September 2015

Novel protective salivary peptides and the chitosan nanoparticles delivery system

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Graduate Program in Medical Biophysics

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

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NOVEL PROTECTIVE SALIVARY PEPTIDES AND THE CHITOSAN NANOPARTICLES DELIVERY SYSTEM

(Thesis format: Integrated article)

by

Flavia, Zaidan Cardoso Salina

Graduate program in Medical Biophysics

Submitted in partial fulfillment
of the requirements for the degree of

Master of Science

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

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ABSTRACT

As a result of its protein composition, saliva performs many essential functions that contribute to oral homeostasis. However, salivary proteins suffer quick degradation in the oral cavity, which decreases the protective potential of that fluid (Chapter 2). Using scanning electron microscopy (SEM), we evaluated the effect of our novel salivary peptides DR9, DR9-DR9, DR9-RR14 and RR14 on the crystallization of calcium oxalate (CaOx). All peptides containing DR9 showed significant inhibitory effect on the crystallization of CaOx, whereas RR14 showed no effect (Chapter 3). Next, we tested the adsorption behavior of our chitosan nanoparticles (CHNP) delivery system to hydroxyapatite (HA) surface and to a protein-coated-HA surface using x-ray photoelectron spectroscopy (XPS) and confocal microscopy. According to our results, CHNP are capable of adsorbing to HA and more evenly to a protein-coated-HA (Chapter 4). Our results bring us closer to developing a reliable delivery system for our protective peptides.

Keywords: saliva, salivary proteins, scanning electron microscopy, x-ray photoelectron spectroscopy, confocal microscopy, chitosan nanoparticles, carrying system, preventative dentistry.
CO-AUTHORSHIP

As the principal author, I performed and collected the vast majority of experimental data and also drafted the entire original manuscript. The co-authors listed below made significant contributions to this work.

Dr. Walter L. Siqueira, DDS, PhD, as the principal investigator, he led the study design, helped to determine project objectives, provided mentorship, assisted with the interpretation of results, and provided editorial assistance as well as overall guidance throughout the study.

Dr. Bernd Grohe, PhD offered guidance and support on data collection and interpretation.

Rajesh K. Gupta, MSc, offered knowledge into the matter of chitosan nanoparticles carrying system, as well as providing technical support for acquiring experimental data.

Vitali Veramkovich, provided great support on data collection and helped with editing the manuscript.

Dr. Yizhi (Cindy) Xiao, MSc, PhD, taught me most of the experimental techniques used in this project and helped in the analysis of experimental data. She also provided guidance in all aspects of my laboratory work.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my husband, Renato Salina for his encouragement, love, affection and respect that were essential for my effort and performance. Words are not enough to express my gratefulness.

Thank you to my mother, father and sisters for providing me with the constant love and support that allowed me to complete this journey.

Thank you to my supervisor Dr. Walter L. Siqueira; I am sincerely thankful for his patience, help and opportunities he has given me. His belief in my abilities, our thought-provoking conversations and his emphasis on productivity have truly inspired me to do my best. I am very thankful for the guidance, support and encouragement he has provided me over the past couple years.

Thank you to the members of my advisory committee (Dr. Amin Rizkalla and Dr. Ruth Martin) and co-authors for their guidance, contributions, edits and for making this thesis possible.

To my lab mates who have greatly helped me throughout this long journey. Thanks for the afternoon coffee breaks, for the relaxing chats during lunch and for many good laughs just when I needed it. Thanks for some great memories and all the wonderful discussions that got me through the past couple years!

Finally, I am truly grateful for the financial support I received during my graduate studies that enabled me to completely focus my time and energy on my research. I acknowledge the funding I received from the Ontario Graduate Scholarship and the Schulich Graduate Scholarship programs.
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<th>Description</th>
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<tbody>
<tr>
<td>AEP</td>
<td>acquired enamel pellicle</td>
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<tr>
<td>sAEP</td>
<td>simulated acquired enamel pellicle</td>
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<tr>
<td>HA</td>
<td>hydroxyapatite</td>
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<tr>
<td>CaOx</td>
<td>calcium oxalate</td>
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<td>COM</td>
<td>calcium oxalate monohydrate</td>
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<td>COD</td>
<td>calcium oxalate dihydrate</td>
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<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
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<td>XPS</td>
<td>x-ray photoelectron spectroscopy</td>
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<td>CHNP</td>
<td>chitosan nanoparticles</td>
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<td>mm</td>
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<td>g</td>
<td>gravitational constant</td>
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<tr>
<td>PPB</td>
<td>potassium phosphate buffer</td>
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<td>eV</td>
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<td>mA</td>
<td>miliampere</td>
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<td>BE</td>
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1 Chapter 1

Salivary proteins and the novel chitosan delivery system

1.1 Introduction

Saliva is a complex biological fluid that is greatly involved in oral health and homeostasis. It consists of secretions from the major salivary glands (parotids, submandibular, and sublingual glands) and from hundreds of minor salivary glands (Carpenter, 2013; Dawes, 2008; Edgar, 1992; Lendenmann et al., 2000). Saliva helps maintaining the integrity of the oral cavity by providing tissue lubrication, aiding in mastication and speech, modulating demineralization and remineralization processes, promoting bacterial and viral clearance, among others (Carpenter, 2013; Lendenmann et al., 2000; Wong, 2008). It is also responsible for the formation of the acquired enamel pellicle (AEP), a protective protein film formed as a result of selective adsorption of salivary proteins onto the enamel surface (Lendenmann et al., 2000; Siqueira et al., 2007a; Siqueira et al., 2012; Vukosavljevic et al., 2011; Vukosavljevic et al., 2014a; Yao et al., 1999).

It achieves these and other roles due to its complex composition and physical properties. The protein and ionic components turn this 99% water solution into a versatile fluid, capable of displaying functions such as: antimicrobial activity, lubricating properties, aiding digestion, and facilitating taste. In the absence of saliva, all oral structures become more vulnerable to bacterial, viral and fungal infections. In other words, individuals with hyposalivation (decreased salivary production) are much more likely to develop dental caries, oral infections and combined dental wear (e.g. dental erosion, abrasion and attrition) (Carpenter, 2013; Dawes, 2008; Magalhaes et al., 2009; Shellis and Addy, 2014; Wong, 2008).

Saliva is also responsible for the formation of the acquired enamel pellicle (AEP), which is fundamentally a protein film that overlays the dental surfaces. The formation of the AEP is a highly selective and dynamic process (Lendenmann et al., 2000; Siqueira et al., 2012; Wong, 2008). In only seconds of exposure to saliva, the initial phase of pellicle formation over the tooth enamel begins (Hannig and Balz, 1999; Siqueira et al., 2012). In this process, precursor proteins (e.g. statherin, histatins, acidic proline-rich proteins) selectively adhere to the surface, forming the initial protein layer (Hannig and Joiner, 2006). The subsequent stage of pellicle formation results in a rapid increase in pellicle thickness.
AEP is comprised of proteins, peptides, carbohydrates and lipids (Hannig and Joiner, 2006; Siqueira et al., 2007a; Siqueira et al., 2012). Its composition and structure regulate all processes that happen at the unique interface between the teeth and the oral environment. The protective character of this integument is largely associated to its proteic composition and, as a result, classic AEP proteins have been further studied in order to fully understand the mechanisms involved in their functions.

Our group has discovered naturally occurring peptides in saliva derived from the degradation of histatin-3 and statherin: RR14 and DR9, respectively. These peptides comprise the active site of the original proteins, and thus exhibit their biological functions. In brief, histatins are salivary proteins that exhibit strong antimicrobial activity (Brand et al., 2014; Edgerton and Koshlukova, 2000; Imamura and Wang, 2014; Oppenheim et al., 2012). Whereas statherin is a largely studied salivary protein that is able to modulate important biomineralization processes (Raj et al., 1992).

Using genetic engineering, our group has created the doubled and the hybrid peptides DR9-DR9 and DR9-RR14 in the hopes that these would conveniently have their activities enhanced and combined. Therefore, the primary objective of the study described in Chapter 2 was to test the crystal-inhibitory activity of DR9 in our novel peptides combinations.

The salivary proteins unfortunately have their clinical use impaired by the quick degradation that they suffer as soon as they are released in the oral cavity (McDonald et al., 2011). That happens due to the highly proteolytic environment of the mouth, which results in a significant decrease of active proteins/peptides (Helmerhorst and Oppenheim, 2007). Various approaches have been proposed to overcome this challenge and attain better oral bioavailability, including the use of surfactants, permeation enhancers, protease inhibitors, etc (Zhang et al., 2010).

Chitosan is a linear polysaccharide that is composed of copolymers of glucosamine and N-acetyl glucosamine. Pharmaceutical and biomedical research has been conducted for many years on chitosan due to its advantageous biological properties, such as biodegradability and biocompatibility. Recently, it has also caught attention in the dentistry field, not only for its antimicrobial activity, but as a result of its drug-carrying-system property, which can be achieved by shaping the chitosan molecule into micro and/or nanoparticles, encapsulating active components that can then be delivered at specific sites of the body (Giunchedi et al., 1998; Mandala Rayabandla et al., 2010; Zhang et al., 2010).

Our group has focused on developing a new protein delivery system made of
chitosan nanoparticles (CHNP), expecting that by using it, protective peptides could be delivered at specific sites of the mouth before being completely degraded by salivary and bacterial enzymes. In order to successfully achieve that goal, our CHNP should be able to adsorb to either hydroxyapatite (dental tissue) or to the acquired enamel pellicle.

Previous studies have shown that chitosan is able to adsorb to various surfaces, including hydroxyapatite (Guo and Gemeinhart, 2008; Lee et al., 2012). However, the behavior of CHNP in relation with oral integuments has not yet been tested. The method we chose to investigate the adsorption of our CHNP to HA and to a simulated acquired enamel pellicle (sAEP) film was x-ray photoelectron spectroscopy (XPS), which is a surface-sensitive quantitative technique that can be used to analyze the surface composition of a material in its as-received state, or after some treatment.

In order to visualize our nanoparticles, these surfaces were submitted to adsorption treatment of CHNP loaded with Alexa F488-tagged DR9, which could then be visualized by confocal microscopy imaging. Both experiments are described in chapter 3.

The results presented in chapters 2 and 3 open new frontiers for the exploration of mechanisms that can enhance the protective character of the AEP and aid in the prevention of some oral diseases.

In this first chapter we discuss saliva’s and AEP’s properties, as well as the influence of synthetic salivary proteins on the protective nature of the AEP. We also consider CHNP as a strong candidate for a future protein delivery system, along with its potential preventive treatment option for some oral diseases.

1.2 General considerations about saliva

Saliva is a complex biological fluid that, under normal conditions, bathes the oral cavity and has several important functions in the oral homeostasis. Its production depends largely on the major salivary glands (parotid, submandibular and sublingual). However, the contribution of the minor salivary glands (labial, palatal, lingual, Von Ebner, Ebner, Blandin-Nuhn), of crevicular ginvival fluid, squamous epithelial cells and plasma components is also important for its formation and final composition (Carpenter, 2013; Edgar, 1992; Wong, 2008). In addition to the glandular secretions, whole saliva contains exogenous components, such as food residues and microorganisms.

As their name suggests, major salivary glands are the major producers of saliva. The parotid glands produce serous saliva, which has watery consistency and a high concentration of enzymes (Edgar, 1992; Hannig et al., 2005; Jenkins, 1978). Despite of having low
viscosity (similar to water’s), it possesses important features and viscous qualities (Proctor et al., 2005) that allow humidification of both hydrophobic as hydrophilic surfaces. Submandibular and sublingual glands produce a mixed secretion, i.e. serous and mucous saliva, and the percentage of production of each type of saliva can vary depending on the amount and type of cells that form the glands.

As for the production of saliva the percentage of glandular secretion is approximately 95.6%, the contribution of gingival crevicular fluid is 2.4%, 1% microorganisms, and epithelial cells 1% (Wong, 2008).

As forementioned, the functions of saliva are essential for maintaining oral health and homeostasis. In the absence of saliva, both teeth and the oral mucosa, become more vulnerable to bacterial, viral and fungal infections. In other words, saliva protects the oral tissues in several ways.

The continuous cleansing that the salivary flow promotes in the oral cavity results in the ingestion of most of the food debris, decreasing the overall amount of substrate for oral bacteria.

The ionic components (particularly bicarbonates and phosphates) promote buffering of the oral environment, protecting the dental structures against demineralization and also working in the dental remineralization process.

Its lubricating properties aid in the bolus formation and humidification, assists in taste and decreases the tooth-to-tooth friction.

Amylases and lipases present in saliva initiate the digestion of starch and fat, respectively.

Another important aspect is the presence of specific proteins that provide saliva with antibacterial, antiviral and antifungal properties.

Saliva also assists in the formation of a protein film called the acquire enamel pellicle (AEP) that overlies the surface of all teeth (Carpenter, 2013; Siqueira et al., 2007a; Siqueira et al., 2012), preventing their direct contact with acids (endogenous or exogenous) (Hannig and Balz, 1999; 2001; Hannig and Joiner, 2006; Juriaanse, 1979; Lendenmann et al., 2000; Siqueira et al., 2007a; Siqueira et al., 2012; Vukosavljevic et al., 2014a; Wiegand and Attin, 2011).

1.3 General considerations about the acquired enamel pellicle (AEP)

Upon exposure to the oral environment, the tooth surfaces acquire a thin protein coating called AEP (Juriaanse, 1979; Lendenmann et al., 2000; Siqueira et al., 2012). Its formation depends on the selective adsorption of salivary macromolecules on the dental tissues
(Mayhall, 1970; Siqueira et al., 2012; Vukosavljevic et al., 2014b) and is a very dynamic process (Lee et al., 2013). It is composed primarily of proteins (in special phosphoproteins) secreted from the salivary glands and also from other sources such as mucous cells, plasma, etc (Siqueira et al., 2012).

Knowledge about the protein composition of saliva and AEP has boomed in the past few years due to advances in proteomics techniques for separation and identification (Neyraud et al., 2006; Siqueira and Dawes, 2011; Siqueira et al., 2012; Yao et al., 2003; Zimmerman et al., 2013). A study using LC-ESI-MS/MS has identified a total of 130 different proteins in AEP formed in vivo over human enamel (Siqueira et al., 2007a; Siqueira et al., 2007b).

Several studies have focused on analyzing the protective impact of the acquired pellicle formed in situ over the enamel (Hannig and Balz, 1999; 2001; Hannig et al., 2003; Hannig et al., 2004). This structure has 3 main known functions: it retards enamel demineralization (Hannig and Balz, 1999; Hannig et al., 2003; Hannig et al., 2004; Juriaanse, 1979; Lendenmann et al., 2000; Vukosavljevic et al., 2014a), prevents crystals growth from supersaturated saliva onto the enamel surface (Hay, 1975; Moreno et al., 1979) and modulates the early adhesion of microorganisms to the tooth surface (Vukosavljevic et al., 2011; Vukosavljevic et al., 2012).

It has been suggested that only mature, several day-old pellicles are capable of preventing enamel demineralization (Zahradnik et al., 1978). However, studies have demonstrated that pellicles developed over one hour offered maximum protection against demineralization, with no increased protection when compared to longer maturation times (Amaechi et al., 1999; Hannig et al., 2003; Nieuw Amerongen et al., 1987). Hannig et al. (2004) found no difference in the protective effect of a pellicle formed over a three-minute period compared to a pellicle formed over a two-hour period, which could be attributed to the fact that the pellicle formation begins within seconds of exposure to the oral environment (Hannig et al., 2004). This first basal protein layers is electron dense and is formed after one minute of exposure (Ericson et al., 1982). Subsequent pellicle layers are much less electron dense and are much more loosely arranged compared to the initial basal pellicle layer (Hannig and Balz, 1999), offering little additional protection against acidic attack (Hannig and Balz, 1999; Hannig et al., 2003; Hannig et al., 2004).

These and other functions of the AEP are directly associated to the numerous proteins found in this oral integument.
1.4 Salivary proteins

The salivary proteins are the highlights in saliva’s and AEP’s composition. Each protein has specific biological functions that contribute to most of saliva’s and AEP’s biological functions. Next are some examples of important proteins and their functions in the oral environment.

Cystatin, are cysteine-rich proteins that exhibit significant inhibitory activity on proteases. This feature makes cystatin capable of attenuating deleterious effects of inflammatory processes, reducing tissue damage associated to proteolysis (Robinson et al., 1997).

Mucins are high molecular weight proteins that are highly glycosylated. These proteins can auto-aggregate, forming large structures that contribute significantly to the viscous texture of saliva (except for MUC 5B, which does not possess this feature) (Culp et al., 2015; Frenkel and Ribbeck, 2015; Siqueira et al., 2008; Wong, 2008).

Salivary amylases are proteins that can be found in 6 described isoforms (Edgar, 1992; Jenkins, 1978). They are the most abundant proteins in saliva and participate actively in the digestion of maltose. A possible explanation for the fact that these are the most abundant proteins in saliva is their important role in oral clearance.

Histatins are a group of histidine-rich salivary proteins that exert important antimicrobial activities in the oral cavity (Helmerhorst and Oppenheim, 2007; Oppenheim et al., 1988). They are also capable of neutralizing endotoxic lipopolysaccharide located in the membrane of gram-negative bacteria. Histatins are also inhibitors of host and bacterial enzymes involved in the destruction of the periodontium. In addition to its antimicrobial activities, histatins are involved in the inhibition of the release of histamine from mast cells, affecting the outcome of oral inflammatory processes (Gusman et al., 2001). It has been reported that the histatins have proven effect against fungi growth in the oral cavity (Vukosavljevic et al., 2012). Moreover, histatins are known for being precursors of the acquired enamel pellicle.

Statherin is a low molecular weight phosphoprotein that is rich in tyrosine, proline and glutamic acid. Its name derives from the Greek "Statheropio" which means stabilizing. This protein is able to bind to calcium (Ca^{2+}) ions present in saliva, balancing the remineralization process and calcium deposition onto the teeth surfaces. It is the only salivary protein capable of modulating primary and secondary precipitation of calcium/phosphate onto the dental surfaces (Raj et al., 1992). Both are crucial processes related to the formation of dental calculus and the remineralization of incipient carious
lesions (Oppenheim et al., 2007). Moreover, it has been reported that statherin facilitates the binding of Actinomyces viscosus (Clark et al., 1986) and Fusobacterium nucleatum (Xie et al., 1991) to the hydroxyapatite surface, which suggests that this protein participates in the modulation of initial bacterial colonization of the acquired enamel pellicle (AEP).

Proline-rich proteins (PRPs) are a group of salivary proteins that is divided into acidic and basic families. Basic PRPs do not adhere to teeth, but bind to bacteria (Matsumoto-Nakano et al., 2008). The acidic PRPs (aPRPs) possess a 30-amino acid N-terminal domain rich in aspartate, glutamate, and containing a few serine phosphate residues. This domain strongly adheres to hydroxyapatite; in other words, this protein is an AEP precursor. Acidic PRPs have important biological functions related to providing a protective environment for the teeth, and appear to possess other activities associated with modulation of bacterial colonization of the oral surfaces and preventing calcium phosphate deposition onto the enamel surface once adsorbed to it (Hay et al., 1987; Moreno et al., 1979).

The human salivary peroxidase system (SPS) contributes in several ways to the maintenance of good oral health. The SPS is one of the non-immunoglobulin defense factors that regulate the quantity and species distribution of oral microorganisms. Peroxidase, for example, is a salivary protein from the SPS that also prevents toxic accumulations of hydrogen peroxide (H$_2$O$_2$) and it inactivates many carcinogenic and mutagenic compounds (Mansson-Rahemtulla et al., 1990; Tenovuo and Pruitt, 1984). It also reduces acid production from bacteria in dental biofilm, decreasing plaque accumulation and establishment of gingivitis and caries (Guven et al., 1996).

Lactoperoxidase is a member of the heme peroxidase family of enzymes. It is a potent antimicrobial agent that is effective in killing a range of aerobic and certain anaerobic microorganisms (Courtois et al., 1992; van ’t Hof et al., 2001).

Immunoglobulins (Ig) are important specific defense factors of saliva. Of the different classes of immunoglobulins, IgA (being the predominant Ig), IgG, and IgM influence the oral microbiota by interfering in the adherence of bacteria or by inhibiting bacterial metabolism. IgA is an agglutinating factor, and also the main secretory protein that represents the adaptive immunity in saliva. After secretion, IgA binds selectively to antigens; this binding prevents antigens, such as microbes and viruses, from attaching to mucosal and dental surfaces.

The salivary proteome comprises 2290 proteins (Siqueira and Dawes, 2011). With such a vast array of proteins within the dynamic oral cavity, it is very likely that many of
these proteins interact with one another, synergistically enhancing their diverse functions (e.g., maintaining the integrity of oral cavity, lubricating hard and soft tissues, inhibiting microbial growth, aiding taste, and facilitating food digestion, etc). 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.

1.5 Protein degradation in the oral cavity

Peptides and proteins have become the drugs of choice for the treatment of numerous diseases as a result of their incredible selectivity and their ability to provide effective and potent action (Frokjaer and Otzen, 2005). In spite of having such advantageous properties, all synthetic and/or natural proteins have their clinical use jeopardized by the degradation they suffer in the oral environment.

A recent study showed that the adsorption of histatin 1 onto hydroxyapatite has provided some resistance against proteolytic degradation (McDonald et al., 2011). Other phosphoproteins with high affinity to hydroxyapatite, such as statherin and aPRPs, have also demonstrated significant resistance against proteolytic degradation when adsorbed onto hydroxyapatite (unpublished observations by Siqueira Lab).

Various approaches have been proposed to overcome this challenge and to obtain better oral bioavailability such as including the use of surfactants, permeation enhancers, protease inhibitors, etc (Zhang et al., 2010). Amongst these, the use of colloidal polymeric particulate delivery systems, particularly mucoadhesive nanoparticles, represents a promising concept (Takeuchi et al., 2001).

1.6 Synthetic salivary proteins

Biosynthesis of proteins is a new and rapidly expanding field with remarkable implications for pharmaceutical companies. Synthetically creating proteins allows for precise specific manipulation of active sites with the desired properties, that is, synthetic proteins (Kosoric et al., 2007) can modify the AEP, enhancing its protective character.

For example, the addition and/or selective alteration of a protein’s functional group with anti-cariogenic properties could tremendously improve the physiological function of the protein and increase its efficiency as a therapeutic agent for tooth decay. Accordingly, in addition to naturally occurring salivary proteins, protective synthetic proteins have been
designed. Another possible advantage of creating synthetic proteins is that, by having a smaller peptide sequence (comprising the active site only), these proteins may also represent a smaller target for enzymatic degradation in the oral environment. Further research is required to prove this assumption.

An example is StN21, which is a synthetic peptide designed to have an amino acid sequence identical to the N-terminal of statherin, a salivary protein that inhibits primary and secondary precipitation of crystals (Kosoric et al., 2007). StN21 is a peptide shown to effectively reduce mineral loss, thus possessing therapeutic potential (Kosoric et al., 2007).

Our group has discovered peptides derived from the degradation of salivary statherin and histatin-3: DR9 and RR14, respectively. Both peptides comprise the active site only of their origin proteins, which means that they are able to exhibit the function of their original proteins. Synthetically duplicating and a combining these peptides (DR9-DR9 and DR9-RR14 respectively) should double and combine their supposed activities.

1.7 Chitosan in medicine and dentistry

Chitosan is a linear cationic polysaccharide composed by copolymers of glucosamine and N-acetyl glucosamine. It is obtained by the controlled deacetylation of chitin, which is a characteristic component of the cell walls of fungi, the exoskeletons of arthropods, and in the cuticles of insects (Chae et al., 2005; Lee et al., 2012). The resulting polycation is soluble in aqueous solutions of small organic acids (Orienti et al., 2002; Ritthidej et al., 2002; Tengamnuay et al., 2000), such as acetic and lactic acids, and can be cross-linked in the presence of polyvalent anions such as phosphates. Furthermore, it is a non-toxic and biocompatible polymer that has known antimicrobial activity (Feng, 2004). Its convenient biological properties (i.e. biodegradability and biocompatibility) have given rise to pharmaceutical and biomedical research over the past years (Giunchedi et al., 1998; Mandala Rayabandla et al., 2010; Mitra and Dey, 2011; Takeuchi et al., 2005).

Recently, the molecule has also caught attention in the medical and dentistry fields, interestingly not for its favorable antimicrobial activity, but actually as a consequence of its encapsulating feature. Chitosan’s encapsulation can be achieved by cross-linking the molecule into micro and/or nano-particles. During this process, the particles being formed trap (on their inside) active components that can then be delivered at specific sites of the body (Giunchedi et al., 1998; Mandala Rayabandla et al., 2010; Zhang et al., 2010). The use of nano or microparticles as drug/protein carriers has recently gained substantial attention,
since they are designed to improve the pharmacological and therapeutic effects by reducing toxic side effects and increasing the lifetime of the active component (Feng, 2004).

Chitosan particles exhibit a pH-sensitive behaviour due to the large number of amino groups on its chains, which makes it suitable transporters of macromolecules such as proteins and peptides. The optimal pH for the release of its contents is between pH 3 – 5 (Aydin and Puylat, 2012). This feature is extremely important from the applicability point-of-view, since major oral complications such as dental caries and dental erosion occur under acidified conditions reflected by pH < 5.0. Future research will focus on describing the release mechanisms of the components encapsulated in our chitosan nanoparticles.

1.8 Hypotheses and objectives

Our first hypothesis was that our new peptides maintain the activity of their original proteins (sthaterin and histatin-3) when duplicated (DR9-DR9) and when conjoint (DR9-RR14).

Our second hypothesis was that CHNP is capable of adsorbing to HA, and at a greater level to AEP-coated-HA.

For this study, our objectives were:
- To test the crystal-inhibitory effect of all our novel peptides, which in other words would mean to test if the activity of DR9 is preserved in all our peptides.
- To evaluate the adsorption of CHNP to HA in the presence and absence of a simulated AEP coating using x-ray photoelectron spectroscopy and confocal microscopy techniques.
1.9 References


CHAPTER 2

Effect of novel salivary peptides on the formation of calcium oxalate crystals

2.1. Chapter summary

Statherin is a salivary protein that modulates biomineralization processes in the oral cavity, such as the deposition of electrolytes onto the dental plaque surface (Raj et al., 1992). Histatins are salivary proteins that exhibit strong antimicrobial activity (Brand et al., 2014; Edgerton and Koshlukova, 2000; Imamura and Wang, 2014; Oppenheim et al., 2012). These proteins (amongst others) help maintain oral homeostasis (Edgar, 1992; Hannig and Balz, 1999; Hannig et al., 2003; Helmerhorst and Oppenheim, 2007; Lendenmann et al., 2000; Siqueira and Dawes, 2011; Siqueira et al., 2012; Vukosavljevic et al., 2014a). Our group has discovered peptides derived from the degradation of salivary statherin and histatin-3: DR9 and RR14, respectively. Both peptides comprise the active site only of the original proteins and maintain their biological functions. We hypothesized that by duplicating (DR9-DR9) and combining these peptides (DR9-RR14) the activity of DR9 would be maintained. The aim of this study was to evaluate the effect of our novel salivary peptides (DR9, DR9-DR9, DR9-RR14 and RR14) on calcium oxalate (CaOx) crystals nucleation and growth onto a smooth mineral (mica) surface. To do so, we promoted the formation of calcium oxalate onto freshly cleaved mica in the absence and in the presence of 0-16 nmol/ml of DR9, DR9-DR9, DR9-RR14 and RR-14. The surfaces containing the precipitated crystals were then imaged by scanning electron microscopy (SEM) and analyzed. As expected, RR14 did not show significant alterations in shape or volume of crystals per microscopic field when compared to the control group (no peptides added). All other tested peptides showed significant inhibitory effect on CaOx. In addition, the shape of the crystals was also drastically affected by the addition of any peptides that contained DR9. In conclusion, our study shows that DR9 does carry the inhibitory effect of its origin protein (statherin). Its activity was not impaired by the combination with other peptide (DR9-RR14) and its duplication (DR9-DR9) has synergistically increased its function.
2.2. Introduction

Calcium oxalate (CaOx) crystals are the primary components of the kidney stones and salivary stones (sialoliths). In addition, calcium oxalate also compose the dental calculus (Socransky et al., 1991; Wahl and Kallee, 1994) together with other types of precipitates such as phosphates, brushite and hydroxyapatite (Yamamoto et al., 1983). Among these, calcium oxalate crystals are the largest crystals present in the dental calculus (Wahl and Kallee, 1994), therefore providing clearer microscopic visualization of its units.

The calculus is formed by nucleation, growth and aggregation of crystals present in saliva that eventually precipitate onto the biofilm surface. Meantime, besides of containing crystal promoters (e.g. calcium and phosphate ions), saliva also contains thousands of proteins, each with specific and distinct functions that largely contribute to oral homeostasis (Carpenter, 2013; Edgar, 1992; Helmerhorst and Oppenheim, 2007; Lendenmann et al., 2000; Vukosavljevic et al., 2011; Wong, 2008).

Statherin, for example, is an acidic phosphoprotein of unique composition that modulates biomineralization processes in the oral cavity (Raj et al., 1992). Its active domain appears to be a 9 amino acid sequence (DpSpSEEKFRL), named DR9, discovered and characterized by our group (Xiao et al., 2015). Another example is histatin 3, which exhibits strong antimicrobial and antifungal activity. Its active domain appears to be a 14 amino acid sequence (RKFHEKHHSHRGYR) also discovered by our group and named RR14. These functional domains are naturally occuring peptides in the oral cavity, products of proteolytic degradation of the original proteins. Despite of being cleaved out from the intact protein, DR9 should be able to modulate biomineralization processes as does the intact statherin, and the RR14 sequence should be able to exhibit the antimicrobial activity of its origin protein histatin-3. Both are very important functions that are closely related to oral health and homeostasis. These findings led our group to the development of the doubled domain peptide DR9-DR9 and the hybrid peptide DR9-RR14, in order to respectively double and combine their functions.

Previous studies analyzed changes in calcium oxalate formed in the presence of citrates and rat osteopontin (OPN) and showed that the crystallization of calcium oxalate (CaOx) can be modified in many aspects by the addition of such compounds (Grohe et al., 2006; O’Young et al., 2009). Therefore, our aim was to analyze structural changes in CaOx, and also to evaluate their precipitation behaviour onto a mineral surface (mica) in the presence and absence of our novel peptides DR9, RR14, DR9-DR9 and DR9-RR14.
2.3. Materials and Methods

**Chemicals and reagents**
Analytical grade calcium nitrate tetrahydrate (Ca(NO$_3$)$_2$·4H$_2$O; Sigma-Aldrich, Steinheim, Germany), sodium oxalate (Na$_2$C$_2$O$_4$; J.T. Baker), sodium chloride (NaCl; J.T. Baker) and sodium acetate (CH$_3$COONa, anhydrous; Sigma) were used as obtained.

**Synthetic peptides**
Four different peptides: DR9 (Sequence: DpSpSEEKFLR, 1270.1 g/mol), RR14 (Sequence: RKFHEKHHSHRGYR, 1875.1 g/mol), DR9-DR9 (Sequence: DpSp-SEEKFLR—DpSpSEEKFLR, 2522.38 g/mol) and DR9-RR14 (Sequence: DpSpSEEKFLR—RKFHEKHHSHRGYR, 3127.29 g/mol) were acquired from ChinaPeptides (Shanghai, China). All peptides presented a purity degree > 95%. In addition, the molecular weight (Mr) of each peptide was verified by mass spectrometric analysis.

**Crystallization experiments**
For the crystallization experiments, calcium and oxalate stock solutions (pH 7.3 and 6.1, respectively) were prepared using deionized water purified with a Milli-Q water system and filtration through a 0.2 mm pore size membrane MF-Millipore™ filters. In addition, aqueous stock solutions of 50 µg/ml of each peptide (DR9, RR14, DR9-RR14, DR9-DR9) were prepared accordingly.

To initiate crystallization, 1 ml of preheated (37°C) solution containing 1 mM calcium nitrate tetrahydrate, 1 mM sodium oxalate, 10 mM sodium acetate and 150 mM sodium chloride were added to preheated (37°C) wells of tissue-culture plates (24-well, FALCON, Becton Dickinson) containing freshly cleaved mica disks (diameter: 9.5 mm, V-1 grade, SPI Supplies). The oxalate solution was added first followed by water and then calcium solution. If peptide was added to the wells, the volume of water was accordingly reduced. After 30 minutes of incubation at 37°C, the disks were rinsed with deionized water and stored in a vacuum desiccator for further drying overnight.

The experiments were conducted with peptide concentrations ranging from 0 to 25 µg/ml (0 to approximately 8 nmol/ml peptide) at pH 6.65–6.75. A total of three replicates for each concentration of each peptide was obtained.

**Imaging and data processing**
Scanning electron microscopy (SEM; LEO 1540XB, Carl Zeiss, Germany) was used to analyze the resulting precipitates over the mica surface without any metal coating, at an acceleration voltage of 1 kV and a working distance of 3.5 - 4.0 mm.

Imaged crystal faces and morphologies were evaluated using crystallographic data (morphologies, crystal symmetry, angles between crystal faces, etc.) available from literature (Grohe et al., 2007; Grohe et al., 2009). X-ray diffraction analyses were not performed.

At least three clear overview images were obtained from each sample. The overviews should not be near the edges of the mica discs, contain visible scratches or impurities. From those images, at least 3 measurements were made for each direction of the crystals.

The crystals were counted and measured as described by Grohe et al. (2009). In brief, <001> and <010> dimensions were measured from {100}-nucleated crystals, and <001> and <100> dimensions were measured from {010}-nucleated crystals. Volumes were calculated as mean area of the {100} face x <010> dimension x number of crystals/mm² (Grohe et al., 2009). The individual mean values (± standard deviation) were obtained from 6 to 8 microscopic fields (3 to 4 from each sample) per calculated volume. To test if sample values are significantly different from corresponding controls, one-way ANOVA and Dunnett’s multiple-comparison tests were carried out.

Figure 2.1 shows the growth habit of calcium oxalate monohydrate (COM) crystals formed under the conditions used in the present study. These are monoclinic penetration twins with {010}, {100}, and {121} faces developed. Also shown in Figure 2.1 are the major crystallographic directions, <010>, <100>, and <001>. Note that lattice-ion addition to {010} and {100} faces results in crystal growth in <010> and <100> directions, respectively; however, ion addition to {121} faces results in growth in <001> directions. The <010> and <100> dimensions of a crystal represent the distance between its {010} and {100} faces, respectively. The <001> dimension is the largest distance between points on the crystal as measured along the <001> axis.

Figure 2.1 Faces and crystallographic directions of calcium oxalate monohydrate crystals.
2.4. Results

The crystals formed were highly prismatic calcium oxalate monohydrate (COM), with <100>, <010>, and <121> faces developed.

Substantial effects on crystal growth habit were observed upon the addition of DR9, DR9-RR14 and DR9-DR9, including inhibition of growth in <100> directions, rounding of edges and significant thinning of {010} faces (Figure 2.2). Calcium oxalate dihydrates (COD) were observed with increased concentrations of peptides, especially DR9-DR9.

Figure 2.2 Effect of peptides on the growth habit of calcium oxalate (COM – calcium oxalate monohydrate; and COD – calcium oxalate dihydrate). Crystallization was performed in absence or in the presence of our novel peptides DR9, DR9-DR9, DR9-RR14, and RR14, and then imaged by scanning electron microscopy. a,b. control (no peptide added). c. RR14 (7.95 nmol/ml). d,e. DR9 (d. 0.90 nmol/ml; e. 7.95 nmol/ml) f,g. DR9-RR14 (f. 0.90 nmol/ml; g. 7.95 nmol/ml) h,i. DR9-DR9 (h. 0.90 nmol/ml; i. 7.95 nmol/ml).

For each effector concentration tested, as well as for control experiments, growth measurements were made in the <010> and <001> directions for at least three crystals nucleated from a {100} face and in the <100> and <001> directions for three crystals nucleated from a {010} face. From the crystal’s measurements and the numbers of crystals
present in the microscopic fields, total volumes of precipitate were estimated (Table 2.1).

Table 2.1. Effect of salivary peptides on calcium oxalate monohydrate precipitation. Concentration of added peptide; D - size of each measured dimension of COM precipitates; * P < 0.05, # P < 0.01 – significantly different from corresponding DR9-DR9 affected precipitate (Dunnet’s multiple comparison test).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>C [nmol/mL]</th>
<th>$D_{100}$ [µm]</th>
<th>$D_{010}$ [µm]</th>
<th>$D_{001}$ [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.03 ± 0.82</td>
<td>6.93 ± 1.28</td>
<td>22.97 ± 2.63</td>
<td></td>
</tr>
<tr>
<td>RR14</td>
<td>0.90</td>
<td>5.28 ± 0.63</td>
<td>6.25 ± 0.73</td>
<td>19.80 ± 0.95</td>
</tr>
<tr>
<td></td>
<td>1.98</td>
<td>6.57 ± 1.02</td>
<td>7.58 ± 0.50</td>
<td>23.69 ± 1.60</td>
</tr>
<tr>
<td></td>
<td>3.97</td>
<td>5.32 ± 1.33</td>
<td>5.83 ± 1.65</td>
<td>18.62 ± 4.58</td>
</tr>
<tr>
<td></td>
<td>7.95</td>
<td>6.94 ± 1.44</td>
<td>6.81 ± 1.36</td>
<td>20.90 ± 3.06</td>
</tr>
<tr>
<td>DR9</td>
<td>0.90</td>
<td>3.77 ± 1.02*</td>
<td>8.13 ± 0.72</td>
<td>21.59 ± 2.08</td>
</tr>
<tr>
<td></td>
<td>1.98</td>
<td>2.50 ± 0.55*</td>
<td>8.30 ± 0.85</td>
<td>21.34 ± 1.75</td>
</tr>
<tr>
<td></td>
<td>3.97</td>
<td>2.30 ± 0.29*</td>
<td>7.36 ± 0.98</td>
<td>17.55 ± 0.87*</td>
</tr>
<tr>
<td></td>
<td>7.95</td>
<td>2.27 ± 0.42*</td>
<td>7.36 ± 0.88</td>
<td>17.79 ± 1.55*</td>
</tr>
<tr>
<td>DR9-RR14</td>
<td>0.90</td>
<td>3.71 ± 1.24*</td>
<td>7.12 ± 0.86</td>
<td>21.05 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>1.98</td>
<td>2.65 ± 0.28*</td>
<td>6.75 ± 1.72</td>
<td>18.57 ± 2.35#</td>
</tr>
<tr>
<td></td>
<td>3.97</td>
<td>2.27 ± 0.30*</td>
<td>6.37 ± 1.14</td>
<td>16.89 ± 1.79*</td>
</tr>
<tr>
<td></td>
<td>7.95</td>
<td>2.08 ± 0.61*</td>
<td>5.82 ± 1.18</td>
<td>16.37 ± 1.77*</td>
</tr>
<tr>
<td>DR9-DR9</td>
<td>0.90</td>
<td>2.79 ± 0.26*</td>
<td>4.58 ± 1.54#</td>
<td>12.45 ± 2.18*</td>
</tr>
<tr>
<td></td>
<td>1.98</td>
<td>1.61 ± 0.30*</td>
<td>2.90 ± 1.64*</td>
<td>7.65 ± 1.54*</td>
</tr>
<tr>
<td></td>
<td>3.97</td>
<td>1.10 ± 0.32*</td>
<td>2.13 ± 0.06*</td>
<td>5.89 ± 0.45*</td>
</tr>
<tr>
<td></td>
<td>7.95</td>
<td>0.82 ± 0.10*</td>
<td>1.85 ± 0.24*</td>
<td>5.16 ± 0.70*</td>
</tr>
</tbody>
</table>

All groups in which crystallization was performed under the presence of peptides containing the statherin derivated DR9 evidently show a decrease of calcium oxalate volume onto a given surface area (Figure 2.3). As expected, RR14 did not show significant changes on the crystals’ growth and precipitation habits when compared to the control group (no peptides added).

The duplicated peptide DR9-DR9 showed most effect over calcium oxalate crystals, not only decreasing the volume of precipitates over the given area, but also inhibiting growth on different dimensions of the crystals.
2.5. Discussion

Approximately 50% of the American adult population has gingivitis, which is the first stage of periodontal disease (Albandar et al., 1999; Brown et al., 1996; Grossi et al., 1994; Grossi et al., 1995; Mandel and Gaffar, 1986; Mandel, 1995; Oliver et al., 1998). Periodontal disease is a multifactorial condition, however an important factor to consider is the presence of dental calculus, which is basically the mineralized dental biofilm that is formed due to the precipitation of electrolytes present in saliva onto the plaque surface. This mineralized layer represents an aggravating factor for the progression of the periodontal disease since its rough surface increases the plaque accumulation (Mandel and Gaffar, 1986; Mandel, 1995) as well as causes mechanical damage on the soft tissue of the periodontium.

The most common precipitates in the dental calculus are the phosphates, such as brushite, dicalcium phosphate dihydrate, octacalcium phosphate and hydroxyapatite (Yamamoto et al., 1983). Calcium oxalate (CaOx) crystals, known for comprising kidney stones and salivary stones, can also be found in saliva and in the dental calculus (Socransky et al., 1991; Wahl and Kallee, 1994). Calcium oxalate crystals are much larger compared to the other types of precipitates in dental calculus, providing a much clearer visualization of its faces by scanning electron microscopy (SEM) imaging (Grohe et al., 2006). For that reason, we chose to work with calcium oxalate instead of other more commonly found crystals in dental calculus. Moreover, previous studies have shown that these crystals have their
formation affected by proteins such as osteopontin (OPN) and its phosphorylated isoforms (bOPN and kOPN) (Grohe et al., 2007; Grohe et al., 2009; Grohe et al., 2011; O'Young et al., 2009). Studies on the inhibition of COM growth demonstrated that OPN, as well as aspartic acid, poly-aspartic acid, and aspartic acid-rich oligopeptides, affects the growth of COM crystal steps in face- and direction-specific fashions (De Yoreo et al., 2006; Orme et al., 2001; Qiu et al., 2004; Wang et al., 2006).

Statherin is a 43-residue acidic proline and tyrosine-rich phosphopeptide of unique composition (Proctor et al., 2005; Raj et al., 1992) that is the only salivary protein capable of modulating primary and secondary precipitation of calcium/phosphate onto the dental surfaces. The negatively charged N-terminus region has been proved crucial for its activity (Raj et al., 1992).

Our group has discovered DR9 (DpSpSEEKFLR), a naturally occurring 9-aminoacid (N-terminal) sequence derived from statherin’s degradation in the oral environment. Being approximately one fifth of the size of the entire statherin molecule, this peptide sequence is less prone to degradation in the oral cavity. It is a negatively charged and phosphorylated molecule, which contributes to its interaction with specific dimensions of the forming calcium oxalate crystals.

It is well established that substances adsorbing to a crystal face will inhibit subsequent growth of that face (Addadi and Weiner, 1985). Our study shows that this small peptide chain possesses potent effect on both the nucleation and growth of calcium oxalate crystals, especially on the <100> dimension and consequently on the {010} face.

We also tested RR14 (RKFHEKHHSHRGYR), another naturally occurring peptide chain derived from histatin-3. No effect was observed on the crystallization process that was performed, which was expected since this peptide’s supposed activity is antimicrobial. Research on this peptide’s activity is being conducted by our group. The purpose of adding this peptide to our experiments was to certify that its combination with DR9 (DR9-RR14 – group 3) did not interfere with DR9’s expected activity. In other words, not only DR9 is a non-toxic and biocompatible molecule, but it could also be combined with other natural protective peptides (i.e. RR14) without having its activity compromised.

The duplicated peptide (DR9-DR9) showed increased inhibitory effect over COM nucleation and growth; interestingly, increased concentrations of this peptide promoted the formation of COD, which is known for forming less aggregates than COM (Wesson et al., 1998).

These data are particularly important for dentistry, since it could potentially lead to
the development of natural oral care products that could ultimately aid in the prevention of some oral diseases.

### 2.6. Conclusion

In summary, our data suggest that our novel salivary peptides that contain DR9 may not only influence the development of calculus by inhibiting nucleation or crystal growth, but that they may also interfere with crystal aggregation by altering the structure of crystals.

Furthermore, this peptide was shown to have considerable influence on the structure of the calcium oxalate crystal formed, tending (in high concentrations) to favor COD formation in preference to COM.

Clinically, the process of calculus formation is certainly multifactorial and critical steps in the process must be identified to propose effective preventative mechanisms. However, the addition of these peptides to daily oral hygiene products may be a valid alternative for future natural prevention of calculus build-up.
2.7. References


CHAPTER 3

Adsorption of chitosan nanoparticles onto hydroxyapatite coated with enamel pellicle

3.1. Chapter summary

Caries is an oral condition that remains a great concern in dentistry and is fundamentally dependent on the dysregulation of biomineralization processes. Acquired enamel pellicle (AEP) is a protective protein layer formed by selective adsorption of salivary proteins to the enamel surface. Individual proteins that comprise the AEP have important physiological properties. Statherin, for example, is a salivary protein that is known for modulating these important biomineralization processes. Unfortunately, the highly proteolytic environment cleaves this and other protective proteins as soon as they are released into the oral cavity. Our group has discovered the peptide sequence called DR9, derived from statherin, that is able to display the same functions as its original protein. However, once released in the oral cavity, it is also subject to further degradation, and consequently, loss of function. In order to overcome that challenge, our group developed the novel delivery system of chitosan nanoparticles. These cross-linked polymers should be able to encapsulate and deliver proteins of interest at specific sites of the mouth. Thus, our aim was to verify if chitosan nano-particles (CHNP) are able to encapsulate our smallest protective peptides (DR9) and bind to both HA and AEP. For this purpose we first promoted blank CHNP (empty particles) adsorption onto a synthetic HA surface and onto an in vitro formed AEP over synthetic HA and analyzed it by x-ray photoelectron spectroscopy (XPS). Results showed that blank CHNP are able to adsorb to both HA and AEP. Next, we used CHNP loaded with Alexa F488-tagged DR9 for the same adsorption experiments, and analyzed the specimens by confocal microscopy imaging. The images proved that DR9 can successfully be encapsulated by CHNP. Furthermore, results showed that CHNP’s adsorption to AEP is greater than its adsorption to the clear HA surface.
3.2. Introduction

Dental caries is a complex multifactorial disease, characterized by the demineralization of the dental tissues. It is initiated underneath dental biofilm when acidogenic microorganisms are exposed to fermentable carbohydrates (Fejerskov, 2004; Kidd and Fejerskov, 2013). This causes the pH in the plaque fluid to drop (down to approximately 5.5), resulting in release of the phosphate and calcium ions from the dental tissue in an attempt to buffer the oral environment. Since its occurrence depends on the combination of many factors (e.g. biofilm, time, diet, saliva, etc), several strategies have been studied and applied in order to help prevent the incidence and/or progression of the disease. Such strategies include dentifrice enrichment with anticariogenic agents, water fluoridation, topical application of high concentration fluoride (gels or varnishes), etc. (Buzalaf et al., 2011; Cardoso et al., 2014; Kidd and Fejerskov, 2013; Pessan et al., 2011; Pessan et al., 2015)

However, according to the World Health Organization (WHO), dental caries still remains a major public health problem in many countries, affecting 60–90% of school-age children and the vast majority of adults.

Another concern in dentistry is the periodontal disease (PD), also a multifactorial disease, usually aggravated by the accumulation of calculus around the dental tissue. The calculus is the mineralized dental biofilm, formed as a result of to the precipitation of electrolytes present in saliva onto the dental plaque surface. This mineralized layer represents an aggravating factor for the development of the periodontal disease because of its rough surface, which increases the biofilm accumulation (Albandar et al., 1999; Brown et al., 1996; Grossi et al., 1994; Grossi et al., 1995). Over 50% of the adult population in the United States is affected by the initial stage of PD, while 35% is affected by chronic periodontitis (Mandel and Gaffar, 1986; Mandel, 1995; Oliver et al., 1998).

Hydroxyapatite (HA) is the main component of the human tooth enamel. In spite of its apparent direct contact with all structures present in the oral cavity, there is a thin, yet important, integument that interposes the enamel and the oral environment: the acquired enamel pellicle (AEP). It has been well established that the AEP is formed by selective adsorption of proteins from saliva, crevicular gingival fluid, microorganisms and mucous cells to the HA surface (Lee et al., 2013; Siqueira et al., 2007a; Siqueira et al., 2012; Zimmerman et al., 2013). Besides acting as a physical-mechanical barrier to the tooth surface, the proteins that compose the AEP have distinct biological functions that protect the dental tissue.
Recent developments in sensitive proteomic methodologies have made possible to obtain a trustworthy characterization of very-low-abundance biological samples. Over 130 proteins have been identified in AEP (Siqueira et al., 2007a). As mentioned above, not only they provide a mechanical barrier between hydroxyapatite (HA) and the oral environment, but they also play key roles in oral homeostasis by regulating (to a certain extent) processes such as demineralization and remineralization, and modulating the early microbial colonization of the dental plaque (Siqueira et al., 2007a; Siqueira et al., 2012; Wong, 2008). Each of these proteins has specific properties that contribute with AEP’s protective character and as a result, classic AEP proteins have been further studied in order to fully understand the mechanisms involved in their functions. Statherin for example is a low molecular weight phosphoprotein that efficiently binds to calcium (Ca^{2+}) ions present in saliva. It is capable of balancing remineralization processes and thus, excessive calcium deposition onto the teeth surfaces. In addition, it is the only salivary protein that is able to modulate spontaneous and secondary precipitation of calcium/phosphate onto the dental surfaces (Raj et al., 1992). Both are crucial processes related to the formation of dental calculus and the remineralization of incipient carious lesions.

DR9 (DpSpSEEKFLR) is a naturally occurring peptide derived from statherin that was discovered by our group. It comprises the active site (N-terminal) of its original protein only and thus is believed to possess the same function of the intact statherin.

Despite of having such desirable properties, these proteins/peptides’ clinical use is jeopardized by the quick degradation they suffer in the highly proteolytic oral environment. Various approaches have been proposed to overcome barriers and to attain better oral bioavailability, including the use of surfactants, permeation enhancers, protease inhibitors, etc (Zhang et al., 2010).

Chitosan is a linear polysaccharide that composed of copolymers of glucosamine and N-acetyl glucosamine. It is obtained by the deacetylation of chitin, which is a characteristic component of the cell walls of fungi, the exoskeletons of arthropods, and the cuticles of insects. Pharmaceutical and biomedical research has been conducted for many years on chitosan due to its advantageous biological properties, such as biodegradability and biocompatibility. Moreover, chitosans are also reported to have antimicrobial properties. Recently, the molecule has also caught attention in the dentistry field, not only for its antimicrobial activity, but as a result of its drug-carrying-system property, which can be achieved by shaping the chitosan molecule into micro and/or nano-particles, encapsulating active components that can then be delivered at specific sites of the body (Giunchedi et al.,...
1998; Mandala Rayabandla *et al.*, 2010; Mitra and Dey, 2011; Zhang *et al.*, 2010). By using this novel carrying system of chitosan nanoparticles (CHNP), protective peptides can be delivered in a specific site of the mouth before being completely degraded by salivary and bacterial enzymes.

Previous studies have shown that chitosan molecules are able to adsorb to various surfaces, including hydroxyapatite (Guo and Gemeinhart, 2008; Lee *et al.*, 2012). However, the behavior of chitosan nanoparticles in relation with oral integuments has not yet been tested.

In this study we evaluated the adsorption behavior of blank and loaded CHNP to simulated dental integuments using X-ray photoelectron spectroscopy and confocal microscopy.

### 3.3. Material and Methods

Three healthy (systemically and oraly) individuals, from both genders ranging from 18 to 35 years old, were selected to participate in this study. All volunteers gave their informed consent to saliva donation, in agreement with the Health Sciences Research Ethics Board of the University of Western Ontario (16181E). Stimulated (sugar-free hard candy) parotid secretion was collected using a special intraoral device. Samples were kept on ice during the collection and total protein concentration was measured by the Bicinchoninic Acid Assay (BCA, Pierce Chemical, Rockford, IL, USA) with bovine serum albumin used as standard. The samples acquired from the 3 subjects were then pooled and kept at -20°C.

**Chitosan nanoparticles**

Chitosan nanoparticles (0.5%) were obtained by ionic gelation. Chitosan was dissolved in acetic acid (1.75 times the concentration of chitosan) and Tween-80 (0.5%). Next, the cross-linker sodium tri-polyphosphate (STPP) solution (w/v) was added to the chitosan solution at a concentration of 0.84 mg/ml, and stirred overnight. Zeta sizer was employed for characterization of size range of the chitosan particles. A range particle size of 10-15 nm was observed. This was performed by the PhD candidate Rajesh Kumar Gupta, who is currently standardizing the CHNP construction process.

The solution containing the nanoparticles was aliquoted in 2 mL microtubes and then centrifuged at 16,000 x g for 35 minutes and the supernatant was removed, leaving a wet CHNP pellet at the bottom of the tubes. The wet pellets were resuspended in 1 mL of 1 mM
potassium phosphate buffer (PPB) and sonicated for 5 minutes prior to use.

**sAEP and CHNP adsorption**

Hydroxyapatite discs (3 mm radius, Clarkson Chromatography products, Inc.) were carefully cleaned with a previously described protocol (Grohe et al., 2006) in order to assure that no foreign residue would interfere with our adsorption protocol. The HA discs were cleaned by 10 minutes of sonication in a series of solutions (distilled water, methanol, acetone, hexane, acetone, methanol and isopropanol respectively). After dried, the discs were then treated with oxygen plasma at low pressure for approximately 15 minutes to ensure that possibly remaining particles were removed from the surface.

Four groups were prepared: 1- Pure HA (no treatment); 2- HA + sAEP; 3- HA + CHNP; and 4- HA + sAEP + CHNP. Two discs of each group (except group 1) were separately placed into a 24 multi-well plate, and incubated with one drop (that covered the entire discs’ surface) containing 100ug of either parotid saliva (sAEP) or CHNP (for 120 minutes and 60 minutes, respectively) at room temperature.

After incubation, the samples were washed in 1 mL of deionized water and allowed to dry overnight in a vacuum desiccator. For group 4, in which both treatments were performed, the wash and overnight dry took place in between and after treatments.

**X-ray Photoelectron Spectroscopy (XPS)**

The XPS analyses were carried out with a Kratos Axis Ultra spectrometer using a monochromatic Al K (alpha) source (15 mA, 14 kV). XPS can detect all elements except hydrogen and helium, probes the surface of the sample to a depth of 5–10 nm, and has detection limits ranging from 0.1 to 0.5 atomic percent depending on the element. The instrument work function was calibrated to give a binding energy (BE) of 83.96 eV for the Au 4f7/2 line for metallic gold and the spectrometer dispersion was adjusted to give a BE of 932.62 eV for the Cu 2p3/2 line of metallic copper. The Kratos charge neutralizer system was used on all specimens. Survey scan analyses were carried out with an analysis area of 3006700 mm and a passing energy of 160 eV. Spectra were analyzed using CasaXPS software (version 2.3.14).

**Fluorescence labeling, CHNP encapsulation and adsorption**

DR9 was labeled with the green Alexa fluorochrome according to the manufacturer's recommendations. Briefly, 5 µL of AlexaFluor-488 in dimethylformamide (10 µg/µL; high
performance liquid chromatography grade, 99.9 %) was added to 200 µL of polypeptide in phosphate-buffered saline (2.5 mg/ml) and 20 µL of 1 M disodium carbonate (Na$_2$CO$_3$), pH~8.3, and incubated for 1 h at room temperature. Unconjugated label was removed by dialysis through 4 changes of 2 L of Tris-buffered saline, pH 7.4, for 4 h each using 3.5-kDa dialysis tubing (Spectra/Por 3, Spectrum Laboratories, Rancho Dominguez, CA).

For the encapsulation, the tagged-DR9 solution was simply mixed with the chitosan (0.5%) solution and CHNP were constructed as described above.

The same adsorption protocol described above (for XPS analysis) was also performed over synthetic HA discs, but with the loaded CHNP. To avoid losing fluorescence, the labeling, encapsulation and adsorption were performed in the dark.

**Confocal microscopy imaging**

A 63× oil-immersion objective, a 90/10 mirror as a beam splitter and LSM 510 DUO Vario software (Carl Zeiss) were used. All procedures were carried out in the dark to avoid the effects of scattered light on the loaded chitosan nanoparticles imaging and to prevent the fluorochrome from bleaching. The focus was adjusted before and during image acquisition, if required. To excite the fluorochrome, a krypton/argon laser ($\lambda = 488$ nm) was used. Fluorescence images were obtained using a BP 500-550 IR emission filter (Chroma Technology, Rockingham, VT). Gain and offset were adjusted to provide optimal contrast, and the pinhole was adjusted accordingly (256-360).

### 3.4 Results

One representative XPS spectrum of each group is shown in Figure 3.1

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Figure 3.1 representative XPS wide scan spectrum of A. hydroxyapatite disc; B. hydroxyapatite surface coated with sAEP; C. hydroxyapatite surface coated with blank chitosan-nanoparticles. D. hydroxyapatite disc coated with sAEP followed by additional CHNP coating with blank chitosan-nanoparticles.
The widescan spectrum of the control specimens shows the presence of C 1s (280.85 eV), Ca 2p (343.85 eV) O 1s (527.25 eV) and P 2p (129.65 eV). The HA discs undergone any of the treatments show additional N 1s peak at 396.35 eV binding energy, which could indicate a bond made by nitrogen in amino groups.

The HA discs’ surface composition was markedly changed upon exposure to the chosen treatments. XPS determination of elemental HA discs’ surface composition, as well as of the adsorbing AEP, CHNP and the combination of both (AEP+CHNP) is shown in Table 3.1.

Table 3.1 XPS-determined atomic percentages of the components of the surface of clear hydroxyapatite (HA) discs and hydroxyapatite (HA) discs treated with: chitosan nano-particles (CHNP), human parotid saliva as simulated acquired enamel pellicle (sAEP), and the combination of sAEP and chitosan nano-particles (PS+CHNP).

<table>
<thead>
<tr>
<th>Samples</th>
<th>C</th>
<th>Ca</th>
<th>N</th>
<th>O</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>23.5</td>
<td>17.3</td>
<td>-</td>
<td>28.5</td>
<td>5.3</td>
</tr>
<tr>
<td>HA</td>
<td>23.4</td>
<td>16.8</td>
<td>-</td>
<td>29.0</td>
<td>5.3</td>
</tr>
<tr>
<td>HA+sAEP</td>
<td>51.2</td>
<td>5.4</td>
<td>8.2</td>
<td>25.8</td>
<td>2.4</td>
</tr>
<tr>
<td>HA+sAEP</td>
<td>52.2</td>
<td>4.2</td>
<td>9.8</td>
<td>25.0</td>
<td>2.1</td>
</tr>
<tr>
<td>HA+CHNP</td>
<td>24.6</td>
<td>15.0</td>
<td>-</td>
<td>49.3</td>
<td>9.7</td>
</tr>
<tr>
<td>HA+CHNP</td>
<td>38.2</td>
<td>8.4</td>
<td>4.1</td>
<td>38.1</td>
<td>6.9</td>
</tr>
<tr>
<td>HA+sAEP+CHNP</td>
<td>54.8</td>
<td>2.3</td>
<td>8.4</td>
<td>30.0</td>
<td>3.8</td>
</tr>
<tr>
<td>HA+sAEP+CHNP</td>
<td>55.4</td>
<td>2.4</td>
<td>8.9</td>
<td>27.5</td>
<td>2.9</td>
</tr>
</tbody>
</table>

The analyzed surfaces (10 nm in depth) showed a significant decrease in percentage of calcium, and phosphate in groups 2 (HA+sAEP) and 4 (HA+sAEP+CHNP). Group 3 (HA+CHNP) showed the most inconsistent results.

Our second set of experiments (loaded-CHNP’s adsorption to coated and non coated HA) was evaluated by analysis of the confocal microscopy images obtained from different areas of each disc. The confocal laser microscope was able to detect a distinct green signal (from chitosan nanoparticles loaded with alexa-tagged DR9), confirming that the encapsulation process had successfully occurred. Groups 1 and 2 (HA and HA+sAEP, respectively) showed absence of signal (Figure 3.2; A and B), which was expected since these groups did not contain CHNP and, therefore, no fluorescent tag.

A higher signal can be observed on group 4 (Figure 3.2;D). In addition, this CHNP adsorbed layer appeared to be uniformly distributed all over the specimen’s surface.
Figure 3.2 Confocal micrograph images of A. hydroxyapatite disc; B. hydroxyapatite disc coated with simulated acquired enamel pellicle; C. hydroxyapatite disc coated with loaded (DR9-Alexa) chitosan nanoparticles (2 hours). D. hydroxyapatite disc coated prior with simulated acquired enamel pellicle (2 hours) followed by additional coat (1 hour) of DR9-loaded chitosan nanoparticles.

3.5 Discussion

Salivary proteins have gained special attention over the past years due to their vast and effective properties in preventing and/or mitigating certain oral diseases. Although their functions have been long known and described, their clinical applications are still a great challenge for researchers around the world.

Our group has been studying chitosan nanoparticles as a novel carrying and delivery system for salivary proteins/peptides, which seem to be a fairly good “capsule” for protecting our salivary proteins of choice against salivary or bacterial enzymes.

On this study, we focused on testing the adsorption of the chitosan nanoparticles to clear and to AEP-coated hydroxyapatite.

Previous studies have shown that chitosan molecules are able to adsorb to various surfaces, including hydroxyapatite (Guo and Gemeinhart, 2008; Lee et al., 2012). However, the behavior of chitosan nanoparticles in relation with oral integuments (e.g. hydroxyapatite and acquired pellicle) was still unknown.

As stated, an additional N 1s peak (at 396.35 eV) was observed in coated groups (2, 3 and 4), which is consistent with nitrogen present in both CHNP and AEP. Moreover, a higher concentration of oxygen (O 1s) can be seen upon chitosan adsorption, especially in
the functionality expressed in XPS at 527.95 eV and as a result of the presence of –C–O–groups. In our study, O/N ratios were 2.82 for acquired enamel pellicle coated HA (group 2), 5.26 for CHNP coated HA (group 3), and finally, 3.32 for CHNP+AEP-coated HA (group 4). This is again closer to theoretical values, proving that the performed treatments did change the surface being analyzed (10 nm depth). Based on our XPS results, CHNP do adsorb to both coated and non-coated HA, although at different levels.

Nonetheless, it is worth to note that the results obtained from group 3 (HA+CHNP) demonstrated the most inconsistent values among the studied groups. A non-uniform coat of CHNP could be the explanation for such results, but this could only be confirmed by a visual parsing. This led us to our next set of tests with confocal microscopy imaging. This technique was chosen because of the possibility of answering two other queries at once: is the DR9 encapsulation possible with nanoparticles ranging from 10 to 15 nm; how does the loaded CHNP coating look over clear HA surface and over AEP-coated HA surface.

To do so, the peptides had first to be tagged with the fluorescent tag (Alexa F488, Sigma-Aldrich, Oakville, Canada). The solution containing the tagged peptides was bright neon yellow and, once encapsulation took place, the final product was a completely transparent solution. This was the first suggestion that the peptides had been successfully encapsulated by the chitosan nano-particles. Samples from groups 1 and 2 (clear HA and AEP-coated HA, respectively) were used as controls, since no signal was supposed to be detected from their surfaces. After observing each sample under the laser microscope, we had the confirmation that encapsulation did occur. The fluorescent signal from the encapsulated peptides was high and clear.

The CHNP coating over clear HA (group 3) was not uniform and had different thicknesses throughout the surface. This explains this group’s conflicting results obtained from the XPS data and confirms the hypothesis of an incomplete/sparse bind of CHNP to HA. The signal observed on group 4 (CHNP+AEP-coated HA) was expressively higher. Furthermore, the CHNP coating over AEP-coated HA was evenly spread over the surface. This suggests that the chitosan nanoparticles’ adsorption to salivary proteins is greater than its adsorption to a clear HA surface. This outcome can be explained by physical parameters, such as net charge of the AEP and chitosan nanoparticles. Acquired enamel pellicle presents a negatively charged film, while our chitosan nanoparticles present a positively charged surface.

These results provide a significant appeal to the preventive dentistry area, since the teeth surfaces are always covered by AEP, and thus are prone to the adsorption of CHNP.
containing protective proteins/peptides. This approach could aid in the prevention of dental caries and/or dental calculus formation.

### 3.6 Conclusion

The present study proves that CHNP are able to bind to both HA and AEP-coated-HA. However, their bind to the surface is significantly improved when HA is previously coated with salivary proteins. This has significant clinical importance since it mimics the real scenario in the oral cavity of having the salivary proteins cover all teeth surfaces. Future research will focus on verifying and describing the mechanisms involved in the protein/peptides release from the CHNP. Our results bring us one step closer to developing a reliable novel carrying/delivery system for proteins/peptides, increasing their bioavailability on the desired site of action, which may ultimately assist in the prevention of some oral diseases.
3.7 References


CHAPTER 4

4.1 General Conclusions

Dental caries and periodontal disease still remain major public health problems around the world. This fact reinforces the need for the development of novel preventative treatments.

Advances in laboratorial instruments have enabled scientists to gain micro-scale imaging of samples of interest (e.g. scanning electron microscopy) and allow for analysis of surface composition of a material (e.g. x-ray photoelectron microscopy). These technological advancements can now be applied to the salivary biology research field to help develop effective preventive treatment options for oral diseases.

Over 2290 salivary proteins have been identified in the complex salivary fluid, 130 of which are involved in the formation of the acquired enamel pellicle (Siqueira et al. 2007a). The protein composition along with the physical properties of both saliva and AEP provides protection against dysregulated demineralization (i.e. dental erosion and/or dental caries) and remineralization over the dental tissues (i.e. dental calculus formation).

Our group discovered naturally occurring peptides derived from statherin and histatin 3. Statherin modulates biomineralization processes while histatins display strong antimicrobial activity. Both these functions are closely related to the development of dental caries, calculus formation and oral infections (e.g. oral candidiasis). The statherin peptide was named DR9 and the histatin peptide, RR14.

For this study we synthetically designed combination of these peptides that could possibly have combined or enhanced activities (DR9-RR14, DR9-DR9). Gaining strong understanding about these peptides’ therapeutic potential is essential for researchers to develop natural oral hygiene products with enhanced protective properties.

The study described in Chapter 3 shows the in vitro effects of our novel peptides on calculus formation. We demonstrated for the first time that DR9 retains the important mineralization modulation function of its mother protein sthaterin. In other words, our DR9 peptide and the synthetic peptides combinations are able to inhibit crystal formation and precipitation onto a mineral surface.

Chapter 4 described a possible tool for increasing the bioavailability of these peptides in the oral cavity, our novel delivery chitosan nanoparticles (CHNP) system. Through this in vitro study we were able to prove that these particles adsorb to the dental tissue (hydroxyapatite) and to acquired enamel pellicle (sAEP-coated-HA) at a greater and
more uniform level. We were also able to show that encapsulation of DR9 in CHNP was successfully achieved.

4.2 Limitations

The limitations of this study are:

- The use of calcium oxalate and mica for the simulation of dental calculus. Calcium oxalate was chosen because of its size, which provided clear visualization of its units. However, these crystals are not always present in dental calculus. In addition, we used mica as a plain mineral surface for the precipitation of the crystals. However, an enamel-like surface such as hydroxyapatite would be more appropriate to mimic the dental tissue. Future research should focus on analyzing the effect of our peptides in other more abundant types of precipitates in dental calculus (e.g. hydroxyapatite, brushite, etc) over enamel-like surfaces (e.g. synthetic hydroxyapatite, ex vivo bovine or human enamel).

- The small sample size for XPS. In this study we have analyzed the surface composition of two samples per group. Group 3 (HA+CHNP) showed conflicting results, which could not be explained with certainty due to the small number of samples in each group. Larger sample sizes should be used for more significant and trustworthy results.

- Our experiments were all in vitro. In situ and in vivo/clinical trials should be carried out in the future in order to confirm these findings.

- We found that the activity of DR9 was not compromised by its combination with RR14. However, we have not assessed the activity of RR14 in. Another student from our group is currently testing its activity.

4.3 Future directions

The full exploration and understanding of our peptides and how they interact with the oral environment is still ongoing. Current research from our group is focusing on testing the activity of the histatin derivated peptide RR14 and its combination with DR9 (DR9-RR14), and on understanding and describing the release mechanisms of the components encapsulated in our chitosan nanoparticles.
Our DR9-containing peptides appear to be a promising alternative for regulating crystal precipitation. Moreover, our CHNP showed signs of success in relation to encapsulation of the protective agents (peptides) and adsorption to the surfaces of interest (synthetic HA and simulated AEP). These results contribute to our group’s goal of developing new oral hygiene product using natural protective components.

4.4 Final conclusions

This study is an initial step towards our final goal of using these peptides and the CHNP delivery system in preventive dentistry. Although these are early stages of a major research project, the results obtained herein are favorable and promising. The data presented here will serve as a base for the steps to follow.
5 Appendix

Appendix A: Health Sciences Research Ethics Board approval (16181E)

Office of Research Ethics
The University of Western Ontario
Room 4180 Support Services Building, London, ON, Canada N6A 5C1
Telephone: (519) 861-3036 Fax: (519) 850-2466 Email: ethics@uwo.ca
Website: www.uwo.ca/research/ethics

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. W.L. Siqueira
Review Number: 16181E
Review Date: March 24, 2010

Review Level: Expedited
Revision Number: 1
Approved Local # of Participants: 100

Protocol Title: Composition, Structure and Function of Salivary Proteins
Department and Institution: Dentistry, University of Western Ontario
Sponsor: NSERC-NATURAL SCIENCES ENGINEERING RSRC COU

Ethics Approval Date: April 20, 2010
Expiry Date: June 30, 2016
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;

b) all adverse and unexpected experiences or events that are both serious and unexpected;

c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.
Curriculum Vitae

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Honours and Awards:
2007-2009 – Recipient of FAPESP (Fundacao de Amparo a Pesquisa do Estato de Sao Paulo) undergraduate scholarship.

2011-2013 – Recipient of FAPESP (Fundacao de Amparo a Pesquisa do Estato de Sao Paulo) graduate scholarship.

2012-2013 – Recipient of BEPE/FAPESP (Bolsa Estagio de Pesquisa no Exterior/Fundacao de Amparo a Pesquisa do Estato de Sao Paulo) exchange graduate scholarship.

2013-2015 Province of Ontario Graduate Scholarship

2009 - Myaki Issáo award for poster presentation of the study “Effect of abrasion of enamel previously eroded with light Cola drink: in situ study.” on SBPqO (Brazilian society of dentistry research)


Publications:
