

Figure A.3. Normalized electron density profile of Si(100) and three cytochrome films relative to the air/Si(100) interface.

A.3.2 Au(111) substrates

The reflectivity spectra for cytochrome films on Au(111) are presented in Figure A.4. These spectra are much more difficult to interpret in comparison to Si(100) based on the very slight deviations in reflectivity from bare Au(111) films. The lack of any spectral features or significant difference in reflectivity is most likely due to the strong contrast in electron density between Au and organics wherein the Au shields the organics. We were therefore unable to model the reflectivity with any degree of confidence. This severely limits our ability to extract virtually any useful conclusions from reflectivity measurements on Au(111) and for all intents and purposes, cannot resolve any structural information for cytochrome films on Au(111).
Figure A.4. Representative reflectivity spectra for Au(111) and three cytochrome films.

A.3.3 Implications for future work

Although the results from the reflectivity measurements were not successful in achieving submolecular resolution of the cytochrome films (i.e. heme position relative to substrate), information about the overall film structure on Si(100) was still learned. The cytochrome films show good coverage and thicknesses near their expected values. Unfortunately, the results on Au(111) were inconclusive because the electron contrast was too high between the organic protein matrix and Au whereas this effect is not expected for films on Si(100) due to similar electron contrast between Si and C. Future experiments using neutron sources might minimize this effect on Au(111) but difficulties remain in sample preparation (i.e. very large and flat substrates are needed). Alternatively, another way to take advantage of the more compatible Si(100) substrate while still probing ordered cytochrome films would be to functionalize Si(100) with molecules that would facilitate oriented cytochrome binding (e.g. self-assembled...
monolayers of organothiol molecules). This could also be done on Au(111) to shield the strong differences in electronic density between protein and substrate but it is unknown how thick the SAM would have to be in order for it to be an effective electron shield. In either of these cases, the cytochromes wouldn’t necessarily be interacting with a solid surface, which is what we’re most interested in, but these experiments might allow for the detection of ordering in the cytochrome monolayers with the possibility of the measurement of vertical heme distribution. These would be important quantities for coupling experimental STM/TS data on cytochrome monolayers with theoretical calculations—a challenge the lies ahead for further molecular scale investigations of mineral-microbe interactions.

ACKNOWLEDGEMENTS

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REFERENCES


Appendix B: Further investigations of decaheme cytochrome monolayers with STM

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INTRODUCTION

This appendix describes three scanning tunneling microscopy (STM) experiments on decaheme cytochromes from Shewanella oneidensis MR-1 that produced interesting results but that did not fit into any of the above chapters. Here, we document the rare observance of submolecular resolution within OmcA monolayers, the self-assembly of soluble MtrA molecules without a recombinant tetracysteine sequence, and STM imaging of mixed monolayers of OmcA and MtrC in hopes of understanding how they behave as a protein complex.

B.1 SUBMOLECULAR RESOLUTION OF OMCA

The STM imaging efforts for OmcA monolayers suggested a film ‘activation’ process lowering the bias voltage to increasingly negative values. Once a threshold was reached (~-1.5 V), the film morphology was drastically altered and showed good resolvable protein molecules instead of terraces with low conductivity ‘pits’ (See Fig. 2C for illustration of this process). This was reproducible in over 90% of the imaging experiments and led us to hypothesize a model for the structure of the cytochrome films, which, for the case of OmcA and MtrC, encompassed a detergent overlayer above the immobilized cytochromes.

However, in a few rare instances, a different yet related activation procedure was possible when imaging at low current setpoints. This involved repeated scans over a particular area on ‘unactivated’ terraces until the progressive revelation of cytochrome molecules occurred. An example of this process is shown in Fig. B.1. Here, this figure shows eight consecutive scans across an area, with Au(111) steps and ‘pit’ features clearly visible on terraces. Upon several scans, molecules slowly come into view,
initially on the uppermost terraces, leading to the speculation that progressive scans slowly shed away the detergent overlayer. During this prolonged activation process, submolecular features are clearly evident.

![Image of STM scans](image)

*Figure B.1. A series of eight consecutive low-current scans (A-H) of OmcA monolayers progressively transitioning from ‘unactivated’ to ‘activated’ terraces on Au(111). Scale bar in all images in 25 nm. Bias voltage +1.08 V, tunneling current setpoint 50 pA.*

A higher-resolution image (Fig. B.2) shows localized bright spots within individual OmcA molecules, which are believed to be heme centers. The number of resolvable hemes in this image ranges from one to five during activation.

![Image of zoomed STM scan](image)

*Figure B.2. Zoomed in area of STM image (same as Fig.1c) showing several OmcA molecules with localized areas of increased conductivity (A). Estimated outlines of ~10 nm OmcA molecules that have yet to become activated (B). Scale bars 5 nm.*
If this process is made reproducible, it would be an unprecedented observation with STM on multicenter metalloproteins. Rarely have submolecular features been observed with STM on simple single-center metalloproteins (FRIIS et al., 1999), but in those instances, they have resulted in major breakthroughs in the field. Unfortunately, further efforts to reproduce these observations on OmcA were unsuccessful, and no similar effect was seen for any other multiheme cytochrome from *S. oneidensis* including MtrC, MtrA, or STC.

**B.2 MTR A SELF-ASSEMBLY**

To compare with our STM/TS experiments on MtrC and OmcA, we attempted to image another decaheme cytochrome, the periplasmic MtrA. No tetracysteine sequence was needed for purification (SHI et al., 2005), so the construct we had available was native MtrA.

As was previously reported in Chapter 2, MtrA (as well native MtrC without a recombinant C4 tag) did not form stable monolayers. However, sub-monolayers were observed for MtrA (Fig. B.3). Interestingly, upon successive scans, the molecules appear to self-assemble into wire-like structures on Au(111) terraces. This could perhaps be the result of MtrA molecules stabilizing themselves to reduce unfavorable hydrophobic interactions with Au(111). However, this is unlikely as MtrA is a soluble cytochrome and should remain stable with a monolayer of adsorbed water.

![Figure B.3. Series of four consecutive scans (A-D) of MtrA monolayers self-assembling into 'wire'-like morphologies on Au(111). Scale bar in all images in 50 nm. Bias voltage +2.0 V.](image)

It remains to be seen why exactly MtrA molecules self-assembled, but the fact that *S. oneidensis* produces extracellular nanowire appendages rich in cytochromes to transfer electrons could reveal a plausible explanation (GORBY et al., 2006). Perhaps
MtrA molecules form the core of these nanowires and are designed to self-assemble into a wire-like protein supercomplex when a critical mass of MtrA molecules is reached.

**B.3 OMCA/MTRC MIXED MONOLAYERS**

OmcA and MtrC from an active protein complex that has been shown to be more active (i.e. higher reduction rates) than OmcA or MtrC alone (Shi et al., 2006). We attempted to not only resolve this complex with STM imaging, but to use tunneling spectroscopy to interrogate its electron transfer mechanisms in comparison to either cytochrome alone. We made equimolar mixtures of OmcA and MtrC and incubated Au(111) following the procedure in Chapter 2.

STM images show roughly two molecular sizes groups within the mixed monolayer: ~5 nm and ~8 nm (Fig. B.4A). These very closely match the molecular sizes of MtrC (5 nm) and OmcA (8 nm) observed with STM in Chapter 2. This is convincing evidence to support the claim that mixed monolayers are forming on Au(111). However, no evidence of OmcA/MtrC protein complexes were observed despite multiple preparation procedures and repeated STM experiments.

![Figure B.4](image)

*Figure B.4. STM image of OmcA/MtrC mixed monolayer (A). Scale bar 50 nm, imaging conditions 1.08 V, 0.5 nA. Cross section across four molecules showing apparent STM height for larger OmcA molecules and smaller MtrC molecules (B). Histogram of all protein molecules in image showing a bimodal height distribution.*
As predicted from the results in Chapters 2 and 3, MtrC and OmcA should have markedly different tunneling behaviors. Specifically, OmcA is suggested to mediate tunneling more efficiently to Au(111). This should be evident in two separate but related lines of evidence: apparent height contrast in STM images and 2) differences in tunneling spectra. We first examined the apparent height distribution of the image shown in Fig. B4. Based on cross-section analysis (taken at the green line shown in 4A), the larger (i.e. 8 nm) proteins appear to be about ~20% ‘taller’ than the smaller (i.e. 5 nm) proteins. The histogram shows a bimodal distribution of the molecular height suggesting both molecules are significantly present in the image.

If the smaller/shorter proteins are indeed MtrC and the taller/higher proteins are OmcA, then the theory of OmcA’s “enhanced conductivity” might seem appropriate, especially considering that differences in tunneling barrier heights are expected to be minimal between MtrC and OmcA. However, the height contrast between the two proteins may also simply be a function of their molecular size.

Preliminary $I-V$ spectra collected over several molecules all appear to only resemble that of MtrC (i.e. smooth, exponential behavior in the negative bias region, multiple inflection points in the positive bias region). No spectra resembled OmcA-type tunneling in the positive bias range. Similarly, the features in $(dI/dV)/(I/V)$ almost perfectly mirror that of MtrC (Fig. B.5). The exclusive appearance of MtrC-like spectra could suggest intra-protein electron transfer and demonstrate the functionality of the OmcA/MtrC protein complex, but that remains to be inconclusively determined.

### B.4 SUGGESTIONS FOR FUTURE WORK

Each of these studies could warrant further investigations. For instance, if a reproducible imaging mode is developed for the submolecular resolution of OmcA, it would be a major breakthrough in molecular electronics and biophysical chemistry. One way to optimize the progressive film activation process is by testing various surface coatings, detergents, and buffer molecules. Some of these may be more or less prone to form stable overlayers on the cytochrome monolayers. Additional comparison of MtrC and OmcA tunneling mechanism to the decaheme MtrA could potentially be aided by designing a C4 construct for MtrA to help it efficiently bind to Au(111) and form complete monolayers. For mixed monolayers of OmcA and MtrC, various solutions
could be tested to ensure a formation of a protein complex. Our repeated efforts to demonstrate a stable OmcA/MtrC complex both with STM and with electrophoresis and Western blots were inconclusive. Certain detergents and/or solution conditions may be more conducive to protein complex stabilization. Additionally, imaging sub-monolayers of OmcA/MtrC ‘complexes’ may be useful for determining the quaternary structure of the cluster; this could be done by exposing a more dilute solution of proteins to Au(111).

Figure B.5. Normalized differential conductance [(dI/dV)/(I/V)] for OmcA/MtrC mixed monolayers (orange line) showing similar spectral features to MtrC monolayers (blue line).

REFERENCES


Appendix C: Scanning tunneling microscopy of the small tetraheme cytochrome c from *Shewanella oneidensis*

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**ABSTRACT**

We performed a scanning tunneling microscopy (STM) study of the small tetraheme cytochrome c (STC) from the metal-reducing bacterium *Shewanella oneidensis* MR-1. STM images of STC monolayers—which contained a recombinant tetracysteine sequence for efficient chemisorption to Au(111)—show good coverage and size expected from the crystal structure. Rigorous tunneling spectroscopy (TS) measurements taken at various bias voltages and distances from the initial position defined by the current setpoint were taken on individual molecules to determine the interfacial electron transfer mechanism to Au(111). Based on interpretation of the tunneling spectra, electrons are transferred between STC and Au(111) through a fast superexchange mechanism. This is similar to another multiheme cytochrome from *S. oneidensis*, OmcA, which suggests that these two cytochromes similarly electronically coupled to solid surfaces through one or more of their many heme groups. This study has implications towards determining the mechanisms of protein-mediated electron transfer at solid surfaces.

**C.1 INTRODUCTION**

Protein electron transfer to solid surfaces is of interest to a number of fields, from molecular electronics to environmental science. Many studies that seek to interrogate interfacial electron transfer mechanisms immobilized proteins do so with bulk electrochemical techniques such as protein film voltammetry (PFV). Although PFV is important for determining electrochemical reaction rates, it does not provide a molecular scale understanding of the processes that are occurring. When native proteins are in contact with a solid substrate, electronic tunneling occurs instead of electron hopping. One method that has been used recently to interrogate electron tunneling at protein/substrate interfaces is scanning tunneling microscopy (STM) and its associated tunneling spectroscopy (TS).
STM/TS has the ability to not only measure interfacial tunneling currents between an adsorbed protein and substrate, but allows for high-resolution imaging of the structure of protein adlayers. Several recent studies have used STM to study protein/substrate interactions for a number of protein classes and in a number of environments. Most often these studies focus on metalloproteins naturally engineered to transfer electrons in their respective organisms. Of these used in STM studies, most often the protein structure has been determined using crystallographic methods. As single-center metalloproteins are more common and much simpler, very little STM work has focused on multi-center metalloproteins. However, multi-center proteins are remarkable enzymes and often exhibit truly unique chemical and biological properties.

This study is to expand upon our earlier work of STM on decaheme cytochrome proteins OmcA and MtrC from *Shewanella oneidensis* MR-1. Neither of these two metal-reducing outer-membrane associated enzymes have been crystallized despite a significant amount of effort in that regard. This has slowed the interpretation of STM/TS data, despite recent theoretical treatments, because the molecular orientations of the molecules are unknown when chemically adsorbed to the surface. To complement these studies, we have examined another multiheme cytochrome from *S. oneidensis*, the periplasmic small tetraheme cytochrome c, which has a well-characterized crystal structure (LEYS et al., 2002). We report detailed STM images of STC monolayers and detailed tunneling spectra in hopes of not only understanding how this enzyme transfers electrons, but with the potential in mind of expanding these results to the decaheme cytochromes that are more interesting in terms of biological reduction of solid-phase electron acceptors.

### C.2 METHODS

Following the procedure outlined in Chapter 2, we prepared monolayers of STC (with a recombinant tetracysteine tag) on Au(111). Images were collected at 0.5 nA setpoint with variable bias voltages. Tunneling spectra collection differed slightly from Chapter 2 whereas $I-V$ curves were collected at a number of bias voltages (+1 V, +0.75 V, +0.5 V, +0.25 V, -0.25 V, -0.5 V, -0.75 V, and -1.0 V). Each file collected was the average of 100 individual $I-V$ spectra which amounts to ~2000 spectra used for each condition to construct curves. Additional $I-V$ spectra were also collected at one
Ångstrom increments above the initial height set by the bias voltage to test for true exponential tunneling. All spectra presented were measured on one particular STC monolayer but with multiple Pt-Ir tips. Additional experiments were performed to test for reproducibility on several additional monolayers. Modeling fitting to a modified Simmons model followed procedures outlined in Chapter 3.

C.3 RESULTS AND DISCUSSION

STM imaging

STM images of STC monolayers show excellent coverage on Au(111) with a uniform shape/size distribution. Figure C.1 shows typical STM images at three different resolutions with Au(111) step edges and individual STC molecules clearly visible in all three images. Figure C.2 shows a 3D representation of a typical 150 nm x 150 nm scan. The height scale in the image is arbitrary and should only be used to measure relative, and not absolute, heights.

![STM images](image)

*Figure C.1 Series of STM images collected for STC monolayers on Au(111) and various resolutions. Scale bars are as follows: (A) 100 nm, (B) 50 nm, and (C) 15 nm.*

Additionally, because the crystal structure of STC is known, it is possible to compare the diameters obtained from STM with the actual molecular size of STC. Figure C.3 shows a typical STM image with a comparison of the crystal structure of STC. STM images show STC is roughly 3–4 nm in diameter which matches very well to its actual size.
The STM imaging results verify that STC retains a stable conformation on Au(111) and is immobilized on the surface by Au-thiol linkages through the tetracysteine tag. The good correlation with the actual molecular size as well as the excellent resolution of molecular boundaries within the monolayer suggest that denaturation of STC is not occurring. Because STC monolayers were stable during imaging, TS measurements on single molecules were permitted.
Tunneling spectroscopy of STC

Tunneling spectra were systematically collected at eight bias voltages to measure the effect of electronic coupling (i.e. the lower the bias voltage, the lower the tip-sample separation distance). Figure C.4 shows predictable $I-V$ behavior for these iterations, where the slope is steepest at lower bias voltage/tip-sample distance. Tunneling spectra were collected for both positive and negative tip bias to measure the effect of the direction of electron flow i.e. at positive tip bias, electrons flow from sample to protein to tip, and the reverse is true for negative tip bias). A slight difference was observed for tunneling spectra of opposite signs, as shown in Figure 1, with positive bias voltage setpoint $I-V$ curves slightly steeper than those collected at negative bias voltage setpoints.

![Tunneling spectra for various bias voltage setpoints at positive and negative tip bias.](image)

Figure C.4 Tunneling spectra for various bias voltage setpoints at positive and negative tip bias.

Spectra were also collected at 1 Å increments above the initial bias setpoint height to ensure exponential-like tunneling. The results for each bias voltage setpoint are shown in Figures C.5 and C.6 (e.g. each curve in Fig. C.4 is shown in its own plot as $z=0$ along with four additional $z$ heights). As expected, tunneling current decays from $z=0$ to $z=4$. At slices through each of these plots, it is possible to view the current-distance profile at any particular bias voltage to see if tunneling is exponential. An example of one such
plot taken at many points along the $I-V$ curve is shown in Figure C.7. Here, the exponential-like tunneling profile is clearly visible showing that true electronic tunneling, as opposed to electrochemical/faradaic currents, is the primary contributor to the current signal.

**Figure C.5** Tunneling spectra for four positive bias voltage setpoints at various heights above the initial imaging setpoint height.

The tunneling spectra follow a similar trend as that observed with the outer-membrane decaheme cytochrome OmcA, wherein the $I-V$ curves are smooth and featureless in both positive and negative bias voltage ranges. As such, modeling of the STC tunneling spectra was carried out with a coherent elastic tunneling model to obtain electron transfer parameters for STC.
Figure C.6 Tunneling spectra for four negative bias voltage setpoints at various heights above the initial imaging setpoint height.

Figure C.7 Example current-distance profile for +0.25 bias voltage setpoint showing exponential-like tunneling within four Ångstroms of initial imaging height.
Tunneling mechanism: Modeling I-V curves

Following the procedure outlined in Chapter 3, average $I$-$V$ curves for each bias condition were fit to a functional form for coherent elastic tunneling through a thin insulating film originally proposed by Simmons (1963). The two primary physical output parameters defined by the Simmons form are the tunneling barrier width, $L$, and height, $\phi$. A modified Simmons model for protein monolayers in a scanning probe molecular junction was developed by Zhao et al. (2004). This modification allows for an asymmetric voltage drop across the tunneling junction with the voltage ratio parameter, $\alpha$. In this case, the current density ($i$) is defined as:

$$i = \frac{e^2}{2\pi h L^2} \left[ (\phi - \alpha V) \exp\left(-K\sqrt{\phi - \alpha V}\right) - (\phi + (1-\alpha)V) \exp\left(-K\sqrt{\phi + (1-\alpha)V}\right) \right]$$

and

$$K = \frac{4\pi L}{h} \sqrt{2me}$$

where, $e$ and $m$ are the electron charge and mass, and $h$ is Planck’s constant.

The outputs from the Simmons fits are shown in Figure C.8. Here we show the parameter change with $z$ for barrier height, width, and asymmetry. Barrier length is predictably strongly dependent on $z$ but not as affected by bias voltage at close distances to the surface. However, when the tip is significantly far from the sample, barrier length becomes more dependent on bias voltage. The opposite is true for barrier height; at larger distances away from the surface, bias voltage has very little effect. The voltage ratio parameter appears also to be moderately bias-dependent, especially at closer distances to the surface. It is also clear that the $I$-$V$ curves become more asymmetric with distance away from the surface. This can also be observed in Figures C.5 and C.6.

Additional information that can be obtained by minor treatments of the tunneling spectra is the zero-bias conductance, $G_0$. This is simply done by looking at the ohmic (i.e. linear) portion of each $I$-$V$ curve (i.e. near zero bias voltage) and measuring the slope in nA/V. The results for each bias voltage setpoint and each $z$ height above the surface are shown in Table C.1.
Figure C.8 Simmons model fits for $I$-$V$ curves at various heights above the surface as a function of imaging bias voltage setpoint.

Table C.1 $G_0$ values calculated from low-bias region of $I$-$V$ curves at various heights above STC molecules on Au(111)

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C.4 CONCLUSION

Relate to OmcA, MtrC results i.e. STC looks like OmcA—could be because of electronic coupling to Au(111) which facilitates superexchange tunneling. The calculation of \( G(0) \) is a step towards connecting tunneling measurements with electron transfer quantities when the participation of redox centers in the \( I-V \) spectra is not detected. This procedure is similar to that taken by Nitzan (2001) and can be used to estimate the rate constant for electron transfer, \( k_{ET} \), and the decay length parameter, \( \beta \). These quantities are then comparable with those calculated in molecular modeling simulations. A similar task has already been conducted on STC electron transfer to hematite surfaces (Kerisit et al., 2007), and an extension to Au(111) is presently under investigation.

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Appendix D: Aquatic Environmental Nanoparticles

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ABSTRACT
Researchers are now discovering that naturally occurring environmental nanoparticles can play a key role in important chemical characteristics and the overall quality of natural and engineered waters. The detection of nanoparticles in virtually all water domains, including the oceans, surface waters, groundwater, atmospheric water, and even treated drinking water, demonstrates a distribution near ubiquity. Moreover, aquatic nanoparticles have the ability to influence environmental and engineered water chemistry and processes in a much different way than similar materials of larger sizes. This review covers recent advances made in identifying nanoparticles within water from a variety of sources, and advances in understanding their very interesting properties and reactivity that affect the chemical characteristics and behaviour of water. In the future, this science will be important in our vital, continuing efforts in water safety, treatment, and remediation.

D.1 INTRODUCTION
Environmental nanoparticles are nanometer-sized (~1–100 nm) crystalline to amorphous solid materials formed in nature. Scientists in the last 20 years have shown that environmental nanoparticles are quite literally everywhere in natural environments. They exist stably in nearly all components of the Earth including the oceans, atmosphere, and subsurface. The most important of these occurrences, however, is probably in the Earth’s so-called “critical zone.” According to a recent National Research Council report, the critical zone of our planet extends from the topmost forest canopy down to the deepest groundwater aquifer (2001). It is the portion of the Earth that provides or strongly influences nearly all of our most vital resources including fresh water, air, and
soil. Environmental nanoparticles, in a vast variety of forms, exist in virtually all of these resources (Brantley et al., 2006), including freshwater resources such as groundwater, lakes, and rivers. Although these water resources comprise less than 1% of the planet’s total water supply, they are the most indispensable because we are critically reliant on them for drinking water and agricultural use for a rapidly expanding population. This article concerns nanoparticles formed by natural geochemical (abiotic) and biogeochemical (biotic) processes in water, as well as those formed in natural aqueous environments as an unintended consequence of human activity in those environments. As an example of the latter, as we will see later, aquatic environmental nanoparticles can form as a result of mining activities and subsequent waste generation. This article does not, however, include intentionally (i.e., industrially) synthesized nanoparticles that have recently been the focus of the booming nanotechnology industry (e.g. CdSe, Fe(0), C60). Although such synthetic nanoparticles are undoubtedly being released into the environment—and understanding the consequences of such releases are of major importance to environmental sustainability (Guzman et al., 2006)—they still only represent a miniscule fraction of the nanoparticulate matter in the environment at this time. Recent reviews on nanoparticles and environmental nanoscience have received great interest in this field (Banfield and Zhang, 2001; Biswas and Wu, 2005; Gilbert and Banfield, 2005; Hochella, 2002a; Hochella, 2002b; Hochella and Madden, 2005). This additional review should be specifically useful for at least two reasons. First, when manufactured nanoparticle introduction to the environment becomes more and more of a concern in the future, as is expected, it will be important to know what nanoparticles were there to start with, equivalent to having a baseline measurement. Second, our writing in this review is based on research that has focused on two separate fronts: finding and characterizing nanoparticles in nature using high spatial-resolution instrumentation, and studying their synthetic analogues through controlled experimental investigations to better understand how they behave in the environment. Based on this research, in this review, we first present recent progress in our understanding of the unique properties, growth conditions, and reactivity of environmental nanoparticles, and
then shift focus towards their characterization and determination of the environmental processes they impact in water-based environmental settings.

**Why environmental nanoparticles are of such great interest**

The reason nanomaterials, in general, have created so much interest is because materials behave much differently when at least one spatial dimension is constrained in the nanoscale size regime. This effect is really what has driven the great majority of the nanotechnology boom. For example, synthetic Fe-oxide nanoparticles have several important industrial applications (Kay et al., 2006; Kim et al., 2003; Miser et al., 2004). But over billions of years, nature has also had the capability of producing its own Fe-oxide nanoparticles. On Earth, they act as carriers of elements and compounds in rivers and groundwater over long distances. They also serve as one of the primary constituents of the “critical zone” that contribute to such dynamic environmental processes as soil genesis, element cycling, and water quality.

The wide-ranging contribution to biogeochemical processes by Fe-oxide nanoparticles is just one example of the importance of nanoparticles. Many other phases exist in nature as nanoparticles and affect a great number of environmental processes. Table D.1 highlights a few of the major mineral classes that exist as nanoparticles and the environmental processes they influence. These nanoparticles, as a part of so many environmental processes, are behaving very differently when compared to particles of the same mineral but at a larger size. They are everywhere, and they account for a disproportionately large amount of potentially reactive surface area in the environment.

As we will see, researchers are now discovering that environmental nanoparticles contribute much more than a auxiliary role to the processes listed in Table D.1. Systems that were once considered to be well-characterized must be reexamined with an eye towards nanoscience. For some systems, what once was considered to exist in the dissolved fraction of water can no longer be viewed as such.

We presently know comparatively little about under what natural conditions environmental nanoparticles form, how long they persist, or in what cases their interaction with contaminants affects overall environmental quality. However, one thing is certain: humans are unintentionally contributing to the natural formation of countless
additional nanoparticles in virtually every environment on the planet. As will become apparent throughout this manuscript, this is not a situation limited to adverse human activity such as pollution of air and water resources, mining and mineral processing, or nuclear waste generation and storage; actions that we perceive to be environmentally beneficial (e.g. drinking water treatment and environmental remediation) can also influence nanoparticle distribution.

<table>
<thead>
<tr>
<th>Mineral class (example phases)</th>
<th>Environmental processes affected (examples)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-oxides (goethite, hematite)</td>
<td>metal/contaminant uptake catalysis organic transformation</td>
<td>(STIPP et al., 2002; WAYCHUNAS et al., 2005) (FREDRICKSON et al., 2004; MADDEN and HOCHELLA, 2005) (CHUN et al., 2006; KLUPINSKI et al., 2004; MCCORMICK and ADRIAENS, 2004; VIKESLAND et al., 2007)</td>
</tr>
<tr>
<td>Mn-oxides (vernadite, birnessite)</td>
<td>metal-binding contaminant uptake</td>
<td>(HOCHELLEA et al., 2005; VILLALOBOS et al., 2005b) (O'REILLY and HOCHELLA, 2003; VILLALOBOS et al., 2005a)</td>
</tr>
<tr>
<td>heavy metal oxides (uraninite)</td>
<td>contaminant immobilization</td>
<td>(FREDRICKSON et al., 2000; O'LOUGHLIN et al., 2003; SUZUKI et al., 2002; SUZUKI et al., 2005)</td>
</tr>
<tr>
<td>metal sulfides (sphalerite, pyrite, galena)</td>
<td>metal immobilization contaminant uptake</td>
<td>(LABRENZ et al., 2000; ROZAN et al., 2000) (MOREAU et al., 2004; WATSON et al., 2000; WATSON et al., 2001)</td>
</tr>
</tbody>
</table>

Not only are we causing such a change in the natural nanoparticle distribution in the environment, but we have virtually no idea what the corresponding consequences will be. For instance, invasive clean-up efforts of contaminated riverine sediment may cause the mobilization of very high levels of contamination release downstream through nanoparticle transport. And because those contaminants are bound to or within nanoparticles, the stability and means of their interaction are likely to be very different than what was once predicted.

Overall, the need for understanding the role of environmental nanoparticles is important to understanding how we are changing the planet, and how we choose to address minimizing (or maximizing) those changes.

**D.2 THE ORIGINS OF ENVIRONMENTAL NANOPARTICLES**

Environmental nanoparticles are commonly formed as either weathering byproducts of minerals, as biogenic products of microbial activity, or as growth nuclei in super-saturated fluids. Concerning the latter, all materials precipitating directly out of solution must start in the nanoparticle size range. Certain phases, based on environmental
conditions and growth kinetics, quickly surpass this size region and form much larger particles. But a large fraction of solid-phase materials exist at this size-range for extended periods of time. In the simplest systems, many inorganic growth mechanisms are responsible for nanoparticle formation including classic crystal growth (Waychunas, 2001), aggregation (i.e. ripening) (Navrotsky, 2004), and redox-triggered crystallization based on changes in mineral solubility (Zachara et al., 2007). Examining how nanoparticles are formed and sustained in natural waters is key to understanding their possible roles in environmental processes such as the transport and ultimate fate of contaminants associated internally or on the surface of the particles.

In many environments, certain microorganisms induce the formation of nanoparticles. Biogenic nanoparticles are sometimes formed directly by the organism as a metabolic requirement (e.g. magnetite, Fe₃O₄, produced intracellularly by magnetotactic bacteria is required for motility (Bazylinski and Frankel, 2004)). Nanoparticles also form as an indirect result of microbial activity. For example, when a microorganism induces the redox transformation of a metal, the solubility may significantly change causing the precipitation of a new nanocrystalline mineral phase of Fe-oxides (Banfield et al., 2000; Glasauer et al., 2002; Hansel et al., 2003; Hansel et al., 2004; Kukkadapu et al., 2005; Lloyd et al., 2000) and Mn-oxides (Tebo et al., 2004; Villalobos et al., 2005a; Villalobos et al., 2005b; Villalobos et al., 2006). With Mn-oxidizing bacteria, for example, the final oxidation product is Mn(IV), which is insoluble and will interact with existing mineral phases, other aqueous metal species, or the cell wall, to form nanoparticles. Mineralization can also be promoted by other metabolites (e.g. electron shuttles) or by microbial cell surfaces acting as organic templates (Chan et al., 2004; Nesterova et al., 2003).

Understanding the precise growth mechanism of nanoparticles has recently become of high importance because this may be strongly correlated to particle reactivity. For example, Fe-oxide nanoparticles grown both abiotically and biotically show different optical properties (Oremland et al., 2004), and rates of heterogeneous catalytic efficiency (Jung et al., 2007). Additionally, defects and phase transitions of abiotically grown hematite (-Fe₂O₃) nanoparticles depends largely on growth kinetics of the particles.
Phase transitions on such a scale are often directly correlated to surface energy and thermodynamics of growth (Navrotsky, 2003).

Delineating the origin of nanoparticles from natural samples, however, is often a very challenging task. For example, when determining the origin/biogenicity of magnetite, criteria such as oxygen isotope fractionation, magnetic properties, particle morphology, and crystal size are often too ambiguous to be used individually (Faivre et al., 2004; Faivre et al., 2005). A much more rigorous characterization using a combination of such methods can sometimes allow for the accurate determination of the origin (Faivre and Zuddas, 2006). Indeed, more understanding of the growth mechanisms for both inorganic and biogenic nanoparticles will undoubtedly aid in the efforts to understand the origin of nanoparticulate phases. Thus far, laboratory studies examining the growth mechanisms of environmentally-relevant nanoparticles have predominately focused on various sulfide (Ciglenecki et al., 2005; Labrenz et al., 2000), Fe-oxide (Banfield et al., 2000; Guyodo et al., 2003; Penn et al., 2006; Waychunas et al., 2005), and other metal oxide phases (Penn, 2004; Penn and Banfield, 1998; Ranade et al., 2002; Zhang and Banfield, 2000).

Nanoparticle formation associated with metal contaminants

In systems with heavy metal and/or radionuclide contamination, nanoparticles are often the byproducts of remediation efforts. One system of high interest for countries with a history of nuclear weapons manufacturing and nuclear power is that of uranium contaminated soils and groundwater aquifers. For example, a primary goal of the United States Department of Energy is to address the nuclear legacy of the weapons program in the U.S., including the remediation of uranium-contaminated subsurface sites. One of the most promising means of non-invasive clean-up is through the bioremediation of soluble U(VI) by microorganisms such as metal-reducing bacteria (Finneran et al., 2002; Wall and Krumholz, 2006). This involves microbial-induced redox-transformations from U(VI) to an insoluble U(IV) phase. Thus the hope is for the uranium to be immobilized within the contaminated aquifer. However, one caveat to this argument is that these precipitates have been shown to predominately exist as nanoparticles (Fayek et al., 2005; Fredrickson et al., 2002; Marshall et al., 2006; Suzuki et al., 2002). In fact, nanoparticles
of uraninite (UO₂) have been shown to form abiotically when U(VI) is reduced by Fe(II)-oxides (Boyanov et al., 2007; O'Loughlin et al., 2003).

Disregarding reoxidation of the solid-phase products (Wan et al., 2005) for the moment, one issue of great concern for the stability of the product is how unreduced aqueous U(VI) interacts with the precipitating nanoparticles. One field study showed that a solid-phase U(IV) precipitate actually contained a large fraction of unreduced U(VI) (Ortiz-Bernad et al., 2004).

The complexities behind the fate of metal and radionuclide contaminants during nanoparticle formation make predicting the final products very difficult. Several examples in the literature of different end-products for various metal/metalloid contaminants highlight the fact that we do not yet fully understand the fate of the contaminant with respect to the precipitating nanoparticle, but a lot of progress is being made. For example, a recent study by Zachara and colleagues showed that the abiotic reduction of Tc(VII) by Fe(II) formed relatively stable iron oxide nanoparticles with homogeneously distributed Tc(IV) in the crystal structure, effectively stabilizing the Tc (Zachara et al., 2007). The opposite can be true with the metalloid As. Tadanier and colleagues showed that the microbial-reduction of Fe-oxide aggregates with adsorbed As(V) caused the deflocculation of As-bearing ferrihydrite (Fe₁₀O₁₄(OH)₂) nanoparticles, subsequently increasing the mobility of As (Tadanier et al., 2005).

When relying on the reductive transformation of metals and subsequent growth of nanoparticles as a means for remediation, the problem of nanoparticle stability must be a chief concern. However, as we will see later, nanoparticles can be incredibly mobile in the environment; understanding the transport mechanisms of nanoparticles in subsurface contamination plumes is also very important for analyzing the practicality of such remediation efforts. One recent study, however, circumvented the need for understanding transport because they simply removed metal- and nanoparticle-bearing water from a contaminated mining site and used it to create additional nanoparticles ex-situ (Wei and Viadero, 2007). Magnetite nanoparticles were grown using ferric iron in the acid-mine drainage waters that also contained trace amounts of other metals including Zn, Ni, and Cu. This presents an intriguing remediation case where contamination species are removed from the site and transformed ex-situ.
D.3 REACTIVITY OF NANOPARTICLES: INSIGHT FROM THE LABORATORY

As alluded to in the Introduction, the principal reason for the recent trend in the characterization of nanoparticles is that their physical and chemical properties are often much different than larger particles of the same material. This is because, as we will soon see, the nanoscale represents a ‘transition zone’ between the behavior of atomic and bulk-like states. Changes are observed, for instance, in the atomic structure, electronic, magnetic, and optical properties of the material. If these changes are significant (and they typically are), then the chemical reactivity of the particle should also be significantly affected. In an environmental context, this becomes especially important when considering the chemical reactions that involve contaminant compounds.

The reasons for changes in reactivity at the nanoscale can be rationalized through four interrelated mechanisms: 1) as nanoparticles get smaller and smaller, the proportion of atoms at the surface or near-surface regions increases dramatically, often causing an increasing reactive surface area depending on the change in the distribution of surface edges, steps, kinks, and terraces, 2) as a result, the surface free energy of the particle will change as a function of particle size, thus influencing the thermodynamics of chemical reactivity, 3) atomic structure variations occur, in terms of changes in bond lengths, bond angles, and vacancies and other defects near and on surfaces, and 4) size-quantization effects modify the electronic structure of the material as the band structure well known in bulk materials begins to resemble discrete energy states of small molecules. Depending on the material and its size range, one or a combination of all four of these factors will contribute to the size-dependent change in the properties and chemical reactivity of that material. The changes observed may be dramatic. For example, even if the bulk material is a good conductor, the smallest nanoparticles may be semiconductors, or even insulators.

The transition from macroscopic to nanoscale properties is not necessarily easy to predict. Where it has been carefully measured, the change in reactivity is often abrupt and significant as size decreases towards atomic clusters or individual atoms (Fig. D.1). One common misconception is that the reactivity tends to increase with decreasing particle size, but that is certainly not always the case.
Environmental nanoparticles are intrinsically complex based on their mode of formation and chemical heterogeneity. Even ‘well-defined’ synthetic nanoparticles tend to be difficult to study because of laborious or complicated synthesis methods which are never ideal, and/or the challenge of chemically and physically characterizing exceptionally small pieces of matter. Recent progress has been made in the laboratory, however, in synthesizing and characterizing environmentally-relevant nanoparticles. Although synthetic analogues are not necessarily ‘environmental nanoparticles,’ they hold an important key to unraveling the mysteries behind nanoparticles in the environment.

One class of minerals—the Fe-oxides—have arguably seen the most focused environmental nanoparticle research (Waychunas et al., 2005). Because iron is by far the most abundant transition metal on Earth, and oxygen is the most abundant element in the crust by far, iron oxides are found in virtually all natural water and soil systems across a wide spectrum of pH, salinity, and geologic settings. Not surprisingly, iron oxides exist, sometimes exclusively, as nanoparticles. For instance, ferricydrite is found in nearly every near-surface aquatic system, but due to its characteristic nanoparticulate nature, has variable structural details and stability. One recent study, however, has presented a

![Image](image_url)

**Fig. D.1** Generalized trend for size-dependent reactivity change of a material as the particle transitions from macroscopic (bulk-like) to atomic. Reactivity can increase or decrease depending on the material and the chemical reaction involved.
detailed crystal structure for this mineral which is a major breakthrough for understanding its reactivity in the environment (Michel et al., 2007).

What else controls the growth, durability, and reactivity of nanoparticles such as ferrihydrite? Why should ferrihydrite and other important nanophases be found in many aquatic systems instead of rapidly transitioning to other larger, more thermodynamically favourable phases? Moreover, how do nanoparticles of more stable Fe-oxide phases like hematite, goethite (\(-\text{FeOOH}\)) and magnetite react compared to ferrihydrite and how do their reactivities change with size? These are important questions that need to be addressed in the context of nanoscience in order to be more fully understood. Fortunately, recent advances in the laboratory have started to provide answers to some of these questions.

Hematite’s catalytic efficiency (surface area normalized rates) in Mn-oxidation reactions increases by between one and two orders of magnitude when going from 37 nm particles to 7 nm in size (Madden and Hochella, 2005). This may be due to the distortion of hematite surface binding sites as the nanoparticles become smaller, which in turn affects the local bonding of the adsorbing aqueous Mn ions, a step necessary in the oxidation process. This, in turn, affects the kinetics of the oxidation reaction. This seems to indeed be the case, as Cu(II), which has an affinity for distorted surface binding sites, sorbs more aggressively on smaller hematite nanoparticles than larger ones (Madden et al., 2006). This becomes increasingly important with nanoparticles less than \(~10\) nm in size because the percentage of atoms on the surface increases exponentially as particle diameter decreases (Madden et al., 2006), providing many more sites for distorted metal bonding to the surface. Indeed, the structure of hematite nanoparticles has been shown to be size-dependent (Chernyshova et al., 2007).

Although these studies are specific to hematite, these effects are likely to be common in many environmental nanoparticles, including other Fe-oxides. Examples of other experimental and/or computational investigations of the reactivity of Fe-oxide nanoparticles include studies that concern goethite (Klupinski et al., 2004; Rustad and Felmy, 2005; Waychunas et al., 2005) and ferrihydrite (Anschutz and Penn, 2005; Poulson et al., 2005). One important consideration when undergoing comparative studies of various Fe-oxides is that although they all exhibit size-dependent changes in reactivity,
they may not change in a uniform way relative to each other. This has been addressed by three compelling studies comparing abiotic dissolution (Larsen and Postma, 2001), Fe(II)-induced transformation (Pederson et al., 2005), and microbial reduction (Bonneville et al., 2004) for various Fe-oxide nanoparticle phases. Although these studies suggest that the bulk solubility of a particular mineral is a good predictor for nanoparticle reactivity, differences in particle morphologies, surface area, and structural defects do not always allow direct comparisons between similar mineral phases. Size-dependent experiments for each mineral phase, such as two recent studies of bacterial reduction of hematite nanoparticles, have begun to address some of these concerns (Bonneville et al., 2006; Bose et al., submitted), but difficulties remain as reduction rates for similar minerals are also highly dependent on growth conditions (Glasauer et al., 2003).

**Focus on direct environmental remediation**

Although the reactivity and stability of nanoparticles is important in determining the baseline behavior for aquatic systems, their reactivity with toxic contaminants is of chief interest for environmental remediation. In these instances, abiotic and biotic processes contribute to sequestration or transformation of such compounds.

Mayo et al. showed that 12 nm particles of magnetite are roughly 200 times more efficient at removing As(III) and As(V) from water than 20 and 300 nm particles, an increase much greater than is predicted based only on an increase in surface area (Mayo et al., 2007). Moreover, the adsorption complex associated with the smaller nanoparticles is much more stable than with the larger particles. Next, it may be possible for the magnetic characteristics of magnetite to be utilized in remediation strategies for arsenic contaminated aquifers. However, the size-dependent variation in the magnetic properties of magnetite need to be considered before the implementation of such schemes can occur.

Magnetite is also important for the degradation of organic contaminants. For example, Vikesland et al showed that magnetite reactivity with carbon tetrachloride is not only size-dependent, but strongly influenced by the degree of particle aggregation (Vikesland et al., 2007). Here, 9 nm particles were over an order of magnitude more reactive than 80 nm particles, again, an effect much larger than predicted based solely on
surface area. However, contrary to the arsenic example mentioned above, the Vikesland et al study showed a pronounced effect on nanoparticle aggregation. Very few examples exist that explore environmental nanoparticle aggregation, which presumably affects the reactive surface area of the component nanoparticles. Determining the effect of aggregation on other systems is very important in understanding how environmental nanoparticles behave in aquatic systems. Recent studies of the aggregation kinetics of alginate-coated hematite provide some of the first steps towards understanding the reactivity consequences of environmental nanoparticle aggregation (Chen et al., 2006; Chen et al., 2007).

D.4 ANALYTICAL TOOLS AND DETECTION METHODS
Several recent reviews in the literature highlight many aspects of the detection and characterization of environmental nanoparticles (Burleson et al., 2004; Contado et al., 2003; Lead and Wilkinson, 2007; Leppard et al., 2004). The purpose of this section is only to briefly mention 1) important environmental nanoparticle characterization methods for completeness of this review, and 2) techniques that will be referred to throughout the remainder of this article. While not all of the studies that are cited below deal specifically with aquatic environmental nanoparticles, the methods employed can certainly be applied and/or adapted to studying them.

Most often there are multiple parameters which need to be analyzed in order to determine the presence and effects of nanoparticles in the environment. These parameters include size distribution, surface area, direct visualization, and the chemical makeup and/or mineral phase of the particles (Table D.2).

When characterizing natural samples, nanoparticles are not initially separated from larger particles and, dependent on the subsequent analysis methods, the sample may need to be size fractionated. Common fractionation methods include centrifugation (Lead et al., 1999), ultrafiltration (Benoit and Rozan, 1999; Wang et al., 2003), tangential-flow (cross-flow) ultrafiltration (TFF) (Doucet et al., 2004; Doucet et al., 2005; Hill and Aplin, 2001; Vignati et al., 2005), and field-flow fractionation (FFF) (Baalousha et al., 2006; Baalousha et al., 2005; Baalousha and Lead, 2007; Lyven et al., 2003; Stolpe et al., 2005; Thang et al., 2000).
Size distribution of particles can be accomplished using a variety of techniques, such as laser or diffuse light scattering (LLS or DLS), laser-induced breakdown detection (LIBD) (Baalousha et al., 2005; Thang et al., 2000; Walther et al., 2006), X-ray diffraction, (XRD) (Huber, 1997; Tarassov et al., 2002), atomic force microscopy (AFM) (Doucet et al., 2004; Doucet et al., 2005; Lead et al., 2005; Madden and Hochella, 2005; Muirhead and Lead, 2003), and transmission electron microscopy (TEM) (Anschutz and Penn, 2005; Bose et al., submitted; Madden and Hochella, 2005; Madden et al., 2006).

Surface area can be determined indirectly by using the diameter of particles obtained with AFM or TEM measurements or directly by the Brunauer-Emmett-Teller (BET) method (Anschutz and Penn, 2005; Madden and Hochella, 2005; Madden et al., 2006).

Table D.2: Analytical tools and detection methods used to characterize environmental nanoparticles. See text for abbreviations and citations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Analytical tool</th>
<th>sample analysis scale</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Whole sample</td>
</tr>
<tr>
<td>size fractionation</td>
<td>centrifugation</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>ultrafiltration</td>
<td>X</td>
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<tr>
<td></td>
<td>TFF</td>
<td>X</td>
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<tr>
<td></td>
<td>FFF</td>
<td>X</td>
</tr>
<tr>
<td>size distribution</td>
<td>LLS or DLS</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>LIBD</td>
<td>X</td>
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<td></td>
<td>XRD</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>AFM</td>
<td>X</td>
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<tr>
<td></td>
<td>TEM</td>
<td>X</td>
</tr>
<tr>
<td>surface area</td>
<td>AFM</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>TEM</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>BET</td>
<td>X</td>
</tr>
<tr>
<td>chemical analysis</td>
<td>AAS</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>ICPMS/ICPAES</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>XRD</td>
<td>X</td>
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<tr>
<td></td>
<td>S/TEM EDS</td>
<td>X</td>
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<tr>
<td></td>
<td>S/TEM EELS</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>NanoSIMS</td>
<td>X</td>
</tr>
<tr>
<td>direct visualization</td>
<td>AFM</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>EM</td>
<td>X</td>
</tr>
<tr>
<td>mineral phase/internal</td>
<td>TEM SAED</td>
<td>X</td>
</tr>
<tr>
<td>structure</td>
<td>FFT of HR-TEM image</td>
<td>X</td>
</tr>
</tbody>
</table>

Once the size parameters have been obtained it is advantageous to have the particle chemistry, as this is a large factor in determining reactivity in the environment. Frequently used quantitative methods for nanoparticle chemical analysis include atomic adsorption spectroscopy (AAS) (Balaram, 2002; Ebdon et al., 1998), inductively coupled
plasma (mass or atomic emission) spectroscopy (ICPMS/ICPAES) (Balaram, 2002; Gomez et al., 2002), XRD (Gomez et al., 2002; Hochella et al., 2005a; Hochella et al., 1999; Hochella et al., 2005b; Zanchet et al., 2000), X-ray energy dispersive spectroscopy (EDS) (Chanudet and Filella, 2006; Glasauer et al., 2002; Gomez et al., 2002; Hochella et al., 2005a; Hochella et al., 1999; Hochella et al., 2005b; Jackson et al., 1999; Labrenz et al., 2000; Mavrocdatos et al., 2004; Utsunomiya and Ewing, 2003; Utsunomiya et al., 2004; Utsunomiya et al., 2003a), electron energy loss spectroscopy (EELS) (Daulton et al., 2001; Daulton et al., 2002; Hochella et al., 1999; Hochella et al., 2005b; Jackson et al., 1999; Labrenz et al., 2000; Mavrocdatos et al., 2004; Utsunomiya and Ewing, 2003; Utsunomiya et al., 2004; Utsunomiya et al., 2003a), electron energy loss spectroscopy (EELS) (Daulton et al., 2001; Daulton et al., 2002; Hochella et al., 1999), and secondary ion mass spectroscopy with a lateral resolution of less than 50 nm (NanoSIMS) (Fayek et al., 2005; Novikov et al., 2006).

It is very important in many cases to directly visualize the particles of interest. Two common visualization methods are AFM (Lead et al., 2005; Mavrocdatos et al., 2004; Plaschke et al., 2002) and electron microscopy. While scanning electron microscopy (SEM) can image nanoparticles and is very useful in nanoscience in general, it does not have the resolution necessary for extensive analysis on the smallest end of the nano-scale. TEM and scanning transmission electron microscopy (STEM) are the best tools for direct visualization on this scale. Not only do the best S/TEMs available now have sub-Ångstrom image resolution, but when combined with their accompanying analytical techniques (EDS, EELS, elemental mapping), they have the best nanoparticle characterization potential. The atomic structure of crystalline particles can also be determined using TEM by either selected area electron diffraction (SAED) (Banfield et al., 2000; Chanudet and Filella, 2006; Hochella et al., 2005a; Hochella et al., 1999; Hochella et al., 2005b; Labrenz et al., 2000; Mavrocdatos et al., 2004) or the Fast Fourier Transform (FFT) (Benoit and Rozan, 1999; Utsunomiya and Ewing, 2003; Utsunomiya et al., 2004; Utsunomiya et al., 2003b) of high resolution (HR) images. Combined with chemical information, this allows for the direct determination of the mineral phase of individual nanoparticles.

There has also been a number of excellent papers which describe optimal TEM and AFM sample preparation procedures for retrieval of particles from water samples (Lienemann et al., 1998; Nomizu and Mizuike, 1986; Perret et al., 1991). Often when
doing this type of work, the quality of the sample preparation will determine the quality
of the results.

D.5 EXAMPLES OF NANOPARTICLES IN THE ENVIRONMENT
Now that scientists know why nanoparticles are important in many aquatic systems, and
how to find them, there has been a recent surge in the documented identification of
environmental nanoparticles in the literature. In aquatic systems, this includes
nanoparticles from surface waters to the deep subsurface. We begin by examining the
environmental nanoparticles that have been found in groundwater and sediments and then
shift focus to examples of nanoparticles in surface waters such as rivers and lakes.
Finally, we describe ongoing work in our group that has sought out nanoparticles in both
of these settings.

Nanoparticles in subsurface aquifers and sediments
As we saw earlier, nanoparticles form naturally without the intervention of human
activity. Based on laboratory experiments with synthetic analogues of such
nanoparticles, they should be able to mediate contaminant transport. Indeed, field studies
present similar implications. For example, recently identified nanocrystalline Fe-oxide
grain coatings on sediments (Penn et al., 2001; Poulton and Raiswell, 2005; van der Zee
et al., 2003) have implications for trace metal and contaminant transport including U(VI)
(Davis et al., 2004) and As(V) (Kent and Fox, 2004). Fe-oxide nanoparticles also affect
metal concentrations such as Zn in soils (Isaure et al., 2005).

Conversely, in not all cases do environmental nanoparticles play a direct role in
mediating contaminant transport. The reason for this is related to the mode of formation
of the nanoparticles and the speciation of the contaminant. At one particular inoperative
Hg mine, particle size-dependent Hg concentrations suggested transport through the
nanoparticles found in the sediment (Lowry et al., 2004). However, Hg was shown to
exist almost exclusively as larger HgS colloids (~500 nm) that formed mechanically from
existing Hg ore (Lowry et al., 2004). In this case, physical processes caused native ore to
break apart into stable colloids, leaving little opportunity for aqueous Hg to become
associated with nanoparticles in the sediment. However, in systems where the
contaminant exists, at least initially, as aqueous ions or complexes, nanoparticles may become very important mediators of their transport.

For heavy metals or radionuclides, nanoparticle transport becomes increasingly important because radionuclides like U or Pu do not simply weather from metal-bearing rocks (ores) as was the case in the Hg mine example above. For example, Novikov and colleagues showed that Fe-oxide nanoparticles contribute to the groundwater transport of Pu and U up to 3 km away from a nuclear waste reprocessing plant in Russia (Novikov et al., 2006). Detailed HR-TEM and NanoSIMS studies showed that the Fe-oxide nanoparticles most responsible for Pu transport were less than 5 nm in size. Nanoparticles also contribute to Pu transport in groundwater below the Nevada Test Site, U.S.A., with a significant portion associated with nanoparticles between 7 and 50 nm in size (Kersting et al., 1999). Uraninite from spent nuclear fuel also contains nanoparticles of radionuclides like Tc (Utsunomiya and Ewing, 2006).

Nanoparticles in surface waters

Typically, any solid phase from ~1 nm to ~1 µm in surface waters is considered an aquatic colloid (Buffle and Leppard, 1995; Lead and Wilkinson, 2006). However, the term ‘colloid’ is generally used to describe not only inorganic solid-phase materials such as minerals, but other environmental molecules and materials such as microorganisms, biomacromolecules, and humic/fulvic acids. An excellent review of the aquatic colloid field is given in a recent book by Lead and Wilkinson (Lead and Wilkinson, 2007). But given what we now know, it becomes necessary to consider nanoparticles in surface waters separately from other aquatic colloids. Conveniently, due to fractionation techniques, nanoparticle fractions of water samples can usually be separated from other colloids and analyzed.

For example, recently researchers were able to detect high concentrations of particles less than 5 nm in diameter from lake and river samples near Birmingham, U.K. (Baalousha and Lead, 2007). Additional studies have shown that nanoparticles exhibit dramatic size-dependent element-binding capacities (Lyven et al., 2003; Stolpe et al., 2005). Although nanoparticles are predicted to travel extremely long distances in river water, their stability can be drastically altered if water chemistry changes. For example, seawater mixing with river water was shown to remove almost all inorganic nanoparticles.
from the suspended load due to salt-induced aggregation (Stolpe and Hassellov, 2007). Nanoparticle stability may also be affected by abundant dissolved organic matter. Conversely, nanoparticles from river samples may be stabilized when in close association with organic colloids or thin-film coatings on other mineral surfaces (Lead et al., 2005; Muirhead and Lead, 2003).

Although bulk chemical methods like ICPMS help determine what colloid size-fraction from the water sample contains specific elements, studies showing the size, composition, and morphology of individual nanoparticles are highly complementary. High-resolution studies become especially important when determining the exact association of contaminants within a size fraction. Baalousha et al combined bulk techniques with TEM/EDS to determine the elemental distribution among/within nanoparticles (Baalousha et al., 2006), but difficulties remain in determining low concentrations of contaminants. A recent study by Allard et al determined through the use of HR-TEM, combined with bulk chemical analysis, that 2-5 nm ferrihydrite nanoparticles contribute to the transport of iron and organic matter in tropical river systems (Allard et al., 2004). High-resolution studies using HR-TEM and EDS seem to hold great potential for complementary studies of nanoparticles in surface water. NanoSIMS may also provide high-resolution chemical distribution of nanoparticles from surface waters, but to our knowledge, no examples exist in the literature.

**Case Study: Clark Fork River, Montana, USA**

Due to almost 150 years of copper and silver mining and smelting activities, the Clark Fork River (Fig. D.2) has been contaminated with dangerously high levels of toxic metals including As, Pb, Zn and Cu. More than 100 million tons of mine waste has been introduced directly into the river and surrounding floodplains (Andrews, 1987; Axtmann and Luoma, 1991). As a result, it is estimated that greater than 2,000,000 m3 of contaminated sediments are stored in the floodplains of the Clark Fork River and its tributaries (Moore and Luoma, 1990).

Although much effort and money has been put forth in remediating this area, elevated levels of metals as far as 500 km downstream from the mining source still exist (Axtmann and Luoma, 1991; Moore and Luoma, 1990). It is assumed that metals
traveling that far within the river water must be either in the aqueous phase and/or occurring within or sorbed onto particles. Although aqueous phase transport cannot be ruled out, it is less likely because the circumneutral pH of the river water would favor precipitation of the metals as insoluble metal hydroxides. During flooding, the river transports large particles which contributes a great deal to the metal load; however, it can only transport these particles during rare high water events. The bulk of long-distance metal transport must then occur during times of low flow-velocity.

![Fig. D.2](image)

*Fig. D.2* The Clark Fork River in western Montana, USA (a). HR-TEM image of ~5 nm – 15 nm particles taken directly from the surface water of the river (b). Crystallinity is apparent based on the presence of well-defined lattice fringes.

In order to determine where the contaminants reside within the river system, the riverbed and floodplain sediments have been sampled and extensively studied using TEM. Toxic heavy metals were found to be structural components of several nanocrystalline phases. Zn alone was found to be present as sulfate, oxide, and sulfide mineral phases (Hochella et al., 2005a; Hochella et al., 1999; Hochella et al., 2005b).
Other environmental nanoparticles were found to have a high sorption capacity for Zn, As and Pb, including Mn-oxides, Fe-oxides, and sulfides.

To get a more complete picture of nanoparticle transport, we sampled the river water at various locations. These water samples have recently been studied using HR-TEM/EDS, and we have identified several metal-bearing nanoparticles (Wigginton et al.). An example of a typical particle is given in Figure D.2 showing 5-15 nm crystalline titanium oxides particles with distinct lattice fringing present in the high resolution images. The existence of such fine-grained phases supports the hypothesis that contaminant transport is facilitated by nanoparticles. Preliminary EDS investigations into the metals associated with such particles has verified that there are various trace metals associated with them. Complementary work is being conducted to determine total metal content in the nanoparticulate fraction of the river water for metals of interest using various filtration and ultra-filtration methods combined with ICPMS.

D.6 THE FUTURE OF THE FIELD: LEARNING FROM NATURE

As we begin to learn more and more about how environmental nanoparticles behave in natural aquatic settings, we can begin to apply that knowledge to other systems, including engineered systems. For example, there is considerable interest in employing synthetic nanoparticles for the remediation of environmental contaminants. One such material which has attracted a great deal of interest is nanocrystalline Fe(0), also known as zero-valent iron (ZVI).

Recent reviews on nanocrystalline ZVI highlight the current state and potential/current uses of this material (Li et al., 2006a; Li et al., 2006b; Nurmi et al., 2005; Tratnyek and Johnson, 2006; Zhang, 2003). Despite the abundance of Fe at Earth’s surface, ZVI appears very rarely in the environment in a natural form. Due to the high amounts of oxygen in Earth-surface environments, most Fe is converted from weathering silicates to either Fe-oxides or Fe-containing clay minerals. Although ZVI nanoparticles appear to be more reactive for many types of reactions than environmental Fe-oxide nanoparticles, their long-term stability and transport mechanisms appear to be relatively untested. Tratnyek and Johnson estimated transport distances through porous media to be, for common ZVI nanoparticles, on the order of a centimeter or less unless fracturing and high groundwater velocity perturb the system (Tratnyek and Johnson,
The risk becomes much higher, however, if a contaminated aquifer were to reach surface waters. Transport then could essentially be limitless and ZVI nanoparticles could unintentionally infiltrate other aquatic systems including sources for drinking water.

Addressing these types of questions remain integral to understanding the relative benefits and risks of nanotechnology. It is also important to note that by keeping a close watch on how naturally occurring nanoparticles behave, we are afforded the opportunity to start to understand the consequences of using synthesized nanoparticles in the environment.

**Nanoparticles in drinking water systems**

Particulate species in drinking water are considered anything greater than 0.45 μm, colloids from 0.1–0.45 μm, and soluble fraction is anything less than 0.1 μm (i.e. 100 nm) (McNeill and Edwards, 2004). But we have just seen that environmental nanoparticles (1-100 nm) are present in many, if not all, natural aquatic systems from subsurface aquifers to surface lakes and rivers. We now pose the following questions based on what has been made apparent in this review: Do nanoparticles from source waters affect drinking water treatment processes? Can environmental nanoparticles (which have been shown to both transport and break-down contaminants) in water distribution systems significantly affect water quality? Should drinking water be considered safe or unsafe for human consumption if environmental nanoparticles are present in treated waters? How well do our present treatment methods remove environmental nanoparticles from source water? Most of these questions have yet to be addressed, but some recent work has provided a few answers. We begin by addressing the seemingly contradictory behavior of nanoparticles able to break-down or transport certain contaminants in aquatic systems.

Fe-oxide nanoparticles have been shown to efficiently sequester As from drinking water in engineered exchange columns (DeMarco et al., 2003; Sylvester et al., 2007). However, if Fe-oxide nanoparticles from the source water make it through the treatment system, they could potentially be effective transporters of contaminants such as As or Pb, especially if those metals are leached from piping in the distribution system. Additionally, once in distribution systems, certain bacterial species could promote the
desorption of adsorbed metals (Tadanier et al., 2005), thus increasing the metal bioavailability.

To address the uncertainties behind these questions, we need to be able to identify nanoparticles in drinking water and then determine their source. A large scale study by Wagner and colleagues of solid phases in water pipelines and bottled water is an excellent example of characterizing nanoparticles in drinking water (Wagner et al., 2004). They were able to detect particles down to 50 nm using LIBD. The detection limits of their method are near this particle diameter so it is unknown if smaller particles exist, but another recent study detected individual nanoparticles down to 15 nm using a similar technique coupled with single-particle counting (Walther et al., 2006). Identification of the particles, however, often proves to be much more difficult, especially in drinking water systems when there are presumably very small concentrations of nanoparticles (as compared to source waters). But it is the chemical identification of the particle that provides the best insight into how it will behave in the system.

As a model system for identifying the mineral phase(s) of nanoparticles extracted from treated waters, we recently examined tap water from Washington, D.C., USA. The Washington, D.C. area recently had a significant problem with dangerously high Pb concentrations in drinking water—much higher than governmental drinking water standards—likely due to leaching of Pb from Pb-bearing pipes promoted by breakdown products of disinfection agents (Edwards and Dudi, 2004). After fractionation through centrifugation, our HR-TEM data showed the presence of many nanoparticles of various compositions, sizes, and morphologies (Wigginton et al., unpublished). Fig. D.3 shows a particularly interesting sample of 20 nm particles that were found to contain (using EDS) high amounts of Fe and Pb. Electron diffraction suggests the Fe phase to be similar to magnetite. A more detailed study is underway.

Although very few studies have been done to address the origin of nanoparticles in drinking water systems, it is relatively safe to assume one of three mechanisms: 1) the particles themselves (not necessarily the sorbed species that they are carrying) are native to the source water and are resilient enough to withstand filtration and chemical processing steps at the treatment facility, 2) nanoparticulate phases precipitate once inside the treatment plant and/or distribution system in response to changing chemical
conditions, or 3) corrosion of pipes promoted by disinfectants and/or their degradation byproducts could cause nanoparticles attached to the piping material to detach. The origin of nanoparticles in Washington, D. C. drinking water is not yet known, but our preliminary evidence suggests Pb transport is influenced by environmental nanoparticles from the source water that have made it through the treatment facility and into the distribution system. Understanding transport mechanisms, as well as how contaminants such as Pb interact with nanoparticles present in these systems, is of utmost importance for maximizing water quality and maintaining safe drinking water practices.

Fig D.3. HR-TEM image of nanoparticulate Fe oxides found in a drinking water sample taken from a metropolitan water source in Washington, D.C. Many particles like these have been found (using EDS) to contain a substantial amount of Pb.

**Perspectives on the advancement of the field**

Government agencies around the world now invest billions of dollars annually in nanoscience and technology research and development. Commercial funding of this field is just as great. However, the amount of funding (and attention) that environmental nanoscience receives represents only a small fraction of this massive overall effort. As a
result, advancement of environmental nanoscience has lagged significantly behind other sub-disciplines in the nanoscience field. Yet environmental and health aspects of nanoscience and technology will eventually gain traction, as the costs of ignoring or under-funding these subfields are much too high. The future of environmental nanoscience looks very bright.

With regards specifically to environmental nanoparticle science, as this area of research grows, it will have to advance aggressively along at least two research paths, as follows: 1) fundamental research on the physical property and chemical reactivity variability of nanoparticles as a function of their size, and 2) the detailed study and understanding of the influence of nanoparticles on aqueous chemical processes. The thesis, based on the vast development of the general field of nanoscience over the last few decades, as well as recent findings with regards to environmental nanoparticles themselves, is that these nanoparticles, in the size range of roughly 1 to 100 nanometers, show size dependent property and reactivity variability that can significantly influence the aqueous chemistry of natural and engineered waters. It is important to remember that this thesis goes beyond the effects of increased surface area as particles get smaller. As experiments have shown, even after surface area normalization, measured rates of reaction change, often dramatically, as a function of the nanoparticle size. The intrinsic properties of the material itself are changing; the material is behaving differently as it transitions from the bulk material to a molecular state.

When looked at in this way, environmental nanoparticle science goes well beyond the traditional boundaries of aqueous colloid science, but clearly these fields are highly complementary and are already beginning to merge. The result will be a better understanding of how natural and engineered waters behave as they do, and important continued development in the fields of water safety, treatment, and remediation.

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