A SPECTROSCOPIC STUDY OF BACTERIAL POLYMERS MEDIATING
CELL ADHESION AND MINERAL TRANSFORMATIONS

by

Sanjai Jagadeep Parikh

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CELL ADHESION AND MINERAL TRANSFORMATIONS

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Final approval and acceptance of this dissertation is contingent upon the candidate’s
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ABSTRACT

Current understanding of molecular-level interactions is inadequate to explain the initial moments of bacterial adhesion. Such information is required to develop appropriate models for bacteria-surface interactions and predictions of cell transport in subsurface environments. Bacterial adhesion is influenced by bacterial surfaces, substratum physical-chemical characteristics, and solution chemistry. Extracellular polymeric substances (EPS), surface proteins, and lipopolysaccharides (LPS) mediate cell adhesion and conditioning film formation via direct bonding to a substrate. The goal of this dissertation is to probe molecular-scale interactions of cell surface macromolecules at mineral surfaces under environmentally-relevant conditions.

Four primary investigations are presented in this dissertation. The first study uses in situ attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy to reveal that prior to Mn-oxidation via Pseudomonas putida GB-1, cell adhesion to ZnSe is favorable. Subsequent Mn-oxidation results in increased extracellular proteins expression. Conversely, planktonic cell adhesion is inhibited for Mn-oxide coated cells via blocking of surface proteins.

The second investigation reveals the formation of inner-sphere complexes between bacteria surface phosphoryl groups and nanohematite (\(\alpha\)-Fe\(_2\)O\(_3\)). Spectra of bacteria (P. aeruginosa PAO1, Shewanella oneidensis MR-1, and Bacillus subtilis) on \(\alpha\)-Fe\(_2\)O\(_3\) contain peaks indicative of P-OFe inner-sphere bonding. Spectra collected for oxide-adsorbed model P-containing compounds give spectral signatures similar to those P-OFe bonding interactions observed for whole cell and EPS.
The behavior of *P. aeruginosa* serotype 10 LPS in aqueous solutions was investigated in the third study. Ionic strength, pH, and electrolyte composition were varied during collection of ATR-FTIR and dynamic light scattering (DLS) data. Results reveal stable aggregate Na-LPS aggregates, whereas binding of Ca$^{2+}$ to phosphate groups in the lipid A region leads to aggregate reorientation and increased interaction with ZnSe (hydrophobic). DLS data demonstrate decreasing hydrodynamic radius of LPS aggregates with increasing $I$ and decreasing pH.

In the fourth investigation, ATR-FTIR was used to probe the solid-solution interface of LPS on surfaces of ZnSe, Ge, $\alpha$-Fe$_2$O$_3$, and $\alpha$-Al$_2$O$_3$ in solutions of varying ionic composition and pH. Na-LPS aggregates remain stable and spectra are biased towards solution phase LPS. Ca-LPS aggregates are disrupted, leading to enhanced interaction with surfaces via hydrophobic (lipid A- ZnSe) and electrostatic (O-antigen- hydrophilic surfaces) interactions.
CHAPTER 1

INTRODUCTION

Explanation of Dissertation Format

This dissertation is comprised of three chapters and nine appendices. Chapter one introduces the research problem and gives a general review of related current literature. Chapter two provides a summary of the present study and an overview of the detailed research presented in Appendices A through F. Chapter three brings together the major findings and highlights the importance of the dissertation research to the field of environmental chemistry. Appendices G through J provide preliminary data and additional information that were not included in other parts of the dissertation.

Four manuscripts for publication are found in appendices A through D. Appendices A, B, E, and G pertain to the adhesion of whole bacterial cells to surfaces. Additionally, biomineralization and biodissolution are addressed in Appendices A and E, respectively. Appendix A has been published in Geomicrobiology Journal and Appendix B will be submitted to Langmuir for publication. Prior to submission, Appendix B will be slightly reduced in length for journal publication. Appendix E describes interesting initial findings regarding bacterial adhesion and biocatalyzed Fe(III) reduction, however further research is required before a manuscript can be prepared for journal publication. Appendix G is provided to show preliminary data and method development for bacterial adhesion studies. Appendices C, D, and F examine the behavior of lipopolysaccharides (LPS) as a function of molecular composition, solution chemistry, and the presence of...
surfaces. Appendices C and D will be submitted to *Colloids and Surfaces B: Biointerfaces*. Minor changes to these extended manuscripts will be made prior to submission. Appendix F demonstrates the development of methods for LPS extraction and analysis. Appendix H provides an introduction to attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy, which is the primary experimental tool used in this dissertation. The reputability of ATR-FTIR spectroscopy is explored in Appendix I. Finally, a discussion on the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of colloid stability as it applies to bacterial adhesion is given in Appendix J.

My advisor, Dr. Jon Chorover, and others provided guidance and intellectual discussion regarding my research. However, the ideas addressed in the manuscripts (Appendix A through D), including experimental design, execution, and data analysis are my own and represent my original work. For Appendix A, Hanna L. Gilbert and Dr. Sunkyung Choi conducted SEM-EDS and TEM-EDS analyses, respectively. An NSF REU student working in the Chorover Lab, Mr. David A. White II, conducted the critical aggregate concentration measurements and some LPS extractions under my direct supervision (Appendix F).

**Explanation of Problem**

Contamination of soil and water from increased urbanization and industrialization threaten both human and environmental health. Understanding bacterial adhesion is critical for addressing environmental phenomena associated with the fate and transport of bacteria and anthropogenic contaminants. Soils and groundwater supplies face
contamination via migration of pathogenic bacteria (Matthess and Pekdeger, 1981; Corapcioğlu and Haridas, 1984; Abu-Ashour and Lee, 2000; Banning et al., 2003), and the task of purifying ground- and sewage water depends, in part, on bioadhesion of bacteria to limit transport (Powelson and Mills, 2001; Stevik et al., 2004; Auset et al., 2005). However, minimizing bacterial adhesion to membranes of water treatment facilities is also critical to prevent contamination of water supplies over long-term periods (Stevik et al., 2004). Soil contamination from fossil fuels, industrial waste, and chemical spills require rapid and cost effective methods for clean-up. The use of bacteria to remediate such sites (i.e., bioremediation) is an innovative and rapidly evolving technique (Bodour et al., 2003; Demnerova et al., 2005; Filonov et al., 2006; Lee et al., 2006). However, adhesion of bacteria to soil particles often diminishes the capacity for bioaugmentation of impacted subsurface zones and limits the use of this remediation strategy (Schafer et al., 1998; Mehmannavaz et al., 2001; Wick et al., 2004).

Bacteria that actively oxidize or reduce metals possess great potential for bioremediation strategies. Biomineralization and biodissolution reactions require interactions of bacteria with existing or newly-formed surfaces. These reactions can be very important for contaminant sequestration/release. During biogenic mineral formation environmental contaminants are often incorporated into, or adsorbed onto, the mixed organic-mineral phase (Nelson et al., 1999; Nelson et al., 2002; Tani et al., 2003; Tani et al., 2004; Tebo et al., 2004), thus limiting both contaminant and bacterial transport. Bacterial adhesion to surfaces followed by mineral dissolution results in a release of
contaminants from the solid. Using information regarding the adhesion of bacteria to surfaces bioremediation strategies can be exploited for increased efficiency.

Unfortunately, the molecular-level interactions between bacteria and environmental surfaces during bacterial adhesion have not been adequately explained. Understanding these interactions is required to manipulate and control the adhesion process. These reactions are driven by chemical interactions between the exterior of bacterial cells, the physiochemical characteristics of the substrate surface, and water chemistry. The exterior surfaces of bacterial cells are complex, comprised of surface proteins, nucleic acids, extracellular polymeric substances (EPS), lipopolysaccharides (LPS) (Gram negative bacteria), teichoic acids (Gram positive bacteria), and other surface biosynthetic molecules that likely are involved in the adhesion process (Wingender et al., 1999).

Microbial cells attach readily to submerged aquatic surfaces, but efficiency of adhesion is strongly dependent on surface charge and functionality (Marshall, 1992; Poortinga et al., 2002). Assuming nutritional and environmental conditions are satisfactory, bacteria will bind to surfaces, grow, reproduce, and synthesize extracellular polymers leading to biofilm formation (Characklis and Marshall, 1990). Biofilms can be beneficial, as they are important for sewage treatment and biological removal pollutants from natural water (Nivens et al., 1993). However, they can also have negative effects leading to biofouling of plumbing systems, pipelines, and ship hulls (Wingender et al., 1999). Biofilms also impact mammalian health, via infection and chronic disease (Costerton et al., 1999; Hentzer et al., 2001).
Biofilms vary greatly according to constituent bacterial species, water chemistry, shear stress, and environmental conditions (Christensen and Characklis, 1990; Heydorn et al., 2000). The heterogeneity of biofilms makes it difficult to give a precise definition to the term. To summarize the lengthy definition given by Characklis and Marshall (1990), a biofilm is “a surface accumulation, which is not necessarily uniform in space or time, which comprises cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin.”

Bacterial adhesion leads to biofilm formation through a series of specific development steps (Davies, 1999; Cheung et al., 2000; Watnick and Kolter, 2000; Hentzer et al., 2001). The general sequence of events can be summarized as follows: (1) planktonic cells move toward the surface by Brownian motion or cell-mediated motility; (2) cells and biomolecules passing the surface make contact leading to conditioning film formation on the substratum to enhance attachment; (3) “reversible” attachment of cells to the surface; (4) “irreversible” attachment of cells to the surface and an upgrade in production of EPS. In the last step cells no longer exhibit Brownian motion and the biofilm is formed as EPS connects many cells together reinforcing their place on the substratum. Biofilm formation of *P. aeruginosa* has been shown to involve a cell-to-cell signaling which is required for the differentiation of individual cells to form into biofilms (Davies et al., 1998). Bacterial adhesion in the case of *P. aeruginosa*, and representative time-scales for each stage of biofilm growth are shown in Figure 1.
Bacterial surface characteristics are important factors in the adhesion process (van der Mei et al., 1989). Some of the important surface characteristics include hydrophobicity (Rosenberg and Kjellberg, 1986; van Loosdrecht et al., 1987), surface free energy (Busscher et al., 1984), surface charge (van Loosdrecht et al., 1987), and specific biomolecular composition of EPS (Wingender et al., 1999), LPS or teichoic acids (Williams and Fletcher, 1996), proteins, and surface appendages (e.g. fimbrae) (Scannapieco et al., 1983).

The chemical properties of both bacteria and abiotic environmental surfaces influence adhesion of microorganisms to environmental particles. Long-range forces, including van der Waals induced dipole and electrostatic interaction are important to the adhesion process (Marshall et al., 1971), and these forces are typically those that are incorporated into predictive models of bacterial transport via DLVO (Derjaguin, Landau, Verwey, and Overbeek) theory of colloid stability (Derjaguin and Landau, 1945; Verwey

Figure 1. Schematic representation of the attachment of *P. aeruginosa* to a germanium crystal (approximate time frame of reaction given in parenthesis).
and Overbeek, 1948; Stumm and Morgan, 1996). Short-range interactions including: 1) chemical interactions (covalent bonding, ionic bonding, hydrogen bonding), 2) dipole interactions, and 3) hydrophobic interactions are also involved (Marshall, 1986). Additionally, microbial adhesion is affected by steric interactions, particularly important for overlapping regions of polymer segments (Harris and Mitchell, 1973; Neu and Marshall, 1990). Steric interactions can promote adhesion via bridging of surface macromolecules, or inhibit it because of entropic effects manifest as steric repulsion (Rijnaarts et al., 1999).

Bacteria and many environmental particles exhibit a net negative charge at environmental pH values (Rijnaarts et al., 1995). For example, silica is negatively charged at pH > 2.0 to 3.0 (Sposito, 1989). However, in weathering environments, many siliceous surfaces become coated with a veneer of hydrous Al and Fe oxide, which can confer net positive charge even at circumneutral pH (Sposito, 1989). Bacterial adhesion to metal-oxide-coated surfaces is often greater than what is observed for negatively charged surfaces and the difference is often attributed to electrostatic attraction (Truesdail et al., 1998; Bolster et al., 2001; Deo et al., 2001).

The DLVO theory as applied to cell adhesion is often used for predicting the fate and transport of bacteria in aqueous systems (Appendix J). However, in its original form, DLVO theory accounts only for the attractive van der Waals dispersion force and diffuse double layer repulsion (Stumm and Morgan, 1996; Poortinga et al., 2002), and it is not sufficient to account for the more complex macromolecular features of adhesion of biological surfaces (Hermansson, 1999; Poortinga et al., 2002). Previous research has
shown that additional non-DLVO forces including covalent bonding, hydrophobic interaction, Lewis acid-base interactions (polar interactions), (van Oss, 1994), and steric interactions (Rijnaarts et al., 1995) are important in bacterial adhesion. The extent to which specific DLVO and non-DLVO interaction are involved in bacterial adhesion is not known.

This dissertation focuses on elucidating the molecular-level interactions of cell surface biomolecules in bacterial adhesion and related biomineralization/dissolution reactions. The research hypotheses are that, (1) molecular-scale interactions of bacterial surface and extracellular biopolymers control adhesion to environmental surfaces, and (2) Fourier transform infrared (FTIR) spectroscopy will provide information regarding the molecular-scale interactions of cell adhesion. An introduction to FTIR sampling techniques is provided in the methods section of Appendix A, and a detailed description of ATR-FTIR spectroscopy is given in Appendix E.

This dissertation addresses bacterial adhesion and conditioning film formation in natural aqueous environments. Experiments were carried over two size scales, the first (µm scale) representing adhesion and reactions of whole bacteria cells with surfaces. The second scale (nm) investigates the behavior of LPS molecules in solutions and at solid-liquid interfaces as a function of solution chemistry and surface composition.
CHAPTER 2
PRESENT STUDY

The dissertation appendices contain detailed methods, results, and conclusions for this study. General descriptions of the most important findings are presented in the following summary.

Summary

The overall objective of the research was to investigate the molecular-level interactions of bacteria during adhesion, and associated biomineralization and biodissolution. This was achieved via a series of experiments to examine cell-bound and extracted biomolecules (i.e., lipopolysaccharides [LPS]). Spectroscopic techniques, primarily Fourier transform infrared (FTIR), was used to elucidate functional group reactions for bacteria and LPS subjected to a range of aqueous solution chemistries and in the presence of numerous different surfaces. The specific projects designed to investigate these interactions are detailed in the following objectives.

Objective #1

The primary objective of the first paper in this dissertation (Appendix A) was to use FTIR spectroscopy in several complementary sample introduction modes (attenuated total reflectance [ATR], diffuse reflectance [DRIFT], and transmission) to investigate the processes of cell adhesion, biofilm growth, and biological Mn-oxidation by Pseudomonas
*Pseudomonas putida* strain GB-1. It was hypothesized that FTIR spectroscopy could be used to probe changes of both organic (bacteria) and inorganic (MnO$_x$) composition resulting from biocatalyzed Mn oxidation.

Adhesion of cells to Ge, ZnSe, and CdTe transmission windows was monitored by suspending crystals in a bioreactor of *P. putida* at the steady state (30°C, 150 rpm, aerobic). These experiments were carried out with (GB-1/MnO$_x$) and without (GB-1) the presence of Mn(II). Adhered cells were examined via transmission FTIR spectroscopy and scanning electron microscopy (SEM) in “nature” mode (i.e., in the presence of water). Flow-through experiments were also conducted to observe *in situ* adhesion and biofilm growth of *P. putida* on a cylindrical ZnSe internal reflection element (IRE). These experiments also used the bioreactor to maintain cell growth conditions, and were conducted using ATR-FTIR spectroscopy (GB-1 and GB-1/MnO$_x$ from beginning and with addition of Mn(II) after stable GB-1 biofilm growth) to determine the effect of Mn oxidation on adhesion and biomass accumulation at the IRE-liquid interface. Characterization of GB-1 and GB-1/MnO$_x$ was performed using ATR-FTIR spectroscopy after deposition onto a horizontal ZnSe IRE. DRIFT spectra were acquired on freeze-dried GB-1 and GB-1/MnO$_x$ cells. GB-1/MnO$_x$ complexes were washed to diminish the organic component signal in order to increase ability to identify the biogenic mineral phase.

Evident differences in adhesion of GB-1 and GB-1/MnO$_x$ were observed as a response to Mn oxidation. Adhesion of GB-1 cells to a ZnSe, Ge, and CdTe was observed, however adhesion was inhibited for GB-1/MnO$_x$ cells to a ZnSe IRE. Upon Mn-
oxidation, increased cell biomass and contributions from surficial or extracellular proteins (amide I and II) were observed via ATR-FTIR and diffuse reflectance FTIR (DRIFT) spectra. DRIFT and transmission spectra of biogenic Mn oxide reveal peaks corresponding to Mn-O stretching vibrations characteristic of common Mn(IV) oxides (e.g., “acid” birnessite, romanechite, todorokite). However, due to incorporation of biomolecular constituents spectra are not identical to known synthetic solids. The results suggest that, when biogenic MnOx accumulates on the surfaces of planktonic cells, adhesion of the bacteria to other negatively-charged surfaces is hindered via blocking of surficial proteins.

**Objective #2**

The second objective, addressed in the paper presented in Appendix B, was to test the hypothesis that interactions between bacteria and metal oxide surfaces are mediated by inner-sphere complexation at biomolecular phosphate groups. This hypothesis derives from previous work on EPS-Fe oxide sorption processes conducted in our research group. Whole cell-metal oxide adhesion was probed using ATR-FTIR spectroscopy of Gram negative (*Pseudomonas aeruginosa* PAO1, *Shewanella oneidensis* MR-1) and Gram positive (*Bacillus subtilis*) bacterial cells in the presence of ZnSe, α-Fe₂O₃, and α-Al₂O₃ surfaces. To improve data analysis and further investigate P-O-Fe bond formation, interactions of selected organic compounds with P-containing functionalities (phenylphosphonic acid [PPA], adenosine 5′-monophosphate [AMP], 2′-
deoxyadenylyl(3′→5′)-2′-deoxyadenosine [DADA], deoxyribonucleic acid [DNA]) with these same surfaces were examined.

Bacteria were grown in the appropriate growth media to the late exponential phase and washed via centrifugation (100 mmol L⁻¹ NaCl, pH 7) to remove nutrient rich media. Cells were resuspended in either 100 mmol L⁻¹ NaCl (pH 7) or colloidal suspensions of α-Fe₂O₃ or α-Al₂O₃. P-containing compounds were dissolved in 1 mmol L⁻¹ NaCl (pH 7). Three distinct experimental designs were employed to study interactions with each surface; bacterial cells/P-containing compounds were deposited on (1) clean ZnSe, (2) coated-ZnSe (α-Fe₂O₃ or α-Al₂O₃), and (3) clean ZnSe after aggregation with suspended α-Fe₂O₃ or α-Al₂O₃.

In the case of bacterial interactions with α-Fe₂O₃, the hypothesis was confirmed via the appearance of strong peaks corresponding to P-O-Fe and saccharide ring structures. Analogous emergent peaks were not observed in the case of α-Al₂O₃. The latter spectra were similar to the interactions of bacteria with the ZnSe surface, and show higher relative protein absorbance that those collected with α-Fe₂O₃ present. The formation of P-O-Fe bonds was also established for DNA, DADA, AMP and PPA upon abiotic reaction of these model compounds with α-Fe₂O₃. ATR-FTIR analysis gives rise to peak locations consistent with previous studies conducted in our lab of extracellular polymeric substances (EPS), which indicates a combination of similar phosphate/phosphonate groups are present in EPS, including extracellular DNA, and are likely involved in cell adhesion to Fe-oxide surfaces. Reaction of free DNA alone with α-Fe₂O₃ resulted in Fe(III)-induced oxidation and cleavage of ribose rings.
Although, it is known that strong associations between Fe and phosphate occur in natural systems they have not been shown previously to play an important role in adhesion of metabolizing bacteria. This paper demonstrates bacterial phosphate/phosphonate groups – either membrane bound or part of the EPS – are involved in adhesion to Fe-oxides through formation of inner-sphere Fe-phosphate complexes.

**Objective #3**

The third objective was to elucidate the behavior of LPS macromolecules as a function of solution chemistry (Appendix C). LPS are amphiphilic molecules on the surface of Gram negative bacteria, which are released into the environment via cell turnover and lysis. LPS contain a hydrophobic lipid A region (2.4 nm) and a hydrophilic O-antigen region (≤ 40 nm) that promote aggregation via formation of micellar structures. Dynamic light scattering (DLS) and ATR-FTIR spectroscopy were employed to determine LPS (*Pseudomonas aeruginosa* ser 10) aggregate size and conformational changes resulting from changes in solution pH (3, 6, and 9), ionic strength ([I]; 1, 10, and 100 mM), and electrolyte composition (NaCl and CaCl₂).

DLS experiments were performed with LPS concentrations of 0.25, 0.5, and 1 mg mL⁻¹. DLS results indicate decreasing particle hydrodynamic diameters with increasing I. Particle size was unchanged when decreasing pH from 9 to 6, however further decrease to pH 3 resulted in decreased aggregate size. LPS concentration did not influence aggregate size under the conditions of this study.
ATR-FTIR data reveal relatively stable Na-LPS aggregates. In contrast, Ca-LPS aggregates were easily disrupted and interaction with the ZnSe internal reflection elements (IRE) was observed under these conditions. Increased spectral contributions from the lipid A are observed for Ca- relative to Na-LPS. These effects become increasingly apparent with time (up to 2 h), indicating lipid A (Ca-LPS) migration towards the ZnSe surface. Time- and cation-dependent changes in ATR-FTIR data suggest that LPS aggregates are perturbed by Ca$^{2+}$ complexation at lipid A phosphoryl groups, thus leading to reorientation of the lipid A at the surface of the ATR IRE.

The data highlight the potential for ATR-FTIR to reveal information on the solution phase behavior of these amphiphilic molecules that, in turn, influences their interaction with surfaces (e.g., the IRE). The acquired spectra reveal a very important role of background cation (Ca$^{2+}$ vs. Na$^{+}$) on the conformation of LPS aggregates in aqueous solutions. Bivalent Ca forms strong complexes with phosphate groups in the lipid A, leading to weaker aggregation and subsequent interaction of the lipid A moieties with the hydrophobic ZnSe surface. This paper demonstrates the impact of solution chemistry on LPS aggregation, conformation, and surface activity.

**Objective #4**

Solution chemistry influences LPS aggregation and subsequent interaction/adhesion with surfaces. The fourth objective was to examine further the influence of solution chemistry on adsorption of LPS to mineral surfaces. This objective was accomplished (Appendix D) through exploration of Ca-LPS and Na-LPS ($I = 10$
mmol L⁻¹; pH 3, 6, 9) interactions with surfaces of varying charge (pH dependent), hydrophobicity, and chemical composition via ATR-FTIR spectroscopy. The study also probed LPS-surface complexes (ZnSe and Ge IREs) via variable angle ATR (VATR)-FTIR spectroscopy (I = 10 mmol L⁻¹; pH 6), which permits spectral acquisition as a function of IR beam penetration depth and helps to understand LPS aggregate conformation at the solid-liquid interface. The relative affinity of Na-LPS and Ca-LPS for surfaces was determined via batch sorption experiments with colloidal Ge, α-FeOOH, and α-Al₂O₃ solids.

Na-LPS spectra collected on ZnSe, Ge, α-Fe₂O₃, and α-Al₂O₃ surfaces were similar, suggesting weak surface interaction. ATR-FTIR and batch experiments both indicated greater interaction between Ca-LPS and the mineral surfaces. Ca-LPS interactions with ZnSe are controlled primarily by hydrophobic interactions with the lipid A, whereas pH-dependent surface charge plays a key role in Ca-LPS bonding through the O-antigen to the more hydrophilic surfaces (Ge, α-Fe₂O₃, α-Al₂O₃). Increased interaction was observed at lower pH values, where surfaces are generally positively charged.

VATR-FTIR analyses reveal penetration-depth-dependent trends in IR spectra, reflecting complex molecular conformations occurring on the spatial scale of LPS aggregates, particularly in the case of Ca-LPS. However, the specific orientation of LPS molecules cannot be determined via techniques employed in this study.

These results verify reorientation of LPS aggregates in the presence of Ca²⁺ at a solid-liquid interface. The background cation (i.e., Ca²⁺, Na⁺) strongly influences aggregate structure and surface interactions. This work demonstrates the importance of
surface and solution chemistry to LPS behavior at a solid-liquid interface. However, further work is required to understand LPS orientation at these interfaces.

**Objective #5**

The fifth objective was to examine the adhesion of the dissimilatory iron reducer *Shewanella oneidensis* (MR-1) at ZnSe and nanohematite (α-Fe₂O₃) surfaces under fluid flow conditions (Appendix E). *In situ* monitoring of biofilm formation on a cylindrical ZnSe IRE was performed via ATR-FTIR spectroscopy. Steady-state *S. oneidensis* cells were pumped out of a bioreactor (30° C, pH 7.4, 100 rpm) to the ATR cell (0.5 mL/min) and spectra were collected as a function of time. After biofilm formation (0-24 h) anoxic conditions were imposed on the flow cell (24-48 h) to observe biocatalyzed Fe(III) reduction via *S. oneidensis*. An additional experiment was performed to monitor cell adhesion to ZnSe with anoxic conditions from time zero.

Successful biofilm formation was achieved for bacteria on ZnSe and α-Fe₂O₃-coated ZnSe surfaces. Adhesion to both surfaces indicates an important role of surface proteins, as inferred from large IR spectral contributions from amide I and II vibrations. Close examination of spectra reveal difference in the mixed carbohydrate and phosphate region. Adhesion of bacteria to α-Fe₂O₃ appears also to involve bacterial phosphate groups, as peaks corresponding to P-O-Fe were identified in the acquired spectra.

A striking visible color change (grey-brown to red) was observed when the bioreactor was switched from oxic to anoxic conditions. This strong visual effect has been reported elsewhere and results from changes in conformation of heme proteins on
the surface of *S. oneidensis*. Unfortunately, this change was not detectable via ATR-FTIR spectroscopy. However, under anoxic conditions, reduction in peaks corresponding to Fe-O vibrations was observed. This may indicate biocatalyzed Fe(III) reduction at the bacteria-α-Fe₂O₃ interface. Although the bioreduction of Fe-oxides by *S. oneidensis* is well documented, further experiments are required to confirm that these changes can be observed via *in situ* FTIR studies.

**Objective #6**

A sixth objective of this dissertation was the development of experimental methods for studying LPS (Appendix F). Both rough and smooth LPS were extracted from *P. aeruginosa* PAO1 (smooth LPS), *Escherichia coli* JM109 (smooth LPS), and *E. coli* D21 (rough LPS). The extracted LPS, along with purchased LPS from *P. aeruginosa* ser 10 (smooth LPS), *Salmonella typhimurium* TV119 (rough LPS), and *S. minnesota* Re595 (deep rough) were analyzed via transmission FTIR to determine the effectiveness of this technique for quantifying relative contributions of lipid A and O-antigen moieties to the extracted material. Methods for measuring the critical aggregate concentration (CAC) of these samples were also explored.

CAC was measured by both electrical conductivity (EC) and ultraviolet-visible spectroscopy (UV-Vis, with 8-anilino-1-napthalensulfonic acid) techniques. The EC method is limited to measuring CAC in pure water, as EC changes resulting from LPS aggregation become increasingly difficult to detect in high ionic strength background solutions. The UV-Vis spectroscopy technique is unaffected by this interference, but it
needs further refinement to more accurately determine inflection points corresponding to the CAC. If this technique is determined to be reliable, it could provide information regarding the CAC for LPS in a variety of different liquid systems.

The CAC of LPS is expected to depend on the relative predominance of the hydrophobic lipid A and hydrophilic O-antigen, which differentiates smooth and rough LPS. In this study transmission FTIR was successfully utilized for evaluating these relative contributions, and differentiating between rough and smooth LPS. This information can be directly related to LPS size and CAC values.
CHAPTER 3

CONCLUSIONS

Predictions of bacterial adhesion to soils and sediments are limited by insufficient understanding of the underlying molecular mechanisms. As a result, standard models (e.g., DLVO theory) do not adequately describe cell adhesion in natural environments (Hermansson, 1999; Poortinga et al., 2002). Increased application of bioremediation to soils, sediments, and water supplies has produced a greater need for answers as to how and why bacteria attach to surfaces. Adhesion is affected by environmental conditions, which vary due to geography and location in the environment (e.g., soil, colloidal freshwater particles, deep-sea sediments). Manifold uncertainty also results from the heterogeneous nature of bacteria and environmental surfaces, thus leading to a complex set of numerous inter-related variables that control cell adhesion. Investigations require a systematic approach where general trends leading to adhesion events can be elucidated.

This thesis research was designed to derive information regarding the primary bacteria-surface functional groups involved in adhesion to surfaces representative of natural particles. While part of the study focuses on living bacteria and determining general trends of cell adhesion, additional experiments were designed to specifically examine free lipopolysaccharides (LPS) in solution and the role of LPS in conditioning film formation and cell adhesion.

This dissertation contributes to the field of environmental biogeochemistry, most significantly through three major findings. The first illustrates a critical role of
*Pseudomonas putida* GB-1 driven mineral oxidation for cell adhesion and biofilm development (Appendix A). Inhibition of cell adhesion resulted from Mn-oxide coatings on cell surfaces, whereas adhesion and biofilm formation was observed for Mn-free bacteria. The formation of Mn-oxide coatings on GB-1 cells has been documented (Okazaki et al., 1997; Brouwers et al., 2000), and it has been hypothesized that these coatings protect cells from metal toxicity, UV irradiation, or other potential threats (Brouwers et al., 2000; Tebo et al., 2005). Our work now indicates that this “armoring” also diminishes cell immobilization on surfaces that may be present in metal contaminated systems. This is the first study to indicate increased biofilm growth as documented by protein accumulation during biocatalyzed Mn-oxidation.

The second major finding reveals formation of Fe-phosphate complexes during cell adhesion to Fe-oxides (Appendix B). Binding of orthophosphate (Tejedor-Tejedor and Anderson, 1990; Persson et al., 1996; Wigand et al., 1997; Arai and Sparks, 2001), organic-bound phosphate (Miller et al., 2001), and phosphate associated with bacterial extracellular polymeric substance (Omoike et al., 2004) to Fe-oxides has been previously shown. However, this dissertation presents the first direct evidence of living bacteria binding to Fe-oxides via formation of inner-sphere Fe-phosphate/phosphonate complexes.

The third major finding demonstrates a strong solution chemistry (primarily cation valence) dependence on the aggregation and surface interactions of LPS in environmentally relevant aqueous solutions (Appendix C and D). Previous research has focused on the behavior of LPS in relation to human-health, but there are few prior studies pertaining to conditions that represent the natural aqueous environment
(Brandenburg et al., 1993; Paradis et al., 1994; Zdorovenko and Veremeichenko, 2001). The destabilization of LPS aggregates in the presence of Ca\(^{2+}\) is in agreement with previous studies (Coughlin et al., 1983; Wang et al., 2005). However, new information regarding the interaction of Ca-LPS with surfaces of varying hydrophobicity and charge is provided through the present work. The new results contribute to our ability to predict sorption of LPS as a function of solution and surface chemistry.

As a whole, the results from this dissertation support the established perspective that electrostatic and hydrophobic interactions do impact biomolecular interactions in bacterial adhesion. The involvement of these “mean field” forces in adhesion has been noted previously (Marshall et al., 1971; Marshall, 1986). The current work, however, also highlights the importance of molecular-scale bonding interactions between substrate sites and specific cell surface functional groups (e.g., phosphoryl) that constitute only a small fraction of the total charged moieties of the cell surface. Contributions resulting from steric interactions may also occur (Rijnaarts et al., 1995; Jucker et al., 1997), but based on the research conducted for this dissertation they are not believed to be the primary forces involved for adhesion of bacteria and LPS. Increased understanding of the primary functional groups on bacterial surfaces involved in cell adhesion allow emphasis to be placed on specific interactions when developing models to predict bacterial adhesion.

While the data and analysis presented in this dissertation do not conclusively solve the problems associated with predicting bacterial adhesion in natural environments, the information within contributes to an increased understanding of molecular mechanisms involved. Further investigation into the surface properties of bacteria and
surfaces and how they relate to adhesion under different environmental conditions is required for development of accurate models pertaining to bacterial adhesion.
REFERENCES


APPENDIX A

FTIR SPECTROSCOPIC STUDY OF BIOGENIC MN-OXIDE FORMATION BY PSEUDOMONAS PUTIDA GB-1

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Abstract

Biomineralization in heterogeneous aqueous systems results from a complex association between pre-existing surfaces, bacterial cells, extracellular biomacromolecules, and neoformed precipitates. Fourier transform infrared (FTIR) spectroscopy was used in several complementary sample introduction modes (attenuated total reflectance [ATR], diffuse reflectance [DRIFT], and transmission) to investigate the processes of cell adhesion, biofilm growth, and biological Mn-oxidation by Pseudomonas putida strain GB-1. Distinct differences in the adhesive properties of GB-1 were observed upon Mn oxidation. No adhesion to the ZnSe crystal surface was observed for planktonic GB-1 cells coated with biogenic MnOx, whereas cell adhesion was extensive and a GB-1 biofilm was readily grown on ZnSe, CdTe, and Ge crystals prior to Mn-oxidation. IR peak intensity ratios reveal changes in biomolecular (carbohydrate, phosphate, and protein) composition during biologically-catalyzed Mn-oxidation. In situ monitoring via ATR-FTIR of an active GB-1 biofilm and DRIFT data revealed an increase in extracellular protein (amide I and II) during Mn(II) oxidation, whereas transmission mode measurements suggest an overall increase in carbohydrate and phosphate moieties. The FTIR spectrum of biogenic Mn oxide comprises Mn-O stretching vibrations characteristic of various known Mn oxides (e.g., “acid” birnessite, romanechite, todorokite), but it is not identical to known synthetic solids, possibly because of solid-phase incorporation of biomolecular constituents. The results suggest that, when biogenic MnOx accumulates on the surfaces of planktonic cells, adhesion of the bacteria to other negatively-charged surfaces is hindered via blocking of surficial proteins.
Introduction

Manganese is the second most abundant transition metal in the earth’s crust behind Fe and, like Fe, its oxidation-reduction reactions are largely mediated by biological activity (Lovley, 2000). Microbial catalysis is known to accelerate the kinetics of Mn(II) oxidation and promote the formation of Mn(IV) oxide minerals in natural waters (Nealson et al., 1988; Tebo et al., 1997). Manganese oxides (MnO\textsubscript{x}) are produced biogenically by numerous species of bacteria, including \textit{Pseudomonas putida} strain GB-1, a fresh-water, facultative-aerobic, gram-negative bacteria. The formation of MnO\textsubscript{x} can influence the environmental fate of other metals (e.g., Cu, Co, Cd, Zn, Ni, and Pb) through co-precipitation and adsorption reactions (Nelson et al., 1999; Nelson et al., 2002; Tani et al., 2003; Tani et al., 2004; Tebo et al., 2004). The physiological basis for bacterially-mediated Mn oxidation is not known, but it is thought that biogenic MnO\textsubscript{x} may serve to protect cells from Mn or other metal toxicity (e.g., heavy metals), UV irradiation, or other potential threats (Brouwers et al., 2000).

The identity of biogenic MnO\textsubscript{x} phases is diverse with apparent dependence on the type of microbial catalyst and conditions of formation. For example, Mandernack et al. (1995) reported mixed phase minerals (hausmannite, Mn\textsubscript{3}O\textsubscript{4}; feiknechtite, \(\beta\)-MnOOH; manganite, \(\gamma\)-MnOOH, and Na-buserite) following Mn(II) oxidation by a marine \textit{Bacillus} strain SG-1, whereas a nanocrystalline todorokite-like mineral was produced by \textit{Leptothrix discophora} strain SP-6 (Kim et al., 2003). The MnO\textsubscript{x} formed by \textit{P. putida} strain MnB1 was found to be most similar to “acid” birnessite (Villalobos et al., 2003), whereas Mn oxide crusts from Pinal Creek, AZ comprise a mixture of todorokite and
birnessite or takanelite/ranci ete, possibly deriving from a buserite precursor (Bilinski et al., 2002) (Gilbert, 2003).

The mechanisms of biotic Mn(II) oxidation and the subsequent binding of MnO_x to cell surfaces are not known. One or more enzymes is likely responsible for catalyzing Mn(II) oxidation. Recent research indicates that the *cumA* gene, a multicopper oxidase (Brouwers et al., 1999) (Francis and Tebo, 2001), and/or a general secretion pathway (*xcp* in *Pseudomonas* species) gene (de Vrind et al., 2003) are integrally involved in Mn oxidation. However, these genes have not been identified unambiguously. Although great progress has been made in identifying the enzymes involved in Mn oxidation (Tebo et al., 2004), the specific macromolecules (e.g., protein or protein/carbohydrate complex) have not been determined (de Vrind et al., 1998).

Upon oxidation by GB-1, MnO_x are precipitated on the cell surface in intimate association with surficial macromolecules (Okazaki et al., 1997). These surface macromolecules may serve to template the oxide nucleation process and influence subsequent crystal growth in a manner that has been observed, for example, with Fe oxyhydroxides (Chan et al., 2004) (Nesterova et al., 2003). When MnO_x encrustations are formed on the surface of planktonic cells, they are expected to alter the cell surface charge and hydrophobicity, thus altering adhesion and transport properties of the cells (Rosenberg and Kjellberg, 1986; van Loosdrecht et al., 1987; van der Mei et al., 1989). The solids may also be formed by intact biofilms that are already attached to stable substrata. The process of biogenic MnO_x formation in heterogeneous aqueous systems may, therefore, be considered a ternary interaction among bacterial cells, neoformed
MnO\textsubscript{x}, and existing surfaces (substrata) for biofilm growth. The favorability of these various interactions is governed to a large degree by the respective surface chemistries. The charge and hydrophobicity of bacterial cells are affected by the composition of surficial macromolecules including extracellular polymeric substances (EPS; i.e., extracellular polysaccharides, proteins, and nucleic acids) (Wingender et al., 1999), lipopolysaccharides (LPS) (Williams and Fletcher, 1996), and surface appendages such as fimbrae (Scannapieco et al., 1983). The degree to which biomolecular composition may be altered in response to environmental cues - such as availability of dissolved Mn(II) and the formation of MnO\textsubscript{x} - is not well known, but it is expected to influence the molecular-scale mechanisms of mineral-microbe interaction.

An improved understanding of the process of biogenic MnO\textsubscript{x} formation should follow from \textit{in-situ} investigation into the production, composition, and structure of biogenic MnO\textsubscript{x} and the co-evolving biomolecular matrix, referred to hereafter as GB-1/MnO\textsubscript{x}. Fourier transform infrared (FTIR) spectroscopy is well-suited for such studies, because it provides simultaneously molecular-scale information on both organic and inorganic constituents of a sample. FTIR spectroscopy uses polychromatic radiation to measure the excitation of molecular bonds whose relative absorbances provide an index of the abundance of various functional groups (Griffiths and Haseth., 1986). Since it can be used to probe distinct vibrations arising from both biomolecules and inorganic solids, it is emerging as a useful tool for investigating processes at the bacteria-mineral and biomolecule-mineral interface (Deo et al., 2001; Benning et al., 2004; Omoike et al., 2004). In this work, FTIR spectroscopy was used to determine whether Mn(II) oxidation
results in a detectable change in the biomolecular composition of GB-1 suspensions and biofilms. Since prior observations suggest a strong association between *P. putida* EPS and neoformed biogenic Mn oxides (Okazaki et al., 1997), we hypothesized that Mn biomineralization results in IR detectable changes in the relative proportion of polysaccharide and protein constituents external to the cell. Complementary modes of FTIR sample introduction were employed to resolve changes in molecular composition of the mineral-microbe interface relative to those occurring in the bulk.

**Experimental Methods**

**Bacterial strain, media and growth conditions**

*Pseudomonas putida* strain GB-1 was generously provided by B. M. Tebo (Scripps Institute of Oceanography). Bacteria were plated on *Leptothrix discophora* agar (Boogerd and Devrind, 1987) at pH 7.5 with 0.2 mM MnSO₄ to ensure the Mn oxidizing factor was present. Bacteria were grown in 250 mL polycarbonate screw cap Erlenmeyer flasks (Nalgene), and subjected to orbital mixing at 100 rpm in an environmental shaker at 30°C. The growth medium contained the following concentrations of mineral salts, trace elements, and glucose (MSTG) dissolved in Barnstead nanopure water: 2 mM \((\text{NH}_4)_2\text{SO}_4\), 0.25 mM \(\text{MgSO}_4\), 0.4 mM \(\text{CaCl}_2\), 0.15 mM \(\text{KH}_2\text{PO}_4\), 0.25 mM \(\text{Na}_2\text{HPO}_4\), 10 mM HEPES, 0.01 mM \(\text{FeCl}_3\), 0.01 mM EDTA, 1 mM glucose, and 1 mL of trace metal solution (10 mg/L \(\text{CuSO}_4 \cdot 5\text{H}_2\text{O}\), 44 mg/L \(\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}\), 20 mg/L \(\text{COCl}_2 \cdot 6\text{H}_2\text{O}\), and 13 mg/L \(\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}\)). Prior to autoclaving, the pH was adjusted to 7.4 with 2 M NaOH. Solutions of \(\text{FeCl}_3\) and trace metals were filter sterilized (0.2 µm) and added to
autoclaved MSTG media. For Mn oxidation experiments, MnSO₄ (0.2 mM) was added to MSTG media. In the study of adhesion of GB-1 cells to transmission windows, Rein’s vitamin solution (for 200 mL: 40 mL of 1 mg mL⁻¹ biotin, 4 mg nicotinic acid, 2 mg, thiamin, 4 mg p-aminobenzoic acid, 2 mg calcium pantothenate, 20 mg pyridoxine, 2 mg cyanobalamin, 4 mg riboflavin, and 4 mg folic acid) was used (1 mL L⁻¹) instead of the trace metal solution (mineral salts, vitamins, glucose; MSVG). A leucoberbeline blue (LBB) assay was used to confirm Mn oxidation (Boogerd and Devrind, 1987). Growth of all bacteria was carried out with autoclaved materials under pure culture conditions. Growth curves were determined from UV absorbance (λ = 600 nm) of cell suspensions using replicate samples.

**Electron microscopy**

GB-1 cells were harvested from the late exponential phase (24 h) and stained using 0.5 % ammonium molybdate (pH 7.56). Samples were dried onto carbon and piloform coated copper grids and observed at 200 kV with a Hitachi H8100 LaB₆ transmission electron microscope (TEM) equipped with energy dispersive x-ray spectroscopy (EDS) and electron diffraction (ED). GB-1/MnOₓ collected from the stationary phase, were also examined without the negative stain to observe electron dense MnOₓ coatings. EDS was performed to verify the composition of Mn coatings. GB-1 biofilms on transmission windows were analyzed with a Hitachi S-2460N natural scanning electron microscope (SEM) with EDS in NatureMode (~40 Pa, 25 kV, working distance 21mm) with a back-scattered electron Robinson detector.
Methods of sample introduction for FTIR spectroscopy

All FTIR spectra were collected using a Nicolet 560 Magna IR spectrometer (Madison, WI). Transmission, diffuse reflectance, and attenuated total reflection sample introduction techniques were employed to characterize the bulk and extracellular composition of GB-1 and GB-1/MnOx (Fig. 1). Each technique has inherent strengths and weaknesses, and provides complementary data. Transmission mode (Fig. 1a) provides data on the bulk sample (e.g., whole cell). One disadvantage of transmission mode is the need for sample desiccation, possibly creating artifacts, such as the dehydration of surface complexes. In diffuse reflectance FTIR (DRIFT; Fig 1b) radiation penetrates the sample to a depth that depends on the reflective and absorptive characteristics of the sample. Re-emission of this radiation occurs in all directions (i.e., “diffusely”). Upon collection, the emitted radiation produces an “absorbance spectrum” that is comparable to that produced in transmission mode, but more dependent on the spectral properties of the sample interface (Griffiths and Haseth., 1986).

Attenuated total reflectance (ATR) FTIR spectroscopy (Fig 1c, 1d) provides nondestructive, in-situ information on aqueous phase samples, including microbial cells and biofilms (Nichols et al., 1985; Nivens et al., 1993; Schmitt et al., 1995; Schmitt and Flemming, 1998). This technique interrogates IR-absorbing functional groups of the sample that reside in close proximity (ca. ≤ 1 µm) to the interface with an IR-transparent
Figure 1. Schematic illustration demonstrating the basic concepts of: (a) transmission spectroscopy, (b) DRIFT spectroscopy, (c) ATR (ARK) spectroscopy, and (d) ATR (circle cell) spectroscopy (d.p. = depth of penetration).

internal reflection element (IRE) (Nivens et al., 1993). ATR-FTIR techniques are particularly useful for examining changes in amide, carbohydrate and other polar functional groups resulting from biofilm evolution (e.g., (Nichols et al., 1985)), biomolecular composition/conformation (e.g., (Omoike and Chorover, 2004)) and surface complexation ((Omoike et al., 2004)). As a result of the limited beam penetration depth, ATR collects information on the composition of the bacteria-IRE interface, giving rise to spectra that emphasize the molecular composition of bacterial surfaces. While the reflected beam may penetrate into cells if the outer cell membrane is adhered directly to the crystal surface, this effect is diminished when EPS is interposed between cells and the
IRE. The acquired spectra thus contain peaks deriving from vibrational modes of both surficial and internal cell material but, relative to transmission data, there is clearly bias toward surface composition. ATR data were collected either in batch mode (e.g., using the ARK cell; Fig. 1c) or flow-through mode (e.g., using the cylindrical circle cell; Fig. 1d).

**Transmission FTIR spectroscopy of GB-1/MnO\textsubscript{x}**

GB-1 cells were grown in both the presence and absence of MnSO\textsubscript{4} (MSTG media) and harvested at 24 h. A 250 mL volume of cell suspension was centrifuged at 5000 relative centrifugal force (RCF) for 20 min (4° C) and cells were concentrated to a 10 mL volume. 100 µL of sample were transferred onto IR transmission windows (ZnSe, CdTe or Ge) and dried under vacuum (340 mbar) overnight. The accessible wavenumber ranges for ZnSe, CdTe, and Ge windows are 20,000 to 454 cm\(^{-1}\), 20,000 to 360 cm\(^{-1}\), and 5500 to 475 cm\(^{-1}\) respectively; with the CdTe crystal being more effective for data collection in the Mn-O stretching region (750 to 200 cm\(^{-1}\); Julien et al. 2004). Transmission spectra were collected with a minimum of 400 scans at a 4 cm\(^{-1}\) resolution.

**Diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy of GB-1/MnO\textsubscript{x}**

GB-1 cells were grown both in the presence and absence of MnSO\textsubscript{4} (MSTG media) and harvested in the stationary phase (24 h). GB-1 and GB-1/MnO\textsubscript{x} were washed twice with 0.1 M NaCl (pH 7.0) by vortex mixing, shaken for 60 s, and then centrifuged (5000 RCF, 20 min, 4° C). Pelleted cells were lyophilized and then diluted with KBr to
approximately 10% (w/w) by gently mixing 39 mg of sample with 30 mg of KBr for 40 s, then folding in an additional 390 mg of KBr to homogenize the samples. DRIFT spectra were collected with a minimum of 400 scans at 4 cm\(^{-1}\) resolution (Fig. 1b).

DRIFT spectra were also collected on washed GB-1/MnO\(_x\) samples generated from cells grown in the presence of MnSO\(_4\) and harvested at 24 h. The washing procedure, which is a modification of more aggressive methods that employ organic solvents and/or oxidants (Madernack et al. 1995; Villalobos et al. 2003), was intended to reduce biomass concentration without altering the MnO\(_x\) structure. In this case, the use of oxidants and solvents was avoided. GB-1/MnO\(_x\) were centrifuged and washed twice with 0.1 M NaCl (pH 7.0, 25,000 RCF, 20 min, 4° C). GB-1/MnO\(_x\) were resuspended in 0.1 M NaCl by vortex mixing and sonicated for 30 min. The MnO\(_x\) mixture was then washed an additional five times (0.1 M NaCl, pH 7.0, 25000 RCF, 20 min, 4° C). After each wash procedure, the organic material that had settled onto the MnO\(_x\) was physically removed with a spatula via gentle scraping. Washed MnO\(_x\) samples were freeze-dried and the lyophilized material was again subjected to removal of any visible organic matter. Samples (5% w/w) were prepared and analyzed by DRIFT as described above. Spectra of acid birnessite were also collected by DRIFT. Acid birnessite was synthesized by adding concentrated HCl to a boiling solution of KMnO\(_4\) (McKenzie, 1971).

*Attenuated total reflectance (ATR) FTIR spectroscopy of GB-1/MnO\(_x\)*

GB-1 cells were grown in MSTG media and harvested at 24 h. Cells were concentrated as described for transmission FTIR, transferred (1 mL) to a ZnSe ARK
internal reflection element (IRE), and allowed to settle onto the IRE for 4 h. Spectra were collected with a minimum of 400 scans at a 4 cm⁻¹ resolution using the growth medium as background for subtraction (Fig. 1c).

**Cell adhesion to FTIR transmission windows**

The adhesion of GB-1 cells to IR-transparent crystal surfaces was assessed in the presence and absence of MnSO₄ (MSVG media) by conducting two separate batch experiments, each with three types of window materials (ZnSe, Ge, and CdTe) suspended in cell culture suspensions. A Virtis Omni Culture Plus bioreactor (Gardiner, NY) was used to maintain constant temperature (30°C), pH (7.5), mixing (100 rpm) and aeration over the course of the experiment. The bioreactor (1.8 L) was inoculated with 15 mL of preculture harvested at the early stationary phase of growth. Influx of fresh media to the bioreactor (beginning at 24 h) was equivalent to cell suspension efflux (0.25 mL/min). Transmission windows were suspended in the bioreactor for the duration of the experiment (85 h). Adsorbed biofilms were dried on the same windows where they formed, and spectra were collected in transmission mode as described previously. Biofilm-coated transmission windows were then analyzed by SEM-EDS. FTIR peak ratios were determined using maximum IR absorbance values for specified wavenumbers.

**In-situ monitoring of biofilm growth and Mn oxidation**

Cell adhesion, biofilm growth, and the effects of Mn oxidation were studied in real-time using a flow-through ATR-FTIR method. The Virtis bioreactor (growth
conditions as described for transmission FTIR studies of cell adhesion) containing 1.8 L of MSTG media was inoculated with 15 mL of GB-1 preculture in the absence of MnSO₄. For the first 24 h, solution was pumped out of the bioreactor, through the flow cell (Fig. 1d; ZnSe ATR IRE), and then re-circulated back into the bioreactor. At 24 h fresh media was added into the bioreactor as cells passing the flow cell were pumped to waste (0.25 mL/min). Once a stable biofilm spectrum was observed (79 h), MnSO₄ (200 µM) was added by mixing to combine with the cell suspension (ca. 10⁹ cells/mL) at a Y connection placed into the effluent tube of the bioreactor. The combined cell suspension/MnSO₄ was then introduced into the ATR-FTIR flow cell (with ZnSe IRE) and out to waste (Fig. 1d). Using this approach, no MnSO₄ was introduced into the bioreactor and all Mn(II) oxidation occurred downstream of the Y connection. The flow rate was maintained at 0.25 mL/min, and spectra were collected as a function of time during flow. This experimental design probes the effects of Mn oxidation by cells adhered to the IRE in the FTIR flow cell, therefore making it possible to observe resultant changes in the biofilm. Spectra were corrected for growth media as background. In-situ flow through experiments were also conducted in the absence of Mn(II), and for cells actively oxidizing Mn in the bioreactor prior to introduction to the FTIR flow cell/ZnSE IRE.

Results

Cell growth, biogenic MnOₓ production, and TEM analysis

Growth curves of GB-1 indicate the early stationary phase is reached at 16 h in MSTG and 24 h in MSVG. When using MSVG, Mn(II) oxidation is first observed around
20 h, and with MSTG the time is approximately 12 h. The presence of MnO\textsubscript{x} was confirmed through a positive reaction to the LBB assay. Addition of MnSO\textsubscript{4} to cells in the late exponential phase leads to Mn oxidation and formation of MnO\textsubscript{x} aggregates.

GB-1 cells required negative staining for TEM observation. Micrographs acquired on GB-1 cells comprise ammonium molybdate-stained GB-1 cells with flagella (not shown). TEM of GB-1 cells with MnO\textsubscript{x} coatings precipitated on cell surfaces did not require stain, due to high electron density of the Mn-coatings (Fig. 2, inset a). The presence of Mn in the solids is evident from EDS results (Fig 2; EDS spot size is 100 nm). The data also indicate the presence of K, Ca, and Na in the biogenic solids, which is characteristic of naturally-occurring birnessites. Electron diffraction patterns (Fig. 2 inset b; ED spot size is 1 µm) indicate that the biogenic MnO\textsubscript{x} exhibits poor crystallinity relative to synthetic analogs (Drits et al., 1997; Bilinski et al., 2002), consistent with the X-ray diffraction studies of (Villalobos et al., 2003).
Figure 2. EDS Spectrum of *P. putida* GB-1/MnOₓ harvested in the late exponential phase. Inset a) TEM micrograph of unstained GB-1/MnOₓ (bar = 0.5 µm); inset b) Electron diffraction pattern.

**IR spectral characterization of GB-1 and GB-1/MnOₓ**

*Transmission spectroscopy*

Spectra of GB-1 cells dried onto ZnSe and CdTe windows (Fig. 1a) are shown in Figure 3. The main difference between GB-1 and GB-1/MnOₓ is observed on the CdTe window, with peaks at 431, 418, 408, 399, 377, and 369 cm⁻¹, corresponding to IR absorbances of MnOₓ (Julien et al., 2004). Unlike ZnSe, the use of CdTe crystals allows spectra to be collected to low wavenumbers (i.e., 360 cm⁻¹) where Mn-O stretching occurs. A spectral downshift to lower wavenumbers of several biomolecular peaks is observed in the presence of MnOₓ (e.g., 1652 to 1648, 1540 to 1535, 1039 to 1033, 1170 to 1166, 1085 to 1074). FTIR assignments for GB-1 and GB-1/MnOₓ systems are
provided in Table 1. A decrease in peak intensity at 1390 cm\(^{-1}\) (\(\nu_{\text{sym, COO}}\)) is observed with Mn-oxidation, as is the formation of a doublet (at 1658 and 1648 cm\(^{-1}\)) from the amide I peak (at 1652 cm\(^{-1}\) in the absence of MnO\(_x\)).

![Transmission FTIR spectra of GB-1 cells dried onto ZnSe and CdTe crystals.](image)

**Figure 3.** Transmission FTIR spectra of GB-1 cells dried onto ZnSe and CdTe crystals.

IR spectral effects of MnO\(_x\) were quantified using peak intensity ratios determined by comparison of maximum IR absorbance values at selected wavenumbers (Niemeyer et al., 1992; Shick et al., 1993; Wander and Traina, 1996) (Gallé et al., 2004). Values appear to be independent of the type of transmission window and the amide II (1540 cm\(^{-1}\)) / amide I (1650 cm\(^{-1}\)) ratio is not affected by Mn oxidation (Table 2). The increased ratio of both phosphate (1220 cm\(^{-1}\); \(\nu_{\text{as}}\) P=O of phosphodiesters) and
carbohydrate (1168, 1120, 1085, and 1035 cm\(^{-1}\)) to amide I indicate a relative increase in 
polysaccharides relative to proteins (amide I and II) with Mn oxidation.

Table 1. Pertinent IR assignments for GB-1 and GB-1/MnO\(_x\) systems.

<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>IR Band Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1652-1637</td>
<td>Amide I: C=O, C-N, N-H(^{a,c})</td>
</tr>
<tr>
<td>1550-1540</td>
<td>Amide II: N-H, C-N(^{a-c})</td>
</tr>
<tr>
<td>1460-1454</td>
<td>C-H: CH(_2) (scissor)(^{a,c})</td>
</tr>
<tr>
<td>1400-1390</td>
<td>C-O: (\nu)(COO(^{-}))(^{a,c})</td>
</tr>
<tr>
<td>1316-1306</td>
<td>Amide III: C-N, N-H, C=O(^c)</td>
</tr>
<tr>
<td>1240</td>
<td>Metal-complexed P=O(^{a,e})</td>
</tr>
<tr>
<td>1220</td>
<td>Hydrated P=O(^{a,e})</td>
</tr>
<tr>
<td>1168</td>
<td>(\nu)(C-O)(^d)</td>
</tr>
<tr>
<td>1112-1114</td>
<td>C-O-P, P-O-P, ring vibrations(^{a,d})</td>
</tr>
<tr>
<td>1126-1119, 1085, 1040-1035, 1020</td>
<td>C-O-C, C-C, C-O ring vibrations (sugars)(^a)</td>
</tr>
<tr>
<td>750-200(^f)</td>
<td>MnO(_x) stretching, bending, and wagging vibrations(^f)</td>
</tr>
<tr>
<td>650-450</td>
<td>CH(_2) vibrations of polysaccharides(^g)</td>
</tr>
</tbody>
</table>

\(^{a}\) Schmitt and Flemming (1998), \(^{b}\) Sockalingum et al. (1997), \(^{c}\) Nivens et al. (1993) \(^{d}\) Brandenburg and Seydel (1996), \(^{e}\) Brandenburg et al. (1997), \(^{f}\) Julien et al. (2004), \(^{g}\) Deo et al. (2001).

**DRIFT spectroscopy**

Spectra collected on lyophilized GB-1 and GB-1/MnO\(_x\) in DRIFT mode (Fig. 4) are similar to the transmission spectra (Fig. 3). However, unlike transmission results, DRIFT spectra show a *decrease* in the ratio of carbohydrate (1085 cm\(^{-1}\)) to amide I (Table 2) for biomineralized systems. A large Mn-O stretching peak is also observed at 433 cm\(^{-1}\) for GB-1/MnO\(_x\). The similarity of DRIFT spectra for GB-1/MnO\(_x\) before and after washing (Fig. 4) indicates that the organic components of the GB-1/MnO\(_x\) complex is resistant to desorption and removal by the relatively mild treatment employed; both spectra exhibit prominent biomolecular peaks. The spectral subtraction of GB-1 from
GB-1/MnOₓ gives the difference spectrum of biogenic MnOₓ shown in Figure 5. This spectrum, which shows Mn-O vibrations at 349, 323, 302, and 271 cm⁻¹ (Julien et al., 2004) is comparable, but not identical to, that of acid birnessite (shown for comparison in Fig. 5) that was synthesized abiotically in the laboratory according the method of McKenzie (1971).
Table 2. FTIR absorption ratios via various sample introduction techniques.

<table>
<thead>
<tr>
<th>IR Method</th>
<th>IR Window and Sample</th>
<th>( \nu_{\text{amII}} : \nu_{\text{amI}} )</th>
<th>( \nu_{\text{P=O}} : \nu_{\text{amI}} )</th>
<th>( \nu_{\text{carb}} : \nu_{\text{amI}} )</th>
<th>( \nu_{\text{carb}} : \nu_{\text{amI}} )</th>
<th>( \nu_{\text{carb}} : \nu_{\text{amI}} )</th>
<th>( \nu_{\text{carb}} : \nu_{\text{amI}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transmission</td>
<td>ZnSe: GB-1</td>
<td>0.68</td>
<td>---</td>
<td>0.71</td>
<td>0.75</td>
<td>0.61</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>ZnSe:GB-1 with MnO(_x)</td>
<td>0.71</td>
<td>---</td>
<td>0.89</td>
<td>1.0</td>
<td>0.81</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>CdTe: GB-1</td>
<td>0.75</td>
<td>---</td>
<td>0.73</td>
<td>0.72</td>
<td>0.67</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>CdTe: GB-1 with MnO(_x)</td>
<td>0.69</td>
<td>---</td>
<td>0.84</td>
<td>1.0</td>
<td>0.77</td>
<td>0.92</td>
</tr>
<tr>
<td>DRIFT</td>
<td>GB-1</td>
<td>0.69</td>
<td>0.27</td>
<td>---</td>
<td>0.03</td>
<td>---</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>GB-1 with MnO(_x)</td>
<td>0.69</td>
<td>0.22</td>
<td>---</td>
<td>0.06</td>
<td>---</td>
<td>0.18</td>
</tr>
<tr>
<td>ATR (ARK)</td>
<td>GB-1</td>
<td>1.0</td>
<td>0.66</td>
<td>---</td>
<td>0.33</td>
<td>0.62</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>GB-1 with MnO(_x)</td>
<td>0.82</td>
<td>0.52</td>
<td>---</td>
<td>0.26</td>
<td>0.49</td>
<td>0.81</td>
</tr>
</tbody>
</table>

*Wavenumber values are approximate, and exact peak location varies between spectra.*
**Figure 4.** DRIFT Spectra of GB-1, GB-1/MnO₅, washed GB-1/MnO₅, and synthetic acid birnessite.

**Figure 5.** Subtraction result of DRIFT spectra for GB-1/MnO₅ minus GB-1 and spectra of synthetic acid birnessite.
**ATR (ARK) spectroscopy**

ATR (ARK, Fig. 1c) spectra for GB-1 and GB-1/MnO$_x$ (Fig. 6) show an increase in amide I relative to carbohydrate, phosphate, and even amide II absorbances in the presence of MnO$_x$ (Table 2).

**Figure 6.** ATR-FTIR spectra of GB-1 cells deposited on a ZnSe. (ARK) IRE.

**Adhesion to transmission windows**

Transmission spectra of GB-1 biofilms adhered from cell culture suspension to IR windows are similar for all crystal types (ZnSe, Ge, and CdTe) (Fig. 7). SEM analyses showed a comparable extent of cell adhesion regardless of crystal material and EDS confirmed the presence of Mn particles in biofilms grown with MnSO$_4$ (not shown). Spectral differences are observed, however, between GB-1 cells that oxidized Mn and those that did not. There was a downshift in wavenumber of the P=O absorbance with
Mn oxidation (1238 cm$^{-1}$ to 1220 cm$^{-1}$), which is accompanied by a relative increase in phosphate absorbance intensity (Fig. 7). Peak shifts of similar magnitudes have been observed upon inner-sphere complexation of phosphate at Fe-oxide surfaces (Tejedor-Tejedor and Anderson, 1990) (Tejedor-Tejedor and Anderson, 1986; Arai and Sparks, 2001) and with dissolved Mg$^{2+}$ (Brandenburg et al., 1997). In the present case, this shift may result from phosphate bonding to Fe or Mn bearing surfaces.

Figure 7. Transmission FTIR spectra of GB-1 biofilms on ZnSe, Ge, and CdTe crystals suspended in bioreactor.

The intensity ratio of phosphate to amide I increased in the presence of MnO$_x$ (Table 3). A relative increase in peak intensity at 1038 cm$^{-1}$ indicates polysaccharide
enrichment. Spectra collected using the CdTe window reveal a small peak at 588 cm\(^{-1}\), attributed to Mn-O stretching vibrations. Although Mn is detected in biofilms (via EDS) and brown precipitates on biofilms were visible, the concentration of MnO\(_x\) is evidently too low to give IR absorption peaks comparable to those observed for the more concentrated samples dried onto transmission windows (GB-1 cells deposited and dried onto transmission windows).

**In situ monitoring of GB-1 biofilm growth and Mn oxidation**

When GB-1/MnO\(_x\) (cells already oxidizing Mn in the bioreactor) were passed over a clean ZnSe IRE (Fig. 1d), no adhesion or biofilm growth was detected even after > 65 h of reaction. However, in the absence of Mn, adhesion and biofilm growth (evident from a time-dependent increase in IR absorbances of biomolecular constituents) were detectable at much earlier times (Fig. 8). Bacteria adhere to the ZnSe crystal surface early in the experiment, and a mature biofilm is formed well after the cell suspension enters the stationary phase (> 50 h). Upon addition of MnSO\(_4\) (79 h) a dramatic increase in the IR intensity for major biofilm peaks was observed (t = 88 h) (Fig. 8 and 9). MnO\(_x\) particles were visible in the flow cell approximately 9 h after MnSO\(_4\) addition. Biofilm growth may be quantified on the basis of time-dependent changes in absorbance of protein regions of the IR spectrum (Fig. 9). Addition of MnSO\(_4\) resulted in a *ca.* 100% increase in absorbance intensity for both amide I and amide II (increasing from approximately
Table 3. IR absorption ratios: Adhesion of GB-1 to transmission windows.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IR Window and Sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnSe: GB-1</td>
<td>0.66</td>
<td>0.37</td>
<td>---</td>
<td>0.29</td>
<td>0.44</td>
<td>0.43</td>
</tr>
<tr>
<td>ZnSe: GB-1 with MnOx</td>
<td>0.61</td>
<td>---</td>
<td>0.41</td>
<td>0.42</td>
<td>0.53</td>
<td>0.59</td>
</tr>
<tr>
<td>CdTe: GB-1</td>
<td>0.67</td>
<td>0.42</td>
<td>---</td>
<td>0.30</td>
<td>0.46</td>
<td>0.43</td>
</tr>
<tr>
<td>CdTe: GB-1 with MnOx</td>
<td>0.60</td>
<td>---</td>
<td>0.47</td>
<td>0.48</td>
<td>0.50</td>
<td>0.59</td>
</tr>
<tr>
<td>Ge: GB-1</td>
<td>0.62</td>
<td>0.37</td>
<td>---</td>
<td>0.31</td>
<td>0.41</td>
<td>0.41</td>
</tr>
<tr>
<td>Ge: GB-1 with MnOx</td>
<td>0.56</td>
<td>---</td>
<td>0.38</td>
<td>0.37</td>
<td>0.43</td>
<td>0.43</td>
</tr>
</tbody>
</table>

*Wavenumber values are approximate, and exact peak location varies between spectra.*
**Figure 8.** ATR (circle cell) spectra for *in situ* monitoring of GB-1 adhesion, biofilm growth, and Mn-oxidation on a ZnSe IRE.

**Figure 9.** Amide I and II maximum IR absorbance as a function of time. MnSO₄ was added to the flow cell at 79 h and visible MnOₓ were observed at 88 h.
0.015 to 0.033 absorbance units for both the amide I and II. In a MnSO₄-free control (no MnSO₄ added), maximum absorbance values for the amide peaks remained lower, even at long times (137 h; \( \text{Abs}_{(\text{amide I})} = 0.0198 \) and \( \text{Abs}_{(\text{amide II})} = 0.0204 \)).

Time-dependent changes in IR absorbance ratios for GB-1 biofilm growth and subsequent Mn oxidation are shown in Figure 10. The trends include a relative decrease in amide II, phosphate (1240 cm⁻¹) and polysaccharide (1166 and 1085 cm⁻¹) peaks relative to amide I over the reaction time. Although the plotted ratios all decrease prior to addition of Mn(II), the rate of decrease is faster after addition of Mn(II) (Table 4). A large increase in protein relative to other biomolecular constituents following MnSO₄ addition is, therefore, evident from the real-time ATR studies.

![Graph showing IR peak intensity ratios versus time](image)

**Figure 10.** Plot of IR peak intensity ratios versus time from GB-1 flow through experiment. MnSO₄ was added to the flow cell at 79 h. After addition of Mn(II) a slope change is observed.
### Table 4. Regression data from IR absorption ratio plot (Figure 10): GB-1 growth and Mn-oxidation on ZnSe IRE (circle cell).

<table>
<thead>
<tr>
<th></th>
<th>Regression Equation</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carb: Am I (1085:1637)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-79 h</td>
<td>$Y = 1.82 - 0.011X$</td>
<td>0.94</td>
</tr>
<tr>
<td>89-120 h</td>
<td>$Y = 2.34 - 0.015X$</td>
<td>0.99</td>
</tr>
<tr>
<td>P=O: Am I (1240:1637)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-79 h</td>
<td>$Y = 1.40 - 0.009X$</td>
<td>0.97</td>
</tr>
<tr>
<td>89-120 h</td>
<td>$Y = 1.75 - 0.011X$</td>
<td>0.99</td>
</tr>
<tr>
<td>Carb: Am I (1166:1637)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-79 h</td>
<td>$Y = 1.07 - 0.007X$</td>
<td>0.99</td>
</tr>
<tr>
<td>89-120 h</td>
<td>$Y = 1.52 - 0.012X$</td>
<td>0.99</td>
</tr>
</tbody>
</table>

### Table 5. Location of maximum amide I IR absorbance.

<table>
<thead>
<tr>
<th>IR Method</th>
<th>IR Window and Sample</th>
<th>Max. Amide I (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transmission</td>
<td>ZnSe: GB-1</td>
<td>1652</td>
</tr>
<tr>
<td></td>
<td>ZnSe: GB-1 with MnO$_x$</td>
<td>1658 &amp; 1648</td>
</tr>
<tr>
<td></td>
<td>CdTe: GB-1</td>
<td>1652</td>
</tr>
<tr>
<td></td>
<td>CdTe: GB-1 with MnO$_x$</td>
<td>1658 &amp; 1648</td>
</tr>
<tr>
<td>DRIFT</td>
<td>GB-1</td>
<td>1656</td>
</tr>
<tr>
<td></td>
<td>GB-1 with MnO$_x$</td>
<td>1660</td>
</tr>
<tr>
<td>ATR (ARK)</td>
<td>GB-1</td>
<td>1637</td>
</tr>
<tr>
<td></td>
<td>GB-1 with MnO$_x$</td>
<td>1637</td>
</tr>
<tr>
<td>Transmission: GB-1 Adhesion</td>
<td></td>
<td>1656</td>
</tr>
<tr>
<td></td>
<td>ZnSe: GB-1</td>
<td>1656</td>
</tr>
<tr>
<td></td>
<td>ZnSe: GB-1 with MnO$_x$</td>
<td>1656</td>
</tr>
<tr>
<td></td>
<td>CdTe: GB-1</td>
<td>1654</td>
</tr>
<tr>
<td></td>
<td>CdTe: GB-1 with MnO$_x$</td>
<td>1654</td>
</tr>
<tr>
<td></td>
<td>Ge: GB-1</td>
<td>1654</td>
</tr>
<tr>
<td></td>
<td>Ge: GB-1 with MnO$_x$</td>
<td>1654</td>
</tr>
<tr>
<td>ATR (Circle Cell): GB-1 Adhesion</td>
<td></td>
<td>1656</td>
</tr>
<tr>
<td></td>
<td>1548 min</td>
<td>ZnSe: GB-1</td>
</tr>
<tr>
<td></td>
<td>3000 min</td>
<td>ZnSe: GB-1</td>
</tr>
<tr>
<td></td>
<td>4680 min</td>
<td>ZnSe: GB-1</td>
</tr>
<tr>
<td></td>
<td>5214 min</td>
<td>ZnSe: GB-1 with MnSO$_4$</td>
</tr>
<tr>
<td></td>
<td>6090 min</td>
<td>ZnSe: GB-1 with MnO$_x$</td>
</tr>
<tr>
<td></td>
<td>7230 min</td>
<td>ZnSe: GB-1 with MnO$_x$</td>
</tr>
</tbody>
</table>
Discussion

**Bulk versus extracellular composition**

In contrast to the transmission results (Fig. 3, Fig. 7), which show enrichment in phosphate and polysaccharide relative to protein during Mn(II) oxidation, ATR (Fig. 6) data indicate a relative increase in protein (Table 2). This apparent discrepancy in effects of Mn(II) oxidation on biomolecular composition is resolved by considering the fact that ATR methods probe preferentially the surficial regions of the GB-1/MnOₓ samples (cell/MnOₓ interface), whereas transmission mode is a bulk (whole particle absorption) technique. Therefore, the data suggest that Mn oxidation by GB-1 cells is accompanied by an increase in bulk polysaccharide and phosphate, whereas the extracellular environment is enriched in proteinaceous constituents. Evidently, the presence of Mn(II) in solution results in an increased expression of extracellular proteins that promote the biomineralization of MnOₓ.

**Effect of Mn-oxidation on amide groups**

The decreased intensity ratio of amide II (1540 cm⁻¹) / amide I (1650 cm⁻¹) in ATR spectra (Fig. 6, Table 2) indicates that, although the overall protein content of GB-1 cells may not change, the expression of surficial protein is indeed altered upon Mn oxidation. Changes in the amide II / amide I intensity ratio suggest variation in protein secondary structure (Ishida and Griffiths, 1993). The amide I ATR peak of GB-1 cells does not shift upon Mn-oxidation (Table 5). Second derivative analysis of GB-1 and GB-1/MnOₓ ATR spectra in Figure 6 both reveal a protein mixture comprising β-sheet (1629
and 1637 cm\(^{-1}\), random coil (1645 cm\(^{-1}\)), and \(\alpha\)-helical (1652 cm\(^{-1}\)) conformations (Buijs et al., 1996). The amide I peak maximum for hydrated samples (ATR) occurs at 1637 cm\(^{-1}\), corresponding to \(\beta\)-sheets (Table 5). A shift in the amide I region to higher wavenumber in DRIFT and transmission spectra (Table 5) is consistent with previous studies (Hübner and Blume, 1998) indicating that this change reflects the impact of sample dehydration. The presence of a small doublet peak in transmission spectra at 1658 (\(\alpha\)-helical) and 1648 cm\(^{-1}\) (random coil/\(\alpha\)-helical) (Buijs et al., 1996; Jung, 2000) suggest a change in protein conformation due to Mn-oxidation/-binding (Fig. 3, Table 5). The peak shift from 1652 cm\(^{-1}\) (\(\alpha\)-helical) represents an increase in randomly-coiled proteins after Mn-oxidation.

**Characterization of biogenic MnO\(\chi\)**

The fact that DRIFT spectra of GB-1/MnO\(\chi\) show no effect of repeated washing and density separation in electrolyte solution indicates a strong, intimate association between MnO\(\chi\) and biomolecular material. Given that organic complexation of neoformed mineral colloids inhibits their crystallization, it is not surprising that biogenic MnO\(\chi\) exhibits short-range crystal order (Mizukami et al., 1999; Villalobos et al., 2003). Transmission (Fig. 3, CdTe) and DRIFT spectra (Fig. 5) of GB-1/MnO\(\chi\) show numerous vibrations in the Mn-O stretching region. Since IR absorbance of polysaccharides also occurs below 700 cm\(^{-1}\) (Deo et al., 2001), Mn-O absorbances are assessed after subtraction of bands arising from cell constituents. The MnO\(\chi\) DRIFT difference spectrum (Fig. 5) is comparable to those observed for Mn oxides (e.g. birnessite,
Based on extensive mineralogical analyses following more aggressive removal of bound organic matter with phenol/chloroform and NaOCl, Villalobos et al. (2003) reported that physico-chemical properties of the biogenic MnO$_x$ produced by *P. putida* strain MnB1 are intermediate between synthetic vernadite ($\delta$-MnO$_2$) and randomly stacked acid birnessite. The latter was synthesized in their study and the present one using the HCl-induced KMnO$_4$ reduction method of McKenzie (1971). Differences between the FTIR spectra of synthetic acid birnessite and GB-1 biogenic MnO$_x$ (Fig. 5) may be due to our less aggressive oxide cleaning procedure and the associated preservation of mineral-bound biomolecular material. As noted by Villalobos (2003), aggressive removal of cellular material may affect the structure of the MnO$_x$ product, but its retention in the solid hinders a direct comparison with products synthesized abiotically in the absence of organic matter. Incorporation of growth media constituents (e.g., K, Fe, Zn, Cu, Co, Mo) into the biogenic MnO$_x$ may also be responsible for differences relative to the acid birnessite sample.

**Effect of Mn-oxidation on GB-1 adhesion and biofilm growth**

GB-1 cells adhere effectively to ZnSe, Ge, and CdTe surfaces to form intact biofilms prior to Mn-oxidation. Furthermore, subsequent oxidation of Mn(II) by the intact biofilms bound to the crystal surfaces did not diminish adhesion once the biofilms were formed. Adhesion is likely mediated by favorable bonding interactions (e.g., cationic proteins, hydrogen bonding, hydrophobic interaction, surface roughness)
between bacterial biomolecules and the substrata that overcome the repulsion induced by net negative charge of bacterial and crystal surfaces (Marshall et al., 1971; Truesdail et al., 1998; Deo et al., 2001; Appenzeller et al., 2002).

Importantly, GB-1 adhesion to negatively-charged crystal surfaces was negligible for cells encrusted with biogenic MnO\textsubscript{x}. These results indicate that bacterial surface chemistry is altered significantly by the presence of surficial biogenic precipitates. Given that the point of zero net charge for birnessite is 1.5 to 2.5 (Sposito, 1989), it is expected to carry a net negative charge at the experimental pH (7.4). The coating of bacterial surface macromolecules by negatively charged MnO\textsubscript{x} and, in particular, the indication that MnO\textsubscript{x} interacts preferentially with the extracellular protein components of the GB-1 surface, suggests that the MnO\textsubscript{x} binds these cell surface proteins and diminishes their availability for subsequent adhesion to negatively charged surfaces. Since most silicate surfaces are negatively charged, environmental mobility in geomedia is expected to be significantly enhanced for MnO\textsubscript{x}-coated cells.

Conclusions

FTIR spectroscopy with several modes of sample introduction was useful for monitoring compositional changes of cells before, during and after biologically-catalyzed oxidation of Mn(II). An increased contribution to the FTIR spectra of surficial proteins is associated with Mn-oxidation during production of a poorly-crystalline Mn(IV) phase. Changes in protein (amide) regions of the spectra result from protein-MnO\textsubscript{x} interaction and/or conformational and compositional changes associated with Mn oxidation. The
apparent surface protein-biogenic MnO\textsubscript{x} association greatly reduces cell adhesion to negatively-charged substrata in aqueous environments. GB-1 cells adhere effectively to negatively-charged ZnSe, Ge, and CdTe crystal surfaces prior to biogenic MnO\textsubscript{x} formation to produce intact biofilms. These biofilms exhibit the capability for effective Mn(II) oxidation, which then results in an accumulation of biomass at a faster rate than in the absence of Mn(II). However, planktonic GB-1 cells exposed to Mn(II) in the absence of crystal surfaces develop biogenic MnO\textsubscript{x} coatings that preclude their subsequent adhesion to a negatively-charged ZnSe IRE. The data suggest an important role of surficial proteins in bacterial adhesion to both the experimental crystal surfaces and also to biogenic MnO\textsubscript{x}.

**Acknowledgements**

We thank Dr. Bradley M. Tebo and Brian Clement for donation of GB-1 cells and providing information critical for cell growth and Mn-oxidation. We also thank Martha Conklin for useful discussions at early stages of this research and Hanna L. Gilbert for her assistance with SEM-EDS analysis. TEM-EDS assistance and analysis was provided by Sunkyung Choi and David Bentley. This research was supported by the National Science Foundation CRAEMS program (Grant CHE-0089156).
References


APPENDIX B

ATR-FTIR INVESTIGATION OF BACTERIAL ADHESION TO
NANOHEMATITE (α-Fe₂O₃), CORUNDUM (α-Al₂O₃), AND ZnSe

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Abstract

The contribution of various bacterial surface functional groups to mineral binding was examined using attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy. When live *Shewanella oneidensis*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* cells were introduced to a horizontal nanohematite (α-Fe₂O₃) coated internal reflection element (IRE), FTIR peaks emerged that corresponded to bacterial phosphate group binding. These IR peaks were not observed when bacteria were introduced to an uncoated ZnSe IRE or to a corundum (α-Al₂O₃) coated IRE. When bacterial cells were introduced into colloidal suspensions of α-Fe₂O₃ and α-Al₂O₃, spectra contain peaks corresponding to P-Of and P-OAl, respectively. Mixing of colloidal α-Fe₂O₃ with bacterial cells at pH 7 also resulted in IR peaks corresponding to ν(COOH) (pKa <5), attributed to binding of carboxylate at mineral surface OH groups. Selected organic compounds with P-containing functionalities (phenylphosphonic acid [PPA], adenosine 5’-monophosphate [AMP], 2’-deoxyadenyl(3’→5’)-2’-deoxyadenosine [DADA], deoxyribonucleic acid [DNA]) on α-Fe₂O₃ produce spectra with peaks corresponding to P-Of interactions. Reaction of DNA with α-Fe₂O₃ results in Fe(III)-induced oxidation via cleavage of ribose rings. The data indicate that both terminal phosphate/phosphonate and phosphodiester groups, either exuded from the cell or present as surface biomolecules, are involved in bacterial adhesion to Fe-oxides through formation of inner-sphere Fe-phosphate/phosphonate complexes.
1. Introduction

The chemical properties of both bacteria and abiotic environmental surfaces influence adhesion of microorganisms to particulate matter in soil and aquatic systems. Long-range forces, including van der Waals and electrostatic forces are important to the adhesion process (Marshall et al., 1971). Short-range interactions are controlled by three major forces: 1) chemical interactions (covalent bonding, ionic bonding, hydrogen bonding), 2) dipole interactions, and 3) hydrophobic interactions (Marshall, 1986). Microbial adhesion is also affected by steric interactions, important for overlapping regions of polymer segments (Neu and Marshall, 1990). The steric interactions can promote adhesion via bridging of surface macromolecules, or inhibition by steric repulsion (Rijnaarts et al., 1999).

Bacteria and many environmental surfaces exhibit a net negative charge at environmental pH values (Rijnaarts et al., 1995). For example, silica is negatively charged at pH > 2.0 to 3.0 (Sposito, 1989). However, in weathering environments, many silicaceous surfaces become coated with a veneer of hydrous Al and Fe oxide, which can confer net positive charge at circumneutral pH (Sposito, 1989). As a result, bacterial adhesion to Fe-oxide surfaces is often greater than what is observed for negatively charged surfaces and the difference is often attributed to electrostatic attraction (Truesdail et al., 1998; Bolster et al., 2001; Deo et al., 2001).

In addition to electrostatic interactions, direct bonding of cell surface macromolecules at mineral surface functional groups may play a role in bacterial adhesion (Arredondo et al., 1994; Makin and Beveridge, 1996; Wingender et al., 1999;
Gomez-Suarez et al., 2002; Poortinga et al., 2002). The exterior surface of bacterial cells are comprised of extracellular polymeric substances (EPS), teichoic acids (Gram-positive bacteria), and lipopolysaccharides (LPS; Gram-negative bacteria). EPS is heterogeneous mixture of polysaccharides, proteins, lipids, and nucleic acids (Wingender et al., 1999). The presence of nucleic acids in EPS and biofilm structures results from extracellular production (Demain et al., 1965; Muto and Goto, 1986; Kadurugamuwa and Beveridge, 1995; Whitchurch et al., 2002) and cell lysis (Sutherland, 2001). Using Fourier transform infrared (FTIR) spectroscopy and quantum chemical calculations it was determined that EPS from *Pseudomonas aeruginosa* and *Bacillus subtilis* bind to Fe centers on goethite (α-FeOOH) via inner-sphere complexation of phosphate-bearing macromolecules (Omoike et al., 2004). A subsequent study showed that phosphate group binding also affects the quantitative partitioning of EPS macromolecules in aqueous goethite suspensions such that P-bearing macromolecules are adsorbed preferentially (Omoike and Chorover, 2006). While these results are highly suggestive of the role of phosphoryl groups in bacterial cell binding to oxide surfaces, and indeed the formation of inner-sphere orthophosphate complexes at Fe oxide surfaces is well known (Tejedor-Tejedor and Anderson, 1990; Persson et al., 1996; Arai and Sparks, 2001), the binding of bacterial cells to Fe oxide surfaces via phosphoryl groups has not been demonstrated previously. If adhesion to Fe oxides of live bacterial cells does indeed involve the formation of Fe-phosphoryl complexes, this may help to explain the common observation of extracellular nucleic acids observed during incipient biofilm formation (Whitchurch et al., 2002; Steinberger and Holden, 2005), bound to soil particles (Ogram et al., 1994; Cai
et al., 2005), and in association with fresh (Ogram et al., 1987; Ogram et al., 1988), brackish (Ogram et al., 1987), and deep-ocean sediments (Dell'Anno and Danovaro, 2005).

FTIR spectroscopy provides molecular-scale information on both organic and inorganic constituents involved in bacterial adhesion (Deo et al., 2001; Benning et al., 2004; Parikh and Chorover, 2005). Attenuated total reflectance (ATR)-FTIR spectroscopy probes infrared-absorbing groups in close proximity (ca. \( \leq 1 \) µm) to the interface between a sample and an internal reflection element (IRE) (Nivens et al., 1993), thereby giving information relevant to surface functional groups involved in adhesion to the IRE. ATR-FTIR permits nondestructive, in-situ interrogation of functional group composition and binding mechanisms of bacterial cells (Nichols et al., 1985; Naumann et al., 1991; Sockalingum et al., 1997), biofilms (Nichols et al., 1985; Nivens et al., 1993; Schmitt and Flemming, 1998; Parikh and Chorover, 2005), and extracted bacterial surface biomolecules (Brandenburg, 1993; Omoike and Chorover, 2004; Parikh and Chorover, 2006) in aqueous systems.

In this study, we used ATR-FTIR spectroscopy to investigate the adhesion of live Gram-negative (\( P. \) aeruginosa, \( Shewanella \) oneidensis) and Gram-positive (\( B. \) subtilis) bacterial cells to a ZnSe IRE, and to the same IRE coated with colloidal corundum (\( \alpha \)-\( Al_2O_3 \)) or hematite (\( \alpha \)-\( Fe_2O_3 \)). We also examined spectra obtained when the bacterial cells were subjected to heterocoagulation with the colloidal oxides in aqueous suspension. The primary objective was to elucidate the cell surface functional groups involved in bacterial adhesion to these different surfaces, and to test the postulated role of phosphate
groups. At circumneutral pH the uncoated ZnSe IRE, isoelectric point (IEP) < 4, serves as a model for a negatively charged surface (Tickanen et al., 1997). Whereas α-Al₂O₃ (IEP = 9.1) (Sparks, 1995), which has similar surface charge properties to α-Fe₂O₃ (IEP = 8.0-8.5) (Sposito, 1989), provides a test of cell binding to Al versus Fe bearing oxide surfaces. Phosphate group binding was most apparent during cell adhesion to the α-Fe₂O₃-coated ZnSe. Band assignment and the role of phosphate/phosphonic groups in bacterial adhesion was further facilitated by collection of ATR-FTIR spectra on selected model compounds.

2. Experimental Procedures

2.1. Bacteria and growth conditions

Bacteria were grown aerobically at 30° C to the early stationary phase (10⁵ cells mL⁻¹) in appropriate growth media as follows: Shewanella oneidensis (MR-1) - tryptic soy broth, 22 h; Pseudomonas aeruginosa (PAO1) and Bacillus subtilis (ATCC 7003) - Luria broth, 24 h. Cells were harvested by centrifugation (3000 RCF, 20 min, 4°C) and washed once (100 mmol L⁻¹ NaCl, pH 7) to remove growth media and free EPS. Cells were then resuspended in 100 mmol L⁻¹ NaCl (pH 7) for FTIR experiments.

2.2. Chemicals and solutions

All solutions were prepared in acid-washed glass vials using Barnstead nanopure (BNP) water with pH adjustment via 100 mmol L⁻¹ NaOH/HCl. Solutions were prepared, stored overnight at 4° C, and brought to room temperature before use. Phenylphosphonic
acid (PPA; Sigma) was dissolved in 1 mM NaCl (8 mg mL^{-1}, pH 7). Adenosine 5′-monophosphate (AMP; Sigma) was dissolved in 1 mM NaCl (4 mg mL^{-1}, pH 7). The oligonucleotide, 2′-deoxyadenyl (3′→5′)-2′-deoxyadenosine (DADA; Operon), is comprised of two deoxyadenosines linked by a phosphodiester bond. DADA was dissolved in 1 mM NaCl (~3.5 mg mL^{-1}, pH 7). Chemical structures for these compounds are given in Figure 1. Deoxyribonucleic acid (DNA; MP Biomedicals) from *Escherichia coli* was dissolved in 1 mg mL^{-1} NaCl (5 mg/mL).

2.3. Mineral adsorbents

Corundum (α-Al₂O₃) (Alfa Aesar) was suspended in 1 mmol L^{-1} NaCl (25 g L^{-1}; pH 6) for ATR experiments. Characterization of α-Al₂O₃ was provided by the manufacturer (size: 1 µm, surface area: 6-8 m² g⁻¹).

Colloidal α-Fe₂O₃ particles were synthesized using the method of Schwertmann and Cornell (1991). Briefly, 100 mL of 1 mol L⁻¹ Fe(NO₃)₃ were added dropwise to 1 L of boiling BNP water over a 4 h period. The solution was allowed to cool overnight at room temperature. Removal of NO₃⁻ from the suspension required flocculation and four repeated washing steps in 100 mmol L⁻¹ NaCl. The α-Fe₂O₃ was resuspended in BNP water adjusted to pH 4 and dialyzed against the same with exterior solution changed twice daily (Spectra/Por 7 1000 MWCO, Spectrum) (Liger et al., 1999). Dialysis was considered complete when pH and electrical conductivity (EC) were unchanged over a 12 h period (pH 4, EC ~ 110 µs cm⁻¹). The concentration of α-Fe₂O₃ in suspension was
determined via freeze-drying an aliquot of the colloidal suspension and measuring mass of $\alpha$-Fe$_2$O$_3$ remaining.

![Chemical structures of P-containing compounds used for ATR-FTIR experiments](image)

**Figure 1.** Chemical structures of P-containing compounds used for ATR-FTIR experiments.
Mineral composition and purity for both $\alpha$-Fe$_2$O$_3$ and $\alpha$-Al$_2$O$_3$ were confirmed by collection of X-ray diffraction (XRD) patterns on powdered specimens using a Scintag XDS 2000 with a Cu x-ray source (40 kV and 40 mA), scan speed of 2° 2θ min$^{-1}$, and a step width of 0.03° 2θ. Since $\alpha$-Fe$_2$O$_3$ was not purchased (e.g., $\alpha$-Al$_2$O$_3$ characterization provided) additional mineral characterization was required. To determine particle size, $\alpha$-Fe$_2$O$_3$ was analyzed by transmission electron microscopy (TEM) with particles mounted on 200 mesh copper grids by placing carbon coated mica into a drop of suspension and floating carbon/$\alpha$-Fe$_2$O$_3$ onto the grid. Samples were observed at 60 kV with a Japanese Electron Optical Laboratories JEM-100CX II electron microscope. Diffuse reflectance (DRIFT) FTIR spectra were also collected to characterize the $\alpha$-Fe$_2$O$_3$ and $\alpha$-Al$_2$O$_3$. Freeze dried $\alpha$-Fe$_2$O$_3$ and $\alpha$-Al$_2$O$_3$ were diluted with KBr to approximately 10% (w/w) by gently mixing 39 mg of sample with 30 mg of KBr for 40 s, then folding in an additional 390 mg of KBr to homogenize the samples. DRIFT spectra were collected using a Nicolet 560 Magna IR spectrometer (Madison, WI) with 400 scans at 4 cm$^{-1}$ resolution.

2.4. ATR-FTIR spectroscopy and analysis

ATR-FTIR spectra were collected using a 45° ZnSE IRE (Spectra-Tech ARK ATR cell) at pH 7, with 100 or 1.0 mM NaCl as background electrolyte. Spectra were acquired for each bacterial strain added as a cell suspension: (i) deposited directly onto the IRE surface, (ii) mixed with colloidal $\alpha$-Fe$_2$O$_3$ and then deposited onto the IRE surface, (iii) mixed with colloidal $\alpha$-Al$_2$O$_3$ and then deposited onto the IRE surface, (iv) deposited onto a $\alpha$-Fe$_2$O$_3$-coated IRE surface, and (v) deposited onto a $\alpha$-Al$_2$O$_3$-coated
IRE surface. Spectra for DNA, DADA, AMP, and PPA solutions were collected using the ZnSe IRE, an α-Fe₂O₃-coated IRE, and an α-Al₂O₃-coated IRE. A schematic of the various ATR-FTIR sample collection modes is given in Figure 2.

For sample introduction modes (ii) and (iii), where cell-oxide contact was made prior to introduction into the ATR compartment, samples were prepared by adding cells to a suspension of colloidal α-Fe₂O₃ or α-Al₂O₃ to give a final suspension comprising 1.96 g L⁻¹ oxide and 5 × 10⁵ cells mL⁻¹ at pH 7.1. The suspension was immediately placed on the IRE for data acquisition. For sample introduction modes (iv) and (v), where cell-oxide contact was made in the ATR cell, metal oxide IRE coatings were prepared by drying the appropriate suspension (6 mL of α-Fe₂O₃, 1.96 g L⁻¹, pH 4; or 1 mL of α-Al₂O₃, 25 g L⁻¹, pH 6) on the ZnSe IRE under vacuum (10 mm Hg) overnight. New metal oxide coatings were prepared for each experiment and spectra of dry oxide films were acquired each time to determine consistency of coatings and to permit calculation of

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<tr>
<th>ZnSe</th>
<th>Metal Oxide</th>
<th>Metal Oxide Coating</th>
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<tbody>
<tr>
<td>· Bacteria in 100 mM NaCl, pH 7</td>
<td>· Bacteria in 2 g/L α-Fe₂O₃, pH 7</td>
<td>· Coatings: α-Fe₂O₃ and α-Al₂O₃</td>
</tr>
<tr>
<td>· DNA and P-containing compounds in 1 mM NaCl, pH 7</td>
<td>· Bacteria in 2 g/L α-Al₂O₃, pH 7</td>
<td>· Samples: Bacteria (100 mM NaCl, pH 7), DNA (1 mM NaCl, pH 7), and selected P-containing compounds (1 mM NaCl, pH 7)</td>
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</table>

Figure 2. Schematic illustration of ATR-FTIR sample collection methods. Bacteria studied include B. subtilis, P. aeruginosa, and S. oneidensis. For specific P-containing compounds used see Figure 1.

For sample introduction modes (ii) and (iii), where cell-oxide contact was made prior to introduction into the ATR compartment, samples were prepared by adding cells to a suspension of colloidal α-Fe₂O₃ or α-Al₂O₃ to give a final suspension comprising 1.96 g L⁻¹ oxide and 5 × 10⁵ cells mL⁻¹ at pH 7.1. The suspension was immediately placed on the IRE for data acquisition. For sample introduction modes (iv) and (v), where cell-oxide contact was made in the ATR cell, metal oxide IRE coatings were prepared by drying the appropriate suspension (6 mL of α-Fe₂O₃, 1.96 g L⁻¹, pH 4; or 1 mL of α-Al₂O₃, 25 g L⁻¹, pH 6) on the ZnSe IRE under vacuum (10 mm Hg) overnight. New metal oxide coatings were prepared for each experiment and spectra of dry oxide films were acquired each time to determine consistency of coatings and to permit calculation of
difference spectra. Spectra were collected at 0, 15, 30, 45, 60, 90, 120, 150, 180, 210, and 240 min after sample introduction. All FTIR spectra were collected with 400 scans at a 4 cm$^{-1}$ resolution using the corresponding electrolyte solution as background. Metal oxide spectra have been subtracted from the results presented below. Time-dependent changes in spectral data were assessed by comparing relative changes in peak intensities of IR-absorbing functional groups. Peak fitting for quantitative comparisons was performed using GRAMS/AI (Thermo Electron Co.) software. The areas of Gaussian fitted peaks were used to determine relative contributions of specific bacterial components. DNA removal from the ATR crystal employed a cleaning solution of 10 mM Tris HCl (pH 8), 5 mM NaCl, and 1 mM EDTA at approximately 40° C (Stepanyugin et al., 2005).

3. Results

3.1. Metal oxide characterization

Synthesis of $\alpha$-Fe$_2$O$_3$ was confirmed via XRD, TEM, and DRIFT analysis (data not shown). Diffraction patterns show eight peaks, which closely match reference spectra (Blake et al., 1966). Broad XRD peaks (indicating small particle size) matched hematite diffraction patterns (Blake et al., 1966). TEM micrographs reveal unidimensional crystals of rhomboid to hexagonal geometry measuring 10-20 nm across. The $\alpha$-Fe$_2$O$_3$ DRIFT spectra (data not shown) have prominent peaks at 454 and 458 cm$^{-1}$ corresponding to the Fe-O vibrations of hematite (Schwertmann and Taylor, 1989). The spectra include distinct Fe-OH stretching (894 cm$^{-1}$) and bending (806 cm$^{-1}$) vibrations resulting from the high specific surface area and surface hydroxyl group density of the nanoscale
precipitates (Farmer, 1974; Schwertmann and Cornell, 1991). A broad peak at 3340 cm\(^{-1}\) corresponds to OH stretching frequencies (Farmer, 1974).

Purchased Al\(_2\)O\(_3\) (size: 1 µm, surface area: 6-8 m\(^2\) g\(^{-1}\)) was verified to be corundum (\(\alpha\)-Al\(_2\)O\(_3\)) via XRD and DRIFT analysis. DRIFT spectra reveal a broad peak between 560 and 790 cm\(^{-1}\) and additional peaks between 375 and 520 corresponding to Al-O vibrations of corundum (Gadsen, 1975; Danchevskaya et al., 2004).

### 3.2. ATR-FTIR spectra of bacteria

Figure 3 shows the region from 1750-1300 cm\(^{-1}\) (representing the protein and carboxyl region) for the three bacterial strains and five different treatments. The most prevalent features in these spectra correspond to proteins as reflected in the amide I and amide II vibrations at ca. 1640 and 1550 cm\(^{-1}\) respectively (Table 1). For the Gram negative strains, PAO1 and MR-1, a smaller peak at \(~1720\) cm\(^{-1}\), corresponding to protonated carboxyl groups, is present for cells aggregated with colloidal \(\alpha\)-Fe\(_2\)O\(_3\) (Fig 3b), but this peak is not observed for \(B.\) subtilis. The COOH peak was also not observed when PAO1 and MR-1 were introduced to the \(\alpha\)-Fe\(_2\)O\(_3\)-coated IRE irrespective of ionic strength (identical spectra were obtained in 1 and 100 mM NaCl), nor in any of the other treatments.

In the presence of \(\alpha\)-Fe\(_2\)O\(_3\), particularly when cells are introduced to the \(\alpha\)-Fe\(_2\)O\(_3\)-coated IRE (Fig. 3c), a decrease in peak intensity at \(~1400\) (COO\(^-\)) and \(~1454\) cm\(^{-1}\) (CH\(_2\)) is observed (to a greater extent for \(S.\) oneidensis) relative to the case with ZnSe IRE only (Fig. 3a). For all three bacteria there is a decrease in intensity of the amide II band (\(~1550\) cm\(^{-1}\))
cm\(^{-1}\)) relative to amide I (~1640 cm\(^{-1}\)) upon adhesion to the \(\alpha\)-Fe\(_2\)O\(_3\)-coated IRE. Contact with \(\alpha\)-Fe\(_2\)O\(_3\) also results in a frequency upshift of the amide I peak for all three strains. There is no apparent relation between the magnitude of the shift and contact with \(\alpha\)-Fe\(_2\)O\(_3\) via aggregation versus colloidal film. No shift in the amide II region is observed.

Table 1. IR assignments for bacteria and biomolecules

<table>
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<th>Wavenumber (cm(^{-1}))</th>
<th>IR Band Assignment</th>
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<tbody>
<tr>
<td>1720-1729</td>
<td>(\nu_{as}(\text{COOH})) ($^{a,b})</td>
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<tr>
<td>1652-1637</td>
<td>Amide I: C=O, C-N, N-H (b^{d})</td>
</tr>
<tr>
<td>1550-1540</td>
<td>Amide II: N-H, C-N (a,c,d)</td>
</tr>
<tr>
<td>1454-1482</td>
<td>(\delta(\text{CH}_2)) (a,b,e)</td>
</tr>
<tr>
<td>1360-1450</td>
<td>(\nu_d(\text{COO})) (a,c,e,f)</td>
</tr>
<tr>
<td>1170</td>
<td>(\nu(\text{C-O})) (b)</td>
</tr>
<tr>
<td>1137</td>
<td>(\nu_(\text{PO}_2^-)) (j,k)</td>
</tr>
<tr>
<td>1114-1118</td>
<td>(\nu(\text{C-O-P}, \text{P-O-P})), ring vibrations (a,b)</td>
</tr>
<tr>
<td>1106-1108</td>
<td>(\nu_{as}(\text{PO}_3^{2-})) (l)</td>
</tr>
<tr>
<td>1084-1094</td>
<td>(\nu_d(\text{PO}_2^-)) (i,k,m), ring vibrations (a), (\nu(\text{C-O})) (i)</td>
</tr>
<tr>
<td>1078</td>
<td>(\nu_d(\text{C-O-C}, \text{C-C})) (m), (\nu(\text{PO}_3^{2-})) (e)</td>
</tr>
<tr>
<td>1048-1060</td>
<td>(\nu(\text{C-O-C}, \text{C-C})) (a,d)</td>
</tr>
<tr>
<td>1042-1046</td>
<td>(\nu_(\text{PO}_3^{2-})) (l)</td>
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<tr>
<td>1039-1043</td>
<td>(\nu(\text{P-OH}, \text{P-OF})) (k,n,o)</td>
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<tr>
<td>1016-1020</td>
<td>(\nu(\text{P-OF})), ring vibrations (a)</td>
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<td>979</td>
<td>(\nu(\text{PO}_3^{2-})) (o)</td>
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<td>974</td>
<td>(\nu(\text{P-OH})) (o)</td>
</tr>
<tr>
<td>962-970</td>
<td>(\nu(\text{PO}_2^-)) (e,o)</td>
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\(\$\). \(\nu_{as}\) = asymmetric stretching vibration, \(\dagger\). \(\delta\) = bending vibrations, \(\ddagger\). \(\nu_s\) = symmetric stretching vibration.


Spectra of bacteria reacted with \(\alpha\)-Al\(_2\)O\(_3\) (Fig. 3d, 3e) are similar to those collected on the ZnSe IRE. Some differences are observed in the spectrum of \(P.\) aeruginosa on the \(\alpha\)-Al\(_2\)O\(_3\)-coating (Fig. 3e). In this case, small peaks (COO\(^{-}\)) at 1413,
1403, and 1375 cm$^{-1}$ are observed. The amide I and II bands do not appear to be affected via bacteria interaction with $\alpha$-Al$_2$O$_3$.

The polysaccharide and phosphate region (1300-950 cm$^{-1}$) of the ATR spectra is shown in Figure 4. Spectra for cells on ZnSe (Fig. 4a) are similar with the exception of the broad shoulder at 1045 cm$^{-1}$ (C-O, C-O-C) for $B$. subtilis. Only a slight change in the spectra of Gram negative bacteria ($P$. aeruginosa, $S$. oneidensis) is observed when cells are aggregated with colloidal $\alpha$-Fe$_2$O$_3$ and placed on the ZnSe IRE (Fig. 4b; $\alpha$-Fe$_2$O$_3$ subtracted). In this case, a small peak at 1018 cm$^{-1}$ (P-OFe) is observed and the broad peak from 1200-1260 cm$^{-1}$ (PO$_2^-$) is better defined. When $B$. subtilis cells are mixed with $\alpha$-Fe$_2$O$_3$ a sharp and intense peak (P-OFe) emerges at 1016 cm$^{-1}$ and the peak at $\sim$1041 cm$^{-1}$ (P-OFe) is increased relative to 1085 cm$^{-1}$ (ring vibrations or PO$_2^-$ \[\nu(\text{ring-PO}_2\text{)}\]). In contrast, spectra of bacteria introduced onto $\alpha$-Fe$_2$O$_3$-coated IRE ($\alpha$-Fe$_2$O$_3$ subtracted) are very similar for all three bacteria (Fig. 4c), and very different from spectra collected under all other conditions. These spectra are characterized by a reduction in peak intensity for the phosphate region present between 1200 and 1260 cm$^{-1}$ (particularly for Gram negative cells) and spectra are dominated by C-C, C-O-C ring vibrations, PO$_2^-$ (1087 cm$^{-1}$), and P-OFe complexes (1041 cm$^{-1}$). Spectra for cells contacting $\alpha$-Al$_2$O$_3$ by either hetero-aggregation (Fig. 4d) or introduced to the Al oxide coated IRE (Fig. 4e) are generally similar to those collected on ZnSe. In the case of cells aggregated with $\alpha$-Al$_2$O$_3$, a peak at $\sim$1020 cm$^{-1}$ is observed, which is similar to with the case for aggregation with colloidal $\alpha$-Fe$_2$O$_3$. Another notable difference is observed in the case of $B$. subtilis on $\alpha$-Al$_2$O$_3$-coated IRE (Fig. 4e), where a broad peak extending from 1079 to 1027 cm$^{-1}$ is
observed. Consistency in spectra for bacterial interactions under the various conditions indicates a strong degree of reproducibility in these experiments. In cases where striking differences are observed in spectra (e.g., Fig 4b, *B. subtilis* aggregated with $\alpha$-Fe$_2$O$_3$) experiments were repeated to verify results.
Figure 3. ATR-FTIR spectra (1750-1300 cm\(^{-1}\)) of \textit{S. oneidensis}, \textit{P. aeruginosa}, and \textit{B. subtilis} on a) ZnSe, b) mixed with \(\alpha\)-Fe\(_2\)O\(_3\) on ZnSe, c) on \(\alpha\)-Fe\(_2\)O\(_3\), d) mixed \(\alpha\)-Al\(_2\)O\(_3\) on ZnSe, and e) on a \(\alpha\)-Al\(_2\)O\(_3\) surface (all spectra collected after 240 min). Reaction with \(\alpha\)-Fe\(_2\)O\(_3\) leads to decreased amide I and amide II contributions to the spectra accompanied with protein conformational change.
Figure 4. ATR-FTIR spectra (1300-950 cm\(^{-1}\)) of *S. oneidensis*, *P. aeruginosa*, and *B. subtilis* on a) ZnSe, b) mixed with α-Fe\(_2\)O\(_3\) on ZnSe, c) on a α-Fe\(_2\)O\(_3\)-coated ZnSe, d) mixed with α-Al\(_2\)O\(_3\) on ZnSe, and e) on a α-Al\(_2\)O\(_3\)-coated ZnSe surface (all spectra collected after 240 min). Reaction with α-Fe\(_2\)O\(_3\) results in growth in the 1087 cm\(^{-1}\) peak (ring vibrations/phosphate) and inner-sphere P-OF\(_e\) complexation as observed in the 1041 cm\(^{-1}\) peak.
3.3. ATR-FTIR spectral analysis

Peak fitting was performed on spectra presented in Figures 3 and 4 to determine the relative contributions of specific functional groups under the conditions studied. For example, peak fitting for *P. aeruginosa* on the ZnSe IRE is shown in Figure 5. Since interaction with Fe gave rise to the most significant spectral changes, we focus on those effects here. Chi-squared values ranged from 0.0004 to 0.2483 and $R^2$ values were consistently 0.99 to 1.00. Peak area ratios were determined using Gaussian fitted peaks for major IR absorbing functional groups (Table 2). For cells on the ZnSe IRE, these ratios are quite similar, but values for *B. subtilis* suggest a higher contribution of sugar rings to the spectra. The amide I:amide II ratio is ~1.0 for ZnSe (even for cells aggregated with colloidal $\alpha$-Fe$_2$O$_3$). However, an increase in the amide I:amide II ratio is observed upon cell adhesion to the $\alpha$-Fe$_2$O$_3$ IRE coating (1.27 to 3.45). For all bacteria, the $\nu$(ring-PO$_2^-$):amide II ratio increases upon bacterial interaction with $\alpha$-Fe$_2$O$_3$ coated ZnSe. A large decrease in the $\nu_{as}$(PO$_2^-$)amide II and $\nu$(ring-PO$_2^-$):amide II is observed for *B. subtilis* when aggregated with colloidal $\alpha$-Fe$_2$O$_3$, whereas changes for the Gram negative bacteria are much smaller. After aggregation of cells with colloidal $\alpha$-Fe$_2$O$_3$, the contribution of Fe-phosphate complexes (~1016 cm$^{-1}$) is greatest for *B. subtilis*. 
Table 2. ATR-FTIR peak area ratios for major bacterial functional groups after 240 min following (i) addition to ZnSe, (ii) aggregated with colloidal α-Fe$_2$O$_3$ and added to ZnSe, and (iii) added to α-Fe$_2$O$_3$-coated ZnSe.

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<tr>
<td><em>S. oneidensis</em></td>
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<td>0.22</td>
<td>0.17</td>
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<tr>
<td><em>P. aeruginosa</em></td>
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<td><em>B. subtilis</em></td>
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$^a$ Phosphate peak areas used correspond only to peaks present between 1200 and 1260 cm$^{-1}$.

$^b$ Ring vibrations and/or PO$_2^-$; $\nu$(ring-PO$_2^-$)

$^c$ Fe-Phosphate peak correspond to 1016-1018 cm$^{-1}$ for bacteria/α-Fe$_2$O$_3$ mix on ZnSe and 1041 cm$^{-1}$ for bacteria on α-Fe$_2$O$_3$-coated ZnSe.
Kinetic data were collected to observe time-dependent spectral changes associated with bacterial adhesion under the five conditions studied (spectra not shown). Analysis of these data employed the use of peak intensity ratios (Fig. 6-8). Data for bacterial adhesion to α-Al₂O₃ was not included because only small effects of Al binding were observed. Minor time-dependent changes were observed for bacterial cells placed on the ZnSe IRE (Fig. 6a, 7a, 8a). For *B. subtilis* (Fig. 8a), there is a slight decrease in the intensity of the amide II to νₐs(PO₂⁻), ν(C-O-C, C-C), and ν(ring-PO₂⁻), revealing a slight increase of the amide II with time. The greatest time-dependency of intensity ratios occurred for bacterial cells aggregated with colloidal α-Fe₂O₃ (Fig. 6b, 7b, 8b). For *S. oneidensis* (Fig. 6b) and *P. aeruginosa* (Fig. 7b) calculated ratios either remain constant or increase. With the exception of amide II:amide I, all ratios decrease for *B. subtilis* (Fig. 8b) under the

**Figure 5.** ATR-FTIR spectra of *P. aeruginosa* on ZnSe showing typical peak fits. The gray lines indicate Gaussian fits to spectra and dotted lines represent data fits based on peak deconvolution.
same conditions. In this case there is a decrease of amide peaks relative $\nu_{\text{as}}(\text{PO}_2^-)$ and $\nu$(C-O-C, C-C) with time. When bacteria are placed on the $\alpha$-Fe$_2$O$_3$-coated IRE, intensity ratios generally decrease (or remain the same) indicating increased polysaccharide and phosphate at the solid-liquid interface. The amide II: $\nu$(ring-$\text{PO}_2^-$) and amide II: $\nu$(P-Of) (1041 cm$^{-1}$) for bacteria on $\alpha$-Fe$_2$O$_3$ films plot nearly on top of each other suggesting that Fe-complexed phosphate groups are part of phosphodiester ring structures.
Figure 6. IR peak intensity ratios as a function of reaction time for prevalent bacterial surface functional groups of *S. oneidensis*: a) on ZnSe, b) mixed with nanohematite (α-Fe₂O₃) on ZnSe, and c) on a α-Fe₂O₃-coated ZnSe surface (note a different scale is used for 5b.)
Figure 7. IR peak intensity ratios as a function of reaction time for prevalent bacterial surface functional groups of *P. aeruginosa*: a) on ZnSe, b) mixed with nano-hematite (α-Fe₂O₃) on ZnSe, and c) on an α-Fe₂O₃-coated ZnSe surface.
Figure 8. IR peak intensity ratios as a function of reaction time for prevalent bacterial surface functional groups of *B. subtilis*: a) on ZnSe, b) aggregated with nanohematite (α-Fe₂O₃), and c) on an α-Fe₂O₃-coated ZnSe surface.
3.4. ATR spectra of DNA and model P-containing molecules

ATR-spectra (1300-950 cm$^{-1}$) for DNA, DADA, AMP and PPA solutions on ZnSe, α-Al$_2$O$_3$, α-Fe$_2$O$_3$ are shown in Figure 9. The spectra are generally similar for solutions contacting ZnSe and α-Al$_2$O$_3$. However, data collection after reaction with α-Fe$_2$O$_3$ results in peak shifts and new peak production for each compound studied.

**Figure 9.** ATR-FTIR spectra (1300-950 cm$^{-1}$) of a) DNA, b) DADA, c) AMP, and d) PPA on ZnSe, α-Fe$_2$O$_3$, and α-Al$_2$O$_3$ (all spectra collected after 120 min).
In the case of DNA (Fig. 9a), spectra collected on ZnSe and α-Al₂O₃ appear to be nearly the same. However for α-Al₂O₃, there is a loss of the peak at 1016 cm⁻¹ (C-C, C-O ring vibrations), accompanied by a slight reduction in the intensity of the 970 cm⁻¹ peak [ν(P-OH)]. When contacted with the α-Fe₂O₃ coating, the spectrum of DNA shows small peaks at 1022 (C-O, C-C) and 998 cm⁻¹ [ν(P-OFe)] (Sheals et al., 2002; Omoike et al., 2004). In the amide region (~1700-1500 cm⁻¹) for DNA on ZnSe and α-Al₂O₃ (Fig. 10) there are strong amide I peaks (1641 and 1650 cm⁻¹, respectively). In the case of DNA on α-Fe₂O₃ films an amide I peak remains (1639 cm⁻¹), accompanied by a larger peak at 1577 cm⁻¹ (amide II). Interaction of DNA with α-Fe₂O₃ also produces a new peak at 1400 cm⁻¹, resulting from νₕ(COO⁻). This experiment was performed three times to verify the result.

**Figure 10.** ATR-FTIR spectra (1750-1300 cm⁻¹) of DNA on ZnSe, α-Fe₂O₃, and α-Al₂O₃ (all spectra collected after 120 min).
The spectrum of the oligonucleotide, DADA, on the α-Fe$_2$O$_3$ film contains a strong peak at 989 cm$^{-1}$ [ν(P-OF$_2$)] (Barja et al., 1999; Sheals et al., 2002) which is absent for other surfaces studied (Fig. 9b). There is also a loss of the peak at 1093 cm$^{-1}$ (ring vibrations/PO$_{-}$) for DADA on the α-Fe$_2$O$_3$ film. The spectra for AMP on all three surfaces (Fig. 9c) are similar to that observed for DADA. AMP on ZnSe has a strong peak at 977 cm$^{-1}$ [ν(PO$_3^{2-}$)/P-OH]. Upon reaction with α-Al$_2$O$_3$ there is reduction of this peak (979 cm$^{-1}$) and a small peak is observed at 995 cm$^{-1}$ [ν(P-OAl)] (Sheals et al., 2002). AMP reacted with α-Fe$_2$O$_3$ produces a peak shift from 977 cm$^{-1}$ (on ZnSe) to 993 cm$^{-1}$ ([ν(P-OF$_{Fe}$)]. This is accompanied by a shift of the 1083 cm$^{-1}$ peak to 1074 cm$^{-1}$, both of which correspond to ring vibrations and/or PO$_2^{-}$.

Spectra of PPA on ZnSe and α-Al$_2$O$_3$ are nearly identical (Fig. 9d). Reaction of PPA with α-Fe$_2$O$_3$ leads to numerous changes in the acquired spectra. New peaks are observed at 1091 cm$^{-1}$ (ring vibrations/PO$_2^{-}$) and 1049 cm$^{-1}$ (C-O-C, C-C). There is a large increase in the peak at ~1137 cm$^{-1}$ (PO$_2^{-}$) and a shift in the 968 cm$^{-1}$ peak to 975 cm$^{-1}$ is observed, indicating binding of the phosphonic group to Fe.

4. Discussion

ATR-FTIR studies of bacterial adhesion under different conditions permits elucidation of molecular mechanisms. Spectral changes in bacterial cells and model compounds were most evident in the presence of α-Fe$_2$O$_3$ surfaces. Conversely, α-Al$_2$O$_3$ did not greatly influence spectra of bacteria, DNA, or selected phosphorylated compounds. Hence, hereafter we focus on comparison of spectra collected for bacterial
cells contacted with ZnSe and α-Fe₂O₃. Analysis of model P-containing compounds provides a set of references for Fe surface interaction with organic phosphate/phosphonate under simpler abiotic conditions.

4.1. Interpretation of spectral changes of bacterial amide groups

IR peak areas normalized to that of amide II are used to determine protein conformational changes and changes in composition or bonding of biomolecules at the solid-liquid interface (Table 2). The amide I:amide II ratio can be used to differentiate changes in protein secondary structure (Ishida and Griffiths, 1993). For example, a conformational change in surface proteins is evident when bacteria bind to the α-Fe₂O₃-coated IRE. For bacteria on ZnSe (including cells aggregated with colloidal α-Fe₂O₃) the ratio is close to unity (0.92 to 1.06), adhesion to α-Fe₂O₃ films leads to an increase of this ratio (1.27 to 3.45). Also, the observed amide I peak shift to higher wavenumber when cells come into contact with α-Fe₂O₃ surfaces (Figure 2), particularly for B. subtilis (1635 cm⁻¹ to 1652 cm⁻¹ [α-Fe₂O₃ mix] and 1656 cm⁻¹ [α-Fe₂O₃α-Fe₂O₃ film]), represents a change in protein conformation from β-sheet to α-helix structure (Byler and Susi, 1986; Buijs et al., 1996). The shift for the Gram negative bacteria is smaller (S. oneidensis: 1640 cm⁻¹ to 1650 cm⁻¹ [α-Fe₂O₃ mix] and 1643 cm⁻¹ [α-Fe₂O₃ film]; P. aeruginosa: 1639 cm⁻¹ to 1639 cm⁻¹ [α-Fe₂O₃ mix] and 1643 cm⁻¹ [α-Fe₂O₃ film]) and represent a change from β-sheet to random coil structures (Buijs et al., 1996). EPS extracted from B. subtilis and P. aeruginosa exhibited very similar shifts in the amide I peak; from 1643 to 1652 cm⁻¹ and 1648 to 1657 cm⁻¹, respectively, after adhesion to goethite (Omoike et al.,
2004). From analysis of amide I:amide II area ratios and peak shifts of the amide I it is apparent that when bacteria interact with the $\alpha$-Fe$_2$O$_3$ surface, the proteins involved are of different composition or altered conformation relative to the case for interaction with ZnSe surfaces.

Along with changes in protein conformation/composition there is also a decrease in overall protein absorbance (relative to polysaccharide and phosphate) upon adhesion to $\alpha$-Fe$_2$O$_3$-coated IRE as compared to adhesion to the ZnSe IRE (Table 2). This suggests that cell adhesion to Fe oxide is not dominated by cell surface proteins, whereas these constituents play a more prominent role in deposition on ZnSe. The isoelectric point for ZnSe is pH < 4 (Tickanen et al., 1997), therefore at pH 7 the IRE is negatively-charged and adhesion of charged amino moieties would be favored. Indeed, surface proteins of $P$. putida (GB-1) have been shown to play an important role in adhesion and biofilm growth on ZnSe under flow through conditions (Parikh and Chorover, 2005). For GB-1 cells, a dramatic decrease in adhesion was observed via protein blocking from biogenic Mn-oxides. Although electrostatics are not solely responsible for cell adhesion, bonding interactions (e.g., protein binding, hydrogen bonding, hydrophobic interaction) must overcome the repulsion between like-charged bacteria and surfaces in order for adhesion to occur (Marshall et al., 1971; Truesdail et al., 1998; Deo et al., 2001; Appenzeller et al., 2002).

Time-dependent functional group peak intensities (normalized to amide II) remain fairly constant for $P$. aeruginosa and $S$. oneidensis (with exceptions, particularly COOH) (Fig. 5,6). However, $B$. subtilis IR intensity ratios show a distinct increase in the relative
contribution of polysaccharides and phosphates. While this trend is observed under all three conditions studied, it is minimal for bacteria introduced to the ZnSe IRE. Particularly in the case of *B. subtilis* adhesion to α-Fe₂O₃, surface proteins do not appear to play an important role.

### 4.2. Sorption induced carboxyl protonation

Carboxylic groups are dissociated at pH 7 (pKa <5) (Martell and Smith, 1977). Therefore, the presence of a peak at ~1720 cm⁻¹ corresponding to νₐs(COOH) is somewhat surprising for the circumneutral conditions of this study (pH ~7). Nonetheless, distinct peaks (~1720 cm⁻¹) are observed for *S. oneidensis* and *P. aeruginosa* aggregated with colloidal α-Fe₂O₃ (Fig. 2b). Triplicate experiments validated the presence of this peak as a reproducible result, although replicate spectra (not shown) showed variable νₐs(COOH) peak intensity and in one case the peak appeared as a shoulder on the amide I peak. In the case of *B. subtilis*, a peak at 1720 cm⁻¹ appeared once but the intensity was low relative to background. The point of zero net proton charge of α-Fe₂O₃ is between 8.0 and 8.5 (Sposito, 1989) and, therefore, it is positively-charged at pH 7. The emergence of a peak at 1720 cm⁻¹ suggests H-bridging from the mineral surface to bacterial COO⁻. This may occur via binding α-Fe₂O₃ surface OH groups, or through water bridging between COO⁻ and α-Fe₂O₃. Since carboxylic groups are deprotonated at pH 7, conditions are favorable for electrostatic interaction between carboxylate and protonated surface hydroxyls of α-Fe₂O₃. In other ATR-FTIR studies similar results have been reported. Duckworth and Martin (2001) observed a peak at 1720 cm⁻¹ for oxalate
(pH 5.0) bound to $\alpha$-Fe$_2$O$_3$, which was assigned to $\nu_{as}$(COO$^-$). Carbonate surface complexes on $\alpha$-Fe$_2$O$_3$ (pH 6.38) produced a small peak (1713 cm$^{-1}$) (Bargar et al., 2005).

4.3. Bacteria adhesion mediated via Fe-phosphate complexes

In all spectra where $\alpha$-Fe$_2$O$_3$ was present, the appearance of Fe-complexed phosphate groups (~1016, 1041 cm$^{-1}$) and increased contributions from ring structures/phosphate (~1085 cm$^{-1}$) implicate their involvement in the binding to $\alpha$-Fe$_2$O$_3$ films and/or colloidal particles (Fig. 4, Table 2). Spectra of cell-mineral ($\alpha$-Fe$_2$O$_3$/$\alpha$-Al$_2$O$_3$) colloid aggregates (Fig. 4b, 4d) show bands at ~1016 cm$^{-1}$. Methylphosphonic acid interaction with goethite particles also produced a $\nu$ (P-OFe) peak at this frequency (1015 cm$^{-1}$), which was attributed to a bridging bidentate complex (Barja et al., 1999). The peak at ~1016 cm$^{-1}$ cannot unambiguously be assigned to P-OFe; it may also represent ring vibrations of DNA sugars. However, since the peak is absent for bacteria on ZnSe, we interpret it as signaling P-OFe bonding. We also observed similar modes in spectra of EPS bonded to Fe-oxides (Omoike et al., 2004), and noted high preferential sorption of P-containing moieties in batch uptake studies (Omoike and Chorover, 2006). In the case of $\alpha$-Al$_2$O$_3$ this peak is similarly attributed to $\nu$(P-O-Al). For Gram positive $B. subtilis$ aggregated with $\alpha$-Fe$_2$O$_3$, the large spectral contribution of this peak (confirmed with triplicate experiments) likely results from binding of Fe metal centers to bacterial teichoic acids. These molecules, which occupy a significant fraction of the cell external surface in Gram positive bacteria, comprise terminal phosphate groups, glycerol and a C side chain (Tallaro and Talaro, 1999). Emergence of a much smaller peak on $B. subtilis$
interaction with α-Al₂O₃ is consistent with the same trends observed for the model P-containing compounds. In prior studies of Fe adsorption to *B. subtilis* cell walls, a high capacity of aqueous Fe removal was observed and attributed to sorption via phosphodiester, carboxylic, phosphoric, and hydroxyl sites (Fein et al., 1997; Wightman and Fein, 2005).

Interaction of bacteria with α-Fe₂O₃ films resulted in very similar spectra for all three bacteria with all showing strong $\nu$(P-OFe) modes (1041 cm⁻¹). The similar peak intensity ratios and time-dependent trends for amide I to ring vibrations and Fe-phosphates (Fig, 5c, 7c, 8c) imply that these peaks arise from a single group of molecules comprised of both ring structures and phosphate groups, the most likely candidate being phosphodiester groups of nucleic acids (Stryer, 1965). Peaks in this region (~1030 to 1045 cm⁻¹) have been attributed to inner-sphere monodentate (and possibly, bidentate bridging) complexes arising from glycophosphate and aminomethylphosphonic acid adsorption to goethite (Sheals et al., 2002; Barja and Afonso, 2005). The growth of IR bands (1085, 1037 cm⁻¹) corresponding to monodentate inner-sphere complexation of phosphodiester groups was confirmed via quantum chemical calculations in conjunction with experimental studies of *B. subtilis* and *P. aeruginiosa* EPS adhesion to goethite (Omoike et al., 2004). The results presented here show that whole cell adhesion to α-Fe₂O₃ occurs via similar mechanisms.

*B. subtilis* contacted with α-Al₂O₃ films produces a broad peak extending from 1079 to 1027 cm⁻¹ which may represent P-OAl bonding, analogous to that assigned to P-OFe in the case of Fe oxide. Guan et al. (2005) examined the interaction of sodium
pyrophosphate (SP) and sodium tripolyphosphate (TP) with aluminum hydroxide and observed $\nu_{as}(\text{Al-PO}_3^\cdot)$ peaks at 1140-1144 (PP) and 1165 cm$^{-1}$ (TP). The absence of peaks in this region indicates that, in the very least, P-OAl interactions are not as strong as those observed for P-OFe.

### 4.4. Interaction of DNA and model P-containing compounds with $\alpha$-$\text{Fe}_2\text{O}_3$

Bacteria samples are chemically heterogeneous and, as a result, weak IR vibrations may be masked by others giving rise to stronger modes at similar frequency. This likely contributes to the spectral structure of the mixed phosphate/polysaccharide region (1200-900 cm$^{-1}$), where several overlapping bands occur. From the data presented here and in Omoike et al. (2004) it is apparent that interactions of bacterial cells or their extracellular polymers with Fe-oxide surfaces are unique relative to surfaces of Ge, ZnSe, and $\alpha$-$\text{Al}_2\text{O}_3$. In particular, it is apparent that phosphate groups play an important role in this difference.

DNA, DADA, AMP and PPA were used as models for extracellular P-containing biomolecules that could contribute to the observed differences in IR spectra. DNA and DADA both contain phosphodiester bonds, AMP has a terminal phosphate group, and PPA has a phosphonic (phosphonate) group (Fig. 1). The terminal phosphate group ($\text{PO}_4^{2-}$) differs from the phosphonic group ($\text{PO}_3^{2-}$) in that phosphorus is attached to the carbon ring through a phosphodiester bond (Fig. 1). Though such differences are also reflected in IR spectra, we do not know if they translate to different reactivities toward Fe oxide surfaces. In any case, examination of IR spectra resulting from reaction of these
model compounds with $\alpha$-Fe$_2$O$_3$ surfaces under conditions similar to those used for the cell-mineral studies facilitates confirmation of peak assignments and bonding mechanisms.

DNA is a component of EPS (Wingender et al., 1999) and could potentially play a valuable role in conditioning film formation and cell adhesion to Fe-oxide surfaces (Omoike et al., 2004). Extracellular DNA may result from cell lysis (Sutherland, 2001) or exudation (Demain et al., 1965; Brown et al., 1969). DNA molecules are comprised of two strands of nucleotides linked via phosphodiester bonds to form a double helix configuration. Purine and pyrimidine bases are within the double helix, while phosphate and deoxyribose units are on the outside of the structure (Stryer, 1995). In this conformation DNA bases are not available for direct interaction with surfaces. However, ATR-FTIR spectra in Figure 10 clearly show a change in the amide region (1500-1700 cm$^{-1}$) upon DNA reaction with $\alpha$-Fe$_2$O$_3$. Strong interaction between aqueous Fe(III) and calf-thymus DNA base groups has also been reported (Ouameur et al., 2005). This interaction was attributed Fe(III)-induced auto-oxidation of DNA and the subsequent availability of bases for reaction with Fe(III). Cleavage of DNA via Fe(III) is documented in the literature (Adachi et al., 1994; Hemmert et al., 2001; Neves et al., 2001). The spectrum of DNA on $\alpha$-Fe$_2$O$_3$ also contains a large peak at 1400 cm$^{-1}$ [$\nu_{s}$(COO$^-$)]. This peak provides further evidence for DNA oxidation in that it likely derives from ribose ring cleavage and the subsequent interaction of COO$^-$ with $\alpha$-Fe$_2$O$_3$.

The IR studies with model P compounds also shed some light on the results of Omoike et al. (2004). When EPS from B. subtilis and P. aeruginosa were reacted with
goethite, new IR peaks emerged at 1137, 1037, and 997 cm\(^{-1}\). These peaks were accompanied by growth in the peak at 1085 cm\(^{-1}\). As noted above, the peaks at \(\sim\)1085 and \(\sim\)1037 cm\(^{-1}\) were also observed in the present study upon introduction of whole bacterial cells to \(\alpha\)-Fe\(_2\)O\(_3\). The peak at \(ca.\) 1137 cm\(^{-1}\), corresponding to \(\nu(P=O)\), is also observed in all three spectra of PPA (Fig. 9d). Upon reaction with \(\alpha\)-Fe\(_2\)O\(_3\) there is a slight shift (to 1135 cm\(^{-1}\)) and a relative increase in peak intensity. This likely signals the bonding of phosphonate to the \(\alpha\)-Fe\(_2\)O\(_3\) surface. The peak located at 1045 cm\(^{-1}\) for PPA reacted Fe\(_2\)O\(_3\) is assigned to monodentate P-OFe bonding based on previous quantum chemical calculations (Omoike et al., 2004). This peak may represent a similar P-funcionality binding to Fe as the P-OFe peak observed for bacteria reacted with \(\alpha\)-Fe\(_2\)O\(_3\) (1041 cm\(^{-1}\)) (Fig. 4c). Strong binding of phosphonate with Fe(III) has been demonstrated previously (Barja et al., 1999; Barja et al., 2001). If phosphonate moieties are present in bacterial samples (Fig. 4c) their IR signal may be swamped by the large contribution of the \(\sim\)1085 cm\(^{-1}\) peak.

The peak at 997 cm\(^{-1}\), also observed for EPS interaction with FeOOH (Omoike et al., 2004), is present in IR spectra of DNA (998 cm\(^{-1}\)), DADA (989 cm\(^{-1}\)), and AMP (993 cm\(^{-1}\)) sorbed to the \(\alpha\)-Fe\(_2\)O\(_3\)-coated IRE (Fig. 9). For these same compounds the peak is absent for spectra collected on ZnSe and \(\alpha\)-Al\(_2\)O\(_3\)-coated ZnSe. These results strongly suggest a unique role for Fe in the bonding of phosphate-containing EPS and cell surface moieties at mineral surfaces. The electron density of P-OFe and P-OAl exceeds that of P-OH, and hence gives rise to higher vibrational frequencies (Barja et al., 1999). As a result, a shift to higher wavenumber is observed for peaks located between 970 and 1000 cm\(^{-1}\).
when these compounds adsorb to $\alpha$-Fe$_2$O$_3$. In the case of AMP (Fig. 9c), the P-OH peak (977 cm$^{-1}$ on ZnSe) is shifted to 993 cm$^{-1}$ (P-OFe). Upon reaction with $\alpha$-Al$_2$O$_3$, there is production of a small peak at 995 cm$^{-1}$ (P-OAl) also indicating the formation of inner-sphere complexes between AMP phosphate groups and Al, though in smaller proportion than observed for Fe.

Spectra for bacteria and P-containing compounds on $\alpha$-Fe$_2$O$_3$ films give absorbances corresponding to the four major peaks observed during EPS adsorption to $\alpha$-FeOOH (Omoike et al., 2004). In all cases, the most likely explanation for the observed results are strong interactions between Fe-centers and phosphate groups. Discrimination of monodentate versus bidentate complex formation would require additional data such as molecular modeling (Persson et al., 1996; Barja et al., 1999; Sheals et al., 2002), which is beyond the scope of this paper. However, the present work provides clear evidence that inner-sphere phosphate complex formation mediates adhesion of cell surface biomolecules to Fe oxides.

5. Conclusions

Nucleic acids and/or surface proteins play a role in bacterial adhesion to ZnSe surfaces, whereas adhesion to $\alpha$-Fe$_2$O$_3$ films involves a change in protein conformation and formation of P-OFe bonds. Cleavage of the ribose ring structures of DNA when reacted with $\alpha$-Fe$_2$O$_3$ may contribute to adhesion via interactions between the cleaved ring and/or nucleic acid bases with $\alpha$-Fe$_2$O$_3$. Aggregation of bacterial cells in colloidal suspension with $\alpha$-Fe$_2$O$_3$ or $\alpha$-Al$_2$O$_3$ gives rise to P-OFe and P-OAl bond formation. P-
OFe interactions are more evident than those of P-OAl under the conditions of this study, for both model compounds and bacterial cells. Thus, phosphate groups present in cell surface molecules and bacterial EPS, including nucleic acids, likely mediate the adhesion process.

The interaction of bacteria with colloidal $\alpha$-Fe$_2$O$_3$ results in sorption induced protonation of surface COO$^-$ groups at pH values above the pKa. The high specific surface area of colloidal $\alpha$-Fe$_2$O$_3$ results in a large mass density of surface OH groups, with a propensity for bridging of COO$^-$ to the mineral surface. The data presented here suggest the biomolecular moieties employed in cell adhesion depend on mineral surface chemistry. Most importantly, the data provide support for the idea that strong interactions between Fe-oxides and bacterial cells are mediated by cell surface phosphate/phosphonate groups.

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APPENDIX C

INFRARED AND PHOTON CORRELATION SPECTROSCOPY
STUDIES OF PSEUDOMONAS AERUGINOSA LIPOPOLYSACCHARIDES
IN AQUEOUS SYSTEMS

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Abstract

The conformation of amphiphilic lipopolysaccharides (LPS) influences the behavior of free and cell-bound LPS in aqueous environments, including their adhesion to surfaces. Conformational changes in *Pseudomonas aeruginosa* serotype 10 LPS aggregates resulting from changes in solution pH (3, 6, and 9), ionic strength \([I]\) 1, 10, and 100 mmol L\(^{-1}\), and electrolyte composition (NaCl and CaCl\(_2\)) were investigated via attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy and dynamic light scattering (DLS). Changes in solution chemistry affected LPS aggregate size and stability, and macromolecular conformation. ATR-FTIR data indicate that LPS forms more stable aggregates in solutions of NaCl relative to those of CaCl\(_2\). Time- and cation-dependent changes in ATR-FTIR data suggest that LPS aggregates are perturbed by Ca\(^{2+}\) complexation at lipid A phosphoryl groups, which leads to reorientation of the lipid A at the surface of a ZnSe ATR internal reflection element (IRE). Aggregate sizes, measured by DLS, typically indicate decreasing hydrodynamic diameter with increasing \(I\). Particle size was unchanged when decreasing pH from 9 to 6, however further decrease to pH 3 resulted in decreased aggregate size. The results indicate that changes in solution chemistry strongly impact the conformation and intermolecular behavior of LPS in aqueous systems.

**KEYWORDS:** LPS, ATR-FTIR spectroscopy, dynamic light scattering, lipid A
1. Introduction

Lipopolysaccharides (LPS) are amphiphilic molecules (Fig. 1a) anchored to the surface of Gram-negative bacteria (Seltmann and Holst, 2002). The hydrophobic lipid A portion of the molecule (Fig. 1b) is imbedded in the outer membrane, with very little structural variation between different bacteria (Seltmann and Holst, 2002). The O-antigen (hydrophilic polysaccharide region) extends from the bacterial surface and may facilitate adhesion to environmental surfaces. For example, Jucker et al. (1998) have shown that H-bonding interactions of O-antigen with oxide surfaces contribute to bacterial adhesion. Most LPS research has been motivated by the immunoreactive properties of these biomacromolecules (Lam et al., 1992; Brandenburg, 1993; Kastowsky et al., 1993; Paradis et al., 1994; Aurell et al., 1999; Zdorovenko and Veremeichenko, 2001) that are thus also referred to as endotoxins. Given the importance of surficial macromolecules to bacterial deposition in environmental media (e.g., mineral and organic particles in soils, water filtration systems), there is a need to elucidate the role of LPS in environmental processes such as conditioning film formation and bacterial adhesion.

Bacterial LPS occur in either “smooth” or “rough” form (Seltmann and Holst, 2002). LPS in the rough form lack the O-antigen. The O-antigen (20 to 70 repeating units of three to five sugar molecules) of the smooth form can protrude up to 30 or more nanometers from the cell surface. The O-antigens of Pseudomonas aeruginosa LPS are believed to extend up to 40 nm from the cell, depending on solution chemistry (Lam et al., 1992). The core region of LPS (present in both the rough and smooth form) consists of five to ten negatively charged sugar units (Jucker et al., 1997). Due to longer protrusion
from the cell, LPS with the O-antigen (i.e., smooth LPS) are likely responsible for interactions of gram-negative bacteria with environmental surfaces (Makin and Beveridge, 1996; Jucker et al., 1997). Surface affinity of LPS is variable, depending on bacterial strain and substrate composition. Specifically, LPS with longer O-antigens are adsorbed more extensively and less reversibly (Jucker et al., 1997). Although negatively-charged bacteria exhibit electrostatic repulsion with many like-charged environmental surfaces, it has been suggested that polymer bridging by LPS can transcend the energy barrier by forming hydrogen bonds with mineral surfaces (Jucker et al., 1997).

As a result of cell turnover and lysis, LPS occurs in both “cell-bound” and “free” forms in natural aquatic systems (Rietschel et al., 1994). The lipid A of cell-bound LPS is contained within the outer membrane of the cell and, therefore, it does not participate

Figure 1. Schematic diagram of (a) smooth lipopolysaccharide and (b) lipid A. (Seltmann and Holst, 2002)
directly in cell adhesion to other surfaces. Conversely, adsorption of free LPS to surfaces may be mediated by functional groups associated with either hydrophilic or hydrophobic portions of the molecule. Bacterial extracellular polymeric substances, which include free LPS (Sutherland, 1985), contribute to the formation of “conditioning films” on environmental surfaces (Frank and Belfort, 1997; Bos et al., 1999; Beech et al., 2000), which in turn modify subsequent cell adhesion processes (Schneider et al., 1994; van der Aa and Dufrêne, 2002). Even in free LPS, however, exposure of the lipid A is limited by LPS amphiphilic properties that promote intermolecular associations and the formation of supramolecular structures above a critical aggregation concentration (CAC) (Seydel et al., 1993; Aurell and Wistrom, 1998; Santos et al., 2003). Indeed, such aggregates of free LPS have been used above the CAC to represent cell-bound forms under the assumption that, in both cases, only the O-antigen is exposed for interaction with environmental surfaces. For example, Jucker et al. (1998) measured the adsorption of LPS micelles to TiO₂, Al₂O₃, SiO₂, and glass beads. However, the nature of restructuring of such aggregates that may occur upon association with surfaces is unknown.

LPS contains weakly acidic (hydroxyl, phosphoryl, amide) functional groups associated with the lipid A, core and O-antigen (Fig. 1a). Changes in aqueous phase pH, ionic strength and ionic composition are expected to affect LPS ionization and background ion complexation reactions in a manner that is comparable to that for model polyelectrolytes (Doty et al., 1957; Barrat and Joanny; Ito, 1998). Conformation of individual LPS molecules (i.e., monomers) is likely to be affected by electrostatic repulsion between anionic functional groups on the O-antigen. Increased background
electrolyte concentration, decreased pH and increased prevalence of bivalent (relative to monovalent) background counterions are all expected to diminish such repulsion and to promote coiling.

Human-health-related studies have shown the importance of calcium in the conformation and aggregation of LPS, however none of these studies have attempted to model the behavior of LPS under conditions representing natural waters. Crystalline calcium silicate hydrate is effective for removing LPS from solution (Wang et al., 2005; Zhang et al., 2005), and the strong affinity of Ca$^{2+}$ for phosphate groups in the lipid A region has been implicated as a key mediator of this process (Wang et al., 2005). The presence of Ca$^{2+}$ has also been shown to increase LPS aggregation (compared to Na$^+$), even acting as a “bridge” between LPS subunits to form supramolecular vesicle structures (Schindler and Osborn, 1979; Li and Luo, 1998; Li and Luo, 1999). Other researchers have also demonstrated a strong affinity between Ca$^{2+}$ and the phosphate groups in the lipid A region of rough LPS (Coughlin et al., 1985; Seydel et al., 1993; Obst et al., 1997). Studies on rough LPS show that at low pH insoluble complexes result from diminished hydration and charge repulsion of the lipid A region. At high pH, diminished hydrogen bonding and increased hydration of the lipid A results in micellar aggregates (Coughlin et al., 1985; Din et al., 1993). It is not clear how changes in pH affect the solubility of smooth LPS, as the solubility of these molecules is dictated largely by the hydrophilic O-antigen chain. Coughlin et al. (1985) also suggest that pH and salt content can influence aggregate structure of rough LPS. These solution chemistry effects are likely to also affect smooth LPS aggregation and surface interaction but there are few reports in the
literature. Kastowsky et al. (1992) performed a molecular modeling study on the conformation of smooth LPS that indicated a high degree of flexibility for the O-antigen chain, with the maximum length of four repeating units (sugar rings) measuring 9.6 nm. However, in this study the effect of solution chemistry was not investigated.

Other studies of smooth LPS conformation have been limited to aggregation without considering the effects of pH, I, or ion composition (Aurell and Wistrom, 1998; Santos et al., 2003; Stoica et al., 2003). There have been LPS conformational studies focused on rough LPS (Brandenburg, 1993; Kastowsky et al., 1993), some of which did investigate the effect of solution chemistry (Coughlin et al., 1985; Din et al., 1993; Seydel et al., 1993; Obst et al., 1997). While comparisons to these studies are appropriate, due to the greater flexibility and greater ionic nature of the O-antigen it is expected that smooth LPS will vary their conformation to a much greater extent with changes in solution chemistry. Few studies have investigated these effects for smooth LPS under conditions representative of natural aqueous systems (Jucker et al., 1997; Jucker et al., 1998; Langley and Beveridge, 1999; Burks et al., 2003; Walker et al., 2004). Changes in LPS monomer conformation and aggregation are expected to have a significant effect on adhesion of both free and cell-bound LPS to environmental surfaces.

2. Research Approach

2.1. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy

Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) spectroscopy can provide nondestructive, molecular-scale information on biofilms
(Nichols et al., 1985; Nivens et al., 1993; Schmitt and Flemming, 1998; Parikh and Chorover, 2005), bacterial cells (Nichols et al., 1985; Naumann et al., 1991; Sockalingum et al., 1997; Wei et al., 2004), and biomacromolecules, such as LPS (Brandenburg, 1993) immersed in aqueous solutions of variable chemistry. We previously used ATR-FTIR to determine the effects of solution chemistry on functional group ionization and associated conformational change in extracellular bacterial polymers (Omoike and Chorover, 2004) and to assess mechanisms of their adsorption at the α-FeOOH surface (Omoike et al., 2004). The spectrum derives from infrared-absorbing moieties that are probed by an evanescent wave. The wave propagates ca. 10^2-10^3 nm (depending on crystal type, incident beam angle, and wavelength) beyond the interface of the crystalline internal reflection element (IRE) and into an aqueous suspension of biomacromolecules (Table 1).

Beam penetration depth ($dp$) varies according to Eq. 1:

$$dp = \frac{\lambda}{2\pi \left(\sin^2 \theta - \left(\frac{n_1}{n_2}\right)^2\right)^{\frac{1}{2}}}$$

where $\gamma$ is wavenumber (cm$^{-1}$) of incident radiation, $n_1$ and $n_2$ are the refractive index (RI) values for the IRE and sample, respectively, and $\theta$ is the effective angle of incidence (Mirabella, 1985). The RI value for the ZnSe IRE used in the present study ($n_{\text{ZnSe}}$) is 2.4. The RI range for LPS ($n_{LPS}$) likely falls in the range reported for lipid A (1.50 for pure lipid A; 1.33 with 90% water) (Seydel et al., 2000), bacterial cells (1.38) (Jonasz et al., 1997; Katz et al., 2005), proteins (1.5) (Chittur, 1998), and polymer bilaminate films (1.52) (Chen et al., 1994). As a result of the limited wave penetration depth, ATR spectra
pertain to the region in close proximity to the sample-IRE interface. For example, the $dp$ for bacteria samples on a 45° ZnSe IRE is 552 nm at 2920 cm$^{-1}$, 1299 nm at 1240 cm$^{-1}$, and 1520 nm at 1060 cm$^{-1}$.

2.2. Dynamic Light Scattering (DLS)

The influence of solution chemistry on the size of LPS was elucidated using photon correlation spectroscopy, i.e., dynamic light scattering (DLS). The DLS approach reveals the mean hydrodynamic diameter of particles in a suspension of macromolecules. The particles may comprise individual monomers or aggregates thereof (Mazer, 1985; Schmitz, 1990). The mechanics of DLS involve measurement of time-correlated fluctuations in the intensity of monochromatic light scattered from the suspension ($I_s$) in order to measure the hydrodynamic diffusion coefficients ($D$) of the particles (Pecora, 1985; Schurtenberger and Newman, 1993). The extent to which $I_s$ values are correlated over time is related inversely to $D$. Small particles with high rates of diffusion are characterized by rapidly diminished time-correlation in $I_s$, while larger particles with low rates of diffusion show correlation in $I_s$ over longer time scales. Diffusion coefficients determined from DLS measurements are converted to yield the hydrodynamic diameter ($D_H$) of the suspended particles using the Stokes-Einstein relation:

$$D = \frac{kT}{3\pi\eta D_H} \quad [2]$$

where $k$ is the Boltzmann constant, $T$ is absolute temperature and $\eta$ is sample viscosity (Schmitz, 1990). In addition to providing the distribution of diffusion coefficients and
hydrodynamic size of scattering particles, DLS experiments give suspension polydispersity, which is analogous to the variance of the particle size distribution. DLS was previously used by Santos et al. (2003) to determine the CAC for *Escherichia coli* serotype 026:B6 LPS. The objective of the current study was to employ ATR-FTIR and DLS in a complementary manner to elucidate the effects of solution chemistry on LPS functional group chemistry, molecular conformation and aggregation.

3. Experimental Methods

3.1. LPS preparation

A single batch of freeze-dried *Pseudomonas aeruginosa* serotype 10 LPS (batch 123K4144; Sigma Inc.) was used for all experiments. Analysis of LPS by size exclusion high performance liquid chromatography revealed high purity and very low protein contamination. Triplicate measurements of the LPS critical aggregation concentration (CAC) were determined via both electrical conductivity (EC) (Prieto et al., 1994) and ultraviolet visible spectroscopy (UV-Vis) (Beyaz et al., 2004) techniques for LPS dispersed in Barnstead nanopure (BNP) water. LPS solutions were prepared above the CAC by dissolving 4.0 mg of freeze-dried LPS in 1.0 g of NaCl or CaCl₂ electrolyte solution at the target ionic strength (I) with pH adjusted to specific values using HCl or NaOH at the same I. Samples were vortexed, sonicated for 10 min, and stored overnight at 4°C prior to re-equilibration the following day to room temperature. All measurements were carried out at 23 ± 2°C, three different values of pH (3, 6 and 9), and three different values of I (1, 10 and 100 mmol L⁻¹). This experimental matrix produced nine replicated
samples that were examined by ATR-FTIR and DLS for each of the two background electrolyte compositions (NaCl and CaCl$_2$), resulting in a total of 18 different aqueous chemistry conditions. To investigate threshold spectral behavior resulting from changes in cation composition, an additional sample set was prepared for infrared studies at 10 mmol L$^{-1}$ ionic strength and pH 6 with incremental variation in the aqueous phase charge fraction of Na$^+$ versus Ca$^{2+}$.

3.2. ATR-FTIR Spectroscopy and Analysis

FTIR spectra were collected using a Nicolet 560 Magna IR spectrometer (Madison, WI). A 1 mL aliquot of LPS solution (4 mg mL$^{-1}$ representing all solution chemistries discussed above) was deposited on a 45° ZnSe IRE (Spectra-Tech ARK ATR cell). Spectra were collected beginning at 0, 15, 30, 60, and 120 min after introduction of LPS solution into the ATR cell. All FTIR spectra were collected with 400 scans at a 4 cm$^{-1}$ resolution (collection time: 495 s) using the corresponding LPS-free electrolyte solution as background. Peak locations were verified via second derivative analysis and peak areas were determined via curve fitting using Grams/AI software (Salem, NH). Spectral areas of Gaussian/Lorentzian fitted peaks or peak intensities were used to quantify the effect of solution chemistry on LPS conformation and aggregation.

3.3. Dynamic Light Scattering Measurements

All laboratory materials were carefully cleaned with detergent, 10% HCl, ethanol, and then rinsed extensively with BNP water prior to experiments to eliminate interfering
dust particles and lipid materials from solutions and surfaces. Samples were prepared at an LPS concentration of 1 mg mL$^{-1}$ and dilutions were made for 0.5 and 0.25 mg mL$^{-1}$ samples. All samples were syringe filtered (Pall Acrodisc 13 mm syringe filter with 0.8 µm Supor Membrane) directly into light-scattering cuvettes to remove any dust particles that may have been present in the LPS solution (Santos et al., 2003).

DLS measurements were carried out using a Brookhaven Instruments goniometer (BI-200) with a BI-8000AT digital correlator and a BI-MDO main detector (Holtsville, NY). Light scattering data were collected at 24° C for 5 min using 15 channels at a scattering angle of 90° relative to the incident beam. Results were processed using the CONTIN (B.I.C. ver 2.0) program to determine z-average $D_H$ and size distribution. Polydispersity ($p$) was calculated using the following formula:

$$p = \frac{<D_z^2> - <D_z>^2}{<D_z>^2}$$

[3]

where $D_z$ represents the z-average diffusion coefficient and $<>$ indicate mean values. Samples are considered monodisperse if $p$ is less than or equal to 0.02.(1982)

4. Results

4.1. Critical Aggregate Concentration Measurements

Excellent agreement between EC and UV-Vis methods was observed for P. aeruginosa ser 10 LPS CAC measurements. EC measurements in BNP water yielded a CAC of 12.7 mg L$^{-1}$ with a standard deviation of 0.47. Corresponding UV-Vis determination resulted in a CAC value of 13.2 mg L$^{-1}$ with a standard deviation of 0.88.
These values ensure that all DLS and ATR-FTIR experiments were performed above the CAC.

4.2. ATR-FTIR Spectroscopy

ATR-FTIR spectra show absorbances corresponding to vibrational modes of distinct ser 10 LPS moieties (Table 1). The lipid A is represented by stretching vibrations of C-H (2820 to 2940 cm\(^{-1}\); 1460 to 1470 cm\(^{-1}\)) and phosphate (1200 to 1265 cm\(^{-1}\), 1106 cm\(^{-1}\), and 960 to 983 cm\(^{-1}\)). The C-O-C stretching of polysaccharides on the O-antigen occurs at 1050-1085 cm\(^{-1}\). Spectral data between 2700 and 1500 cm\(^{-1}\) have been removed from all figures; this region is devoid of useful information and its deletion permits an expanded view of more important data.

### Table 1. Pertinent IR assignments for LPS

<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>IR Band Assignment</th>
<th>Assignment Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>2848-2854</td>
<td>(\nu_{as}(\text{CH}_3))(^{\delta,a})</td>
<td></td>
</tr>
<tr>
<td>2915-2923</td>
<td>(\nu_{as}(\text{CH}_2))(^{\beta,a,b})</td>
<td></td>
</tr>
<tr>
<td>1460-1470</td>
<td>(\delta(\text{CH}_2))(^{\delta,c})</td>
<td></td>
</tr>
<tr>
<td>1250-1265</td>
<td>Metal-complexed/dehydrated (\nu_{as}(\text{PO}_2))(^{c-f})</td>
<td></td>
</tr>
<tr>
<td>1230-1245</td>
<td>Metal-complexed/hydrated (\nu_{as}(\text{PO}_2))(^{c-f})</td>
<td></td>
</tr>
<tr>
<td>1200-1225</td>
<td>Hydrated (\nu_{as}(\text{PO}_2))(^{c-f})</td>
<td></td>
</tr>
<tr>
<td>1106</td>
<td>(\nu(\text{PO}_2))(^{c,d})</td>
<td></td>
</tr>
<tr>
<td>1020, 1050-1085</td>
<td>(\nu(\text{C-O, C-O-C}))(^{a,g})</td>
<td></td>
</tr>
<tr>
<td>960-983</td>
<td>(\nu(\text{PO}_2))(^{h})</td>
<td></td>
</tr>
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</table>

\(^{\delta}\): \(\nu_{as}\) = asymmetric stretching vibration, \(^{\beta}\): \(\nu_s\) = symmetric stretching vibration, \(^{\delta}\): \(\delta\) = bending vibrations.

The type of background cation (Na⁺ versus Ca²⁺) strongly affects ATR-FTIR results (Fig. 2 and 3). Relative to Na-LPS, spectra for Ca-LPS exhibit several sharper and more intense absorbances in the region 1500 to 900 cm⁻¹, and they also show significant changes in relative intensity of various bands. When LPS are immersed in a NaCl background, ATR-FTIR spectra show strong pH dependence, and a smaller effect of I (Fig. 2). The distinct peak at 1060 cm⁻¹ (Fig. 2a) indicates a greater relative contribution of polysaccharides (O-antigen; C-O, C-O-C) at pH 3 relative to pH 6 and 9. Na-LPS spectra also show a relative increase in fatty acid (CH₂) and phosphate (PO₄⁻) group absorbances of the lipid A with increased I. Other notable effects include resolution of a triplet band (1263, 1228, 1207 cm⁻¹) in the phosphate (PO₄⁻) region of the spectrum at pH 9 in 100 mmol L⁻¹ NaCl (Fig. 2c).
Figure 2. ATR-FTIR spectra of *P. aeruginosa* ser 10 LPS in 1, 10, 100 mmol L⁻¹ NaCl at: a) pH 3, b) pH 6, and c) pH 9 (spectra collected at 120 min).
Figure 3. ATR-FTIR spectra of *P. aeruginosa* ser 10 LPS in 1, 10, 100 mmol L$^{-1}$ CaCl$_2$ at: a) pH 3, b) pH 6, and c) pH 9 (spectra collected at 120 min).
The solubility of LPS in CaCl₂ solution decreased at high I and low pH. White precipitates were formed in 100 mmol L⁻¹ I CaCl₂ at pH 3 and, to a lesser degree, at pH 6. Strong PO₂⁻ (1243, 1213, 1106, 964 cm⁻¹) and CH₂ vibrations (~2920, ~2852, 1471 cm⁻¹) arising from lipid A are present in all Ca-LPS spectra (Fig. 3). Contributions of the O-antigen are relatively diminished, with only small peaks observed at 1085, 1060, 1018, and 983 cm⁻¹. Exceptions are 100 mmol L⁻¹ I CaCl₂ (pH 3 and 6) samples where broad bands with peaks at 1085 and 1060 cm⁻¹ that emerged (Fig. 3a, 3b) coincident with the precipitates, are indicative of O-antigen polysaccharides. In contrast, spectra for 1 mmol L⁻¹ CaCl₂ (pH 6 and 9) show a large contribution from ν₁as(CH₂) (2920 and 2852 cm⁻¹) with much lower absorbances from ν₁as(PO₂⁻) and ν(C-O, C-O-C) (Fig. 3b and 3c).

Representative peak-fitting results are shown in Figure 4, with peak locations selected on the basis of known vibrations. Peak area ratios were determined by integrating the fatty acid region (2980-2800 cm⁻¹), and by summing the integrated fits for PO₂⁻ (1200-1265 cm⁻¹) or polysaccharide (1085-1020 cm⁻¹). The larger contribution of CH₂ and PO₂⁻ (lipid A) groups in spectra for Ca-LPS relative to those for Na-LPS is illustrated by the IR peak area ratios depicted in Figure 5. These ratios also show a general trend of increasing lipid A [ν(CH₂, CH₃)] relative to polysaccharide, with increasing pH and I for NaCl solutions (Fig. 5a), and with increasing pH for CaCl₂ solutions (Fig. 5c). The ratio of PO₂⁻ to polysaccharide for Ca-LPS (Fig. 5d) increases with increasing pH for 10 and 100 mmol L⁻¹ I samples, but decreases from pH 3 to pH 6 and 9 for the 1 mmol L⁻¹ I solution.
Figure 4. ATR-FTIR spectra for *P. aeruginosa* ser 10 LPS in 10 mmol L\(^{-1}\), pH 6 solution for a) NaCl and b) CaCl\(_2\) (collected at 120 min). The gray lines indicate Gaussian fits to spectra and dotted lines represent data fits based on peak deconvolution.
Maximum absorbance values for Ca-LPS were significantly higher than for Na-LPS. For example, at $I = 10$ mmol L$^{-1}$, pH 6 and equivalent LPS concentrations, absorbance at 2920 cm$^{-1}$ was 0.010 for Na and 0.082 for Ca, at 1240 cm$^{-1}$ absorbance was 0.007 for Na and 0.213 for Ca, and at 1060 cm$^{-1}$ absorbance was 0.008 for Na and 0.019 for Ca. In addition, Ca-LPS spectra showed greater time-dependency than did Na-LPS spectra. Absorbance values for Na-LPS spectra are constant from 0 to 120 min (Fig. 6a) whereas the Ca-LPS absorbance values increased with time (Fig. 6b).

**Figure 5.** IR peak area ratios for fatty acid:polysaccharide and phosphate: polysaccharide of *P. aeruginosa* ser 10 LPS as a function of pH and ionic strength in NaCl (a,b) and CaCl$_2$ (c,d) (spectra collected at 120 min).
Variation in the charge fraction of Ca (E_{Ca}, defined as the moles of Ca charge normalized to the total moles of cation charge) in CaCl_2/NaCl solutions at pH 6 and at constant I (10 mmol L^{-1}) shows increasing IR absorbance values with increasing charge fraction of Ca when spectra are plotted on a common scale (Fig. 7a). Spectral changes are apparent when data are plotted on non-common scale to maximize absorbance values for all spectra (Fig. 7b). The latter clearly shows the evolution from a broad phosphate peak (1248 to 1204 cm\(^{-1}\)) when Na\(^+\) is the predominant cation (E_{Ca} \leq 0.07) to a doublet (1243 and 1213 cm\(^{-1}\)) when Ca\(^{2+}\) is present (E_{Ca} \geq 0.08). Increasing charge fraction of Ca\(^{2+}\)
leads to greater peak separation and intensity, and distinct peaks are observed at $E_{Ca}$ values as low as 0.08.

**Figure 7.** ATR-FTIR spectra (collected at 120 min) for *P. aeruginosa* ser 10 LPS at pH 6, 10 mmol L$^{-1}$ ionic strength and variable charge fraction ($E_{Ca}$) of Ca relative to Na: a) y-axis (absorbance) constant for all spectra (common scale), and b) y-axis manipulated to show major peaks in spectra (non-common scale).
4.3. Dynamic Light Scattering

Particle size distributions and z-average hydrodynamic diameters ($DH$) were calculated using the CONTIN method (Provencher, 1982a; Provencher, 1982b; Santos et al., 2003). Figure 8 shows representative particle size distributions for Na-LPS (Fig. 8a) and Ca-LPS (Fig. 8b). All samples were polydisperse, but greater polydispersity ($p$) values were obtained for Ca-LPS samples. For example, at 10 mmol L$^{-1}$ $I$, pH 6 and 1 mg LPS mL$^{-1}$, $p_{Na-LPS}$ was 0.01 (±0.09) and $p_{Ca-LPS}$ was 0.28 (±0.18). The DH values of LPS aggregates showed little dependence on LPS concentration (0.25, 0.5, 1 mg mL$^{-1}$ LPS, data not shown). Therefore, mean values of DH based on four replicated measurements at LPS concentrations of 1 mg mL$^{-1}$ (closest to the concentrations used in the FTIR studies) are plotted for Na–LPS and Ca-LPS as a function of pH and $I$ in Fig. 9a and b, respectively. Due to diminished solubility of LPS in 100 mmol L$^{-1}$ $I$ CaCl$_2$ at pH 3, only one replicate is shown for this case, and the precise LPS concentration after filtration for that sample is not known. Overall, the data show a decreased size of LPS aggregates with increased $I$. The only exception to this trend is for Na-LPS at pH 9, where mean DH values are statistically equivalent, irrespective of $I$. Careful inspection of Fig. 9 indicates that DH values of Ca-LPS aggregates are smaller than those of Na-LPS at pH 3, but they show a much steeper increase in size with increasing pH to 6, particularly at 1 and 10 mmol L$^{-1}$ $I$.  


Figure 8. Representative particle size distributions for *P. aeruginosa* ser 10 LPS (10 mmol L\(^{-1}\), pH 6) aggregates as measured using dynamic light scattering (CONTIN method, 90°) in (a) NaCl and (b) CaCl\(_2\) solutions (\(p^\infty\) polydispersity calculated according to Eq. 3).
5. Discussion

The results suggest that solution-chemistry-induced changes in conformational properties of smooth LPS are intermediate between those reported for rough LPS (Coughlin et al., 1985; Din et al., 1993; Seydel et al., 1993; Obst et al., 1997) and those expected for acidic polyelectrolytes (Schneider and Doty, 1954; Doty et al., 1957; Skolnick and Fixman, 1977; Barrat and Joanny, 1996; Ito, 1998) – presumably because of the prevalence of O-antigen saccharides. The aggregation of LPS monomers is driven by the hydrophobic effect whereby the favorable energetics of H-bonded water promotes the

Figure 9. Average particle diameter measured by dynamic light scattering for 1 mg mL\(^{-1}\) \textit{P. aeruginosa} ser 10 LPS at pH 3, pH 6, pH 9 in a) 10 mmol L\(^{-1}\) NaCl and b) 10 mmol L\(^{-1}\) CaCl\(_2\). Error bars represent standard deviation with \(n \geq 4\).
exclusion non-polar moieties and the coalescence of the amphiphilic molecules (Tanford, 1973). During aggregation, LPS molecules are expected to internalize the hydrophobic lipid A because contact between the non-polar lipid tail and water molecules is minimized while hydration of the O-antigen is promoted (Seydel et al., 1993; Aurell and Wistrom, 1998; Jucker et al., 1998; Santos et al., 2003; Stoica et al., 2003). Counter ion (e.g., Na\(^+\), Ca\(^{2+}\))-dipole and H-bonding at O-antigen saccharide groups should also contribute to aggregate stability. H-bonding between LPS monomers of ionizable groups of the KDO (ethanolamine and carboxyl groups) or phosphate groups of the lipid A are believed to stabilize rough LPS aggregates in NaCl (Coughlin et al., 1985), whereas the formation of cation-phosphate bonds (e.g., in CaCl\(_2\)) could potentially disrupt aggregate structure (Wang et al., 2005). Steric and electrostatic repulsion between the saccharide groups must be overcome to promote aggregation. Thus, increasing \(I\) or decreasing pH are expected to facilitate this process.

5.1. Effect of solution chemistry on Na-LPS conformation

With increasing pH and \(I\), Na-LPS spectra show increased relative band intensities for \(\nu(CH_2, CH_3)\) compared to \(\nu(C-O, C-O-C)\) (Fig. 2, 5a, 5b), indicating a progressively larger relative contribution from the lipid A moiety. One possible explanation is that increasing \(I\) resulted in an increase in accumulation of LPS monomers and, therefore, in lipid moieties bonded at the IRE interface. However, the CAC of lipids generally decreases with increasing \(I\) (Elworthy et al., 1968; Tanford, 1973; Lindman and Wennerström, 1980), which should result in a lower concentration of free monomers in
solution. Thus, the negative effect of $I$ on CAC would be expected to result in an increase in the $\nu(CH_2, CH_3)$ at lower $I$, whereas the opposite is observed here. In addition, no time-dependent changes in Na-LPS spectra were observed (as might be expected for macromolecular bonding to the IRE). Alternatively, increasing pH and $I$ both contribute to proton dissociation of weakly acidic hydroxyls on saccharides and this may diminish IR absorbance in the polysaccharide region (Omoike and Chorover, 2004).

The DLS data are consistent with increased coiling of the O-antigen at high $I$ and low pH (Fig 9a). The O-antigen exhibits greater conformational lability than the lipid A and core, and is most susceptible to conformational change (Kastowsky et al., 1992; Seydel et al., 1993), thus it is most likely responsible for the observed decrease in aggregate size. With increasing pH from 3 to 9, the effect of Na$^+$ concentration on aggregate size is diminished relative to the case for Ca$^{2+}$, presumably because the monovalent ion is less effective at LPS charge attenuation (Figure 9).

5.2. Effect of solution chemistry on Ca-LPS conformation

Na-LPS ATR-FTIR data were unchanged with time (Fig. 6a), suggesting that LPS structures pre-equilibrated in NaCl solution are not affected by introduction to the IRE. Conversely, Ca-LPS results showed time-dependent changes that were indicative of conformational restructuring in the ATR cell (Fig. 6b). Indeed, the integrated ratios of peaks assigned to $\nu_{as}(CH2)$ or $\nu_{as}(PO4-)$ to $\nu(C-O, C-O-C)$ increased with time for Ca-LPS samples, while remaining constant for Na-LPS (Fig. 10). Time-dependent changes in conformation of the lipid A itself are also evident when Ca$^{2+}$ is present. Examination
of the phosphate to fatty acid absorbance ratio for 10 mmol L\(^{-1}\) CaCl\(_2\) (Fig. 11) shows a progressive increase in the contribution of \(\nu_{as}(\text{PO}_4^-)\), relative to \(\nu(\text{CH}_2, \text{CH}_3)\) up to ca. 3000 s and then, although spectral areas of both functional groups are still increasing with time, their respective rates of increase are equivalent so that their absorbance ratio is unchanged. The ratio of Ca\(^{2+}\)-bound to hydrated PO\(^2^-\) generally increased with increasing Ca\(^{2+}\) concentration (Table 2). The only exception to this trend was the 100 mmol L\(^{-1}\) CaCl\(_2\) sample at pH 3, which is also the sample that showed limited solubility.

**Figure 10.** ATR-FTIR spectral kinetics: Absorbance ratios for *P. aeruginosa* ser 10 LPS in 10 mmol L\(^{-1}\), pH 6 solution (NaCl and CaCl\(_2\)).
Table 2. FTIR absorbance ratio of Ca$^{2+}$-$\nu_{as}(PO_2^-)$: H$_2$O-$\nu_{as}(PO_2^-)$ for *P. aeruginosa* ser 10 LPS in CaCl$_2$ (data collection at 2 h).

<table>
<thead>
<tr>
<th></th>
<th>pH 3</th>
<th>pH 6</th>
<th>pH 9</th>
</tr>
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<tbody>
<tr>
<td>1 mM CaCl$_2$</td>
<td>0.90</td>
<td>0.90</td>
<td>0.99</td>
</tr>
<tr>
<td>10 mM CaCl$_2$</td>
<td>1.00</td>
<td>0.98</td>
<td>1.06</td>
</tr>
<tr>
<td>100 mM CaCl$_2$</td>
<td>0.91$^\dagger$</td>
<td>1.10$^\dagger$</td>
<td>1.09</td>
</tr>
</tbody>
</table>

$^\dagger$ Low solubility samples, may not be representative.

Since the beam penetration depth exceeds the size of LPS monomers, the relative intensity increase for CH$_2$ and PO$_2^-$ groups of the lipid A (Fig. 10) can not be attributed only to their preferential accumulation in proximity to the IRE; a concurrent increase in absorbance for O-antigen functionalities should also result. However, the results do suggest that LPS micelles/tubes are destabilized in the presence of Ca$^{2+}$ (relative to Na-LPS), which is in agreement with previously published studies investigating aggregation of rough LPS (Coughlin et al., 1983). In a molecular modeling study, Obst et al. (1997) reported that Ca$^{2+}$ ions bind to both phosphate and carboxyl groups of rough LPS to form a stable bidentate complex. This fixes the distance between the ionized groups, stabilizes
and rigidifies the head group (inner core and glucosamine backbone), and changes the conformation of the glucosamine backbone of the lipid A (Coughlin et al., 1985; Seydel et al., 1993; Obst et al., 1997).

Our current interpretation of the Ca-LPS ATR results is that after deposition on the IRE, LPS aggregates disassemble such that the lipid A re-orients on the IRE surface. This may be facilitated by lipid A adsorption to the ZnSe IRE either by hydrophobic interaction or, more likely, by Ca$^{2+}$ bridging between the negatively-charged ZnSe IRE surface and PO$_2^-$/COO$^-$ groups. Since the isoelectric point for ZnSe is pH < 4 (Tickanen et al., 1997), the IRE is negatively-charged at pH 6 and 9, which would favor cation bridging interactions. Wang (2005) suggested that LPS aggregates are re-organized in the presence of a Ca silicate surface to form two-dimensional micelles anchored at the interface by Ca$^{2+}$ binding to phosphate groups. In this scenario, Ca-LPS molecules exhibit conformational change giving rise to their adsorption to the IRE surface on the time scales of minutes to hours (Fig. 10-11).

The relative hydrodynamic diameters of LPS aggregates, for Na-LPS and Ca-LPS samples, are similar in size. However, unlike Na-LPS results, the size of Ca-LPS aggregates was affected by changes in $I$ even at high pH (Fig. 9). At pH 9, most ionizable groups on the O-antigen are deprotonated. Under these conditions Ca$^{2+}$ can bind to one or two ionized groups on O-antigen chains, perhaps bridging and coalescing LPS monomers, or binding to deprotonated sites on the same chain, causing it to coil. Monovalent Na$^+$ exhibits lower affinity for weakly acidic functional groups, which is likely the reason for its smaller effect on aggregate hydrodynamic diameter at pH 9.
5.3. Phosphate group hydration

Our results suggest that phosphate groups of the lipid A (Fig. 1b) play a central role in LPS conformation and aggregation in CaCl₂ solution. Calcium (along with Mg²⁺) has been shown to have a strong affinity for lipid A phosphates (Schindler and Osborn, 1979; Coughlin et al., 1981). Molecular dynamics simulations have revealed that Ca²⁺ ions are very strongly bound to the negatively charged head groups (PO₂⁻ and COO⁻) of rough LPS (Obst et al., 1997). We suggest that key differences in behavior of Na- and Ca-LPS can be attributed to the stronger binding of Ca²⁺ with PO₂⁻.

Binding of metal cations can lead to diminished hydration of PO₂⁻, (Brandenburg et al. 1997) and this is reflected in FTIR spectra. PO₂⁻ vibrations are observed in the region from 1200-1265 cm⁻¹, with specific frequencies correlating inversely with the degree of functional group hydration (Fringeli and Günthard, 1981). Highly hydrated PO₂⁻ groups give rise to peaks at 1200-1225 cm⁻¹, whereas moderately hydrated and non-hydrated groups produce peaks at 1230-1245 and 1250-1265 cm⁻¹, respectively (Brandenburg et al., 1997). Brandenburg et al. (1997) showed PO₂⁻ frequency shifts to higher wavenumber resulted from Mg²⁺ binding to PO₂⁻, which was thought to result in displacement of solvation waters. In the present work, binding of Ca²⁺ to phosphate groups results in the formation of a strong doublet peak (Fig. 3). Relative to Na-LPS (Fig. 2), Ca-LPS show increased absorbance in the PO₂⁻ region and reduction in the signal of moderately-hydrated phosphate. This implies that binding of Ca²⁺ to phosphate groups displaces hydration waters and results in the strong band at 1243 cm⁻¹. PO₂⁻ groups that are not bonded to Ca²⁺ remain hydrated and give rise to the absorbance at 1213 cm⁻¹. This
strong binding of Ca$^{2+}$ to the lipid A region is expected to have a substantial effect on conformation of LPS monomers and aggregates. Spectra of Na/Ca-LPS with varying Ca$^{2+}$ charge fraction in solution reveal its strong influence LPS aggregation/conformation (Fig. 7). Figure 12 shows the ratio of LPS PO$_2^-$ to saccharide groups as a function of $E_{Ca}$. With $E_{Ca}$ values greater than 0.18 there is a large increase in spectral contributions from PO$_2^-$. Although the ratio is sensitively dependent on $E_{Ca}$ even at low values, the lack of a plateau in Figure 12 indicates that competition from Na$^+$ for LPS binding sites persists to $E_{Ca}$ values up to at least 0.40 (equimolar contributions of Ca$^{2+}$ and Na$^+$).

Our data are complementary to those of Coughlin et al. (1983), who determined that equimolar Ca$^{2+}$ converted Na-LPS tubes into bilayers. Variation in the contribution of Na$^+$ and Ca$^{2+}$ to background electrolyte at constant $I$ (10 mmol L$^{-1}$) and pH 6 reveals that the emergent impacts of Ca-PO$_2$ bonding on ATR-FTIR spectra occurs even at low charge fraction of the bivalent ion (Fig. 7). Increased charge fraction of Ca$^{2+}$ leads to progressive reorientation of LPS (CH$_2$ and PO$_2^-$ absorbances increase) (Fig. 7a) and separation of the broad phosphate peak into two sharp absorbances assigned to hydrated (1213 cm$^{-1}$) and Ca-bound (1243 cm$^{-1}$) PO$_2^-$ (Fig. 12). The persistence of hydrated PO$_2^-$ (1213 cm$^{-1}$), even at high $E_{Ca}$ (Fig. 12), results from increased residence time of water molecules (due to the presence of Ca$^{2+}$) in the inner core and glucosamine backbone of the molecule (Obst et al., 1997). This indicates incomplete shielding of the Ca$^{2+}$ ions, required for intermolecular binding via bivalent cations (Obst et al., 1997). However, as Ca$^{2+}$ concentrations are increased there is an increase in the ratio of Ca$^{2+}$-bound to
hydrated phosphate (Table 2 and Fig. 12), indicating removal of H$_2$O and increased Ca$^{2+}$ binding.

Figure 12. IR absorbance ratio of phosphate to fatty acid versus charge fraction of Ca (E$_{Ca}$) in pH 6, $I$ of 10 mmol L$^{-1}$ (varying contributions of NaCl and CaCl$_2$) *Pseudomonas aeruginosa* ser 10 LPS samples.

6. Conclusions

Changes in LPS aggregation as a function of solution chemistry were observed using ATR-FTIR spectroscopy and DLS. Overall, aggregate size was smallest at high $I$ (100 mmol L$^{-1}$) and low pH, for both NaCl and CaCl$_2$ solutions. ATR-FTIR spectra reveal strong effects of counterion composition on the conformational properties of LPS in proximity to a ZnSe IRE. Increased spectral contributions from the lipid A are observed for Ca- relative to Na-LPS, and these effects become increasingly apparent with time (up to 2 h) that Ca-LPS is in the presence of the ZnSe surface. We propose that LPS less stable in the presence of Ca$^{2+}$, as the bivalent ion forms strong complexes with phosphate groups of the lipid A. This complex formation apparently results in a reorientation of LPS at the negatively-charged ZnSe surface and this also enhances
absorbance of lipid A functionalities within the region probed by the incident IR beam. However, the specific orientation of LPS molecules in the bulk solution or at the IRE interface cannot be determined via techniques employed in this study. The results indicate that bivalent ions strongly affect the conformational properties of LPS, even when they are present at relatively low charge fraction in aqueous solution.

Acknowledgements

We are grateful to David A. White II for performing the CAC measurements. We also thank Dr. Craig A. Aspinwall, Assistant Professor of Chemistry at The University of Arizona, for use of light scattering instrumentation housed within his laboratory. This research was supported by the National Science Foundation CRAEMS program (Grant CHE-0089156).

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APPENDIX D

ATR-FTIR STUDY OF PSEUDOMONAS AERUGINOSA
LIPOPOLYSACCHARIDE-SURFACE INTERACTIONS

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Abstract

Cation valence influences lipopolysaccharide (LPS) aggregate stability and surface interactions. The presence of Ca$^{2+}$ (vs. Na$^+$) in LPS solutions leads to aggregate reorientation and increased sorptive reaction with ZnSe, GeO$_2$, $\alpha$-Fe$_2$O$_3$, $\alpha$-FeOOH, and $\alpha$-Al$_2$O$_3$. ATR-FTIR spectra of Na-LPS systems demonstrate reduced surface activity and are representative of solution phase LPS. Ca-LPS spectra reveal that hydrophobic interactions with the lipid A region occur on a ZnSe IRE. However, for hydrophilic surfaces (GeO$_2$, $\alpha$-Fe$_2$O$_3$, and $\alpha$-Al$_2$O$_3$) pH-dependent charge controls Ca-LPS interactions that are mediated principally via O-antigen-surface reaction. As a result of accumulation at the solid-liquid interface, spectra of Ca-LPS represent primarily surface-bound LPS. Variable-angle ATR-FTIR spectra of Ca-LPS systems show depth-dependent trends occurring at the spatial scale of LPS aggregates.

1. Introduction

The interactions between free- and membrane-bound lipopolysaccharides (LPS) with environmental surfaces contribute to bacterial adhesion (Sutherland, 1985; Makin and Beveridge, 1996; Jucker et al., 1997; Jucker et al., 1998a). LPS are amphiphilic molecules with a hydrophobic lipid A region embedded in the outer membrane of Gram negative bacteria (Seltmann and Holst, 2002). Beyond the lipid A is a core sugar region, and the O-antigen. The portion of the molecule comprising the O-antigen is present in “smooth” LPS, whereas it is absent from “rough” LPS. The O-antigen is hydrophilic and extends outward from the intact cell into aqueous solution (See Appendix C, Fig. 1). It is
is composed of 20 to 70 repeating units of three to five sugars (Seltmann and Holst, 2002). Some bacteria, such as *Pseudomonas aeruginosa*, possess LPS with O-antigens extending up to 40 nm from the cell surface (Lam et al., 1992). Since cell turnover and lysis results in the presence of both “cell-bound” and “free” LPS in natural aquatic systems (Rietschel et al., 1994), LPS may promote bacterial adhesion by sorption of free LPS molecules to surfaces during conditioning film formation, or through cell adhesion mediated by membrane bound LPS (Makin and Beveridge, 1996; Jucker et al., 1997). It has been suggested that during cell adhesion to negatively-charged surfaces, the O-antigen may extend beyond the electrostatic energy barrier and become adsorbed in a secondary minimum in close proximity to the surface (Jucker et al., 1997).

Adhesion of both rough and smooth LPS has been observed to occur on metal oxide surfaces (Jucker et al., 1998a; Jucker et al., 1998b), crystalline calcium silicate hydrate (Wang et al., 2005; Zhang et al., 2005), GeO₂ crystal, positively charged lipids and polymers (Reiter et al., 2002), and to bovine lung and tracheal tissue samples (Paradis et al., 1994). Adhesion of *P. aeruginosa* ser 10 LPS (in ultrapure water) bound more strongly to a positively charged surface (aminopropyltriethoxysilane polymers) than to hydrophilic (GeO₂) or hydrophobic (dipalmitoylphosphatidic acid monlayer) surfaces (Reiter et al., 2002).

Adsorption of free LPS to surfaces may be mediated by functional groups associated with either hydrophilic or hydrophobic portions of the molecule. However, in free LPS, exposure of the lipid A is limited by LPS amphiphilic properties that promote intermolecular associations and the formation of supramolecular structures above a
critical aggregation concentration (CAC) (Seydel et al., 1993; Aurell and Wistrom, 1998; Santos et al., 2003). Dynamic light scattering measurements indicate that LPS aggregate sizes (4 mg mL\(^{-1}\) LPS, \(I\) of 10 mM, pH 6) range from 325 to 400 nm for Na-LPS and from 400 to 475 nm for Ca-LPS (Appendix C). LPS aggregates have been used above the critical aggregate concentration (CAC) to represent cell-bound forms under the assumption that only the O-antigen is exposed for interaction with environmental surfaces. For example, Jucker et al. (1998a) measured the adsorption of phosphate-buffered LPS aggregates at various ionic strengths (NaCl, KH\(_2\)PO\(_4\), K\(_2\)HPO\(_4\)) to TiO\(_2\), Al\(_2\)O\(_3\), and SiO\(_2\). The results reveal much greater adhesion to TiO\(_2\) and Al\(_2\)O\(_3\) surfaces. In some cases irreversible adhesion was observed, particularly for LPS with long O-antigens regions. However, the possible surface interactions of monomeric LPS (in thermodynamic equilibrium with aggregates) and/or the potential restructuring of LPS aggregates that may occur upon association with a surface were not investigated.

Adhesion to environmental surfaces of membrane-bound LPS is primarily controlled via the O-antigen region. The adsorption of three different O-antigens was investigated to elucidate information on LPS adhesion (Jucker et al., 1997). O-antigens from *Escherichia coli* and *Citrobacter freundii* had high affinity for TiO\(_2\) and low affinity for Al\(_2\)O\(_3\). Adsorption of O-antigen from *Stenotrophomonas maltophilia* was low for both surfaces. All O-antigens exhibited low sorption affinity for SiO\(_2\). The sorption of *E. coli* and *C. freundii* O-antigen to TiO\(_2\) and Al\(_2\)O\(_3\) was irreversible, whereas the sorption of *S. maltophilia* O-antigens was partially reversible (Jucker et al., 1997). Differences in O-antigen length were responsible for variation in adhesion. The study also demonstrated
that the reversibility of dextran (surrogate for O-antigens of varying lengths) adhesion decreased with increasing molecular weight (Jucker et al., 1997), suggesting that adhesion of bacteria with smooth LPS would exceed that of cells with rough LPS.

*P. aeruginosa* (PAO1) adhesion is affected by the O-antigen length. Cells with primarily long O-antigen preferentially adhere to hydrophilic surfaces and cells with shorter O-antigen have a higher affinity for hydrophobic surfaces (Makin and Beveridge, 1996). Thus, the ability of a cell to mediate O-antigen length might confer a capacity to influence adhesion in dynamic environments. However, adhesion of free-LPS to surfaces may be quite different, particularly if LPS aggregates are disrupted and interaction between the lipid A region and a substratum is favorable.

Although LPS has been shown to contribute to cell adhesion, instances where adhesion is diminished by LPS may also occur. Partial LPS removal from *Thiobacillus ferrooxidans* led to an increase in adhesion (50%) to hydrophobic sulfur prills (Arredondo et al., 1994), a result that was attributed to increased protein exposure and cell surface hydrophobicity. Bacterial adhesion is a function of cell surface composition (e.g., LPS, EPS, teichoic acids, surface proteins, flagella), substratum surface chemistry (e.g., hydrophobicity, surface charge), environmental conditions (e.g., pH, I), and conditioning film formation. No single controlling factor facilitates bacterial adhesion, and therefore each individual component should be investigated under a variety of conditions. This study seeks to investigate the interactions of free LPS with surfaces, contributing to increased understanding of cell adhesion.
Like bacterial adhesion, the interaction of free LPS with surfaces is influenced by aggregate structure, substratum surface chemistry, and solution chemistry. Whereas previous work in this thesis examined LPS-solution interactions (Appendix C), this study focuses on the interactions of Na-LPS and Ca-LPS with various surfaces. It has been suggested that the presence of Ca$^{2+}$ (vs. Na$^+$) increases LPS aggregation (Schindler and Osborn, 1979; Naumann et al., 1989; Li and Luo, 1998; Li and Luo, 1999). This may result from the strong affinity between Ca$^{2+}$ and the phosphate groups in the lipid A region (Coughlin et al., 1985; Naumann et al., 1989; Seydel et al., 1993; Obst et al., 1997). Our previous results reveal a strong influence of cation composition on the attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectra of LPS samples (Appendix C). Spectra show increased phosphate and fatty acid contributions (relative to carbohydrate) for Ca-LPS samples. From these results we postulate that LPS aggregates in the presence of Ca$^{2+}$ are, in fact, disrupted as the cation complexes with phosphate groups in the lipid A region. A similar suggestion was put forth by Wang et al. (2005). Their studies suggested that Ca$^{2+}$ may disrupt LPS aggregates causing reorientation on calcium silicate hydrate (Wang et al., 2005).

### 2. Research Approach

Using attenuated total reflectance ATR-FTIR spectroscopy we probed the interactions of Na-LPS and Ca-LPS ($I = 10$ mmol L$^{-1}$, pH 3, 6, 9) on ZnSe, GeO$_2$, $\alpha$-Fe$_2$O$_3$, and $\alpha$-Al$_2$O$_3$ surfaces. Detailed descriptions of ATR-FTIR spectroscopy and its relevant applications can be found in Appendix H.
ATR-FTIR spectra represent infrared-absorbing moieties at the liquid-IRE interface. An evanescent wave propagates ca. 10^2-10^3 nm (depending on crystal type, incident beam angle, and wavelength) beyond the interface of the IRE and into an aqueous suspension. Beam penetration depth ($dp$) varies according to Eq. 1:

$$d_p = \frac{\lambda}{2\pi \left( \sin^2 \theta \right) - \left( \frac{n_1}{n_2} \right)^2}^{1/2}$$

[1] where $\lambda$ is wavelength (cm) of incident radiation, $n_1$ and $n_2$ are the refractive indices (RI) for the IRE and sample, respectively, and $\theta_{eff}$ is the effective angle of incidence (Mirabella, 1985). The RI values for the ZnSe and GeO$_2$ IRE used in the present study are 2.4 ($n_{ZnSe}$) and 4.0 ($n_{Ge}$). The RI range for LPS ($n_{LPS}$) likely falls in the range reported for lipid A (1.50 for pure lipid A; 1.33 with 90% water) (Seydel et al., 2000), bacterial cells (1.38) (Jonasz et al., 1997; Katz et al., 2005), and proteins (1.5) (Chittur, 1998). As a result of the limited wave penetration depth, ATR spectra pertain to the region in close proximity to the sample-IRE interface.

As a result of $\theta_{eff}$-dependence (Eq. 1), variable angle VATR-FTIR permits depth-profiling of samples. By systematically varying $\theta_{eff}$, $dp$ can be varied over length scales varying up to hundreds of nanometers (Table 1), which is comparable to the size of LPS aggregates as measured by dynamic light scattering (Appendix C). In the present study, depth profiling of Na-LPS and Ca-LPS on GeO$_2$ and ZnSe IREs was conducted via variable angle ATR (VATR)-FTIR spectroscopy. For the VATR cell used in this study
(ATRMAX II Variable Angle Horizontal ATR Accessory, PIKE Technologies, Inc.), \( \theta_{\text{eff}} \) was determined according to Eq. 2 (Pereira and Yarwood, 1994):

\[
\theta_{\text{eff}} = \theta_{\text{fix}} - \sin^{-1}\left[ \frac{\sin(\theta_{\text{fix}} - \theta_{\text{var}})}{n_1} \right]
\]

where \( \theta_{\text{fix}} \) is the angle of the crystal face \((45^\circ)\), and \( \theta_{\text{var}} \) is the scale angle set on the VATR accessory. The effect of variation in the \( dp \) as a function of wavenumber, \( \theta_{\text{eff}} \), IRE composition is shown in Table 1.

Batch experiments were conducted to measure the sorption of Na-LPS and Ca-LPS to GeO\(_2\), \( \alpha \)-Al\(_2\)O\(_3\), and \( \alpha \)-FeOOH surfaces. These experiments were conducted to provide complementary macroscopic information on LPS surface affinity, thus improving our interpretation of the ATR-FTIR data.

Based on previous work it is clear that ATR-FTIR spectra do not provide information representative of only solution phase macromolecules. Under certain conditions spectra can provide information on interactions of compounds with the IRE. The objective of this study was to use ATR-FTIR to investigate the interactions of LPS with different surfaces. It was our goal to elucidate the affinity of LPS for these surfaces, and to determine the effect of electrolyte chemistry (NaCl vs. CaCl\(_2\)).
Table 1. Depth of penetration at wavenumbers pertinent to LPS samples for ATR-FTIR with ZnSe (refractive index = 2.4) and GeO₂ (refractive index = 4.0) IRE.

<table>
<thead>
<tr>
<th>Scale Angle (°)</th>
<th>ZnSe Depth of Penetration (nm) for selected wavenumbers (cm⁻¹)</th>
<th>GeO₂ Depth of Penetration (nm) for selected wavenumbers (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Effective Angle (°)</td>
<td>2920</td>
</tr>
<tr>
<td>30</td>
<td>38.8</td>
<td>911</td>
</tr>
<tr>
<td>37</td>
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<td>622</td>
</tr>
<tr>
<td>45</td>
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<td>552</td>
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<td>52</td>
<td>47.1</td>
<td>502</td>
</tr>
<tr>
<td>60</td>
<td>51.2</td>
<td>432</td>
</tr>
</tbody>
</table>

† The refractive index of LPS was assumed to be close to that of bacterial samples, and therefore a value of 1.38 was used (Jonasz et al. 1997; Katz et al. 2003).
3. Experimental Methods

3.1. LPS preparation

A single batch of freeze-dried *Pseudomonas aeruginosa* serotype 10 LPS (batch 123K4144; Sigma Inc.) was used for all experiments. For FTIR experiments, LPS solutions were prepared above the critical aggregate concentration (Appendix G) by dissolving 4.0 mg of freeze-dried LPS in 1.0 g of NaCl or CaCl₂ electrolyte solution that was at an ionic strength \( I \) of 10 mmol L\(^{-1} \) and pH was adjusted to 3, 6, and 9 using 0.01 M HCl or 0.01 M NaOH. Samples were vortexed, sonicated for 10 min, and stored overnight at 4°C prior to re-equilibration the following day at room temperature.

The C content of LPS was determined via total organic carbon analysis (Shimadzu TOC-V CSH TOC/TN analyzer, Columbia MD) to be 33.3%. For batch LPS sorption experiments stock solutions were prepared at 1 mg C mL\(^{-1} \). Freeze dried LPS was dissolved in the appropriate background solution chemistry (1 mmol L\(^{-1} \) NaCl of CaCl₂), vortexed, and sonicated for 10 min. A total of 10 LPS stocks solutions were made to accommodate pre-equilibration of LPS of two ionic compositions (NaCl and CaCl₂) at 5 different pH values (3, 4.5, 6, 7.5, 9). The pH was adjusted using 0.01 M HCl or 0.01 M NaOH. Stock LPS solutions were stored overnight at 4°C prior to re-equilibration the following day to room temperature.

3.2. Metal oxide analysis

GeO₂ powder (100 mesh; Sigma, Inc.) was sent to Micromeritics Analytical Services (Norcross, GA) for multipoint BET (N₂) surface area analysis. Diffuse
reflectance FTIR (DRIFT) spectroscopy was used to determine the presences of (hydr)oxide groups on the Ge surface. For DRIFT, freeze dried $\alpha$-Fe$_2$O$_3$ was diluted with KBr to approximately 10% (w/w) by gently mixing 39 mg of sample with 30 mg of KBr for 40 s, then folding in an additional 390 mg of KBr to homogenize the samples. DRIFT spectra were collected using a Nicolet 560 Magna IR spectrometer (Madison, WI) with 400 scans at 4 cm$^{-1}$ resolution. Characterization of $\alpha$-Al$_2$O$_3$ was provided by the manufacturer (Alfa Aesar; size: 1 µm, surface area: 6-8 m$^2$ g$^{-1}$). X-ray diffraction (XRD) and DRIFT spectroscopy were performed to verify composition of purchased $\alpha$-Al$_2$O$_3$. Diffraction patterns were collected on powdered $\alpha$-Al$_2$O$_3$ using a Scintag XDS 2000 (Scintag, Inc.) with a Cu x-ray source (40 kV and 40 mA), scan speed of 2° 2θ min$^{-1}$, and a step width of 0.03° 2θ. DRIFT spectroscopy was used to confirm goethite synthesis via the methods previously described for GeO$_2$.

Colloidal hematite ($\alpha$-Fe$_2$O$_3$) particles were synthesized based on the method of Schwertmann and Cornell (1991). The procedure required adding 100 mL of 1 mol L$^{-1}$ Fe(NO$_3$)$_3$ dropwise to 1 L of boiling Barnstead nanopure (BNP) H$_2$O over a 4 h period. The solution was allowed to cool overnight at room temperature. Removal of bound NO$_3^-$ from suspension required precipitation with 100 mmol L$^{-1}$ NaCl. After flocculation and settling, the supernatant was removed, and the washing step was repeated four times. The $\alpha$-Fe$_2$O$_3$ was resuspended in 100 mmol L$^{-1}$ BNP water (pH 4) and dialyzed (Spectra/Por 7 1000 MWCO, Spectrum) against BNP water (changed twice daily) (Liger et al., 1999). Dialysis was complete when pH and electrical conductivity (EC) remained constant for a 12 h period (pH 4, EC ~ 110 µs cm$^{-1}$). The concentration of $\alpha$-Fe$_2$O$_3$ in suspension was
determined by freeze-drying an aliquot of the suspension and measuring mass of $\alpha$-Fe$_2$O$_3$ remaining.

XRD was performed to verify composition of synthesized $\alpha$-Fe$_2$O$_3$ via the same methods used for $\alpha$-Al$_2$O$_3$. For transmission electron microscopy (TEM) analysis, samples of $\alpha$-Fe$_2$O$_3$ were mounted on 200 mesh copper grids by placing carbon coated mica into a drop of suspension and floating carbon/$\alpha$-Fe$_2$O$_3$ onto the grid. Samples were observed at 60 kV with a Japanese Electron Optical Laboratories JEM-100CX II electron microscope. DRIFT spectroscopy was used to confirm hematite synthesis via the methods previously described for GeO$_2$.

Goethite ($\alpha$-FeOOH) synthesis, followed the methods of Schwertmann and Cornell (1991). 100 mL of 1 mol L$^{-1}$ Fe(NO$_3$)$_3$ was added to a 2 L polyethylene bottle with rapid stirring. 180 mL of 5 mol L$^{-1}$ KOH were added to the flask. The suspension was immediately brought to a volume of 2 L with BNP water. The bottle was sealed tightly and placed in a 70° C oven for 60 h. The suspension was then centrifuged (10000 RCF, 20 min) and supernatant discarded. The pelleted goethite was washed repeatedly (via centrifugation) with 1 mmol L$^{-1}$ HCl until the supernatant solution was pH 4. The goethite was then washed (via centrifugation) with BNP water until the electrical conductivity was equal to that of pure BNP water. The goethite was freeze-dried for use in sorption experiments. This method produces goethite crystals with a surface area of 20 m$^2$ g$^{-1}$ (Schwertmann and Cornell, 1991). DRIFT spectroscopy was used to confirm goethite synthesis via the methods previously described for GeO$_2$. 
3.3. ATR-FTIR spectroscopy and analysis

FTIR spectra were collected using a Nicolet 560 Magna IR spectrometer (Madison, WI). Spectra of LPS were collected using both a 45° ZnSe and 45° GeO\textsubscript{2} IRE (Spectra-Tech ARK ATR cell). Although marketed as a Ge IRE the surface is likely (hydr)oxylated (as shown via DRIFT spectroscopy of “pure” Ge, Fig. 3) and is therefore referred to as GeO\textsubscript{2} throughout this study. Metal oxide coatings were made by drying the appropriate suspension (6 mL of $\alpha$-Fe\textsubscript{2}O\textsubscript{3}, 1.96 g L\textsuperscript{-1}, pH 4; 1 mL $\alpha$-Al\textsubscript{2}O\textsubscript{3}, 25 g/L, pH 6) on the ZnSe IRE under vacuum (10 mm Hg) overnight. Spectra of dry metal oxide films were acquired to determine consistency of colloidal coatings. For experiments with metal oxides, spectral subtractions were made to remove spectral contributions of the metal oxide films.

For experiments utilizing the ARK ATR-FTIR accessory, a 1 mL aliquot of LPS solution (4 mg mL\textsuperscript{-1} representing all solution chemistries discussed above) was deposited on the appropriate IRE. Spectra were collected at 0, 15, 30, 60, and 120 min after introduction of LPS solution into the ATR cell.

VATR-FTIR measurements were made using an ATRMAX II Variable Angle Horizontal ATR Accessory (PIKE Technologies, Inc.) with a 45° ZnSe and 45° GeO\textsubscript{2} IRE. Spectra were acquired at scale angles of 30, 37, 45, 52, and 60°, and as a function of time (0, 15, 30, 45, 60, 90, and 120 min) after sample (0.6 mL of 4 mg mL\textsuperscript{-1} LPS, 10 mM, pH 6 in both NaCl and CaCl\textsubscript{2}) introduction. All FTIR spectra were collected with 400 scans at a 4 cm\textsuperscript{-1} resolution (collection time: 495 s) using the corresponding LPS-free electrolyte solution as background.
Peak locations were verified via second derivative analysis and peak areas were
determined via curve fitting using Grams/AI software (Salem, NH). In some cases,
spectral areas of Lorentzian fitted peaks were used in quantitative analysis and peak area
ratios.

3.4. LPS sorption experiments

Sorption of ser 10 LPS to GeO$_2$, $\alpha$-FeOOH, and $\alpha$-Al$_2$O$_3$ was measured in
duplicate via batch experiments. Stock mineral solutions were pre-equilibrated overnight
at the appropriate pH (3, 4.5, 6, 7.5, 9) in 1 mmol L$^{-1}$ NaCl and CaCl$_2$, for a total of 30
stock mineral solutions. The pH was adjusted using 0.01 M HCl or 0.01 M NaOH.
Reactions were carried out in 2 mL polypropylene vials. Using the pre-equilibrated LPS
stock solutions, the initial LPS concentration was set at 100 mg C L$^{-1}$. The mineral
surface area for each sample was normalized to 8 m$^2$ L$^{-1}$. A total of 60 LPS-mineral
samples were run, along with 20 blanks (no mineral present) for the 10 different solution
chemistries. Samples were reacted for 2 h on an end-over-end shaker at 7 rpm. Following
reaction samples were centrifuged (3675 RCF, 10 min) and aliquot of the supernatant
solution was immediately removed for further analysis. The final solution pH for each
sample was measured. LPS remaining in solution was measured on a C-basis using
acidified samples via a total organic carbon (TOC) analyzer (Shimadzu TOC-V CSH
TOC/TN analyzer, Columbia MD). The solution phase was also analyzed for GeO$_2$, Fe,
and Al using an inductively coupled plasma mass spectrometer (ICP-MS, Pelkin-Elmer
DRC-II, Shelton, CT) with a PFA nebulizer in self-aspirating mode, a cyclonic quartz
spray chamber, and Pt sample and skinner cones. ICP-MS was run in peak hopping acquisition mode with an RF power of 1350 w, dwell time of 50 ms, using 3 replicates (40 sweeps per replicate), and a sample uptake rate of 0.4 mL min⁻¹. The Ar flow rates were 0.86 L min⁻¹ for the nebulizer, 15 L min⁻¹ for the coolant, and 1.2 L min⁻¹ for the auxiliary.

4. Results

4.1. Metal oxide characterization

BET analysis of colloidal GeO₂ revealed a surface area of 0.1571 m² g⁻¹. The DRIFT spectrum of purchased “pure” Ge indicate the presence of both hydroxide and oxide groups (Fig. 3d). Both the Ge IRE and purchased Ge powder are labeled without indicating the presence of surface (hydr)oxide groups, however the spectrum indicates these surfaces are not pure Ge. A sizable peak at 3357 cm⁻¹ corresponds to ν(OH) and peaks between 600 and 1000 cm⁻¹ result from ν(Ge-O) (Abo-Naf et al., 2002; Mei et al., 2004). The mineralogy of colloidal α-Al₂O₃ was confirmed via XRD. Successful synthesis of colloidal α-Fe₂O₃ was confirmed via XRD, TEM, and DRIFT analysis. Diffraction patterns (Fig. 1) show eight peaks, which closely match reference data (Blake et al., 1966). TEM micrographs (Fig. 2) reveal unidimensional crystals of rhomboid to hexagonol geometry measuring 10-20 nm across. DRIFT spectra (Fig. 3a) have prominent peaks at 454 and 458 cm⁻¹ corresponding to the Fe-O vibrations of hematite (Schwertmann and Taylor, 1989). DRIFT spectra of synthetic goethite confirmed successful synthesis of α-FeOOH with diagnostic IR vibrations present at 892 and 792
cm\(^{-1}\) (Fig. 3b) (Schwertmann and Taylor, 1989; Schwertmann and Cornell, 1991). DRIFT analysis of the purchased Al\(_2\)O\(_3\) reveal a broad peak between 560 and 790 cm\(^{-1}\) and additional peaks between 375 and 520 corresponding to Al-O vibrations of corundum (Gadsen, 1975; Danchevskaya et al., 2004).

**Figure 1.** XRD pattern for synthesized \(\alpha\)-Fe\(_2\)O\(_3\).

**Figure 2.** TEM micrograph of synthesized \(\alpha\)-Fe\(_2\)O\(_3\).
Distinct differences between ATR-FTIR spectra for LPS in NaCl and CaCl$_2$ are observed for all surfaces studied (Fig. 4-7). IR band assignments can be found in Appendix C, Table 1. It has previously been shown that Ca-LPS samples on ZnSe show time-dependent changes (increased spectral absorbance for 120 min) whereas Na-LPS samples on ZnSe show no time dependent changes after 15 min (i.e., reach apparent equilibrium within this time; Appendix C, Fig. 6). This same effect is observed less dramatically for LPS samples on a GeO$_2$ IRE. However, both Na-LPS and Ca-LPS

### 4.2. ATR-FTIR spectra of interfacial LPS

Figure 3. DRIFT FTIR spectra of synthesized a) α-Fe$_2$O$_3$ and b) α-FeOOH, and purchased c) α-Al$_2$O$_3$ and d) GeO$_2$. 

![ATR-FTIR spectra of synthesized a) α-Fe$_2$O$_3$ and b) α-FeOOH, and purchased c) α-Al$_2$O$_3$ and d) GeO$_2$.](image-url)
samples show unchanging spectra within 15 min when in contact with $\alpha$-Fe$_2$O$_3$ and $\alpha$-Al$_2$O$_3$ (data not shown).

Visual examination of spectra allows for qualitative analysis of trends regarding contributions from fatty acid [$v_{as}(\text{CH}_2)$], phosphate [$v_{as}(\text{PO}_2^-)$], and carbohydrate [$v$(C-O, C-O-C)] moieties. Spectra of Na-LPS show some effect of surface composition, but strong similarities overall. Typically Na-LPS spectra contain a single broad peak corresponding to [$v_{as}(\text{PO}_2^-)$; 1220-1260 cm$^{-1}$] and a relatively large peak in the carbohydrate (O-antigen) region [$v$(C-O, C-O-C); $\sim$1060 cm$^{-1}$]. Fatty acid [(v$_{as}$(CH$_2$); i.e., lipid A)] contributions, relative to v$_{as}$(PO$_2^-$) and v(C-O, C-O-C), are greatest in Na-LPS spectra collected on ZnSe (Fig. 4a) and $\alpha$-Fe$_2$O$_3$ (Fig. 6a). An increased contribution of v$_{as}$(PO$_2^-$) and v(C-O, C-O-C) [relative to v$_{as}$(CH$_2$)] is observed with decreasing pH for Na-LPS samples.
Figure 4. ATR-FTIR spectra of ser 10 LPS on ZnSe as a function of pH (3, 6, 9) in a) 10 mmol L$^{-1}$ NaCl and b) 10 mmol L$^{-1}$ CaCl$_2$. 
Figure 5. ATR-FTIR spectra of ser 10 LPS on GeO$_2$ as a function of pH (3, 6, 9) in a) 10 mmol L$^{-1}$ NaCl and b) 10 mmol L$^{-1}$ CaCl$_2$. Decreased peak intensity with increased pH was observed, therefore the Y-axis scale is expanded to show spectra details (non-common scale).
Figure 6. ATR-FTIR spectra of ser 10 LPS on α-Fe₂O₃-coated ZnSe as a function of pH (3, 6, 9) in a) 10 mmol L⁻¹ NaCl and b) 10 mmol L⁻¹ CaCl₂. Decreased peak intensity with increased pH was observed, therefore the Y-axis scale is expanded to show spectra details (non-common scale).
The spectra of Ca-LPS samples are strongly dependent on crystal surface chemistry (Fig. 4b, 5b, 6b, 7b). Ca-LPS spectra acquired using a ZnSe IRE (Fig. 4b) show relatively small contributions arising from $\nu$(C-O, C-O) (~1060 cm$^{-1}$). In contrast, when a GeO$_2$ IRE is used for Ca-LPS (Fig. 5b) there is a large $\nu$(C-O, C-O) peak at pH 3. This O-antigen contribution is diminished with increasing pH, however strong $\nu_{as}$(PO$_2^-$) peaks remain. Regardless of pH, $\nu_{as}$(CH$_2$) peaks remain small (Fig. 5b), and contribute

**Figure 7.** ATR-FTIR spectra of ser 10 LPS on $\alpha$-Al$_2$O$_3$-coated ZnSe as a function of pH (3, 6, 9) in a) 10 mmol L$^{-1}$ NaCl and b) 10 mmol L$^{-1}$ CaCl$_2$. Decreased peak intensity with increased pH was observed, therefore the Y-axis scale is expanded to show spectra details (non-common scale).
less to the spectra than is the case for Ca-LPS on ZnSe (Fig 4b). Ca-LPS spectra acquired on α-Fe₂O₃-coated ZnSe and α-Al₂O₃-coated ZnSe have strong ν(C-O, C-O-C) peaks, more like the GeO₂ case. There is also an increase in the ~1085 cm⁻¹ peak (relative to 1060 cm⁻¹) with increased pH, attributed to either νₐₛ(PO₂⁻) or ν(C-O, C-O-C) ring vibrations.

In order to show details of all spectra, the Y-axis in Figures 5, 6, and 7 are not on a common scale. Figure 8 shows the actual absorbance values of νₐₛ(CH₂), νₐₛ(PO₂⁻), and ν(C-O, C-O-C) for Ca-LPS and Na-LPS on the four different surfaces. With the exception LPS on ZnSe (Fig. 8a), there is a general trend of decreasing absorbance with increasing pH (particularly from pH 6 to 9).

LPS spectra acquired on α-Fe₂O₃ contain peaks at ~1407 cm⁻¹ (Fig. 6) corresponding to νₛ(COO⁻). In LPS spectra collected on α-Al₂O₃ (Fig. 7), a less prominent peak at 1417 cm⁻¹ is also assigned to νₛ(COO⁻). LPS samples on the hydrophobic (ZnSe) surface exhibit a distinct difference in IR absorbance based on the background electrolyte (Fig. 8a), with increased absorbance intensity for Ca-LPS samples. On the hydrophilic surfaces (GeO₂, α-Fe₂O₃, and α-Al₂O₃; Fig. 8b, 8c, 8d) no distinguishable effect of background electrolyte on IR absorbance is observed. It is important to note that the Y-axis scales are not the same for each part of Figure 8. The greatest absorbance values correspond to low pH samples of Ca-LPS on ZnSe (Fig. 8a) and LPS (Na and Ca) on α-Fe₂O₃.
4.3. VATR-FTIR spectroscopy of LPS on ZnSe and GeO$_2$

VATR spectra of NaCl-LPS and CaCl$_2$-LPS solutions on ZnSe and GeO$_2$ crystals are shown in Figure 9. In order to quantify spectral differences, Lorentzian peaks were fit to spectra and peak areas were quantified. These areas and area ratios of major functional groups reveal slight depth-dependent changes for Ca-LPS solutions on ZnSe and GeO$_2$. 

**Figure 8.** Raw IR absorbance values corresponding to $\nu_{\text{as}}$(CH$_2$), $\nu_{\text{as}}$(PO$_2^-$), and $\nu$(C-O, C-O-C) for Na-LPS (solid symbols) and Ca-LPS (open symbols) collected on a) ZnSe, b) GeO$_2$, c) $\alpha$-Fe$_2$O$_3$, and d) $\alpha$-Al$_2$O$_3$ as a function of solution pH.
No time dependent changes were observed for Na-LPS solutions at any of the angles examined.

Ca-LPS samples on ZnSe exhibit increased contributions from $\nu$(CH$_2$, CH$_3$) and $\nu_{as}$(PO$_4$) at both the high and low angles, with the greatest contribution from $\nu$(C-O, C-O-C) occurring at 45°. This trend is observed less dramatically for Ca-LPS samples on GeO$_2$. Greater relative $\nu$(C-O, C-O-C) intensities are observed for Ca-LPS samples on GeO$_2$ than on ZnSe. Unlike NaCl-LPS solutions, Ca-LPS spectra exhibit increased $\nu$(CH$_2$, CH$_3$) and $\nu_{as}$(PO$_4$) contributions.

Figure 9. VATR-FTIR spectra of ser 10 LPS (pH 6) on a) ZnSe in 10 mmol L$^{-1}$ NaCl, b) ZnSe in 10 mmol L$^{-1}$ CaCl$_2$, c) GeO$_2$ in 10 mmol L$^{-1}$ NaCl, and d) GeO$_2$ in 10 mmol L$^{-1}$ CaCl$_2$. The effective angles are labeled below spectra. Due to the variation in IR absorbance as a function of depth of penetration the Y-axis has expanded to show detail (non-common scale). Spectra collected at 120 min.
CH₃) and νₐₛ(PO₄⁻) peak areas with increased time (e.g., Fig. 10a-2, 10b-2). Plots of major LPS band intensities versus the square root of time (t, diffusion limited sorption model) reveal near linear trends, particularly for Ca-LPS on ZnSe. The goodness of fit (R²) for regressions of ν(CH₂, CH₃) versus t¹/₂ ranged from 0.93 to 0.97, from 0.80 to 0.94 for νₐₛ(PO₄⁻), and from 0.94 to 0.99 for ν(C-O, C-O-C). For Ca-LPS samples collected on GeO₂, R² values for absorbance versus t¹/₂ ranged from 0.50 to 0.96 for ν(CH₂, CH₃), from 0.88 to 0.99 for νₐₛ(PO₄⁻), and from 0.14 to 0.67 for ν(C-O, C-O-C). The slope of each line is plotted against θₑffective in Fig. 10. Error bars represent 95% confidence intervals, and when not seen this indicates error bars are smaller than the symbol size. Each point on the graph represents the slope taken for peak area as a function of collection time; n=7 for each data point. Because of the much lower absorbance of Ca-LPS on GeO₂ relative to ZnSe [i.e., ~10 times for νₐₛ(PO₄⁻) and ν(CH₂, CH₃), ~2 times for ν(C-O, C-O-C), the slope values for spectra acquired on ZnSe are much greater.
Figure 10. VATR-FTIR data for *P. aeruginosa* ser 10 LPS in 10 mM CaCl₂ at pH 6 showing the regression slope of peak areas vs. (time)⁰.⁵ at five different angles for fatty acid, phosphate, and carbohydrate moieties on a) ZnSe and b) GeO₂ IRE. Error bars represent 95% confidence intervals and n = 7 for each point, representing data collection at 7 different times. The insets are given as examples of data used for linear regression analysis. This data represent the three points corresponding to the three functional groups examined at an effective angle of 45°.
4.4. LPS sorption experiments

Na-LPS sorption to colloidal GeO₂ in aqueous suspension is observed at pH 3, however no sorption is detected at pH 4.5 through 9 (Fig 11a). The α-FeOOH shows greater Na-LPS sorption (Fig. 11b), particularly in the neutral pH range. Sorption of Na-LPS to α-Al₂O₃ is minimal (< 5%) through the pH range of 4.5 to 9. High variation in the data for Ca-LPS sorption is observed (Fig. 11) indicating that a larger set of experimental replicates may be required to diminish coefficients of variation. However, the results do indicate that sorption of Ca-LPS to all three surfaces studied is greater than what is observed for Na-LPS. For all samples, sorbed LPS sorption remained below 30% of the total. ICP-MS results show centrifugation successfully separated the colloidal solids from suspension, and that LPS did not promote significant mineral dissolution (Table 2).

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Metal</th>
<th>% Metal Remaining in Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GeO₂</td>
<td>Ge</td>
<td>0.398 ± 0.317</td>
</tr>
<tr>
<td>α-FeOOH</td>
<td>Fe</td>
<td>0.044 ± 0.049</td>
</tr>
<tr>
<td>α-Al₂O₃</td>
<td>Al</td>
<td>0.018 ± 0.017</td>
</tr>
</tbody>
</table>

Table 2. Mean percent of metal remaining in solution after centrifugation for Na-LPS and Ca-LPS samples reacted with minerals (± standard deviation).
Figure 11. Ser 10 LPS sorption to a) GeO$_2$, b) $\alpha$-FeOOH, and c) $\alpha$-Al$_2$O$_3$. Sorption reactions were carried out in 1 mmol L$^{-1}$ NaCl.
Blank samples, containing no mineral phase, were also run to determine loss of LPS from solution via sorption to polypropylene vials or macromolecular sedimentation during centrifugation. For Na-LPS samples, the recovery for blank samples was > 93% (Table 3). The recovery for Ca-LPS ranged from ~64-93%, which certainly contributes to the high variability in surface excess calculations. Preliminary experiments were also conducted to determine if LPS loss from solution in blank samples occurs from sorption to reaction vessel or sedimentation via centrifugation. The results revealed > 92% recovery for Na-LPS samples, with no increased loss from centrifugation. In the case of Na-LPS samples recovery was ~92% for non-centrifuged samples, and ~68% for centrifuges samples.

Table 3. Mean percent LPS loss from solution for experimental blank vials (± standard deviation).

<table>
<thead>
<tr>
<th>pH</th>
<th>Percent LPS Loss for Blank Vials (no mineral)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaCl</td>
</tr>
<tr>
<td>3.0</td>
<td>5.44 ± 1.06</td>
</tr>
<tr>
<td>4.5</td>
<td>5.72 ± 0.86</td>
</tr>
<tr>
<td>6.0</td>
<td>1.92 ± 0.23</td>
</tr>
<tr>
<td>7.5</td>
<td>0.23 ± 3.73</td>
</tr>
<tr>
<td>9.0</td>
<td>6.21 ± 3.05</td>
</tr>
</tbody>
</table>

5. Discussion

The results show distinct differences between Na-LPS and Ca-LPS samples when examined via ATR-FTIR. The effect of counterions on aggregate stability is consistent with our previous findings, where LPS interaction with Ca²⁺ resulted in aggregate reorientation and increased interaction with a ZnSe IRE (Appendix C). LPS aggregates in NaCl solutions are stabilized via H-bonding between saccharide groups (Coughlin et al.,
1985), whereas the formation of Ca-phosphate bonds in the lipid A region cause LPS aggregates to break apart (Wang et al., 2005).

Analysis of ATR-FTIR spectra presented in this study confirms LPS aggregate interaction with surfaces. The surface properties influence interactions occurring between LPS and the IRE surface. Na-LPS spectra exhibit more similarity between samples on different surfaces, indicating less interaction between Na-LPS aggregates and the IRE.

5.1. Chemical properties of surfaces

The surface properties of the IREs used in this study influence the ATR-FTIR LPS spectra. ZnSe is relatively hydrophobic (Reiter et al., 2002). Conversely, GeO$_2$ has been referred to as a hydrophilic IRE (Snabe and Petersen, 2002), presumably because of the formation of an hydroxylated interface similar to that observed on silica. The metal oxides ($\alpha$-FeOOH, $\alpha$-Fe$_2$O$_3$, and $\alpha$-Al$_2$O$_3$) are also relatively hydrophilic due to the presence of hydroxyl groups at their surface. These surfaces all exhibit pH-dependent charge, which influence LPS spectra. The pH where a surface exhibits no net charge is defined as the point of zero charge (PZC). The PZC for ZnSe is < 4 (Tickanen et al., 1997). Although a value for GeO$_2$ could not be found in the literature, the isoelectric point (IEP) is estimated to < 4. This is based on the similarity in structure between GeO$_2$ and SiO$_2$ (Gun'ko et al., 1998), which has an IEP between 2.2 and 2.5 (Gun'ko et al., 1998) and similarly, a PZC = 2.0 (Sparks, 1995), and on experimental results by Gun’ko et al (1998). In that study, the IEP of SiO$_2$ was measured with increasing GeO$_2$ contributions (up to 20%). The results show increasing IEP (2.2 to 3.4) for 0 to 7 % GeO$_2$,
followed by a decrease in the IEP for 12 and 20% GeO₂ (IEP = 2.6). The PZC for the metal oxides are 8.0 to 8.5 for α-Fe₂O₃ (Sposito, 1989), 7.0 to 8.0 for α-FeOOH (Sposito, 1989), and 9.1 for α-Al₂O₃ (Sparks, 1995). ATR-FTIR experiments were carried out at pH 3, 6, and 9 where surface charge of the IREs and metal oxides is variable. A summary of the pH-dependent charge and hydrophobicity of LPS and surfaces is given in Table 4.

Table 4. pH-dependent Charge and hydrophobicity of LPS, IREs, and metal oxide coatings.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Surface Charge and Hydrophobicity</th>
<th>pH 3</th>
<th>pH 6</th>
<th>pH 9</th>
<th>Hydrophobicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>Neutral-Negative</td>
<td>Neutral</td>
<td>Negative</td>
<td>Negative</td>
<td>Amphiphilic</td>
</tr>
<tr>
<td>ZnSe</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>GeO₂</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>α-Fe₂O₃</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>α-FeOOH</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Neutral</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>α-Al₂O₃</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Neutral</td>
<td>Hydrophilic</td>
</tr>
</tbody>
</table>

5.2. LPS sorption to GeO₂, α-FeOOH, and α-Al₂O₃

Sorption experiments to ZnSe surfaces were not conducted due to high toxicity of ZnSe powder. The small size of the (nano) α-Fe₂O₃ used for ATR studies prohibited its use in batch sorption experiments as efficient centrifugation would not be possible. Therefore α-FeOOH, which has a similar surface chemistry (Table 4), was used as a surrogate for α-Fe₂O₃.
5.2.1. Na-LPS sorption

Sorption envelopes for LPS with GeO$_2$, $\alpha$-FeOOH, and $\alpha$-Al$_2$O$_3$ (Fig. 11) reveal relatively low affinity of Na-LPS aggregates for surfaces. Sorption to GeO$_2$ occurs at low pH, where the surface is positively charged. As the pH increases dissociation of carboxyl (pH 2-6) and hydroxyl (pH 9-10) groups (Martinez et al., 2002) leads to increased negative charge on the O-antigen. Although most ionizable functional groups on LPS are protonated at low pH, limited dissociation of carboxyl groups (pK$_a$ 2-6) at pH 3 may lead to favorable electrostatic interactions between O-antigens and GeO$_2$.

In the case of $\alpha$-FeOOH some sorption is observed over the entire pH range, with maximum sorption occurring between pH 4.5 and 7. Sorption decreases as the pH increased to 9. The pH-dependent charge properties of $\alpha$-FeOOH and LPS are likely responsible for the observed result. At pH 3, LPS carries a neutral (or slightly negative charge) and $\alpha$-FeOOH is positively charged. Resulting, interactions are not strongly favored between LPS and $\alpha$-FeOOH are minimal. For the pH range of 4.5 to 7.5, $\alpha$-FeOOH is positively charged, and dissociation of O-antigen functional groups leads to favorable electrostatics for LPS sorption. However, as the pH is increased to 9 both $\alpha$-FeOOH and LPS are negatively charged and charge repulsion leads to minimal sorption. These results are in contrast to what has been observed for the sorption of extracellular polymeric substances (EPS) to $\alpha$-FeOOH (Omoike and Chorover, 2006). In that study EPS sorption exhibited a near linear decrease with increasing pH (pH 3-9). However, <10% of Na-LPS was sorbed in this study, whereas nearly 100% of EPS was adsorbed at pH 3 and 20% was adsorbed at pH 9. The discrepancy in observed trends may be due to a
higher concentration of reactive functional groups in the case of EPS (including carboxyl, phospholipid and amide groups) and the tendency of LPS to form aggregates, which does not occur in the case of EPS.

Although less sorption was observed for Na-LPS to $\alpha$-Al$_2$O$_3$ the trends are comparable to those observed for $\alpha$-FeOOH. The exception here is that the PZC for $\alpha$-Al$_2$O$_3$ is 9.1 (7.8 for $\alpha$-FeOOH). Therefore at pH 9, $\alpha$-Al$_2$O$_3$ is net neutral and charge repulsion does not occur.

5.2.2. Ca-LPS sorption

The batch sorption data for Ca-LPS uptake show greater variation than Na-LPS results (Fig. 11). The reason for this discrepancy is evident in the percent recovery for blank reaction vessels (Table 3). LPS recovery for Na-LPS is $> 93\%$ for all pH values. Conversely, Ca-LPS recovery ranges from 64-93% with large standard deviations. This indicates Ca-LPS are either binding to the polypropylene tubes or are being pelleted via centrifugation. Preliminary data indicate that centrifugation of Ca-LPS leads to pelleting of LPS. This method of measuring Ca-LPS sorption to mineral surfaces is not appropriate and further experiments (e.g., quartz crystal microbalance) are required to determine the affinity of Ca-LPS for the various surfaces. Unfortunately, this means that Ca-LPS data are not reliable. However, from the data it can be gleaned that Ca-LPS sorption is generally greater than what is observed for Na-LPS. It must be stressed that before this conclusion can be drawn further experiments are required.
Based on evidence for Ca\(^{2+}\) leading to disruption of aggregate structures presented in Appendix C and in previous studies (Coughlin et al., 1983; Obst et al., 1997; Wang et al., 2005), greater sorption of Ca-LPS is predicted to hydrophobic surfaces. Therefore, if surfaces with more hydrophobic characteristics were used for batch experiments it is possible increased hydrophobic Ca-LPS interactions with surfaces would lead to even greater sorption.

5.3. ATR-FTIR: LPS interactions with ZnSe, GeO\(_2\), \(\alpha\)-Fe\(_2\)O\(_3\), and \(\alpha\)-Al\(_2\)O\(_3\)

Although, striking differences between the spectra of Ca-LPS and Na-LPS are observed, similarities in the IR absorbance trends are observed between these samples (Fig. 8). LPS interaction with hydrophilic surfaces (GeO\(_2\), \(\alpha\)-Fe\(_2\)O\(_3\), \(\alpha\)-Al\(_2\)O\(_3\)) exhibits decreased IR absorbance with increasing pH, particularly for \(\nu\) (C-O, C-O-C) and \(\nu_{as}(PO_4^-)\). This trend is consistent with previous studies, showing decreased peak intensity of bacterial polysaccharides upon proton dissociation (Omoike et al., 2004). Surfactant adsorption to surfaces is controlled by ion exchange, ion pairing, hydrophobic bonding, polarization of \(\pi\) electrons, and dispersion forces (Somundaran and Huang, 2000; Paria and Khilar, 2004). These forces of adsorption typically involve monomers, as opposed to aggregates (Griffith and Alexander, 1967). Amphiphilic LPS adsorption to surfaces should follow similar behavior. As will be discussed below, hydrophobic interactions dominate LPS-ZnSe spectra, whereas pH-dependent charge properties are significant for LPS interactions with the more hydrophilic surfaces.
5.3.1. Na-LPS interactions

Na-LPS spectra are generally similar regardless of IRE surface (Fig. 5-8). Trends in pH are similar, with increased $\nu$(C-O, C-O-C) and $\nu_{as}$(PO$_4^{2-}$) peaks relative to $\nu_{as}$(PO$_4^{2-}$) at lower pH values. Also, pH 3 spectra show a sharp peak at $\sim$1060 cm$^{-1}$ that masks other peaks on the region. The O-antigen is more susceptible to changes in conformation than the lipid A and core regions (Kastowsky et al., 1992; Seydel et al., 1993). Variation in the Na-LPS spectra as a function of pH results from conformational changes due to protonation/dissociation of ionizable functional groups in the O-antigen region. The lack of influence resulting from different IRE and metal oxide coatings indicate minimal interaction between Na-LPS and the selected surfaces. The only evidence of surface interactions for Na-LPS is with $\alpha$-Fe$_2$O$_3$, and, to a lesser extent, with $\alpha$-Al$_2$O$_3$, where the 1409 cm$^{-1}$ [$\nu_s$(COO)] likely represents bonding between the O-antigen chain and Fe(III) or Al(III). This peak is smallest at pH 9 where $\alpha$-Fe$_2$O$_3$, carries a negative charge and $\alpha$-Al$_2$O$_3$ is approximately neutral. Adhesion of negatively-charged rough LPS to a positively-charged surface (aminopropyltrihexoxysilane polymers) in ultrapure water has been previously observed (Reiter et al., 2002). The lack of change in ATR spectra due to the presence of different surfaces gives further evidence that Na-LPS aggregates are stable in solution and do not re-assemble at the solid-liquid interface. The spectra of Na-LPS may represent solution phase spectra. Due to smaller penetration depths for the GeO$_2$ IRE (Table 1) lower absorbance values are expected.
5.3.2. Ca-LPS interactions

While Na-LPS spectra remain relatively unchanged as a function of IRE surface chemistry, Ca-LPS spectra show distinct differences (Fig 4-7). Greater interaction of Ca-LPS with surfaces is consistent with sorption results (Fig 11). Noticeable difference in IR spectra are observed for Ca-LPS on ZnSe compared to GeO$_2$, $\alpha$-Fe$_2$O$_3$ and $\alpha$-Al$_2$O$_3$. ZnSe is the only hydrophobic surface used, and spectra show diminished $\nu$(C-O, C-O-C) peaks relative to $\nu_{as}$(PO$_4^{3-}$) and $\nu$(CH$_2$, CH$_3$), both of which are functional groups within the lipid A region. Under these conditions LPS aggregate stability is apparently decreased by Ca$^{2+}$ binding to PO$_4^{3-}$ in the lipid A region. This is in agreement with previously published studies on rough LPS (Coughlin et al., 1983; Obst et al., 1997; Wang et al., 2005). Figure 12 presents a proposed model for LPS aggregation (Na$^+$ and Ca$^{2+}$) on ZnSe. This model is in agreement with general characteristics of surfactants, tending to become oriented on surfaces to which they adsorb (Paria and Khilar, 2004). Since it is assumed that micelles comprising amphiphiles do not adsorb to hydrophobic surfaces (Paria and Khilar, 2004), the release of monomers from LPS aggregates to the surface is likely the key factor governing adsorption. The importance of surface hydrophobicity for LPS adhesion has been demonstrated for membrane-bound rough LPS (Jucker et al., 1997). Adhesion of membrane-bound LPS (Makin and Beveridge, 1996) and LPS in ultrapure water (Reiter et al., 2002) was low for hydrophobic surfaces. Direct comparisons between these studies and the free Na-LPS and Ca-LPS surface interactions in this study cannot be made due the effect of cation valence on LPS aggregation and surface interactions.
While the effect of Ca\(^{2+}\) on LPS aggregation remains the same for Ca-LPS samples regardless of IRE surface composition, differences in the interaction of the lipid A and O-antigen regions with surfaces are apparent in the collected IR spectra (Fig. 5-7). GeO\(_2\), α-Fe\(_2\)O\(_3\), and α-Al\(_2\)O\(_3\) are hydrophilic and possess different pH-dependent charge properties (Table 4). The hydrophilic nature of these surfaces translates into greater interaction with O-antigen chains, as evidenced by increased \(\nu(C-O, C-O-C)\) peaks, compared to the case for ZnSe spectra. The influence of pH on Ca-LPS spectra collected on ZnSe is minimal as hydrophobic interactions primarily control the interactions. However, the pH-dependent charge on hydrophilic surfaces, and Ca-LPS, greatly influence the collected spectra. Bonding of the hydrophilic region of amphiphilic molecules to hydrophilic surfaces may occur via ion exchange, ion pairing, polarization of \(\pi\) electrons, dispersion forces (van der Waals) (Paria and Khilar, 2004), and hydrogen bonding (Jucker et al., 1997).
Ca-LPS spectra on GeO\textsubscript{2} have relatively large $\nu$(C-O, C-O-C) contributions at pH 3, where GeO\textsubscript{2} is positively charged. Decreased interactions between the O-antigen [$\nu$(C-O, C-O-C) rich] and the GeO\textsubscript{2} IRE are observed at higher pH values (negative charge on GeO\textsubscript{2}). The pH-dependent charge properties of the O-antigen have been previously discussed (see section 5.2). Both $\alpha$-Fe\textsubscript{2}O\textsubscript{3} and $\alpha$-Al\textsubscript{2}O\textsubscript{3} are positively charged at higher pH values, and therefore larger contributions from the O-antigen (negative charge) are observed in these spectra (Fig. 6, 7).

Increased contributions arising from $\nu$(CH\textsubscript{2}, CH\textsubscript{3}) are observed in $\alpha$-Fe\textsubscript{2}O\textsubscript{3} at pH 3 and 6 (Fig. 6). The high affinity of Fe for PO\textsubscript{4}\textsuperscript{3-} (Tejedor-Tejedor and Anderson, 1990; Persson et al., 1996; Arai and Sparks, 2001) may play a role in the increased $\nu$(CH\textsubscript{2}, CH\textsubscript{3}) signal. The $\alpha$-Fe\textsubscript{2}O\textsubscript{3} surface is positively charged (pH 3, 6) and P-OFe binding between the lipid A region and the surface may cause the fatty acid region to migrate in close proximity to the IRE, thus increasing $\nu$(CH\textsubscript{2}, CH\textsubscript{3}) absorbance. This is observed to a much smaller degree for Ca-LPS on $\alpha$-Al\textsubscript{2}O\textsubscript{3} (Fig. 7).

The proposed model for Ca-LPS interaction with ZnSe indicates that the lipid A orients itself on the hydrophobic ZnSe surface (Fig. 12). However, spectra of Ca-LPS on GeO\textsubscript{2}, $\alpha$-Fe\textsubscript{2}O\textsubscript{3}, and $\alpha$-Al\textsubscript{2}O\textsubscript{3} indicate less interaction between fatty acid groups and the surface. Increased $\nu$(C-O, C-O-C) contributions to the spectra have lead to the proposed model for Ca-LPS interaction on these hydrophilic surfaces (Fig. 13.) In this model there is a reduced importance to the lipid A, as O-antigen regions interact more at the solid-liquid interface. These hydrophilic interactions likely result from hydrogen bonding (Jucker et al., 1997) and bridging of O-antigens to surfaces (Jucker et al., 1998a). The
precise arrangement of LPS at this interface is not known, and the schematic diagram represents only hypothetical structures.

![Figure 13. Proposed model of LPS aggregation in the presence of Na\(^+\) and Ca\(^{2+}\) on a GeO\(_2\), α-Fe\(_2\)O\(_3\), and α-Al\(_2\)O\(_3\).](image)

5.4. VATR spectra of Na-LPS and Ca-LPS

The sorption of polymeric molecules to surfaces can be rate-limited by diffusion to the interface or by surface induced transitions of conformation and/or orientation (Brusatori and van Tassel, 1999). Plots of Ca-LPS peak areas, for ν(CH\(_2\), CH\(_3\)), ν\(_{as}\)(PO\(_2\))\(^-\), ν(C-O, C-O-C) versus the square-root of time for five angles of IR beam incidence (all conducted as separate experiments) show a near linear increase (R\(^2\) near unity, e.g., Fig. 10a inset, 10b inset) indicating Ca-LPS interactions with ZnSe are consistent with diffusion-limited kinetics (Kookana et al., 1992; Jacobsen et al., 1997). The slopes of these regressions are plotted against angle of incidence in Figure 10a. Similar trends are observed for Na-LPS on GeO\(_2\) (Fig 10b), however slopes are much smaller and R\(^2\) values show greater variation and are consistently near unity only for ν\(_{as}\)(PO\(_2\))\(^-\). The lack of correlation indicates Ca-LPS aggregates do not exhibit the same rate-limited behavior.
upon adsorption to the GeO$_2$ IRE. VATR data are collected at pH 6, where interactions between Ca-LPS and GeO$_2$ are minimal (Fig. 5). If VATR spectra were collected at pH 3, greater reaction between the O-antigen region and the positively charged GeO$_2$ surface would likely demonstrate increased surface interaction and variation with depth of penetration. Still, data show PO$_4^-$ interactions with the GeO$_2$ IRE (not observed for Na-LPS).

Data for Ca-LPS on ZnSe reveal different slopes for the three functional groups (Fig 10a inset), but have similar trends in $\theta_{eff}$ for $\nu$(CH$_2$, CH$_3$) and $\nu_{as}$(PO$_2^-$). At low angle (high $d_p$), steep slopes for $\nu$(CH$_2$, CH$_3$) and $\nu_{as}$(PO$_2^-$) indicate strong time-dependence. Slopes decrease with increasing angle followed by a slight increase at shallowest depth measured ($\theta_{eff} = 51.2^\circ$). Although we cannot determine the precise orientation of Ca-LPS at the ZnSe IRE interface, depth-dependent variation in LPS structure is apparent. It is interesting to note that peak areas corresponding to $\nu$(C-O, C-O-C) of O-antigen remain relatively unchanged, whereas lipid A peak areas vary with penetration depth. This suggests that variation of $d_p$ is monitoring different layers of the aggregate structure, each of which may have different organization of LPS monomers and associated subunits. Importantly, the length scale over which depth-dependent changes in IR spectra are significant (hundreds of nanometers) is consistent with the size of aggregates measured by DLS (Appendix C).
6. Conclusions

The influence of substratum hydrophobicity, surface charge, and chemical composition on ATR-FTIR spectra of Na-LPS and Ca-LPS were investigated. Surface activity of LPS is strongly influenced by the cations present. Greater interaction between Ca-LPS (vs. NaCl) and surfaces (ZnSe, GeO₂, α-Fe₂O₃, α-FeOOH, α-Al₂O₃) was determined through batch sorption experiments and ATR-FTIR investigations. Na-LPS spectra on all surfaces were similar, indicating little surface interaction and spectra biased towards solution phase LPS. Ca-LPS interactions with ZnSe are controlled primarily by hydrophobic interactions (lipid A moieties), whereas pH-dependent surface charge plays a key role in Ca-LPS reactions (O-antigen moieties) on more hydrophilic surfaces (GeO₂, α-Fe₂O₃, α-Al₂O₃). Increased interactions were observed at lower pH values, where surfaces are generally positively charged.

VATR-FTIR spectra indicate variation in Ca-LPS structure as a function of distance from ZnSe and GeO₂ IRE surfaces (~500 to 2500 nm). However, the specific orientation of LPS molecules cannot be determined via techniques employed in this study. Na-LPS spectra exhibit minimal depth-dependent changes, revealing limited surface interactions with GeO₂ and ZnSe IREs at pH 6.

These results complement our previous work (Appendix C), where we propose reorientation of LPS aggregates in the presence of Ca²⁺ at the solid-liquid interface. The nature of cations present strongly influences aggregate structure and surface interactions. Additional work is needed to determine LPS orientation on surfaces, particularly for LPS in the presence of divalent cations.
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References


APPENDIX E

IN SITU INVESTIGATION OF SHEWANELLA ONEIDENSIS
MR-1 BIOFILM GROWTH AND Fe(III) REDUCTION
ON ZnSe AND NANOHEMATITE SURFACES

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Abstract

Adhesion of *Shewanella oneidensis* MR-1 cells and subsequent biofilm formation were monitored *in situ* to ZnSe and nanohematite (α-Fe$_2$O$_3$) using attenuated total reflectance (ATR) Fourier transform infrared spectroscopy (FTIR). Initial biofilm formation was carried out under oxic conditions, followed by anoxic conditions to allow observation of potential bioreduction of α-Fe$_2$O$_3$. Extensive bacterial adhesion was observed on both surfaces. Large contributions to the spectra arising from amide I and II vibrations implicate surface proteins in cell adhesion. Adhesion to α-Fe$_2$O$_3$ produced additional peaks corresponding to P-OFε interactions, highlighting the importance of bacterial phosphate groups for adhesion to Fe-oxides surfaces. Under anoxic conditions, slight spectral changes in Fe-O vibrations may be a result of biocatalyzed Fe(III) reduction.

Introduction

Interactions between bacteria and mineral surfaces play an important role in cell adhesion, biomineralization, and biodissolution reactions. The physiochemical properties environmental surfaces influence mechanisms of bacterial adhesion and may provide for unique biocatalyzed reactions at this interface. *Shewanella oneidensis* MR-1 (previously *S. putrefaciens* MR-1) is a facultative Gram negative bacteria capable of dissimilatory Fe$^{III}$ reduction under anoxic conditions (Heidelberg et al., 2002; Kostka et al., 2002; Neal et al., 2003). Under oxic conditions, oxygen is commonly used as the terminal electron acceptor. However, under anaerobic conditions, this bacterium can utilize Fe$^{III}$ as a
terminal electron acceptor (Myers and Myers, 1994; Caccavo and Das, 2002; Zachara et al., 2002; Neal et al., 2003). MR-1 is also capable of using Mn$^{III}$, Mn$^{IV}$, Cr$^{VI}$, U$^{VI}$, fumurate, nitrate, trimethyamine N-oxide, dimethyl sulfoxide, sulfite, thiosulfate, and elemental sulfur as terminal electron acceptors (Myers and Nealson, 1988; Nealson and Saffarini, 1994; Heidelberg et al., 2002).

The environmental consequences of dissimilatory Fe reduction by MR-1 include Fe-cycling, toxic heavy metal transport, and mineral phases in the environment (Heidelberg et al., 2002; Liu et al., 2002; Gonzalez-Gil et al., 2005). Therefore MR-1 has been proposed for bioremediation of metal/organic-contaminated anoxic zones (Heidelberg et al., 2002). The use of bacteria for bioaugmentation of contaminated soils and sediments requires cell adhesion to mineral surfaces to develop a microbial community in the contaminated zone. However, it is important that bacteria are capable of migrating with contaminant plumes for increased efficiency of remediation.

The primary objective of this study is to use in situ attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy to examine the adhesion of MR-1 to both negative (ZnSe) and positive (nanohematite; $\alpha$-Fe$_2$O$_3$) charged surfaces. During anoxic biofilm growth on $\alpha$-Fe$_2$O$_3$, reduction of Fe$^{III}$ will occur and changes in the Fe-oxide and biomolecular IR signal may be observed.
Methods

Synthesis and Analysis of Nanohematite (α-Fe₂O₃)

Colloidal α-Fe₂O₃ particles were synthesized based on the methods of Schwertmann and Cornell (1991). The synthesis and analysis (x-ray diffraction [XRD], diffuse reflectance [DRIFT] FTIR spectroscopy, and transmission electron microscopy [TEM]) of α-Fe₂O₃ are discussed in detail in Appendix B.

Bacteria and Growth Conditions

*Shewanella oneidensis* MR-1 (ATCC 700550) was grown in 250 mL polycarbonate screw cap Erlenmeyer flasks (Nalgene), and subjected to orbital mixing at 100 rpm in an environmental shaker at 30°C. Bacteria growth was under aerobic conditions in triptic soy broth (TSB) to the early stationary phase (22 h; 10⁹ cells mL⁻¹). Cells were harvested by centrifugation (3000 RCF, 20 min, 4°C) and washed with MR-1 growth media (30 mmol L⁻¹ pipes, 1 mmol L⁻¹ NaCl, 30 mmol L⁻¹ lactic acid, 28 mmol L⁻¹ NH₄Cl, 28 mmol L⁻¹ CaCl₂, 1.34 mmol L⁻¹ KCl, and 100µmol⁻¹ AQDS in Barnstead nanopure water [BNP]) at pH 7.4 (Zachara et al., 2002) to remove TSB. Cells were resuspended in MR-1 growth media (10⁹ cells mL⁻¹) for use in flow experiments.

ATR-FTIR Flow Experiments

The cylindrical ZnSe internal reflection element (IRE) was coated with α-Fe₂O₃ for experiments monitoring adhesion of MR-1 to α-Fe₂O₃. A suspension of colloidal α-Fe₂O₃ (2 g L⁻¹; pH 4.3) was pumped over a ZnSe IRE (0.5 mL/min) for 24 h. The flow
cell was emptied, allowed to dry, and BNP water was pumped over the IRE to remove loosely bound $\alpha$-Fe$_2$O$_3$.

Cell adhesion, biofilm growth, and Fe-reduction were studied in real-time using a flow-through ATR-FTIR method (see Appendix A, Fig. 1). A Virtis Omni Culture Plus bioreactor (Gardiner, NY) was used to maintain constant temperature ($30^\circ$C), pH (7.4), mixing (100 rpm) and oxygen content over the course of the experiment. The bioreactor was filled with 1.8 L of MR-1 cells ($10^5$ cells mL$^{-1}$) in MR-1 growth media.

Three distinct flow through experiments were conducted to study the adhesion of MR-1 and subsequent Fe reduction. The basic experimental parameters for each were: (1) ZnSe IRE with oxic (air purge) conditions for 24 h, followed by anoxic ($N_2$ purge) conditions for the remainder of the experiment (48 h), (2) $\alpha$-Fe$_2$O$_3$-coated ZnSe IRE with oxic (air purge) conditions for 24 h, followed by anoxic ($N_2$ purge) conditions for the remainder of the experiment (48 h), and (3) ZnSe IRE with anoxic ($N_2$ purge) MR-1 pumped over the IRE for 258 h. For all experiments MR-1 cells were pumped from the bioreactor over the IRE (0.25 mL min$^{-1}$) and recirculated back to the bioreactor. Spectra were collected as a function of time during flow. Spectra were collected on Nicolet 560 Magna IR spectrometer (Madison, WI) with 400 scans at a 4 cm$^{-1}$ resolution and corrected for growth media as a background.
Results

Nanohematite Characterization

Successful synthesis of (nano) $\alpha$-Fe$_2$O$_3$ was confirmed via XRD, TEM, and DRIFT FTIR analysis. Diffraction patterns (Fig. 1) show eight peaks corresponding to hematite (Blake et al., 1966). TEM micrographs reveal unidimensional crystals of rhomboid to hexagonal geometry measuring 10-20 nm across, consistent with published data (Schwertmann and Cornell, 1991). DRIFT spectra have prominent peaks at 454 and 458 cm$^{-1}$ corresponding to the Fe-O vibrations of hematite (Schwertmann and Taylor, 1989). Small peaks at 806 and 892 cm$^{-1}$ may indicate the presence of a light mixed-phase with goethite (Schwertmann and Taylor, 1989).

Figure 1. XRD diffraction pattern of freeze-dried $\alpha$-Fe$_2$O$_3$. 
Figure 2. TEM micrograph of $\alpha$-Fe$_2$O$_3$.

Figure 3. DRIFT spectra of $\alpha$-Fe$_2$O$_3$. 
Monitoring Cell Adhesion to ZnSe and Nanohematite via ATR-FTIR

MR-1 adhesion to both ZnSe and \( \alpha \)-Fe\(_2\)O\(_3\)-coated ZnSe was observed, leading to stable biofilm development (Fig. 4 and 5). Assignments for IR peaks are given in Table 1. Initially cells were grown under oxic conditions and the cell suspension had a grey-brown color. When N\(_2\) was bubbled into the bioreactor a dramatic color change to a bright red suspension was observed within 1 to 2 min. ATR-FTIR spectra do not show noticeable changes accompanying this change in cell-suspension color (Fig. 4 and 5). Both the background media and \( \alpha \)-Fe\(_2\)O\(_3\)-coated ZnSe have been subtracted from the spectra. Differences between MR-1 adhesion to ZnSe and \( \alpha \)-Fe\(_2\)O\(_3\) are seen by looking at relative contributions from proteins (\(~1500\text{-}170 \text{ cm}^{-1}\)) to polysaccharides and phosphate groups (\(~1000\text{-}1250 \text{ cm}^{-1}\)). There is a greater contribution for IR absorbing protein moieties (relative to polysaccharides and phosphate groups) for MR-1 cells on the \( \alpha \)-Fe\(_2\)O\(_3\) surface, compared to ZnSe.
Table 1. FTIR peak assignments for bacteria on ZnSe and α-Fe₂O₃ surfaces.

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>IR Band Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1720-1729</td>
<td>νₛ(COOH)ᵃ</td>
</tr>
<tr>
<td>1652-1637</td>
<td>Amide I: C=O, C-N, N-Hᵇᵈ</td>
</tr>
<tr>
<td>1550-1540</td>
<td>Amide II: N-H, C-Nᵃᶜ</td>
</tr>
<tr>
<td>1460-1454</td>
<td>δ (CH₂)ᵃᶜ</td>
</tr>
<tr>
<td>1400-1390</td>
<td>νₛ(COO⁻)ᵃᶜ</td>
</tr>
<tr>
<td>1220-1260</td>
<td>νₐs(PO₂⁻) of phosphodiesters (hydrated)ᵃ⁺ₑᶠ</td>
</tr>
<tr>
<td>1170</td>
<td>ν(C-O)ᵈ</td>
</tr>
<tr>
<td>1114-1118</td>
<td>ν(C-O-P, P-O-P), Ring vibrationsᵃᵈ</td>
</tr>
<tr>
<td>1084-1088</td>
<td>νₐs(PO₂⁻) of phosphodiesters⁻¹, ring vibrationsᵃ, ν(C-C)⁶</td>
</tr>
<tr>
<td>1024, 1045, 1052,</td>
<td>ν(C-O-C, C-C) from polysaccharidesᵃ⁺ᵍ</td>
</tr>
<tr>
<td>1058</td>
<td></td>
</tr>
<tr>
<td>1039</td>
<td>ν(P-OH, P-O-Fe)ʰ⁻ˡ</td>
</tr>
<tr>
<td>962-968</td>
<td>ν(PO₂⁻)ʰ⁻ᵏ</td>
</tr>
<tr>
<td>800, 894</td>
<td>Fe-Oᶠ</td>
</tr>
<tr>
<td>650-450</td>
<td>ν(CH₂)ᵐ</td>
</tr>
</tbody>
</table>


Figure 4. ATR-FTIR spectra of S. oneidensis MR-1 cell adhesion and biofilm growth on ZnSe (0-24 h oxic, 24-48 anoxic).
Examination of the mixed polysaccharide and phosphate region (1000-1250 cm\(^{-1}\)) reveals differences in cell adhesion to ZnSe and \(\alpha\)-Fe\(_2\)O\(_3\) surfaces (Fig. 6 and 7). No difference between adhered cells under oxic conditions (24 h; Fig 6) and anoxic conditions (48 h; Fig 7) was observed. MR-1 cells at the ZnSe surface produce strong IR peaks at 1236 \(\nu_{\text{as}}(\text{PO}_2^-)\) and 1083 \(\nu_s(\text{PO}_2^-),\) sugar ring vibrations, and 1024 cm\(^{-1}\) \(\nu(C-O, C-O-C)\). For MR-1 cells on \(\alpha\)-Fe\(_2\)O\(_3\), there is production of IR peaks at 1170 \(\nu(C-O)\), 1052 \(\nu(C-O, C-O-C)\), and 1039 \(\nu(P-O-Fe)\). There is also a sizable reduction in relative contributions from the 1081 cm\(^{-1}\) \(\nu_s(\text{PO}_2^-),\) sugar ring vibrations] peak.
Figure 6. ATR-FTIR spectra of *S. oneidensis* MR-1 on ZnSe and α-Fe$_2$O$_3$-coated ZnSe at 24 h (oxic conditions).

Figure 7. ATR-FTIR spectra of *S. oneidensis* MR-1 on ZnSe and α-Fe$_2$O$_3$-coated ZnSe at 48 h (anoxic conditions, oxic from 0-24 h).
New peaks, corresponding to Fe-O vibrations (750-900 cm\(^{-1}\)), resulting from Fe\(^{III}\) are not observed in ATR-FTIR spectra presented in Figure 5. In Figure 5 some the presence of small peaks from 800 to 825 cm\(^{-1}\) likely represent \(\nu\)(Fe-O), but have a poor signal to noise ratio. The background \(\alpha\)-Fe\(_2\)O\(_3\)-coated ZnSe has been subtracted from this figure.

Figure 8, represents the region of 750 to 1000 cm\(^{-1}\) for the 258 h anoxic flow experiment of MR-1 cells to \(\alpha\)-Fe\(_2\)O\(_3\)-coated ZnSe. Good resolution of these peaks is observed (\(\alpha\)-Fe\(_2\)O\(_3\) not subtracted). In this figure the peaks at 800 and 894 cm\(^{-1}\) correspond to \(\nu\)(Fe-O), and the peak at 969 cm\(^{-1}\) results from \(\nu\)(PO\(_4^2-\)). Although small to begin with, the peaks at 894 and 969 cm\(^{-1}\) disappear with increased time.

Figure 8. ATR-FTIR spectra of \textit{S. oneidensis} MR-1 on \(\alpha\)-Fe\(_2\)O\(_3\)-coated ZnSe (anoxic for entire experiment).
Absorbance intensity reduction of peaks at 894 and 969 cm\(^{-1}\) is best observed via a plot of absorbance intensity versus time (Fig. 9). Within the first 50 h these peaks are reduced to near zero absorbance. There is a large amount of variation for IR absorbance of the 800 cm\(^{-1}\) peak, whereas peak intensities for \(\nu(\text{PO}_2^\cdot)\) decrease, but do not fluctuate.

![Graph showing absorbance intensity reduction over time](image)

**Figure 9.** IR peak absorbance from ATR-FTIR spectra of *S. oneidensis* MR-1 on \(\alpha\)-Fe\(_2\)O\(_3\)-coated ZnSe (anoxic for entire experiment).

**Discussion**

The presence of heme proteins on the surface of *S. oneidensis* MR-1 is well documented (Dichristina et al., 2002; Meyer et al., 2004; Mowat et al., 2004). Changes from oxic to anoxic conditions (and vice versa) resulting in a dramatic color change of the cell suspension are likely caused by changes in heme group conformation. However, it appears that this conformational change is not detected via ATR-FTIR (Fig. 4 and 5). The presences of numerous other surface proteins are likely preventing observable changes in the IR spectra.
The relative increase of protein contributions to spectra for MR-1 adhesion to α-Fe₂O₃-coated ZnSe is somewhat surprising and in contrast to previous findings (Appendix B), where a reduction in protein content was observed when bacteria were reacted with a α-Fe₂O₃ surface. In this case it appears that surface proteins are playing an important role in cell adhesion to the α-Fe₂O₃ surface. One possible reason is that the α-Fe₂O₃-coating on the cylindrical IRE may be somewhat patchy (an artifact from different coating procedures; flow versus deposition). If uncoated portions of the ZnSe IRE are present surface proteins may be interacting strongly at these sites. Consistent with the finding presented in Appendix B, is the presence of Fe-O-P (1039 cm⁻¹) in the IR spectra (Fig. 6 and 7). Peaks in this region (~1030 to 1045 cm⁻¹) have been attributed to inner sphere monodentate complexes (possibly, bidentate bridging) arising from glycophosphate and aminomethylphosphonic acid adsorption to goethite (Sheals et al., 2002; Barja and Afonso, 2005). These results are also in agreement with the interaction of extracted extracellular polymeric substances with a goethite (α-FeOOH) surface (Omoike et al., 2004).

It was hypothesized that Fe³⁺-reduction would lead to noticeable changes in the IR spectra. Unfortunately ATR-FTIR spectroscopy does not permit data collection at low wavenumbers that are diagnostic for goethite (~470 and 540 cm⁻¹). However, other peaks corresponding to ν(Fe-O) are visible in the spectra. The results reveal no new peaks corresponding to ν(Fe-O) for all experiments. However, for the 258 h anoxic experiment there appears to be a slight reduction in the ν(Fe-O) peak located at 894 cm⁻¹ (Fig. 8). This, along with the fluctuation of IR peak intensities for ν(Fe-O) (800 and 894 cm⁻¹),
may indicate changes in the Fe-oxide coating with time. However without further experiments this remains speculative.

Summary

Flow experiments with *S. oneidensis* MR-1 lead to significant biomass accumulation on cylindrical ZnSe and α-Fe₂O₃-coated ZnSe surfaces. IR peaks corresponding to amide groups are dominant in all ATR-FTIR spectra, and implicate proteins as playing an important part of MR-1 adhesion to these surfaces. Adhesion of MR-1 to α-Fe₂O₃-coated ZnSe resulted in peaks attributed to Fe-O-P bond. Likely these peaks arise from the interaction of phosphodiesters with α-Fe₂O₃. Under anoxic conditions, the disappearance of a Fe-O peak (894 cm⁻¹) and the oscillation in absorbance intensity for another Fe-O peak (800 cm⁻¹) may indicate Fe³⁺ reduction.

References


APPENDIX F

DEVELOPMENT OF EXPERIMENTAL TECHNIQUES FOR
INVESTIGATIONS OF LIPOPOLYSACCHARIDES: LPS EXTRACTION,
CRITICAL AGGREGATE CONCENTRATION, AND THE USE OF FTIR FOR
COMPARING ROUGH AND SMOOTH LPS

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The material in this appendix includes studies of LPS that were not included in published manuscripts. The work is included in this dissertation as it provides some valuable preliminary data that could be used for additional studies of LPS. Many of the measurements involving LPS aggregation were performed by David A. White III (with supervision from S.J. Parikh).

Introduction

Gram-negative cells have lipopolysaccharides (LPS) anchored to cells with the lipid portion imbedded in the outer membrane. The polymers extend from the bacterial surface and may adhere to environmental surfaces. (Jucker et al., 1998) have shown that short-range interactions of LPS with solid surfaces contribute to bacterial adhesion. Bacterial LPS can be in either the “smooth” or “rough” form (Appendix B, Fig. 1), respectively). The O-specific polysaccharide (O-antigen) is not present for LPS in the rough form. The smooth form contains an O-antigen (20 to 70 repeating units of three to five sugars) protruding up to 30 or more nanometers from the cell surface. The core region of LPS (present in both the rough and smooth form) consists of five to ten negatively charged sugar units (Jucker et al., 1997). Due to the extension of the LPS with the O-antigen, LPS in the smooth form is likely responsible for interactions of gram-negative bacteria and surfaces (Jucker et al., 1997; Makin and Beveridge, 1996). “Deep rough” LPS present on some Gram negative bacteria, lack both the O-antigen and the outer core region.

As a result of cell turnover and lysis, LPS occurs in both “cell-bound” and “free” forms in natural aquatic systems (Rietschel et al., 1994). In the case of free LPS, exposure of the lipid A (hydrophobic region) is limited by LPS amphiphilic properties that promote intermolecular associations and the formation of supramolecular structures.
above a critical aggregation concentration (CAC; Figure 1) (Aurell et al., 1999; Santos et al., 2003; Seydel et al., 1993). Due to the prevalence of LPS in the environment it is important to understand their behavior in soil and aquatic environments.

![Figure 1. Schematic diagram illustrating the process of LPS aggregation.](image)

The critical aggregate concentration of LPS is strongly influenced by the presence/length the O-antigen. Resulting typical CAC values for rough LPS (~ 1 to 5 mg L\(^{-1}\)) are lower than for smooth LPS (~10 to 35 mg L\(^{-1}\)) (Aurell and Wistrom, 1998). It is therefore expected that the behavior of rough and smooth LPS in aquatic systems will be quite different.

Development of an analytical technique to identify the presence of rough or smooth LPS from extracted LPS is desirable. Comparison of relative contributions of fatty acids to polysaccharides of LPS samples can be used to determine the presence, or extent of, O-antigen present. Since little variation of the lipid A region (Appendix B, Fig. 1) is observed for different bacteria and forms of LPS (Kastowsky et al., 1992; Seydel et al., 1993), changes in the ratio of fatty acids to polysaccharides represent varying amounts of O-antigen present.
Transmission Fourier transform infrared (FTIR) spectroscopy is a bulk sampling technique, where IR light passes through the sample providing information corresponding to the entire sample composition. For this reason it was chosen to investigate contributions arising from the lipid A and O-antigen regions of selected LPS.

The research presented in this appendix evaluates the CAC for selected smooth LPS. Also included are preliminary results of selected rough and smooth LPS that compare ratios of lipid A and polysaccharide absorbing moieties.

**Methods**

**Extraction of LPS from Bacterial Cells**

Extraction of LPS first involves removal of extracellular polymeric substances (EPS) from cell surfaces. This is done using a method adopted from Chen and Strevett (2001). Cells are harvested from the cultures at the stationary phase of growth by centrifugation (5,000 RCF, 10 min, 4°C). The loosely bound EPS is removed from the cell during this step. Next, a 0.1 mol L⁻¹ solution of NaCl is used to wash the precipitated cells. To extract the bound EPS 0.1 mol L⁻¹ NaCl is added to cells and solution heated at 30°C for 1 h. The solution is centrifuged to precipitate EPS-free cells.

Extraction of smooth LPS is performed using standard methods (hot water/phenol extraction) (Hancock and Poxton, 1988). Phenol (65°C) is added to EPS-free cells suspended in Barnstead nanopure (BNP) water (65°C) and stirred vigorously for 15 min. The solution is cooled to 10°C and centrifuged (5,000 RCF, 10 min), and the water layer is saved. Fresh water is added to the phenol residue and the process repeated. The water
layer, containing the LPS, is dialyzed against Milli-Q water for 4 days (water change twice daily). Purification continues by centrifugation to remove any insoluble deposits (10,000 RCF, 15 min). Rotary evaporation is used to reduce the volume. Collection of LPS is performed by high-speed centrifugation (100,000 RCF, 3 h). The pelleted LPS is then freeze-dried for experimental use.

Rough LPS was extracted via the phenol-chloroform-petroleum spirits method (Hancock and Poxton, 1988). The extraction solvent is comprised of 90% phenol, chloroform, and petroleum spirits in the proportion of 2:5:8 (by volume). Freeze-dried cells (5 g with EPS removed) are added to 20 mL of extractant solvent, stirring below 20°C for 2 minutes. Solution is then centrifuged (Teflon tube) at 10,000 RCF for 15 min, followed by filtering supernatant into a round bottom flask through Whatman No. 1 filter paper. This extraction process is repeated on the pellet. Chloroform and petroleum are removed by rotary evaporation. After removal of chloroform and petroleum spirits, water is added (dropwise) until LPS precipitates. No more water should be added when LPS starts to settle on standing 1-2 minutes. Solution is then centrifuged 5,000 RCF for 10 min. Supernatant is removed the pellet is washed 2 times with 80% phenol, centrifuge at 5,000 g for 10 minutes. The final pellet is washed twice with ether and dried under vacuum. The dry material is taken up in BNP water using a 23 gauge needle. Finally the solution is centrifuged at 100,000 RCF for 4 h and pellet freeze-dried.
Determination of Critical Aggregate Concentration (CAC)

CACs for LPS were measured by employing electrical conductivity (EC) and ultraviolet visible spectroscopy (UV-Vis, with 8-anilino-1-naphthalensulfonic acid), methods previously used to study lipids. The EC methods are similar to published techniques (Prieto et al., 1994). The EC probe is submersed in a sample vial containing 12 mL BNP water with continuous stirring and 100 to 200 µL aliquots of LPS solution are added. After each aliquot the solution is stirred for 10 min. The amount of LPS solution added and EC values are recorded. This process was repeated until a desired concentration of LPS is reached within the sample vial (concentration greater than the CAC).

The CAC was determined by plotting the concentration of LPS versus its corresponding EC. Increased concentrations of LPS increase EC values. Aggregation of LPS results in a reduction in the rate at which conductivity increases. Therefore, the point where a change in slope occurs on an EC versus LPS concentration plot represents the CAC.

Determination of CAC via UV-vis spectroscopy using 8-Anilino-1-naphthalenesulfonic acid (ANS) follows the methods of Beyaz et al. (2004). A 1 mmol L⁻¹ solution of ANS was prepared in BNP water and stirred for 30 min. Measurements were determined by adding 50 µL aliquot of 1mM ANS prepared in BNP water to the sample cuvette containing 3 mL of BNP water. The same ratio of dye to water added in the sample cuvette was also added to the stock solution of LPS. All spectra were collected from 200 to 400 nm. LPS was added in 15 to 25 µL increments to the sample cuvette, and
mixed thoroughly. The sample was then allowed to equilibrate for five min and absorption spectra collected. This process was repeated until the desired final concentration of LPS was reached (concentration greater than the CAC). These measurements were also performed in NaCl (10, 100, and 1000 µmol L\(^{-1}\)) at pH values of 3, 6, and 9 to observed changes in CAC as a function of solution chemistry.

The absorbance spectra for LPS with no ANS added reveals maximum absorbance at 270 nm and as the concentration of LPS is increased the absorbance also increases in a linear fashion. When ANS is added to LPS solutions the maximum absorbance is present at 360 nm. Plotting the concentration of LPS with its corresponding absorbance at 360 nm allows determination of the CAC. The point on this plot with the greatest increase in absorbance value (change in slope) is the CAC.

**Transmission FTIR Analysis of LPS**

Both purchased and extracted LPS were used for transmission FTIR analysis (See Appendix A, Fig 1 for technique illustration). Smooth LPS studied include *Pseudomonas aeruginosa* ser 10 (Sigma), *P. aeruginosa* PAO1 (extracted), and *Escherichia coli* JM109 (extracted). The rough LPS examined were from *E. coli* D21 (extracted) and *Salmonella typhimurium* TV119 (Sigma). The deep rough LPS used originate from *S. minnesota* Re595 (Sigma).

LPS solutions (1 mg mL\(^{-1}\) in BNP water) were sonicated for 10 min. 100 µL of each solution was deposited onto ZnSe transmission windows and dried under vacuum
(340 mbar) for 24 h. Transmission spectra were collected with 400 scans at a 4 cm\(^{-1}\) resolution.

**Results and Discussion**

**Aggregation of Lipopolysaccharides**

CAC values for smooth LPS extracted from *Pseudomonas aeruginosa* (ser 10), *P. aeruginosa* (PAO1), and *Escherichia coli* (JM109) in BNP water using both EC and UV-vis techniques are displayed in Table 1.

<table>
<thead>
<tr>
<th>LPS</th>
<th>CAC (mg/L)</th>
<th>EC</th>
<th>UV-vis</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109</td>
<td>2.63 ± 0.25</td>
<td>3.11 ± 0.88</td>
<td></td>
</tr>
<tr>
<td>Ser 10</td>
<td>12.7 ± 0.47</td>
<td>13.2 ± 0.88</td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>6.89†</td>
<td>6.06†</td>
<td></td>
</tr>
</tbody>
</table>

† based on one observation

Ser 10 LPS was studied in further detail using the UV-vis method to examine the effect of solution chemistry on the aggregation process. Using NaCl, \(I\) was varied (10, 100, 1000 µM) and pH (3, 6, and 9) and measured the CAC values. A typical plot of LPS concentration vs UV absorbance is shown in Figure 2 (*P. aeruginosa* ser 10 LPS, 100 µmol L\(^{-1}\) NaCl, pH 3). The CAC is determined using STATA software (ver 9.0) using breakpoint regression for two linear regions to determine the point of inflection (Jones and Molitoris, 1984). This analysis gives one \(R^2\) value (0.9578) and one RMSE value (0.0003) indicating the goodness of fit for the breakpoint at 5.65 mg L\(^{-1}\) LPS.
Figure 2. Break point regression analysis for *P. aeruginosa* ser 10 LPS in 100 µmol L⁻¹ NaCl at pH 3.

Figure 3 shows the results from UV-vis CAC measurements for *P. aeruginosa* ser 10 LPS. Increasing the I decreases the CAC. A near linear trend is observed for LPS in H₂O, where CAC increases with increasing pH. The same trend is observed for LPS solution in NaCl when increasing from pH 3 to pH 6, however there is a decrease in the CAC for pH 9. Although the results shown in Figure 3 have a small degree of error, the statistical analysis using breakpoint regression analysis did reveal some uncertainty in CAC values for some samples. Further investigations and analysis are required to verify the validity of these results.
Increasing the ionic strength of aqueous solutions has been shown to decrease the CAC of ionized amphiphilic compounds (Elworthy et al., 1968; Lindman and Wennerström, 1980; Tanford, 1973). The same relationship is observed here with ser 10 LPS. The repulsive forces between the O-antigen components of LPS molecules (via electrostatic interactions) are lowered by the charge-screening action of the counterions. The CAC is therefore decreases as less energy is required for micelle formation. The pH of the aqueous solution also affects the aggregation of LPS by changing the charge properties of the O-antigen. Upon protonation of ionizable functional groups (decreased pH), coiling of the LPS molecule occur and a relative decrease in the size of the O-antigen takes place. The coiling results in LPS molecules having a greater relative contribution from the lipid A and thus more hydrophobic, leading to increased aggregation (decreased CAC). It is not clear why a decrease in CAC is observed when the
pH is increased from 6 to 9. It is possible that at high pH, elongation of the O-antigen occurs allowing tighter packing and less steric interactions of LPS monomers, thus lowering the CAC.

**Transmission FTIR for Determination of Lipopolysaccharide Composition**

Transmission FTIR results for selected LPS are shown in Figure 4. The spectra reveal higher IR absorbance values for polysaccharides in smooth LPS samples. This is consistent with increased O-antigen contributions for smooth LPS. The spectra for the rough and deep rough LPS are dominated by peaks corresponding to fatty acid (CH$_2$ and CH$_3$) – representative of the lipid A region.

![Figure 4. Transmission FTIR spectra of smooth, rough, and deep rough LPS.](image-url)
Quantification of contributions arising from lipid A and O-antigen regions of LPS can be made through comparison of peak intensity and peak area ratios for the fatty acid and polysaccharide regions of the spectra. Figure 5 shows IR peak absorbance ratios for fatty acids (2923 cm$^{-1}$) to polysaccharides (1074 cm$^{-1}$).

Figure 5. IR peak absorbance ratios of fatty acid to polysaccharide regions of transmission spectra for selected LPS samples (smooth LPS: Ser 10, PAO1, JM109; rough LPS: D21, TV119; deep rough LPS: Re595).

Peak areas were determined by integrating peak areas corresponding to fatty acids (2800-3000 cm$^{-1}$) and polysaccharides (984-1184 cm$^{-1}$). The results of this integration and the area ratios of these regions are shown in Figure 6.
Figure 5. IR peak area ratios of fatty acid to polysaccharide regions of transmission spectra for selected LPS samples (smooth LPS: Ser 10, PAO1, JM109; rough LPS: D21, TV119; deep rough LPS: Re595).

Results for peak area and peak intensity ratios are similar. Trends do generally confirm decreased polysaccharide contributions for rough LPS. For both quantification methods ratios of JM109 (smooth LPS) and D21 (rough LPS) are approximately the same. These strains of *E. coli* have been documented to have different lengths of LPS (Boman and Monner, 1975; Burks et al., 2003) corresponding to rough and smooth LPS, contradicting the results shown here. The differences may result from LPS extraction procedures.

This data suggests that transmission FTIR can be used to evaluate the relative contributions arising from the O-antigen and lipid A region of LPS. However, additional
samples should be examined of known smooth and rough LPS, with precise information regarding the length of the O-antigen region to develop a system for estimating contributions of O-antigens to LPS via FTIR analysis.

Summary

The results presented in this appendix provide preliminary data for additional evaluation of physicochemical properties of bacterial LPS. The UV-vis spectroscopy technique needs further refinement to more accurately determine inflections points corresponding to the CAC. However, once this technique is determined reliable it can provide information regarding the CAC for LPS under a variety of different liquid environments. Directly related to the CAC, is the contribution of O-antigen to LPS monomers. Transmission FTIR has shown to be a useful tool for evaluating the relative contributions of lipid A and O-antigen regions of LPS, which can directly be related to LPS size and CAC values.

References


APPENDIX G

IN SITU MONITORING OF PSEUDOMONAS AERUGINOSA BIOFILM GROWTH

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The work presented in Appendix G served as preliminary data for much of the research performed for this dissertation. The results are presented here as they do provide meaningful results, consistent with the major findings of there research presented in this dissertation.

**In Situ Monitoring of Pseudomonas aeruginosa Biofilm Growth**

Strains of *Pseudomonas aeruginosa*, PAO1 (wild type) and PDO300 (mutant, EPS over-producer), were used to compare bacterial adhesion based on the quantity of extracellular polymeric substances (EPS). *In situ* ATR-FTIR flow cell experiments were conducted using *P. aeruginosa* (PAO1 and PDO300). The methods used for data collection are rudimentary versions of the methods used to study *P. putida* (GB-1) (Appendix A). A Virtis Omni Culture Plus bioreactor (Gardiner, NY) was used to maintain constant temperature (30°C), pH (7.0), mixing (100 rpm) and aeration over the course of the experiment. The bioreactor (1.8 L of Luria broth) was inoculated with 15 mL of preculture harvested at the early stationary phase of growth. Influx of fresh media to the bioreactor (beginning at t = 0h) was equivalent to cell suspension efflux (0.25 mL/min).

Adhesion of bacterial cells and biofilm growth on a Ge ATR crystal were observed for 120 h. Figure 1 compares the spectra of PAO1 at 480 and 5880 min, showing a large increase in the amide II region of the spectra (1550 cm⁻¹), representing protein adhesion to the Ge surface. Other increases in peak intensities are seen in the CH₂ and CH₃ symmetric and asymmetric stretch regions. Based on peak intensities the results for PDO300 reveal less adhesion of *P. aeruginosa* (Figure 2), despite the fact that it is the
EPS over producer. This suggests that EPS may not enhance (i.e., may hinder) initial adhesion to a negatively charged surface (i.e., Ge).

**Figure 1.** IR Spectra of PAO1 adhesion to Ge crystal at 480 and 5880 min.

**Figure 2.** IR Spectra of PDO300 adhesion to Ge crystal at 495 and 5735 min.
Figure 3. IR Spectra of adhesion kinetic to GE ATR crystal for a) PAO1 and b) PDO300.
Figure 4. a) IR Spectra of PDO300 adhesion to Ge crystal at 495 and 5735 min; b) IR Spectra of PDO300 adhesion to Ge crystal at 495 and 5735 min.

The increase in peak intensities is much more noticeable for PAO1 and little increase of the amide II peak in PDO300 is observed (Figure 3). Peak oscillations are
attributed to the variations in cell density (Figure 4) resulting from daily temperature fluctuations that will be eliminated in future experiments. Figures 3a and 4a shows a general trend of increased intensities for PAO1, particularly for the amide I (1650 cm$^{-1}$) and amide II (1550 cm$^{-1}$) regions of the spectra. PDO300 intensities do not increase significantly, which is consistent with less adhesion (Figures 3b, 3b).

The over production of EPS (ionic biopolymer, predominately negatively charged at pH 7) by PDO300 reduces the initial attachment of \textit{P. aeruginosa} to the Ge substratum. Attachment of \textit{P. aeruginosa} can be monitored by ATR/FTIR, particularly by observing absorbance at 1550 cm$^{-1}$, indicating the attachment of proteinaceous material. For PAO1, increased cell density is well correlated with increased IR absorbance of bacterial surface functional groups. An inverse relationship is observed between absorbance from cell adhesion and those from water hydrating the Ge crystal surface (indicate pertinent water absorption frequencies) that suggests that hydration water must be displaced during EPS sorption to the crystal surface.

The preliminary work presented here indicates that real-time monitoring of microbial adhesion and biofilm formation can be observed using ATR-FTIR spectroscopy. The data demonstrated an important role of bacteria surface proteins in adhesion to a negatively charged substratum (i.e., Ge). One shortcoming of this set of experiments is that adhesion was monitored with bacteria in Luria broth. This rich media is not representative of natural waters and therefore not representative of environmental conditions. This research was used as major foundation for developing methods and hypothesis for future experiments.
APPENDIX H

ATTENUATED TOTAL REFLECTANCE FOURIER TRANSFORM INFRARED SPECTROSCOPY

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Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy

Most microbial methods for investigating properties of microorganisms are destructive and not appropriate for studying bacteria and biofilm characteristics. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy is a tool that provides nondestructive in situ studies of bacteria and microbial biofilms (Nichols et al., 1985) Nivens (Nivens et al., 1993a) Schmitt (Schmitt et al., 1995) Schmitt (Schmitt and Flemming, 1998). ATR-FTIR spectra provide information about functional groups near the surface (~ 1 µm) of an internal reflection element (IRE) (Nivens et al., 1993a). ATR-FTIR techniques are particularly useful in examining biofilm structure where amide and carbohydrate groups are important (Nichols et al., 1985). FTIR spectroscopy uses polychromatic radiation to measure the excitation of molecular bonds whose relative absorbances provide an index of the abundance of various functional groups (Griffiths and Haseth, 1986). Since it can be used to probe distinct vibrations arising from both biomolecules and inorganic solids, it is emerging as a useful tool for investigating processes at the bacteria-mineral and biomolecule-mineral interface (Deo et al., 2001; Benning et al., 2004; Omoike et al., 2004).

Water absorbs strongly (1640 cm⁻¹ and 3300 cm⁻¹) in the mid infrared range, common to vibrational frequencies of many biomolecule functionalities (Suci et al., 1998). ATR-FTIR avoids the masking of signals by water by sending IR light through a highly refractive index prism (IRE). The refracted IR light travels beyond the IRE surface in evanescent waves, probing the solid-liquid interface without penetrating into the bulk solution (Suci et al., 1998).
ATR-FTIR is limited in that only a few crystal materials can be used effectively (e.g., ZnSe, Ge). The IRE has a high refractive index and infrared light passing through the crystal is refracted within the crystal. The light traveling from the optically dense medium (IRE) into the rare medium (bacteria/liquid medium) will totally reflect at the interface if the angle of incidence is greater then the critical angle (Chittur, 1998a). The critical angle is calculated using the following equation:

\[
\theta_{\text{critical}} = \sin^{-1} \frac{\text{refractive index of rare medium}}{\text{refractive index of dense medium}}
\]  

where \( \theta \) is the incident angle (Chittur, 1998b). A high refractive index of the IRE and increasing \( \theta \) will result in a decreased depth of penetration (Schmitt and Flemming, 1998). The IR beam is able to penetrate into the rare medium (biofilm) allowing a sample spectrum from the thin layer attached the crystal surface (Schmitt and Flemming, 1998).

The refractive index (RI) of the IRE \((n_1)\) and sample \((n_2)\) governs the depth of beam penetration. The depth of penetration \((d_p)\) is calculated using the following equation (Mirabella, 1985):

\[
d_p = \frac{\lambda}{2\pi \left[ \left( \sin^2 \theta \right) - \left( \frac{n_1}{n_2} \right)^2 \right]^{1/2}}
\]  

where \( \lambda \) is the wavelength (cm) and \( \theta \) is the angle of incidence. The intensity of reflected light traveling through the IRE will be reduced with interactions with IR absorbing material in the rare medium (Chittur, 1998b). The IR light is absorbed by bacteria on the
surface and the IR detector will record the amount of light absorbed from the original IR source, thus producing IR absorption bands and an IR spectrum (Nivens et al., 1993a). A schematic representation of this process is shown in Figure 1.

![Figure 1](image)

**Figure 1.** Schematic diagram illustrating the basic concepts of ATR/FTIR spectroscopy. IR light passes through the crystal, reflecting just beyond the surface, measuring the IR absorbance of material touching the surface.

Figure 1 shows ATR-FTIR in a flow cell, however this technique can also be used using an ARK cell where a material is placed on trapezoidal crystal and observations are made in regard to the composition of the material at IRE surface. As shown in equation 1, the depth of penetration is also dependent on $\theta$. Variable angle ATR (VATR)-FTIR permits depth-profiling of samples at the IRE-liquid interface. By varying $\theta$ of the IR beam into the sample, the $d_p$ into IR absorbing moieties is altered. This technique gives information about the spatial arrangement of samples at the IRE interface on small length scales. The effective angle of incidence ($\theta_{\text{eff}}$) is determined using the following equation (Pereira and Yarwood, 1994):
\[ \theta_{\text{eff}} = \theta_{\text{fix}} - \sin^{-1}\left(\frac{\sin(\theta_{\text{fix}} - \theta_{\text{var}})}{n_1}\right) \]  

where \( \theta_{\text{fix}} \) is angle of the crystal face (commonly 45°), and \( \theta_{\text{var}} \) is the scale angle set on the VATR accessory. As an example of how \( d_p \) varies as a function of \( \theta_{\text{eff}} \) the depth of penetration for bacteria on a ZnSe IRE is shown in Figure 2. The RI of ZnSe \( (n_1) \) is 2.4, and the RI of bacterial cells (sample, \( n_2 \)) is commonly reported as 1.38 (Jonasz et al., 1997; Katz et al., 2003).

![Figure 2](image-url)

**Figure 2.** Depth of penetration vs. wavenumber showing how angle of incidence affects depth of penetration for VATR-FTIR spectroscopy (ZnSe IRE).

Interpretation of ATR-FTIR spectra requires a thorough review of previous studies. Researchers have determined assignments for characteristic bands of biofilms and bacterial samples, which can be used to aid in band assignment (Table 1).
Table 1. Pertinent IR assignments for bacteria samples

<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>IR Band Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1720-1729</td>
<td>(\nu_{as}(\text{COOH})^a)</td>
</tr>
<tr>
<td>1652-1637</td>
<td>Amide I: C=O, C-N, N-H(^{b-d})</td>
</tr>
<tr>
<td>1550-1540</td>
<td>Amide II: N-H, C-N(^{a-c})</td>
</tr>
<tr>
<td>1460-1454</td>
<td>(\delta (\text{CH}_2)^{a,c})</td>
</tr>
<tr>
<td>1400-1390</td>
<td>(\nu_{as}(\text{PO}_2^-)^{a,c})</td>
</tr>
<tr>
<td>1220-1260</td>
<td>(\nu_{as}(\text{PO}_2^-)) of phosphodiesters (hydrated)(^{a,c,f})</td>
</tr>
<tr>
<td>1170</td>
<td>(\nu(\text{C-O})^{d})</td>
</tr>
<tr>
<td>1114-1118</td>
<td>(\nu(\text{C-O-P, P-O-P}), \text{Ring vibrations}^{a,d})</td>
</tr>
<tr>
<td>1084-1088</td>
<td>(\nu_{s}(\text{PO}_2^-)) of phosphodiester(^{i}), \text{ring vibrations}^{a,\nu(\text{C-C})^{g}})</td>
</tr>
<tr>
<td>1045, 1052, 1058</td>
<td>(\nu(\text{C-O-C, C-C})) from polysaccharides(^{a,g})</td>
</tr>
<tr>
<td>962-968</td>
<td>(\nu(\text{PO}_2^-)^{h,i})</td>
</tr>
<tr>
<td>650-450</td>
<td>(\nu(\text{CH}_2)^f)</td>
</tr>
</tbody>
</table>

\(^{a}\text{Brandenburg and Seydel (1996)}, \; ^{b}\text{Schmitt and Flemming (1998)}, \; ^{c}\text{Sockalingum et al. (1997)}, \; ^{d}\text{Nivens et al. (1993b)}, \; ^{e}\text{Brandenburg et al. (1997)}, \; ^{f}\text{Naumann et al. (1991)}, \; ^{g}\text{Fringeli and Günthard (1981)}, \; ^{h}\text{Barja et al. (1999)}, \; ^{i}\text{Quiles et al. (1999)}, \; ^{j}\text{Deo et al. (2001)}.\)

References


APPENDIX I

REPRODUCIBILITY OF ATTENUATED TOTAL REFLECTANCE FOURIER TRANSFORM INFRARED SPECTROSCOPY

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Reproducibility of Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy

This dissertation relies on attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy as the primary analytical tool for investigating bacterial adhesion and the behavior of lipopolysaccharides (LPS) at the solid-solution interface. Replicate ATR-FTIR samples were acquired in many cases to ensure reproducibility of spectra. However, averaging multiple spectra is not appropriate as absolute baseline and peak intensity values are not always constant due to slight differences in instrument (e.g., alignment, age of source) and sample (e.g., concentration, pH) variation.

The reproducibility of ATR-FTIR spectra can be observed qualitatively from the data presented throughout this dissertation. For example, the high degree of similarity between spectra bacteria acquired on hematite indicates that repeated sample collection would result in similar results (Appendix B, Fig. 4c). This can also be inferred by observation of VATR data for LPS on both ZnSe and Ge IREs (Appendix D, Fig. 9). These spectra are acquired represent different samples of the same concentration and solution chemistry prepared independently of one another. Although samples are collected at different angles of incidence the resulting spectra are similar, and differences result only from variation in depth of penetration.

To confirm sample reproducibility it is not appropriate to only compare similarities between spectra of slightly different samples. In Appendix B, it is presented that the ribose ring of DNA is oxidized via interaction with nanohematite (α-Fe₂O₃) leading ring cleavage and the production of an IR peak corresponding to ν₅(COO⁻). The
spectrum of *Escherichia coli* DNA on $\alpha$-Fe$_2$O$_3$ is distinctly different from spectra collected on ZnSe and corundum ($\alpha$-Al$_2$O$_3$) (Appendix B, Fig. 10). To demonstrate this as a reproducible result, spectra were acquired numerous times. Figure 1 is presented to show that multiple experiments, with replicate samples of DNA, produce the same results. These spectra are very similar to one another and in each case production of a large peak corresponding to $\nu_s$(COO') is observed.

![Figure 1. Replicate ATR-FTIR spectra of *E. coli* DNA on $\alpha$-Fe$_2$O$_3$. Spectra represent different samples of DNA reacted with pristine $\alpha$-Fe$_2$O$_3$ coatings collected after 240 min.](image)

In the case of *Pseudomonas aeruginosa* serotype 10 LPS, a great deal of importance has been placed on the relative differences between spectra as a function of ionic strength ($I$), pH, and electrolyte composition. Confirming the reproducibility of spectra presented in Appendices C and D is therefore very important. Figure 2 presents
spectra from Appendices C and D to demonstrate that reproducibility is achieved not only between samples of similar solution chemistries, but that it also transcends different ATR-FTIR sample collection methods. The spectra for samples collected using the ARK and VATR accessories (both ATR-FTIR) of the same solution chemistries have been paired with one another to qualitatively confirm reproducibility of sample collection.

Figure 2. ATR-FTIR spectra of *P. aeruginosa* ser 10 LPS with *I* = 10 mM, pH 6, in both NaCl and CaCl₂ collected on ZnSe and GeO₂ IRE using the ARK and VATR accessories. Spectra are paired based on solution chemistry and IRE material to demonstrate data reproducibility between samples and data collection techniques (non common scale).
Based on my experience collecting ATR-FTIR spectra for a variety of samples and using different collection techniques I believe that with careful experimental techniques the acquired spectra have a high degree of reproducibility. I have presented examples of spectra in this appendix to demonstrate this. The trends in sample reproducibility extend beyond the few cases presented here and apply to all spectra presented in this dissertation.
APPENDIX J

THE DLVO THEORY OF COLLOID STABILITY

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The DLVO Theory of Colloid Stability

Classical Derjaguin-Landau-Verwey-Overbeek (DLVO) theory is used to describe colloidal interactions, accounting for the combination of repulsive and attractive forces (Stumm and Morgan, 1996). The theory was developed independently by two research groups (Derjaguin and Landau, 1945; Verwey and Overbeek, 1948; Stumm and Morgan, 1996). The DLVO theory states that adhesion is controlled by long-range interactions between the adhering particle and the macroscopic substratum (Poortinga et al., 2002). According to the theory the total energy of adhesion ($V_T$) results solely from van der Waals interactions ($V_W$) and diffuse double layer repulsions ($V_R$) (Rijnaarts et al., 1995; Stumm and Morgan, 1996; Oliveira, 1997; Hermansson, 1999; Poortinga et al., 2002):

$$V_T = V_W + V_{DL}$$  \[1\]

$V_W$ is defined by:

$$V_W = -\frac{Ar}{6d}$$  \[2\]

where $A$ is the Hamaker constant, $d$ is the distance between the two surfaces, and $r$ is the radius of the adhering particle. The force of adhesion due to the diffuse double layer interactions ($V_{DL}$) originates from the Coulomb interaction between charged molecules (Hermansson, 1999). $V_{DL}$ can be simply stated as:

$$V_{DL} \propto \Psi^2 e^{-\kappa d}$$  \[3\]
where \( \Psi \) represents the surface potential and the Debye length is \( \kappa \) (reciprocal double layer thickness). If the surface potential is different for the two surfaces then \( \Psi^2 = (\Psi_1 + \Psi_2)^2 \). Equation 3 can be given in more detail and for cases where the potential energies of interaction are at constant potential (\( V_{\text{DL}}^\Psi \)) and at constant charge (\( V_{\text{DL}}^\sigma \)) by:

\[
V_{\text{DL}}^\Psi = \varepsilon \pi r \left( (\Psi_1 + \Psi)^2 \ln |1 + e^{-\kappa d}| + (\Psi_1 - \Psi)^2 \ln |1 - e^{-\kappa d}| \right) \tag{4}
\]

\[
V_{\text{DL}}^\sigma = -\varepsilon \pi r \left( (\Psi_1 - \Psi)^2 \ln |1 + e^{-\kappa d}| + (\Psi_1 + \Psi)^2 \ln |1 - e^{-\kappa d}| \right) \tag{5}
\]

where \( \varepsilon \) is the electrical permittivity of the medium.

The DLVO theory has been shown to be less accurate in explaining the coagulation of colloids in the presence of divalent ions (Sposito, 1989; Stumm and Morgan, 1996). Also at distances below 5 nm the DLVO theory fails (Sposito, 1989), apparently due to non-DLVO forces such as solvation, and capillary effects (Stumm and Morgan, 1996).

Bacterial adhesion has been thought to be governed by forces covered by the DLVO theory (Hunter 1989; Israelachvili 1992). However, when using this theory to describe bacterial adhesion, surfaces are treated as homogenous and non-biological, resulting in a poor explanation of bacterial adhesion (Hermansson, 1999; Poortinga et al., 2002). It is important to note that DLVO is a “mean-field” theory that does not account for molecular scale heterogeneity and patchiness, both of which are important in bacterial adhesion. DLVO theory does not take into account short-range interactions that are involved in adhesion and polar interactions (e.g. hydrophobic interactions) (Azeredo et al., 1999). The extended DLVO (XDLVO) theory has been proposed to describe bacterial
adhesion, accounting for short-range interactions (van Oss, 1994). The XDLVO theory goes beyond the classical DLVO theory by including polar (acid/base) interactions ($V_p$):

$$V_T = V_w + V_{DL} + V_p$$  \[6\]

These additions improve the ability to predict the adhesion of bacteria (Hermansson, 1999; Brant and Childress, 2002a; Brant and Childress, 2002b). Brownian movement forces ($V_B$) have also been included in the XDLVO theory, distinguishing them as separate forces from $V_{DL}$ (Azeredo et al., 1999). This discrepancy stems from terminology used by van Oss (1994), where electrostatic interactions are used as a general term, including $V_{DL}$, and distinguished as separate from $V_B$. AFM measurements between a single colloid and a membrane surface in strongly hydrophobic systems showed that the XDLVO was better at predicting experimental results than DLVO theory; however for hydrophobic systems the predictions were about the same (Brant and Childress, 2002a). The XDLVO theory may be an improvement, but other interactions outside van der Waals interactions, diffuse double layer repulsions, Brownian motion, and hydrophobic interactions can be involved in bacterial adhesion. Steric interactions between bacteria and substratum have also been shown to play an important role in adhesion (Rijnaarts et al., 1995). Steric forces can be either attractive or repulsive. If in aqueous environments cell-surface macromolecules are hydrophilic and have no affinity for the substratum then steric repulsion will occur (Rijnaarts et al., 1999). However, if the macromolecules have an affinity for the substratum then bridging may occur, generally
taking place when both adsorbing bacteria and substratum are hydrophobic (van Loosdrecht et al., 1990; Rijnaarts et al., 1993).

Surface roughness also affects the adhesion of colloids to surfaces. The DLVO theory assumes perfectly smooth surfaces (Hermansson, 1999). Interaction energies between the substratum and a particle can be significantly altered by a surface roughness in the range of 0 – 0.05 µm (Czarnecki and Warszynski, 1987).

The bacterial cell is a biological heterogeneous surface and should be considered as such. Bacterial surfaces have charged layers of non-zero thickness that can vary over the surface (Rijnaarts et al 1999). Current models of colloid stability are not applicable to bacterial adhesion and further work is required to develop a model that does apply.

Increase in ionic strength lowers the energy barrier for adhesion and favors adhesion (Oliveira, 1997). Rijnaarts (1999) determined that in aqueous environments at low ionic strengths the deposition of bacteria to is inhibited by classical DLVO type interaction and/or steric interactions. In domestic waste-waters and saliva, classical DLVO type interaction and/or steric interactions control bacterial deposition. And only in saline environments (milk, blood, sea water) do steric interactions controlled adhesion. The observed trend of bacterial deposition inhibited by classical DLVO interactions at low ionic strength and dominates by steric interactions at high ionic strength (Rijnaarts et al., 1999).
References


