University of Alberta

The Influence of EPS Conditioning Films on *Pseudomonas aeruginosa* Adhesion to Solid Surfaces

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Environmental Engineering

Civil and Environmental Engineering

Fall, 2011
Edmonton, Alberta

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ABSTRACT: Bacterial adhesion to inert surfaces in aquatic environments is highly dependent on the surface properties of the substratum, which could be altered significantly by the formation of conditioning films. The impacts of extracellular polymeric substances (EPS) and its several representative components of conditioning films on the initial adhesion of the wild type *Pseudomonas aeruginosa* PAO1 were investigated under four different conditions of ionic strength. Our results showed that bacterial adhesion to bare slides and slides coated with alginate or humic substances increased with the ionic strength. Conversely, BSA and extracted EPS coating enhanced bacterial adhesions only under low ionic strengths, but hindered their adhesion at higher ionic strengths. In addition, during the experiments using the components of *P. aeruginosa* PAO1 EPS, proteins seem to dominate the impact of EPS on bacterial adhesion. The extended Derjaguin-Landau-Verwey-Overbeek (DLVO) theory was applied to explain the adhesion of *P. aeruginosa* PAO1 to solid surfaces.

KEYWORDS: adhesion; ionic strength; conditioning film; EPS; extended DLVO theory.

Acknowledgements

I am more than grateful to all the people who supported me during the course of the research implementation and thesis writing. I am deeply indebted to my supervisors, Dr. Yang Liu and Dr. Seoktae Kang who offered invaluable guidance and suggestions during the whole process. Without their continuous help, the thesis would never have become what it should be.

I would like to express my gratitude to all the following professors and technicians who provided various help throughout these experiments. Dr. Jonathan J. Dennis from the Department of Biological Sciences provided the *Pseudomonas aeruginosa* (PAO1) bacteria strain. Dr. Ming Chen from the Surgical Medical Research Institute helped me a lot in performing SEM imaging in high quality with his expert advice and skills. Thanks to the laboratory technicians Jody Yu, Jela Burkus and Maria Demeter for their help and patience in chemicals orders and equipments trainings.

I would also like to thank my colleagues, Zhiya Sheng, Xuejiao Yang, who have provided me with immense help in acquiring the microbiological experiment skills and understanding of the day to day running of a laboratory; Geelsu Hwang, who often worked with me and offered great amounts of time and efforts for my papers, as well as in conducting my experiments.

Finally, I feel thankful to my parents, my brother and his wife, and my girlfriend (Wang, Weiwei) for their unconditional support and continuous encouragement in my oversea postgraduate studies. Last but not least, I would like to thank every friend I met in the University of Alberta.

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List of Symbols, Nomenclature, or Abbreviations

EPS: extracellular polymeric substances

SEM: scanning electron microscope

DLVO: Derjaguin, Landau, Verwey and Overbeek theory

BSA: bovine serum albumin

HA: humic acid

FA: fulvic acid

Chapter 1 Introduction

1.1 Overview

In aqueous natural and engineered environments, bacteria have a tendency to form microbial consortia, also widely known as biofilms on solid surfaces. Indeed, at least 90% of aquatic bacteria adhere to solid surfaces and attach to each other rather than remain as single, suspended, and planktonic (Hall-Stoodley et al., 2004; Klemm et al., 2010). The significance of bacterial adhesion to solid surfaces has been noted in several processes including the remediation of contaminated soil and groundwater, biological wastewater treatment, and microbial-facilitated transport of contaminants. In aquatic systems, various habitat conditions (e.g., the presence of biofilms, natural organic matter and solid surface heterogeneity) may significantly impact the deposition and transport of bacterial cells.

As a result, it is beneficial to develop the theory or mechanism that could predict bacterial initial adhesion for a better management of biofilm formations and removals. To date, a lot of studies have been done on investigating the principle and mechanism of bacterial adhesion. In fact, some theories have been proposed to predict bacterial initial adhesion such as the DLVO and the thermodynamic theory. However, due to the secretion of cell surface polymer, those theories usually fail in making predictions (Tsuneda et al., 2003a; Uyen et al., 1988).

Extracellular polymeric substance (EPS) is one of the main cell surface polymers, which has been of great interest for decades. The EPS matrix typically consists of a mixture of macromolecules, including proteins, polysaccharides, nucleic acids, humic substances, lipids, and other polymeric compounds. It is believed to have profound effects on bacterial adhesion and the subsequent biofilm formation (Frank and Belfort, 2003). Previous studies mostly focused on the EPS bound to a cell's surface and indicated that EPS encouraged bacterial adhesion on solid substratum. However, very few studies concerned the effect of EPS pre-coating on substratum. Indeed, EPS would exist as a part of dissolved organic matter and probably pre-coat on substratum as a conditioning film before the bacteria attached. Few reported studies showed that EPS conditioning film discourages bacterial adhesion, which is contrary to the EPS on cell surfaces. The exact role of EPS conditioning films on bacteria initial adhesion has not been elucidated and more research is desired.

The impact of EPS conditioning films on bacterial attachment to solid surfaces will be investigated and reported in this thesis. The influences of each EPS component as well as the ionic strength of the solution are also topics of this dissertation.

1.2 Objectives

The purpose of this research is to investigate the role of EPS conditioning films in bacterial initial adhesion under different ionic strengths. Specifically, the objectives are to:

- Examine the influences of EPS conditioning film on initial adhesion to solid surfaces.
- Compare and determine the effects of each EPS component on bacterial adhesion.
- Investigate the impact of ionic strength on each conditioning film with respect to bacterial adhesion.
- Model bacterial initial adhesion mechanisms using extended DLVO theory.

1.3 Organization of the dissertation

Following the introduction, Chapter 2 provides a review of previous publications related to bacterial adhesion on the surfaces covered by conditioning films, especially

the EPS components. The significance, process, mechanism, and model of bacterial initial adhesion are also included within this chapter.

Chapter 3 will explain the experimental setup idea of this study and the selection of bacteria and representative EPS components. The materials and equipment used in the experiments and their characteristics are also introduced.

The results of experiments and the consequent modeling data are present in Chapter 4, and each will be discussed in more depth and individually in Chapter 5. Finally, the implications of this study and suggestions for future directions in which to take such studies are given in Chapter 6.

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Chapter 2 Literature Review

2.1 Biofilm and Bacterial adhesion

2.1.1 The significance of biofilms

Biofilms can be defined simply and broadly as communities of microorganisms that are attached to a surface (O'Toole et al., 2000). Biofilms have significant impacts on both bacteria itself and the environment that have been widely and deeply investigated for decades.

As for bacteria, biofilms serve to protect the producer microbes from hostile environments, so they strengthen the resistance of bacteria to shear forces and/or antibiotics (Costerton et al., 1999; Klemm et al., 2010; Mattilasandholm and Wirtanen, 1992). Specifically speaking, the biofilm matrix protects organisms against desiccation, oxidizing or charged biocides, some antibiotics and metallic cations, ultraviolet radiation, many protozoan grazers and host immune defenses (Flemming and Wingender, 2010).

As for the environment, biofilms have various effects on aqueous systems, which could be either profitable or deleterious (Rodrigues and Elimelech, 2009). For example, on one hand, biofilms are applied to remove dissolved and particulate contaminants from natural streams in wastewater treatment plants. They are also

required in paints, coatings, adhesives, fiber-matrix composites, and bioremediation (Camesano and Logan, 1998; Parent and Velegol, 2004; Salerno et al., 2007; Steffan et al., 1999). On the other hand, biofilms are also believed responsible for contaminating work surfaces, the clogging of tubes and machinery in industrial areas (Liu et al., 2008a), reducing the effectiveness of membranes and filters, accelerating the corrosion of medical implants (Habash and Reid, 1999; Salerno et al., 2007) and deterioration (Kumar and Anand, 1998; Mattilasandholm and Wirtanen, 1992). In addition, due to the release of pathogens and the reduction of water quality in drinking water distribution systems, biofilms could cause more serious microbial risks.

2.1.2 The formation of biofilms

Biofilm formation is a multistep process (Dufrene et al., 1996b): (1) cells contact a solid surface through diffusion, sedimentation, and convection, (2) cells initially adhere to the surface, and facilitate the arrival of following cells (3) extracellular polymeric substances (EPS) are excreted by bacteria to strengthen their attachment to the surface, (4) cells multiply to produce a mature biofilm.

Scientists and engineers have spent huge efforts on managing the formation of biofilms, and found that bacterial initial adhesion is the first crucial step in biofilm

formation. Therefore, understanding the mechanisms of initial adhesion is beneficial and critical for biofilm management.

2.1.3 The mechanisms of bacterial adhesion

Bacterial adhesion could be described as a two phase process including 1) initial, instantaneous, and reversible physicochemical interactions, and 2) time-dependent and irreversible molecular and cellular interactions (An and Friedman, 1998; Dan, 2003). The first stage is mainly controlled by physical forces and interactions between the cell and the substrate, such as Brownian motion, van der Waals attraction forces, gravitational forces, electrostatic, and acid-base interactions, and highly dependent on physicochemical characteristics between the cells and the surface, such as cell motility, surface charges, hydrophobicity, and surface roughness (An and Friedman, 1998; Busscher et al., 1992; Dufrene et al., 1996b; Rodrigues and Elimelech, 2009).

The exhibition of Brownian motion is the evidence of first phase adhesion, during which the bacteria could be easily removed by rinsing (Carpentier and Cerf, 1993; Marshall et al., 1971). In the second stage, the bacteria no longer show Brownian motion, and this implies a firmer adhesion to surface. Bacteria usually could not be simply removed by rinsing. Much stronger forces such as scraping and scrubbing are required to remove bacteria (Carpentier and Cerf, 1993).

As to the second phase, molecular or cellular reactions such as polymer bridging or binding by means of surface adhesins and the substratum receptors are more important (Jones and Isaacson, 1983). Usually, phase one is considered more of a physicochemical process while phase two is more concerned with biological factors.

The forces involved in bacterial adhesion could be divided into non-specific forces and specific interactions. Generally speaking, specific interactions are much stronger than non-specific forces, but non-specific forces happen more frequently. Once the bacteria come close enough to a surface (<3 nm), acid-base bonding, hydrogen bonding, ionic and dipole interactions dominate the adhesion (An and Friedman, 1998; Araujo et al., 2010). These forces are so-called non-specific interactions, which are governed by macroscopic surface properties such as hydrophobicity and charge. The adhesion is finally determined according to the net sum of attractive or repulsive forces generated between the bacteria and the surface (Dunne, 2002). Among these forces, electrostatic interactions tend to favor repulsion, because most bacteria and inert surfaces are negatively charged (Jucker et al., 1996), while Van der Waals forces are attractive. Hydrophobic interaction is considered an important factor for bacterial adhesion due to it being of a polar origin that may be up to two orders of magnitude higher than Van der

Waals or electrostatic forces, and it could be attractive or repulsive (Carpentier and Cerf, 1993; Olofsson et al., 1998; Ubbink and Schar-Zammaretti, 2007).

As to specific interactions, they only exist between specific bacterial surface adhesins and the corresponding substratum receptors such as antigens and antibodies, carbohydrates and lectins, substrates and enzymes (An and Friedman, 1998; van Oss, 1995). They are less influenced by environmental factors among which are salts, pH, temperature, and soluble sugar.

2.2 Bacterial surface polymers

2.2.1 Description

Besides the interactions discussed above, bacteria would consolidate the adhesion through the binding of the surface and cell surface polymers such as exopolysaccharides (Czaczyk and Myszka, 2007), proteins (Dufrene et al., 1996a), pili or fimbriae (Krogfelt, 1991), fibrillae (Weerkamp et al., 1986), and flagella (McClaine and Ford, 2002). The term “exopolysaccharides” mainly refers to the extracellular polymeric substances (EPS) and lipopolysaccharide (LPS), which are excreted by bacteria as well as the extracellular proteins. Their compositions and amounts are variable depending on the environment’s

conditions (Vandevivere and Kirchman, 1993). As a result, the interactions described above are significantly affected by various environmental parameters (Araujo et al., 2010). The presence of cell surface polymers makes the adhesion behavior of bacteria much more complex and unpredictable than the adhesion of inert particle (Tsuneda et al., 2003a; Uyen et al., 1988).

For example, LPS is the major components of the outer membrane of Gram-negative bacteria, which cover approximately 45% of the surface of Gram-negative bacteria (Cox et al., 1999; Jucker et al., 1997; Paradis et al., 1994). The main components of LPS include Lipid A, core and O-polysaccharide. Lipid A supports the structure of LPS and plays an essential role in biological activities; core serves as the functional group which makes connection between lipid A and O-polysaccharide; O-polysaccharide have a chain structure consisting of repeating oligosaccharide units (Kabanov and Prokhorenko, 2010). Pili are adhesive hair-like organelles that protrude from the surface of bacteria, and they can be used as appendages for transfer of genetic material during bacterial conjugation. Fimbria refers to the pili that devoted to attach bacteria to a surface. It was found that type IV pili not only mediate adhesion, but also provide bacteria with powerful tools to enhance their contact with target surfaces (Pizarro-Cerda and Cossart, 2006).

2.2.2 Roles in bacterial adhesion

Biopolymers modify the adhesion process through altering the hydrophobicity, charge, topography, and chemical composition of cell surface (Prince and Dickinson, 2003; Schaer-Zammaretti and Ubbink, 2003; Xu and Logan, 2006). For example, EPS, LPS, and proteins contain different functional groups including carboxylic, phosphoric, phosphodiester, amino, hydroxyl that can interact with the surface (Adoue et al., 2007). As to the cell surface appendages (fimbriae and flagella etc), they are able to encourage bacterial adhesion by enhancing the mobility of bacteria and/or binding with substratum through either specific or nonspecific interactions (de Kerchove and Elimelech, 2008; Stenstrom and Kjelleberg, 1985).

The lipid A and core region of LPS had been found to have strong affinity for bovine serum albumin (BSA) due to hydrogen bonding, which dominate the association between *P. aeruginosa* and protein-coated surfaces other than electrostatic or steric interactions (Atabek et al., 2008). In addition, the length of LPS may vary the cell surface charge character and/or hydrophobicity, which could affect the cell's ability to adhere to a surface. Arredondo et al. (1994) found an increase of *T. ferrooxidans* adherence to hydrophobic sulfur prills when part of its LPS was removed. It was reported an attractive interaction between the

wild-type strain (with the longer LPS chain) and a glass surface. In contrast, the mutant with the truncated LPS chain was repelled by the glass surface (Razatos et al., 2000; Razatos et al., 1998).

Adhesion pili are specialized surface structures responsible for the successful recognition and binding of these bacteria to their host receptors. They are reported to be responsible for maintaining this contact during the first stages of bacterial colonization (Bullitt and Makowski, 1998). The presence of fimbriae increased hydrophobicity as well as increased negative and positive surface charge of the cell surface, and maybe resulting in greater bacterial adhesion (Stenstrom and Kjelleberg, 1985). Repulsive steric interactions were detected between the alginate brush structure and bacteria flagella, which prevent the development of attractive interactions. Steric interactions between the flagella and the alginate brush may cause the sudden reversal of the swimming direction of the cell, which is a common swimming response of other flagellated bacteria confronting obstacles (de Kerchove and Elimelech, 2007). Actually, this ability to reverse direction of flagellar rotation could allow flagella to attach to the surface (and stabilize adsorption) (McClaine and Ford, 2002).

The role of EPS in bacterial adhesion is going to be discussed thoroughly in the following section.

2.3 Conditioning film and EPS

2.3.1 Definition and characterization

Prior to bacterial adhesion, conditioning films are usually formed on surfaces in aqueous environments due to the adsorption of dissolved organic matter (Jain and Bhosle, 2009). Besides dissolved organic matter, surfaces may be covered by the "footprints" of previously attached organisms. These footprints can include cell surface components, excreted biosurfactants, and EPS (Gomez-Suarez et al., 2002).

EPS consists of various macromolecular materials including polysaccharides, proteins, humic substances, nucleic acids, glycoproteins, phospholipids, lipid, and other polymeric compounds (Tsuneda et al., 2004). The compositions of EPS are variable in amount and type depending on the environmental conditions (Sutherland, 1983). According to its physical state, EPS could be further divided into "bound EPS" and "soluble EPS" (Nielsen et al., 1997). The soluble EPS increase the chance of EPS conditioning film formation since they exist as a part of dissolved organic matter (Lignell, 1990).

2.3.2 Impact on initial adhesion

It has been found that conditioning films affect initial bacterial adhesion dramatically through altering the surface tension, charge, roughness, and hydrophobicity of the substratum (Bakker et al., 2004; Bakker et al., 2003; de Kerchove and Elimelech, 2007; Jain and Bhosle, 2009). The impact of bovine serum albumin (BSA) coating on solid surfaces has been studied by Grzeskowiak et al. (in press) and Vendrell et al. (2009). Their studies showed that non-specific forces such as hydrophobic interactions were involved in bacterial adhesion on BSA coated surfaces. The atomic force microscopy (AFM) study performed by Xu and Logan (2005) indicated that when the colloid probe was coated with BSA, bacterial adhesion forces were reduced as the ionic strength increased. Abu-Lail et al. (2007) found that adhesion forces between *P. aeruginosa* PAO1 and Suwannee River humic acid (HA) coated probes were twice as large as adhesion forces between *P. aeruginosa* PAO1 and bare silica. Additional studies also showed that higher bacterial adhesion can be achieved when surfaces are coated with humic acid (Johnson and Logan, 1996; Parent and Velegol, 2004). The impact of alginate conditioning film was investigated using both motile and nonmotile *P. aeruginosa* strains (de Kerchove and Elimelech, 2007), which showed that the adhesion of both types of bacteria was improved in the presence of an alginate conditioning film. Later, they also demonstrated that the presence of divalent

cations enhanced bacterial adhesion on the alginate conditioning film (de Kerchove and Elimelech, 2008).

EPS have profound effects on bacterial initial adhesion to solid substratum and the subsequent biofilm formation, which have been widely discussed in previous studies (Burdman et al., 2000; Liu et al., 2007; Long et al., 2009). More research proved that the presence of EPS on cell surfaces improves initial adhesion through all kinds of interactions. Rijnaarts et al. (1993) indicated that outer cell surface macromolecules would enhance bacterial adhesion due to a polymer-bridging effect. EPS could also serve as a ligand and provide binding sites for other charged molecules (Decho and Moriarty, 1990), and its functional groups also contribute to more adhesion (Leone et al., 2006; Parikh and Chorover, 2006). In addition, cell surface EPS influence bacterial adhesion through altering the physicochemical characteristics of a surface such as its hydrophobicity, surface charge, topography, chemical composition and polymeric property (Mikkelsen and Keiding, 2002; Suci et al., 1995; Tsuneda et al., 2003b). However, few studies reported that the EPS hinder bacterial adhesion through steric repulsion or that they change the acidic nature of a cell's surface (Kim et al., 2009; Kuznar and Elimelech, 2005; Kuznar and Elimelech, 2006; Liu et al., 2008b; Rijnaarts et al., 1999).

Also, very few investigators have studied the effects of EPS pre-coated on bacterial initial surface adhesions to date. A few studies showed that EPS conditioning film may discourage bacterial adhesion (Gomez-Suarez et al., 2002; Liu et al., 2007). They believed that some surface-active exopolymers in EPS attribute to the reduction of bacterial adhesion. This phenomenon and hypothesis were also reported and supported by earlier studies (Busscher et al., 1997; McGroarty and Reid, 1988; Pringle et al., 1983; van Hoogmoed et al., 2000; Velraeds et al., 1998). Recently, Liu and Li (2008) observed that EPS pre-coating on solid surfaces has a profound impact on bacterial initial adhesion. They found that alginate EPS from *P. aeruginosa* PDO300 biofilms reduced *E. coli* bacterial initial adhesion while EPS from *P. aeruginosa* PAO1 biofilms enhanced *E. coli* bacterial adhesion.

2.4 Extended DLVO model

Thousands of researches have been done that study adhesion, and several mechanisms have been proposed to explain the adhesion of bacteria to surfaces, as well as biofilm formation (Araujo et al., 2010), such as the Derjaguin, Landau, Verwey and Overbeek (DLVO) model and its extensions (Azeredo et al., 1999; Hermansson, 1999; Ong et al., 1999) the thermodynamic (surface tensions) model

(Absolom et al., 1983), and the steric model (Rijnaarts et al., 1999). In this study, the extended DLVO model was applied to explain cell adhesion to all tested surfaces.

The DLVO model is based on the DLVO theory describing the forces between charged surfaces interacting through a liquid medium (Hermansson, 1999). It accounts for the effects of the van der Waals attraction and the electrostatic repulsion. The electrostatic part of the DLVO interaction is computed in the mean field approximation in the limit of low surface potentials and the radius of the approaching spheres. van Oss (2006) proposed an extension of the DLVO theory, generally known as extended DLVO theory. This new approach considers that the total free energy of interaction between two surfaces immersed in an aqueous medium is the sum of the Lifshitz–van der Waals forces, polar interactions, electrical double layer interactions and Brownian movement forces.

2.5 Experiment design and chemical selection

In order to clarify the role of EPS conditioning films on bacterial adhesion to solid surfaces, an EPS producer *P. aeruginosa* was selected and the main components of its EPS were investigated individually. *P. aeruginosa* is widely present in

natural environments and extremely active in the biofouling events in natural and engineered aqueous systems, infecting both humans and plants (de Kerchove and Elimelech, 2007; Liu et al., 2007). Alginate is a naturally occurring polysaccharide commonly found in marine environments (Chen et al., 2006), and is the major EPS component of *P. aeruginosa* biofilms (Liu and Li, 2008). It represents the polysaccharides of bacterial EPS as well. Humic acid (HA) and fulvic acid (FA) are the major humic substances in aqueous environments (Amal et al., 1992), and are therefore used as model humic substances in EPS. Bovine serum albumin (BSA) was selected as the representative protein in EPS since it has been extensively studied as a model protein for its strong adsorption capacity for many types of materials (Caro et al., 2009; Valle-Delgado et al., 2006). By comparing bacterial adhesion on each EPS component and the original EPS conditioning films, the dominant component in EPS on initial adhesion could be determined.

The electrolytic environment is another critical factor in cell-surface interactions (Mercier-Bonin et al., 2009; Rodrigues and Elimelech, 2009). In conditioning films, the conformation of polymeric layers can be changed under certain ionic strength conditions (low or high, monovalent or divalent cations), which may alter steric interactions between outer cell surface macromolecules and the substratum surface, resulting in more, or less, adhesion (Chen and Walker, 2007). Thereby,

all adhesion tests were performed under four ionic strength conditions. These were 1mM, 10mM, 100mM KCl and 8.5mM KCl + 0.5mM $CaCl_2$ (abbreviated to "Ca^{2+}" in subsequent text).

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Chapter 3 Methodology

3.1 Materials

3.1.1 Bacteria culture

The *P. aeruginosa* PAO1 culture was obtained from Dr. Jonathan Dennis, department of Biological Science at the University of Alberta, which was maintained in glycerol at -80°C. To get a working colony, the cultures were streaked onto Luria–Bertani (LB) agar plates and incubated at 37°C overnight. Then, a single colony from the fresh agar plate was transferred to 50 mL LB broth in a 150 mL Erlenmeyer flask. The PAO1 culture was shaken at 200 rpm at 37°C in a shaker incubator (New Brunswick Scientific Co., NJ) for 18 hours to reach the stationary phase. Cells were harvested by centrifugation (Centrifuge 5415R Eppendorf, CA) at 3000 g at 4°C for 10 minutes. The growth medium was decanted and the pellet was resuspended in salt solutions at different ionic strengths. Electrolyte solutions were prepared with reagent grade salt (Fisher Scientific Inc., U.S.) and ultrapure water, and sterilized by autoclave before use. Centrifugation and resuspension were repeated to remove any traces of the growth medium. A final cell density of approximately 10^7 cells/mL was obtained by

measuring the optical density (OD) at 600 nm with a UV spectrophotometer (Varian Inc., U.S.).

3.1.2 EPS extraction and characterization

The isolation of EPS from *P. aeruginosa* PAO1 is summarized as below. Harvested bacterial cells were washed twice with an isotonic solution (0.9% NaCl), then the pellet was suspended in ultrapure water and heated for 10 min at 80 ºC (Zhang et al., 1999). After this treatment, the samples were centrifuged at 15000 g at 4 ºC for 15 min, and the supernatants were collected as EPS extracts. The EPS were further filtered through a 0.22 µm membrane, and stored at -20 ºC.

The composition of the EPS extracts was investigated using colorimetric methods performed with a UV-Vis spectrophotometer (Varian Inc., US). Protein and polysaccharide quantifications were performed by the bicinchoninic acid assay (Frolund et al., 1995; Lowry et al., 1951) and the phenol-sulfuric acid assay (Dubois et al., 1956), respectively. BSA (96%, Sigma, MO) and glucose (Sigma, MO) were used as the standards for the protein and the polysaccharide, respectively, using an absorbance at 480 nm for both assays.

3.1.3 Conditioning films and substratum preparation

Microscope glass slides (Fisher Scientific Inc., U.S.) were selected as the inert solid substratum for bacterial adhesion in this study. The slides were cut into small pieces (approximate size: 15mm × 12mm × 1mm) so that they could fit in the wells of the 24-well plate. Prior to experiments, the slide pieces were rinsed with deionised (DI) water thoroughly to remove visible impurity (large particles). Subsequently, the slides were immersed in 1N HCl and sonicated for 10 minutes for grease removal. After sonication, the slides were rinsed with sterilized ultrapure water, 70% ethanol, and finally sterilized ultrapure water. Finally, drying process was achieved in a biosafety cabinet (CLASS II Type A2, Microzone Cor., Canada). The clean slides were reserved as model bare slides.

A chemical modification of slide surfaces was necessary before conditioning films coating. The clean bare slides were submerged in the 0.01% poly-L-lysine (PLL) (Sigma, MO) solution (pH 5.6) at 4°C overnight to form a PLL layer. Then PLL slides were rinsed with sterilized ultrapure water and dried under vacuum overnight before use.

The formation of the conditioning films was achieved by immersing the PLL slides in the corresponding solutions. The required immersion time and solution concentrations are 15 minutes and 0.1 g/L for HA (IHSS Suwannee River Humic

Acid Standard II 2S101H), FA (IHSS Suwannee River Fulvic Acid Standard 1S101F), and alginate (sodium alginate, Sigma, MO). BSA (Sigma, MO) coatings required a longer time and a higher concentration, which are 30 minutes and 1 g/L respectively. To apply the EPS conditioning film, bare slides were dipped into the extracted EPS solution and remained overnight. After immersion, the coated slides were rinsed with sterilized ultrapure water to remove traces of the coating solution and dried in the biosafety cabinet. All substratums were freshly prepared and dried before each adhesion test.

3.2 Experiment protocols

3.2.1 Bacterial adhesion

Bacterial adhesion on solid surfaces was investigated as a function of ionic strength in several concentrations of monovalent salt (1 mM KCL, 10 mM KCl, and 100 mM KCl) and in a 10 mM equivalent solution containing mono and divalent salts (8.5 mM KCl ＋ 0.5 mM $CaCl_2$). Electrolyte solutions were prepared by mixing reagent grade salt (Fisher Scientific Inc., U.S.) and ultrapure water, and were sterilized by the autoclave before use. For each adhesion test, fresh *P. aeruginosa* PAO1 bacterial suspension was prepared as described in

section 3.1.1 and 2.5 mL was distributed in each well of a 24-well plate (Corning Inc., U.S.). The slides were completely submerged in the bacterial suspension for 30 minutes and kept still. After adhesion, the slides were gently rinsed with the electrolytic solution being tested to remove loosely attached cells.

3.2.2 Contact angle measurement

Sessile drop method (Vandermei et al., 1995) was applied to measure the contact angles on the microbial surface in this study. The bacteria cells harvested by centrifugation were washed with ultrapure water three times to remove any cell surface particles. In order to create a bacterial lawn, the cells suspended in the ultrapure water were vacuum filtered through a 0.45 μm pore size nitrocellulose membrane (type HA, Millipore®, USA). This bacterial layer was air-dried at room temperature until the measured water contact angle reached the maximum (plateau) value. The maximum contact angles of water, diiodomethane, and glycerol were measured at 20ºC using an FTA-200 system (First Ten Angstroms, USA).

3.2.3 Zeta potential measurement

The determination of the zeta potentials of each slide surface is achieved by measuring the zeta potentials of bare and conditioning film coated silica particles (prepared using the same coating methods as described in section 3.1.3). As to the

zeta potential of bacteria cells, it was measured using bacteria suspension. All measurements were preformed in 1 mM, 10 mM, 100 mM KCl, and 8.5 mM KCl + 0.5 mM $CaCl_2$ using a Brookhaven ZetaPALS (Brookhaven Instruments Corporation, USA). Each condition was repeated for at least 5 runs of 20 cycles at 25 °C, and the results were averaged over.

3.2.4 Surface roughness measurement

The surface roughness of each conditioning film coated slide was analyzed using an atomic force microscope (MFP-3D, Asylum Research) at the nanometer scale. An AC mode AFM was performed and the cantilever was made out of Si with a spring constant of 2 N/m and a nominal tip apex radius of less than 10 nm. The root-mean-squared (RMS) roughness (Boussu et al., 2005) was determined over areas of 5 μm^2 for each sample.

3.2.5 Fluorescent microscopy

The visualization and quantification of attached cells on slides were completed by fluorescent microscopy. After each adhesion test, the bacteria coated slides were placed on clean microscope slides and stained with SYTO® 9 green fluorescent nucleic acid dye for 15 minutes. An epifluorescent microscope (DMRXA, Leica Inc., Germany) fitted with a camera (DXM 1200, Nikon Cor., Japan) was used to

take five images of randomly chosen different areas of each slide. The size of each image was 0.0034813 cm^2. The number of bacteria in each image was obtained through counting five areas (4 corners plus the center) of the image. The total number of adherent cells was calculated using those amounts.

3.2.6 Scanning electron microscope (SEM)

The size and the shape of *P. aeruginosa* PAO1 were visualized using SEM (S-2500, Hitachi, Japan). Prior to imaging, some treatments on the samples are required. Cell coated slides were firstly fixed in 2.5% glutaraldehyde in Millonig's ($NaH_2PO_4 \cdot H_2O$ 1.8g + $Na_2HPO_4 \cdot 7H_2O$ 23.25g + NaCl 5.0g) buffer (pH 7.2) at room temperature for 1 hour. The samples were then washed in the same buffer 3 times, for 10 minutes each. Secondly, the slides were further fixed in 1% OsO_4 in the Millonig's buffer at room temperature for another 1 hour. After washing them with distilled water briefly, the dehydration was finished in a series of ethanol (50-100%) for 10 minutes each and two additional, absolute ethanol, 10 minutes each again. Subsequently, the slides were critical point dried with CO_2 at 31 °C for 5 minutes. Finally, the samples were sputter coated with gold (Edwards, Model S150B Sputter Coater).

3.3 Extended DLVO model development

van Oss et al. (1986) added a term for polar (Lewis acid–base) interfacial energy into the classical DLVO theory and developed the so-called extended DLVO theory:

$$\Delta G^{TOT}(d) = \Delta G^{LW}(d) + \Delta G^{EL}(d) + \Delta G^{AB}(d) \qquad (1)$$

where $\Delta G^{LW}(d)$, $\Delta G^{EL}(d)$ and $\Delta G^{AB}(d)$ denote Lifshitz-van der Waals, electrostatic, and Lewis acid–base interaction energies. For a sphere opposed to a semi-infinite plate by the distance *d*, $\Delta G^{LW}(d)$, $\Delta G^{EL}(d)$, and $\Delta G^{AB}(d)$ are then expressed as:

$$\Delta G^{LW}(d) = -\frac{A}{6}\left[\frac{a}{d} + \frac{a}{d+2a} + \ln\left(\frac{d}{d+2a}\right)\right]$$
$$\Delta G^{EL}(d) = \pi\varepsilon a(\zeta_1^2 + \zeta_2^2)\left[\frac{2\zeta_1\zeta_2}{\zeta_1^2+\zeta_2^2}\ln\frac{1+\exp(-\kappa d)}{1-\exp(-\kappa d)} + \ln\{1-\exp(-2\kappa d)\}\right] \qquad (2)$$
$$\Delta G^{AB}(d) = 2\pi a\lambda\Delta G_{adh}^{AB}\exp[(d_0 - d)/\lambda]$$
$$A = -12\pi d_0^2 \Delta G_{adh}^{LW}$$

where *a* is the radius of a sphere.

In Eq. (2), *A* is the Hamaker constant; ε, ζ, and κ^{-1} are the permittivity of the medium, the zeta potential, and the double-layer thickness, respectively; λ is the correlation length of molecules in a liquid medium; and d_0 is the distance of the

closest approach between the plate and the sphere in Eq. (2). ε is usually expressed as the product of the permittivity of a vacuum (ε_0, 8.854×10^{-12} $C^2/J \cdot m$) and the relative permittivity (formerly called the dielectric constant) of the medium ε_r, which is 80 for water at 20°C. κ^{-1} is expressed as:

$$1/\kappa = \left[(\varepsilon kT)/\left(e^2 \sum v_i^2 n_i\right)\right]^{1/2} \qquad (3)$$

where k and e are Boltzmann's constant $\left(k = 1.38 \times 10^{-23}\, J/K\right)$ and the charge of an electron $\left(e = 1.602 \times 10^{-19}\, C\right)$, respectively; T is the absolute temperature in K; v_i and n_i are the valency and the number density (per ml of bulk liquid) of each ionic species, respectively. ΔG_{adh}^{LW} and ΔG_{adh}^{AB} (see Eq. (2)) have been derived from the LW-AB approach (van Oss et al., 1987a; van Oss et al., 1986; van Oss et al., 1987b) as follows:

$$\Delta G_{adh}^{LW} = -2\left(\sqrt{\gamma_b^{LW}} - \sqrt{\gamma_l^{LW}}\right)\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_l^{LW}}\right) \qquad (4)$$

$$\begin{aligned}\Delta G_{adh}^{AB} = {} & +2\left(\sqrt{\gamma_b^+} - \sqrt{\gamma_s^+}\right)\left(\sqrt{\gamma_b^-} - \sqrt{\gamma_s^-}\right) - 2\left(\sqrt{\gamma_b^+} - \sqrt{\gamma_l^+}\right)\left(\sqrt{\gamma_b^-} - \sqrt{\gamma_l^-}\right) \\ & - 2\left(\sqrt{\gamma_s^+} - \sqrt{\gamma_l^+}\right)\left(\sqrt{\gamma_s^-} - \sqrt{\gamma_l^-}\right)\end{aligned} \qquad (5)$$

where the subscripts *b*, *l*, and *s* denote a bacterial cell, water, and a solid substrate, respectively. The surface tension components for the solid are determined from the following equation:

$$(1 + \cos\theta)\gamma_l = 2\left(\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + \sqrt{\gamma_s^+\gamma_l^-} + \sqrt{\gamma_s^-\gamma_l^+}\right) \qquad (6)$$

To determine the surface tensions for the solid (γ_s^{LW}, γ_s^+, and γ_s^-), contact angles (θ) should be measured with at least three different liquids. In this study, κ^{-1} was calculated to be 9.63 nm, 3.04 nm, and 0.96nm for 1mM KCl, both 10mM KCl and 8.5mM KCl + 0.5mM $CaCl_2$, and 100mM KCl solution, respectively. 0.6 nm and 0.157 nm were given as the values of λ and d_0, respectively (van Oss, 1990; van Oss, 2006).

3.4 Statistical analysis

Physicochemical properties, bacteria size, and degree of bacterial adhesion were analyzed with a one-way analysis of variance (ANOVA) and reported as p-values. ANOVA was performed using Microsoft Excel software. P-values of less than 0.05 suggest differences are statistically significant.

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Chapter 4 Results

4.1 Surface properties of bacteria and solid substrates

Figure 4-1 summarizes the zeta potentials of bacteria and the solid substratum as a function of ionic strength. The absolute magnitude of *P.aeruginosa* PAO1's cell surface potential was reduced with the increasing electrolyte concentration, which can be attributed to the compression of the double layers under high ionic strength.

The negative values of zeta potentials indicate that all tested substratum were negatively charged under all ionic strength conditions. The presence of conditioning films enhanced the zeta potential of bare slide surfaces under all conditions other than 100mL KCl. BSA and FA coated slides had a similar surface charge ($p = 0.6$) and were the least negatively charged surfaces. Alginate coated and bare slides were observed to be less negatively charged in the presence of calcium ions compared to 10 mM KCl, which is due to the fact that calcium ions neutralize the surface charge through a screening effect (Simoni et al., 2000) and/or binding with polymers and altering their conformations (Chen and Walker, 2007). However, this phenomenon was not observed for HA, FA, BSA and EPS coated slides. It is also noted that the impact of conditioning films with respect to the zeta potential is more significant at lower ionic strength conditions.

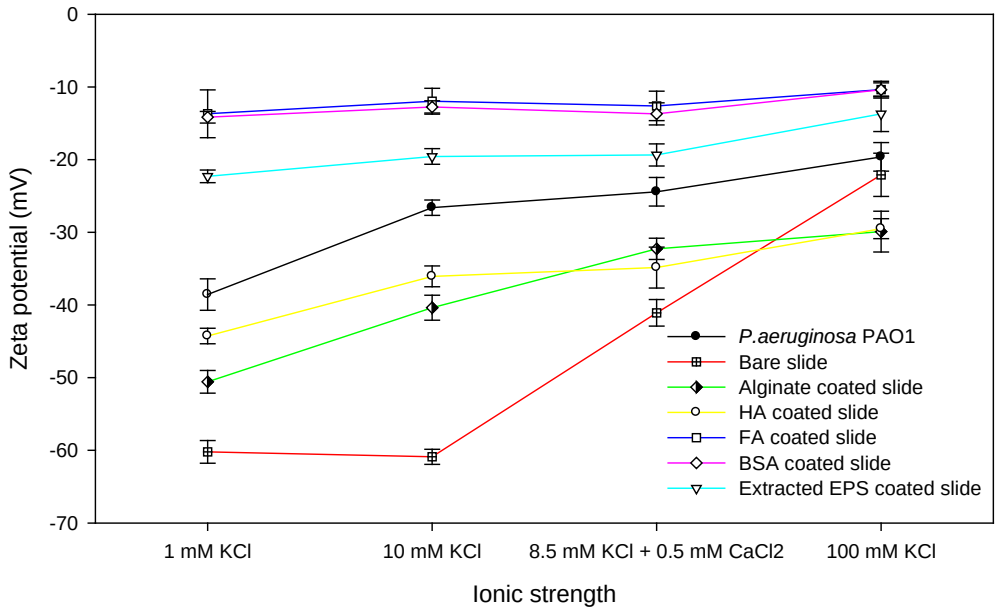


Figure 4-1 Zeta potentials of surfaces as a function of ionic strength. Error bars represent one standard deviation of triplicate experiments

The extracted EPS of *P. aeruginosa* PAO1 showed similar amounts of protein and polysaccharides, which were 6.02 ± 1.77 and 6.75 ± 1.65 mg/dry cell g, respectively.

The contact angles of *P. aeruginosa* PAO1 and various slides are summarized in Table 4-1. The water contact angle of *P. aeruginosa* PAO1 was 21.9 ± 2.4, which indicates the hydrophilic property of the cell surface. According to the polar liquid (i.e., water and glycerol) contact angles, all of the coated slides were more hydrophobic than the bare slides. The EPS coated slide was the most hydrophobic of all the slides tested.

Table 4-1 Contact angles of *P. aeruginosa* PAO1, bare slides, and conditioning films in various media, and root-mean-squared (RMS) roughness of bare and conditioning film coated slides

Solid	Contact angles (°)			Roughness RMS (nm)
	Water	Diiodo-met-hane	Glycerol	
P. aeruginosa PAO1	21.9 ± 2.4	41.5 ± 3.6	70.6 ± 3.7	—
Bare slide	10.6 ± 0.9	36.1 ± 2.3	31.8 ± 2.0	0.36 ± 0.23
Alginate coated slide	35.8 ± 1.2	30.5 ± 0.2	37.5 ± 3.7	0.85 ± 0.38
HA coated slide	24.2 ± 2.4	29.1 ± 1.4	39.6 ± 3.9	1.56 ± 1.06
FA coated slide	25.5 ± 1.3	28.9 ± 2.0	36.5 ± 1.7	2.83 ± 1.97
BSA coated slide	54.9 ± 3.6	26.1 ± 0.9	61.2 ± 1.8	0.57 ± 0.34
EPS coated slide	75.5 ± 2.6	28.0 ± 1.4	63.5 ± 2.8	1.78 ± 0.68

The surface morphology of bare and coated slides was studied by AFM. Fig. 4-2 shows AFM (three dimensional 3D) images of bare and coated slides. RMS values of bare and conditioning film coated slides are presented in Table 4-1. Our results indicate that the surface roughness increased slightly in the presence of the conditioning films. FA coated slides had the highest surface roughness; however the differences between the surface roughness values of FA coated slides and other slides with and without conditioning film coatings were significantly less than the sizes of *P. aeruginosa* PAO1 bacteria (see Table 4-1). It has been reported that the impact of surface roughness on bacterial adhesion should be considered only when the surface roughness is greater or similar to the size of the bacterial cell (Flint et al., 2000; Pons et al., 2011). In this study, it is expected that the surface roughness of substrates had little impact on bacterial initial adhesion.

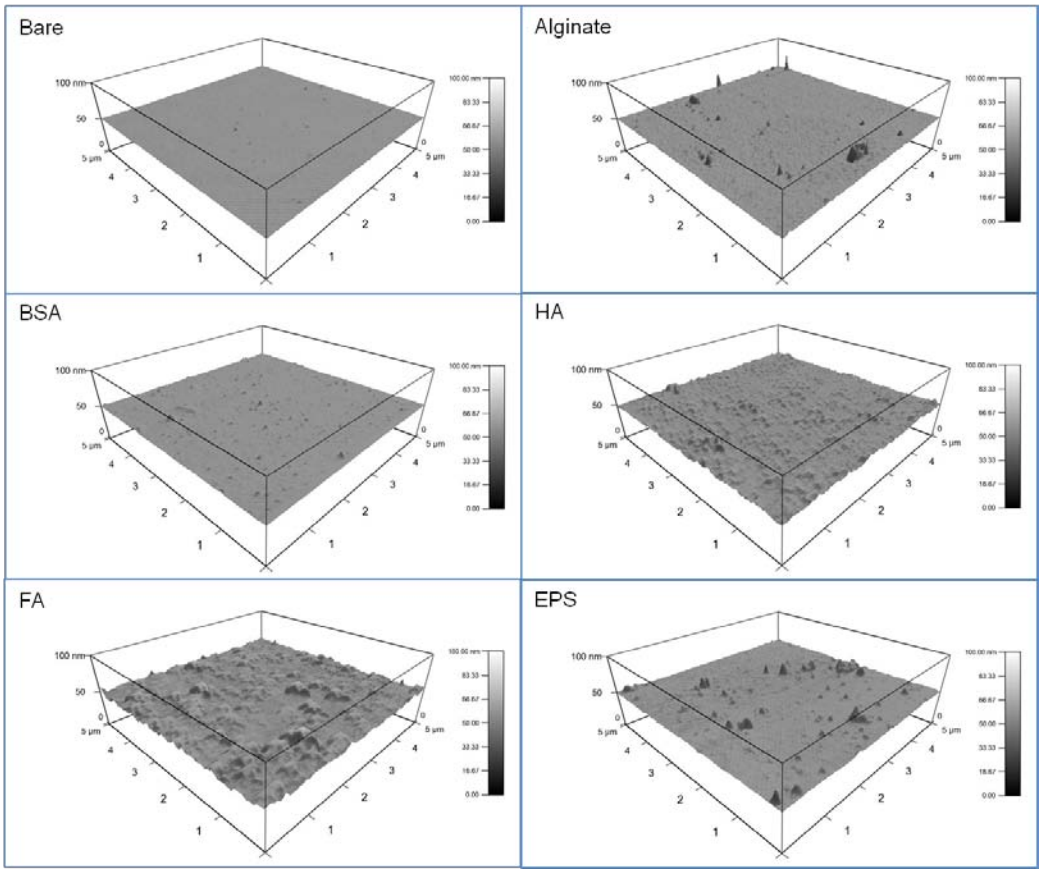


Figure 4-2 AFM 3D images of bare glass slides and slides coated with alginate, HA, FA, BSA and extracted EPS conditioning films

4.2 Bacterial adhesion to alginate or humic substance coated slides

Representative fluorescence microscope images of cell attached surfaces are shown in Fig. 4-3. The adhesion results of *P. aeruginosa* PAO1 to bare and coated slides at different ionic strengths are presented in Fig. 4-4. Cell adhesion increased with the ionic strength on bare slides and on slides coated with alginate or humic substances. Since *P. aeruginosa* PAO1 and the substratum are both negatively charged (Fig. 4-1), as the ionic strength increases the thickness of the electrostatic double layer is compressed so that the repulsive force is weakened. Consequently, more cells adhered to the substratum at the higher ionic strengths.

In the presence of 1 mM KCl, the number of cells attached to the slides coated with alginate or humic substances was 3 or 4 times the number of cells attached to the bare slides (Fig. 4-4). At high ionic strength conditions (100 mM KCl), bacterial adhesion test results showed only slight differences among bare, alginate coated, and humic substance coated slides (Fig. 4-4).

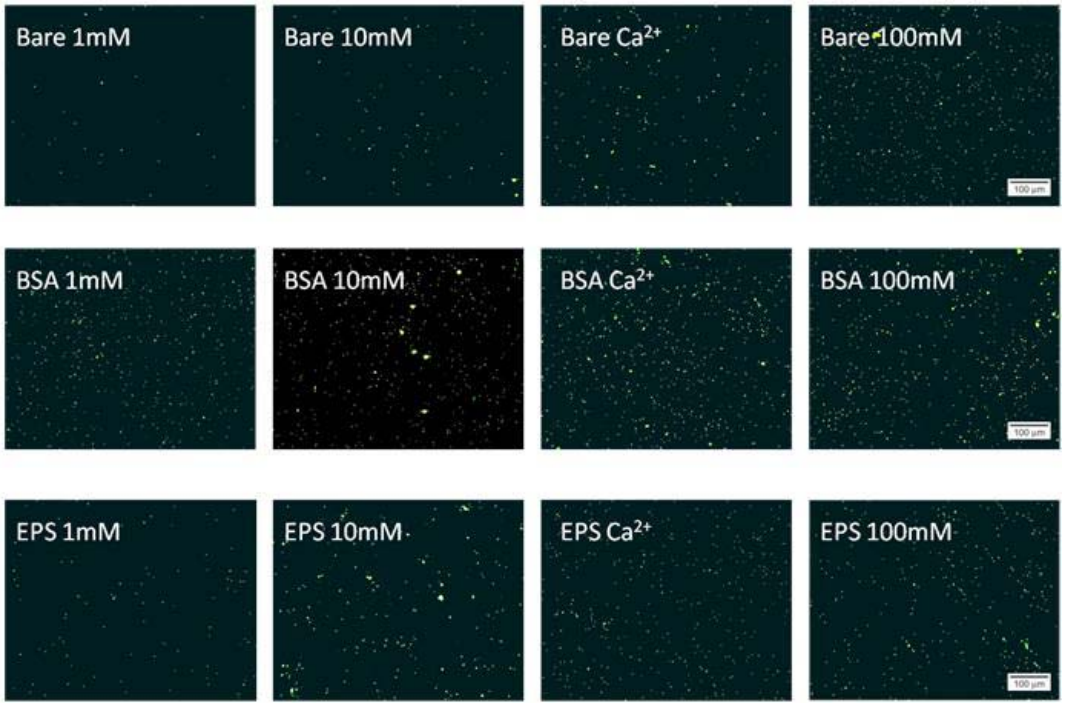


Figure 4-3 Representative fluorescence microscopy images of *P. aeruginosa* PAO1 adhesion on bare, BSA and extracted EPS coated slides under 1mM, 10mM, 100mM KCl and 8.5mM KCl + 0.5mM $CaCl_2$ (abbreviate to Ca^{2+} in figure) media

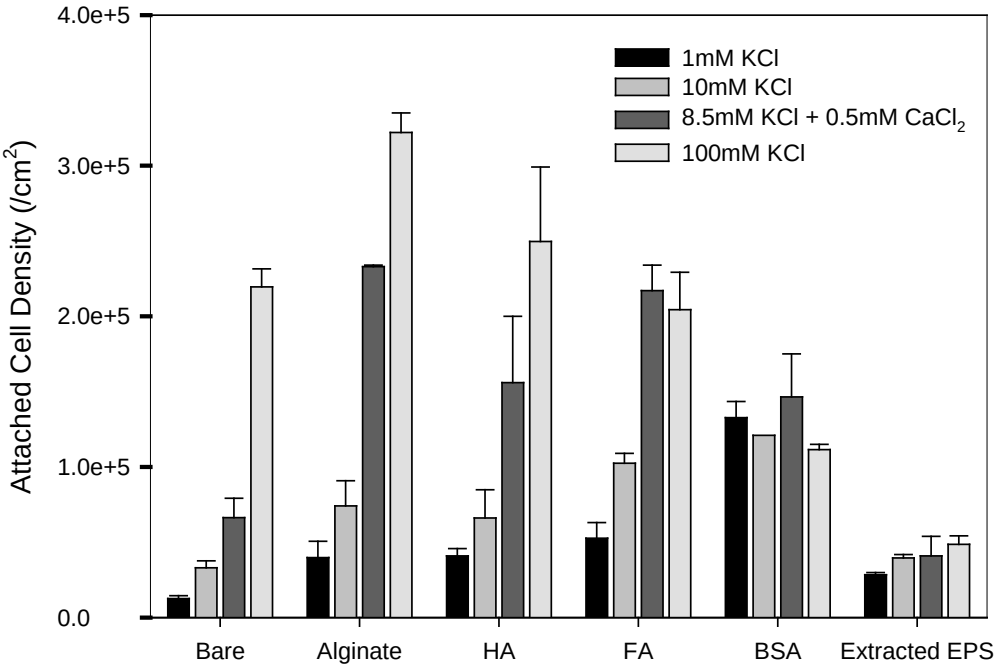


Figure 4-4 *P. aeruginosa* PAO1 adhesion on bare slide and slides coated with alginate, HA, FA, BSA and extracted EPS conditioning films and under different ionic strength conditions. Error bars represent one standard deviation of triplicate experiments

To better evaluate the effects of conditioning film on bacterial adhesion, we introduced adhesion factors that were calculated by dividing the number of attached cells on the conditioning film by the number of attached cells on a bare slide at each ionic strength tested (Fig. 4-5). The adhesion factors could reflect to what degree the conditioning films enhance or hinder bacterial attachment. The adhesion factors of alginate and humic substance conditioning films were similar, decreasing and approaching 1 as the ionic strength increased, indicating that the impact of alginate and humic substance coating on bacterial adhesion become less important as the ionic strength increases.

In the presence of calcium ions, bacterial adhesion was enhanced on bare, alginate coated and humic substance coated slides. As shown in Figures 4-4 and 4-5, the number of attached cells is about double the number of attached cells in 10 mM KCl for bare slides and slides coated with humic substances and triple for slides coated with alginate. The influence of calcium ions on improving bacterial initial adhesion is more significant in the presence of alginate.

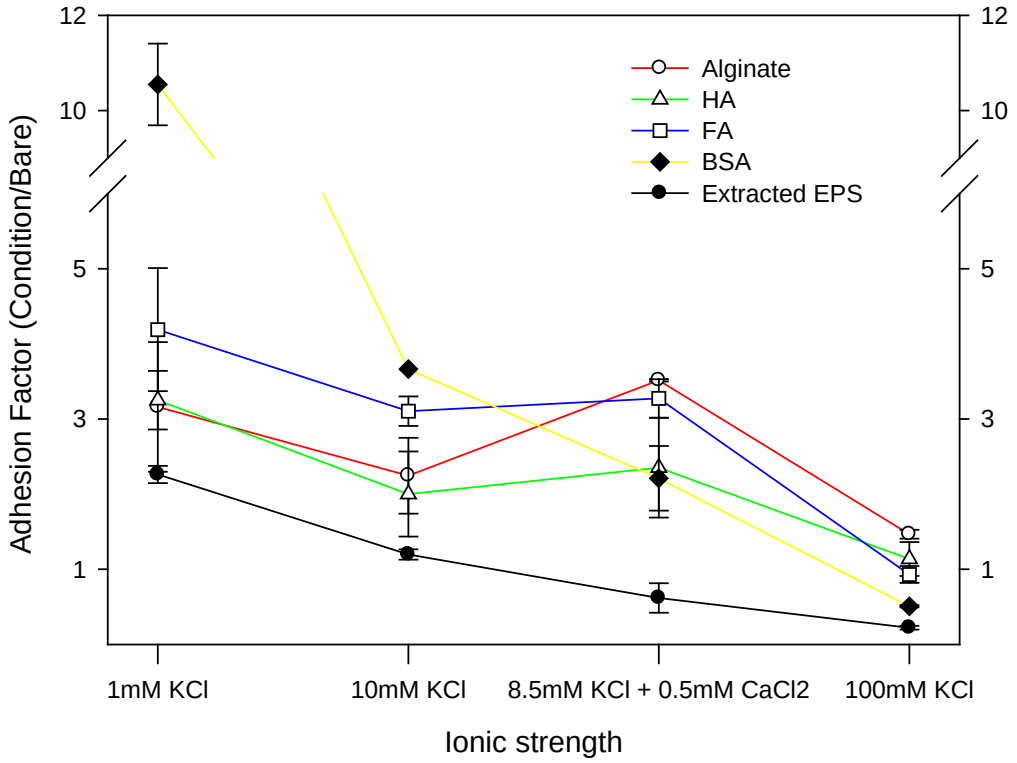


Figure 4-5 Adhesion factors of slides bare and coated with conditioning films alginate, HA, FA, BSA and extracted EPS as a function of ionic strength. Error bars represent one standard deviation of triplicate experiments

4.3 Bacterial adhesion to BSA and EPS coated slides

Bacterial adhesion on BSA or extracted EPS coated slides as a function of ionic strength is shown in Fig. 4-4. For both BSA and extracted EPS coated surfaces, an increase in bacterial adhesion with the ionic strength was not observed ($p = 0.09$). In addition, the effect of the calcium ions is not significant. This coincides with the narrow variations in zeta potentials of BSA and extracted EPS coated slides in the four different ionic strength conditions.

Compared to bare surfaces, BSA and extracted EPS coated surfaces reduced bacterial adhesion at the high ionic strength condition (100mM KCl) and enhanced their adhesion at the low ionic strength condition (1mM KCl), and the presence of calcium ions did not improve cell adhesion to the EPS coating (Fig. 4-5).

4.4 Extended DLVO modeling data

In the extended DLVO approach, the adhesion energy should be calculated at the closest approach and as a function of the separation distance (Bayoudh et al., 2006). Eq. (2), which is the governing equation of the extended DLVO theory, is designed for the adhesion of spherical particles. Cells of the *P. aeruginosa* PAO1

strain used in this study are not spherical, but cylindrical (rod-type) (see Fig. 4-6). Hence, as a first approximation to the use of a real sphere, we calculated the radii of a sphere with the same volume as *P. aeruginosa* PAO1. The average length and diameter of the rod-shaped cells are 1.29 ± 0.28 μm and 0.53 ± 0.06 μm, respectively, which is equivalent to a spherical volume with a radius of 0.40 μm.

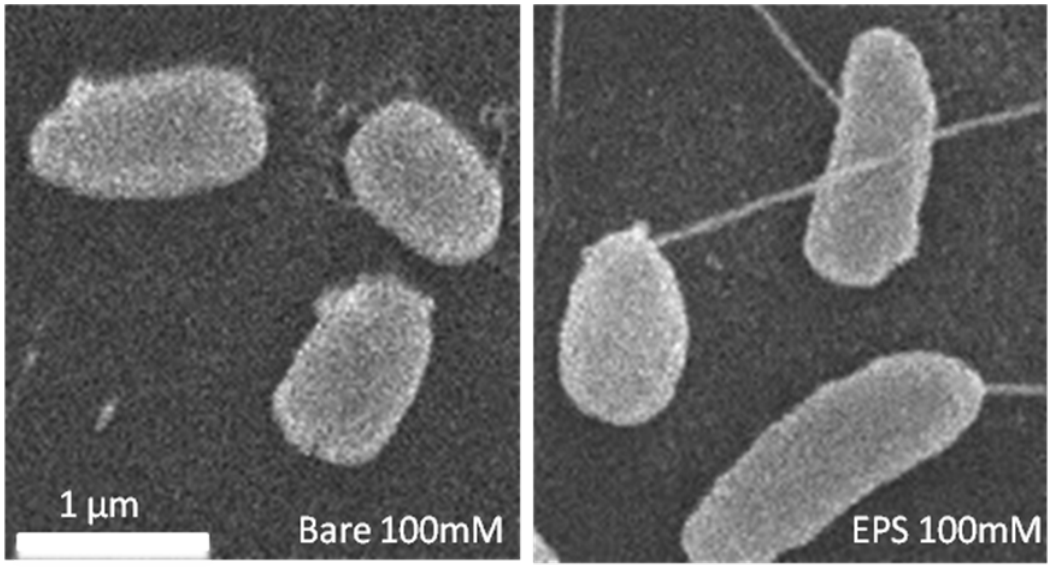
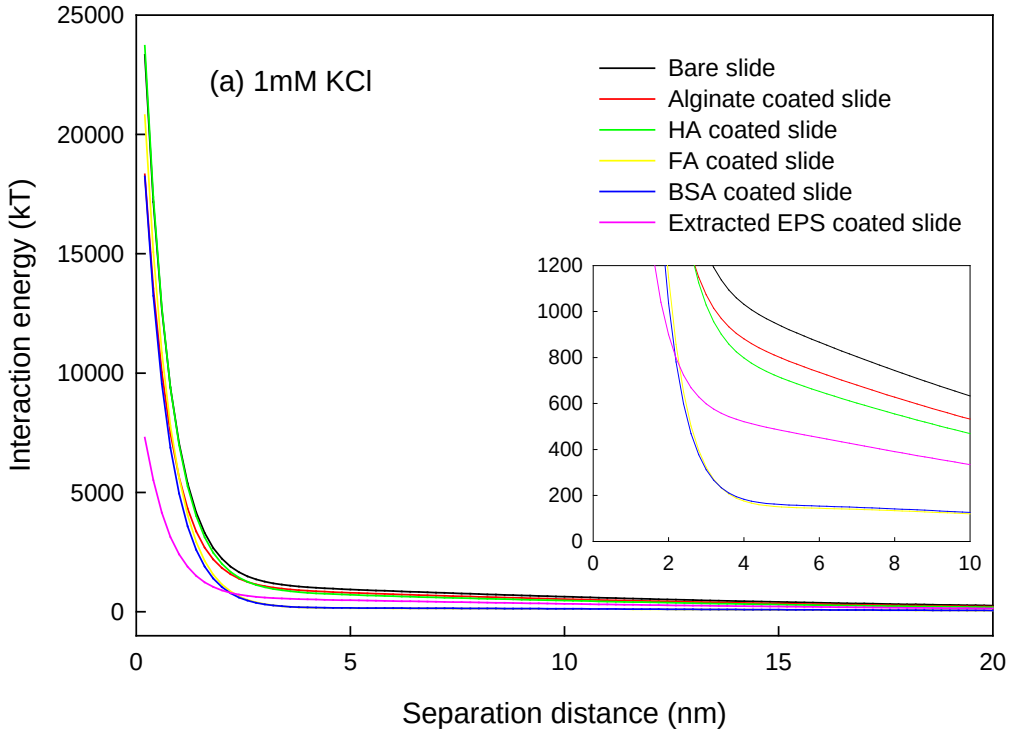
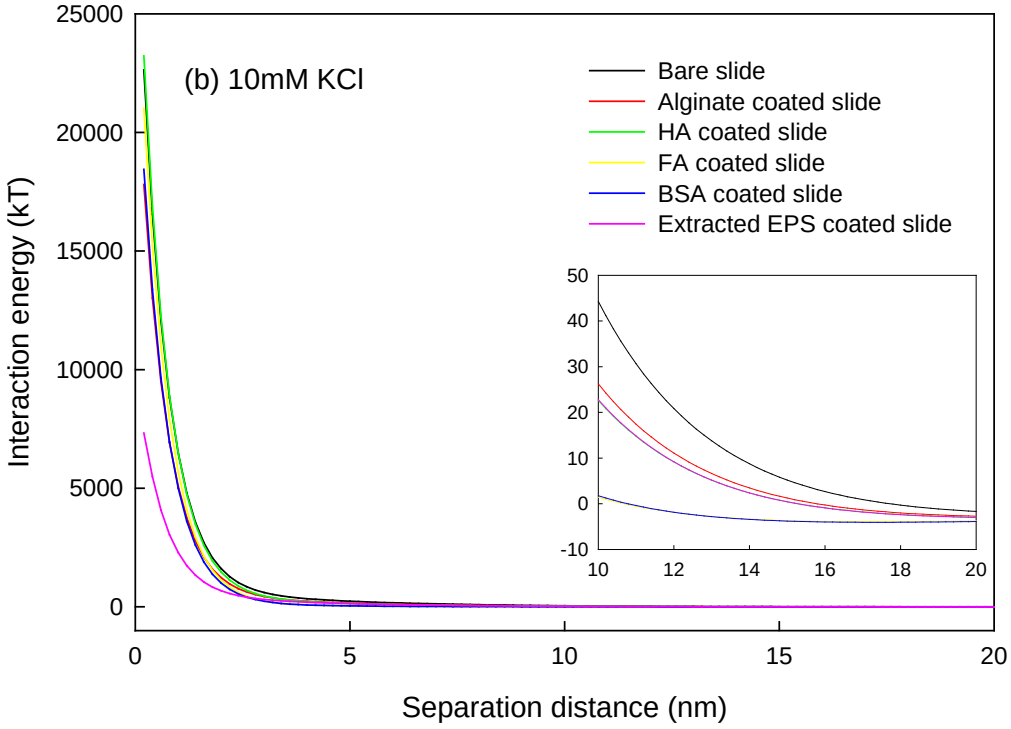
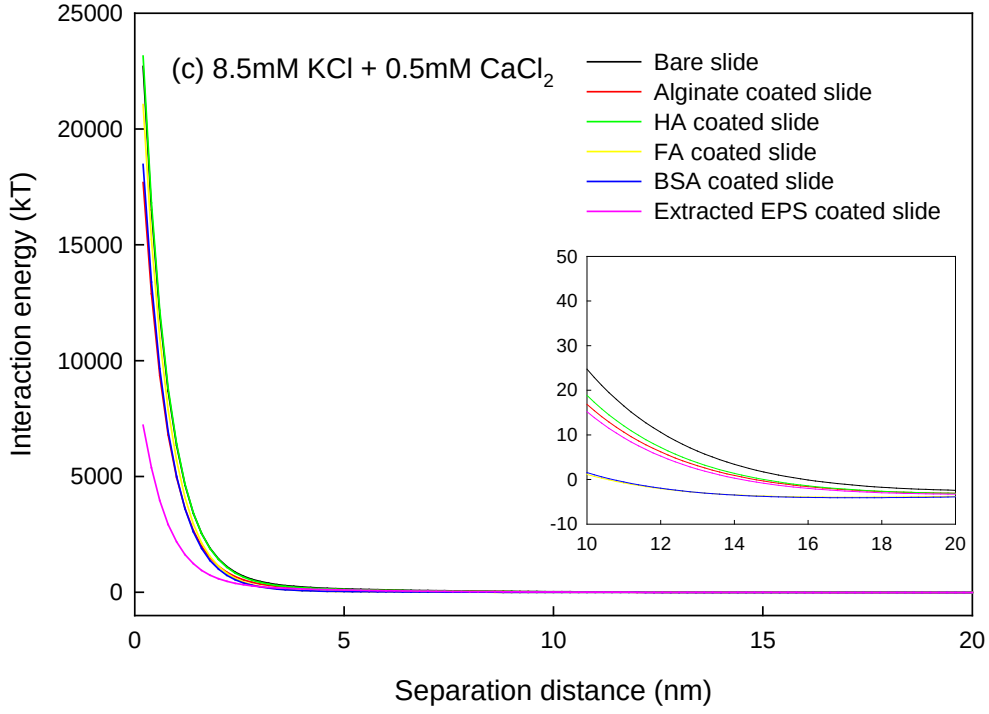


Figure 4-6 Representative SEM images of *P. aeruginosa* PAO1 adhesion on bare and extracted EPS coated slides under 100mM KCl media.

Using Eqs. (1) and (2) in section 3.3, the total Gibbs free energies ($\Delta G^{TOT}(d)$) for the adhesion of *P. aeruginosa* PAO1 to the various slides in various electrolytes (1mM, 10mM and 100mM KCl, and 8.5mM KCl＋0.5mM $CaCl_2$) were plotted as a function of the separate distance in Fig. 4-7. The region for the secondary minimum was magnified and inserted into the lower-right portion of each figure. One of the characteristic features of the extended DLVO approach is the presence of a secondary minimum in the curves of potential energy versus the separation distance (Fornes, 1985). This secondary minimum can be used to determine, not only whether the interaction would occur, but also whether the interaction is reversible or irreversible.


(a) 1mM KCl
Bare slide
Alginate coated slide
HA coated slide
FA coated slide
BSA coated slide
Extracted EPS coated slide
Interaction energy (kT)
Separation distance (nm)
25000
20000
15000
10000
5000
0
0
5
10
15
20
1200
1000
800
600
400
200
0
0
2
4
6
8
10


(b) 10mM KCl
Bare slide
Alginate coated slide
HA coated slide
FA coated slide
BSA coated slide
Extracted EPS coated slide
Interaction energy (kT)
Separation distance (nm)
25000
20000
15000
10000
5000
0
0
5
10
15
20
50
40
30
20
10
0
-10
10
12
14
16
18
20


(c) 8.5mM KCl + 0.5mM CaCl2
Bare slide
Alginate coated slide
HA coated slide
FA coated slide
BSA coated slide
Extracted EPS coated slide
Interaction energy (kT)
Separation distance (nm)
25000
20000
15000
10000
5000
0
0
5
10
15
20
50
40
30
20
10
0
-10
10
12
14
16
18
20

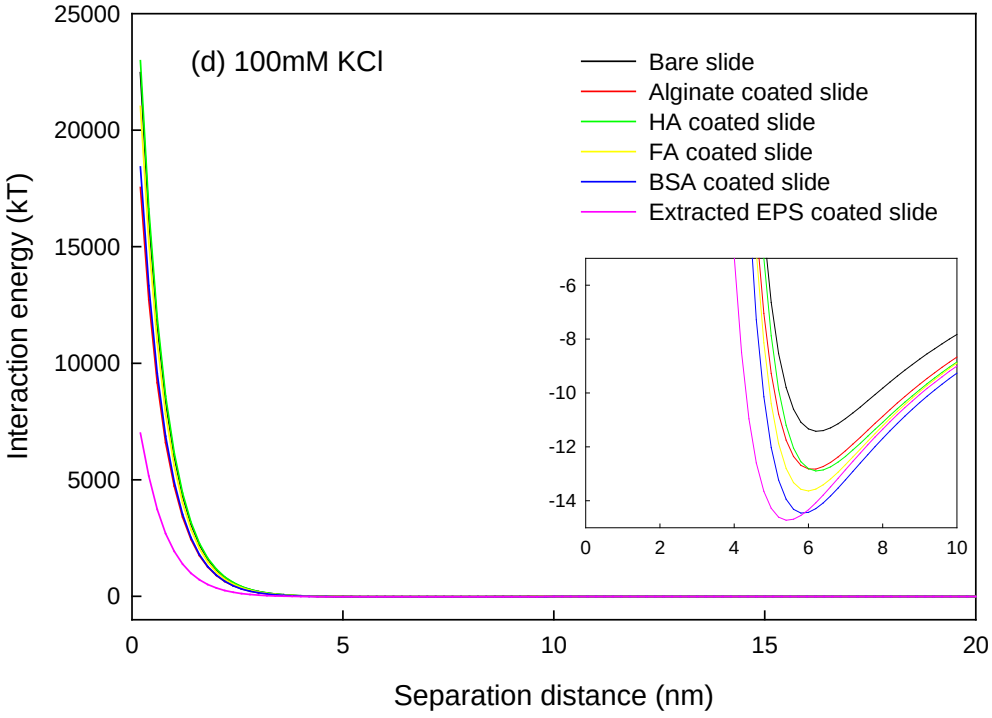

(d) 100mM KCl
Bare slide
Alginate coated slide
HA coated slide
FA coated slide
BSA coated slide
Extracted EPS coated slide
Interaction energy (kT)
Separation distance (nm)
25000
20000
15000
10000
5000
0
0
5
10
15
20
-6
-8
-10
-12
-14
0
2
4
6
8
10

Figure 4-7 Profiles of the total Gibbs free energies for the adhesion of *P. aeruginosa* PAO1 to slides bare and coated with conditioning films alginate, HA, FA, BSA and extracted EPS as a function of the separation distance in (a) 1 mM KCl, (b) 10 mM KCl, (c) 8.5mM KCl + 0.5mM $CaCl_2$, and (d) 100mM KCl. The region for the secondary minimum was magnified and inserted in the lower-right portion of each figure.

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Chapter 5 Discussion

5.1 Bacterial adhesion to alginate or humic substance coated slides

Improved bacterial adhesion on alginate coated surfaces and humic substances was also observed in previous studies (de Kerchove and Elimelech, 2007). The enhanced bacterial adhesion may be explained by the reduced negative surface charge of alginate and humic substance coated surfaces. Further, the improved bacterial adhesion on alginate coated surfaces has been attributed to the extended alginate polymers (Chen and Elimelech, 2008). In the present study, the occurrence of the polymeric interactions between the alginate or humic substance coating and the bacterial surfaces may have caused the enhanced bacterial attachment. Previous studies showed that the alginate film folds and retracts toward the slide surface by electrostatic shielding at a higher ionic strength (Chen et al., 2006). Consequently, the alginate coated surface might become smoother and similar to a bare slide (Chen and Elimelech, 2008). It is believed that similar changes occur on humic and fulvic acid films (Hong and Elimelech, 1997). Therefore, the effects of alginate and humic substance layers become less important to bacterial adhesion as the ionic strength increases.

The enhancement of bacterial adhesion by calcium ions has been reported to be due to the screening and neutralization of surface charges (Chen and Walker,

2007; Grobe et al., 1995; Simoni et al., 2000) and the formation of covalent bridges specifically for negatively charged functional groups (de Kerchove and Elimelech, 2008). Enhanced adhesion might also be explained by the increased thickness and fluidity of an alginate film in the presence of calcium ions (De Kerchove and Elimelech, 2008).

5.2 Bacterial adhesion to BSA or EPS coated slides

Our observations are in agreement with previous studies, which reported that stronger adhesion forces between colloids and protein coatings were apparent in solutions of low ionic strength, which was proposed to be due to the protein conformation (Xu and Logan, 2005). It has been demonstrated that proteins can be compressed and folded into a denser core at high ionic strengths, resulting in fewer interaction sites and, therefore, weaker adhesion forces (Xu and Logan, 2005). In addition, previous studies showed that an EPS coating on substrata may discourage bacterial adhesion due to the highly surface-active groups of uronic acid in the EPS (Gomez-Suarez et al., 2002; Liu et al., 2007), where macromolecules (such as proteins) in EPS generate steric repulsive forces to hinder bacterial adhesion (Kuznar and Elimelech, 2005; Kuznar and Elimelech, 2006; Tong et al., 2010).

5.3 Explanation of bacterial adhesion based on the extended DLVO theory

As shown in Fig. 4-7a, the secondary minimum was not found in the 1mM KCl condition for all substrates and the energy differences among the slides are relatively high. Hence, it is expected that the degree of adhesion would be low in 1mM KCl and the range of adhesion factors would be relatively broad. When the concentration of KCl was increased to 100 mM, a secondary minimum was found for all substrates and both the shape of the curves and the depth of the secondary minimum of all the substrates climbed to parity (Fig. 4-7d). Consequently, higher bacterial adhesion on the substratum and smaller deviations of the adhesion factor are expected in the presence of 100mM KCl. These extended DLVO results are in agreement with our batch of adhesion test results observed for all surfaces except for the BSA and EPS coated slides (Figures 4-4 and 4-5).

It should be noted that the degree of adhesion on BSA coated slides was much higher than on other surfaces in 1mM KCl despite no secondary minimum being found; even though high bacterial adhesions on the BSA and EPS coated slides in 100mM KCl were predicted by the extended DLVO theory, both the degree of adhesion and the adhesion factors of BSA and EPS coated slides in 100 mM KCl were lower than with the other conditioned slides. These findings imply that the adhesion of *P. aeruginosa* PAO1 on the conditioned slides was generally

governed by electrostatic interactions (as shown in cases of bare slides and alginate, HA and FA coatings), and that interactions other than hydrophobic and electrostatic interactions, perhaps interactions specific to BSA and EPS, affected bacterial adhesion to the BSA and EPS coated slides.

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Chapter 6 Conclusions and Recommendations

The roles of EPS conditioning films in bacterial initial adhesion were investigated through precoating the microscope slides with different components of *P. aeruginosa* PAO1 EPS. In addition, the effects of ionic strength, including both monovalent and divalent cations, were also studied in this research. All adhesion results were explained with the extended DLVO model.

The roles of EPS in bacterial adhesion have been widely discussed in literature, but findings are controversial due to the complex composition of EPS and the heterogeneity of each study. We found that bacterial adhesion to bare slides and to slides coated with alginate or humic substances increased as the ionic strength of the media increased. At low ionic strength (below 10 mM KCl) conditions, the bacterial adhesion to slides coated with BSA was greater than the adhesion to slides coated with alginate or humic substances. Compared to other conditioning film coatings, the slides coated with extracted EPS showed the lowest cell adhesion rate under all tested conditions.

According to the adhesion factor results, the presence of conditioning films significantly improved bacterial initial adhesion at a lower ionic strength, but became less important in higher ionic strengths. Notably, the adhesion factors of

BSA and extracted EPS in 100mM KCl showed values much less than 1, which implied that these layers hindered the attachment of cells.

Nevertheless, profiles of the total cell adhesion Gibbs energy predicted favourable adhesion for BSA and extracted EPS in 100mM KCl. These findings imply that forces other than hydrophobic and electrostatic interactions were involved in controlling bacterial adhesion on BSA and extracted EPS coated surfaces, and that protein played a major role for the inhibition of the initial bacterial adhesion at a high ionic strength.

Our results demonstrate that the impact of conditioning films on bacterial adhesion is more significant in the aqueous environment of low ionic strength, such as freshwater and groundwater. We propose that the steric interaction and polymer bridging in bacterial adhesion to conditioning films should be tested in the future work. It would be beneficial to explore the mechanisms of bacterial adhesion to conditioning films and the biofilms formations or removals.