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Investigating the Therapeutic Potential of Salivary Proteins for Oral Diseases

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Engineering Science

INVESTIGATING THE THERAPEUTIC POTENTIAL OF SALIVARY PROTEINS FOR ORAL DISEASES

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ABSTRACT

Saliva is responsible for the formation of the acquired enamel pellicle (AEP), a protein integument formed as a result of selective adsorption of salivary proteins to the enamel surface. The AEP demonstrates an important role for modulating dental erosion as a result of its physical properties, along with its salivary and exogenous protein composition (Chapter 2). In addition, individual proteins that comprise the AEP have important physiological functions. Histatin 5 (H5) has potent antifungal effect against *C. albicans*, the yeast responsible for the initiation of oral candidiasis. We designed an *in vitro* model and found, for the first time, that H5 adhered in the form of pellicle retains its antifungal activity on *C. albicans* (Chapter 3). As a pellicle precursor protein, H5 demonstrates high affinity for hydroxyapatite, the primary mineral component of enamel. We used atomic force microscopy (AFM) to determine adhesion forces between H5 and the hydroxyapatite surface to be stronger compared to our protein control, albumin. This knowledge can be applied in the design of therapeutic proteins, and the methodology that we developed can be used for measuring adhesion forces between various other proteins and substrates of interest (Chapter 4). Finally, with the development of proteomics instruments, researchers have identified some protein biomarkers, hidden within salivary fluids. These can be used for diagnostic dentistry, in a clinical setting to identify patients' susceptibility of developing oral diseases. In addition, the delivery proteins with antimicrobial properties via toothpastes or oral rinses can have tremendous therapeutic potential for a multitude of oral diseases (Chapter 5).

Keywords: saliva, acquired enamel pellicle, salivary proteins, histatin 5, albumin, dental erosion, atomic force microscopy, adhesion forces, *C. albicans*, oral candidiasis, salivary diagnostics, proteomics

CO-AUTHORSHIP

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Paper 1. Acquired Enamel Pellicle as a Modulator for Dental Erosion, submitted for publication to Journal of Dentisry in November 2012, located in Chapter 2
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LIST OF ABBREVIATIONS AND SYMBOLS

AEP	Acquired enamel pellicle
AFM	Atomic force microscopy
HA	Hydroxyapatite
H5	Histatin 5
kV	Kiliovolt
nm	Nanometer
nN	Nanonewton
PMMA	Poly(methyl methacrylate)
SEM	Scanning electron microscope
spp.	Species
µL	Microlitre
µm	Micrometer
µM	Micro molar
^{o}C	Celsius (degrees)

CHAPTER 1

1.1 Introduction

The human body contains a wide array of fluids that circulate throughout the body and have important roles in maintaining the overall health of the individual. Saliva is a very important fluid that continually bathes the oral cavity. Saliva consists of secretions from the salivary glands, including parotid, submandibular, and sublingual glands, along with hundreds of minor salivary glands (Dawes, 2004). In addition saliva contains several non-salivary components, which include gingival crevicular fluid, serum and blood derivatives, bronchial and nasal secretions, microorganisms, food debris, and desquamated epithelial linings (Kaufman and Lamster, 2002).

In terms of physiological functions, saliva provides tissue lubrication, aids in mastication and speech, protects enamel from demineralization, aids in remineralization, plays an important role in bacterial and viral clearance; ultimately maintaining the integrity of the oral cavity (Mandel, 1987). In addition, saliva is responsible for forming the acquired enamel pellicle (AEP), an organic protein layer formed *in vivo* as a result of selective adsorption of 130 salivary proteins to the enamel surface (Dawes, 1963; Siqueira et al., 2007). Pellicle precursor proteins play an important role in AEP formation since they demonstrate a high affinity for hydroxyapatite (HA), the inorganic mineral component of teeth. Pellicle precursor proteins include statherin (Hay, 1973), prolin-rich proteins (Oppenheim et al., 1971), cystatins (Larkin et al., 1991), and histatins (Hay, 1975; Oppenheim et al., 1986).

Pellicle formation is a highly selective process since only a fraction of proteins in human saliva (130/2290 proteins) are present within the *in vivo* AEP (Siqueira et al., 2007; Siqueira and Dawes, 2011). Within seconds of enamel exposure to saliva, the initial phase of pellicle formation occurs (Hannig, 1999; Smith et al., 2000), during which precursor proteins adhere to the surface, forming a 10–20 nm-thick protein layer (Hannig and Joiner, 2006). The second stage of pellicle formation (30-90 min) involves the adsorption of protein aggregates, resulting in an increase of pellicle thickness to 100-1000 nm, and a plateau in pellicle formation (Skjorland et al., 1995).

The AEP demonstrates anti-erosive properties within the oral cavity due to its physical structure and protein composition. As a result, AEP possesses tremendous

therapeutic potential for preventing dental erosion, a multifactorial condition that can result in the loss of tooth structure and function, potentially increasing tooth sensitivity (Imfeld, 1996). The progression of dental erosion is caused as a result of enamel exposure to acids from non-bacterial sources (Imfeld, 1996). These erosive challenges are counteracted by the anti-erosive properties of the AEP. The protective nature of AEP is affected by pellicle thickness (Amaechi et al., 1999; Milosevic et al., 1994 Bartlett et al., 1996), maturation time (Nieuw Amerongn et al., 1987; Amaechi et al., 1999; Hannig et al., 2003) and site of development (Carlen et al., 1998). The pellicle contains naturally secreted salivary proteins embedded within its structure that demonstrate anti-erosive properties. However, rather than individual proteins, protein-protein interactions may play a fundamental role in the protective nature of the AEP (Cheaib and Lussi, 2011). In addition, dietary (Herod, 1991) and synthetic proteins (Kosoric et al., 2007) can modify the pellicle, enhancing its protective efficiency against dental erosion. In addition, the salivary composition of the AEP and its corresponding protein-profile may be employed as a diagnostic tool, since it likely contains salivary biomarkers for oral diseases that initiate at the enamel surface, including dental erosion. By modifying the composition and structure of the AEP, this protein integument has the potential to be used as a target-specific treatment option for dental erosion (Vukosavljevic et al., 2011).

Salivary proteins that are members of the AEP can assume an important role in preventing the colonization of pathogenic microorganisms within the oral cavity. For instance, histatins demonstrate potent antifungal effects against *C. albicans*, a pathogenic yeast responsible for initiating oral candidiasis. Histatins are cationic, histidine-rich salivary proteins (Oppenheim et al., 1986). The main members of the histatin family include histatin 1, 3, and 5, consisting of 38, 32, and 24 amino acids, respectively (Oppenheim et al., 1988). At concentrations found in salivary secretions of healthy individuals (15-30 μM), the carboxyl-terminal of histatin 5 (H5) demonstrates the most potent fungistatic and fungicidal affects, followed by histatin 3, and histatin 1 (Oppenheim et al., 1986; Xu et al., 1991). The antimicrobial effect of H5 is likely due to its composition of multiple basic amino acid residues (arginine and lysine), which enables it to interact with the negatively charged lipid bilayer. It has been suggested that the basic character of histatin 5 disrupts the cell membrane by forming membrane pores,

thus inducing membrane permeability (increased loss of K^+ from cell) and resulting in cell death (Pollock et al., 1984). In addition, histatin 5 inhibits the transformation of *C. albicans* to its virulent, filamentous form (Xu et al., 1991), therefore preventing invasion of mucosal membranes.

Although fluid-phase H5 demonstrates antifungal effect on *C. albicans* in planktonic phase (Gyurko et al., 2000; Pusateri et al., 2009), and on *C. albicans* biofilms (Konopka et al., 2010), its inhibitory effect when adhered to hydroxyapatite and polymethacrylate (PMMA) surfaces, resembling conditions of the *in vivo* pellicle, remains unexplored. Therefore, the primary objective of the *in vitro* study described in Chapter 3 was to design a model system to investigate whether surface-adhered H5 inhibits the colonization of *C. albicans* on HA and/or PMMA. The limited number of treatments for oral candidiasis resulted in the emergence of azole-resistant *Candida albicans* strains, thus enforcing the need for novel antifungal treatments.

Atomic force microscopy (AFM) has been previously used to study the adsorption dynamics of proteins on surfaces including mica and polydimethylsiloxane (PDMS) (Dufrene, 2003; Toworfe et al., 2004; Toscano and Santore, 2006). Although histatins are among the pellicle precursor proteins possessing a high affinity to HA (Hay, 1975; Oppenheim et al., 1986; Oppenheim et al., 1988), the strength of this affinity has not yet been established. Understanding the adhesive properties of proteins with high affinity for HA (i.e., adhesion forces), would provide fundamental knowledge that can be used in the design of various therapeutic treatments, involving natural or synthetic proteins/peptides. Since adhesion forces can provide a method to determine the affinity of a protein or peptide to the surface in direct contact with the substrate. The experiment that investigates the adhesion force between H5 and HA is described in Chapter 4.

In addition to containing proteins that possess anti-erosive and antimicrobial properties, saliva is an important body fluid to be explored for health and disease surveillance. This is primarily because saliva contains gingival crevicular fluid, which in turn contains a variety of hormones, antibodies, enzymes, cytokines, and antimicrobial constituents (Zelles et al, 1995). Therefore, the gingival crevicular fluid found within whole saliva allows it to contain biomarkers from the body's circulation, thus making saliva an attractive diagnostic fluid for monitoring disease biomarkers that are typically

found in serum (Oppenheim, 2007; Loo et al., 2010). Therefore, saliva contains important information about the physiological state of the body. Saliva can be used for monitoring oral health, including periodontal diseases (Socransky et al, 2000) and to assess caries risk (Baughan et al, 2000), as well as overall systemic health. For instance, saliva has been found to contain biomarkers for cancer (Zhang et al, 2010), bacterial (Lendenmann et al, 2000) and viral (Pozo and Tenorio, 1999) diseases. The development of proteomics has revolutionized the field of salivary diagnostics because it allows researchers to use certain biomarkers to diagnose a disease. The goal in salivary diagnostics is to be able to detect changes in the salivary proteome prior to the onset of clinical symptoms of the disease. It is important to gain an understanding of how to utilize recent technological advances in dental research for predicting, monitoring, and preventing the development of oral diseases by investigating the diagnostic and therapeutic role of salivary proteins.

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CHAPTER 2

Acquired Enamel Pellicle as a Modulator for Dental Erosion

2.1 Introduction

Dental erosion is described as the loss of dental hard tissue due to dissolution and chemical etching by acids of nonbacterial source (Imfeld, 1996). In recent years, the prevalence of dental erosion has significantly increased, particularly in developing countries (Truin et al., 2005), affecting anywhere between 4-82% of adults and 6-50% of children (Jaeggi and Lussi, 2006). The pathophysiology of dental erosion is modulated by multiple factors including host behaviour, salivary flow rate, and the microenvironment surrounding the tooth. As a result of the multifactorial dependence, high prevalence, and potentially rapid and destructive behavior of dental erosion, the development of effective management and preventative approaches to avoid the dissolution of dental enamel is becoming increasingly important. The most common approach to treat and prevent erosive wear is the use of fluoride-containing dental products (Lussi, 2009; Lussi and Jaeggi, 2006). However, low-to-moderate fluoride concentrations are not able to completely prevent the progression of dental erosion (Larsen and Richards, 2002), and there has been concerns with the use of fluoride in terms of its toxicological effects (ten Cate, 1999).

Alternatively, human saliva possesses several natural biological properties that protect tooth surfaces against demineralization (Amaechi and Higham, 2001). For instance, saliva's bicarbonate content supplies a constant source of ions that interact with the tooth surface, acting as a buffer that effectively resists changes in pH, thus neutralizing acids that are responsible for erosion (Lendenmann et al., 2000). Along with buffering capacity, salivary clearance of erosive agents and its remineralizing capacity also contribute to the anti-erosive properties of saliva (Zero, 1996; Sreebny, 2000; Amaechi and Higham, 2001). The protective function of saliva can also be attributed to the formation of the acquired enamel pellicle (AEP), a protein integument formed *in vivo* as a result of selective adsorption of salivary proteins to the enamel surface in the oral cavity (Dawes et al., 1963). Within seconds of enamel exposure to saliva, the initial phase of pellicle formation occurs (Hannig and Balz, 1999), during which precursor proteins

(i.e., statherin, histatins, acidic proline-rich proteins) selectively adhere to the surface, forming a protein layer 10-20 nm thick (Hannig and Joiner, 2006). The rapid increase in pellicle thickness (100-1000 nm) during the second stage of pellicle formation and the presence of adsorbed knotted, globular-like structures *in vivo* suggests that protein aggregates, rather than individual proteins, are responsible for subsequent pellicle development (Hannig et al., 2001).

In this chapter, we discuss the role of AEP physical properties, and the influence of salivary, exogenous, and synthetic proteins, on the protective nature of the AEP (summarized in Figure 1). We also consider the pellicle as a strong candidate as a future proteomics-based diagnostic tool, along with its potential as a target-specific therapeutic treatment option.

2.2 Anti-erosive properties of the pellicle

The AEP protects the tooth from enamel demineralization by acting as a natural diffusion barrier inhibiting the direct contact between the tooth surfaces and dietary acids (Siqueira et al., 2007b). As a result, there is a decrease in diffusion rates of phosphate and calcium ions into the surrounding fluid following exposure to acidic conditions, thus protecting against tooth demineralization (Zahradnik et al., 1976; Hannig and Balz, 1999; Siqueira et al., 2010). More specifically, the AEP significantly inhibits the surface microhardness loss and surface roughness increase on bovine enamel that occurs as a result of exposure to organic acids (i.e., citric acid; Nekrashevych and Stosser, 2003).

The protective efficiency of AEP against erosion is dependent on its physical properties, including pellicle thickness and maturation time. The thickness of the AEP varies widely throughout the oral cavity, with a thicker pellicle exhibiting stronger protective effects against erosion (Amaechi et al., 1999). The AEP is thickest on the lingual surfaces of the lower teeth, since this region is constantly bathed in saliva excreted from submandibular and sublingual glands (Carlen et al., 1998). Meanwhile, the palatal surfaces of upper teeth are exposed to shear forces from the rubbing action of the tongue and these areas are also poorly bathed in saliva, resulting in a thin pellicle

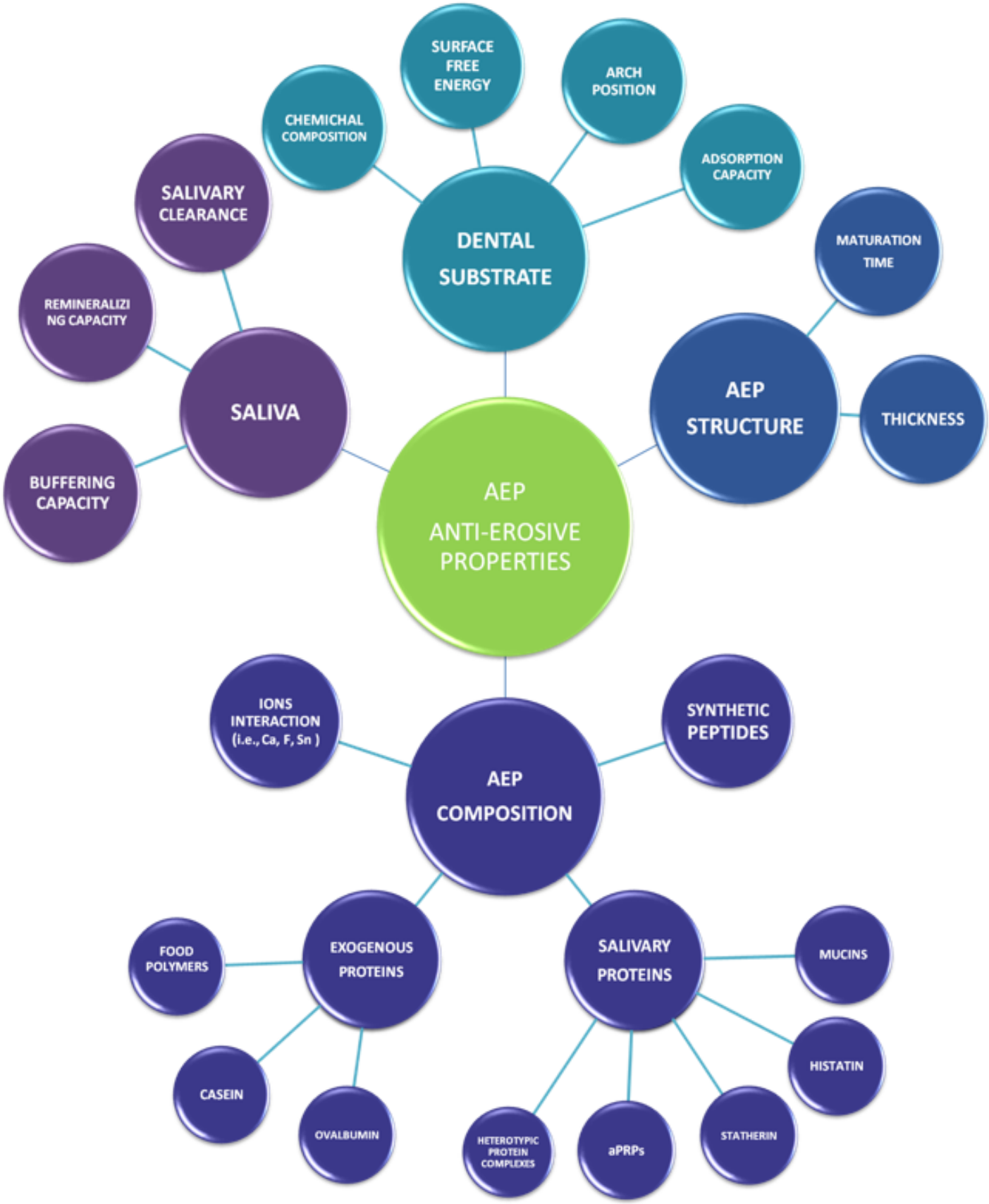

Figure 2.1 The ability of the AEP to reduce/inhibit dental erosion effectively depends on a wide-range of properties within the oral cavity. The schematic figure illustrates the importance of saliva, AEP physical features, AEP composition, and substrate characteristics when considering the anti-erosive property of the AEP.

layer (Amaechi et al., 1999). Therefore, the variation of pellicle thickness within dental arches contributes to site-specificity and severity of erosion, with palatal surfaces as frequent sites of erosion in children (Milosevic et al., 1994) and adults (Bartlett et al., 1996).

It has been suggested that only mature, several day-old pellicles are capable of preventing enamel demineralization (Zahradnik et al., 1978). However, when 24-hour and 7-day-old pellicles were compared, there was no significant difference in the protective ability of the pellicles (Hannig and Balz, 1999). Studies have demonstrated that pellicles developed over one hour offered maximum protection against demineralization, with no subsequent decrease in erosion when using longer maturation times (Nieuw Amerongn et al., 1987; Amaechi et al., 1999; Hannig et al., 2003). Hannig et al. (2004) found no difference in the protective effect of a pellicle formed after three minutes compared to a pellicle formed after two hours. This can be attributed to the fact that pellicle formation, in terms of protein adsorption, begins within seconds of exposure of salivary proteins to the oral cavity (Hannig et al., 2004), producing an electron dense basal pellicle layer after one minute (Ericson et al., 1982). Since subsequent pellicle layers are much less electron dense and are much more loosely arranged compared to the initial basal pellicle layer (Hannig, 1999), they offer little additional protection against acidic attack (Hannig and Balz, 1999; Hannig et al., 2003; Hannig et al., 2004).

2.3 Anti-erosive effect of salivary pellicle proteins

Along with pellicle thickness and maturation time, the incorporation of single host salivary proteins into the pellicle layer can significantly affect AEP function, including its ability to protect against dental erosion and calcium phosphate crystallization (Dickinson and Mann, 2005). Salivary mucins are high-molecular-weight glycoproteins, secreted by the sub-mandibular and sublingual glands, comprising a key component of the pellicle and 7-26% of total salivary proteins (Slomiany et al., 1996). At physiological concentrations *in vitro*, mucins adhered to the enamel surface inhibit enamel demineralization caused by erosive attack (Nieuw et al., 1987; Kielbassa et al., 2001). In addition to mucins, statherin, histatins and acidic proline-rich proteins (PRPs) comprise the basal layer of the AEP and control dental erosion by modulating calcium and

phosphate concentration within the oral cavity (Hay and Moreno, 1989; Hannig et al., 2004; Siqueira et al., 2007a). Calcium and phosphate concentrations modulate the dissolution and remineralization kinetics of enamel (Gao et al., 1991), since a decrease in oral pH requires an increase in phosphate and calcium concentrations in order to maintain saturated fluid that surrounds the tooth. Therefore, statherin and PRPs maintain a state of saturation of calcium and phosphate in the oral cavity by inhibiting their precipitation at oral pH and liberating these ions following acidic attack and during demineralization (Hay and Moreno, 1989). For instance, statherin and acidic PRPs maintain elevated concentrations of calcium by binding it to form calcium-based salivary layers (Proctor et al., 2005) or alternatively micelle-like structures (Rykke et al., 1995).

2.4 The role of dietary proteins on erosion

Exogenous proteins, obtained through dietary means, can interact with salivary proteins within the pellicle to protect the tooth from dental erosion (Barbour et al., 2008). Casein is a phosphoprotein found in bovine milk that binds to amorphous calcium phosphate (ACP) with its multiple phosphoserine residues (Herod, 1991). Upon binding, small casein phosphopeptide (CPP) stabilized ACP clusters become formed that are able to inhibit demineralization and possibly enhance remineralization of the tooth (Reynolds, 1997; Kielbassa et al., 2005). Specifically, CPP-ACP is capable of transporting calcium and phosphate to the tooth surface, localizing it in the pellicle (Reynolds, 2009). Therefore, with a decrease of pH in the oral cavity, calcium and phosphate ions become released, thus inhibiting demineralization by the localized increased amount of mineral ions, and promoting remineralization by diffusion down concentration gradients (Reynolds, 2009). Subfractions of casein that differ in molecular size and chemical characteristics do not show any difference in overall protective ability, thus suggesting that multiple mechanisms likely occur enabling casein to exhibit its inhibitory effects on erosion (Coultate, 2002). *In vitro* studies on the effectiveness of CPP-ACP technology for promoting remineralization and/or inhibiting demineralization of enamel have shown that CPP-ACP can provide significant protection (Cochrane et al., 2008; Lata et al., 2010; Manton et al., 2010; Zhang et al., 2011). However, enamel remineralized with CPP-ACP may interfere with resin bonding, since it has been found to be more resistant to acids

(Iijma et al., 2004). In addition, since CPP-ACP-containing mousse has found to have no protective effect on erosive tooth wear (Wegehaupt et al., 2010), its use as a protective agent against erosion remains controversial.

Along with casein, ovalbumin, a protein found in egg white, can also reduce the erosion of hydroxyapatite in acidic solutions, *in vitro,* once adsorbed to the hydroxyapatite surface in the form of a pellicle (Hemingway et al., 2008). It is likely that both casein and ovalbumin increase the ion-diffusion-restricting properties of the pellicle, thus increasing the ability of the pellicle to prevent erosion of the underlying enamel (Hemingway et al., 2010).

In addition, food polymers such as sodium hexametaphosphate, tri-polophosphate, and xanthan gum possess anti-erosive properties. For instance, the phosphate groups of sodium hexametaphosphate bind to the free calcium sites of the enamel surface. As a result, sodium hexametaphosphate incorporated into the pellicle can protect enamel against erosive agents (Busscher et al., 2002; Hooper et al., 2007). Similarly, when combined, calcium lactate pentahydrate and sodium linear polyphosphate also demonstrate anti-erosive effect on enamel and dentin (Scaramucci et al., 2011).

2.5 Anti-erosive synthetic proteins

In addition to naturally occurring salivary proteins and dietary proteins, synthetic proteins have been designed to inhibit dental erosion. StN21 is a synthetic peptide designed to have an amino acid sequence identical to the N-terminal of statherin, a salivary pellicle protein that inhibits spontaneous and secondary precipitation of enamel (Kosoric et al., 2007). StN21 is a stable peptide shown to effectively reduce mineral loss caused by erosion, thus possessing therapeutic potential (Kosoric et al., 2007).

Biosynthesis of proteins is a rapidly expanding field with tremendous implications for pharmaceutical companies. Synthetically creating proteins allows for precise, site-specific manipulation of active sites with anti-erosive properties. For example, the addition/selective alteration of functional groups with anti-erosive properties could ultimately tremendously improve the physiological function of the protein and increase its effectiveness as a therapeutic agent for dental erosion.

2.6 Effect of protein complexes on erosion

The formation of heterotypic protein complexes results in the formation of micelle-like globules that comprise the mature AEP (Hannig, 1999). Although single proteins have been found to protect against *in vitro* enamel erosion (Kielbassa et al., 2005), it is likely that protein-protein interactions play an important role in enabling the pellicle to exert its erosion-inhibiting properties. A recent study found that the incorporation of mucin or casein alone to the *in vitro* pellicle did not have any effect on the erosion-inhibiting properties of the pellicle. However, experimental treatment of the pellicle with a mixture containing casein and mucin resulted in a significant reduction in enamel softening (Cheaib and Lussi, 2011). This is likely because protein-protein interactions produce additional binding sites, which result in additional interactions with the pellicle (Cheaib and Lussi, 2011). It is important to note that mucin also forms heterotypic complexes with amylase, PRPs, statherin and histatins (Iontcheva et al., 1997) and these complexes could have important impact on the functional role of these proteins in the oral cavity. Mucin heterotypic complexes have an important role in the mature pellicle, which as a result is more effective at preventing enamel erosion compared to an early pellicle coating (Hannig and Joiner, 2006). Therefore, the formation of protein complexes (via protein-protein interactions) likely enhances the anti-erosive properties of the AEP.

Furthermore, when combined with fluoridated dentifrices, the remineralizing effect of mucins becomes enhanced, suggesting that mucin/calcium/fluoride interaction has a strong effect on remineralization (Meyer-Lueckel, 2004). Since fluorides increase calcium diffusion through the mucin film (Alhaique et al., 1986), mucins may promote calcium diffusion into the initial lesion, therefore supporting enamel remineralization (Meyer-Lueckel et al., 2004; Alhaique et al., 1986). Similar to mucins, casein combined with fluoride has an additive effect in decreasing dental erosion compared to casein or fluoride alone (Weiss and Bibby, 1966; White et al., 2010). In addition to protein-protein interactions increasing the protective function of the AEP, the affinity of proteins to hydroxyapatite can be increased as a result of interactions with other proteins (Yin et al., 2006).

With the latest protein-analysis techniques (e.g., mass spectrometry), the field of proteomics has rapidly progressed throughout the last decade, and continues to expand.

We now known that the salivary proteome comprises 2290 proteins (Siqueira and Dawes, 2011), 130 of which are found in the AEP (Siqueira et al., 2007b). With such a vast array of proteins within the dynamic oral cavity, it is very likely that proteins interact with one another to synergistically enhance their diverse functions (i.e., maintaining integrity of oral cavity, lubricating tissues, inhibiting microbial growth, aiding taste, and facilitating food digestion; Siqueira et al., 2007a). Furthermore, there is likely unidentified salivary protein interactions that may demonstrate even more potent anti-erosive effects compared to those currently known. Therefore, it is imperative to continue to investigate the wide range of possible protein-protein interactions to gain further insights into complex proteomes that ultimately control oral physiology.

2.7 AEP as a diagnostic tool for dental erosion

The use of salivary proteins as biomarkers to predict the development of oral diseases has tremendous therapeutic potential since salivary protein composition can be indicative of pathophysiological state of the patient. For example, it is suggested that patients with childhood caries have elevated levels of glycoprotein, and caries-free individuals exhibit elevated salivary proline-rich proteins (Bhalla et al., 2010). Meanwhile, patients with head and neck squamous cell carcinoma have higher levels of salivary isoforms, soluble CD44 (solCD44) compared to cancer-free patients (Franzmann et al., 2007).

When searching for salivary biomarkers for oral diseases that initiate at the enamel surface, such as dental erosion and dental caries, the salivary composition of the AEP and its corresponding protein profile is likely more informative compared to saliva (Siqueira and Oppenheim, 2009). In order to investigate the protein composition of patients' AEP, and subsequently utilize this information for salivary diagnostics, effective sample collection is vital. Sample collection must provide optimal yield and prevent contamination by other materials in the oral cavity. Many different methods for accurate AEP sampling have been employed; however, differences in sample collection methods have produced heterogeneous results for AEP composition (Armstrong, 1966; Eggen et al., 1982; Rykke et al., 1990). The mechanic-chemical harvesting technique developed by Siqueira et al. 2007b has simplified and improved the reproducibility of AEP *in vivo* collection. As a result, AEP proteins can be directly transferred from wick paper to

polyacrylamide stacking gel to perform electrophoretic separation, therefore effectively minimizing sample manipulation and potential protein loss. In addition, the use of soaked paper in 3% citric acid allows for collection of almost all proteins that comprise the *in vivo* enamel surface (Siqueria et al., 2007b).

Combining accurate AEP collection techniques (Siqueira et al., 2007b) with powerful proteomics analytical tools (i.e., mass spectrometry) will allow researchers to more efficiently identify possible changes in AEP protein composition as a result of oral disease. This information can be used to identify disease-specific biomarkers, which can have a powerful role in oral diagnostics. For example, if dental care professionals can effectively utilize the patient's AEP protein profile to predict the development of dental erosion, or even identify a specific stage of erosion, an adequate management plan based on preventative and therapeutic measures can be implemented. Instead of treating advanced stages of diseases, health care professionals could focus on early identification and monitoring prior to their clinical manifestations (Vukosavljevic et al., 2011).

2.8 Future research

Proteins that possess anti-erosive properties include naturally occurring salivary proteins (i.e., mucins, statherin, PRPs), dietary proteins (i.e., casein, ovalbumin), and synthetically formulated proteins (i.e., CPP, ACP, StN21). These proteins are able to control oral homeostasis in solution (saliva) and when adhered to oral surfaces (pellicle). However, proteins incorporated into the pellicle form a microenvironment within the direct vicinity of the tooth, thus controlling conditions favorable for demineralization or remineralization at the interface between the oral cavity and the tooth surface. In order to effectively control demineralization and remineralization processes that occur at this interface, understanding how to modify the pellicle composition to favour anti-erosive properties (i.e., calcium and phosphate diffusion, fluoride retention) is fundamental. This can ultimately be achieved by modifying the *in vivo* pellicle protein composition by incorporating proteins with anti-erosive properties into the pellicle via mouthwashes, toothpastes, or even sports drinks. Finally, since the pellicle contains more than 130 proteins, with 51% of these possessing unknown physiological functions (Siqueira et al., 2007b), future research is warranted to isolate and identify these unknown proteins by

employing the latest proteomics approaches (i.e., mass spectrometry). Performing subsequent assays to determine the anti-erosive properties of these proteins is essential in order to gain a comprehensive understanding of how to use the natural components of the pellicle to develop novel preventative/therapeutic treatments against dental erosion.

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CHAPTER 3

The effect of histatin 5, adsorbed on PMMA and hydroxyapatite, on *Candida albicans* colonization

3.1 Introduction

Oral candidiasis is a severe fungal infection that affects immunocompromised patients, those with reduced salivary flow, diabetics, newborns (Cannon et al., 1995) and approximately 45.3% of acrylic denture wearers (Figueiral et al., 2007). This infection is mainly caused by *Candida albicans*, one of the most common opportunistic and pathogenic microorganisms associated with oral biofilm formation (Reichart et al., 2000). Interestingly, *C. albicans* is a member of the harmless commensal microflora; however, upon suppression of the host immune system, it can utilize its morphological plasticity to cause mucosal and even systemic infections (Kumamoto and Vinces, 2005). Candida albicans is a dimorphic fungus possessing the ability to alternate its morphology from a single-celled, coccial (yeast) form to a filamentous, mycelial form (Niimi et al., 1999). Although both *C. albicans* forms can readily colonize a wide range of hard oral surfaces, including polymethylmethacrylate (PMMA) resin and hydroxyapatite (Chandra et al., 2001; Nikawa et al., 2000), the filamentous form is virulent and essential for invasion of host mucous membranes (Bastidas and Heitman, 2009).

In order to initiate oral infection, adhesion of individual *C. albicans* cells to oral surfaces must occur for successful colonization, because it prevents the cells from being removed by salivary flow (Göcke et al., 2002), thus promoting further sequestration and growth of pathogenic microbial communities. Salivary proteins control the adhesion of *C. albicans* to PMMA and hydroxyapatite surfaces because they are responsible for the development of the acquired pellicle (AP). The AP is an integument formed on oral surfaces comprising more than 130 proteins, along with carbohydrates and lipids, as a result of a selective adsorption process (Siqueira et al., 2007). Within seconds of oral surface exposure to saliva, the initial phase of pellicle formation occurs, during which precursor proteins, i.e. histatin 5 (H5), statherin and acidic proline-rich proteins, adhere to the surface, forming a 10–20 nm-thick protein layer (Hannig and Joiner, 2006).

Controlling the development of oral candidiasis at the pellicle level, by increasing the amount of antifungal pellicle proteins, has been suggested as a novel therapeutic approach (Vukosavljevic et al., 2011). For instance, H5 is prime candidate for candidiasis treatment, since it is a natural salivary protein, capable of exerting inhibitory effects against *C. albicans*, including azole-resistant strains (Tsai et al., 1997). Although previous studies have shown that fluid-phase H5 exhibits antifungal effects on planktonic phase *C. albicans* (Gyurko et al., 2000; Pusateri et al., 2009) and on *C. albicans* biofilms (Konopka et al., 2010), the role of H5 when it is adsorbed to oral surfaces (i.e. PMMA and hydroxyapatite), as an pellicle protein integument, has not been reported.

The objective of this study was to investigate the effect of H5 on *C. albicans* colonization when H5 is adsorbed as a monolayer protein integument to PMMA resin or hydroxyapatite. It was hypothesized that the H5 protein integument on both PMMA and hydroxyapatite will inhibit *C. albicans* colonization on either surface.

3.2 Materials and Methods

For this *in vitro* study, hydroxyapatite discs (Hitemco Medical) and hot water bath polymerized acrylic resin discs (QC-20 PMMA, Dentsply Ltd, Weybridge, UK) were used as substrates (both measuring 5x2mm). The surfaces of both materials were polished by aluminum oxide papers (320, 400 and 600 grit) to standardize surface roughness (SR), aiming to mimic the natural enamel and denture surfaces in the oral cavity (Gomes et al., 2011; Botta et al., 2009). A profilometer (Surface Roughness Tester SJ-210, Mitutoyo; Mississauga, ON, Canada) accurate to 0.01 mm, with a total measurement length of 3.2 x 0.5 mm, was used and showed an average SR 0.31 ± 0.002 mm for PMMA resin and 0.04 ± 0.004 mm for hydroxyapatite discs. The discs were then cleaned by sonication (Digital Ultrasonic Cleaner UD100SH, Kwun Wah International Ltd, Wanchai, Hong Kong, China) in distilled water three times for 20 min each. The discs were randomly divided into a control group (to be coatedwith serum albumin) and an experimental group (to be coated with H5). The number of samples (n=5) for each group ensured that the sample size provided an adequate power for detecting statistically significant differences ($p<0.001$). With five subjects/group, a mean difference of 50 was detected with 80% power, assuming a standard deviation (SD) of 25 and a = 0.05 (two-sided).

Protein film formation

Prior to the *C. albicans* assay, H5 (protein purity 95%, GenScript, Piscataway, NJ, USA) and serum albumin (protein purity>95%, Fisher Scientific, Rochester, NY, USA) were individually resuspended in water (pH 7.4, protein concentration of 100 mM), then placed into 96-well microlitre plates (Corning Inc., Corning, NY, USA). A total volume of 200 ml of either protein solution was added to each well, then the wells were incubated for 2 h at 37ºC under gentle agitation. The discs were then washed with distilled water to remove the non-adsorbed proteins, and subsequently used for the *C. albicans* adherence assay. The amount of H5 or albumin adhered to either PMMA or hydroxyapatite was determined by Micro BCA assay (Pierce, Rockford, IL, USA) in order to confirm protein adsorption on the PMMA and hydroxyapatite surfaces. The amounts of H5 adhered to PMMA and hydroxyapatite were 3.41 and 3.87 mM, respectively, while the amounts of albumin adhered to PMMA and hydroxyapatite were 0.19 and 0.17 mM, respectively.

Candida albicans adherence assay

C. albicans (ATCC 90028) cells were grown on sabouraud dextrose agar (BDTM Difco, Franklin Lakes, NJ, USA) at 37ºC for 24 h. Ten colonies were inoculated into 50 ml YNB broth (BioShopW, Canada Inc., Burlington, Canada) supplemented with 50 mM glucose and incubated overnight on an orbital shaker at 37ºC. The cell culture was then centrifuged (6000 x g for 5 min at 23ºC) and the pellet washed with sterile phosphate-buffered saline (PBS), pH 7.4. The inoculum suspension was prepared by resuspending the washed pellet in YNB broth supplemented with 100 mM glucose to an absorbance of 0.250 ± 0.05 at 520 nm, using an iMark™ spectrophotometer (Bio-Rad Laboratories, Hercules, CA,USA), roughly equivalent to 1 x 10^7 cells/ml. The standardized *C. albicans* inoculum (200 ml) was dispensed into presterilized, polystyrene, flat-bottomed 96-well microtitre plates; individual wells contained either PMMA or hydroxyapatite preconditioned protein discs. The discs were incubated for 5, 30, 90 and 1440 min at 37ºC, with gentle agitation. At each time interval, non-adherent cells were removed from the discs by immersing the samples in distilled PBS as a wash step. The samples were immediately prepared for scanning electron microscopy (SEM) analysis.

Scanning electron microscopy

Samples were fixed with 2% glutaraldehyde for 16 h and then dehydrated in an ethanol series (25%, 50%, 75% and 100%, with three replicates, for 15 min each). Dehydrated samples were critical point-dried (SamDri-PVT-3BW Critical Point Drier, Tousionis, Rockville, MD, USA), secured on 12 mm SEM carbon adhesive tabs and platinum-coated (Denton Vacuum Desk II, Denton Vacuum Inc., Moorestown, NJ, USA). Each sample was analyzed under a scanning electron microscope (LEO 1540XB Field Emission SEM, Carl Zeiss SMT AG, Oberkochen, Germany) at detector beam energy of 3 keV (Power et al., 2009). Microbial counts were obtained for each group at each sampling time interval, by taking SEM micrographs of five randomly chosen regions on each sample. A grid system of 24 000 mm^2 was applied on each micrograph (grid system contained 15 40 mm^2 areas), and the number of cells within each 40 x 40 mm square was counted. This method allowed us to obtain an average number of cells per 24 000 mm^2 area for each sample. Two independent experiments were carried out.

Statistical analysis

Statistical analysis was performed using the SAS/LAB package (SAS v 9.0; SAS Institute, Cary, NC, USA) with a significance limit fixed at 1%. A negative binomial (NB) statistical model was used to compare the differences in microbial counts between substrates, protein treatments and across time. Post hoc comparisons were performed using the Tukey–Kramer test; $p<0.01$ was considered statistically significant.

3.3 Results

Microbial counts indicated that H5-coated PMMA surfaces had significantly lower numbers of cells compared to albumin-coated PMMA surfaces at 30, 90 and 1440 min ($p<0.0001$) (Figure 1A). Similarly, compared to albumin-coated surfaces, H5-coated hydroxyapatite had significantly fewer cells at 90 and 1440 min ($p<0.0001$) (Figure 1B). When comparing differences across time, H5-coated PMMA had a significant decrease in the number of cells at 90 and 1440 min ($p<0.0001$) (Figure 1A,), while the number of cells colonizing H5-coated hydroxyapatite decreased significantly at 30, 90 and 1440 min ($p<0.0001$) (Figure 1B). In Figure 1, upper case letters for albumin and lower case letters

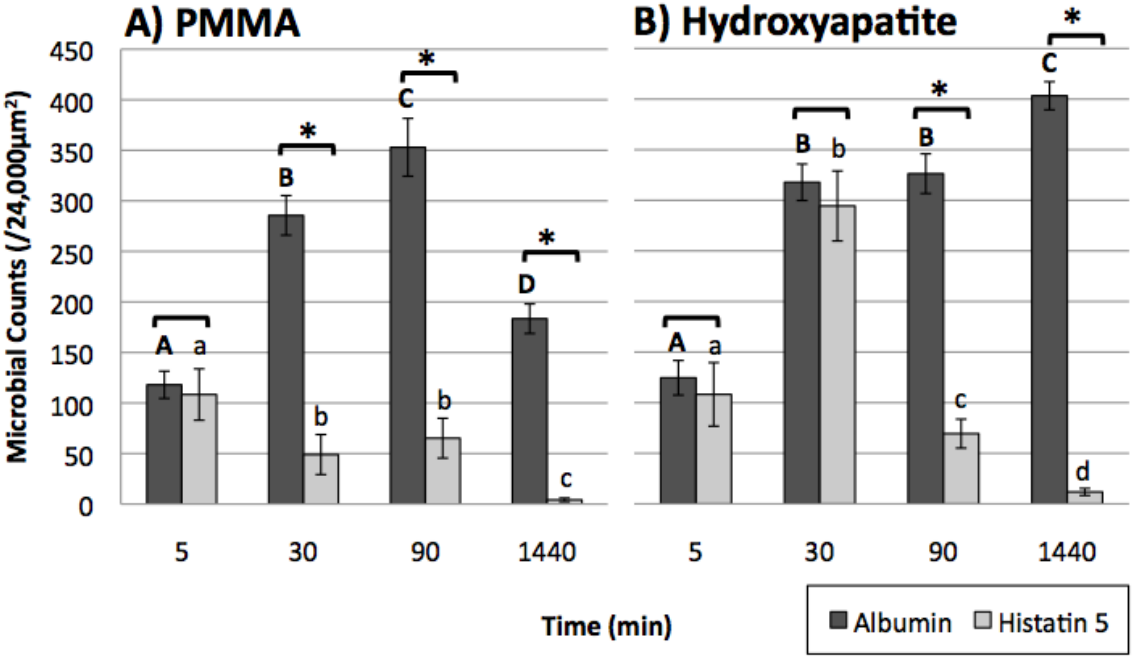

Figure 3.1 Microbial counts of cells on albumin and H5-coated PMMA (A) and hydroxyapatite (B), following 5, 30, 90 and 1440 min exposure periods. *Significant differences between albumin and H5-coated-surfaces; significant differences across time are denoted by letters (upper case, albumin; lower case, H5)

for H5 represent this significant difference across time.

In contrast to albumin-coated PMMA and hydroxyapatite, *C. albicans* exposure to the H5-protein integument results in a time-dependent decrease in the number of cells on both surfaces, with colonization being inhibited most significantly at 1440 min. This time-dependent effect of adsorbed H5 on *C. albicans* colonization is illustrated through a series of SEM micrographs which illustrate *C. albicans* colonizing albumin and H5-coated PMMA surfaces at 5 min (Figure 2A, B), 30min (Figure 2C, D), 90 min (Figure 2E, F) and 1440 min (Figure 2G, H). Similarly, Figure 3 illustrates *C. albicans* colonizing albumin and H5-coated hydroxyapatite at 5 min (Figure 3A, B), 30 min (Figure 3C, D), 90 min (Figure 3E, F) and 1440 min (Figure 3G, H).

In addition, *C. albicans* colonizing albumin-coated PMMA exhibited yeast morphology (Figure 4A), while *C. albicans* formed dense networks of hyphae across the albumin-coated hydroxyapatite surface (Figure 4B) at the 1440 min sampling interval.

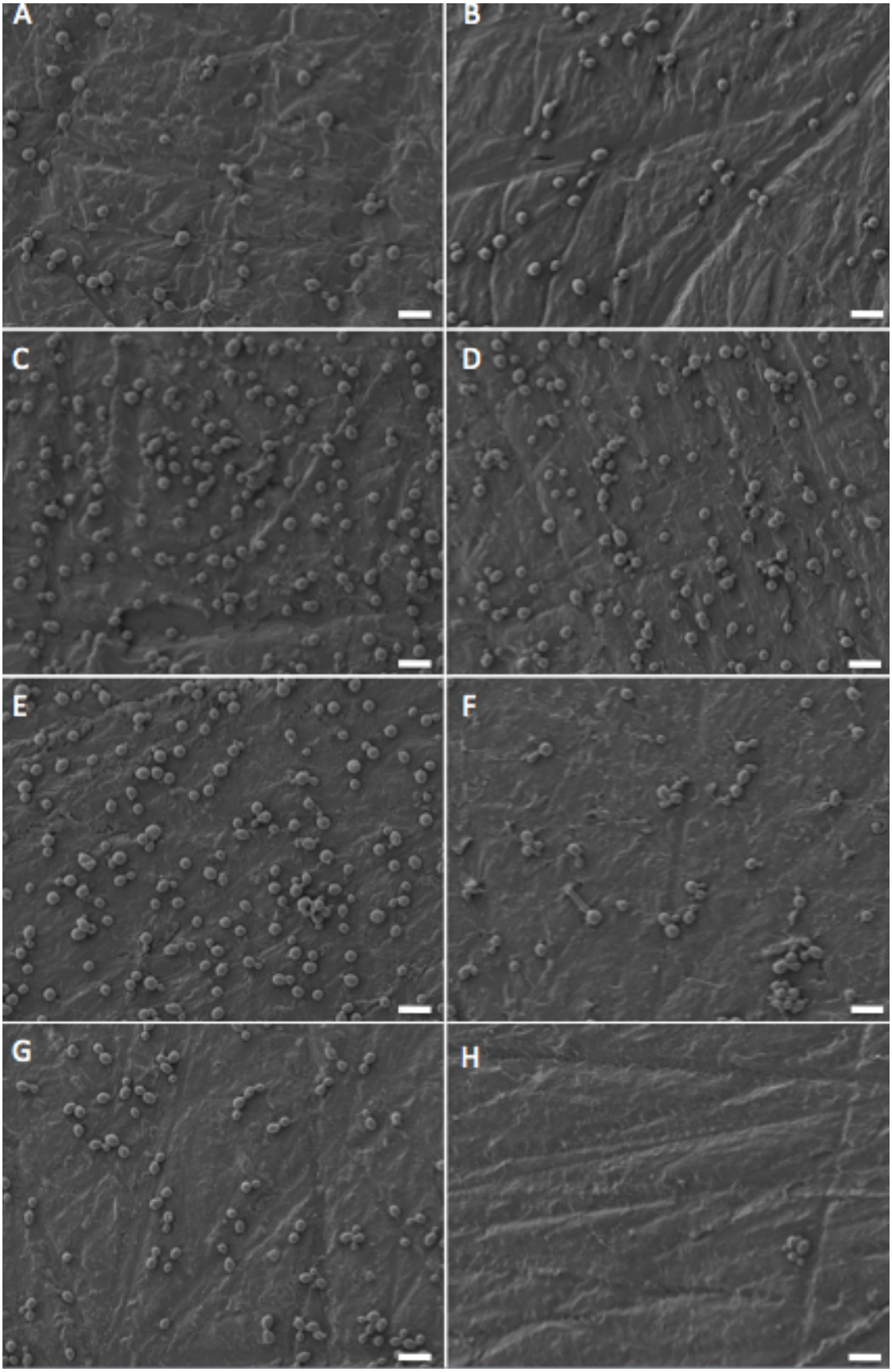

Figure 3.2 SEM micrographs of *C. albicans* colonizing albumin and H5-coated PMMA surfaces at 5 min (A, B), 30 min (C, D), 90 min (E, F) and 1440 min (G, H), respectively. Scale bar = 10 μm

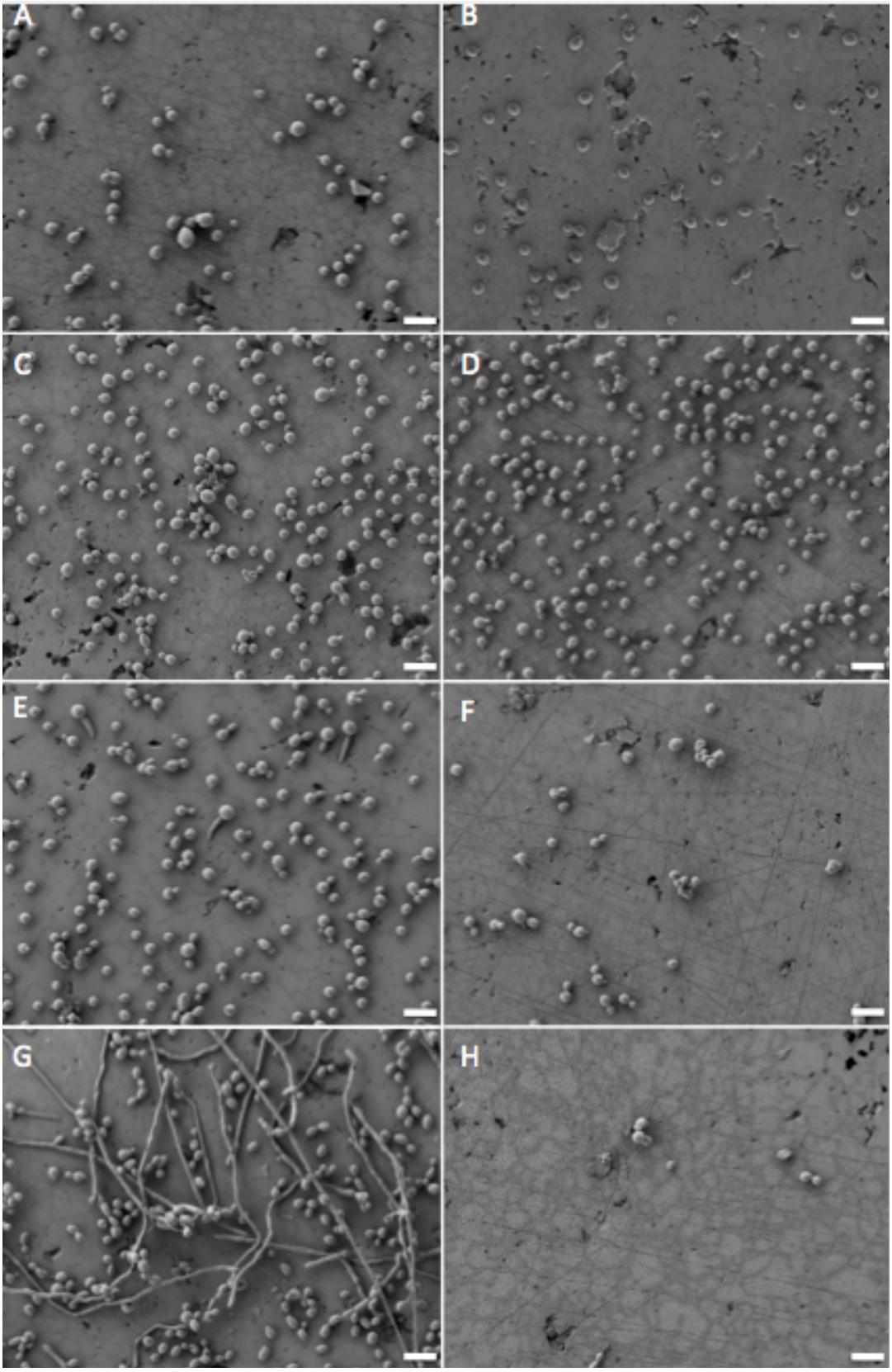

Figure 3.3 SEM images of *C. albicans* colonizing albumin and H5-coated hydroxyapatite surfaces at 5 min (A, B), 30 min (C, D), 90 min (E, F) and 1440 min (G, H), respectively. Scale bar = 10 μm

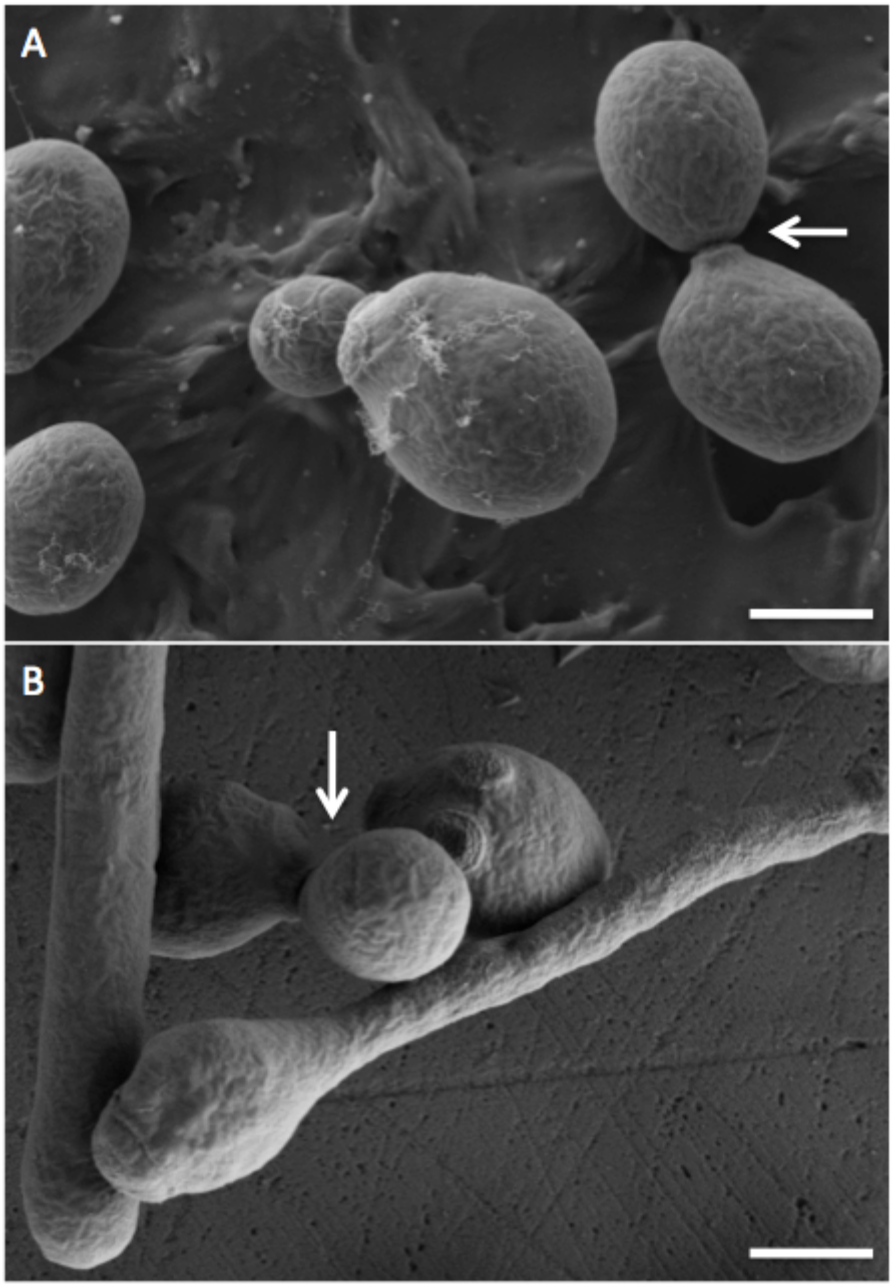

Figure 3.4 SEM images indicate that *C. albicans* colonizing albumin-coated PMMA exhibit yeast morphology (A) at the 1440 min sampling interval, while C. albicans formed dense networks of filaments across the albumin-coated hydroxyapatite surface (B) at the 1440 min sampling interval. Actively dividing cells commonly occur on both surfaces (arrows). Scale bar = 2 μm

3.4 Discussion

Microbial adhesion to hard oral surfaces (e.g. dentures and enamel) is mediated by specific salivary protein receptors in the AP, which therefore have a significant effect on the adhesion process of pathogenic microbial communities responsible for initiating oral

diseases (Buergers et al., 2010). For this study, an in vitro model system was developed, which initially involved developing either an H5 or a serum albumin protein integument on hydroxyapatite and PMMA surfaces, and then inoculating the system with *C. albicans*. Serum albumin was the control treatment, because it is a salivary protein identified within the in vivo pellicle (Siqueira et al., 2007); it serves as a receptor for initial microbial adhesion in the oral cavity (Kohavi et al., 1995) but has not been found to promote or inhibit the microbial adhesion process (Buergers et al., 2010).

Previous studies indicated that fluid-phase H5 exhibits antifungal effects on the planktonic phase of *C. albicans* (Gyurko et al., 2000; Pusateri et al., 2009) and on *C. albicans* biofilms (Konopka et al., 2010). Interestingly, our results demonstrate that H5 adsorbed to PMMA and hydroxyapatite inhibits the colonization of *C. albicans* on these surfaces (Figure 1A, B). Adhesion of individual C. albicans cells to oral surfaces must occur for successful subsequent colonization and initiation of candidiasis (Göcke et al., 2002). We demonstrated that H5 adsorbed as protein integument on PMMA and hydroxyapatite successfully reduces *C. albicans* colonization, which would therefore prevent further sequestration and growth of microbial communities.

The decrease in *C. albicans* cells on both tested surfaces prior to adsorption to H5 suggests that this protein decreases *C. albicans* colonization compared to albumin (Figure 1A, B). Although previous studies have found that the uptake of H5 by *C. albicans* and its killing activity are time-dependent, with the killing efficiency of H5 becoming more pronounced with longer periods of exposure (Gyurko et al., 2000), our results indicate that the time-dependent effect of H5 on *C. albicans* adherence exists when this protein is adsorbed to PMMA and hydroxyapatite surfaces.

In addition, serum albumin, adhered as a conditioning film to the hydroxyapatite surface, retains its ability to induce *C. albicans* to undergo morphological change to its filamentous, virulent form following 1440 min exposure to the protein-conditioning layer (Figure 4). Serum albumin, a major component of the gingival crevicular fluid, has been previously found to promote the shifting from yeast to the filamentous form of *C. albicans* via multiple signaling pathways on serum-coated metallic and non-metallic materials (Frade and Arthington-Skaggs, 2011). However, the specific mechanisms regulating hyphae development remain unclear.

Since patients with periodontal disease demonstrate elevated amounts of albumin in gingival crevicular fluid (Curtis et al., 1990), the present data suggest that the amount of albumin in the acquired pellicle may also modulate the virulence of the pathogenic fungus *C. albicans*.

Although the denture surface is known to be the major reservoir for *C. albicans* (Ramage et al., 2006), the tooth surface may also function as an important reservoir for candidal reinfection, since hydroxyapatite surfaces were much more heavily colonized by virulent *C. albicans* compared to PMMA (Figure 4). Serum albumin's ability to stimulate the morphological change of *C. albicans* may be dependent on the surface available for protein adsorption. It is very possible that a specific conformation of serum albumin may be required to induce *C. albicans* to change into its virulent, hyphal morphology. For example, H5 candidacidal potency is suggested to be dependent on C-terminal exposure, the tendency of H5 to adopt a-helical conformation and the ability of the protein to bind to the glycosylated extracellular receptors of the cell, thus allowing it to exhibit its anticandidal activity (Tsai et al., 1997). Similarly, serum albumin may bind to hydroxyapatite in a conformation that maintains the exposure of its functional domain responsible for the induction of filamentous development. However, this suggestion needs to be evaluated in the future. The present results demonstrate that albumin, adhered as a conditioning film on hydroxyapatite, retains its ability to induce *C. albicans* to undergo morphological change into its filamentous, virulent form following 1440 min incubation. Since the cells on albumin-coated PMMA did not develop extensive hyphal networks, the morphological plasticity of *C. albicans* may be influenced by the surface properties available in the oral cavity for salivary proteins.

Gaining a comprehensive understanding of the key factors that modulate the earliest stages of *C. albicans* adherence and colonization on oral surfaces, such as PMMA and hydroxyapatite, is crucial to effectively develop a new therapeutic approach to preventing or even treating oral candidiasis. One possible therapeutic approach is to load antimicrobial peptides (e.g. H5) via adsorption or chemical crosslink to the PMMA denture surface prior to insertion into the oral cavity (Zasloff, 2002). For instance, modifying the PMMA surface by co-polymerization of methyl methacrylic acid to introduce carboxyl groups provides double the adsorption of H5 per surface area

(Edgerton et al., 1995). Utilizing H5 as a novel treatment technique for oral candidiasis could be an ideal option, since histatins exhibit no or low haemolytic activity to human erythrocytes (Wei and Bobek, 2004) and have low tendencies to induce resistant strains in vitro (Zasloff, 2002). Future studies should investigate whether similar effects of adhered H5 and albumin on *C. albicans* occur in a multi-species biofilm model, to gain a more comprehensive understanding of in vivo conditions.

In conclusion, H5 adsorbed as a protein integument on PMMA and hydroxyapatite effectively inhibits *C. albicans* colonization. In addition, serum albumin adhered as a protein integument retains its effect at inducing *C. albicans* hyphal development. This novel insight reinforces the idea of utilizing antimicrobial salivary proteins such as H5 as a treatment option for preventing the development of pathogenic fungal biofilms, which are resistant to current treatments.

3.5 References

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CHAPTER 4

Adhesion forces between histatin 5 and hydroxyapatite measured by atomic force microscopy

4.1 Introduction

Histatins are a group of low-molecular-weight salivary proteins possessing cationic properties that are secreted by the major and minor salivary glands (Oppenheim et al., 1986; Siqueira et al., 2008). Histatins possess a high affinity to hydroxyapatite (HA), resulting in their selective adherence to the enamel surface (Hay, 1975; Oppenheim et al., 1986; Oppenheim et al., 1988a; Jensen et al., 1992). As a result of being among the first salivary proteins to adhere to enamel, they are among the proteins that are responsible for initiating the development of the acquired enamel pellicle (AEP), a protein integument formed *in vivo* as a result of selective adsorption of salivary proteins to the enamel surface in the oral cavity (Dawes et al., 1963).

It is important to develop an understanding of protein-surface interactions within the oral cavity because many salivary proteins have important biological functions. Understanding the adsorption of histatins, particularly histatin 5 (H5) is particularly important because H5 exhibits an antifungal effect against *Candida albicans*, an opportunistic yeast that induces oral candidiasis (Pollock et al., 1984; Oppenheim et al., 1988; Gyurko et al., 2001). In addition, understanding the adhesive properties of proteins with high affinity for HA (i.e., adhesion forces), would facilitate the design of therapeutic treatments involving natural or synthetic proteins. Although histatins have been found to have high affinity to HA (Oppenheim et al., 1988b; Jensen et al., 1992), the exact strength of adhesion forces between H5 and HA has not yet been established.

Atomic force microscopy (AFM) was originally used as a tool for imaging surfaces at nanometer scales. This was achieved by placing a sharp probe attached to a cantilever in contact with a sample surface and measuring the minute deflections of the cantilever as the probe is moved laterally along the surface (Binnig et al., 1986). Forces between the tip and the surface of the sample are responsible for causing the cantilever to deflect, and a detector measures the cantilever deflection as the tip scans the sample. A contact AFM image is typically obtained of the surface topography by mapping the

vertical displacement of the sample required to maintain a constant cantilever deflection (Muller et al., 1997). AFM is also able to monitor submolecular details of proteins under their physiological conditions (Hoh et al., 1991; Karrasch et al., 1993; Muller et al., 1995). The atomic fore microscope has also been used to study adsorption dynamics of proteins on surfaces including mica and polydimethylsiloxane (PDMS) (Dufrene, 2003; Toscano and Santore, 2006; Holland and Marchant, 2000; Toworfe et al., 2004), and to determine the conformational change of proteins upon their absorption to a surface (Agnihotri and Siedlecki, 2004).

The versatility of AFM can be attributed to the fact that probes can be functionalized to enable researchers to probe specific interactions between two surfaces.

Recently, AFM has been used to measure the adhesion force of a sample, commonly known as the pull-off force needed to separate the AFM tip from the surface of the sample of interest (Pelin et al., 2012). The fundamental factors that control adhesion include adhesive forces between a sample and substrate of interest (Bowen et al., 1998). Adhesion measurements are typically obtained from the force required to detach the AFM tip from the surface by quantifying the difference in approach and retract curves at the point when the two surfaces are separated (Fang et al., 2000). This approach of measuring adhesion forces was used to analyze adhesion between an AFM cantilever and sulfate-reducing bacteria (Fang et al., 2000), and other bacteria (Vadillo-Rodriguez 2003; 2004; 2006). AFM can therefore be used as an important tool for developing the fundamental knowledge of adhesion forces of salivary proteins, e.g., H5, to the enamel surface and consequently provide a better understanding on acquired enamel pellicle formation.

The objective of this study was to determine the adhesion forces between H5 and HA. This would provide fundamental knowledge of molecular forces, which can be used in the development of therapeutic agents for oral diseases. In addition, the novel methodology that was developed for determining adhesion forces between H5 and HA can be utilized by researchers to determine adhesion forces involved in the adhesion of a variety of other proteins to various substrates of interest.

4.2 Methods

Biotinylation of Proteins

H5 (protein purity > 95%, GenScript, Piscataway, NJ, USA) and albumin (Fisher Scientific, Rochester, NY, USA) were individually resuspended in water (pH=7.4, protein concentration of 100 μM). EZ-Link Sulfo-NHS-SS-Biotin (Pierce Biotechnology, Rockford, IL, USA) was equilibrated to room temperature, and dissolved in distilled water to obtain a 10 mM final concentration. Biotin was added to each protein solution at 20-fold molar excess and the reaction was incubated at room temperature for 60 min. To remove excess biotin, the solution was added to a desalting column (3 kDa; Nanosep®), Pall Corporation, NY, USA and centrifuged at 4,000 x *g* for 10 min. The remaining liquid at the surface was collected and the flow-through fluid in the column was discarded. This was repeated for a total of three times to obtain a purified biotinylated protein sample, which was then immediately placed on ice.

The HABA assay (4´-hydroxyazobenzene-2-carboxylic acid; Pierce Biotechnology, Rockford, IL, USA) was performed to quantify biotinylation, the level of biotin incorporation, and ultimately determine the molar ratio of biotin to protein. The biotinylated protein sample (either H5 or albumin) was added to a HABA/Avidin mixture and the absorbance was read at 500 nm using a Bio-Rad iMark™ spectrophotometer *(*Bio-Rad Laboratories Inc, Hercules, CA, USA). In order to calculate the biotin:protein ratio, the absorbance readings of HABA/Avidin and biotinylated protein/HABA/Avidin, along with the molecular weight and concentration of each protein, was applied to the automatic HABA Calculator (Thermo Scientific, Pierce Biotechnology). The Beer-Lambert Law was applied with the known variables to provide an estimate of moles of biotin per moles of protein.

Cantilever system

To obtain adhesion force measurements between our protein of interest and the HA surface, we designed a cantilever system consisting of a Si_3N_4 AFM cantilever (NP-S, Veeco Instruments, NY, USA) with an attached silica microsphere, in turn containing biotinylated protein. To achieve this, ~5 μm-diameter silica microspheres pre-conjugated with streptavidin of (ProActive® Microspheres, Bangs Laboratories, IN, USA) were

acclimatized to room temperature prior to use. A suspension of 0.5 mg/mL of microspheres was washed by vortexing for 20 sec, centrifuging at 1,200 x*g* for 15 min, and re-suspending in a 10x volume of PBS. The supernatant was discarded and the wash step was repeated three times. The pellet obtained (containing microspheres) was re-suspended in a 100 μM solution of either H5 or albumin biotinylated protein. This was incubated at room temperature (22°C) for 30 min on a shaker under gentle mixing. Following incubation, the particles were washed once again, as described earlier. The microspheres were then dried for 2 h on silica wafers. The bond formation between biotin and avidin is rapid and once formed is unaffected by changes in pH, denaturing agents and organic solvents (Green, 1965; Green, 1971; Green, 1975).

Once dry, microspheres were immediately attached individually to the AFM cantilever using Araldite 10-min two-component epoxy resin (Araldite®, Huntsman Advanced Materials, Switzerland) as described in previous studies (Ong and Sokolov, 2007; Lee et al., 2009; Zhang et al., 2009). For each cantilever system, the attachment of each bead to the cantilever was confirmed using optical microscopy (400x magnification).

In addition, six cantilever systems consisting of the biotinylated attached microspheres were sacrificed for SEM microscopy (and not re-used for AFM measurements). They were secured on 12-mm SEM carbon adhesive tabs, and platinum coated (Denton Vacuum Desk II, Denton Vacuum Inc, Moorestown, NJ, USA). The cantilever system was then observed under scanning electron microscopy (SEM; LEO 1540XB Field Emission SEM, Carl Zeiss SMT AG, Oberkochen, Germany) at detector beam energy of 3 keV to confirm the microspheres were successfully attached to the AFM cantilever and that the Araldite was not covering the microspheres.

Optical microscopy was successful in confirming the attachment of each microsphere to each cantilever, and confirmed that the microsphere remained intact even following adhesion measurements. After examining several cantilever systems under SEM, micrographs reveal successful attachment of the microsphere to the cantilever via Araldite (Figure 4.1A), and the fact that Araldite did not coat the surface of the microsphere was also confirmed (Figure 4.1B).

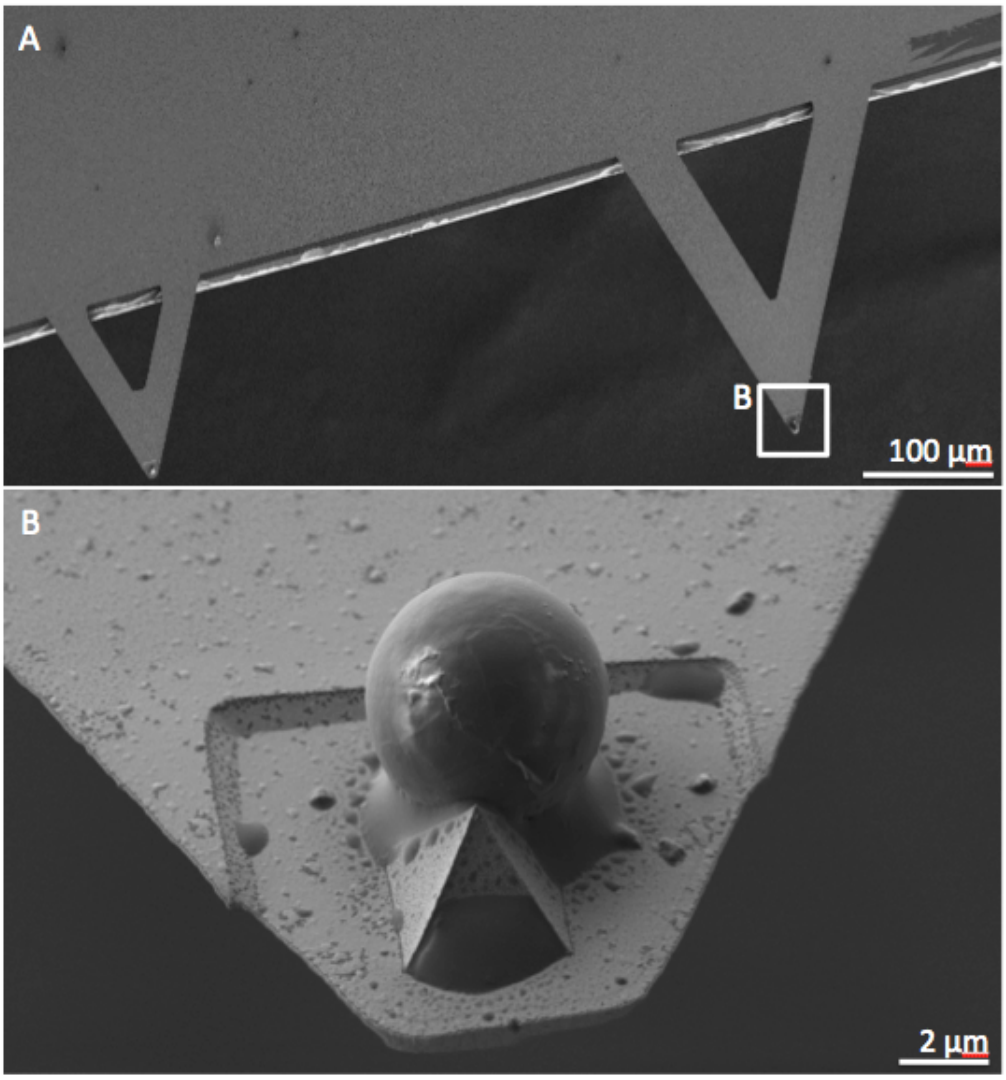

Figure 4.1 SEM micrographs of AFM cantilever with streptavidin pre-coated silica microsphere adhered successfully using Araldite (A). Closer magnification reveals that the surface of the microsphere is not coated in Araldite (B).

Substrate Preparation

HA discs of 5 mm diameter and 2 mm thickness (Hitemco Medical, Old Bethpage, NY, USA) were used as the substrate. Their surfaces were polished using aluminum oxide papers (320, 400, and 600 – grit) to standardize surface roughness (SR), aiming to mimic the natural enamel surface in the oral cavity (Gomes et al., 2011; Botta et al., 2009). A profilometer (Surface Roughness Tester SJ-210, Mitutoyo; Mississauga, ON, Canada), accurate to 0.01 mm with a total measurement length of 3.2 mm x 0.5 mm, was used to measure surface roughness. Discs were then cleaned by sonication (Digital Ultrasonic Cleaner UD100SH, Kwun Wah International Ltd, Wanchai, HK, China) in

distilled water, three times, for 20 min each to obtain clean discs for adhesion measurements.

Adhesion Measurements

A Multimode atomic force microscope (AFM) with Nanoscope IIIa controller (Veeco Instruments, Santa Barbara, CA, USA) was used to measure the adhesion force (force-distance measurements) between the cantilevers containing protein-functionalized silica microspheres and the HA surface. All measurements were performed within a fluid cell containing distilled water. The imaging was performed in tapping mode using Si_3N_4 AFM cantilever (NP-S, Veeco Instruments, Plainview, NY, USA), while the adhesion forces were measured in AFM contact mode using the cantilever system that was created, described earlier. Following each adhesion measurement, the cantilever system was examined using optical microscopy to confirm the bead remained attached to the cantilever.

Adhesion measurements were obtained for two treatments, either biotinylated H5 or albumin microspheres and the HA surface. The control that was used was a microsphere pre-conjugated with streptavidin (containing no protein of interest). Adhesion measurements were made for each treatment, along with the control, on 3 HA discs. A new cantilever system was used for each measurement. For each HA disc, adhesion measurements were obtained on three different areas, each subdivided into a matrix of 32 × 32 points, for a total of ~1000 force-distance curves for each area; therefore ~3000 force-distance curves for HA triplicates (per treatment). The triplicates for each treatment were plotted as histograms, which depicted the range of adhesive forces (nN) measured between H5 functionalized AFM tip and the HA surface or albumin functionalized AFM tip and the HA surface.

In order to obtain the histograms of adhesion forces, adhesion forces were first obtained by mounting the HA disc in the AFM, placing the cantilever system into the fluid cell, positioning the laser to obtain a strong signal, doing a coarse manual approach of AFM cantilever system to the HA surface, followed by an auto approach to the sample, activating the AFM to start a series of approach-retraction cycles.

A typical force-distance curve describing a single approach-retract cycle with the H5 functionalized AFM cantilever and HA surface is illustrated in Figure 4.2. As the AFM tip approaches the sample (Figure 4.2, approach curve), the sample height (height between the cantilever tip and HA surface) decreases. The initial contact between the AFM tip and the surface results in the attraction of the tip towards the surface via van der Waals forces. When the AFM tip makes contact with the HA surface (with a constant force), there is an increase in force, resulting in cantilever deflection (due to stiffness of the surface). During the retraction phase, the AFM tip retracts and tries to break contact with the surface (Figure 4.2, retraction curve). Adhesion forces between the sample and AFM tip attempt to prevent the tip retraction, but the tip eventually overcomes the adhesive forces, withdraws and loses contact with the surface (Shahin et al., 2005). The adhesion force measured is ultimately the force required to detach the AFM tip from the surface by quantifying the difference in approach and retract curves at the point when the two surfaces are separated.

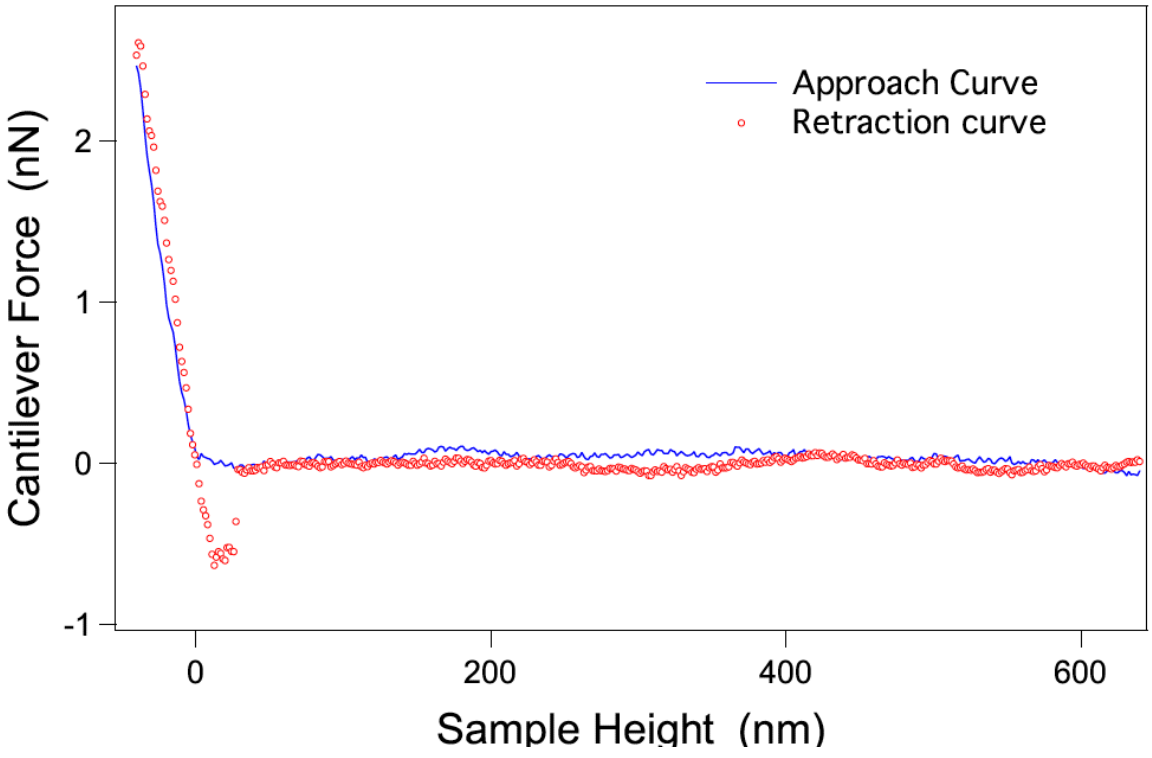

Figure 4.2 A typical force-distance curve, between H5 functionalized AFM cantilever and HA surface, that was used to calculate the adhesion force between H5 and the HA surface. The blue line represents the approach of AFM cantilever to the surface, while the red points represent the retraction of the AFM cantilever from the surface.

Once all adhesion measurements were obtained, each force curve was examined with IGOR Pro macros (Wavemetrics, Tigard, OR, USA), and error force-distance curves were removed. When the adhesion forces were exported to Excel (Microsoft Excel 2008 for Mac, Version 12.3.4), adhesion forces that were equal or less than zero were removed from the data set. Each adhesion force was then multiplied by the spring constant of the corresponding cantilever used to obtain that adhesive force. AFM cantilever spring constant, specific to each cantilever used, was obtained using the Thermal Noise Method. This involved mounting a flat substrate to the AFM (we used silica wafer), mounting the AFM cantilever system into the AFM, performing the approach and recording the deflection data while the force curves are being obtained. Once one cycle is obtained, the AFM cantilever system is withdrawn from the substrate. The data analysis involved a series of steps including measuring the slope of contact portion of force curve and fitting a Lorentzian to the peak, and applying the thermal calibration formula to obtain the spring constant.

After the raw adhesion measurements were corrected with the corresponding cantilever spring constant, bins (ranges) of adhesion forces were created in Excel, and the frequency of data (adhesion measurements) that fell in each bin was obtained. Adhesion measurements were then standardized based on % counts, by dividing each frequency (for each bin) by the total number of adhesion measurements obtained. Only adhesion forces greater than zero were taken into account. Standardizing the adhesion measurements (to obtain % counts) allowed us to combine triplicates for each treatment to form another set of histograms that included all measurements (~9,000 force curves per treatment).

4.3 Results and Discussion

In terms of efficiency of biotinylation, results from the HABA assay indicate the mole:mole ratio of biotin:protein is 0.73 for H5 and 0.71 for albumin. Thus, it can be concluded that biotin was successfully attached to the protein of interest, resulting in biotinylated H5 and biotinylated albumin. Due to the strong avidin-biotin affinity (Green, 1965; Green et al., 1971; Green 1975) we assumed biotinylated proteins were successfully attached to streptavidin pre-conjugated, silica microspheres.

In terms of surface topography of HA, SEM and AFM micrographs, in combination with an AFM three-dimensional image of the HA surface, reveal a visually smooth surface for adhesion measurements (Figure 4.3A, B, C, respectively). In addition, a profilometer (Surface Roughness Tester SJ-210, Mitutoyo; Mississauga, ON, CA) indicated an average surface roughness of 0.040 ± 0.004 (μm), closely mimicking the natural enamel surfaces within the oral cavity determined to be 0.046 ± 0.0107 (Botta et al., 2009).

A collection of force-distance curves (~3,000/replicate) was used to formulate histograms that illustrate adhesion force between the HA surface and control, albumin, and H5 (Figure 4.4A, B, C, respectively). Although Figure 4 indicates variability between replicates for each treatment, when replicates are combined to formulate one histogram (~9,000 force curves), representing the distribution of adhesion forces, a clear difference in histogram distribution can be observed (Figure 4.5). According to the distribution of the histograms, H5 exhibits stronger adhesion forces to the HA surface compared to albumin and the control (Figure 4.5). It can be concluded that the majority of force-distance curves showing non-zero adhesion (90%) are >0.570 nN for the control, >0.282 nN for albumin, and >1.830 nN for H5. Therefore, based on our results, it can be determined that the adhesion force between H5 and HA is stronger than the adhesion between microsphere control and albumin protein control to HA. The histograms were compared in this format because the histograms demonstrate a non-Gaussian distribution and the data sets all demonstrated a high standard deviation. Therefore, the 90% percentile method was used to quantify the histograms for comparison purposes. Comparing the histograms in this way allowed us to determine that the majority of adhesion measurements (90%) fall above a certain value. In addition, since all of the data was standardized (by %counts, as described earlier), histograms were compared with one another directly.

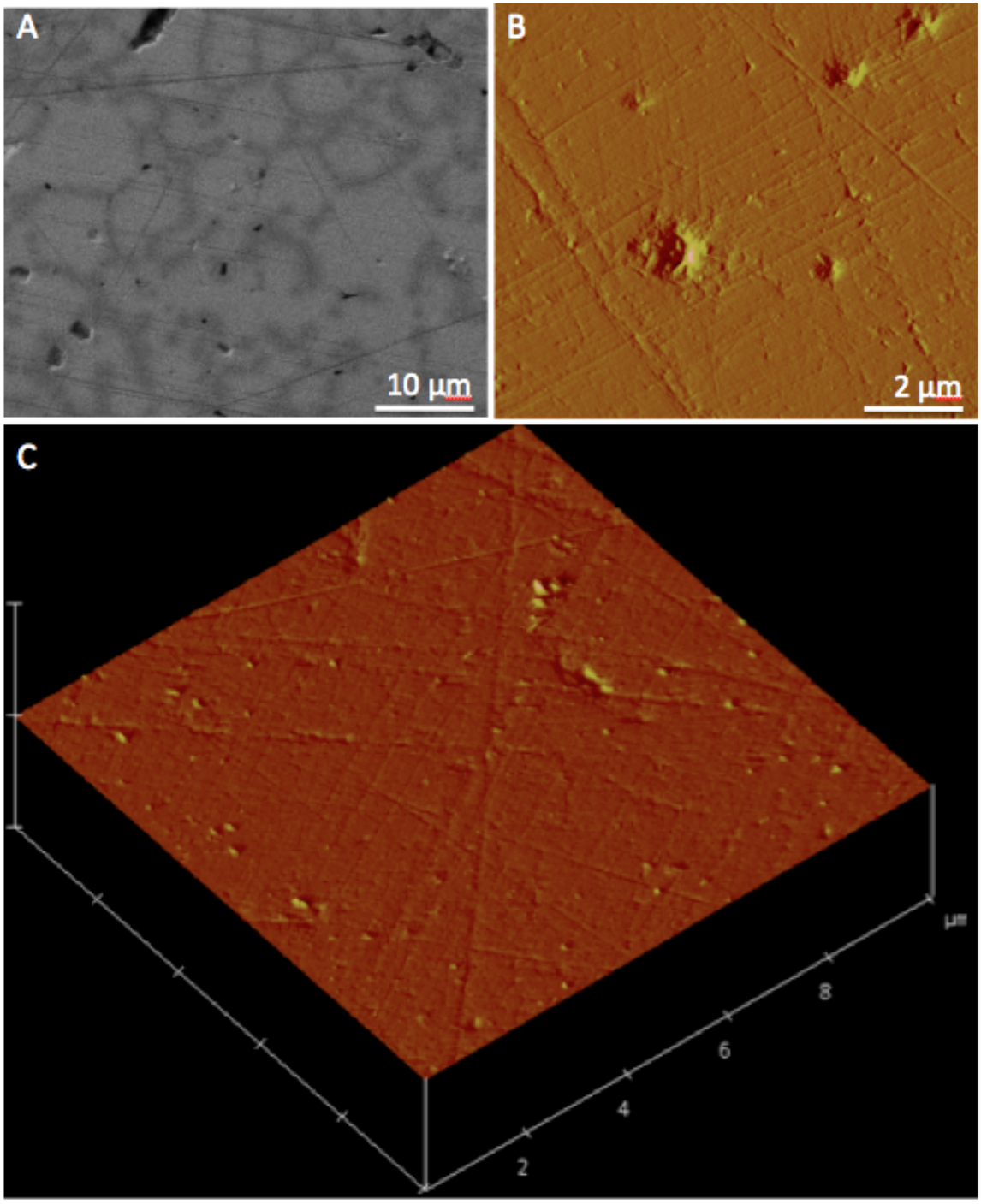

Figure 4.3 The surface topography of the HA disc viewed using SEM, AFM, and AFM three-dimensional surface representation (A, B, C, respectively), reveals a smooth surface for adhesion measurements.

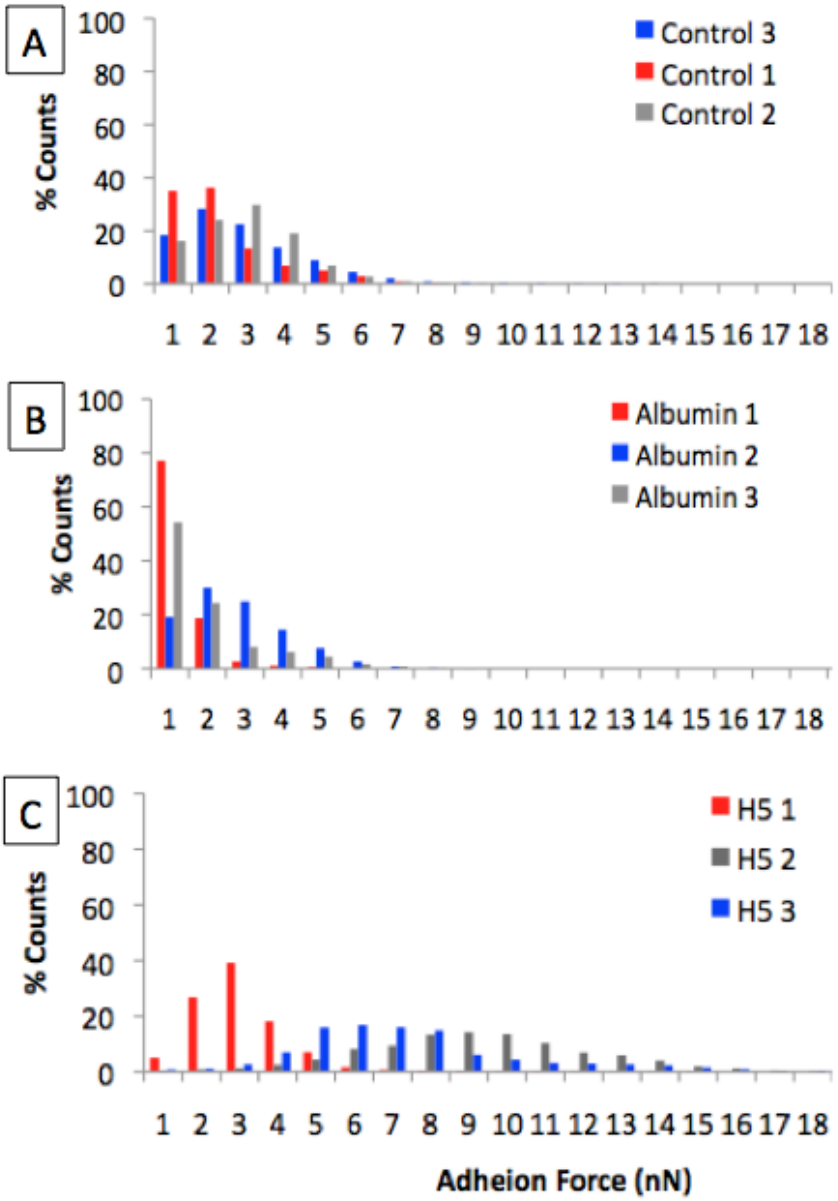

Figure 4.4 Standardized histogram representation of adhesion forces between HA and two treatments albumin, and H5, along with the control (A, B, C, respectively). Each treatment consisted of 3 HA discs, with each disc being sampled in three separate locations and averaged together to ultimately represent one bar (~3,000 force-distance curves). Results show variability between samples, however, adhesion forces between HA and H5 (C) appear to be stronger compared to the control and albumin (A, B).

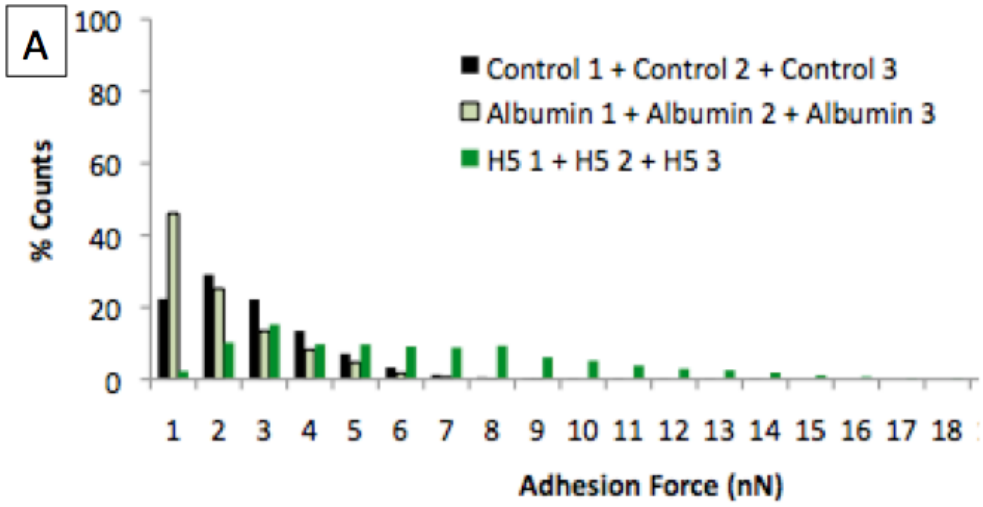

Figure 4.5 Standardized histogram representation of adhesion forces between HA and two treatments albumin, and H5, along with the control. Each bar is a combination of triplicates for each treatment, thus representing an average adhesion force that incorporates ~9,000 force-distance curves. These results indicate H5 demonstrates stronger adhesion forces to HA compared to the albumin treatment, and the control.

It is important to note that due to the high variability between replicates for each treatment (Figure 4.4A, B, C), the experiment should be repeated to obtain more data points in order to see if the results show the same trend.

Gaining an understanding of adhesion forces of salivary proteins to enamel is important to develop a fundamental knowledge of the strength of these forces. The experimental design of this study provided insight as to the adhesion forces of H5 and albumin to HA. Figure 5 indicates that the adhesion force between H5 and HA is stronger compared to the microsphere and albumin controls. These results suggest proteins with high affinity to HA (i.e., H5) have strong molecular adhesive forces to HA. On the other hand, albumin is a salivary protein that does not exhibit high affinity for HA (Carlen et al., 1998), and thus does not demonstrate adhesion forces to HA as large as does H5 (Figure 5).

Proteins with high affinity to HA (i.e, histatins) are able to maintain their intact structure by binding to HA, thus resisting proteolytic degradation within the oral cavity (McDonald et al., 2010). Since we found that a protein's affinity to HA likely coincides with the strength of its molecular-scale adhesion properties, investigating how to modify or enhance the adhesive forces of salivary proteins (natural or synthetic) to inhibit their

degradation in the oral cavity could potentially be used to develop novel therapeutic strategies that focus on the use of salivary proteins.

For instance, several studies indicate that H5 has potent antifungal activity against the pathogenic *C. albicans*, which is the primary pathogen responsible for initiating oral candidiasis (Gyurko et al., 2000; Pusateri et al., 2009). In fact, it has been recently discovered that H5 adhered as a pellicle on a HA surface retains its antifungal effect against *C. albiancs* (Vukosavljevic et al., 2012). Future studies could investigate how to increase the adhesive properties of natural or synthetically formulated proteins that have antimicrobial effects against pathogenic oral microbiota. If a protein that exhibits antimicrobial properties can be developed with stronger adhesive properties, it can potentially adhere to the enamel surface and exhibit a stronger, more potent therapeutic effect.

The overall adsorption behaviour of a protein to a substrate is a complex process modulated by a number of parameters, including structural stability/arrangement of the protein, charge distribution at the interfacial layer, hydrophobicity of the substrate surface, protein-surface polarity, solution pH, ionic strength (Norde and Luklema, 1979; Haynes and Norde, 1994). Therefore, being able to effectively control the process of adsorption of proteins to the enamel surface requires an understanding of the driving adhesion forces for adsorption. Due to these several parameters that control protein adsorption, it becomes a challenge to be able to predict the behaviour of how proteins interact with surfaces within the oral cavity under *in vivo* conditions.

Adhesion forces between the protein of interest and a substrate can be used as an important quantitative measurement to evaluate the adsorption of proteins to the respective surface. It is important to develop an understanding of adhesion forces of physiologically important salivary proteins (i.e., H5, statherin, aPRP) to various oral surfaces. Once this knowledge is obtained, studies should be designed to understand factors that control the strength of these adhesive forces to surfaces within the oral cavity (outlined earlier).

In conclusion, this study provides fundamental knowledge of adhesion forces between H5 and HA, knowledge that can possibly be used in the future to develop stable (proteinase-resistant) synthetic peptides for therapeutic use against various oral diseases

(i.e., dental caries, periodontal disease, candidiasis). In addition, this study demonstrates an experimental design for the measurement of adhesion forces between a protein of interest and a substrate, methodology that can be applied to understand adsorption dynamics of proteins in future studies.

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CHAPTER 5

5.1 General Conclusion

With the increase in prevalence of dental erosion (Truin et al., 2005), and development of resistant strains of *C. albicans* to current treatment methods of oral candidiasis (Tsai et al., 1997), the need for novel therapeutic treatments is becoming increasingly important. Technological advancements of laboratory instruments in recent years now enable scientists to gain micro-scale imaging of samples of interest (e.g., scanning electron microscopy) and allow for measurement of molecular forces between samples (e.g., atomic force microscopy). Researchers can now apply these technological advancements to the field of salivary biology to better understand how to develop effective treatment options for oral diseases.

Within the complex salivary fluids lies more than 2290 salivary proteins, 130 of which are involved in the formation of the acquired enamel pellicle (Siqueira et al. 2007). We now understand that the physical properties of the AEP along with its protein composition can provide protection against enamel demineralization diseases such as dental erosion and/or dental caries. Gaining an in-depth understanding of the details of how the AEP exhibits anti-erosive properties is the first step for researchers to take in order to be able to effectively utilize/manipulate the AEP to modulate dental erosion.

In addition to the therapeutic potential of the AEP against dental erosion, an individual salivary protein that is involved in AEP formation, H5, demonstrates potent antifungal effects on *C. albicans*, which is responsible for development of oral candidiasis (Pusateri et al., 2009; Konopka et al., 2010). We demonstrated for the first time that H5 retains its antifungal effect even when it is adhered to both hydroxyapatite and PMMA as a mono-protein integument. This knowledge can be used in an attempt to modify the AEP in order to increase its therapeutic effect against oral candidiasis.

It is also important to gain a strong understanding of the molecular forces (i.e., adhesion forces) that are involved in the adhesion of salivary proteins to enamel or dental resin. This knowledge would allow researchers to manipulate and alter adhesion forces of proteins with physiologically important characteristics. For instance, if the adhesion forces of H5 to enamel were to be strengthened, more H5 could potentially adhere to the

surface creating a protein layer that would be more effective at preventing the development of oral candidiasis. In addition, the knowledge of how to measure adhesive forces between proteins of interest to clinically important oral surfaces can be used in the development of synthetic proteins with therapeutic potential. For instance, StN21 is synthetic 21-amino acid peptide identical to the N-terminus of statherin, which was shown to be a stable and potent peptide with potential as a therapeutic agent for the treatment of dental caries and dental erosion (Krosic et al., 2007). Once a protein with therapeutic potential is identified or designed, manipulating the protein or external parameters to strengthen adhesion forces between the protein and substrate, could ultimately allow for more physiologically important protein molecules to adhere to the surface and prevent their potential proteolysis within the oral cavity. However, in order to strengthen such forces, they must first be measured effectively. The study in Chapter 4 describes an effective AFM cantilever design to measure adhesion forces between the protein and substrate of interest.

Chapter 5 is concluded with a published commentary article entitle "Salivary proteins as predictors and controls for oral health", addressing how the composition of saliva and the AEP are strong candidates for a future proteomics-based diagnostic tool, along with potential of AEP, in particular as a target-specific therapeutic treatment option.

5.2 Salivary proteins as predictors and controls for oral health: Introduction

Periodontal disease (i.e., gingivitis, chronic periodontitis) and dental caries are the two most globally prevalent chronic oral pathologies that affect children, youth, adults, and elders (Featherstone 2000; Albandar 2002). In the United States, gingivitis affects 50% of adults, while chronic periodontitis affects an estimated 35% of the adult population (Albandar et al. 1999). In addition, each year over 300,000 patients worldwide are diagnosed with oral cancer (Parkin et al. 1988), representing 2–3% of all malignancies (Parkin et al. 2005). More than 90% of these cases are categorized as oral squamous cell carcinoma (SCC), with high metastasis rates, resulting in high patient mortality (Neville

and Day 2002; Parkin et al. 2005).

Recent advances in dental research have enforced the need to gain a more comprehensive understanding of the prevention, treatment, and management of oral diseases. Oral health is an essential component of an individual's well-being because it is very closely related to general health. Throughout the years, oral diseases have been defined as localized oral disturbances, but recent research suggests that they can be considered as general health distal determinants, acting as comorbidities and risk factors for many systemic diseases. For instance, the association between diabetes mellitus and periodontal disease can be considered to be bidirectional: diabetes can be a risk factor for the development of periodontitis (diabetic patients are 2.1–3.0 times more at risk of developing periodontitis; Salvi et al. 1997), while patients with periodontitis are much more likely to develop diabetes (Grossi and Genco 1998; Deshpande et al. 2010). Considering the interconnectedness of oral diseases and general health, we must gain a complete understanding of the pathophysiology of oral diseases within the dynamic, complex oral cavity in order to successfully develop potential treatments and accurate patient risk assessments for the prediction of these diseases (i.e., biomarkers).

Periodontitis and dental caries are multi-factorial diseases primarily dependent on biofilm development. The oral cavity fosters an intricate microbial ecosystem, consisting of more than 700 bacterial species, many of which play an important role in maintaining oral health (Aas et al. 2005). However, when this ecosystem becomes disrupted, an increase in pathogenic microorganisms occurs, resulting in the initiation of disease. To initialize the growth of pathogenic biofilms and therefore the development of oral disease, microbial adhesion to the oral surface, such as dental enamel or denture resin, is the first and essential step to prevent cells from being removed by salivary flow (Whittaker et al. 1996; Jenkinson and Lamont 1997). Human saliva plays a significant role in controlling microbial adhesion since its proteinaceous components, after adsorbed to the oral surface, result in the formation of salivary protein pellicles.

The acquired pellicle (AP) is a protein integument formed on the oral surface immediately after exposure of saliva to the oral environment. This protein film formed on the dental enamel is a result of specific physical bonds (i.e., hydrophobic, hydrogen bonding, ionic, and van der Waals bonds) between the substrata surface and the salivary

molecules (i.e., salivary proteins, peptides, carbohydrates, lipids; Dawes et al. 1963; Rolla et al. 1983; Siqueira et al. 2007a), resulting in the development of a 100–1000 nm protein film on the oral surface for microorganisms to adhere (Kuboki et al. 1987; Skjorland et al. 1995). The AP has important binding sites for oral microbiota; the protein-microbial adhesion process involves stero-specific interaction between receptors on the pellicles and adhesins on the microbial cell surfaces (Scannapieco et al. 1994).

The AP may control the adhesion of pathogenic microbes to oral surfaces because some salivary pellicle proteins found in vivo can inhibit or enhance growth of oral microbiota (Scannapieco et al. 1994). For instance, the carboxylterminus of histatin 5 demonstrates potent fungistatic and fungicidal effects against pathogenic fungi, *C. albicans*, at concentrations found in salivary secretions of healthy individuals (15–30 μM) (Oppenheim et al. 1986; Xu et al. 1991). The antimicrobial affect of histatin 5 is due to its composition of multiple basic amino acid residues (arginine and lysine), allowing this salivary protein to disrupt the cell membrane by forming membrane pores, inducing membrane permeability (increased loss of K+ from cell) and resulting in cell death (Pollock et al. 1984).

In contrast, the carboxyl-terminus of acidic proline-rich proteins (PRPs) promotes the attachment of various oral bacteria (i.e., Streptococcus and Actinomyces spp.) to the AEP, thus enhancing microbial colonization of the tooth surface (Gibbons and Hay 1988). Specifically, the ProGln terminus of acidic PRPs is the preferred protein-binding site for microorganisms including S. gordonii (Gibbons et al. 1991). Similarly to acidic PRPs, the carboxyl-terminus of statherin binds a variety of potentially invasive oral microbiota, including P. gingivalis (Amano et al. 1994) and *C. albicans* (Cannon et al. 1995). In addition, at concentrations of 100 μg/mL (healthy individuals), statherin is capable of inducing the transition from virulent, hyphael *C. albicans* to the cocci form (Leito et al. 2009).

Recent studies have shown that pathogenic microorganisms have increased their resistance to natural host defenses and to antimicrobial treatments, resulting in more persistent and serious infections (Ramage et al. 2006; Tsang et al. 2007). This reinforces the need for the development of novel antimicrobial treatments that would inhibit and/or kill pathogenic microbes, preventing further colonization and development of oral

diseases. Since certain salivary proteins affect the growth of pathogenic oral microbes, their potential role in treatment/prevention of oral diseases must be considered.

5.3 Challenges

In order to evaluate the effectiveness of antimicrobial salivary proteins as a potential novel therapeutical approach for the combat of oral diseases, it is important to gain a comprehensive understanding of the inhibitory effects salivary proteins exhibit on pathogenic oral microbiota.

One approach is to design larger-scale reaction systems that can allow us to control variables of interest (i.e., microbial consortia) and target specific questions about salivary protein-microbial interactions. Throughout the years, many in vitro model systems that model the oral cavity have been designed involving either flow cells (Christersson et al.1987; Larsen and Fiehn 1995; Guggenheim et al. 2001) and even chemostats (Herles et al. 1994; Bradshaw et al. 1996; Kinniment et al. 1996; Bowden 1999). However, some of these models yield contradictory results due to the selection of different parameters. Since the oral cavity is an extremely complex and dynamic system, many different components need to be considered when designing these systems, including multi-species biofilms, flow rate, temperature, pH, nutrient fluxes, and choice of proteins. Considering that human saliva consists of 2290 proteins (Loo et al. 2010) and 130 proteins in the AP (Siqueira et al. 2007b) it becomes incredibly challenging to reproduce the in vivo environment.

Another challenge when investigating the role of salivary proteins on oral biofilms is being able to view the world of a microbe on a small, micro-meter-scale. The advancements in high-resolution microscopy instruments have facilitated the investigation of microbial interactions (i.e., scanning electron microscopy, confocal microscopy, transmission electron microscopy). In addition to these tools, atomic force microscopy (AFM) has revolutionized the field of oral microbiology, enabling us to make a variety of protein/cell surface measurements on the atomic magnitude, directly in aqueous solution. Unlike conventional microscopy, AFM allows us to study adhesive (Lodish et al. 2004), mechanical (Greenleaf et al. 2007), electrostatic (Barkai et al. 2004), and immunochemical (Horber and Miles 2003) nanoscale-level properties. In order to

successfully conduct these measurements, the AFM cantilevertip is typically functionalized with the protein/cell of interest and then used to probe a substrate (Zhang et al. 2009). However, the attachment of a pre-functionalized microsphere to the cantilever provides a much higher surface area when probing the substrate of interest, therefore greatly expanding the spectrum of adhesive interactions that can be obtained by a single cantilever (Ounkomol et al. 2009). For instance, streptavidin-coated microspheres can be attached to the AFM cantilever-tip (Figure. 1), and then reacted with biotinylated protein of interest to obtain an AFM functionalized, high-surface area probe (Zhang et al. 2009). This novel AFM-based force spectroscopy approach allows us to determine biophysical properties of cells and proteins, and also enables us to measure single cell-protein adhesion interactions: the first pathophysiological phenomenon that occurs prior to the development of biofilms on oral surfaces. By manipulating microbial-protein interactions, we could essentially control oral disease development at the pellicle level, to prevent initial microbial adherence that leads to the development of oral diseases.

In addition to the use of these microscopic techniques, the development of revolutionary mass spectrometry has allowed for the investigation of microbial-protein interactions using a proteomic approach, allowing us to understand how salivary proteins affect metabolic pathways inside a cell. Moreover, the analysis of the composition of the AP can lead to the establishment of biomarkers and therefore the prevention of oral diseases that can impact general health. These powerful microscopic and proteomic techniques should be combined when investigating protein-microbial interactions in order to obtain a representative and comprehensive understanding of microbial responses to antimicrobial agents.

5.4 Salivary components as diagnostic tools for oral diseases

The advancement of new technology and instrumentation will enable us to obtain reliable protein fingerprints based on saliva and/or the acquired pellicle. Combining this information with a patient's oral microbiome can provide health care professionals with a comprehensive grasp of each patient's pathophysiological state.

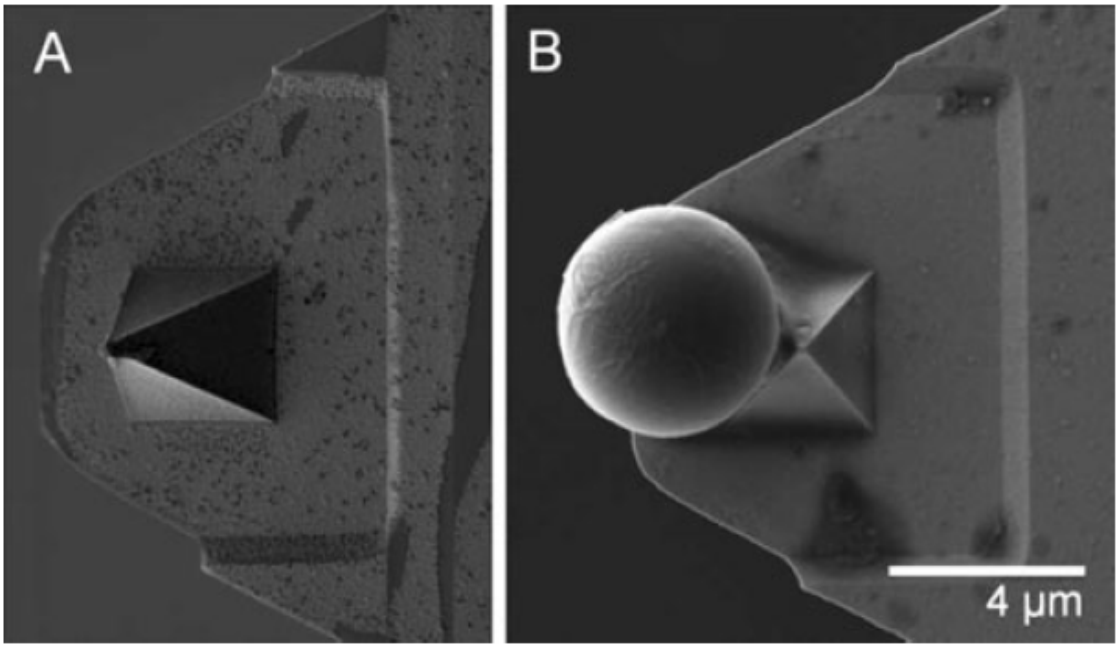

Figure 5.1 Scanning electron micrograph of AFM cantilever (a) and of streptavidin-coated silica microsphere (~5 µm) adhered to AFM cantilever-tip via Araldite® epoxy glue (b). This functionalized microsphere can be coated with biotinylated proteins of interest to create an effective AFM probe for protein-adhesion measurements.

A patient's saliva sample could potentially have individual proteins or groups of proteins that are powerful biomarkers for oral diseases, including oral cancer. This is because saliva contains secretions from gingival crevicular fluid along with major and minor salivary glands, and its harvesting is much less invasive than blood sampling (Spielmann and Wong 2011; Edgar 1992; Siqueira and Dawes 2011). For example, it has been demonstrated that patients diagnosed with head and neck squamous cell carcinoma exhibit elevated levels of soluble CD44 (solCD44), compared to cancer-free patients (Franzmann et al. 2007). In addition, patients with early childhood caries exhibit higher levels of glycoprotein, while caries-free patients demonstrate elevated amounts of proline-rich protein in saliva (Bhalla et al. 2010). Therefore, analyzing the protein composition of a patient's saliva could provide a protein profile for that patient, which could contain information as to which salivary proteins are upregulated/downregulated. This information could ultimately be compared to protein profiles of healthy patients, and those with different stages of various oral diseases, in order to accurately diagnose a patient (Blicharz et al. 2009).

When considering oral diseases that initiate on hard surfaces (i.e., dental caries), sampling the acquired pellicle and obtaining a corresponding protein profile can likely be

much more important compared to saliva (Siqueira et al. 2007a; Siqueira and Oppenheim 2009). Pellicle formation is a highly selective process since only a fraction of proteins found in human saliva (130/2290 proteins) are present in the in vivo AP on the dental enamel surface (Siqueira et al. 2007b). Since more than 51% of the recently identified pellicle proteins have unknown biological functions (Siqueira et al. 2007b), future research should focus on identifying the biological function of the remainder of the pellicle proteins. There may in fact be additional proteins, or protein complexes, that could be even stronger biomarkers/predictors for various oral diseases.

In addition to obtaining saliva and AP protein patient profiles, sampling the oral microbial community (microbes adhered to oral surfaces) could produce a microbial patient profile, which could also be used as a biomarker for oral disease. Certain oral microbes adhered to the pellicle become more prevalent in patients suffering from certain oral diseases. For instance, patients exhibiting dental caries have an oral microbiota dominated by acidogenic and acid-tolerant gram-positive bacteria (i.e., Streptococcus and Lactobacilli spp.) (Marsh 2003). Meanwhile, patients with periodontal disease have an increased proportion of obligately anaerobic bacteria (i.e., gram-negative species) (Socransky et al. 1998). The presence/absence or quantity of certain individual microbes, or even microbial community composition, adhered to the pellicle can correspond to various stages of oral disease development. Health care professionals could employ techniques such as the Human Microbial Identification Microarray to determine the microbial community of the oral cavity, and determine 'predictor' microbes of oral diseases.

5.5 Future direction

Gaining a comprehensive understanding of interactions between oral microbiota and salivary antimicrobial proteins could potentially result in the development of novel treatments for a variety of oral pathologies. It would be very interesting if a potential treatments for oral diseases could be currently in the oral cavity in the form of salivary proteins. Therefore, biofilm-dependent oral diseases can be controlled at the pellicle level - the interface between pathogenic microbes and the solid oral surfaces. By controlling, or perhaps altering, the composition of the pellicle, we could potentially interfere the

adhesion process of the oral microbiota to oral surfaces. Therefore, the future of oral therapeutics should focus on the interaction between salivary proteins and microorganisms.

In addition, the oral cavity contains biomarkers for oral diseases hidden within its complex oral fluids, AP integuments, and microbial consortia. The use of these proposed salivary biomarkers would promote health professionals to change their focus from disease diagnosis to monitoring and detecting oral disease at onset. Longitudinal studies should be conducted to better understand if such biomarkers could be used to identify progression of oral diseases. Ultimately, multiple biomarkers should be combined to achieve optimum specificity and sensitivity for detection of oral diseases.

5.6 Conclusion

Saliva is a complex fluid that possesses many important functions that relate directly to oral health. Accurate analysis of salivary components is a relatively new tool for assessing biological markers (hormones, immunoglobulins and antimicrobial proteins) for oral diseases as dental caries, periodontitis and oral candidiasis. The fingerprint profile of immunological compounds, such as immunoglobulin and other antimicrobial proteins, in saliva samples can be an indicator of the host immune system's stress response to acute systemic disturbances, whereas assessment of the pellicle salivary constituents can identify susceptibility to local infections. Assessing proteins' physical properties (i.e., adhesion forces) on different surfaces, cells or even to other proteins (protein-protein complexes) would assist us in understanding the role of salivary proteins and the pathophysiology of oral diseases. Subsequently, this new knowledge would help in developing innovative and effective therapeutic approaches to maximize the prevention of pathologic biofilm development.

In conclusion, assessing and understanding salivary composition can be applied as a feasible and reliable tool for predicting and treating several oral infections, diagnosing systemic diseases and determining the state of patients' immune systems. Therefore, collecting and analyzing saliva would not only help to better monitor and maintain the oral health of patients, but it could also significantly improve the health care system.

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