Chemical effects of biofilm colonization on stainless steel
by Jyostna Pendyala

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in
Physics

Montana State University

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Abstract:

Microbially induced corrosion (MIC) of metals is a serious problem for industry. Currently the only way to combat MIC is by replacing the affected metal. In order to understand the mechanism of MIC, thus allowing control of biocorrosion, the effects of bacteria on long- and short-term surface chemistry of the metals at a micro scale (i.e., on the scale of atoms and electrons), must be better understood. Although there have been numerous investigations of this problem from a microbiological and engineering aspect, there has been little work investigating the problem from a surface chemistry point of view. The goal of this work is to determine the effects of biofilm colonization on the surface chemistry of stainless steel in the first 20-60 Å. The effects of biofilm (a mixed culture of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Klebsiella pneumoniae*) colonization on 0.05µm- finish polished and unpolished 304 stainless steel was examined using Auger electron spectroscopy (AES), X-ray Photoelectron Spectroscopy (XPS) and Time of Flight Secondary Ion Mass Spectroscopy (TOF-SIMS).

Changes in the surface concentrations of the main alloying elements of an as-received unpolished 304 stainless steel were observed after biofilm colonization. In the oxide film close to the bulk stainless steel, there was an enrichment in the relative concentration of Cr with a corresponding decrease in the relative Fe concentration as compared to a control coupon exposed only to sterile media. There were no changes observed in the relative Ni concentration.

Electrochemically induced localized pits reveal a chemistry similar to unpolished stainless steel surfaces colonized by bacteria. The electrochemically treated surfaces also show the presence of blackened grains and visually unaffected areas whose chemistry has changed significantly.

The activity of the biofilm on the well characterized polished stainless steel surface causes penetration of organic material into the matrix of the surface oxide layer. This penetration is dominated by carbon and nitrogen on surfaces exposed to bacteria. There were no chemical changes observed due to the exposure of the surfaces to sterile media under identical flowing conditions.

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A thesis submitted in partial fulfillment of the
requirements for the degree

of

Doctor of Philosophy

in

Physics

MONTANA STATE UNIVERSITY-BOZEMAN
Bozeman, Montana

September 1996

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ACKNOWLEDGMENTS

I wish to thank Dr. Recep Avci for giving me the opportunity to work on this project and to the rest of my Ph.D. committee; Dr. Gill Geesey, Dr. A. Camper, Dr. R. Smith, and Dr. L. Peterson, and Dr. I. Beech for their guidance and support throughout my research work.

I want to thank Dr. J. Anderson for his willingness to answer all my questions and helping me with my work these last two years. I am indebted to and thank Dr. R. Robiscoe for encouraging me to stay in Physics, and for his belief and faith in me. I thank Dr. W. Costerton and Dr. J. Hermanson for their encouragement and to Dr. D. Mogk for his support and allowing me the use of his polishing equipment.

I thank E. Anderson for providing excellent technical expertise in the care of the instruments. I thank N. Equall for her help with the SEM and TOF SIMS analyses. I thank P. Dirkcx, J. Pennington and Matthew for their help with my presentations.

I thank my friends S. Becker, J. Bruner, R. Kumar, A. LeRoux, R. Srinivasan, P. Devi, M. Baldwin, M. Jarrett, R. Waldon, D. Chard, J. Neumann, and J. Brodowy for their undying friendship, support and encouragement through all my years in Bozeman.

I wish to thank my parents, brothers, and family for their support throughout my studies. Finally, I thank my husband Dev for always being there with reality checks and moral support and encouragement.

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ABSTRACT

Microbially induced corrosion (MIC) of metals is a serious problem for industry. Currently the only way to combat MIC is by replacing the affected metal. In order to understand the mechanism of MIC, thus allowing control of biocorrosion, the effects of bacteria on long- and short-term surface chemistry of the metals at a micro scale (i.e., on the scale of atoms and electrons), must be better understood. Although there have been numerous investigations of this problem from a microbiological and engineering aspect, there has been little work investigating the problem from a surface chemistry point of view. The goal of this work is to determine the effects of biofilm colonization on the surface chemistry of stainless steel in the first 20-60 Å. The effects of biofilm (a mixed culture of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Klebsiella pneumoniae*) colonization on 0.05µm- finish polished and unpolished 304 stainless steel was examined using Auger electron spectroscopy (AES), X-ray Photoelectron Spectroscopy (XPS) and Time of Flight Secondary Ion Mass Spectroscopy (TOF-SIMS).

Changes in the surface concentrations of the main alloying elements of an as-received unpolished 304 stainless steel were observed after biofilm colonization. In the oxide film close to the bulk stainless steel, there was an enrichment in the relative concentration of Cr with a corresponding decrease in the relative Fe concentration as compared to a control coupon exposed only to sterile media. There were no changes observed in the relative Ni concentration.

Electrochemically induced localized pits reveal a chemistry similar to unpolished stainless steel surfaces colonized by bacteria. The electrochemically treated surfaces also show the presence of blackened grains and visually unaffected areas whose chemistry has changed significantly.

The activity of the biofilm on the well characterized polished stainless steel surface causes penetration of organic material into the matrix of the surface oxide layer. This penetration is dominated by carbon and nitrogen on surfaces exposed to bacteria. There were no chemical changes observed due to the exposure of the surfaces to sterile media under identical flowing conditions.

CHAPTER 1

INTRODUCTION

Accumulation of microorganisms and their metabolic products on metal surfaces has been proposed to accelerate and enhance corrosion (Pope *et al.*, 1984; Walsh *et al.*, 1993). This phenomenon has been termed as microbially influenced corrosion (MIC), or biocorrosion, biodeterioration, biofouling and so on. Corrosion is the generic term involving natural, or electrochemical processes that effect the life and utility of materials. MIC on the other hand, can involve a plethora of organisms and mechanisms (Hamilton, 1990) and refers to the undesirable formation of biological deposits on material surfaces leading to a decrease in material performance and the useful life of the material (Characklis, 1988).

As early as 1910, Gaines suggested that iron bacteria and sulfur bacteria were partly responsible for the corrosion of buried ferrous metals. However it was not until two decades ago that MIC was recognized as a contributing factor in several types of routinely observed corrosion (Wolfram, 1991).

MIC has now been recognized as one of the key factors of material failure in heat exchangers, oil pipelines, submarine hulls and even in aircraft and nuclear power plants. MIC has also been implicated in the failure of medical implants leading to infections in the patient. Although biocide treatment has been shown to be effective in some cases, it

is only a temporary measure. The long term and sometimes only alternative is to replace the damaged material. This leads to grave economic consequences in industry. White *et al.* (1990) stated very aptly in their paper that "corrosion (is) the venereal disease of industry (it is painful, incapacitating, and expensive, but usually unmentionable)...". An even greater cause for concern is that even materials such as stainless steels, titanium etc., that were thought to be immune to corrosion attack have been found to succumb to MIC. Thus the industrial sector has an increased interest in understanding the mechanisms contributing to MIC and the interactions of the bacteria with surfaces at the micro- and nanoscales. A search for the "holy grail" of a material resistant to bacterial attachment and attack, has also begun.

Most of the biocorrosion occurs in the form of localized attack and pitting corrosion. The mechanisms through which microorganisms participate in pitting corrosion, however, remain obscure. Studies performed in this field have mainly been microbiological or electrochemical in origin. Although the studies focus on the chemical processes within the biofilm or at the surface, few have attempted to study the micro- and nanoscale chemistry of the liquid-solid interface. Much of the chemical evidence linking pitting corrosion to microbial processes is based on data obtained from surface deposits which have accumulated long after microorganisms exert their initial effects. Studies involving surfaces have mainly investigated bulk properties of the surface, which provides few if any clues to the surface properties involved or contributing to MIC.

Biofilm-forming bacteria are likely to induce subtle effects on the underlying metal surfaces during early stages of colonization and biofilm formation. Detection of near-surface chemical changes mediated by biofilms requires surface-sensitive analytical techniques. Auger Electron Spectroscopy (AES) , X-ray Photoelectron Spectroscopy (XPS) and Time-of-Flight Secondary Ion Mass Spectroscopy (TOF SIMS) obtain elemental information from the top 1-6 nm of the metal surface where biofilm microbial processes are likely to have their greatest influence. Few studies have combined surface spectroscopic analyses such as AES, XPS and TOF SIMS, with biological analyses. Hence there is little understanding of the metal-biofilm interface and the processes occurring there. This work aims to take the first steps in trying to address these issues.

The majority of the research in this topic until now has been concentrated on the microbiological and engineering aspects with little emphasis placed on the biofilm-surface interactions at the microscale. This research integrates the vast surface science resources of Image and Chemical Analysis Laboratory (ICAL) at MSU Physics, together with Microbiology and Engineering. Surface science has long pursued the understanding of solid-solid, solid-liquid and solid-gas interactions at the microscale. The combined interdisciplinary focus is being used to bring a broader perspective and approach to understanding and solving the issues of cell-solid and cell-liquid interactions. A review of the some of the microbiological and engineering work preceding this research will be presented in the following chapter.

Hypothesis and Objectives

The objectives of this thesis are based on the main hypothesis that biofilm colonization causes changes in the elemental chemistry and composition on the surface of stainless steel. Therefore the main objective of this work is to determine the effect of biofilm on the top 50 - 100 Å of the surface of stainless steel after the initial stages of colonization.

In order to achieve this objective, a list of specific tasks to be addressed are :

- (i) Examine and determine surface chemistry of unpolished and polished coupons with the surface analytical techniques before any treatment (or exposure, as-received).
- (ii) Expose unpolished and polished stainless steel coupons ("biocoupons") to biofilm and media under flowing conditions in a controlled environment for a fixed length of time. Simultaneously expose unpolished and polished stainless steel coupons ("control coupons") to sterile media alone under the same conditions as the biocoupons.
- (iii) Reexamine the surface chemistry of these coupons with surface analytical techniques after the above treatments. Establish if there are quantifiable changes in the surface chemistry before and after treatments in terms of the bulk alloying elements of stainless steel, Fe, Cr and Ni, and in terms of changes in the passive layer.
- (iv) Determine if surface chemistry changes can be accelerated electrochemically and whether any useful parallels to the changes occurring due to biofilm colonization can be drawn.

(v) Determine if there are differences in surface chemistry between colonized and uncolonized areas by the biofilm on the biocoupon.

CHAPTER 2

LITERATURE REVIEW

Biofilms and Stainless Steel

This review focuses on work relating to stainless steel, biofilms, MIC and surface analyses. Before reviewing current literature, a brief introduction to stainless steels, bacteria, biofilms, their physiology and interactions with surfaces from both microbiological and engineering standpoints will be given.

Bacteria

The majority of bacteria are unicellular and possess rigid cell walls. They do not possess a true nucleus. The DNA exists as a single circular molecule that is not surrounded by a nuclear membrane. Reproduction occurs through cell division and replication of the DNA and respiration through the plasma membrane (see Fig. 1 below; Characklis and Marshall, 1990). They may be spherical (cocci), rod shaped (bacilli) or spiral shaped (spirilla), and vary in size from ~ 0.2 to $2\ \mu\text{m}$ in width and 1 to $10\ \mu\text{m}$ in length. Due to their small size, bacteria have large surface/volume ratios. Hence the bacteria are easily affected by any unfavorable change in their environment. However, the simplicity of their structure and the rapidity with which they can utilize nutrients enables

Figure 1: Bacterial cell

Figure 2: Microbial Growth Curve

them to multiply (and perhaps to mutate) exceedingly fast (Miller, 1970).

Microbial growth occurs exponentially and consists of four phases (shown in Fig. 2 above): (i) *Lag Phase*: the addition of bacteria to the medium does not result in a doubling of the population, (ii) *Logarithmic Phase*: the cells begin to divide steadily, (iii) *Stationary Phase*: Growth plateaus, and (iv) *Death* (Characklis and Marshall, 1990).

Bacteria can withstand a wide range of temperatures from -10 °C to 90 °C (although most grow between 20-50 °C) and pH from 0 to 11. They can be aerobic, i.e., use oxygen to respire or anaerobic, i.e., they use other electron acceptors. Different groups of bacteria are known to use exclusively one or both mechanisms to respire. Bacteria can exist in two forms, the planktonic form and the sessile form. Planktonic bacteria are freely swimming or floating bacteria while sessile bacteria are attached to a substratum. The two strongest traits of bacteria are their ability to adapt to non-ideal conditions and exist in a state of suspension. In this way the bacteria remain viable (live, but in a frozen state) for long periods in extremely unfavorable conditions (Tatnall, 1991).

Biofilms and their structure

Biofilms are a collection of microorganisms, predominantly bacteria, attached to a surface in an aqueous environment and embedded within a three-dimensional gelatinous organic matrix of extracellular polymers secreted by the bacteria (Bryers, 1993; Characklis and Marshall, 1990). As seen in Fig. 3, the bacterial microcolony is the basic structural and functional unit of the microbial biofilm (Costerton, 1995). These microcolonies can be

Figure 3: Schematic of biofilm (Center for Biofilm Engineering, MSU)

composed of single-species or multiple-species. The microcolonies are interspersed with water channels that are more permeable and contain less dense matrix material (Costerton and Lappin-Scott, 1995). These water channels form a network throughout the biofilm and provide access from the bulk fluid to the colonized surface (Costerton *et al.*, 1994). Researchers argue that the persistence of these water channels deep in the biofilm together with the development of mushroom shaped microcolonies indicates some form of cell-cell communication (Costerton, 1995). The further discovery that convective flow is possible through the water channels and that they allow the passage of large particles has revolutionized the concept of bacterial growth in biofilms. All these findings suggest that the water channels may actually represent a primitive circulatory system (Costerton *et al.* 1995). Thus the biofilm structure possesses a complex architecture and assumes a high degree of cooperation between microorganisms. Bacteria and other microorganisms have often been dismissed as primitive. However they are the most successful lifeform on earth having survived eons in time and many extreme environments. They pose a challenge to scientific understanding.

Advantages of Biofilm Growth Mode

The biofilm mode of growth allows sessile bacteria to trap and retain scarce organic compounds. It also helps in the breakdown of complex nutrients requiring time or the cooperation of other bacterial species. Biofilm formation changes the

microenvironment at the colonized surface and renders its inhabitants less vulnerable to hostile physical, chemical, and biological factors (Costerton and Lappin-Scott, 1995).

Biofilm Accumulation

Biofilms accumulate as a result of the microbes' ability to adsorb to a substratum, replicate and produce extracellular polymers. The initial surface colonization and the subsequent biofilm formation is a complex series of chemical, physical and biological processes (Fig. 4; Bryers, 1994):

1. Macromolecules present in the bulk fluid adsorb to the surface resulting in a "pre-conditioning" of the substratum.
2. Transport of planktonic cells from the bulk fluid to the substratum.
3. Reversible adsorption of cells to the substratum for a finite time.
4. The desorption of cells from the substratum either due to fluid shear forces or other physical, chemical and biological factors.
5. Irreversible adsorption occurs when some cells remain adsorbed permanently to the substratum.
6. The attached cells grow at the expense of the substrate and nutrients in the bulk fluid.
7. Cell replication and extracellular polymer production occur along with the formation of microcolonies and biofilm growth.

Figure 4: Biofilm accumulation (Bryers and Characklis, 1992)

Figure 5: Biofilm accumulation curve (Bryers and Characklis, 1992)

8. Detachment of biofilm material occurs either continuously due to erosion brought about by shear forces, or randomly, where portions of the biofilm detach (sloughing) (Bryers and Characklis, 1992; Characklis and Marshall, 1990).

This progression occurs as a sigmoid curve (shown in Figure 5). The curve is divided into: (i) initial events, (ii) exponential or logarithmic accumulation and (iii) plateau or steady state (Characklis and Marshall, 1990).

Stainless Steel

Stainless Steels are iron based alloys containing at least 10.5% chromium up to 30% chromium. Chromium combines with oxygen to form a transparent chromium oxide film on the surface that makes the steel resistant to corrosion. This film is also known as the passive film (American Iron and Steel Institute (AISI), 1967). In addition to their corrosion resistance, the numerous grades of steel offer many advantageous physical and mechanical properties. Today stainless steel is ubiquitous with uses varying from nuclear fuel storage tanks made of steel to automobiles and aircrafts with steel parts.

Within the stainless steel family, there are martensitic, ferritic, austenitic and precipitation-hardenable steels. Austenitic steels account for two-thirds of the total stainless steel production. Austenitic steels have a face-centered cubic lattice crystal structure and may contain from 6-22% nickel and 16-28% chromium (AISI, 1967). These steels are extremely ductile and strong and hence are extensively used in the piping and

tubing industry. Martensitic steels principally contain 11.5-18% chromium while ferritic steels contain 14 -27% chromium besides iron.

The austenitic steels comprise the AISI 300 series. The most common among these are types 304, 304L, 316 and 316L. These alloys contain mainly iron, chromium, nickel and some manganese, molybdenum and silicon with less than 1% carbon, sulfur and phosphorus depending on the grade. The addition of minor alloys is thought to increase the passivating ability and decrease the activity of weak points in the film (Hermas *et al.*, 1995). Silicon increases oxidation resistance at high temperatures and is thought to improve resistance to pitting corrosion. Molybdenum increases resistance to localized corrosion by reducing the oxidation of iron. However, studies performed by Scott *et al.* (1991) have shown that alloys containing between 6-9% molybdenum have been susceptible to corrosion. Carbon is added as it is a good austenite (phase) stabilizer (Bruemmer, 1992). However if an unstabilized austenitic stainless steel is slowly cooled or reheated in the temperature range 500-800 °C (known as sensitization), diffusion of carbon to the grain boundaries occurs (Laws and Goodhew, 1991). The precipitation of chromium-rich carbides occurs along the grain boundaries, leaving a region depleted of chromium. Impoverishment of chromium is hypothesized to be the reason for poor corrosion resistance (Joshi and Stein, 1972). Hence only a low amount of carbon is added to the alloys. Sulfur is added mainly to improve the machinability of the alloys (ASM, 1977). Corrosion of stainless steel, in general, is fueled by the breakdown of the passive film.

Stainless Steel, Biofilms and MIC

Electrochemical Nature of Corrosion

A corrosion reaction is caused by a flow of electrons from one metal to another, due to the potential difference between them, coupled together in a conducting electrolyte (Miller, 1974). The rate of the overall electrochemical process is determined by current flow. In an electrochemical cell, the dissolution reaction occurs at the anode and the electron consuming reaction occurs at the cathode. At the anode positively charged metal ions enter the electrolyte leaving excess electrons on the metal surface, which becomes negatively charged. The electrons flow to the cathode, which thus receives protection from corrosive action. At the anode, films which make it difficult for the emergence of metal cations may form and slow down the corrosion rate. This is known as anodic polarization. Similarly, if incoming electrons from the anode cannot be released easily, they tend to accumulate at the cathode, reducing the current in the cell and hence the corrosion of the anode. This is known as cathodic polarization (Miller, 1974). Polarization refers to this decrease in the corrosion reaction. If the reaction accelerates then it is called depolarization (Characklis *et al.*, 1988).

Biofilms and Electrochemical Corrosion

When biofilms are formed on a metal surface, the microcolonies in the biofilm are typically distributed unevenly across the surface. This patchiness results in the

formation of differential aeration cells where areas under respiring colonies are depleted of oxygen relative to surrounding areas that are non-colonized. This leads to an oxygen gradient across the surface and the formation of local anodes and cathodes and subsequently to corrosion currents (Characklis *et al.*, 1988; Costerton and Boivin, 1990; Geesey, 1990). Depending on the type of bacteria involved there are different types of corrosion that can occur.

Cathodic Depolarization

Bacteria were first implicated in this process by Von Wolzogen Kuhr and Van der Vlugt in 1924 (Miller, 1970). They noticed that corrosion of buried pipes associated with sulfide occurred in wet anaerobic soil in which polarization of cathodic areas would be expected to inhibit corrosion. They found iron sulfide as an adherent corrosion product and also in the surrounding areas. They detected sulfate-reducing bacteria (SRB) and suggested that these bacteria acted as cathodic depolarizing agents by removing the cathodic (atomic) hydrogen that is then utilized to reduce sulfate to sulfide (Miller, 1970). The accumulation of hydrogen at the cathode leads to cathodic polarization.

Some bacteria produce large amounts of acids as by-products of metabolism. If these acids are not utilized by the bacteria then they create localized pH gradients on the surface. The probability of corrosion thus increases and leads to depolarization of the cathode. This mechanism has been the focus of many studies (Characklis *et al.*, 1991; Geesey, 1990; Little *et al.*, 1986; Pope *et al.*, 1984; Videla and Characklis, 1992).

Hydrogen Embrittlement

The increased concentration of surface hydrogen causes penetration of hydrogen atoms into the metal and loss of ductility (hydrogen embrittlement). This penetration may also induce high internal stresses and cause spontaneous cracking (hydrogen cracking) (Uhlig, 1967). When SRB colonize a metal surface, they produce sulfides. In the presence of sulfides, atomic hydrogen is accumulated at the surface, which eventually leads to hydrogen embrittlement (Geesey, 1990).

Effects of Metal Oxidation Products(Chloride)

and Stress Cracking Corrosion

Metal oxidizing bacteria play an important role in MIC. These organisms oxidize ferrous ions to ferric ions and manganous ions to manganic ions to obtain energy for growth. Acidic ferric and manganic chlorides are highly corrosive to stainless steels (Characklis *et al.*, 1991; Geesey, 1990; Videla and Characklis, 1992). The chlorides accumulate in the grain boundaries and in cracks at pit initiation sites under bacterial deposits and cause chloride stress corrosion cracking (SCC, Stoecker *et al.*, 1986). Tatnall (1991) found that *Gallionella*, metal oxidizing (in particular iron) organisms, cause small pits about the size of a pinhole that later extend into large subsurface cavities. A high amount of chloride is found in pits even when the metal is exposed to extremely low chloride containing water (Borenstein, 1991).

Effects of Inclusions

In pure electrochemical studies (Castle and Ke, 1990; Ke and Alkire, 1992) it has been found that pits form primarily at MnS inclusions. A similar effect has been seen in MIC research. The large number of inclusions on the surface causes the attachment and proliferation of MIC to be more rapid than in the absence of inclusions. The orientation of pits and composition of inclusions are important factors in determining the MIC response of materials. A direct correlation was found between the number of tubercles (localized concentrations of cells) and the sulfur content of inclusions as well as pit initiation at these inclusions (Walsh, 1993).

Effects of Welds

Welded stainless steels are widely used in industry today and seem to be more susceptible to corrosion. Das and Mishra (1986) reported preferential corrosion at welded interfaces of stainless steel surfaces. They used the logic that MIC in stainless steel can be explained by the theory that an excess amount of chromium is precipitated along the grain boundaries. The areas around the grain boundaries lose their chemical resistance, and bacterial and chemical attack occurs preferentially in and around these weak areas. Hence a huge amount of corrosion damage is seen in the heat affected zones (HAZ) near and parallel to weld interfaces where cracks appear during corrosion processes (Cubicciotti and Licina, 1989). Electrochemical studies have shown that the pitting potential for welded stainless steel is much lower (pits quicker) than regular stainless steel (Ibars *et al.*,

1992). Although it has been well documented that MIC is enhanced especially in zones related to the weld, the mechanisms for MIC attack at welds are not well understood.

Surface Analytical Studies of MIC

Castle (1977) and McIntyre (1984) separately reviewed the applications of X-ray photoelectron spectroscopy (XPS) and Auger electron spectroscopy (AES) in corrosion science. Yet almost two decades later, surface analytical studies have not been thoroughly integrated and combined with MIC studies. Chen *et al.* (1988) reported in 1988 to have found no work on the investigation of MIC by surface sensitive techniques, prior to their work. They used AES in their investigations of MIC of condenser tubes. However they did not carry out extensive chemistry studies. They reported that AES spectra and maps corroborated their visual examination of MIC attack on stainless steel metal plates. Jolley *et al.* (1988, 1989) used AES and XPS to study biocorrosion of copper films on germanium crystals by polysaccharides. They were able to show using these techniques that there was a removal of some Cu film by the polymer solution. Lee *et al.* (1992) incorporated electrochemical, microbiological and AES analyses of MIC of mild steel in seawater. AES analysis showed a loose spatial correlation between oxygen and sulfur in the corroded and pitted areas. Metal ions in solution were initially exposed to SRB (sulfate reducing bacteria) by Kearns *et al.* (1992). Dehydrated and dried cells were then examined in XPS for the presence of metal/metal sulfide.

Other research claiming the use of surface analytical techniques in analyzing aspects of MIC have been carried out in the most part using Scanning Electron Microscopy (SEM) (Gouda, 1993; Lee and Characklis, 1993; Lee *et al.*, 1992). However, SEM is not surface sensitive and probes mainly the bulk properties of materials, within a depth of 1 μm . While bulk effects are important, it is the surface effects that hold the key to the puzzle of biofilm interaction with surfaces. This again draws the attention to using surface analytical techniques that probe the top 10-50 Å of the surface. All previous studies that report surface science studies of MIC were carried out in conjunction with electrochemical corrosion. The above review shows that the application of surface analyses to MIC research is just now gaining momentum.

Effects on Surface Chemistry due to Biofilm

Attachment to and Colonization of Surfaces

A crucial initial event in biofilm formation is the adsorption of bacteria to surfaces. If this is successful (irreversible) then the cells will replicate and eventually form a biofilm. Zobell (1943) first suggested that bacteria were not just passively stuck to the surface, but were attaching to the surface and growing on them. He hypothesized that the surfaces provide a means of concentrating the nutrient for the bacteria and hence attachment was advantageous.

The effects of surface charge and degree of hydrophobicity of the substratum influencing bacterial attachment was proposed by Fletcher *et al.* (1979). They first

alluded to the attachment of a bacterium being dependent on not just physical properties but also chemical properties of the surface and the chemical interactions between the surface and the bacterium. Investigations into the hydrophobic properties of the surface led to the discovery that surface tension also has an influence on attachment (Absolom *et al.*, 1983; Van Loosdrecht *et al.*, 1990).

Stone and Zottola (1985) and Zoltai *et al.*, (1981) performed many SEM studies for physical evidence of bacterial attachment to stainless steel surfaces and the effect of static and dynamic environmental conditions. Stanley (1983) showed pH to be another contributing factor to the attachment process. Although all previous studies had alluded to the importance of the chemistry of the surface, this was the first indication of a chemical property of the substratum being a factor in the attachment process. Another reason for this omission is that most studies were performed on glass or polystyrene substrata. The idea was that once the mechanism of attachment was understood on simple surfaces, then chemically diverse substrates could be tackled.

McEldowney and Fletcher (1986) could not attribute electrostatic and hydrostatic interactions to wholly account for the attachment process. Pringle and Fletcher (1986) in following research hypothesized about the importance of conditioning films on the surface in the attachment process. The importance of the chemical composition of the substratum was stated categorically, however it was not until a decade later that this property received attention.

To the best of the author's knowledge, there has been only one study investigating the surface chemistry changes due to bacterial colonization. Geesey *et al.* (1996) investigated the effect of substratum features and their effect on bacterial colonization. They also characterized the surface chemistry of the stainless steel surfaces before and after colonization and discovered that colonization led to changes in the elemental composition that were enhanced with time. They examined the very early stages of biofilm colonization. Their studies involved results mainly of a single species biofilm colonization of the steel, at extremely low flow rates and were terminated when the surface features were occluded from sight.

Present research undertaken by the author involves mixed species biofilm colonization effects on the chemistry of polished and unpolished 304 stainless steel, using surface analytical techniques. This research focuses on the chemical properties of the steel before and after biofilm formation under flowing conditions in a parallel flat plate flow-through reactor. The reactor allows confocal imaging of the biofilm as it develops on the stainless steel coupons *in situ*.

CHAPTER 3

SUMMARY OF INSTRUMENTAL TECHNIQUES

General Aspects of Surface Analysis

Ultrahigh Vacuum

The main reason for performing surface analysis under vacuum is that electrons emitted from a specimen should interact with as few gas molecules as possible on their way to the analyzer, so that they are not scattered and hence lost from analysis. For this condition to be satisfied, pressures in the range of 10^{-5} - 10^{-6} torr would be adequate (Briggs and Seah, 1984). However x-ray photoelectron spectroscopy (XPS), Auger electron spectroscopy (AES) and time-of-flight secondary ion mass spectroscopy (TOF SIMS) are extremely surface sensitive with the photoelectrons, electrons and ions originating from the very top surface layers. These techniques are therefore very sensitive to surface contamination. To characterize the surface, it is necessary to start with a surface that is atomically clean or in some other stable condition. To achieve these clean surfaces ultrahigh vacuum (UHV) conditions ($\leq 10^{-9}$ torr) are needed. For the experiments discussed in this thesis, the typical operating pressures in the case of XPS, AES and TOF SIMS were in the 10^{-9} - 10^{-10} torr range.

Surface Sensitivity and Escape Depth

AES and XPS utilize electrons in the range of 10 - 2000 eV. These low energies make the probability of scattering (due to inelastic collisions) during escape from the sample very high. Escape depth is the average distance that an electron can travel from the point of origin without undergoing any inelastic (loss of energy) collisions. The Auger electron has a probability of $1/e$ of traveling a distance (characterized by the mean free path) before being inelastically scattered (Briggs and Seah, 1984). If these electrons are detected at an energy which is known to be unchanged by passage through the surface region, they could have only traveled through the top surface region (Zangwill, 1988). The mean escape depth of these electrons, in the energy range of interest, can be as small as 1-2 atomic layers and up to 10 atomic layers. This makes AES, XPS, and SIMS extremely surface sensitive techniques (Brundle and Baker, 1977). The dependence of escape depth on the electron energy is shown in Fig. 6 (Briggs and Seah, 1984).

Auger Electron Spectroscopy (AES)

The Auger spectrometer used in the experiments in this thesis was a Physical Electronics instrument (PHI 595) equipped with a lanthanum hexaboride (LaB_6) source. The analyzer was a cylindrical mirror analyzer (CMA). The instrument was also equipped with imaging capability, thus allowing the collection of elemental chemical maps.

Figure 6: Dependence of mean free path on electron energy (Briggs and Seah, 1984)

Figure 7: Auger spectrum (lower curve, $N(E)$) and its derivative (upper curve $dN(E)/dE$).

This spectrum is only a guide to understand the various features present in an Auger spectrum.

The AES operation is described as follows. A high energy beam (1 - 10 keV) is directed at a sample surface and a spectrum of secondary electrons is collected. This spectrum (shown in Fig. 7 above) consists of an elastic peak, which is made up of electrons that have been reflected back from the surface without losing any energy and a long tail of electrons that have either lost energy to the solid or secondary electrons excited from the solid. Part of the secondary continuum is due to the Auger electron emission process which shows up as very small features in the spectrum. Thus it is necessary to take the derivative of this spectrum (inset in Fig. 7) to reveal the characteristic elemental signature of a given sample (Zangwill, 1988). The schematic of the Auger process is shown in Fig. 8, where a core hole is created by the incident electron beam. The energy released in the above process can be taken up either by an x-ray or another electron. In the latter case, the electron fills up the core hole releasing some additional energy. This energy is taken up by a third electron to escape into the vacuum. This electron is called the Auger electron and the process is known as the Auger process.

From energy conservation, the measured kinetic energy of this Auger electron for a given transition say, in the K, L and M levels is given by:

$$E_{KLM}(Z) = E_K(Z) - E_L(Z) - E_M(Z+\Delta) - \Phi_A \quad \text{Equation 1}$$

where K, L, M are the three different energy levels involved in the Auger process. The K level is the level in which the core hole was created. The L level is the level from which the electron filled the core hole and the M level is the level from which the Auger electron is ejected. E_K , E_L , and E_M are the binding energies (typically measured relative to

Figure 8: Schematic energy level diagram illustrating the Auger emission process

the Fermi level E_F , of the analyzer) of these levels for a given atomic number Z . The Δ is a correction to the actual energy of E_M (Buck *et al.*, 1979). The correction arises due to a different chemical environment (since the electron from this level is emitted in the presence of a hole in the energy level E_L , as opposed to emission in the absence of this hole). Since the energy of the Auger electron is obtained from three different energy levels, any changes in the energy due to the chemical environment of the atom is a complex combination of all three energy levels involved. Φ_A is the effective work function of the electron energy analyzer being used.

The outgoing electron's kinetic energy depends only on the properties of that particular atom, as seen from the above equation. This means that for each element in the Periodic Table there is a unique set of Auger energies, since there are no two elements with the same set of atomic binding energies (Briggs and Seah, 1984). Thus all elements in the Periodic table can be easily identified with AES except helium and hydrogen, since they only have K orbital electrons (Buck *et al.*, 1979).

Scanning Auger Microscopy (SAM)

In the scanning electron microscope (SEM), a focused electron beam is scanned across the surface while a synchronous beam is scanned across the face of a display oscilloscope. This enables a one-to-one correspondence to be made between the positions of the probe on the sample and the beam on the oscilloscope face. When a signal is detected from the sample, it can be used to produce an image; for example, scanning the

emitted secondary electrons as the incident beam scans the surface. If an electron spectrometer is inserted between the sample and the electron detector (such as a channeltron) and the spectrometer is tuned for particular Auger electrons, then we have a scanning Auger microprobe (SAM). In case of SAM, images (maps) can be made in which the contrast depends on the concentration of an element in different places (Pruyton, 1984) of the area scanned.

Quantification

The most common method of quantitative analysis is done using the differentiated peak-to-peak height data to define the amount present of a particular element. By this method, the concentration of an element X is given by (Buck *et al.*, 1979):

$$C_x = \frac{I_x / S_x}{\sum_j I_j / S_j} \quad \text{Equation 2}$$

where the sum is over all the n constituents detected by Auger. In the above equation, C_x refers to the concentration of element X, I_x is the peak-to-peak height of an element X in the derivative spectrum, S_x is the sensitivity factor for element X, and j is the summation index (Briggs and Seah, 1984; Buck *et al.*, 1979). Sensitivity factors are tabulated and the ones used in these studies are listed below in Table 1. The incident electron energy used was 3 keV.

Table 1: Sensitivity factors (Davis *et al.*, 1976) for elements detected in the Auger experiments.

Elements	C	O	N	Cr	Fe	Ni
Kinetic Energy (eV)	252	505	380	525	705	850
Sensitivity Factors	0.2	0.5	0.3	0.35	0.2	0.37

Analyzer

The analyzer used in the PHI instrument is a cylindrical mirror analyzer (CMA). The CMA consists of two coaxial concentric metal cylinders. The inner cylinder is grounded and the outer cylinder is placed at a negative potential. The primary electron beam hits the sample and Auger electrons are generated in all directions. The negative potential on the outer cylinder bends (or refocuses) the electrons back into the inner cylinder through a second aperture and then through an exit aperture on the analyzer axis. If the electrons are traveling very fast they will hit against the outer cylinder and if they are traveling very slowly they will hit against the inner cylinder. Thus only electrons within a narrow range of energy (called pass energy) succeed in reaching the detector (ESCA Users group web page, 1996). A channeltron is placed at this exit aperture. The schematic of the CMA is shown in Figure 9.

Figure 9: Schematic of CMA (Charles Evans & Associates Web page, 1995).

Information from AES

(I)Surface Composition: The high ionization efficiency of the incident electrons and the small mean free paths of the Auger electrons give AES a high sensitivity for surface species. Also, through the use of a finely focused electron beam, AES has very high spatial resolution (~ 1000 Å in our case), making it an important tool in the evaluation of cleaning procedures used in thin film fabrication, semiconductor surface doping and corrosion problems (Brundle and Baker, 1977).

(II)Chemical Shifts: The chemical environment of an atom is reflected in the changes in the binding energies of core level atoms. Changes in peak widths in the Auger spectrum (Briggs and Seah 1984) can be related to chemical shifts that arise due to the electronic structure of the atom itself or from interaction with other atoms. Chemical shifts are much harder to interpret because the Auger process is a multielectron process.

(III)Depth Profiling: AES along with ion sputtering can be used to obtain a layer by layer elemental profile of the sample surface. In the sputtering process, the sample is bombarded with Ar ions accelerated in an ion gun to an energy in the range 0.5 - 5 keV. A small fraction of the energy is transferred to surface atoms causing them to leave the surface i.e., they are sputtered away. Spectra are taken alternatively with the sputtering, thus giving a depth profile.

X-Ray Photoelectron Spectroscopy (XPS)

The spectrometer used in the experiments (discussed later) in this thesis was a Physical Electronics instrument (PHI 5600 *ci*) equipped with a standard Mg K $_{\alpha}$ (1253.6 eV) and a monochromatized Al K $_{\alpha}$ (1486.6 eV) source and a concentric hemispherical electron energy analyzer (CHA).

The working of the XPS is summarized as follows. X-ray photons of a known wavelength strike the sample surface. If the photon energy $h\nu$ exceeds the binding energy E_B of a core electron (shown in Fig. 10), it may be absorbed by the core electron. This electron gets excited into a higher energy level above the Fermi level. If the energy of this electron exceeds the work function of the sample, it is emitted into the vacuum (Park and Lagally, 1985).

The spectrum of emitted electrons, known as the Energy Distribution Curve (EDC) displays peaks at appropriate kinetic energies (or binding energies) (Zangwill, 1977). The notation used to identify elements in the spectra is the element name followed by 1s, 2p, 3s, etc., that refer to the different binding orbitals; for example Fe2p. The Einstein photoelectric equation connects the peak positions to specific binding energies as shown in Equation 3 below.

$$E_K = h\nu - E_B - e\phi_{spect}$$

Equation 3

Figure 10 : Schematic energy level diagram illustrating the photoelectron emission process in an XPS experiment.

Two types of measurements were done in the work reported in this thesis:

(I) Binding Energy: This measurement produces an EDC which is measured using an electron energy analyzer. The spectra are measured at fixed angles of electron emission relative to both the x-ray source and the sample.

(II) Angle Resolved Measurements : These measurements are made relative to the axes fixed with respect to the sample. The electrons emitted at take-off angle, perpendicular to the sample surface, are more representative of the bulk of the sample. Electrons emitted at grazing angles (15° from the surface) are more representative of the surface layers. The two schemes are shown in Fig. 11.

In general, both Auger electrons and secondary electrons generated by inelastic scattering processes, will also be emitted from the sample. It is generally possible to distinguish between these electrons and photoelectrons. An Auger peak can be identified in two ways, by : (a) comparing the observed peak energies with Auger energies of the atom present, and (b) changing the incident photon energy by a fixed amount (obtained by switching from one source to another). If the kinetic energy associated with the peak shifts by the same amount, it is a photoelectron peak. If the peak remains fixed in kinetic energy, then it is an Auger peak.

Inelastic scattering or loss peaks can be identified by : (a) identifying nearly identical features at kinetic energies below different no-loss peaks. This is because all high energy electrons will be capable of the same excitations in inelastic scattering,

Figure 11: Angle resolved XPS (MultiTechnique ESCA User's Manual, Perkin Elmer).

The diagram on the left illustrates a situation where the sample is tilted normal to the analyzer for a takeoff angle of 90° . In this situation, photoelectrons traveling along the axis of the analyzer are going normal to the surface and can thus escape from the maximum depth for the greatest depth of analysis. The illustration on the right shows a situation where the sample is tilted to grazing takeoff angles. Here the photoelectrons traveling along the axis of the analyzer can only escape from near the surface of the sample.

although with different probabilities, and (b) comparing the observed spectra with reference energy loss spectra of the same sample.

The sensitivity or elemental detection limit of the XPS technique for a homogenous sample, the elemental detection limit, is usually between 0.1 and 1 atomic percent of a monolayer. The detection limit depends mainly on the photoelectric cross sections of particular elements. The typical escape depth of photoelectrons in XPS is about 1-5 nm due to extremely short scattering lengths for electrons with kinetic energies in the range 10 - 1500 eV.

Quantification

The most common method of quantitative analysis is done by stripping out the background and using the area under the peak to define the amount present of a particular element. By this method, the concentration of an element X is given by (Buck *et al.*, 1979):

$$C_x = \frac{I_x / S_x}{\sum_j I_j / S_j} \quad \text{Equation 4}$$

where the sum is over all the n constituents of the solid. In the above equation, C_x refers to the concentration of element X, I_x is the area under the peak of an element X in the XPS spectrum, S_x is the sensitivity factor for element X (at a particular pass energy), and j is the summation index (Briggs and Seah, 1984; Buck *et al.*, 1979). Sensitivity factors are tabulated and the ones used in these studies are listed below in Table 1.

Table 2: Sensitivity factors for elements detected in the XPS experiments for the pass energy 23.5 eV.

Elements	C1s	O1s	N1s	Cr2p	Fe2p	Ni2p
Binding Energy	270	530	350	575	710	850
Sensitivity Factors	5.214	13.099	8.574	40.91	50.714	66.729

Sources

There are two different sources available in the PHI 5600 *ci* instrument.

(1) Standard Sources: The most commonly used sources for XPS are the K_{α} lines of magnesium (Mg, 1253.6 eV) and aluminum (Al, 1486.6 eV). The line width for these sources are 0.7 eV and 0.85 eV for Mg and Al, respectively. The photon energy of these elements allows the observation of core levels of most elements.

The compactness of the source means that the maximum power dissipation that can be achieved is 350 W. Hence the anode block must have high heat conductance. The block and the water tubes are thus typically made of copper. Due to these constraints, the anode material is deposited on the copper block as a thick film ($\sim 10 \mu\text{m}$). This film thickness excludes copper L_{α} radiation and is still thin enough for heat transfer. Most commercial X-ray sources have two filaments for electron bombardment

and two anode surfaces, one for Mg and the other for Al. For certain applications one source may be better than the other. Also, if peak interferences may be removed by switching between the sources. A thin window is needed between the anode and the sample to screen the sample from any contamination from the source region. In practice, a $\sim 2 \mu\text{m}$ Al foil is used for the window material because it is transparent to the Mg and Al x-ray radiation and does not cause much flux attenuation (Briggs and Seah, 1984).

(2) Monochromatized Sources: The emission spectrum from any material is complex and consists of a broad continuous background, called bremsstrahlung, on which are situated the narrow characteristic lines. The spectrum is also made complex by the presence of satellites and peaks due to multiplet splitting. The removal of these extra features in the spectrum can be achieved by monochromatization of the x-ray sources. This monochromatization depends on the dispersion of the x-ray energies by diffraction on the surface of a crystal. This diffraction is governed by Bragg's law (Briggs and Seah, 1984):

$$n \lambda = 2 d \sin \theta$$

Equation 5

where n = diffraction order and

λ = wavelength of X - ray sources

d = crystal spacing

θ = Bragg angle.

For first order diffraction of Al K_{α} x-rays, it has been found that quartz crystals are suitable. The advantage of quartz is that it is available in many different sizes and in

Figure 12: X-ray monochromatization

perfect crystalline shape. It can also be ground easily and baked to high temperatures. The commonly used method for monochromatization is that of fine focusing. The anode lies on the Rowland circle and is irradiated with a finely focused electron beam. The K_{α} x-rays are dispersed by diffraction and refocused (by the quartz crystal) to another point on the circle where the sample is located (Briggs and Seah, 1984). The schematic of this method is shown in Fig. 12 .

Analyzer

The analyzer used in XPS is a concentric hemispherical analyzer (CHA) also known as the spherical deflector analyzer. The CHA consists of two concentric metal hemispheres. The inner hemisphere having a radius R_1 and the outer radius being R_2 . A potential DV is applied between the surfaces so that the inner hemisphere is at a positive potential relative to the outer hemisphere thus creating an electric field between the two hemispheres. As in the case of the CMA, only electrons in a narrow energy region pass through the hemispheres to the detector. R_0 is the median equipotential surface between the hemispheres, and the entrance and exit slits are centered on R_0 . The schematic of the CHA is shown below in Fig.13.

Information from XPS

(I)Surface Composition: Core levels are characteristic of the atomic species present on the surface. Since XPS is a core level spectroscopy, the XPS spectrum is indicative of the

elements present on the surface of the sample.

(II) Chemical Shifts: (Briggs and Seah, 1984)

(a) *Core Level Spectra:* Differences in the oxidation state or a difference in the molecular environment etc., between atoms of the same element in a solid give rise to core level peaks with measurably different binding energies. This produces different XPS core level spectra for the same element.

(b) *Valence Band Structure:* Valence band spectra are related closely to the occupied density of states. This is very useful when studying the electronic structure of materials. In case of polymers, valence bands reveal structural information that cannot be obtained from core level studies.

(c) *X-ray satellites and ghosts:* Satellites arise from less probable transitions or transitions in a multiply ionized atom. Ghosts are due to excitations of impurity elements in the X-ray source.

(d) *Multiplet splitting:* Multiplet splitting is due to the presence of unpaired electrons in the valence level. Multiplet splitting causes broadening of the peaks with asymmetry leading to apparent variation in the peak maxima.

(e) *Shake-up satellites:* To the valence electrons associated with an atom the loss of a core electron by photoemission appears to increase the nuclear charge. This perturbation gives rise to reorganization of the valence electrons (relaxation) which could cause the excitation of one of them to a higher unfilled level (shake-up). The energy required for

this transition is not available to the primary photoelectron and this two-electron process leads to a discrete structure on the low kinetic energy side of the photoelectron peak.

(f) *Asymmetric metal-core levels*: In a solid metal, there is a distribution of unfilled energy levels above the Fermi level which are available for shake up events after core electron emission. In this case, the core level is accompanied by a low kinetic energy tail.

(g) *Shake-off satellites*: Shake-off refers to valence electrons being completely ionized, leaving behind an ion with vacancies in the core and valence levels. However this process rarely gives rise to satellites as the energy separation from the primary photoelectron peak is so large that the satellites tend to fall in the inelastic tail and also such transitions cause the original photoelectron peak to broaden rather than forming discrete peaks in the spectrum.

(III) Angular Effects: A great deal of chemical information can be obtained nondestructively using angle resolved XPS. As discussed earlier, the surface sensitivity is greatly enhanced at grazing emission angles of the photoelectrons.

(IV) Depth Profiling : Same as in the case of AES.

Secondary Ion Mass Spectroscopy (SIMS) And Time Of Flight Secondary Ion Mass Spectroscopy (TOF SIMS)

The TOF SIMS instrument used in the experiments discussed below was a Charles Evans Trift T-2000 machine. This instrument consists of a cesium source for high mass resolution purposes and a liquid metal (Ga^+) ion gun for obtaining good spatial resolution and to obtain chemical ion maps.

In SIMS, atomic and molecular species are desorbed from the surface under particle bombardment (Benninghoven *et al.*, 1987). An ion gun produces primary ions which strike the surface and produce secondary ions. These secondary ions are detected by a mass spectrometer, resulting in a mass spectrum. This mass spectrum is a plot of detected ion yield versus a measured mass-to-charge ratio, m/e . Most of the time the charge is 1 and the x-axis of the plot is taken to be the mass scale (Spool, 1994). The secondary ion emission includes all emitted ionized surface particles (atomic ions, inorganic cluster ions, organic fragment ions and molecular ions) in the ground state as well as the excited state. The m/e spectrum supplies direct information on the chemical composition of the bombarded area (Benninghoven *et al.*, 1987).

The primary ion strikes the surface with an energy of a several keV and hence penetrates into the sample. The penetration of the primary ion leads to the formation of a collision cascade, shown in Fig. 14, in the target. A few of the displaced atoms are ejected

Figure 14 : Collision cascade (Spool, 1994).

(sputtered) from the surface through this impact. The dimension of this cascade depends on the energy and mass of the primary ion as well as the density and structure of the target material and can extend up to 10-25 nm into the solid (Spool, 1994). Most recoil particles have low energies and thus, only particles from the very top surface with momenta directed towards the surface, can overcome the surface binding energy and leave the target (Benninghoven *et al.*, 1993). SIMS thus has monolayer sensitivity. As the primary ion strikes the surface, the atoms are displaced in all directions (as water is when a rock is thrown in a quiet pool). Atoms near the area of impact arrive with high energies toward the surface and produce sputtered atoms and mononuclear ions. Depending on the probability of ionization and neutralization many ion impacts may occur before a single ion is produced that escapes the sample (Spool, 1994). The removal of ions from the surface results in the erosion of the surface. This erosion rate is determined by the initial primary ion dose. There is minimal damage to the surface in the minimum ion dosage limit. This limit, called the static SIMS limit, is defined as the ion dose at which an alteration of the surface is apparent in the mass spectrum. Below this limit, the probability of a primary ion striking an already bombarded surface is not significant (Spool, 1994). The maximum primary ion dose as established by this limit is 10^{12} ions/cm² which is less than a monolayer (assuming a monolayer is made up of 10^{15} atoms/cm²). The rate of this erosion has resulted in two modes of SIMS:

(a) Static SIMS: This mode consists of the bombardment of a relatively large target area with a low primary ion current density. This results in a low sputtering speed for an

individual monolayer, which implies a long average lifetime for an individual layer (Benninghoven, 1973). For an ion current density of 10^9 atoms/cm² the lifetime of a monolayer is of the order of some hours (Benninghoven *et al.*, 1993). Thus in this mode, samples are unaffected by the ion beam in a representative experiment of a few minutes.

(b) Dynamic SIMS: This mode applies high current densities, up to 10^{17} atoms/cm², which results in a short lifetime of a monolayer down to 10^{-3} seconds range (Cotter, 1992). This mode is useful for depth profiling.

For the detection of elements present in a low concentration in a sample, along with high lateral resolution (static SIMS), then the number of useful secondary ions from an analytical point of view, is limited. This reason for this is that majority of the secondary particles are emitted as neutrals. For maximal use of all secondary ions produced, a time-of-flight (TOF) mass analyzer is very important. The TOF SIMS spectrometer can detect as much as 50% of the ions generated at the sample during analysis (Spool, 1994).

In the TOF spectrometer, the primary ion gun is pulsed. The secondary ions are formed in a short source region in the presence of an electric field which accelerates the ions into a longer field free drift region (Fig. 15). Since all ions are accelerated by the same field, ideally all ions enter the drift free region with the same kinetic energy.

$$K. = z e E s = \frac{1}{2} m v^2 \quad \text{Equation 6}$$

where K = kinetic energy, z = number of charges, e = charge on the electron, E = electric field, s = path length from sample to detector, m = mass, and v = velocity.

Schematic diagram of an imaging TOF-SIMS mass spectrometer

Figure 15: Schematic of the TOF SIMS spectrometer

The velocities of these ions will therefore depend on their masses.

$$v = (2 z e E s / m)^{1/2} = L / t \quad \text{Equation 7}$$

where t = time-of-flight through the field free region, and

L = length of field free region or flight path (=2m, in the Trift instrument).

The arrival time of the ions at the detector is directly proportional to the square root of the mass of the ions, so that a mass spectrum can be obtained from a time spectrum (Cotter, 1992). From equation 7 we get:

$$t = (m / 2 z e E s)^{1/2} L \quad \text{Equation 8}$$

Simplifying Equation 8 we get:

$$m / z = 2 e E s (t / L)^2 \quad \text{Equation 9}$$

To obtain timing information, the time of ion formation must be known. The pulsed primary ion beam makes this possible. Also the current in the primary ion is set low enough so that all of the ions that reach the detector can be individually counted (Spool, 1994). Most systems make use of additional electric sectors to correct for initial kinetic energy distribution of secondary ions to improve mass resolution (Cotter, 1992).

Imaging

In ion mapping the distribution of a particular ion species across the selected area is recorded (Benninghoven *et al.*, 1987). The SIMS imaging system consists of an ion optical collection system that preserves the spatial relationship of the desorbed ions from sample to detector. The system is similar to an optical microscope, except that the detected particles can be mass selected (Winograd, 1993). A routine spatial resolution of $\sim 1\mu\text{m}$ can be achieved. Highly focused liquid metal ion guns such as Ga^+ in scanning modes can achieve submicron spatial resolution while maintaining reasonably good mass resolution.

The ion image can be obtained by modulating the intensity of a cathode ray tube, which is scanned synchronously with the primary ion beam, with the secondary current intensity. The information of all points in the field of view is obtained simultaneously and time sequentially. Thus the data for that area can be viewed in parallel with the image (Benninghoven *et al.*, 1987).

Quantification

In SIMS, varying peak intensities could mean varying concentrations or varying chemistry. The results obtained from a SIMS experiment are also not easily quantifiable for a chemically unique interface due to surface dependent variation in ion yields. However, the results from two similar surfaces differing slightly in their chemistry can be compared to give an idea of relative composition (Spool, 1994). Thus the interpretation of

the results obtained requires an in-depth understanding of the formation mechanisms of the detected secondary ions.

Sources

The basic requirement for static SIMS is a controllable primary beam source of variable density, from 10 pA/cm^2 to 5 nA/cm^2 . For depth profiling in surface studies, a primary beam with pA/cm^2 to nA/cm^2 is advantageous (Vickerman, 1988). The TRIFT-2000 consists of two different sources, cesium and gallium. They are described below:

(a) Cesium Source: An ampoule of pure cesium is introduced into the cesium reservoir and broken under vacuum. The reservoir heater maintains the temperature of the reservoir at about 75°C ; this maintains the appropriate vapor pressure for cesium. The cesium vapor is fed to a porous tungsten ionizer plug. The ionizer plug is kept at about 1100°C , thus causing the cesium vapor to leave the source through the front surface of the tungsten plug where it is fully ionized. The vapor is drawn away as an ion beam by the extractor electrode (Charles Evans Web page, 1995). A schematic is shown in Fig. 16 below (Charles Evans Web page, 1995). The main advantage of this source is that it produces a high negative secondary yield of hydrogen, carbon, oxygen and noble metals and it can be used to produce smaller spot sizes with high current densities.

(b) Liquid Metal Ion Source (LMIS): These sources are metals that are liquid at room temperature, like cesium, indium, gold, gallium etc. Ion beams are generated in two ways. In the first case, as liquid metal flows through a fine aperture in the presence of a high

Figure 16: Schematic of cesium gun (Charles Evans & Associates Web page, 1995).

Figure 17: Schematic of LMIG (Vickerman, 1988).

electrostatic field, it forms a cone. Intense positive ion emission occurs from the apex of this cone. In the second case, a high positive field is established between a needle tip (1-10 μm) and an extractor. As the liquid metal is drawn over the needle by a capillary action. A high voltage between 4 and 10 kV is then applied to the extractor electrode, whereupon the liquid metal is drawn out in a cusp-like protrusion from which positive metal ions are extracted (Vickerman, 1988). A schematic of this source can be seen in Fig. 17 above. These beams can be used to obtain spot sizes of $\leq 1 \mu\text{m}$ with high intensities which makes it possible to investigate small surface features on samples. Chemical ion mapping can also be done with these sources.

Analyzer

In the Trift TOF SIMS instrument the analyzer consists of three identical hemispherical 90° electrostatic analyzers. Electrostatic analyzers are used for high energy resolution since they bend lower energy ions more than higher energy ions. The inner and outer sectors have voltages of opposite polarity which allows for energy selection. The final secondary ions are positioned onto a detector. The start time of the secondary ions is recorded and the resistive anode encoder (RAE) measures the arrival, or stop positions of the secondary ions. These times are correlated back to the masses of the secondary ions to obtain a mass spectrum. Secondary ion images are acquired by setting time windows in the mass spectrum and correlating these with the position information from the RAE (Scheuler *et al.*, 1990).

Trace Analysis And Surface Composition

SIMS offers some unique advantages as compared to AES and XPS, including detailed chemistry information and extremely high sensitivity of many elements, molecules and their fragments which is important to identify the organic present on the surface. Since mass is measured, SIMS can be used for isotope detection along with the added bonus of hydrogen which cannot be detected by either AES or XPS. SIMS has the capability for direct compound (organic species) detection by molecular secondary ion emission. SIMS can be used for chemical ion mapping with short acquisition times (minutes) and high lateral resolution ($\leq 1 \mu\text{m}$).

The application of TOF SIMS to the characterization of bulk polymers allows determination of repeat units, end groups and additives, monitoring of surface modifications, and identification of contaminants (Benninghoven *et al.*, 1987). The unique information available from SIMS complements the other surface analytical techniques and have made it an important tool in adhesion studies as well, from studying the pre-treatment and cleaning methods on the bonding surface to the surface after bonding (Spool, 1994).

Confocal Scanning Laser Microscope (CSLM)

Confocal microscopy enables viewing the formation and development of the biofilm on the stainless steel *in situ*. In a confocal scanning laser microscope (CSLM), light originating from a laser is brought to a focal point by an objective lens. A second (collector) lens is positioned so that the same point is imaged on the detector pinhole, called the confocal condition (Brakenhoff *et al.*, 1979). To build up the image it is necessary to scan the beam across the specimen or vice versa (Bio-Rad's User Manual, 1986). This is because the confocal microscope does not produce a complete optical image of the specimen. The image is then displayed on a cathode ray tube running in synchrony with the object scan (Fig. 18, Brakenhoff *et al.*, 1979). The CSLM used in these experiments was Bio-Rad MRC 600, located in the Center for Biofilm Engineering. In this model the same lens functions as the objective and condenser lens (Fig. 19).

Light microscopes observe live objects in real time and *in situ*. CSLM has definite advantages over conventional microscopy. The CSLM has an extended dynamic range and an improved resolving power in the transverse direction. Also the existence of sophisticated fluorescent probes, enables researchers to view the spatial organization of specific substances inside biological material (live organisms). The CSLM can generate three dimensional images of living cells. Thus, the CSLM is unique in being able to obtain spatial information directly from hydrated material (Van Der Voort *et al.*, 1987).

Figure 18: Conventional CSLM

Figure 19: Bio-Rad CSLM

CHAPTER 4

MATERIALS AND METHODS

Experimental Protocol

Sample Preparation

Two unpolished 304 stainless steel coupons (6.4 mm x 12.7 mm, 0.5 mm thick) were cut from the same sheet metal of 20 gauge (0.9 mm) with a 2B finish (as-received). The sheet metal was obtained from Metal Samples in Munford, Alabama. The stainless steel was hot rolled (1260 °C), pickled, passivated, cold-rolled, solution-annealed at 1121°C, rapidly cooled and subjected to a final cold roll to flatten and shape. The final sheet metal had an ASTM (American Society for Test Methods) #7 grain size (Geesey *et al.*, 1996). In the case of the polished samples, mechanical polishing was done using an automatic polisher located in the Geology department, MSU. The samples were polished using 1µm grit alumina polish till a smooth finish was obtained (after about 10 minutes). The samples were then polished for five minutes each using 0.3µm and 0.05µm grit alumina polish at a speed of 150 rpm.

Both polished and unpolished samples were cleaned ultrasonically using sequential washes with organic solvents: acetone, methanol, hexane and then finally with acetone again. All samples were marked with a x-y coordinate system with a sharp needle before the cleaning procedure. The polished samples were marked with six squares of

dimensions $120\text{ }\mu\text{m} \times 120\text{ }\mu\text{m}$ using the gallium liquid metal ion gun (LMIG) of the TOF SIMS, so as to identify the analysis area between various instruments. The squares could not be marked on the unpolished samples because they could not be located due to the extreme roughness of the unpolished surface. A schematic of the sample marking is shown in Figure 20.

Sample Insertion

As stated earlier, the surface analysis chamber needs to be maintained at ultra high vacuum conditions. In order to insert samples into the UHV chamber without compromising the vacuum totally, a preparation ("prep") chamber or a sample introduction chamber is used where the chamber is pumped down from ambient conditions to at least 10^{-6} torr pressure before inserting the sample into the UHV chamber. During the sample insertion, the pressure in the UHV chamber does rise, but quickly drops back to the UHV regime.

Surface Analysis using Atomic Force Microscopy (AFM)

The surface of the as-received coupons was examined by AFM (Topometrix, TMX 2000) in the "contact mode", to establish the exact surface morphology of the grains and channels. The topography is shown in Fig. 21.

120x120 μ m square
with hole in center

Enlarged view of hole

Fun with the LMIG ion gun

Figure 20: Sample marking scheme

Surface Analysis using AES

Only AES was used in the experiments on unpolished samples since the XPS and TOF SIMS instruments were unavailable at that time. Preselected areas on the biocoupon (coupon exposed to bacteria and media) and on the control coupon (coupon exposed to sterile media alone) were analyzed using AES in the as-received condition (before exposure to biofilm or media) as well as after exposure to the respective media. Data were taken with a 3 keV primary electron beam and at a 1000X magnification. The coupons were initially sputtered in short time intervals using the argon (Ar) ion sputtering technique to remove the surface contamination due to exposure to air and to remove part of the protective oxide layer. The oxide layer was sputtered until approximately 5-14% oxygen was left on the surface in order to determine the chemical changes within this layer close to the bulk stainless steel. After each sputtering an area-averaged spectra from a $100 \times 100 \mu\text{m}^2$ area was taken so as to obtain depth profiles of the various elements on the surface, as shown in Fig. 22. Elemental chemical maps and Scanning Electron Microscope (SEM) images were also taken of these areas. Ten grains on the control coupon were analyzed before and after exposure to the sterile media. The positions of these grains were noted on an SEM picture of the area. Individual grains were also analyzed on the biocoupon before and after exposure to the biofilm.

Figure 21: AFM image of the 304 unpolished surface

Figure 22: Depth profile of an as-received stainless steel coupon

Surface Analysis using XPS

The 2 mm monochromatized Al K_{α} source and 400 μm aperture were used for all analyses. Initially wide area scans or survey (0-1100eV binding energy in XPS and 0-1000eV kinetic energy in AES) and multiplex scans were taken of the analysis area. The multiplex scan consisted of scanning only certain energy windows (and thus taking scans of certain elements only and saving time by not taking data where no element lines exist), instead of the whole range as in a survey scan. The elements used in the multiplex scans were carbon (C1s), oxygen (O1s), nitrogen (N1s), iron (Fe2p), chromium (Cr2p) and nickel (Ni2p). N1s was monitored only after exposures of the samples to biofilm or media, since no nitrogen was present in the as-received samples. The survey scan was taken at 93.6 eV pass energy at 0.2 eV/step with an acquisition time of 10 minutes and the multiplex scan was taken in high resolution mode at 23.5 eV pass energy at 0.2 eV/step with a total acquisition time of 20 minutes. The measurements were performed with the analyzer, ion gun and television camera at 45° with respect to the sample surface

The analysis area was then sputtered using the Ar ion beam and survey and multiplex scans were again acquired. The procedure was repeated at regular time intervals to obtain a depth profile of the elements present on the sample. The sputtering was done initially for 5 seconds with an ion current of 0.01 μA until the surface-bulk interface was reached and then the sputtering time was increased in steps to 15, 30, 45 and 60 seconds. The depth profile is shown in Fig. 22. Sputtering was stopped when the relative O

concentration was at $\leq 10\%$, as this was taken as an indication of approaching the bulk of the sample. In later experiments the analysis area was masked with an Al foil containing a $750\text{ }\mu\text{m}$ hole for the analysis area, so as to be assured that the data was taken well within the analysis area.

Surface Analysis using TOF SIMS

Positive and negative ion spectra were taken from each of the six squares on the polished samples and specific areas marked using a coordinate system on the unpolished samples. The data were taken using the LMIG source at 25kV from an area of $80\text{-x } 80\text{ }\mu\text{m}^2$. This was to ensure that the data was coming from the center of the $120\text{ x } 120\text{ }\mu\text{m}^2$ squares on the polished samples. The data were taken using the retrospective mode which allowed the user to obtain raw data and replay the data later on. This mode records a mass spectrum for each and every point ($256\text{ x } 256$ points total) on the analysis area. Thus, ion maps can be taken and viewed during the replay. This mode allows data portability, as the data can be analyzed on any computer with the TOF SIMS software, leaving the actual instrument free for data acquisition.

Flow-Through Reactor

The flow-through reactor consists of a polycarbonate housing (5 mm wide, 1 mm deep, and 24 mm long) with a viewing window that consisted of a rectangular coverslip (60 mm x 24 mm). A rectangular rubber gasket with an opening over the coverslip

(which formed the viewing window) was placed between the coverslip and the top cover of the reactor. The sample was placed in a channel in the bottom half of the reactor with the coverslip placed directly above the sample. The coverslip sealed the housing by tight contact with the flat face of the polycarbonate holder. The reactor was steam sterilized for 20 minutes at 121°C, before the sample was placed in it.

Inoculum and Media

A mixed culture consisting of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Klebsiella pneumoniae*, which had been previously isolated from a corrosive biofilm (Stoodley *et al.*, 1994) was used in all experiments. This mixed culture was chosen because it had been well studied and characterized in numerous experiments at the Center for Biofilm Engineering. Stocks of the mixed culture were prepared and stored in glycerol peptone solution in vials, in a freezer kept at -70 °C.

The media used in these experiments was Scheusner's minimal salts media. The media consisted of the following constituents in a 1 liter volume:

0.7g	Dibasic Potassium Phosphate (K_2HPO_4)
0.3g	Monobasic Potassium Phosphate (KH_2PO_4)
0.1g	Ammonium Sulfate ($(NH_4)_2SO_4$)
0.01g	Magnesium Sulfate ($MgSO_4 \times 7H_2O$)
0.1g	Glucose (100ppm)

The glucose was filter sterilized using 0.2µm polycarbonate filters (Poretics Corporation,

Livermore, CA) and added after the rest of the media was autoclaved to prevent caramelizing.

System Assembly and Preparation

The reactor was a once through system (Fig. 23), i.e. the media was not recycled through the system after it was passed through the reactor. The media was prepared in 10 liter carboys using deionized water and steam sterilized at 120 °C. The time required for autoclaving was approximated at 15 min/L. However, the sterilization time does not increase linearly with volume. A four hour sterilization was done for 2 10 liter carboys and a six to seven hour sterilization was done for 4 10 liter carboys. The carboy was sealed using a #13 rubber stopper with three holes, all with glass tubes inserted in them. One tube had an air filter attached to it, to allow pressure dissipation during autoclaving. The second tube was sealed with a small rubber stopper and used for inserting glucose into the media after autoclaving without exposing the media to air and other contaminants. The third tube was the influent tube. The glucose and influent tubes were pinched off with stoppers during autoclaving to prevent liquid from boiling off from these outlets. The stopper from the influent tube was removed when the media was used. The glucose stopper was only removed for glucose insertion and then reattached.

The media (influent) from the carboy was pumped by a Masterflex pump and controller through a Norprene size 13 tubing (Cole Palmer) into a mixing vessel. Fluid was then pulled from the mixing vessel through the reactor by means of a Masterflex

Figure 23: Schematic of the flow-through reactor system

pump and controller and then directly into waste (effluent) as shown in Fig. 23. Norprene tubing (black in color) was used instead of silicone tubing to prevent silicon contamination on the sample. However, at all the inlets and outlets a small length of size 20 silicone tubing was used to visually verify media flow. A flow break was placed between the influent and the media and another was placed between the reactor and waste to prevent back growth of the bacteria and to avoid contamination. The mixing vessel was a 250ml flask sealed with a rubber stopper. The stopper had an opening for oxygen, an opening with a rubber tube through which the inoculum was injected, one for the influent and another for the effluent. Air was continuously bubbled into the mixing vessel using a fish tank pump. Pulse dampeners were placed at various points before the reactor to allow air bubbles to escape out into the atmosphere before reaching the reactor.

The entire system, (Fig. 23), with the exception of the sample was steam sterilized in the autoclave at 120 °C. After autoclaving, the sample was removed from UHV and sealed in the channel of the sterile flow-through reactor with the aid of a chemically non-reactive Torr Seal vacuum seal, in a biological hood. The reactor was then reconnected to the system. Sterile media was pumped through this reactor using a Masterflex pump (1-100 rpm speed) and controller at a flow rate of 1.6 ml/min. The residence time (defined as reactor volume / flow rate) of the system was maintained at 10 minutes in order to minimize suspended cell growth. The inoculum was directly injected from the vials into an aerated mixing vessel (located upstream from the reactor). The bacteria were allowed to grow there for a period of twenty four hours without flow in

Scheusner's minimal salts media. Media flow was initiated from the influent carboy to the mixing vessel and from the mixing vessel to the reactor and then to the effluent 24 hours after inoculation so as to allow initial bacterial growth.

After each experiment, the influent and effluent tubing to the reactor were clamped off and the reactor removed. The sample was removed from the reactor and put into the UHV. The reactor was reattached to the system and deionized water was pumped through the entire system to clean out any biofilm remnants in the system.

Confocal Imaging

Confocal Scanning Laser Microscopy (CSLM, Bio-Rad MRC 600) was used to follow the colonization and biofilm formation of mixed bacterial populations on the surfaces. The technique offers reflected white and or red light capabilities to visualize surface features of opaque substrata such as metals under developing biofilms.

Biofilm growth and development in the preselected areas on the coupons was monitored daily with the CSLM. Images were captured and analyzed using the Bio-Rad Comos software. In the unpolished coupon experiments, biofilm growth was monitored until the coverslip became opaque due to thick biofilm growth (after 18 days). At this point the biofilm experiment was terminated and the coverslip was replaced with a new one so as to record areas colonized by the biofilm clearly. The areas which were not colonized by biofilm were marked for analyses and for comparison with those areas colonized by biofilm. In the polished coupon experiment, one of the squares was

monitored throughout the experiment, for 18 days. On the initial and final days, images were taken from all squares. The biofilm was removed from the surface on the final day by pinching the inlet tubes and cleaning the surface with a cotton tipped stick. Earlier work had shown that this procedure did not affect the surface. Images were then again taken from all squares.

CHAPTER 5

UNPOLISHED STAINLESS STEEL

As-Received 304 Stainless Steel

Preselected areas on two unpolished type ("as-received") 304 stainless steel coupons were analyzed using AES, before exposure to bacteria or sterile media (i.e., in the as-received condition). The bulk composition of the 304 stainless steel is determined by x-ray analysis using an SEM and is shown below in Table 3.

Table 3: Composition of 304 stainless steel

Elements	Fe	Cr	Ni	Mn	C	Si	Cu	P	S
Percent	71.22	18.18	8.19	1.51	0.07	0.43	0.36	0.032	0.009

One of the coupons was exposed to sterile abiotic media for 18 days, henceforth referred to as the "control coupon". The other coupon was exposed to media containing a defined mixed culture of bacteria (described in Chapter 4) for 18 days, henceforth referred to as the "biocoupon". The positions of ten grains on the control coupon were noted on an SEM picture of the area and then analyzed. Individual grains were also analyzed on the biocoupon before and after exposure to the biofilm.

Experimental Results And Discussion

Experimental results will be presented in two major groups; the first group of data were obtained from the as-received stainless steel coupons and the second group of results were obtained when the coupons were exposed for a period of 18 days to media with and without bacteria.

Concentrations of the elements are calculated from the peak-to-peak height of each line and the sensitivity factors of each element as given in Table 1 of chapter 4. The concentrations of Fe, Cr and Ni are normalized so that $\text{Fe \%} + \text{Cr \%} + \text{Ni \%} = 100 \%$, which have been referred to as relative concentrations.

As received coupons

The mean relative concentrations in the oxide region close to the bulk, of Fe, Ni, and Cr, respectively, before exposure to either bacteria or sterile media, were $74.9 \pm 2.4 \%$, $12.6 \pm 1.5 \%$, and $12.5 \pm 1.5 \%$. The distribution of these concentrations varied somewhat from sample to sample, and grain to grain but the values stayed within the expected statistical uncertainty in the data. Chemical mapping revealed (shown in Fig. 25) that C was concentrated mainly in the grain boundaries, while Fe and O were concentrated on the grains. Cr was spread uniformly across the surface. There was very little Ni present on the surface. EDAX analysis revealed that about 10% of the surface area was made up of grain boundaries.

Figure 24: Confocal image of biocoupon after two days of biofilm colonization. The dark patches are biofilm colonies.

**SEM Image
of an
unpolished
as-received
stainless
steel**

C Map

Fe Map

Figure 25: Auger image and chemical maps of C, Fe(same as O) of an as-received stainless steel.

Figure 26: Auger spectra of control and biocoupon for 304 unpolished stainless steel.

The 18-day Exposure

Fig. 26 above, shows two Auger spectra comparing the control and biocoupon after 18 days exposure to their respective treatments. Qualitatively, it is observed that the biocoupon spectrum contains nitrogen with some excess Cr in comparison to the control coupon. There is no nitrogen in the control spectrum. The Fe peak is observed to be reduced in the biocoupon spectrum as compared to that in the control spectrum.

Figs. 27 and 28 show, respectively, the relative Fe % vs. the relative Cr % and the relative Fe % vs. the relative Ni % for control and biocoupon. Note that Fig. 27 shows a distinct separation between the two groups of data. There was an overall increase in the relative percentage of carbon on the biocoupon and control coupon after 18 days of exposure as compared to that before exposure. There were marked differences in the Cr and Fe relative concentrations between the biocoupon and the control coupon. Mean relative Cr concentration increased by 7.2 ± 1.7 % in the biocoupon as compared to that of the control coupon. Correspondingly, a 7.0 ± 1.9 % decrease was observed in the mean relative Fe concentration in the biocoupon as compared to the control coupon. There were no differences observed in the mean relative Ni concentrations between the two coupons, as seen in Fig. 28. The mean relative concentrations for the two groups are also shown.

Fig. 29 shows the relative Fe % vs. relative Cr % for colonized and uncolonized areas on the biocoupon selected using the confocal microscope. This comparison is similar to that done in Fig. 27, but the separation of the data groups although not distinct

Figure 27 : Normalized Fe concentration versus the normalized Cr concentration for control and biocoupon after 18 days of exposure. The dotted lines indicate the means of the groups.

Figure 28 : Normalized Fe concentration versus the normalized Ni concentration for control and biocoupon after 18 days of exposure. The dotted lines indicate the means of the groups.

Figure 29: Normalized Fe concentration versus the normalized Cr concentration for biofilm colonized and uncolonized areas on the biocoupon. The dotted lines indicate the mean relative concentration for that group of data.

still shows that the areas colonized by biofilm have more Cr and less Fe.

One difficulty of analyzing the data in terms of the main components of the stainless steel was the question of how to handle the other elements such as C, O, Si, N, and P. The main emphasis here is to determine the statistically meaningful alterations in Fe, Cr, and Ni concentrations in the oxide layer of the surface due to bacteria. This objective was shadowed because of the variation in the elemental composition of the surface from grain to grain and time to time. There are several reasons for this variation. One reason was the variation in the amount of the other elements present (mentioned above). The other reason is the noise in the spectra; particularly at high kinetic energy (Ni region) it causes fluctuations in the measurements of the Ni concentrations. Another reason is the uncertainty associated in assessing exact peak-to-peak determinations. For example Cr and O peaks are very near each other which causes difficulty in determining the exact peak locations.

Let X denote the sum of the absolute concentrations of the rest of the elements ($Si+N+O+Ar+P+S$) except for Fe, Cr and Ni, present on the surface. The absolute Fe concentration versus X for the control and the biocoupon, before and after 18-days exposure, is shown in Fig. 30. The data points fall on two distinct (dotted) lines, the top line in which no bacteria was involved and the bottom line in which the coupon was exposed to the bacteria. The spread in concentrations is due to nonuniformities in the distributions of various elements from grain to grain and time to time on the area of interest. These (dotted) lines are the least-squares fit to the data points. These lines

Figure 30: Absolute Fe concentrations versus the sum of the absolute concentrations of all other elements (except Cr and Ni) present on the control and biocoupon. The dotted lines indicate the linear least square fit to the data.

indicate the linear dependence of the absolute Fe concentrations on X. The same dependence on X was also observed in the case of Cr and Ni .

The linear dependence of the Fe, Cr, and Ni on X suggests that the relative concentrations of Fe, Cr, and Ni do not change with the accumulation of other elements on the surface. This implies that X can be eliminated from the analysis by simply considering the relative concentration of Fe, Cr and Ni. Thus, these concentrations are normalized so that $\text{Fe \%} + \text{Cr \%} + \text{Ni \%} = 100 \%$, which have been referred to as relative percentages and are shown in Figs. 27, 28 and 29.

In order to determine whether the differences observed in the mean relative percentages of Fe, Cr and Ni on the biocoupon and the control coupon (Fig. 27), were statistically significant, a bivariate analysis of variance test (Seber, 1984) was conducted. The calculations were accomplished using the Statistical Analysis System (SAS) procedures PROC MANOVA and PROC DISCRIM (SAS/STAT User's Guide, 1988). The same statistical analysis was applied to the data for the colonized and uncolonized areas on the biocoupon. The main result of the test is a probability value (p). A small p-value indicates that the data contradict the null hypothesis that the true mean of the relative concentrations (of the alloying elements) on the control coupon is identical to the true mean of the relative concentrations on the biocoupon. The true mean is the conceptual mean of the relative concentration if relative concentrations were measured on an infinite number of identically treated grains. For a 95% confidence on the test, the value of p should be less than 0.05 to conclude that there is a statistically significant

difference between the means being compared.

Table 4 shows the mean relative concentrations for the control coupon and the biocoupon, after 18 days and the corresponding p-value ($p = 0.001$). We can conclude that there is a statistically significant difference between the Fe and Cr concentrations on the control coupon and the biocoupon after 18 days. The mean concentrations of Ni between the two coupons are not significantly different. The mean relative percentages of colonized and uncolonized areas on the biocoupon after 18 days and the corresponding p-value ($p = 0.17$) for this comparison are shown in Table 5. We conclude that differences between the colonized and uncolonized areas on the biocoupon are not as significant as that between the biocoupon and control but shows a similar cause and effect by bacteria as in the case of the biocoupon. The reasons for this may be that these areas are several 100 μm outside the area of interest. Hence, the sputtering during the pre-analysis (before exposure) will be inadvertently less in those areas, creating different levels of removal of the passive layer present on the surface. This will give rise to variations in the passive layer present on the surface, creating a different environment for the bacteria on the various areas.

Another reason for the lack of differences between Fe, Cr and Ni relative concentrations in Table 5 could be that the bacteria may have initially attached to an area on the biocoupon, and then subsequently detached from it. This could lead to the idea that a certain area was uncolonized when viewed (under the microscope) after this detachment process. The presence of the bacteria earlier would have lead to chemical changes on the

Table 4: Comparison of mean relative concentrations of Fe, Cr and Ni between the control coupon and biocoupon after 18 days; $p = 0.001$.

ELEMENT	CONTROL COUPON AFTER 18 DAYS	BIOCOUPON AFTER 18 DAYS
Mean Fe (%)	70.7 ± 1.2	63.7 ± 1.7
Mean Cr (%)	13.6 ± 1.5	20.8 ± 2.0
Mean Ni (%)	15.7 ± 2.2	15.5 ± 1.7

Table 5: Comparison of mean relative concentrations of Fe, Cr and Ni between the colonized and uncolonized areas on the biocoupon after 18 days; $p = 0.17$.

ELEMENT	UNCOLONIZED AREA AFTER 18 DAYS	COLONIZED AREA AFTER 18 DAYS
Mean Fe (%)	69.7 ± 2.6	67.9 ± 3.0
Mean Cr (%)	15.4 ± 1.6	16.9 ± 1.8
Mean Ni (%)	14.9 ± 2.3	15.2 ± 2.1

so called uncolonized areas.

The presence of the excess carbon on both samples after the 18-day exposure could be partly due to the bacteria, organic molecules present in the media and partly due to some contamination from air. The mechanism of Cr enrichment and Fe depletion are not clear. One speculation is that Fe may have been carried away by the flowing media and that Cr compounds may have been redeposited on the surface.

Conclusions

Biofilm causes chemical changes on unpolished 304 stainless steel surface in the oxide film close to the bulk. These changes can be summarized as follows: the concentration of Cr is enriched by 7.2 ± 1.7 % relative to that of the control coupon. The relative concentration of iron is depleted by 7.0 ± 1.9 %. The relative Ni concentration showed no noticeable change (0.2 ± 1.6 %). Although not statistically significant, differences were observed in the relative Cr, Fe, and Ni concentrations between the colonized and uncolonized areas due to a patchy biofilm on the biocoupon which support the hypothesis that biofilm cause the removal of Fe in the oxide region close to the bulk.

CHAPTER 6

LOCALIZED PITTING

Biofilms - An Electrochemical Cell ?

Inherent surface topological and chemical features of metals may influence the pattern of colonization of biofilm-forming microorganisms that promote the establishment of surface chemical and electrochemical heterogeneities that, over time, can promote substratum deterioration in the form of localized corrosion. Pitting corrosion of stainless steel alloys often occurs over regions that have been welded and contain a microbial biofilm. Mapping the distribution of microorganism, surface-chemical, electrochemical, and topological features will reveal correlations between biological, physical, chemical and electrochemical phenomena. These phenomena will contribute to substratum deterioration, decreased system performance and lead to costly equipment repairs and replacement.

In this chapter electrochemically induced pits in the presence of sterile media are investigated, so as to assess the role of the media in this process and the resultant chemical deposits relating to the liquid/metal interface. The morphology, density, and chemistry of these localized pits on the surface were also investigated. The objective was to determine if the chemical changes occurring in these experiments can be correlated to those observed due to biofilm colonization.

Experimental Protocol

Preselected areas on three unpolished stainless steel coupons were analyzed by AES before exposure to bacteria or media or electrochemical currents. The flow-through reactor was modified for these experiments to induce pits on the stainless steel coupon as the coupon was exposed to sterile media. The schematic is shown in Fig. 31. A three-electrode system was made, where the 304 stainless steel coupon served as the working electrode. A molybdenum plate with dimensions of the coverslip replaced the coverslip for the electrochemical experiments and served as the counter electrode. A silver/silver chloride (Ag/AgCl) reference electrode completed this three-electrode system. The reference electrode was extremely sensitive to local changes at the surface and could not be used. This problem was overcome by maintaining 1.2 volts continuously between the stainless steel coupon and the molybdenum plate and monitoring the current on the chart recorder. Since the aim of this experiment was to generate pits on the surface, this setup was sufficient. The schematic of this circuit and the graph of the current vs. time are shown in Figs. 32 and 33 respectively.

The second coupon, the biocoupon, was exposed for three days to the mixed culture biofilm (described earlier) alone, no voltage was applied. The third coupon, the control coupon, was exposed to sterile media for three days. The experiments were terminated at the end of the third day when well defined colonies were formed on the surface, as observed by the Confocal microscope. The aim of these experiments was to

Flow-through reactor modified for electrochemical experiments

Figure 31: Schematic of Modified Flow-Through Reactor

Figure 32: Circuit diagram for electrochemical pit induction

Figure 33: Current versus time as monitored in the electrochemical experiment

correlate changes occurring on areas underneath the biofilm to those changes in the pits.

The electrochemical experiment was terminated after 14 hours because there was no change in current observed after 10 hours thus indicating that no more electrochemical changes were occurring on the surface. In order to verify this theory, the experiment was continued for another 4 hours. No change in current was observed and the experiment was terminated.

Results

As-received, Biocoupon and Control coupon:

The coupons were analyzed before and after exposure to biofilm and media, sterile media, and sterile media with electrochemical treatment. Depth profiles of the elements present were taken in the case of the biocoupon and control coupons. The depth profiles of the control after exposure to sterile media and the as-received coupons were similar in all respects, as shown in Fig. 34. The depth profile of the biocoupon after exposure to the biofilm is shown in Fig. 35. An excess amount of carbon was present on the surface of the biocoupon. This carbon persisted through the oxide layer up to a depth of 50 Å, while the carbon present on the control after exposure to sterile media, was sputtered to its bulk level very quickly, by a depth of 20 Å.

Figure 34: Depth profile of control after exposure to sterile media.

Figure 35 : Depth profile of biocoupon after exposure to biofilm for three days

Morphology And Chemistry of Pits

There were striking physical and chemical changes before and after electrochemical treatment. The changes can be grouped into three categories:

(1) localized pits, (2) C-rich grains and (3) O-rich grains.

Fig. 36 (top) shows a typical pit as seen by the Auger microscope on the right and blackened grains on the lower left of the picture. Figures 36(middle) and 36(bottom) are enlarged views of the same areas. The area in Fig. 36(middle) will be referred to as Pit 1 and the area in Fig. 36(bottom) will be referred to as Pit 2 henceforth. Pit 1 represents a typical pit observed across the surface and is about 20 μm in diameter. Pits of this type are scattered throughout the substrate surface with a density of less than 2 parts per thousand of the total area. "Pit" 2 is a misnomer for this feature since there is no loss of material in this area as in the case of Pit 1. This area contained excess carbon deposits most likely due to hydrocarbon chemisorption as in the case of grain boundaries.

Chemical maps reveal the chemistry of Pits 2 and 1 to be very similar, as shown in Figs. 37 and 38. These figures correspond to elemental maps of C, O and Fe corresponding to Pits 2 and 1 respectively. Carbon is concentrated inside the pits or black areas just as it is in the grain boundaries, whereas outside these areas the usual distribution of Fe and O (O-rich areas) are seen. It appears that when the chromium oxide protective layer is bridged, the pitting formations are encouraged and the area is coated with hydrocarbon deposits as in the grain boundaries.

**SEM image
after electro-
chemical
activity
showing
two pits**

Pit #1

Pit #2

Figure 36: Auger normal (top) and enlarged images of Pit1 (middle) and Pit 2 (bottom).

C Map

O Map

Fe Map

Figure 37: Auger elemental maps of Pit2

C Map

O Map

Fe Map

Figure 38: Auger chemical maps of Pit 1

BEFORE

AFTER

ELECTROCHEMICAL TREATMENT

Figure 39: Auger images (top left and right) and oxygen maps (bottom left and right) of an area before (top and bottom left) and after (top and bottom right) electrochemical treatment.

In some areas (C-rich areas), although the carbon deposits cover the majority of the surface, (Fig. 39) there is no physical effects observed on these areas. Figures 39(top, left) and 39(top, right) are the Auger pictures before and after the electrochemical treatment, respectively. These figures show no visible physical differences. Figs. 39(bottom, left) and 39(bottom, right) are the corresponding oxygen maps of the same areas before and after electrochemical treatment. The dark areas in these figures are carbon rich areas.

Depth profiles of a grain at the periphery of Pit 2 were taken. This grain was half black and half white (normal) as seen in Fig. 36(bottom). The depth profile of the white half of the grain, shown in Fig. 40 (except for some carbon diffusion in to the bulk), is the same as Fig. 36(middle), i.e. the control coupon after exposure to sterile media. The depth profile of the black profile, shown in Fig. 41, is similar to the depth profile of the biocoupon after exposure to biofilm (Fig. 36(bottom)).

Discussion

While pitting corrosion is well documented, little is understood about pitting initiation (Castle and Ke, 1990). In the corrosion process, the fundamental reaction of a metal with its environment is the replacement of the metal electrons by anions from the environment as "binders" for the metal cations. The nature of the metal determines if corrosion will continue i.e., if the metal is easily soluble in the liquid the dissolution will continue (Hoar, 1970). When the stainless steel is submerged in the aqueous medium, a

Figure 40: Depth profile of white half of the grain.
Note that the oxygen vanishes after 50 Angstroms.

Figure 41: Depth profile of black half of the grain.

major adsorbate at the metal-liquid interface is water. At negative potentials the water molecules are oriented with the hydrogen end of the dipole next to the metal, and at positive potentials the oxygen is oriented next to the metal. This is shown schematically in the diagram below. The potentials are termed cathodic and anodic respectively.

Positive potentials deprotonate the water very readily since the anodic field can replace them easily with metal cations from the metal lattice and thus begin passivation. However at low positive potentials, the anodic field removes the metal cations without deprotonating the water leading to dissolution of the metal (Hoar, 1970).

Hoar (1970) found that for stainless steels in chloride solutions and in a complex solution the breakdown (pitting) potentials were + 0.2 to + 0.4 V relative to the standard hydrogen (reference) electrode (SHE). He and his coworkers maintained the stainless steel at a constant potential and monitored the passive current. They found an induction period during which no change occurred followed by an extremely high current at breakdown (Hoar, 1970).

Koehler and Evans (1964) found that the growth of an insoluble film on a metal surface maintained at a constant potential, causes a continuous decrease in the current. This is termed as passivation. Passivity is defined as the state of a metal surface

characterized by low corrosion rates in a potential region that is strongly oxidizing to metals (Sedriks, 1986). The net rate of film formation decreases till a steady state is achieved where film formation equals film dissolution rate (Koehler and Evans 1964). Brookes *et al.* (1990) also observed that between 0.5V and 1.2V dissolution of the passive layer occurred along with continuous current decay. They maintained that the current decrease was due to the repassivation of the surface. Also under natural conditions pit initiation can take a long time. However it can be induced by anodic polarization. With these ideas in mind, a positive voltage of 1.2 V was applied to the stainless steel. As seen in Fig. 33 the current starts at a high value and then continuously decreases. This corresponds to local breakdown of the oxide film on the stainless steel with the formation of localized pits. However as the surface is maintained at a constant voltage, the repassivation of the surface occurs completely and no more breakdown is seen on the surface. This is why the current was observed to reach a steady state in this work.

Brown *et al.* (1992) found that laminar flowing conditions increased the stability of the passive film against pit initiation. The entire experiment, in our work, was carried out at a steady laminar flow of the media across the surface. These conditions probably made the surface less susceptible to pitting as well.

As mentioned in the results, there were two types of pits observed, one in which there was dissolution of material and the other in which there was no dissolution observed. The pits in which no dissolution of material is observed may be metastable pits.

All pits start as metastable pits which can either undergo stable pitting or repassivate (Burnstein and Psitorius, 1995). Burnstein and Psitorius (1995) noted that on a rougher surface, there would be more metastable pits due to the many activation sites. However these sites were also less open and hence the metastable pits would not develop into stable pits. Daufin *et al.* (1985) also discuss these types of pits, calling them depthless, repassivated pits. They stated that these pits would have two characteristics: (a) no material dissolution, and (b) a colored ring resulting from the cathodic reduction process would be present to show that corrosion action was in progress (Daufin *et al.*, 1985). The second pit observed in this work satisfies these criteria and further lends credence to the theory that when breaks in the passive film occur, repassivation of these areas may occur. In this case, the pits essentially die (Burnstein and Psitorius, 1995). Scully (1990) adds that pitting occurs in areas where the repair is so slow that the solution changes necessary to stabilize pitting can occur (Scully, 1990). This causes a local breakdown of the passivating film followed by pitting (Frankenthal, 1967).

Once a pit is established, it continues to grow, making the surrounding area a permanent cathode. This protects the surrounding areas by making pits grow at discrete distances from each other (Scully, 1990) and explains why in this work pits were observed to be well separated from each other, and that the surface has local anodic and cathodic sites. During the course of pitting, pits are often covered with a membrane of product which severely restricts the access of oxygen. The oxygen in the liquid in the pit is consumed by reduction and as no further oxygen is available, the attack on the metal

increases within the pit. This we believe, is why Auger chemical maps show the pits to consist primarily of carbon with no oxygen present. Since the attack is localized, the other areas are relatively untouched. Hence the same oxygen and iron patterns are seen in areas around pits as before pitting.

Similarities between pit chemistry and areas colonized by bacteria lend support to the theory that bacteria cause the generation of local anodic and cathodic sites. It is thus not surprising that over time localized corrosion is seen in microbially colonized areas. Since the above comparisons were made between metastable pits on one surface and biofilm colonized areas on another surface, the conditions may be right for pitting of metastable pits to occur, but pitting may or may not occur on the biofilm colonized areas.

Conclusions

The results demonstrate that chemical changes can be observed on surfaces of 304 stainless steel exposed to biofilm and electrochemical pitted corrosion. These changes are most clearly reflected in the abundance of the carbon on the surface, penetrating into the oxide film. There appear to be similarities in the chemical changes on the 304 stainless steel under biofilm and in pits and in surface perturbations induced by electrochemical means. The white half (oxygen rich area) of the grain is the same as the as-received steel (except for some carbon diffusion into the bulk), while the black half (carbon rich area) of the grain in Pit 2 resembles the pitted areas and areas under the biofilm. These carbon

rich areas are metastable or depthless pits just on the brink of pitting. Instead repassivation occurred in these areas, protecting these areas from further breakdown. The results suggest that chemical changes occurring on stainless steel involve organic layers between the top surface and the bulk where a mixed interface is formed. The mechanism of pitting is perhaps associated with the bridging of this interface.

The model stainless steel system proposed by these results is shown in Fig. 42 below. The presence of organic molecules on the surface which are separated from the bulk by a mixed organic-passive layer interface are the observations incorporated from the results into this model. The stainless steel is thus divided into three regions: (i) the very top surface covered by organic molecules, (ii) the mixed interface, and (iii) the bulk material.

Figure 42: Model of the stainless steel as suggested by experimental results

CHAPTER 7

POLISHED STAINLESS STEEL

Surface Chemistry of Steel and its Role in Biofilms and Steel Interactions

The previous two chapters have shown that surface chemistry and morphology play an important role in bacterial and stainless steel interactions. In order to reduce the variables that affect this interaction, mechanically polished surfaces were chosen. The use of polished surfaces removed the problem of grains and grain boundaries which may have an influence on bacterial colonization encountered in unpolished surfaces.

The previous chapters also revealed the importance of understanding surface chemistry. In this chapter the short range chemistry (local environment surrounding an ion) is examined with XPS and the long range chemistry (interrelation between atoms) is examined with TOF SIMS.

Experimental Protocol

The mechanically polished samples were etched with six squares of 120 x 120 μm dimensions using the Ga gun in the TOF SIMS operated at 15 keV. SIMS positive and negative spectra were taken from an area of 80 x 80 μm from the center of each of the squares before and after exposures. Ion images and maps of particular masses were also taken. The samples were then analyzed using XPS. An aluminum mask was used to cover

the analysis area so as to be sure that the data was being taken from that particular square and no other. This also reduced the risk of other squares being sputtered inadvertently during analysis of the previous area. Depth profiles and angular resolved studies were done before and after biofilm and media exposures. The selected squares were sputtered until the oxygen peak had been reduced to $\leq 10\%$ of the oxygen present on the surface. The biocoupon was then exposed to biofilm and media for 18 days and the control was exposed to abiotic (sterile) media for 18 days. The samples were then again examined in SIMS and XPS.

Experimental Results And Discussion

The data will be presented in two sections. The data of the coupons before exposure, "as-received" will be presented, followed by the 18 days exposure data.

As-received coupons

The interaction of the bacteria with the stainless steel involves their interaction with the passive protective layer on the steel. As such, it is essential to these studies to gain an understanding of this layer in terms of the various elemental components, namely, O, Cr, Fe and Ni, involved.

XPS angular resolved studies and depth profile studies were performed. The results of the angular resolved studies are presented followed by depth profile results. The

SIMS results are presented together with the XPS results in order to complement each other.

General interpretation of XPS Spectra:

Selected spectra of O, Cr, Fe and C are presented in Figs. 43-47.

O1s spectra: Three components (Fig. 43) could be easily discerned: (i) O^{2-} with binding energy ~ 529 eV, (ii) OH^- with binding energy ~ 531 eV, and (iii) H_2O with binding energy ~ 533 eV (Brooks *et al.*, 1986; Clayton and Lu, 1986; Fierro *et al.*, 1989; Harrington *et al.*, 1985; Lumsden and Stoecker, 1986).

The H_2O component may be simply chemisorbed to the surface interacting with alloying compounds on the surface. Both H_2O and OH^- were found to be enhanced at smaller angles (Fig. 43). This shows that they are located on the surface and in the outermost layers of the passive film. The H_2O and OH^- peaks diminished at larger angles. However H_2O diminished considerably more than OH^- . The dependence of the hydroxide-water peak on angle is shown in Fig. 48.

Figs. 50-52 reveal that the O^{2-} peak is associated mainly with the Cr_2O_3 and some Fe, while the OH^- and H_2O peaks are associated with Fe species in the outer layers and with some Cr as the inner layers are approached.

Cr $2p^{3/2}$ spectra: In the smaller angles three components (Fig. 44) were found while at larger angles a fourth component at the higher binding energy side was also found. The peaks were (i) Cr metal from the substrate, (ii) Cr^{3+} corresponding to Cr_2O_3 , (iii) Cr^{6+} corresponding to CrO_3 and (iv) Cr^{6+} corresponding to CrO_4^{2-} (Brooks *et al.*, 1986;

Figure 43: O1s spectra at 15 and 55 angles respectively

Figure 44: Cr $2p_{3/2}$ spectra at 15 and 55 angles respectively

Clayton and Lu, 1986; Halada and Clayton, 1991; Lumsden and Stoecker, 1986; Mesawri and Ignatiev, 1990; Olefjord and Elfstorm, 1982; Olefjord *et al.*, 1985; Storp and Holm, 1977).

The main difference between the above references and this work is that while they quote a binding value of 574.1 ± 0.3 eV for Cr metal and 576.6 ± 0.3 eV for Cr_2O_3 , 573.8 ± 0.3 eV was obtained for Cr metal and 575.8 ± 0.3 eV for Cr_2O_3 , in our experiments. However the difference of 2 eV between the metal and oxide is the same in the references and this work. This shift was only noticed in the Cr spectra. All other elements match the values obtained in literature. However the values reported here are within the limits of statistical uncertainty. Two explanations are possible for this discrepancy. The first possibility is that the stoichiometrically correct species is not present. Another possibility is that Cr_2O_3 is absent. Halada and Clayton (1991) have discussed finding CrO_2 at 575.2 eV binding energy. However they also had Cr_2O_3 present at 576.3 eV and Cr metal at 574.1 eV. This leads to the assumption that there is no CrO_2 present in our case. On increasing the peak positions by 0.3 eV: 574.1 eV for Cr metal, 576.1 eV for Cr_2O_3 , 578.1 eV for CrO_3 and 579.1 eV for CrO_4^{2-} were obtained.

The angle resolved studies indicated stronger Cr_2O_3 and CrO_3 at higher angles, suggesting the formation of a mixed phase at the metal film interface (Clayton and Lu, 1986). The presence of some Cr hydroxide species was discussed earlier, but none were actually found on peak fitting. One possible explanation is that these hydroxide species

interact with OH^- to form CrO_4^{2-} in the solid state or dissociate H_2O molecules to form Cr_2O_3 (Brooks *et al.*, 1986; Clayton and Lu, 1986).

or

Brooks *et al.* (1986) proposed that the formation of CrO_4^{2-} in the solid state would result from the formation of a non-crystalline gel-like hydrated $\text{Cr}(\text{OH})_3$ phase. The solid state environment of these Cr^{3+} ions in the gel would be enriched with OH^- ions resulting in the formation of CrO_4^{2-} . They also discuss the lack of CrO_4^{2-} on the surface. The formation of CrO_4^{2-} requires a hydrated layer, and a higher concentration on the surface would be expected. However the lower-angle spectra do not reveal any CrO_4^{2-} , in agreement with Brooks' (1986) results. Brooks' *et al.* (1986) suggest this may be due to the selection of CrO_4^{2-} formed near the solution-film interface. Finally, they propose that the combination of two different oxidation states of Cr would provide for significant bond flexibility that could lead to the formation of a noncrystalline oxide.

Halada and Clayton (1991) discuss finding hexavalent Cr species at low take-off angles and their importance in improving corrosion resistance. This is in agreement with the results of this work, although hexavalent species were not discerned at higher take-off angles as seen in Fig. 44. The hexavalent species were lower at higher angles, with the trivalent Cr species dominating.

Tjong *et al.* (1982) have confirmed that increasing chromium content in the Fe-Cr alloys results in the formation of large amounts of bound water in the form of OH⁻, and found it to be the highest in the case of Fe-18%Cr alloys. Large amounts of OH⁻ are seen in this work past even in the inner layers of the passive film. Tjong *et al.* (1982) proposed that the high chromium content along with the bound water contribute to the low corrosion rate of these alloys.

Fe 2p^{3/2} spectra: Five components were found at smaller angles (Fig. 45) and six at larger angles: (i) Fe metal at 706.7 ± 0.3 eV, and the rest of them being various oxides at (ii) 708.1 ± 0.1 eV, (iii) 709.05 ± 0.1 eV, (iv) 710.02 ± 0.2 eV, (v) 711 ± 0.2 eV, and (vi) 712 ± 0.2 eV. Various researchers have several different explanations for each of these oxide peaks. Clayton and Lu (1986) give binding energies of 706.8 eV for Fe metal, 709.3 eV for Fe²⁺ in FeO and 711 eV for Fe³⁺ in Fe₂O₃. Brundle *et al.* (1977) observed that for Fe²⁺ species have a Fe 2p^{3/2} peak located at ~709.9 eV with a satellite at ~715 eV, while Fe³⁺ species have a Fe 2p^{3/2} peak located at ~711 eV with a satellite at ~719 eV. While the 715 eV satellite was present, the 719 eV satellite was not observed. This does not imply that Fe³⁺ species is not present, only that Fe²⁺ species is definitely present. Pou *et al.* (1984) have reported that iron in the oxidized state, Fe³⁺, is present at 711.5 eV binding energy. Mesarwi and Ignatiev (1990) have reported that Fe2p^{3/2} peaks present at 710.9 eV and 709.3 eV are indicative of Fe³⁺ and Fe²⁺ present in Fe₃O₄. They also found FeCr₂O₄ at binding energy 708.7 eV. McIntyre *et al.* (1979) have discussed that if the

Figure 45: Fe2p_{3/2} spectra at 15 and 55 angles respectively

peak centroid is located at 711.5 ± 2 eV then it is indicative of a ferric compound, probably FeOOH. The chemical state of iron in the oxide is not easy to detect because of the large amount of coupling between the core hole created by photoemission and the high spin states of iron. It is further complicated due to the presence of the hydrated film on the surface. Gimzewski *et al.* (1977) discuss the shift of the oxidized Fe2p_{3/2} from 710.6 eV to 711.1 eV in the course of oxidation. They pointed out that this shift was not only due to the changing ratio of the two overlapping broad iron peaks but also possibly due to a change in the oxidized iron valency. From these references, it is concluded that Fe metal, FeCr₂O₄, Fe²⁺ in FeO, Fe³⁺ in Fe₃O₄, Fe³⁺ in Fe₂O₃, and Fe³⁺ in FeOOH are present. The Fe³⁺ species is more abundant than the Fe²⁺ species. Also from considering the O, Cr and Fe spectra (Figs. 50-52) it is seen that the oxidized iron species dominate on the outer surface of the passive film while Cr³⁺ is the dominating species in the inner layer of the film. Since from the oxygen spectra (Fig. 48) it is observed that the hydroxide and water components dominate on the outermost surface, iron must be present mainly in the hydroxide-oxide phase. This confirms the duplex structure of the passive film with an outer layer of hydrated hydroxide and an inner layer of oxide, the outer one being mainly Fe³⁺ and Fe²⁺ with some Cr³⁺ and the inner one being mainly Cr³⁺ and some Cr⁶⁺ and some Fe³⁺ and Fe²⁺. The same behavior was observed by many other researchers (Ben-Haim *et al.*, 1988; Clayton and Lu, 1986; da Cunha Belo *et al.*, 1977; Olefjord *et al.*, 1985; Tjong *et al.*, 1982).

Ni 2p^{3/2} spectra: Only metallic Ni spectra were recorded. No evidence of oxidized Ni was

Figure 46: Ni2p^{3/2} spectrum at 75° angle.

Figure 47: Cl1s spectrum at 38° angle.

Figure 48 : Angle versus absolute concentration for oxygen components.

found. There was very little Ni present and it could only be seen at the 75° angle (Fig. 46). The results are in good agreement with other research (Brooks *et al.*, 1986; Clayton and Lu, 1986; Olefjord and Elfstorm, 1982; Olefjord *et al.*, 1985). Olefjord and Elfstorm (1982) have suggested that the positive influence of Ni on the corrosion properties of the steels is not in its occurrence in the passive film but in the underlying metal phase.

C 1s spectra: Only pure carbon with a trace of a carboxyl (C=O) group was found (Fig. 47). Some of the carbon may be contamination from the air.

Depth profile: The depth profile of an as-received 304 stainless steel (taken with XPS, Fig. 49) shows that the profile can be divided into three regions: (i) outermost region, (ii) Cr and O rich passive region and (iii) bulk region. The outermost region shows a high amount of C that reduces to ~5% when the protective Cr and O rich region is reached. The outermost region reveals that more of the Fe species than Cr species is present, in agreement with previously presented angle resolved results. The Cr goes through a maximum in the inner passive film that is also mimicked by the O, indicating that they are related. As the bulk region is reached, Cr diminishes slightly to reach its bulk value. The C and O are considerably reduced while Fe increases to its bulk value. Very little Ni is seen. However the Ni concentration seems to go through a maximum at the film-bulk interface. This is in accord with the theory that Ni's enrichment at the bulk is what makes it possible to passivate the alloy (Brooks *et al.*, 1986).

Figure 49: Depth profile of an as-received 304 stainless steel.

Figs. 50-52 show the depth profiles of O1s, Fe2p3/2 and Cr2p3/2 respectively. From these figures it is seen that the outer hydroxide layer in the O1s spectra follows the Fe oxidized peak while the O²⁻ peak in the O1s spectra follows the Cr oxide peak in the Cr2p3/2 spectra. Some of the Cr oxide is also associated with the hydroxide peak. Fig. 53 shows SIMS spectra for the same surface and reveals the presence of FeOH and CrO on the surface, in agreement with the XPS results. On normalizing the Fe, Cr and Ni concentrations to add up to a 100%, it is observed that the relative Fe concentration is maximum on the very top surface, going through a minimum in the chromium oxide layer of the passive film (Fig. 54). There is about 50% relative concentration of Fe present in the chromium oxide layer. The relative Cr concentration is observed to be a minimum initially on the very top surface and goes through a maximum before reducing to the bulk value. The relative Cr concentration is ~45% at its maximum value.

Ramifications of similar results in terms of the structure of the passive film on stainless steel have been discussed by da Cunha Belo *et al.* (1977) and Ben-Haim *et al.* (1988) and have proposed the existence of two zones in the passive film: an inner nonporous oxide which exhibits good electronic conduction and in which cation transport could be expected, and an outer porous oxide in which anions can migrate. They conclude that the outer layers are in an amorphous state. Ben-Haim *et al.* (1988) report the presence of a metal-hydroxide (M-OH) concentration gradient in the passive film, the

Figure 50: Depth profile of O1s after exposure to biofilm

Iron

Figure 51: Depth profile of Fe2p_{3/2} after exposure to film

Chromium

Figure 52: Depth profile of Cr₂p_{3/2} after exposure to film

Figure 53 : SIMS spectrum of as-received stainless steel, showing FeOH and CrO on the surface.

Figure 54: Depth profile of normalized Fe, Cr and Ni concentrations of an as-received stainless steel

concentration decreasing on going inward into the passive film. They propose that the deprotonation process of M-OH bonds near the metal surface induces a local electric field which promotes repassivation. Also they suggest that higher M-OH bond concentrations imply a more amorphous film and higher improved corrosion protection characteristics due to formation of a polymorphic oxide phase and hydrogen bond formation. Brooks *et al.* (1986) also talk about the bipolar nature of stainless steel and indicate that hydrated metal oxides are either anion selective with a positive fixed charge or cation selective with a negative fixed charge, depending on the particular case.

These references and results from our work suggest that the high iron concentration on the outer layer indicates an outer porous layer. The gradual reduction of the hydroxide peak as the film-bulk interface is approached, seen in the results, lends further support to the hypothesis that the structure of the passive film is amorphous. The increasing Fe metal peak relative to the oxide peak also indicates that the film is thin enough to allow photoelectrons from the bulk metal to escape through the film. This is in agreement with theories that increasing chromium content makes the passivating film thin.

A model of the as-received stainless steel is shown in Fig. 55 below. The surface is covered by a thin layer of water molecules followed by a porous iron hydroxide layer. This layer is ~ 30 Å followed by a chromium oxide layer ~ 60 Å. The iron hydroxide layer is intermixed with chromium and iron oxides. The same holds true for the chromium oxide layer. Iron oxides are mixed into the chromium oxide.

Figure 55: Model of a stainless steel surface

18-day Exposures

The control and biocoupon samples were cleaned with deionized water before analysis. The control was exposed to sterile media alone and the biocoupon was exposed to a mixed culture biofilm and media for 18 days. Angle resolved studies and depth profile studies were done with XPS.

Biocoupon: The angle resolved studies showed similar elemental features as before. However, in the $\text{Cr}2\text{p}^{3/2}$ case, no Cr^{6+} species were observed. Instead Cr^{3+} species related to $\text{Cr}(\text{OH})_3$ and CrOOH were observed along with Cr_2O_3 and Cr metal. The concentration of OH^- and H_2O was higher after exposure to biofilm. Carbon was present mainly in its elemental form with some $\text{C}=\text{O}$, $\text{C}-\text{O}$ and $\text{C}-\text{OH}$ present. Again Ni was present only in the metallic form and was seen only at the highest take-off angle. The major difference from the as-received spectra was the presence of nitrogen ($\text{N}1\text{s}$). The angular resolved spectra revealed that nitrogen was present at all angles (Fig. 56), although it decreased at the highest angle of 75° .

The depth profile studies revealed the presence of an excess amount of carbon as well. These studies showed that nitrogen varied from ~5%-1% as the bulk matrix was approached (Fig. 57). The overall depth profile is shown in Fig. 58. On resolving the different oxygen, chromium, and iron peaks from the depth profiling studies before and after exposure to biofilm, some differences were observed. The O^{2-} peak after exposure (Fig. 60) appeared to have decreased both on the outermost layer and the inner layer of the passive film. There was a corresponding increase in the amount of water adsorbed.

Figure 56: N1s emission angle versus area under the peak

Figure 57: Depth profile of N1s on biocoupon after 18 days of biofilm colonization. Note that this data was taken at the 45° angle.

Figure 58: Depth profile of biocoupon after 18 days of biofilm colonization.

Figure 59: Depth profile of O1s components of an as-received stainless steel.

Figure 60: Depth profile of O1s components of biocoupon after 18 days biofilm colonization.

This increase might be due to the fact that now not only is water present, but also some carbon species that bond with oxygen molecules. These two peaks overlap and are difficult to separate. Since an excess amount of carbon is present on the surface, it is possible that the excess water peak adsorbed to the surface may also contain carbon bound to oxygen.

The depth profile of the chromium shows that the concentration of chromium oxide is lower after exposure to biofilm (Fig. 62) than in the as-received case (Fig. 61). The bulk chromium metal values appear to be the same. This trend is observed in the case of iron as well that the overall oxide after exposure to biofilm (Fig. 63) is reduced as compared to the as-received case (Fig. 64). The iron oxide is however present further into the chromium oxide passive layer. The iron hydroxide layer has increased to ~ 60 Å with the chromium oxide layer extending to ~ 80 Å. The depth profile (Fig. 65) of normalized Fe, Cr and Ni such that $\text{Fe} + \text{Cr} + \text{Ni} = 100\%$ shows that the relative concentration of Fe has increased to $\sim 60\%$ in the chromium oxide film whereas the relative Cr concentration has decreased to $\sim 38\%$. There is no change in the relative Ni concentration. These results contradict the results for the unpolished stainless steel coupons where the Cr concentration increased with a corresponding decrease in the Fe concentration. Ramasubramanian *et al.* (1985) attribute the reversal of the trend in the Fe and Cr concentrations to the chemical differences in the properties of the passive layer and that the concentrations depend on the pH of anodizing solutions in case of electrochemical experiments.

Figure 61: Depth profile of $\text{Cr}2p^{3/2}$ components of an as-received stainless steel.

Figure 62: Depth profile of $\text{Cr}2p^{3/2}$ components of biocoupon after 18 days biofilm colonization.

Figure 63: Depth profile of $\text{Fe}2p^{3/2}$ components of an as-received stainless steel.

Figure 64: Depth profile of $\text{Fe}2p^{3/2}$ components of biocoupon after 18 days biofilm colonization.

Figure 65: Depth profile of normalized Fe, Cr and Ni concentrations of biocoupon after 18 days exposure to biofilm.

Control: The depth profile of the control after 18 days (Fig. 66) is the same as in the as-received case, with a little excess carbon on the surface. No nitrogen is seen in this case.

In the case of the biocoupon there appears to be carbon-nitrogen penetration accompanied with a decrease in the oxide components. This penetration can be explained by two different models: (i) Island Formation and (ii) Mixture. The schematic of the island model is shown in Fig. 67. In the island model there are microscopic biofilm remnants interspersed with bare stainless steel and oxide on the surface. The nitrogen is detected from these localized remnants of biofilm. Three reasons exist why this theory does not hold. First, if the nitrogen was obtained from localized biofilm remnant patches on the very top surface, then it should be possible to sputter through the nitrogen. Also when the nitrogen is sputtered off a sharp increase in the oxygen concentration from below the nitrogen should be seen. As observed from Figs. 56-60, the nitrogen persists all the way until the bulk matrix is approached and no pronounced localized increase in oxygen, corresponding with a dramatic decrease in nitrogen, is seen. The nitrogen steadily decreases all the way through. The second reason being that the angular dependence of nitrogen, carbon, and oxygen plotted together reveal that at 75° the nitrogen decrease is accompanied by a corresponding carbon decrease and an oxygen plateau (Fig. 68). The oxygen plateau indicates that the carbon and nitrogen are present only through the top layers of the passive film, while the oxide persists along with the chromium oxide in the deeper layers of the passive film.. The oxygen increases through the chromium oxide

Figure 66: Depth profile of control after 18 days exposure to sterile media.

Model 1: Islands?

Figure 67: Model 1: Islands?

Figure 68: Angular dependence of nitrogen, carbon and oxygen on the biocoupon after 18 days of exposure to biofilm.

layer and then begins to decrease. Although Figs. 68 and 56 were plotted using the area under the peak versus the emission angle, the areas under the peaks have been normalized to account for geometrical effects involved in angular dependent photoemission. The geometrical factors used in the normalization were obtained from angular dependent studies of graphite. Since the graphite sample contained carbon alone, any change in the area under the peak at different angles is attributed to geometrical factors involved in angular dependent photoemission. The area under the peaks from the different angles are normalized to the area under the peak obtained for the highest angle (75°) in these studies. This gives a normalization factor accounting for the geometrical dependence for each angle, shown in Table 6.

Table 6: Geometric factors for different angles in angle resolved photoemission

Angle (degrees)	15	24	38	45	55	75
Geometric Factor	0.13	0.38	0.67	0.77	0.87	1.0

Fig. 68 can be better understood if we assume we have a tube of unit area and of length λ , the mean free path of electrons, henceforth referred to as the photoemission tube from which photoelectrons from the surface area of depth λ , are detected. As we tilt the sample to various angles, this tube detects photoelectrons from various depths. At the grazing angles, the tube probes the very top surface where the intensity of nitrogen is low on account of lower nitrogen concentrations. At the 45° angle into the surface the tube length coincides with the maximum nitrogen concentration, as it probes deeper into

Polished Biocoupon after 18 Days

Figure 69: TOF SIMS Maps of biocoupon

Model 2: Mixture?

Figure 70: Model 1: Mixture?

the surface. Thus, the majority of the photoelectrons detected, are from this area causing an intensity increase in the carbon-nitrogen intensity. At 75° emission angle the photoemission tube probes deeper into the passive film, the tube now detects photoelectrons from the oxide rich layer. This intensity is greater than the carbon-nitrogen intensity. The carbon and nitrogen intensity thus decreases at this point, at the 75° angle. It must be noted that oxygen is present even on the very top surface, but in a lower concentration than the carbon and nitrogen concentration. This discussion relates directly to the workings of angle resolved x-ray photoelectron spectroscopy as described in Chapter 3 and is represented schematically in Fig. 11. The third reason is that ion maps (Fig. 69) of the masses of iron, chromium and two organic molecules, with the TOF SIMS revealed no local patches corresponding to any of these masses. Thus clearly there can be no localized island formation from which the nitrogen may have been detected.

The mixture model shown in Fig. 70 explains the observed chemical changes more closely. The organic molecules diffuse through the upper oxide layer, displacing some of the oxide molecules. This accounts for the reduction in the oxide components seen on the surface.

Halada and Clayton(1993) found an increase in the Cr^{3+} , and in particular $\text{Cr}(\text{OH})_3$ species, after the steel was exposed to a nitrogen atmosphere. They also proposed that there was a substitution of some of the bound water by ammonia in the passive film, resulting in a lower degree of deprotonation and hence a less dense or looser

passive film. The nitrogen in their XPS experiments was found to consist of NH_3 at 400 eV binding energy and nitride species at 399 eV binding energy. Their angle resolved spectra showed the nitride to be strongest at the metal-film interface. They found the passive film on the surface to be thicker after introduction of the steel to a nitrogen atmosphere than in the as-received condition.

Cabrera (1989) studied the effects on the composition of 302 stainless steel in a N_2 atmosphere. Cabrera (1989) reported nitrogen to be bound to chromium in the form of CrN at 575.5 eV binding energy in $\text{Cr}2\text{p}^{3/2}$ spectra and 396.6 eV in $\text{N}1\text{s}$ spectra, followed by a Cr_2O_3 layer. This work also reported an excess amount of Si on the surface, which seemed to inhibit nitrogen adsorption to the surface. Thus, implying more nitrogen will be dissolved in iron containing lower amounts of Si.

There are many similarities between the above research and our work, the primary difference being that this work involves the effects of biofilm colonization on steel as opposed to pure electrochemical effects. However, similarities between electrochemical and biofilm effects on stainless steel have already been demonstrated in Chapter 6. The present work also found an increase in chromium hydroxide, in agreement with Halada and Clayton (1993). The formation of CrN as found by Cabrera (1989) can be ruled out because although CrN could not be distinguished from Cr_2O_3 due to peak overlaps, there was no trace of 396.6 eV peak in the $\text{N}1\text{s}$ spectra. The angle resolved studies showed that the $\text{N}1\text{s}$ peak was centered at 400 eV at low angles and 399 eV at higher angles. This

could indicate that nitrogen is present in the form of NH_3 on the surface and in the form of nitride near the metal-film interface. The handbook of x-ray photoelectron spectroscopy indicates that Cr_2N species have a nitrogen binding energy of 397.4 eV and $\text{Cr}(\text{CO})_5\text{NH}_3$ species have a binding species of 399.5 eV. We may have a combination of these species present in our case.

Recall that the nitrogen originated from an organic source, the biofilm, and could be bound to carbon and oxygen as well. The cited literature (Halada and Clayton, 1993; Cabrera, 1989) indicates that the presence of nitrogen in the passive film may not be an entirely unexpected phenomenon. It has been discussed earlier that the outer layer of the passive film may be porous. This porosity allows the nitrogen and other organic molecules penetrating the passive film. Other studies (Hoar, 1970; Scully, 1990) have discussed about Cl^- penetration of the passive film by displacement of bound water and binding with metal ions. These points become the weak points of the passive film and lead to breakdown. Nitrogen penetration of the passive film could be occurring in the same manner with perhaps the same effects.

The source of the nitrogen is attributed to the bacteria since no nitrogen was found in the control coupon experiments. When bacteria colonize the surface they excrete extracellular proteins which are long chained molecules typically with an amino end group. Normally these amino groups would remain bound to the protein molecules. However on coming into contact with the surface, they may interact with the surface and acquire enough energy to break free of the larger protein molecule and form ammonia.

This ammonia may then dissociate into atomic nitrogen and hydrogen, in the presence of a surface colonized by bacteria. Atomic nitrogen has been implicated (Designer's guide to modern steels, AISI, 1967) in the weakening of the chromium oxide film by preferentially binding with the chromium, for example, in the form of Cr_2N and $\text{Cr}(\text{CO})_5\text{NH}_3$. This causes lesser amounts of chromium to bind with oxygen, thus reducing the effective thickness of the passive layer formed. This comprises the stainless steel leading to eventual degradation of the material.

The ammonia by itself is a small enough molecule to penetrate into the porous hydroxide layer. The diffusion process causes the interaction of ammonia with hydroxide and other molecules present in this layer. The interaction may lead to the dissociation of ammonia forming nitrogen and hydrogen. This is borne out by the fact that the nitrogen peak in this work, shifts between 399 and 400 eV binding values corresponding to nitrogen and ammonia. There is some ammonia present in the media. However the control which is exposed to the sterile media alone reveals no nitrogen diffusion. It is thus assumed that the bacteria are responsible for this nitrogen diffusion. Although at present the mechanism behind this phenomenon is not clear, bacteria may alter the chemical potential in the surface region to cause a chemical gradient for ammonia, and/or for nitrogen to diffuse into the oxide region. Similarly carbon penetration may also be explained in terms of the presence of a chemical gradient for carbon, and or other organic species such as CN, catalyzed by the presence and activity of bacteria.

Conclusions

Biofilm colonization causes a change in the chemistry of the surface and the passive film in terms of the main alloying elements and oxygen. Depth profiles and angle resolved studies reveal nitrogen and carbon penetration of the passive film. The nitrogen and carbon diffusion are attributed to the biofilm colonizing the stainless steel since no such diffusion is seen in the control experiments. The nitrogen penetration causes organic species to intermix with the passive film. The changes in the passive film due to carbon and nitrogen seem to correlate with results obtained from electrochemical and unpolished stainless steel studies. The impoverishment in the chromium concentration could result in a weakening of the passive film leading to an eventual breakdown of the film. This would lead to the eventual degradation of the material. These conclusions lead to a model of the stainless steel surface after biofilm colonization shown in Fig. 71. The model shows a thin water layer with some organic molecules on the very top surface. It is followed by a concentration of carbon-nitrogen molecules at ~ 30 Å, inside the passive film, mixed into the iron hydroxide layer. The iron hydroxide layer is ~ 60 Å thick with the chromium oxide layer extending to ~ 80 Å.

Figure 71: Model of a stainless steel surface after 18 days biofilm colonization.

CHAPTER 8

CONCLUSIONS

The stainless steel surface chemistry is affected by biofilm colonization. The effects were observed after an 18 day colonization period by the bacteria of the surfaces. The role of surfaces has often been neglected in the past, in considering the interaction of biofilms with various materials. This research reveals the importance of understanding the role of surface chemistry towards obtaining a better understanding of biofilm colonization and interaction with surfaces. Sophisticated ultra high vacuum surface systems can not only be used to understand the workings of surfaces of single crystal (ideal) surfaces , but also polycrystalline and hence more complicated real world surfaces of materials of interest to industry.

Biofilm colonization of stainless steel surfaces causes changes in the surface chemistry. This is reflected in the carbon and nitrogen diffusion into the passive oxide film of the surface of polished stainless steel. Although the mechanisms causing this diffusion are not clear, two scenarios may be possible. In the first case, the nitrogen penetration may be directly related to bacteria that excrete extracellular proteins with amino end groups. These amino end groups on interacting with the stainless steel surface may break away from the protein molecules and diffuse into the porous passive layer. The presence of nitrogen and ammonia in the passive layer is in good agreement with this theory. The second scenario explore the establishment of a chemical potential gradient across the

surface layers causing carbon, nitrogen and or organic species like CN, to diffuse into the passive layer.

A good understanding of the passive layers of stainless steel has been obtained, leading to a duplex iron-chromium passive layer in the as-received state and organic diffusion into the iron rich layer after biofilm colonization. The surface oxide reveals the presence of an excess amount of hydroxide extending close to the bulk of the polished stainless steel, with a concentration of carbon-nitrogen at the top edge of this layer close to the surface.

Chemical changes on the unpolished 304 stainless steel surface in the oxide film close to the bulk have been observed after biofilm colonization. These changes have occurred in the relative concentrations of the bulk alloying elements of the unpolished stainless steel. In a normalized scale where relative concentrations of Cr, Fe, and Ni are normalized to 100%, the concentration of Cr is enriched on the biocoupon relative to that of the control coupon. There is a corresponding decrease in the relative concentration of iron. The relative Ni concentration showed no noticeable change. Although statistically not significant differences were observed in the relative Cr, Fe, and Ni concentrations between the colonized and uncolonized areas on the biocoupon in the unpolished stainless steel, that were very similar to the changes observed on the biocoupon.

The localized pits from electrochemical experiments revealed carbon rich areas in the pits and some darkened grains on the surface with no visible deterioration and a few other areas with no visible or physical changes. These darkened grains were metastable

pits just on the brink of pitting, but which did not undergo pitting. The areas with no physical changes only showed a local depletion in oxygen with an increase in carbon. The chemistry of areas colonized by bacteria followed the chemistry of the pits closely, suggesting that bacterial behavior may resemble electrochemical behavior, on attaching to a surface. Chemical changes occurring on unpolished stainless steel involve organic layers between the top surface and the bulk where a mixed interface is formed. The mechanism of pitting may be associated with bridging this interface.

Both polished and unpolished control sample surfaces showed no chemical changes after being exposed to sterile media for 18 days.

CHAPTER 9

RECOMMENDATIONS FOR FUTURE WORK

The role of bacteria in the diffusion of organic matter into the passive oxide film is not well understood. The question arises, from the results presented in this work, whether the bacteria is simply a "source" or a catalyst or both in causing the carbon and nitrogen containing molecules to diffuse into the passive layer of stainless steel. This point needs careful investigation using biofilm, as well as electrochemical techniques. The nature and the chemistry of the organic molecules, whether it is a single species or a combination of molecules made up of carbon and nitrogen, also needs to be resolved.

The results obtained in this research apply to initial colonization events. Further research needs to be done for longer time periods. The mixed consortium (different species of bacteria) used in this research was obtained from a corrosive environment. However the interaction with the stainless steel surfaces revealed little physical change on the surface. A highly corrosive consortia should be used in future experiments so as to accelerate physical and chemical changes on the surface, making it possible to investigate long term surface chemistry changes due to microbially induced corrosion. Visual examination and fixed time periods were used to establish end-points for these experiments in this research. Electrochemical monitoring during biofilm experiments in the future may provide an in situ indicator of changes occurring on the surface, thus allowing experiments to be carried out for longer time periods.

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