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## Evaluation of novel salivary peptides and a new application method for prevention and treatment of oral disease

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science  
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EVALUATION OF NOVEL SALIVARY PEPTIDES AND A NEW APPLICATION METHOD FOR  
PREVENTION AND TREATMENT OF ORAL DISEASE

(Thesis format: Integrated Article)

by

Tayebeh Basiri

Graduate Program in Medical Biophysics

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

The School of Graduate and Postdoctoral Studies  
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London, Ontario, Canada

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## Abstract

Some salivary proteins degrade quickly after secretion into the oral cavity, due to proteases present in the oral environment. DR9, the N-terminal domain of statherin is an example of these peptides, which sustains the activity of the original protein. Inspired by naturally occurred DR9 and the evolutionary pathway taken by proteins; we constructed novel peptides by combining the functional domains of different salivary proteins and investigated the functionality of these peptides. Our results revealed that DR9 duplication could increase its functionality and DR9 combination with RR14, the functional domain of histatin, could combine the functions of both peptides. Furthermore, we explored the possibility of using chitosan, an amino-polysaccharide, as a carrier for salivary peptides. Chitosan nanoparticles showed a significant higher killing effect compared to non-particle chitosan against *Candida albicans*. Moreover, chitosan nanoparticles showed a favorable killing effect in specific pHs, similar to the critical pHs in the oral cavity.

## Keywords

Salivary proteins, chitosan nanoparticles, *Candida albicans*, Acquired enamel pellicle, Statherin, Histatin, Oral candidiasis, Oral homeostasis, Salivary peptides

## Co-Authorship

All chapters were written by Tayebeh Basiri and edited by Walter L. Siqueira, DDS, PhD.

Walter L. Siqueira provided guidance throughout the whole study, including experimental design, data interpretation, statistical analyses and technical advice for all parts of the study.

Yizhi Xiao, MSc, PhD, provided technical support for all the experimental parts and offered generous knowledge and consult throughout the study.

All experiments were performed by Tayebeh Basiri except the followings:

Rajesh Kumar Gupta, MSc, constructed the chitosan nanoparticles for this study and provided substantial guidance during performing chitosan related parts of the study.

Nicole Johnson, DDS, performed the enamel demineralization assay and calcium and phosphate analyses in chapter 3.

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## List of Abbreviations

AEP	Acquired enamel pellicle
PRPS	Proline-rich proteins
GlcN	D-glucosamine
GlcNAc	N-Acetyl glucosamine
MWs	Molecular weights
bPRP	Basic or glycosylated PRP
aPRP	Acidic PRP
HA	Hydroxyapatite
SVs	Statherin variants
MG1, 2	Mucin glycoprotein 1, 2
TEM	Transmission electron microscopy
IgA	Immunoglobulin A
SDA	Sabouraud dextrose agar
TPP	Tripolyphosphate
MRSA	Multidrug-resistant <i>S. aureus</i>
ROS	Reactive oxygen species
HPLC	High performance liquid chromatography
PI	Isoelectric point
OD	Optical density
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
MALDI-TOF-MS	Matrix assisted laser desorption/ionization-time of flight-mass spectrometry

## Chapter 1

### 1.1 Introduction (Problem statement in saliva biology)

Saliva is composed of hundreds of salivary proteins secreted from a few major salivary glands and many minor salivary glands, including submandibular, sublingual and parotid glands and several non-salivary components (Dawes, C et al. 2004). Some salivary proteins are secreted commonly from all major glands, such as the secretory IgA (the main antibody in saliva), but some proteins are exclusive to specific gland such as proline-rich proteins (PRPs), which appear to be secreted only from parotid glands and minor salivary glands (Dawes, C et al. 1963 and Siqueira, W et al. 2008). Saliva is one of the most important fluids in the body that performs critical roles in the oral environment, such as facilitating the taste and detection of foods nutritious to the body and acting as a lubricant and antimicrobial agent. Saliva also prevents the dissolution of teeth and aids digestion. In addition, saliva is responsible for forming the acquired enamel pellicle (AEP), a thin organic layer formed on the enamel surface of the teeth (Dawes, C et al. 1963).

Extensive studies have been done to elucidate the process by which the AEP is formed on the enamel surface of the tooth. Briefly, one minute after exposure to the oral environment, a thin pellicle layer is detected on the enamel (Hannig, M et al. 1999). This pellicle formation is completed after 120 minutes and no further increase in protein/peptide is observed. However, a significant number of bacteria start adhering and forming the so-called oral biofilm or dental plaque (Siqueira W et al. 2012). Extensive electron microscopy experiments have been employed to determine the structure of the

acquired enamel pellicle and the majority of these experiments have shown that the thickness of pellicle is within the range of 30 and 100 nm (Hannig, M et al. 1989; Tinanoff, N et al. 1976).

Over the years, several groups have utilized different experimental models to investigate the composition of AEP. For example, *in vitro* models such as hydroxyapatite (HA) discs incubated with whole saliva and HA powder were used to study the AEP. The use of these models resulted in the identification of some major AEP proteins such as amylase, histatins, albumin, carbonic anhydrase VI, cystatins, statherin, lysozyme and proline-rich proteins (PRPs) (Jensen, JL et al. 1992; Li, J et al. 2004; Hannig, M et al. 1999). More recently, application of sensitive techniques such as LC-ESI-MS/MS and MALDI-TOF-MS has led to the identification of 130 proteins in the AEP composition (Siqueira, W et al. 2007). Of these 130 identified proteins, 113 were novel proteins. Statherin, PRPs, cystatins and histatins are the major components of the AEP and make a substantial portion of proteins present in saliva (Hay, D et al. 1975; Oppenheim, FG et al. 1986; Hay, D et al. 1973; Oppenheim, FG et al. 1971). Additionally, these techniques identified 78 naturally occurring AEP peptide derived from 29 different proteins within the *in vivo* pellicle (Siqueira, W and Oppenheim, FG et al. 2009). Multiple peptides identified in AEP peptidome originate from histatins and statherin.

The AEP plays an important role as a lubricant in the oral environment, which in turn improves the efficiency of speech and chewing (Tabak, LA et al. 1982). Mucins, aPRP1 and statherin are AEP constituents that are involved in this process (Tabak, LA et al. 1995; Vukosavljevic, D et al. 2014; Hahn Berg, IC et al. 2004). Due to its lubrication properties, AEP also plays a protective role against abrasive damage (Joiner, A et al.

2008). Furthermore, several studies have shown that multiple AEP components provide protection against acid-induced enamel demineralization (Hannig, M and Balz, M et al. 2001; Cheaib, Z and Lussi, A et al. 2011; Siqueira, W et al. 2010).

Statherin is a salivary protein that is phosphorylated at serine residues at position 2 and 3. Statherin has highly important physical properties crucial for saliva. This protein is the most surface-active saliva component (Proctor, GB et al. 2005) and functions as an important lubricant in the saliva (Douglas, WH et al. 1991, Harvey, NM et al. 2011). Statherin and aPRPs play a role in inhibiting crystal deposition on enamel surfaces (Hay, DI and Moreno, EC et al. 1979). However, statherin is the most crucial inhibitor of calcium phosphate precipitation, which can serve its inhibitory effect at physiological concentrations (Tamaki, N et al. 2002). Furthermore, recent findings from our laboratory revealed that phosphate groups in the N-terminal domain of statherin fragments are responsible for inhibiting the growth of hydroxyapatite crystal. Moreover, statherin quickly disappear from whole saliva due to proteolytic activity and its affinity for tooth surfaces.

Histatins are a protein family present in the AEP, which are secreted by the major and minor salivary glands. Histatins contain high level of histidine (Oppenheim, FG et al. 1986) and show antifungal activity against *Candida albicans*, the major cause of oral candidiasis. Of the 12 members of histatin family, Histatin 1, 3 and 5 are the major members of the histatin family, forming 80% of this family (Oppenheim, FG et al. 1988). Like statherin, histatin 1 is phosphorylated at serine 2. Histatins show lower concentrations in whole saliva compared to pure glandular secretions likely due to proteolytic activity of saliva resulting the rapid degradation of these proteins after their

release into the oral cavity. Furthermore, histatins play a role in multiple processes, including buffering, modulation of mineral formation and antibacterial activities (Castagnola, M et al. 2004; Groot, F et al. 2006).

Chitosan, an amino-polysaccharide, is a derivative of chitin, which is mainly present in the exoskeletons of arthropods such as insects, in algae and in some fungal cell walls. Chitosan is made from chitin by alkaline deacetylation, achieved by boiling chitin in concentrated alkali such as NaOH for 7-10 hours (Rabea, EI et al. 2003; Kumar, MNV et al. 2000). In terms of chemistry, chitosan is a polycationic heteropolysaccharide, which consists of two monosaccharides, D-glucosamine (GlcN) and N-Acetyl glucosamine (GlcNAc), linked by  $\beta$  (1 $\rightarrow$ 4) glycosidic bonds (Pochanavanich, P and Suntornsuk, W et al. 2002; Singla, AK and Chawla, M et al. 2001). Several features of chitosan such as molecular weights (MWs) viscosities, pKa values may be affected by the relative amount of these two monosaccharides in the composition of chitosan (Singla, AK and Chawla, M et al. 2001; Tharanathan, RN and Kittur, FS et al. 2003).

Chitosan has a higher killing rate and lower toxicity rate compared to other types of disinfectants that make chitosan a better antimicrobial agent (Franklin, TJ et al. 1981; Takemono, K et al. 1989). Multiple mechanisms have been proposed to explain how chitosan serves its antimicrobial activity; however, the exact mechanism remains to be discovered. Chitosan can change cell permeability by interacting with the membrane of the bacterial cell, which leads to the leakage of cellular compartments and the death of bacteria (Seo, HJ et al. 1992; Chen, CS et al. 1998). Chitosan also can inhibit microbial growth and the production of toxins by acting as a chelator that selectively binds to trace metals (Cuero, RG et al. 1991). Other proposed mechanisms for antimicrobial activity of

chitosan include the inhibition of mRNA synthesis and various enzyme activities essential for bacterial survival (Sudarshan, NR et al 1992). It is interesting to mention that chitosan demonstrates its strong antibacterial activity only in an acidic condition because its solubility drastically decreases in pHs above 6.5. Furthermore, different molecular weights of chitosan can have the opposite effect on antimicrobial activity. For instance, chitosan with a molecular weight ranging from 10,000 Da to 100,000 Da inhibited the growth of bacteria, whereas, chitosan with an average molecular weight of 2,200 Da enhanced the growth of bacteria (Tokura, S et al. 1994). Nano and microparticles have recently gained substantial attention as carriers for drugs and proteins. These particles can control the release of drugs and proteins to a specific target site, which minimize nonspecific effects. Chitosan displays some biological features such as biodegradability, biocompatibility and pH sensitivity that make chitosan a suitable carrier for the delivery of drugs and proteins in the biomedical field. Encapsulation of salivary proteins/peptides in chitosan particles provides an opportunity for these proteins/peptides to have a longer lifespan in the oral environments.

The aim of this study was to investigate the novel constructed histatin and statherin peptides' activity, compared to the original proteins and also, to explore the possibility of using chitosan nanoparticles as a delivery system for salivary peptides/proteins. We hypothesized that only the functional domain of selected salivary proteins could have the same function as the original proteins, also, the duplication/combination of protein functional domains could increase/combine their activity. This novel approach of salivary peptides' construction could help us to benefit from the result of evolution millions of years ahead of the time that it would naturally



occur, since these could be the evolutionary pathways that salivary proteins will eventually take. Moreover, the idea of chitosan nanoparticles application for peptides/proteins delivery could be highly beneficial since, not only it will increase the peptides' life span in the oral cavity but it can also provide bonus antimicrobial and antifungal properties.

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## Chapter 2

### 2 Protein adsorption on the enamel and their biological function

#### 2.1 Introduction

The AEP is an organic layer, free of bacteria, that coats the tooth surfaces. AEP consists of proteins, glycoproteins and enzymes (Lendenmann, K et al. 2000), and plays important roles in several processes in the oral cavity. For instance, it is responsible for lubricating tooth surfaces to prevent wear. It also acts as a buffer and anti-erosive barrier. Furthermore, it displays multiple antimicrobial properties (Hannig, M et al. 2001; Al-Hashimi, I et al. 1989; Bradway, SD et al. 1992; Hannig, M et al. 2002; Hannig, M and Balz, B et al. 1999; Amaechi, BT et al. 1999; Hannig, M et al. 1999 and Hahn Berg, IC et al. 2004).

AEP postpones enamel demineralization (Darling, AI et al. 1956; Meckel, AH et al. 1968; Zahradnik, RT et al. 1976; Propas, D et al. 1977; Juriaanse, AC et al. 1979) and impedes the supersaturated saliva to form calcium and phosphate crystal on the tooth surface (Hay, DI et al. 1979; Moreno, EC et al. 1979). It is a key player in the colonization of bacteria on the tooth surface by mediating the selective binding of bacteria (Hillman, JD et al. 1970; McGaughey, C et al. 1971; Gibbons, RJ et al. 1976).

It has been shown that there is no increase in the amount of amino acid formed on the tooth surface after 90 minutes (Sonju, T et al. 1973). X-ray studies have revealed that there is equilibrium between adsorption and re-sorption of proteins on the tooth surface after 90 min (Kuboki, Y et al. 1987).

The majority of research in the salivary protein field has focused on the chemical composition of the AEP with little focus on its ultrastructure. The initial transmission electron microscopic (TEM) studies characterized the AEP as a bacteria-free, homogeneous and amorphous layer (Armstrong, WG et al. 1968; Leach, SA et al. 1966; Meckel, AH et al. 1965 and Tinanoff, N et al. 1976). Further investigations of the AEP at different time points revealed a more complicated structure as the 2-hour AEP demonstrated a fine, uneven organic layer and after the maturation of the AEP during a period of several hours, it forms a compact layer with the granular structure (Berthold, P et al. 1979; Lie, T 1977; Nyvad, B et al. 1987 and Tinanoff, N et al. 1976).

Although these investigations provided helpful insights into the AEP structure, there have been some limitations. For example, they were performed on enamel slabs carried in the buccal sulcus. Since the oral cavity is exposed to different salivary gland secretions with different protein composition, a systematic ultrastructural study investigating site-dependent differences in the structure of the AEP was needed (Dawes, C et al. 1993; Dawes, C et al. 1989; Sas, R et al. 1997; Brookes, SJ et al. 1995 and Veerman, ECI et al. 1996). In 1999, Hannig performed an experiment using TEM to investigate the structure of the AEP at two different oral sites for a period of 24 hours. This experiment demonstrated that a thin layer with thickness of 10–20 nm is formed on the enamel surface after one minute. Further adsorption of salivary proteins was controlled by locally effective shearing forces of the oral cavity. The lingual site of the oral cavity was covered by homogeneous, granular layer with a thickness of 20 to 80 nm in 2 hours, which increased to the thickness of 100–200 nm after 24 hours. In contrast, on the buccal surface, a granular and globular layer with the thickness of 200 and 700 nm

was formed after 2 hours, which increases to a globular layer with the thickness of 1000-1300 nm. Other researchers also confirmed the globular and granular structures of the AEP (Schupbach, P et al. 1996).

In summary, these findings revealed that the AEP formation occurs in two steps: First, salivary proteins are adsorbed on the enamel surface as an electron dense basal layer, subsequently; salivary proteins are adsorbed on the enamel surface forming a globular layer. These investigations also showed that the structural appearance and formation of pellicle changes by site dependent salivary composition, different salivary flow rate and effective shearing forces.

The identification of the AEP protein composition has been the focus of several studies over the years. Three experimental models including *in vitro*, *in situ* and *in vivo* models have been employed to investigate the AEP components. Although the *in vitro* model has some limitation, in terms of mimicking the dynamic environment of the mouth, but it provided some valuable information about the composition of the AEP (Jensen, JL et al. 1992). This model revealed the identity of some of the major components of the AEP such as amylase, carbonic anhydrase VI, histatins, cystatins, lysozyme, albumin, statherin and PRPs. (Hannig, M et al. 1999; Li, J et al. 2004).

## 2.2 AEP Composition

The *in situ* model, which, uses bovine or human enamel slabs mounded in intra-oral appliances provided valuable information about the activity and structure of the enzymes within the AEP such as amylase, peroxidase, transaminases and lysozyme (Hannig, M et al. 2004 and Hara, AT et al. 2006). With the emergence of sensitive proteomics



technology, the *in vivo* AEP composition could be analyzed in a small amount of sample (Siqueira, WL et al. 2007a). These delicate techniques have provided the opportunity to expand our information about the AEP composition. LC-ESI-MS/MS and MALDI-TOF-MS are the examples of these techniques, which have made the possibility to identify 130 proteins in the *in vivo* AEP (Siqueira, WL et al. 2007b). Furthermore, LC-ESI-MS/MS techniques have led to the discovery of 78 peptides that are the result of the cleavage of 29 different proteins present in the AEP (Siqueira, WL et al. 2009). These proteomics techniques have allowed us to design complex experiments, which will lead to a greater understanding of the AEP composition. The better understanding of the AEP components and their activity will provide the opportunity for the development of better therapies for oral diseases.

### 2.2.1 Amylase

The parotid salivary gland is the main source of salivary alpha-amylase, which is the prevailing protein in human saliva. Amylase is produced by both salivary glands and pancreas, providing different levels of starch digestion (Merritt, AD et al. 1973 and Tomita, N et al. 1989). Amylase is encoded by two genes named AMY1 and AMY2. Salivary amylase contains 496 amino acids and shows a highly similar sequence compared to the pancreatic amylase with only 3% variation in their sequence.

Salivary amylases are divided into two families: family A and family B (Keller, PJ et al. 1971). Family A enzymes are glycosylated at Asn 412 residue and consists of three isomers: isoenzyme 1, 3 and 5 which are slightly different in their structure. Family B enzymes, are non-glycosylated, and include isoenzymes 2, 4 and 6 (Takeuchi, T et al. 1979). In terms of function, alpha-amylase is involved in hydrolyzing alpha-1, 4

glycosidic bonds, leading to the first level of starch and glycogen digestion, in the oral cavity. Furthermore, salivary amylases are able to attach to several oral streptococci species, indicating their role in the clearance of bacteria (Scannapieco, FA et al. 1989; Scannapieco, FA et al. 1990; Douglas, CW et al. 1990; Bergmann, JE et al. 1995 and Scannapieco, FA et al. 1993). Amylase is detectable in the AEP, but the exact role of this protein on the tooth surface remains to be investigated (Al-Hashimi, I et al. 1989; Yao, Y et al. 2003; Orstavik, D et al. 1973; Orstavik, D et al. 1974 and Dipaola, C et al. 1984).

### 2.2.2 Histatins

Histatins are a family of salivary proteins that consists of 12 highly similar members. Two distinct genes mapped to chromosome 4q13, HIS1 and HIS2, encode histatin 1 and histatin 3 respectively (Azen, EA et al. 1973 and Sabatini, LM et al. 1989). Other members of histatin family are derived from histatin 1 and 3 by proteolytic cleavage of salivary proteases during secretion. For instance, histatin 3 undergoes a proteolytic cleavage at Tyr-24 within salivary gland cells and creates histatin 5 (Sabatini, LM et al. 1989). In addition, histatin 1 undergoes other post-translational modifications. One such example is the phosphorylation of histatin 1 at serine 2 and sulfation of tyrosine residues at the C-terminal of histatin 1 (Oppenheim, FG et al. 1988 and Cabras, T et al. 2007). These salivary cationic peptides defend the oral environment against oral candidiasis caused by *Candida albicans*. Among all histatins, histatin 5 and 3 have the highest antifungal activity (Xu, T et al. 1991).

Earlier studies revealed that these histidine rich proteins are secreted from parotid and submandibular/sublingual salivary glands. However, recent immunochemistry and mass spectrometry techniques have shown that minor salivary glands also secrete

histatins. Of the 12 members of this family, histatin 1, 3 and 5 constitute about 80% of glandular secretions and contain 38, 32 and 24 amino acid residues, respectively. The concentration of histatins in the saliva ranges from 50  $\mu\text{M}$  to 425  $\mu\text{M}$  (Helmerhorst, EJ et al. 1997).

Previous studies have revealed that the N-terminal domain of these histatins is responsible for antimicrobial activity and the C-terminal domain possesses wound-healing properties (Melino, S et al. 1999; Gusman, H et al. 2001 and Grogan, J et al. 2001).

The oral cavity takes advantage of several defense mechanisms to prevent bacterial and fungal infections. Histatins are the key players in protecting the oral cavity against oral pathogens. Several studies have investigated the antibacterial activity of histatins against different pathogens. Some studies have shown that histatins have low or no effect on *Streptococcus mutans* and *S. milleri* (Helmerhorst, EJ et al. 1997 and Bartie, KL et al. 2008). However, it has been demonstrated that both *S. aureus* and multidrug-resistant *S. aureus* (MRSA) are sensitive to the antimicrobial activity of histatin 5 and its homolog peptides such as Dh5, P-113, Dhvar5 and in particular Dhvar4 which shows an  $\text{IC}_{50}$  of 3.1–4.2  $\mu\text{M}$  in vitro and 1–1.5  $\mu\text{M}$  in vivo, respectively (Welling, MM et al. 2007). In addition, histatin 1 inhibits the adsorption of glycoprotein to hydroxyapatite surfaces, which results in reduction of *S. mutans* adhesion to hydroxyapatite surfaces (Shimotoyodome, A et al. 2006). Histatin 5 is responsible for regulating the periodontopathic bacterium *Porphyromonas gingivalis*, and hindering the induction of inflammatory cytokines in human gingivalis fibroblasts by *P. gingivalis* (Imatani, T et al. 2000; Imatani, T et al. 2004 and Yoshinari, M et al. 2010). Furthermore, histatins show a

strong antifungal effect against *C. albicans*, *C. krusei*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae* and *Neurospora crassa* in vitro (Oppenheim, FG et al. 1988; Rayhan, R et al. 1992; Driscoll, J et al. 1996; Tsai, H et al. 1997 and Vukosavljevic, D et al. 2012). Histatin 5 serves its candidacidal activity through a multistep molecular mechanism. First, histatin 5 binds to the yeast protein receptor Ssa1/2p that activates the internalization of the peptide. Finally, histatin 5 interacts with intracellular targets such as mitochondrial membrane leading to the formation of reactive oxygen species (ROS) and oxidative damage (Edgerton, M et al. 1998; Sun, JN et al. 2008; Baev, D et al. 2001; Xu, Y et al. 1999; Helmerhorst, EJ et al. 1999; Koshlukova, SE et al. 2000; Ruissen, AL et al. 2001 and Isola, R et al. 2007). In addition, it has been demonstrated that histatin 5 induces ATP efflux from *C. albicans* cells by interacting with TRK1p, a potassium transporter, and inducing potassium ion release (Baev, D et al. 2004). The exact and detailed mechanism for antifungal activity of histatins remains to be elucidated. A better understating of these mechanisms might provide better opportunities for the development of new generation antifungal drugs. Histatins are one of the earliest precursors of the AEP due to their high affinity to the hydroxyapatite and they aid the AEP protection of teeth from demineralization (Jensen, JL et al. 1992; Al-Hashimi, I et al. 1989; Siqueira, W et al. 2010). Histatins also contribute to the antimicrobial property of AEP by inhibiting bacteria adhesion to the tooth's surface and providing antimicrobial and antifungal properties in the AEP composition (Xie, H et al. 1991 and Siqueira, W et al. 2007b).

### 2.2.3 Mucin

Mucins are other members of saliva, which are divided into two types of

genetically different salivary mucins: mucin glycoprotein 1 (MG1), also called high molecular weight mucin, encoded by the MUC5B gene (Thornton, DJ et al. 1999), and the MG2 (Mucin glycoprotein 2), or low molecular weight mucin, encoded by the MUC7 gene (Bobek, LA et al. 1993). 11 distinct Mucin genes have been mapped in human so far; each of these different mucins shares several sequences and features.

MG1 is secreted from mucous cells, while MG2 is secreted from serous cells. Mucins contain a high level of carbohydrate chains attached covalently to their polypeptide backbones and are rich in proline and serine/threonine. These carbohydrate chains make up to 60% to 80% of the molecule (Van Klinken, BJ et al. 1995). MG1 is a component of the AEP that is responsible for lubricating the dental surfaces and protecting it against mechanical wear (Nieuw Amerongen, AV et al. 1987). Mucins also participate in the acid resistance of the AEP (Cheaib, Z and Lussi, A et al. 2011). In addition, mucins function as a barrier against penetration of irritants and toxins into mucous cells. They regulate permeability of mucosal surfaces, and play a protective role against proteases generated by bacteria and modulate colonization of the oral cavity by bacteria and viruses (Mandel, ID et al. 1987). 30% of the salivary mucins are secreted from the submandibular glands, while 70% of them are secreted from sublingual, labial and palatal glands. Interestingly, parotid glands do not secrete any mucins (Wu, AM et al. 1994; Thornton, DJ et al. 1999; Nielsen, PA et al. 1996; Troxler, RF et al. 1997; Milne, RW et al. 1973 and Edgerton, M et al. 1992). Furthermore, mucins facilitate speech, chewing and swallowing by the viscoelastic properties. In addition, MG1 has the ability to bind to a variety of microorganisms such as *Porphyromonas gingivalis* and *Candida*

*albicans* and contribute in their clearance from the oral cavity (Amano, A et al. 1994; Edgerton, M et al. 1993).

#### 2.2.4 Proline-rich proteins (PRPs)

PRPs are divided into two subcategories: Basic or glycosylated PRPs (bPRPs) and acidic PRPs (aPRPs). bPRPs are encoded by four loci (PRB1-PRB4), mapped to chromosome 12p13 and only secreted by parotid glands. aPRPs are the product of two loci (PRH1 and PRH2), mapped to chromosome 12p13 close to the bPRPs loci, and are detected in both parotid and submandibular/sublingual secretions (Kauffman, DL et al. 1991 and Inzitari, R. et al. 2005). All PRPs have a repeat region, a signal peptide, a carboxyl terminal region and a transition region in common (Ann, DK et al. 1985)

All aPRPs undergo post-translational modification such as phosphorylation of residues 8 and 22. Acidic PRPs effectively inhibit the calcium phosphate crystal growth, but they do not play a role in crystal nucleation, which occurs by calcium phosphate precipitation at physiological concentrations. The N-terminal domain of aPRPs, which contains 30 amino acids, including two phosphoserine and several negatively charged residues, is responsible for inhibiting the calcium phosphate crystal growth. Studies have shown that the presence of both phosphoserines at the N-terminal domain of aPRPs plays a crucial role in mineral homeostasis. This domain also has a high affinity for binding to the hydroxyapatite (HA). The intact PRPs are absent in saliva indicating their complete cleavage in the oral cavity. More than 20 PRPs have been identified in human saliva (Bennick, A 2002). Several studies have shown that different peptides are derived from the cleavage of basic PRPs including Pe (DEAEII-2), PmS, Con1, Ps, Pc, Con2, PmF, and Po (Kauffman, DL et al. 1979; Azen, EA et al., 1979 and 1980; Anderson, LC et al.

1982; Azen, EA et al. 1984a; Karn, RC et al. 1985). In addition, basic PRPs are cleaved to multiple fragments which include P-D, P-F, P-E, P-I and P-H, II-1, II-2, IB-7, IB-8a and IB-1 fragments (Isemura, S et al. 1982; Saitoh, E et al. 1983a, b, c; Kauffman, D et al. 1982, 1986, 1991). The structure of basic PRPs is well known and characterized but their exact function in the oral cavity is yet to be discovered.

PRPs were identified in human saliva for the first time (Mandel, ID et al. 1965). Further studies showed that these proteins are present in the saliva of several animals as well, such as rat, goat, sheep and mouse. In terms of molecular weight, PRPs range from 5000 to over 25000 Daltons (McArthur, C et al. 1995 and Bennick, A et al. 2002). PRPs produced in some species such as human contained about 40% Proline indicating their unique structure considering the fact that most proteins only contain about 5% proline in their structure (Mole, S et al. 1990; Mehansho, H et al. 1983 and 1985; Schulz, GE and Schirmer, RH et al. 1979).

Parotid saliva possesses the highest concentration of PRPs among all salivary glands (Veerman, EC et al. 1996). Acidic PRPs contain a longer N-terminal region, and their repeated sequence is different compared to basic PRPs (Bennick, A et al. 2002). Acidic PRPs are able to bind to calcium hydroxide and therefore are involved in the formation of AEP on tooth surfaces. PRPs contribute to the lubricating property of AEP and inhibit the calcium and phosphate precipitation on the enamel surface along with some other proteins (Vitorino, R et al. 2008; Tabak, LA et al. 1995; Hahn Berg, IC et al. 2004; Hay, DI and Moreno, EC et al. 1979). Basic PRPs have been also detected in the AEP (Vitorino, R et al. 2008). Acidic PRPs are able to bind to bacteria, whereas basic and glycosylated PRPs have the ability to bind to fungi, viruses and bacteria, which is an

indicative of their role in the exclusion of these microorganisms (Fábián, TK et al. 2008; Fábián, TK et al. 2012; Shugars, DC et al. 1998 and Tenovuo, J et al. 2002).

### 2.2.5 Statherin

Statherin inhibits the nucleation as well as the growth of hydroxyapatite in the supersaturated environment of saliva; therefore, as an AEP component, it avoids the crystal formation on the tooth surface (Schlesinger, DH et al. 1977; Hay, DI Moreno, EC et al. 1979). Statherin also functions as a boundary lubricant and a mediator of bacterial adhesion in periodonto-pathology (Douglas, WH et al. 1991).

The major statherin contains 43 residues and is detectable in human parotid and submandibular salivary glands (Hay, DI et al. 1977). Statherin inhibits the spontaneous precipitation of calcium phosphate salts from their supersaturated solutions (Hay, DI and Moreno, EC et al. 1979). Therefore, statherin, in conjunction with acidic PRPs, is able to inhibit precipitation of calcium phosphate salts in the salivary glands, the mouth and onto the tooth surface ((Hay, DI et al. 1979).

Like other calcium-binding proteins such as Osteopontin and biomineralization proteins regulating calcium carbonate crystallization, statherin contains a pSpSEE (where pS is phosphorylated serine) acidic motif (Waite, JH et al. 2001). Statherin is phosphorylated at two serine residues in position 2 and 3 and has a high level of tyrosine and proline (Schlesinger, DH et al. 1977). Initially, it was believed that statherin exists as a single protein in human saliva until smaller statherin variants (SVs) were identified (Jensen, JL et al. 1991). There are three SVs, including SV1, 2 and 3. SV2 lacks an internal 10-residue segment, which is the result of eliminating exon 4 of statherin gene by



an alternate mRNA splicing event. SV1 and SV3 are similar to statherin except that they are missing a phenylalanine residue at the C terminal domain. The removal of this residue is thought to be a post-translational modification (Jensen JL et al. 1991 and Castagnola, M et al. 2003). Interestingly, similar to histatins, the concentration of statherin in glandular secretions is significantly higher than in whole saliva due to its proteolytic degradation and strong affinity for tooth surfaces. Statherin, an AEP component, inhibits primary as well as secondary calcium phosphate precipitation, and therefore is thought to have a crucial role in maintaining oral fluid, supersaturated with calcium phosphate salts, which is an important function for the remineralization capacity of human saliva (Schlesinger, DH et al. 1977; Hay, DI et al. 1982 and Moreno, EC et al. 1979). Statherin also assists in the protection of tooth mineral tissue to the acid products, along with other members of AEP (Li, J et al. 2004).

### 2.2.6 Lysozyme

Lysozyme is an enzyme present in the secretions of human and many other vertebrates and invertebrates such as, phages, plants and bacteria. Because of its abundance in human secretions, it is considered a part the innate immune system (Goodman, H et al. 1981).

Lysozyme, also called muramidase, is a 14-kDa enzyme with antimicrobial activity in human saliva and AEP (Siqueira, W et al. 2007b). Lysozyme serves its antimicrobial activity through multiple mechanisms. First, it hydrolyzes the cell wall peptidoglycans leading to the death of bacteria (Torsteinsdottir, I et al. 1999). However, most of bacterial species are not directly lysed upon exposure to lysozyme, and further addition of detergents is required to lyse the bacteria (Goodman, H et al. 1981; Cho, MI

et al. 1982 and Pollock, II et al. 1987).

Lysozyme is also able to change intermediate glucose metabolism in sensitive bacteria and, in some cases, to lead to bacterial aggregation, which can contribute to the clearance of bacteria from the oral cavity (Pollock II et al. 1976 and Twetman S et al. 1986). Moreover, it can activate bacterial autolysins (Laible, NJ et al. 1985). In addition to antimicrobial activity, lysozyme may play a role in protection from acute bronchitis and oral candidiasis (Taylor, DC et al. 1995; Yeh, CK et al. 1997; Wu, T et al. 1999). Interestingly, it has been demonstrated that non-enzymatic properties of lysozyme are responsible for antimicrobial activity, since heat-denatured lysozyme maintains antibacterial activity. Further studies have revealed that a small cationic amphipathic sequence (12-15 amino acids) in the C-terminal domain of lysozyme displays antimicrobial activity (Doring, K et al. 1999).

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## Chapter 3

### 3 Duplication and Hybridization of Protein Functional Domains for Oral Homeostasis

#### 3.1 Introduction

Many studies have been devoted to uncovering the nature of the acquired enamel pellicle (AEP), an organic film on the enamel surfaces, formed by the selective adsorption of around 130 proteins, peptides, and other molecules (Hannig, M and Joiner, A et al. 2006; Siqueira, W et al. 2007; Vitorino, R et al. 2007). These proteins, primarily originate from salivary glands, bacterial products, gingival crevicular fluid, or oral mucosa (Siqueira, W et al. 2012). However, salivary peptides are merely products of these proteins after bacterial cleavage and may retain or augment the functional properties of their native proteins (Castagnola, M et al. 2004; McDonald, EE et al. 2011; Siqueira, W and Oppenheim, FG et al. 2009). The AEP plays a crucial role in dental homeostasis by neutralizing acids from bacterial products and acting as a selectively permeable membrane for de-remineralization (Hara, AT and Zero, DT et al. 2010; Siqueira, W et al. 2010; Siqueira, W et al. 2012; Zahradnik, RT and Moreno, EC et al. 1977). It also dictates the composition of early colonizers that ultimately form the microbial biofilm (Chaudhuri, B et al. 2007; Gibbons, RJ and Hay, DI et al. 1989; Li, J et al. 2004).

One of the AEP principal proteins is statherin, which is wholly effective at inhibiting primary and secondary calcium phosphate precipitation (Oppenheim, FG et al. 2007; Schlesinger, DH and Hay, DI 1977) allowing for supersaturated saliva that aids in remineralizing enamel surfaces. Statherin's functional peptide resides at the N-terminal

(Long, JR et al. 1998; Raj, PA et al. 1992). Recently, our group identified as a member of the AEP a naturally occurring peptide from this region (Siqueira, W and Oppenheim, FG et al. 2009). This peptide consists of 9 amino acids, DpSpSEEKFLR (where pS is a phosphorylated serine). This peptide chain, termed DR9, has shown a significant effect ( $p < 0.05$ ) on the hydroxyapatite growth inhibition in all studied concentrations when compared to other native statherin peptides (Xiao, Y et al. 2010).

Frequently, salivary proteins that have existed for millions of years contain one or more repeats of functional domains within their primary structure, thereby improving their functional capacity under evolutionary pressures (Oppenheim, FG et al. 2012; Troxler, RF et al. 1997). Protein diversity may be achieved through allelic variation, gene duplication, splicing events, and post-translational modifications (Helmerhorst, EJ and Oppenheim, FG et al. 2007; Oppenheim, FG et al. 2007). However, statherin is an evolutionarily young protein, and as such, DR9 is the known natural functional domain in relation to the inhibition of primary and secondary calcium phosphate precipitation (Xiao, Y et al. 2010). Artificially duplicating or triplicating this peptide sequence may induce the effects that we would expect to see over the course of evolution, creating a salivary protein with enhanced dental homeostasis, such as enamel demineralization protection.

In addition to multiplying a functional domain, evolutionary processes may also merge functional domains from different proteins for a combinatory effect. Therefore, another AEP protein to be studied is histatin; its benefits include antimicrobial effects, demineralization protection, buffering, and regulation of mineral formation (Edgerton, M and Koshlukova, SE et al. 2000; Helmerhorst, EJ et al. 2006; Siqueira, W et al. 2010; Vukosavljevic, D et al. 2012). Histatins degrade quickly upon secretion by the salivary



glands due to high proteolytic activity in the oral cavity, but the resulting fragments often retain their original function (Castagnola, M et al. 2004; Groot, F et al. 2006). Our group has characterized the peptide RR14, a synthetic analog of histatin's antimicrobial functional domain RKFHEKHSHRGYR, to connect with statherin's DR9. It has been previously shown that the functional domain of histatins could have the same antimicrobial effect as of the original protein. By synthetically combining RR14 with DR9, we hypothesize the creation of a novel protein with superior antimicrobial qualities and enhanced mineral homeostasis. This would represent promising new molecular methodologies for the clinical exploitation in oral health maintenance.

## 3.2 Materials and Methods

### 3.2.1 Protein and peptides characterization

Synthetic statherin, histatin 1 and peptides derived from statherin or histatin 3 were purchased from Chinapeptide (Shanghai, China). All proteins and peptides used in this study are listed in Table 1 (Table 1). Purity (> 95%) and  $M^r$  from each protein and peptide were verified by high performance liquid chromatography (HPLC) and mass spectrometry analysis. Protein/peptide solutions were prepared in 50 mM NaCl, pH 6.8, at a protein concentration of 1 mg/mL for the following experiments.

### 3.2.2 Calculation of protein and peptide isoelectric points

Isoelectric points (pI) of statherin, histatin 1 and their peptides were determined using the calculator developed by Gauci and coworkers. This approach calculates the pI of a protein and peptide at a particular pH using user-specified pK values. The calculation is repeated until the pH corresponding to a net charge of zero is found (Gauci, S et al.

2008). pI values quoted were calculated using the Scansite option (Azzopardi, P et al. 2010).

### 3.2.3 Enamel demineralization assay

Enamel sample preparation was done as previously described (Siqueira, W et al. 2010). Briefly, human permanent first molars without defects were cleaned, rinsed, and sectioned. After having the roots removed, the crowns were sliced sagittally into 4 sections (each with a 300  $\mu\text{m}$  thickness) using a diamond saw, followed by grinding to a thickness of 150  $\mu\text{m}$  using sandpaper. Each specimen was coated with a layer of light-cured dental adhesive (3M ESPE Scotchbond<sup>TM</sup> Universal) and nail varnish, excluding an untouched 2 mm window on the natural surface enamel.

Samples were randomly divided into 6 groups (N = 10 per group), as shown in Table 1. Each specimen was submerged in 1mg/mL peptide/protein solution or distilled water (control group) and incubated for 2 hours at 37°C. After this period, the samples were then submerged in 1mL of demineralization solution (0.05M acetic acid; 2.2mM CaCl<sub>2</sub>; 2.2mM NaH<sub>2</sub>PO<sub>4</sub>; pH 4.5) at 37°C for 12 days. Afterward, enamel slices were removed from the solution and the remaining 1 mL of acidic solution was used to assess the calcium and phosphate concentration released from enamel during the demineralization process.

### 3.2.4 Calcium and phosphate analyses

The calcium concentration of the solution was assessed using a quantitative colorimetric calcium determination assay (QuantiChrom<sup>TM</sup> Calcium Assay Kit, Bioassay Systems, Hayward, Calif., USA) with a UV-visible spectrophotometer determining the optical density at a wavelength of 612nm. The phosphate concentration was also

performed using a colorimetric assay (PiColorLock™ Gold Detection System, Innova Biosciences, Cambridge, U.K.) and UV-visible spectrophotometer, determining the optical density at a wavelength of 635 nm. All samples were analyzed in triplicate.

### 3.2.5 *Candida albicans* killing assay

*Candida albicans* (ATCC 90028) colonies were picked from a Sabouraud Dextrose Agar (SDA) plate (<1 week old) and suspended in 5 mM potassium phosphate buffer, pH 7.0, to a final OD 620 nm of approximately 0.3. From this suspension, 50 µl was added to 50 µl of a serial dilution series of DR9, DR9-DR9, DR9-RR14, RR14 and Histatin 3 in a 96-well polypropylene microtitre plate. The initial concentration during the dilution series of each peptide or protein was 214 µM. After 1.5 h of incubation at 37°C, 50 µl from selected wells were diluted in 9 ml phosphate-buffered saline, pH 7.0 and 25 µl aliquot of the diluted suspension was plated on SDA. After 48 h of incubation at 30 °C, cell viability was assessed by colony counting, using comparisons with the number of cells in a control sample incubated without the presence of peptide or protein. This experiment was carried out three dependent times and each time in duplicate.

### 3.2.6 Statistical analyses

Statistical procedures were performed with the software package Minitab 13.1. After checking for normal distribution, we subjected the data to analysis of variance (ANOVA) followed by Tukey's test. The level of significance was set at a value of  $p < 0.05$ .

### 3.3 Results

Isoelectric points were calculated for each peptide and protein in pH 6.8. Histatin 1, a positive protein, demonstrated a pI of 7.00 while statherin, a negative protein, showed a pI of 4.41. DR9, the natural statherin peptide, showed a pI of 3.63 while DR9-DR9 reached the lowest pI value, 3.44 and RR14 showed the highest value, 11.00, whereas DR9RR14 exhibited an intermediate PI value of 7.16. Values are demonstrated in Table 3-1.

**Table 3-1: Constructed peptides derived from statherin and histatin and their calculated pI. Note. pS is a phosphorylated serine.**

1	DR9	DpSpSEEKFLR	3.63
2	DR9-DR9	DpSpSEEKFLRDpSpSEEKFLR	3.44
3	DR9-RR14	DpSpSEEKFLRRKFHEKHSHRGYR	7.16
4	RR14	RKFHEKHSHRGYR	11.00
5	Statherin	DpSpSEEKFLRRIGRFGYGYQPVPPEQLYPQPYQPQYQQYTF	4.41
6	Histatin 1	DpSHEKRHHGYRRKFHEKHSHREFPFYGDYGSNYLYDN	7.00
7	Distilled water	None	

To gain insight into the biological functions of those new constructed pellicle peptides when adsorbed on the enamel surface, we decided to evaluate the effects of those peptides on *in vitro* enamel demineralization. The amounts of phosphate released from the enamel specimens after 12 days in demineralization solution are shown in Table 3-2. The control group (non-peptide coated) shows the highest phosphate loss. No statistically significant differences were observed between the amount of phosphate released from the groups treated with DR9-RR14 and histatin 1 or between statherin group and its naturally occurring peptide, DR9. On the other hand, statherin and DR9 demonstrated a significantly lower phosphate loss when compared to DR9-RR14 and histatin 1 groups, while DR9-DR9 showed the smallest phosphate loss compared with any other group ( $p < 0.05$ ).

In relation to calcium released, the results were very similar to the phosphate released. DR9-DR9 and statherin showed the lowest calcium loss, followed by DR9 group (Table 2,  $p < 0.05$ ). DR9-RR14 and histatin 1 groups demonstrated no statistically significant differences, but both groups demonstrated a significant calcium loss when compared to DR9-DR9, statherin and DR9 groups which held an intermediate value among the groups. As expected, all peptide/protein groups demonstrated significant difference when compared to the control group (Table 3-2).

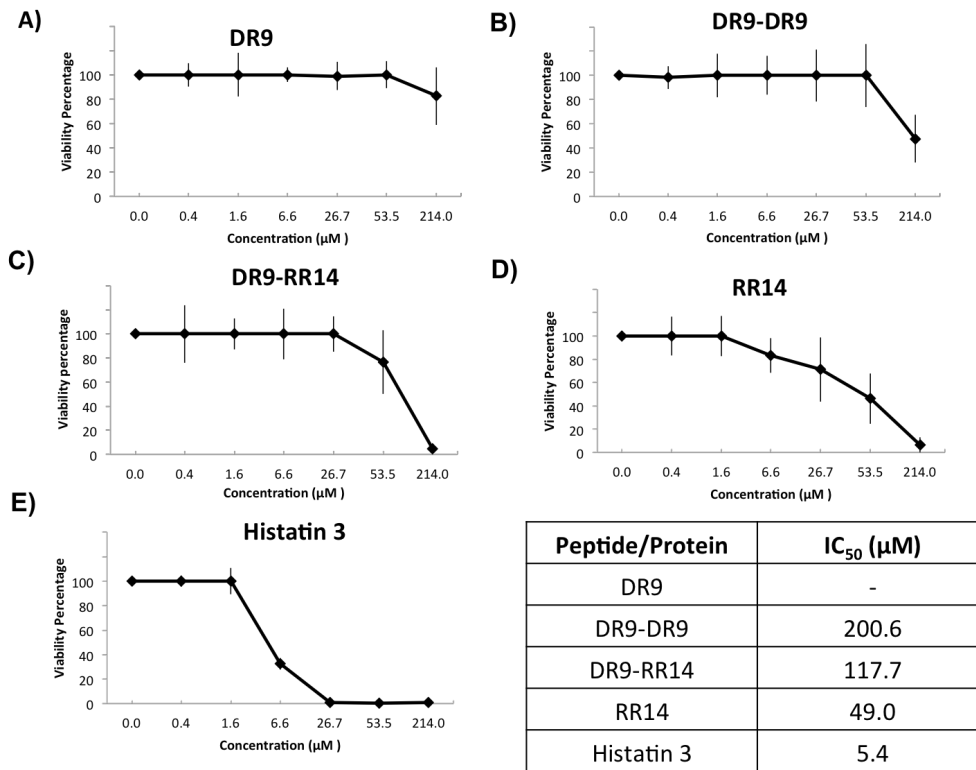
Ca/PO<sub>4</sub> ratio was calculated for each tested group, a percentage variation of less than 9% was observed among the groups. DR9-DR9 group reached the smallest ratio of 1.44 while DR9-RR14 showed the highest ratio of 1.55.

**Table 3-2: Calcium and phosphate release from human enamel sections**

Means and standard deviations of calcium and phosphate released from human enamel sections first exposed to constructed pellicle peptides, proteins or water (control) followed by exposure to the demineralizing solution (n = 10 per group). Different letter superscripts indicate statistical difference, and same letter superscripts indicate no statistical difference within the same column, according to Tukey's test (p<0.05).

<b>Peptide</b>	<b>Mean PO<sub>4</sub> Conc. (mM/mm<sup>2</sup>)</b>	<b>Mean Ca Conc. (mM/mm<sup>2</sup>)</b>	<b>Ca/Po<sub>4</sub> Ratio</b>
Water	3.09 ± 0.52 <sup>A</sup>	4.56 ± 0.49 <sup>A</sup>	1.47
DR9-RR14	2.01 ± 0.71 <sup>B</sup>	3.13 ± 0.46 <sup>B</sup>	1.55
DR9	0.92 ± 0.28 <sup>C</sup>	1.45 ± 0.13 <sup>B</sup>	1.57
Statherin	0.79 ± 0.21 <sup>C</sup>	1.17 ± 0.31 <sup>B,C</sup>	1.48
Histatin 1	1.81 ± 0.22 <sup>B</sup>	2.83 ± 0.17 <sup>B,C</sup>	1.56
DR9-DR9	0.44 ± 0.18 <sup>D</sup>	0.63 ± 0.16 <sup>D</sup>	1.44

In order to evaluate the antimicrobial activity of these new constructed peptides, *Candida albicans* killing assay was performed. IC<sub>50</sub> was calculated for all tested peptides (Figure 3-1). Histatin 3 and its functional domain (RR14) reached IC<sub>50</sub> values of 5.4 and 49.0 μM respectively. Statherin constructed peptides DR9 did not reach IC<sub>50</sub> value while DR9-DR9 reached at 200 μM. Interestingly, DR9-RR14, reached IC<sub>50</sub> at 117 μM, demonstrating a significant antimicrobial activity.



**Figure 3-1: *Candida albicans* killing Assay of newly constructed peptides and histatin3.**

A) DR9, B) DR9-DR9, C) DR9-RR14, D) RR14 and E) histatin 3. The inset Table indicates the  $IC_{50}$  values.



### 3.4 Discussion

Our group has demonstrated that a single functional domain represented by a 14 amino acid residue (RR14) located within the middle portion of histatin 3 keeps higher antifungal activity (Xu, T et al. 1991). Moreover, the multiplication of this functional domain, that in theory anticipate the effects of evolutionary trends and improve functional properties, demonstrated a significantly higher antifungal protection, suggesting a synergistic effect upon active domain multiplication (Oppenheim, FG et al. 2012). In analogy, we hypothesized that recently identified and characterized AEP phosphorylated statherin peptide, when duplicated may show increased biological function property on the enamel surface. The significance of generating novel constructs with increased adhesion to enamel is to increase the degree of substantivity that is related to the rate of clearance of a biologically active molecule from its site of action. It is also well known that salivary molecules that are more retentive to oral surfaces have a lower rate of proteolytic degradation (Gibbons, RJ et al. 1988; McDonald, EE et al. 2011). Apart from domain duplication, another potentially useful approach to generate functionally improved pellicle constructs is the preparation of hybrid molecules. While functional domain repeats are designed to augment a single functional characteristic, the combination of two different functional entities can lead to constructs with multiple functions. The hybrid approach is particularly promising for pellicle components, since some AEP peptides show a high affinity for HA whereas other AEP peptides have different functions such as antimicrobial properties (Siqueira, W et al. 2012; Vukosavljevic, D et al. 2011). Examples of such synthetic bi-functional constructs in the

protein field are cystatin-histatin (Bobek, LA et al. 1993) and statherin-osteopontin (Raj, PA et al. 1990) chimeras.

Thus, this study has pioneered in functionally characterizing the AEP's constructed peptide based on *in vivo* natural proteins/peptides identified by proteomics technology (Siqueira, W and Oppenheim, FG et al. 2009). DR9, a natural functional domain of statherin, was duplicated and/or incorporated to RR14, a functional domain of histatin 3. Our *in vitro* results showed that all constructed peptides have the potential to provide some level of protection against acid injury. Interestingly, a significant functional difference was observed when statherin or its single domain (DR9) was compared to its sibling-duplicated domain (DR9-DR9). The duplication of statherin functional domain resulted in a reduction of more than 50% of mineral loss, when compared with statherin or DR9 groups. We can speculate that this difference is based on the number of phosphorylated sites, where DR9 or statherin are phosphorylated in the amino acid residues 2 and 3; DR9-DR9 is phosphorylated in the amino acid residues 2, 3, 10 and 11. As observed before, the presence or the number of phosphorylation sites can significantly affect the adsorption to the hydroxyapatite and the process of enamel demineralization protection or calcium phosphate crystal growth inhibition (Hunter, GK et al. 2009; Hunter, GK et al. 2013; Siqueira, W et al. 2010). In addition to phosphorylated sites; DR9-DR9 has the lowest pI when compared to statherin and DR9 in pH 6.8, the physiological salivary pH. This biochemical characteristic can facilitate the adsorption on the enamel surface of this peptide (Moreno, EC et al. 1982). It was evident that multiplying the N-terminal functional domain of statherin (DR9-DR9) is evolutionarily

advantageous in promoting dental homeostasis, and it is expected that further studies on peptides of interest will continue to uncover novel peptides for clinical exploitation.

As expected, RR14 showed a significant antimicrobial property compared to DR9 or DR9-DR9. More importantly, our hybrid peptide, DR9-RR14, demonstrated to retain antimicrobial activity, despite 50% less active than RR14. This result confirms that the combination of functional domains of salivary proteins could be part of the evolutionary pathway and artificial development of these peptides can provide a promising new methodology for enhancing prevention/treatment of oral diseases. Based on the limitation of this study, it should be mentioned that the protective/antimicrobial behavior of individual peptides might be different compared to their activity in the presence of other peptides or proteins, as the case of *in vivo* formed AEP. For instance, some studies have revealed differences in the adsorption behavior of histatins in the presence of other proteins (Yin, A et al. 2006). Some other research has shown that casein and mucin, when mixed, were able to increase the erosion-inhibiting properties of the pellicle (Cheaib, Z and Lussi, A et al. 2011). No studies have as yet been carried out to address the functional effect of those newly constructed peptides in such multi-component protein films with the incorporation of other constructed peptides or histatin 1 or statherin. However, a single-component functional exploration represents the first step in gaining insights into the possible role of these peptides in enamel protection as well as antimicrobial/biofilm modulation. Knowledge obtained here may provide a basis for the development of stable (proteinase-resistant) synthetic peptides for therapeutic use against dental caries, dental erosion and/or oral candidiasis.

### 3.5 References

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## 4 The effect of pH on killing properties of chitosan nanoparticles against *Candida albicans*

### 4.1 Introduction

*Candida albicans* plays an important role in the pathogenesis of oral candidiasis and denture stomatitis. It comprises about 80% of all isolated microorganisms from these types of oral lesions (Martinez, RF et al. 2013 and Pereira, CA et al. 2013). Although, *Candida albicans* is abundantly present in the normal flora of the oral cavity, the imbalance between host and fungus can lead to the initiation of oral candidiasis. This imbalance can be resulted from several factors, including diminished salivary flow, immune defects, smoking and poor oral hygiene (Pinelli, LAP et al. 2013). Oral *Candida* infection can be treated by several commercial antifungal agents such as amphotericin B, nystatin, miconazole, itraconazole, clotrimazole and ketoconazole. However, multiple adverse side effects, including bitter taste, allergic reactions, host toxicity, interruption with cellular function and drug interactions are associated with the application of these drugs despite their efficacy (Amanlou, M et al. 2006; Bakhshi, M et al. 2012 and Donnelly, RF et al. 2008). Furthermore, *Candida albicans* is capable of reducing drug accumulation by the expression of efflux pump and therefore resisting to antifungal agents (Sanglard, D et al. 2002). Hence, many studies have focused on the development of natural products to treat oral candidiasis and overcome the weaknesses of these commercial antifungal drugs.

Chitosan is a linear amino-polysaccharide with mucoadhesive properties, which consists of two monosaccharides, GlcNAc (N-Acetyl glucosamine) and D-glucosamine

(GlcN) combined with glycosidic linkages (Sundar, S et al. 2010). Chitosan is an deacetylated form of chitin, which is mostly extracted from crab and shrimp offals. The deacetylation is achieved by boiling chitin at 80-140 °C in concentrated alkali such as sodium hydroxide or potassium hydroxide (30–60% w/v) for up to 10 hours (Kumar, MNV et al. 2000 and Rabea, EI et al. 2003). This amino-polysaccharide possesses several characteristics, including biodegradability, biocompatibility and low toxicity, which make it an effective and suitable tool in many different medical applications, mainly based on its antimicrobial and antifungal properties (Rinaudo, M et al. 2006 and Rabea, EI et al. 2003). Several mechanisms have been proposed to explain how chitosan functions as an antimicrobial agent. These proposed mechanisms include altering cell permeability by interacting with the membrane of the bacterial cell, inhibition of the production of toxin and inhibition of mRNA synthesis and various enzyme activities crucial for bacterial survival (Sudarshan, NR et al. 1992; Cuero, RG et al 1991; Seo, HJ et al. 1992 and Chen, CS et al. 1998). However, the exact mechanisms by which chitosan serve its antimicrobial activity remains to be elucidated. In addition, Chitosan is dissolved in water at acidic pHs since the amino groups can be protonated, enabling the positively charged chitosan molecules interact with the negative charges of the cell surface. However, since chitosan is insoluble in water at neutral and basic pHs that limits its antimicrobial activity at neutral and basic pHs (Agnihotri, SA et al. 2004).

Due to mentioned pH-sensitive behavior, chitosan, in the form of nano- or microparticles, can be used to encapsulate and deliver proteins and/or peptides as well as different drugs. Different approaches have been taken to create chitosan nanoparticles such as emulsion crosslinking, ionic gelation, emulsion-solvent extraction, emulsion-



droplet coalescence and emulsification solvent diffusion. Chitosan nanoparticles used in this study were constructed by the ionic gelation method and with the employment of tripolyphosphate (TPP) as a cross-linker. In this report, we evaluate the antifungal activity of chitosan particles. We also include different initial concentrations of chitosan in the gelation process of constructing particles and its impact on the resulted particle's antifungal activity. Finally, the antifungal effect of chitosan nanoparticles is compared at different pHs.

## 4.2 Material and methods

### 4.2.1 Chitosan nanoparticles' construction

Chitosan nanoparticles were constructed using an ionic gelation method. In summary, chitosan dissolved in 1.75% acetic acid and 0.5% Tween-80, is subject to adding 0.84 mg/ml TPP solution, drop by drop in ratio of 1:4. The mix will be then stirring continuously, overnight, at room temperature. In this process, TPP behaves as a cross-linker in between the chitosan molecules. The result of this interaction will be chitosan nanoparticles with small pores on the surface. For the purpose of this study, chitosan nanoparticles were made with different initial concentration of chitosan in the solution of constructing particles. 0.05%, 0.25%, 0.5% and 1% were the initial concentrations of chitosan, used in this study to produce chitosan nanoparticles. The size and surface charge of the chitosan nanoparticles were measured,  $\approx 10$  nm and +30 to +35 using Zeta sizer nano and Zeta potential analyzer (Brookhaven instruments corp.) instruments respectively. Chitosan nanoparticles were harvested by centrifugation at 16000 g for 30 minutes and then were dried and stored for the following experiments.

#### 4.2.2 *Candida albicans* killing assay

The *Candida albicans* strain used in this study was ATCC90028. Yeast cells were precultured on the Sabouraud Dextrose Agar (SDA) media plates for 48 hours, at 37° C. The SDA powder medium (Catalog number 210950) was purchased from BD Company. Less than one week old, *Candida albicans* colonies were picked from SDA plates and suspended in 5 mM potassium phosphate buffer, pH 7.0, to a final OD of  $\approx 0.3$  at the wavelength of 620 nm. In addition, a serial dilution of chitosan/chitosan nanoparticles was made in a 96-well polypropylene microtitre plate, starting from 2 mg/ml. 50 ul of the yeast suspension made in the previous step, was added to 50 ul of serial dilution series of chitosan/chitosan nanoparticles in the microplate wells. After mixing the cells with the chitosan solution, the plate was incubated for 1.5 hours at 37°C.

#### 4.2.3 Evaluation of cell viability

After the incubation time, 50 ul of the solution, from selected concentrations, was diluted in 9 ml of phosphate-buffered saline, pH 7.0 and 25 ul of the diluted suspension was then plated on SDA plates, after 48 hours incubation at 30°C, number of colonies on each plate was counted and compared to the number of colonies on the control plate that was incubated without chitosan (nanoparticles).

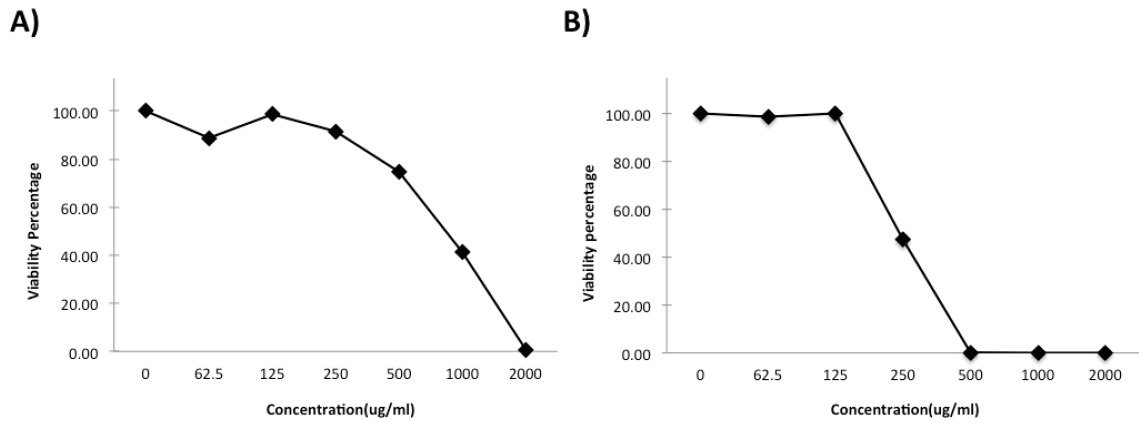
#### 4.2.4 Chitosan nanoparticles suspension

In order to make chitosan nanoparticle suspensions for the killing assay, dried chitosan nanoparticles were measured by weight and added to the buffer required for the experiment. 5 mM potassium phosphate buffer was used for pH 7.0 and acetate buffers were used for pH 4.0, 5.0 and 6.0. In order to maximize the suspension of chitosan nanoparticles into the buffers, 5 minute sonication was used.

## 4.3 Results

### 4.3.1 Chitosan nanoparticles possess a more effective antifungal activity compared to plain chitosan

The killing assay was performed for plain chitosan and chitosan nanoparticles in 5 mM potassium phosphate buffer pH 7.0, for *Candida albicans*. The percentage of cell viability in different concentrations of chitosan and chitosan nanoparticles was calculated compared to the control (Figure 4-1). As shown in Figure 4-1, chitosan nanoparticles was able to kill 100% of the cells in a concentration 4 times less (500 ug/ml) than the concentration (2000 ug/ml) that is required for chitosan (non-nanoparticle) to kill 100% of the cells. Furthermore,  $IC_{50}$ , the concentration in which an antimicrobial agent can kill 50% of the cells, was calculated for non-nanoparticle chitosan and chitosan nanoparticles. Table 4-1 shows the  $IC_{50}$  values, where again chitosan nanoparticles exhibit a killing efficiency four times more effective when compared to non-nanoparticle chitosan.

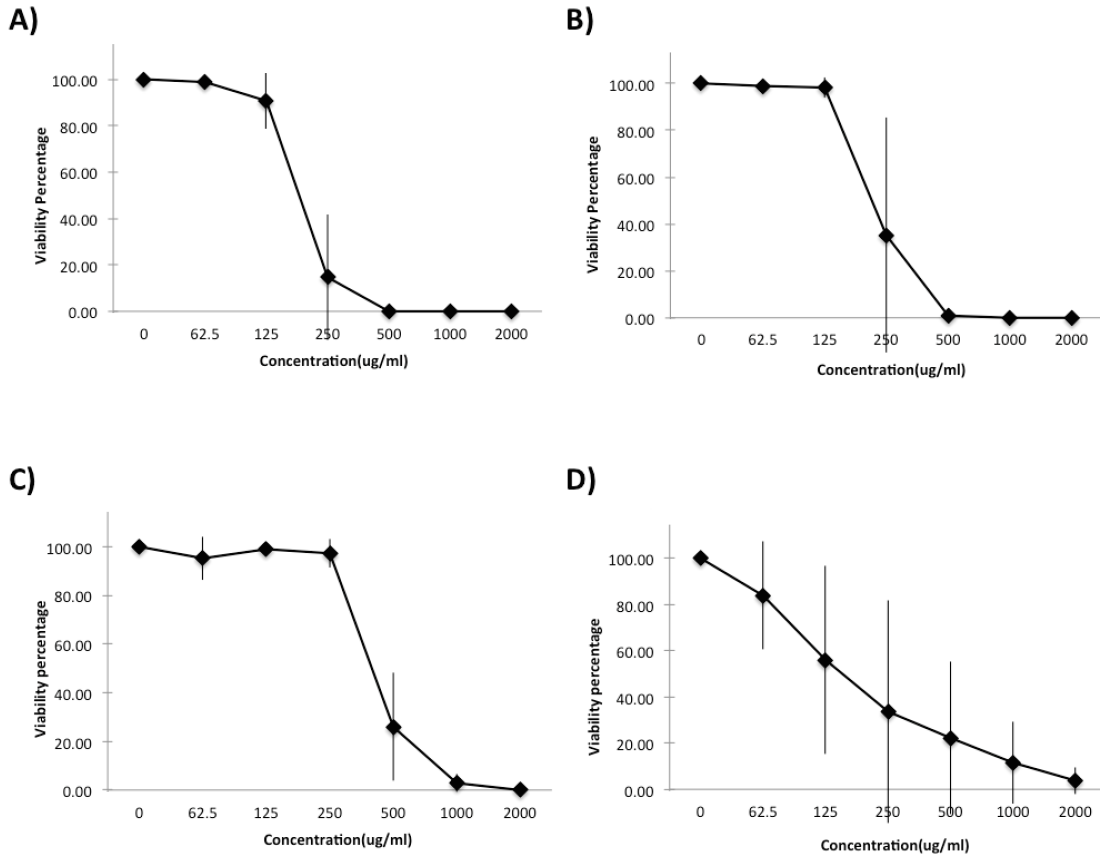


**Figure 4-1: Killing effect of chitosan nanoparticles and non-nanoparticle chitosan against *Candida albicans*.**

*Candida albicans* cells were incubated with different concentrations of chitosan/chitosan nanoparticles for 1.5 hours and the viability of the cells were compared to the controls by plating them on the SDA plates and comparing the number of colonies after 48 hours. Graph A and B represents the results for non-nanoparticle chitosan and chitosan nanoparticles respectively. Both experiments were done in duplicate and the graphs show the average.

#### 4.3.2 The Killing effect of chitosan nanoparticles made with different initial concentrations of chitosan proved to be significantly different

Chitosan nanoparticles were constructed with ionic gelation method initiating with different chitosan concentrations in the solution. The Killing effect of constructed nanoparticles was examined against *Candida albicans* in 5 mM potassium phosphate buffer pH 7.0. The percentage of cell viability compared to the controls was plotted against concentration for each group of nanoparticles (Figure 4-2). This experiment was performed to investigate whether the initial concentration of chitosan used in construction of nanoparticles can affect the antifungal activity of resulted nanoparticles against *Candida albicans*. As shown in Figure 4-2, chitosan nanoparticles showed a more effective antifungal activity when a higher initial concentration of chitosan was used to construct nanoparticles. This can be explained by the fact that chitosan nanoparticles made with 1 or 0.5% of initial chitosan concentration required 4 times less concentrations to kill 100% of the cells compared to the nanoparticles initiated in 0.25 and 0.05% chitosan.



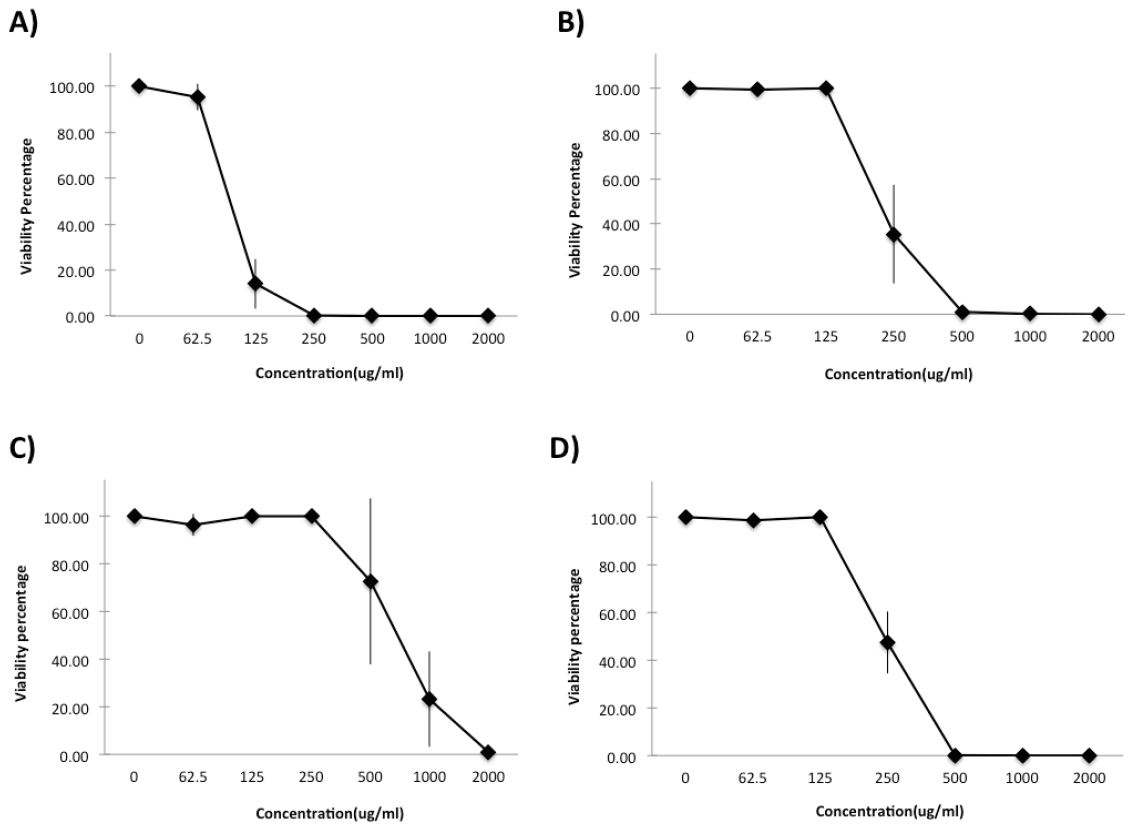
**Figure 4-2: Killing effect of chitosan nanoparticles made with different initial chitosan concentrations.**

*Candida albicans* cells were incubated with different concentrations of non-nanoparticles chitosan/chitosan nanoparticles for 1.5 hours and the viability of the cells were compared to the controls by plating them on the SDA plates and comparing the number of colonies after 48 hours. A) 1% chitosan, B) 0.5% chitosan, C) 0.25% chitosan and D) 0.05% chitosan; each experiment was performed in duplicate for three times. Error bars are based on the standard deviations of replicates.

### 4.3.3 Chitosan nanoparticles show a most efficient killing effect in pH 4

Since chitosan nanoparticles constructed with the initial concentration of 0.5% and 1% showed the highest killing effects among other constructed nanoparticles and to control the viscosity of chitosan solution, we chose 0.5% chitosan solution for future nanoparticle construction. We further investigated the antifungal activity of these nanoparticles in different pHs. To do this, the killing assay was performed in acetate buffer pH4.0, 5.0, 6.0 and 5 mM potassium phosphate buffer pH 7.0. Then, the percentage of cell viability compared to the control was plotted against the concentration of chitosan nanoparticles (Figure 4-3).

As shown in Figure 4-3, chitosan nanoparticles represent a higher antifungal activity in acidic pHs since chitosan nanoparticles require to be two and four times more concentrated in pH 5.0 and 6.0 compared to pH 4 respectively in order to kill 100% of cells. Furthermore, the comparison of the  $IC_{50}$  of chitosan nanoparticles in different pHs confirms that chitosan nanoparticles show the most effective antifungal activity at pH 4, with the  $IC_{50}$  of 39  $\mu$ g/ml.



**Figure 4-3: Killing effect of chitosan nanoparticles in different pHs.**

All the killing assays were performed the same as previous ones, except for the buffers, in which *Candida albicans* cells were exposed to the chitosan nanoparticles. A) pH=4, B) pH=5, C) pH=6 and D) pH=7. (For all the acidic pHs, acetate buffer with different pHs were used). Each experiment was performed in duplicate for three times. Error bars are based on the standard deviations of replicates.



**Table 4-1: IC<sub>50</sub> of different groups against *Candida albicans*.**

<b>Group</b>	<b>IC<sub>50</sub> (ug/ml)</b>
Plain chitosan	875
Chitosan nanoparticles (1% initial chitosan)	200
Chitosan nanoparticles (0.5% initial chitosan)	234
Chitosan nanoparticles (0.25% initial chitosan)	438
Chitosan nanoparticles (0.05% initial chitosan)	167
Chitosan nanoparticles (in pH 7)	244
Chitosan nanoparticles (in pH 6)	750
Chitosan nanoparticles (in pH 5)	109
Chitosan nanoparticles (in pH 4)	39

## 4.4 Discussion

Oral candidiasis is an oral condition that is getting more difficult to treat due to the *Candida albicans*' resistance to the available antimicrobial agents. Studies to develop a new treatment system for this disease are challenging and ongoing. Our findings propose a novel antimicrobial agent for oral candidiasis. These chitosan nanoparticles are novel for this purpose, in the sense that they can be used as a carrier for other drugs and have an efficient synergic effect along with the main drug. The ability of these nanoparticles to serve their most effective antifungal function in both pHs 4 and 7, makes them even more promising, since these two pHs are the most common pHs in the oral cavity. The normal pH of saliva is around 6.8 but following ingesting fermentable carbohydrates the pH of the oral cavity will drop quickly due to the bacteria acid production. Besides, this pH drop is more extreme in individuals with poor oral health. However, the buffering nature of saliva will return the pH to the normal pH quite fast (Stephen, RM et al. 1940 and 1944). Furthermore, chitosan nanoparticles showed a better effect in acidic pH, which is a crucial pH in the oral environment, since the acid will predispose the demineralization of tooth enamel. Moreover, nano-scale size of these particles enables them to penetrate into the biofilm and diminish the organisms both from inside as well as outside of the biofilm. The Polysaccharide nature of chitosan will also assist in particle penetration into the biofilm.

Nano/micro particles have recently drawn significant attention as drug delivery carriers, since they can be targeted directly to the intended tissue, which will reduce the side effects and increase the lifetime of the drug. With nano/micro particles' application, drug release could be activated by chemical or physical factors such as temperature, pH,

ionic strength, biological factors' concentration, and etc. Thus, stimuli dependent particles are promising drug carriers, since they can only respond to a condition which confirms the pathogenic state in which the specific drug should be released.

Chitosan particles exhibit a pH-sensitive property because of the large number of amino groups in the chitosan chain. The optimum pH for a drug encapsulated inside chitosan particles to be released is pH 4-5 (Aydin RST et al. 2012). This feature of chitosan particles is extremely important for oral application, since most of the oral complications such as dental caries and dental erosion initiate in acidic conditions in the oral cavity. This study took the first steps of developing a new therapeutic/preventive drug application in the oral cavity. Penetration of these nanoparticles into the biofilm and controlled release of the encapsulated drug when necessary is an outstanding phenomenon, which if happens can revolutionize the future of dental care and treatment.

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## 5 Conclusion and discussion

### 5.1 General rationale and conclusion

Inefficiency in oral health could be a major threat to an individual's overall health, since insufficient oral care can contribute to the severe health complications, such as stroke, diabetes development, cardiovascular disease, atherosclerosis, adverse pregnancy and many more extreme consequences (Scannapieco, FA et al. 2003).

Because of the increase in prevalence of oral problems and resistance of different bacteria/fungi present in the oral cavity to the current treatments, novel methods of treatment or preventive mechanisms are demanding (Truin, GJ et al. 2005 and Tsai, H. et al. 1997). For example, oral antifungal drugs such as azoles are applied for the treatment of *Candida albicans* infections; however, this method of treatment is becoming very limited due to the ongoing organism resistance to these agents. This case has reached the point that nowadays only a limited number of available antifungal agents such as fluconazole, itraconazole, amphotericin B and a few more, could be registered for this purpose (Carrillo-Munoz, AJ et al. 2006). In addition to organism resistance, continuous use of chemical products will create unpreventable and unwanted side effects that could become more hazardous in special cases. In the case of oral infections, such as Candidiasis, an idealistic antifungal/antimicrobial agent would be the one with a broad-spectrum efficacy and no toxicity to the host (Ito, CY et al. 2004). The idea of using natural materials to treat patients has always been a more promising and safe method to address most of the problems that we are facing with currently available chemical

products (Cruz, MC et al. 2007; Rojas, JJ et al. 2006 and Holetz, FB et al. 2002).

More than 2000 different proteins were detected in human saliva, each serving different functions and properties. Among all the salivary proteins, 130 of them appeared to be part of AEP (Siqueira, W et al. 2007). AEP is an organic layer; formed on the tooth's surface that has precursors from different origins, such as saliva, bacterial products, oral mucosa and gingival crevicular fluid (Siqueira, W et al. 2007 and Siqueira, W et al. 2012). AEP serves a strategic role in the oral cavity by neutralizing the acid products of bacterial metabolism, governing the types of early colonizers on the tooth surface and monitoring the demineralization and remineralization of the enamel (Hara and Zero, et al. 2010; Siqueira, W et al. 2010; Siqueira, W et al. 2012; Zahradnik and Moreno, et al. 1977; Chaudhuri, B et al. 2007; Gibbons and Hay, et al. 1989; Li, J et al. 2004). Therefore, AEP and its composition are a crucial element in preserving the oral health and it is hypothesized that altering its composition could shift the physical and chemical reactions, which will eventually lead to an irreversible dental condition, such as tooth decay.

There are salivary proteins that provide natural antimicrobial/antifungal properties against certain microorganisms in the oral cavity to keep the biological balance between the beneficial and virulence organisms in the oral environment (Tenovuo, J et al. 1989). Most of which, are evolutionary occurred proteins present in different animal species as part of the innate immune system (Hoffmann, JA et al. 1999). These natural antimicrobial proteins usually have wide activity spectra against different bacteria, fungi or viruses. Antimicrobial/antifungal salivary proteins have a small cationic region in common, within their structure, which could provide the killing effect of the protein on target

organisms.

Histatins are a family of salivary proteins with antimicrobial activity. These proteins are all products of 2 gene loci; therefore, all of them possess a common cationic region, which seems to be the functional domain of the protein (Azen, EA et al. 1973 and Sabatini, LM et al. 1989). Histatins vanish immediately after secretion into the oral cavity because of degradation; despite the resulted fragments appear to sustain the activity, while carrying the functional portion of the original protein (Castagnola, M et al. 2004; Groot, F et al. 2006).

Some other saliva components inhibit the calcium and phosphate precipitation in the oral cavity, which is a very essential feature of saliva to keep the oral fluid supersaturated from these minerals that will aid the remineralization of the teeth enamel, when it is required (Gron, P et al. 1976). These members of saliva are usually the proteins that are selectively adsorbed onto the enamel surface and therefore, seem to be AEP precursors (Hay, DI et al. 1973). Examples of such proteins are proline-rich proteins and a small tyrosine-rich acidic protein called Statherin (Oppenheim, FG et al. 1971; Hay, DI et al. 1974; Hay, DI et al. 1973).

Recently, several studies revealed high susceptibility of functional salivary proteins, including histatins and statherin, to the oral proteases and/or bacterial enzymes presented in the oral cavity. Although some of the fractions could remain functional after the cleavage to some point, this immediate degradation causes remarkable decrease in the overall function (Helmerhorst, EJ et al. 2006; Siqueira, W *et al.* 2010; 2007b; Siqueira, W et al. 2009; McDonald, EE et al. 2011; Castagnola, M et al. 2004; Groot, F et al.



2006). Meaning that, if feasible to keep those proteins active in the same scale as they are produced by salivary glands, this could be employed as a natural promising therapeutic method in the dentistry field. To evaluate this hypothesis, this study has pioneered a method of encapsulation for synthetic salivary peptides/proteins to be beneficial for the oral application.

Recently, our group, taking advantage of novel proteomics techniques, has detected a small peptide consisting of only 9 amino acids termed as DR9, in the AEP composition (Siqueira and Oppenheim, et al. 2009). This peptide appeared to be the N-terminal part of statherin, which is believed to be its functional domain (Long, JR et al. 1998; Raj, PA et al. 1992). DR9 peptide naturally occurred in the oral cavity and exhibited the same affinity to the HA and functionality as the original protein, statherin (Xiao, Y et al. 2010). Inspired by this naturally existed peptide in AEP composition, the idea of analyzing other salivary proteins' functional domain and accelerating the evolution pathway by combining them together or duplicating them, came to our mind.

Eventually, from the data collected in this study, we confirmed that only the functional domain could carry the biological function of tested salivary proteins of interest. Also, evolutionary new generation proteins could be constructed artificially by combining different protein functional domains and/or duplicating protein functional domains. This idea was inspired by the fact that ordinarily, proteins over the track of evolution misplace the portions of their sequences that are not essential for their function and replicate the functional portions instead, therefore saving energy and improving their functional capacity (Oppenheim FG et al. 2012; Troxler, RF et al. 1997). Hence, this could be the evolutionary pathway that these salivary proteins might take after millions of

years, which could be induced artificially by synthetic methods and in this way, we could benefit from the result of evolution millions of years ahead of the time that it would naturally occur.

A method of oral application for proteins was also investigated in this study, which proved to be likely, even though we took only the first steps and there are lots of limitations yet to be addressed.

Recently, chitosan, a cationic polysaccharide, has attracted vast attention from the pharmaceutical fields due to its preferential biological properties to deliver different active drugs or proteins to the site of action (Kumar, MNV et al. 2000; Rabea, EI et al. 2003). Chitosan micro/nanoparticles have drawn significant attention recently, since not only they have all the critical features of a drug delivery agent, including nontoxicity, biodegradability and being physically inducible, but also, they have bonus antimicrobial, antifungal and wound healing properties, which could be highly beneficial for the dental application (Rinaudo, M et al. 2006; Rabea, EI et al. 2003). Moreover, chitosan nanoparticles have a pH sensitive behavior, in terms of the size of pores on their surface, which makes them more favorable for the oral application. Since they could only release the proteins/peptides when the pH drops to the acidic point, which is accounted as a red flag in the oral cavity since at the acidic point heavy demineralization assault on the teeth tissue initiates.

Another approving property of chitosan nanoparticles for this application is the extremely small size of the nanoparticles, because of which, it is hypothesized that they could penetrate into the micro-scale pores in between the biofilm components. This

penetration will make the nanoparticles part of the biofilm and the protein/peptide release from nanoparticles will diminish the biofilm from the inside when the pH drops to the acidic point, which is the point that the damage to the teeth tissue upraises. Particles' penetration into the biofilm is highly critical in dentistry because they will be able to reach to the part of the biofilm, which is inaccessible by brushing and other manual types of cleaning. These dental biofilms are also highly resistant to antimicrobial products compared to the individual microorganisms, which is because of the exopolysaccharide matrix produced by microorganisms (Costerton, JW *et al.* 1995). This compact structure of the biofilms makes them resistant to the host immune system as well, causing them to be very challenging to control and eliminate (Lewis, K. *et al.* 2001).

## 5.2 Final Conclusion

- Only the functional domain of statherin and histatin, termed as DR9 and RR14 respectively, showed some level of the original protein's activity.
- Combination/duplication of salivary protein functional domains could increase/combine their activity.
- Chitosan nanoparticles constructed by the ionic gelation method, exhibited a higher antifungal activity compared to the non-nanoparticle chitosan and also they presented the most efficient activity when higher concentrations of chitosan was used to construct the nanoparticles.
- Chitosan nanoparticles exhibited the highest antimicrobial activity in pH 4, which is the critical pH in the oral cavity, at which several dental complications initiate, followed by neutral pH which is the normal pH of the oral cavity.

### 5.3 Challenges and limitations

Similar to any other project, presented one has several limitations to be addressed. One of the major concerns about this project is that since more than 1000 different microorganisms coexist in the human oral cavity (Paster, BJ et al. 2006 and Zaura, E et al. 2009), and chitosan nanoparticles might have different effects, in terms of toxicity, towards them, this could interrupt the natural balance in between the oral microbiota. Moreover, the polysaccharide nature of chitosan might make it consumable for some bacteria, which could massively increase their quantity and proportion in the biofilm, whether the bacteria is an acute virulence or a beneficial normal flora. One approach for this challenge could be investigating the effect of chitosan nanoparticles on all the critical microorganisms presented in the oral microbiota as well as performing multispecies assays to evaluate the effect of chitosan nanoparticles on the multispecies communities, which is mimic to the biofilm condition. This way, there will be the possibility to investigate how different microorganisms will react when exposed to chitosan both individually and in a biofilm format and therefore, if there could be any other approach to make the method more feasible.

### 5.4 Future directions

This could be a novel method in dentistry if a therapy for different oral pathologies could be designed using natural salivary proteins or peptides. Although acquiring a detailed understanding of protein/peptide encapsulation and release into/from chitosan nanoparticles, in different physical conditions, such as different pHs, will be essential to move the project forward. Saliva pH can vary from 3 to 6.8 in different conditions; therefore protein/peptide release from the chitosan nanoparticles should be

examined in buffers with this pH range as well as in saliva itself. In addition, the effect of these particles loaded with the proteins should be investigated in single or multispecies biofilms in different conditions. This will give us an understanding of how the whole biofilm will react to chitosan nanoparticles rather than only a single microorganism. Finally, it has to be tested in vivo in a rat model before being able to move it to the clinical trial.

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