A peptide coating preventing the attachment of *Porphyromonas gingivalis* on the surfaces of dental implants

by

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Abstract

Background:

Most late implant failures are related to peri-implantitis, which is an inflammatory condition involving both soft and hard tissues surrounding the implant. Various factors contributing to peri-implantitis have been identified, such as poor oral hygiene, a history of periodontitis, smoking, and diabetes. However, its primary cause is the microbial biofilm. Most common microorganisms associated with peri-implantitis are gram negative anaerobes, such as *Porphyromonas gingivalis, Tannerella forsythia*, and *Prevotella intermedia*. Moreover, current treatments, including nonsurgical and surgical interventions, are usually unsatisfactory. Therefore, prevention of biofilm formation has been intensively investigated in implant dentistry.

Recently, two peptides that prevent bacterial attachment to surfaces were designed and preliminary tested. They were shown to inhibit the attachment of *Escherichia coli* on the titanium surface and promote proliferation of mammalian cells. However, *Escherichia coli* are not common pathogens associated with peri-implantitis.

Objective:

The objective of this study was to investigate whether these two hexapeptide coatings could prevent the adhesion of *Porphyromonas gingivalis*, one of the key human pathogens associated with peri-implantitis, on the surfaces of dental implants.

Materials and Methods:

Part I preliminary studies: 1) *Porphyromonas gingivalis* were seeded on titanium discs at different densities: 10^3/ml, 10^4/ml, 10^5/ml, and 10^6/ml. After incubation under anaerobic

conditions overnight, the concentration of bacteria was quantified by spectrophotometer readings. 2) Discs with or without hexapeptide coatings were placed on agar seeded with *Porphyromonas gingivalis.* Plates with filter-paper discs soaked in penicillin and streptomycin and plates without *Porphyromonas gingivalis* were included as positive and negative controls, respectively. After incubation under anaerobic conditions for 48 hours, the zone of inhibition was measured. The agar in contact with the surfaces of the discs was harvested and cultured under anaerobic conditions for 24 hours and the number of *Porphyromonas gingivalis* were quantified. Agar which had only bacteria growing was used as negative control. 3) Titanium discs were coated with two novel peptides (hexapeptide 1 and 2). *Porphyromonas gingivalis* were seeded on the discs. After incubation under anaerobic conditions overnight, bacteria were detected with red fluorescent dye and semi-quantified by fluorescence intensity.

Part II: A salivary pellicle was created on the surfaces of hexapeptide 1-coated bare discs and verified with anti-human immunoglobulin G, A and M, and anti-fibrinogen. Early colonizers, *Veillonella parvula* and *Streptococcus sobrinus*, and the later colonizer, *Porphyromonas gingivalis,* were labeled with green and red fluorescent dyes, respectively, and seeded on the discs. Bacterial attachment was semi-quantified by fluorescence intensity.

Results:

In part I preliminary studies: 1) Wells with 10^5/ml of *Porphyromonas gingivalis* had a significant higher bacterial load after overnight incubation when compared with the ones with 10^3/ml and 10^4/ml of *Porphyromonas gingivalis*. However, there was comparable

bacterial load when seeding at 10^6/ml. Therefore, 10^5/ml was considered the optimal seeding density and was used for subsequent experiments.

2) No zone of inhibition was observed around the titanium discs with/without hexapeptide coatings. After culturing the agar contacting the discs, as expected, no bacteria grew out from the antibiotic control disc. The titanium discs with/without hexapeptide coatings had comparable bacterial growth to the control well which had only bacteria growing, indicating that the hexapeptide coatings do not have antimicrobial effects.

3) Hexapeptide 1 coating significantly reduced the attachment of bacteria, while hexapeptide 2 coating resulted in comparable bacterial attachment as control group. We concluded that coating with hexapeptide 1, but not hexapeptide 2, significantly reduced the attachment of *Porphyromonas gingivalis*. Therefore, part II of the thesis was focused on hexapeptide 1.

In part II, we found that the salivary pellicle was evenly distributed on the discs, with or without the peptide coating, with an average thickness of 3.84 μm. These results show that the hexapeptide-1 coating does not disturb the normal formation of the salivary pellicle. A multi-species dental biofilm was created on the salivary pellicle. The peptide-coating resulted in an approximate 25% reduction in the attachment of *Veillonella parvula* and *Streptococcus sobrinus*, and a 50% reduction in *Porphyromonas gingivalis*, when compared to control, uncoated implant discs.

Conclusion:

The novel hexapeptide coating does not kill bacteria, but can inhibit the attachment of *Porphyromonas gingivalis* and prevent the formation of the dental biofilm. It may have the potential to impede the development of peri-implantitis.

Preface

This thesis is an original work by Dongdong Fang. The preliminary studies on the optimization of bacterial seeding density and the antimicrobial effects of hexapeptides were performed by Raisa Catunda and Ji Min Lim (Part I; 1&2).

The protocol of the research project in this thesis has been approved the University of Alberta Research Ethics Board, "Effect of peptide coating of dental implant on the formation of the dental plaque", No. 00083117.

I was responsible for the literature review and manuscript writing. Both Dr. Levin and Dr. Febbraio were the supervisory authors and were involved with concept formation and manuscript writing.

The part II of manuscript has been published as D. Fang, S. Yuran, M. Reches, R. Catunda, L. Levin, and M. Febbraio. A peptide coating preventing the attachment of *Porphyromonas gingivalis* on the surfaces of dental implants. *J Periodontal Res*. 2020 Feb 24. I participated in the study design, conducted experiments, analyzed the data, and drafted the manuscript. R. Catunda was involved in the study design and conducted preliminary experiments. S. Yuran and M. Reches were involved in the concept of the study, provided the peptide, and revised the article critically. L. Levin and M. Febbraio supervised this study, and were responsible for the concept/design, analyzed the data, revised the manuscript critically.

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List of Abbreviations

A. a	*Aggregatibacter actinomycetemcomitans*
°C	Celsius
AMPs	Antimicrobial peptides
aPRP1	Acidic proline-rich protein 1
ATCC	American Type Culture Collection
AU	Arbitrary unit
BL	Bone loss
BOP	Bleeding on probing
CHX	Chlorhexidine
CIST	Cumulative interceptive supportive therapy
CTRL	Control
DOPA	3,4-dihydroxyphenylalanine
DTT	Dithiothreitol
E. coli	*Escherichia coli*
Er:YAG	Erbium doped yttrium aluminum garnet
F. nucleatum	*Fusobacterium nucleatum*
FITC	Fluorescein isothiocyanate
FPD	Fixed partial denture
G	Gram
Hexa	Hexapeptide
Hexa	Hexapeptide
hLF1-11	Human lactoferrin-derived peptide 1-11
IAN	Inferior alveolar nerve
Ig GAM	Immunoglobulin G, A, and M
K_2CO_3	Potassium carbonate
LraI	Lipoprotein receptor antigen
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar
N	Number
NaCl	Sodium chloride
NHANES	National Health and Nutrition Examination Survey
nm	Nanometer
OD	Optical density
P. gingivalis	*Porphyromonas gingivalis*
P. intermedia	*Prevotella intermedia*
P. nigrecens	*Prevotella nigrecens*
PBS	Phosphate buffered saline

PD	Probing depth
PEN	Penicillin
PLL-g-PEG	Poly(L-lysine)-grafted-poly(ethylene glycol)
RGB	Arginine-Glycine-Aspartic acid
S. sobrinus	*Streptococcus sobrinus*
SB	Schaedler Broth
SLA	Sandblasting-large grit-acid etching
Staph. anaerobius	*Staphylococcus anaerobius*
Staph. aureus	*Staphylococcus aureus*
Strep. intermedius	*Streptococcus intermedius*
Strep. mitis	*Streptococcus mitis*
T. denticola	*Treponema denticola*
T. forsythia	*Tennerella forsythia*
T. socranskii	*Treponema socranskii*
TiO_2	Titanium dioxide
V. parvula	*Veillonella parvula*
ZnO	Zinc oxide
µg	Microgram
µl	Microliter
µm	Micrometer

Chapter 1 Introduction

Dental implant is defined as “any object or material, such as an alloplastic substance or other tissue, which is partially or completely inserted or grafted into the body for therapeutic, diagnostic, prosthetic, or experimental purposes”(1). It has been widely used to replace missing teeth or as an anchorage in orthodontic treatment.

Rationale of dental implant

Due to the increasing aging population, the demand for dental implants is growing and the dental implant market is expanding rapidly. The National Health and Nutrition Examination Survey (NHANES) study reported that more than half of Americans aged 20-64 had experienced at least one tooth loss, and 23% of adults aged above 65 were edentulous in the years 2005-2008(2). Canadian Health Measures Survey 2007-2009 showed that 6.4% of Canadian adults were edentulous with 21.7% in aged group (60-79 years old) and 14.6% of dentate adults had less than 21 teeth(3). The World Workshop of Periodontology in 2017 reported that overall annual tooth loss is 0.2 teeth/year. This increases with age and is doubled in patients diagnosed with periodontitis(4). Additionally, according to statistics from the American Dental Association, over five million implants are placed in the United States each year, with an annual growth of 9.1%(5, 6).

Dental implants have some advantages over other restorative modalities. Firstly, implant supported restoration is a rather predictable therapy with relatively high success rates. Studies reported up to 96.4% 10-year survival rate(7) and 91.5% survival at 16-22 years follow-up(8). Even implants placed in well treated and maintained periodontally compromised patients have shown a 10-year survival rate of up to 90%(9). Fixed partial

denture (FPD), which is another commonly used modality for tooth replacement, has a somewhat lower survival rate in some reports (89.1% at 10 years(10) and 74% at 15 years(11)) and an estimated life-span of 10 years(11). Moreover, preparation and crown of the abutment teeth significantly increases the rate of caries and endodontic issues, leading to the failure of the FPD(11, 12). Splinting of teeth compromises hygiene control, which predisposes the abutments to periodontal diseases. With carefully planned and executed implant treatment, there might be lower risks of caries and endodontic problems for the adjacent teeth, and improved ability for oral hygiene control, since they do not involve the adjacent teeth(13). In addition, implants have the benefits of esthetics, bone maintenance, and patient's psychological health. Due to these advantages, dental implants have been one of the most common treatments for teeth replacement in the last decates. Implants also have some disadvantages, such as higher initial cost, more traumatic procedures, and prolonged whole process and wound healing(14).

Success and complications of dental implant

In 1986, Albrektsson and Zarb proposed the criteria to evaluate the long term outcomes of dental implants(15). They included various parameters, such as implant mobility, radiographic changes, vertical bone loss, and other signs and symptoms (see Table 1). These are the most commonly used criteria to determine osseointegration and implant success.

Table 1:

Success criteria of dental implants (Albrektsson and Zarb 1986)(15)	
1	No mobility when tested clinically
2	No peri-implant radiolucency.
3	Vertical bone loss is < 0.2 mm annually following the implant's first year of service.
4	Absence of persistent and/or irreversible signs and symptoms such as pains, infections, neuropathies, paresthesia, or violation of the mandibular canal.

Table 2:

Most commonly used parameters for implant success criteria(16)	
Categories	**Parameters**
Implant level	Pain, mobility, radiolucency Bone loss <1.5 mm at the first year Annual bone loss <0.2 mm after the first year Infection
Peri-implant soft tissue	Probing depth Suppuration Bleeding Swelling Plaque index Recession Width of keratinized tissue
Prosthetic level	Esthetics Functions Minor or major complications Failures
Patient level	Discomfort/paresthesia Ability to chew/taste General satisfaction Esthetics

With the evolution of implant dentistry, some other parameters have been added to the success criteria, including peri-implant soft tissue, esthetics, and patient's satisfaction(16). A systematic review by Papaspyridakos *et al.* summarized the most commonly used parameters for success criteria, which are categorized into four groups(16) (see Table 2). The reported success rate varies from 73.4%(17) to 100%(18) with an average of 89.7%(19). The new World Workshop of Periodontology proposed the definition of peri-implant health as 1) Absence of visual signs of inflammation; 2) No profuse bleeding on probing (BOP); 3) No increase in probing depth; 4) Absence of bone loss following initial healing(20).

Complications of dental implants are roughly grouped into three categories: mechanical, esthetic, and biological(21, 22). The prevalence and incidence of complications increase as the number of implants being placed grows(22, 23).

Mechanical complications are mainly attributed to biomechanical overloading. There are various contributory factors, including implant malposition, inadequate supporting bone, and bruxism(21). Screw loosening is one of the most common mechanical complications. The incidence is approximately 2.29% at 1 year and 10.8% at 5-year follow-up(22), and could reach up to 59.6% at 15-year follow-up(24). Fracture of implant or screw is another common complication associated with biomechanical overloading. It tends to occur in implants with smaller diameters and vertical bone loss(25). Additionally, fractures of the veneer or the framework of the FPD also occur frequently for implant-supported restorations.

Esthetic outcome has become an essential criterion for implant success. Esthetic complications include loss of inter-implant papilla, gingival recession, metal exposure, and

poor emergence profile of crown(26). Several etiological factors have been reported underlying esthetic complications, such as poor pre-surgical design, inadequate bone, implant malposition, and inadequate provisional restoration(26). Currently, due to the development of digital dentistry and new materials, the incidence of esthetic complications has somewhat decreased (22).

Biological complications are infection or inflammation resulting from bacterial accumulation, and sensory disturbances caused by injury to a critical structure(21, 25). Peri-implant diseases are the most common biological complications of dental implants. They are classified into peri-implant mucositis and peri-implantitis. The etiology, diagnosis, treatment, and prognosis are discussed in detail in the next section. Another common and serious complication is injury of the inferior alveolar nerve (IAN) in the mandible(27). The incidence of IAN injury varies from 0 to 40%(28). Numbness and paresthesia are the most common symptoms(28, 29), but other sensation alterations occur as well, such as allodynia, hyperaesthesia, and hypoaesthesia(28). Implant surgery could also injure other vital structures, like the maxillary sinus, lingual nerve and artery, nasopalatine foramen, and nasal cavity(25).

Table 3

Complications of dental implant	
Category	Complications
Mechanical(21)	• Screw loosening • Fracture of implant or screw • Cement failure • Fracture of the veneer • Fracture of framework of implant supported FPD
Esthetic(26)	• Loss of inter-implant papilla • Gingival recession • Metal exposure • Poor emergence profile
Biological(21)	• Peri-implant mucositis • Peri-implantitis • Sensation alterations

Peri-implant diseases

Peri-implant diseases refer to inflammatory conditions of supporting tissues surrounding dental implants. They can be classified into two categories: peri-implant mucositis and peri-implantitis(30). Peri-implant mucositis was defined as "an inflammatory lesion of mucosa surrounding an implant without loss of supporting peri-implant bone" at the first European Workshop of Periodontology(30, 31). The typical symptoms include inflammation (redness, swelling, and BOP), increased probing depth (PD), with no evidence for supporting bone loss(20). The reported prevalence varies from 19% to 65%, with an average of 43%(32). Peri-implantitis is not only characterized by soft tissue inflammation, but also involves the supporting bone around the dental implants(30). The diagnosis of peri-implantitis requires 1) soft tissue inflammatory signs; 2) increased PD; 3) progressive bone loss, or presence of bone loss ≥3mm and/or PD ≥ 6mm with profuse BOP(20). Peri-implant mucositis has been regarded as the precursor to peri-implantitis, however, the mechanisms of this conversion remain poorly understood(33). Costa and colleagues investigated 80 patients with diagnosed peri-implant mucositis retrospectively, and found that 43% of patients without regular maintenance converted to peri-implantitis over a five-year follow-up, while a maintenance program reduced conversion to 18%(34). BOP and PD were the most significant contributing factors to conversion to peri-implantitis. The average prevalence of peri-implantitis was reported to be 22%(32), but it could reach up to 56%(35).

It has been well-established that the primary cause of peri-implant diseases is dental plaque, which is a biofilm on the surface of the implant(31). There are many other factors, local and systemic, that contribute to these diseases. For peri-implant mucositis, the contributory

factors include smoking, abutment characteristics, radiotherapy, width of keratinized tissue, diabetes, genetics, residual cement, gender, and function time of implant(36). For peri-implantitis, history of periodontitis, smoking, excess cement, and maintenance are the most critical contributory factors(37).

Formation of dental plaque

Dental plaque is a well-organized microbial community composed of multiple species of bacteria and extracellular matrix. Approximately, 80-90% of dental plaque is water, while 70% of its dry weight is bacteria and 30% is matrix(38). With the advancement of genomics, approximately one thousand bacterial species have been identified(39). Rather than randomly attaching to the tooth surface, these bacteria are distributed in a spatially and functionally organized way(40). They can communicate with one another through small molecules diffusible through the matrix, and develop food chains and metabolic cooperation. In addition, the structure of dental plaque facilitates gene transfer among the microorganisms(41, 42).

The pattern of dental plaque formation on the implant surface is comparable to that on the tooth surface(43, 44). The formation of dental plaque is not a random process, but occurs in an ordered sequence(45). The entire process can be distinguished into six stages(46): 1) acquisition of the pellicle; 2) reversible attachment of early colonizers; 3) permanent attachment of early colonizers; 4) attachment of later colonizers; 5) maturation of the plaque; 6) dispersion of the plaque.

The acquired pellicle is an acellular layer composed of specific proteins derived from saliva, gingival crevicular fluid, and cell and micro-organism products(47). The components of the pellicle include immunoglobulins, amylase, fibrinogen, fibronectin, mucins (MUC5B),

histatin1, and more(48). The thickness, varies from 0.1 μm to 90 μm(49). The formation of the acquired pellicle in the oral cavity occurs within one minute after tooth eruption or teeth cleaning, and reaches maturation within two hours(50). Beak and colleagues investigated the real-time formation of a salivary pellicle with an optical approach and found that pellicle "islands" formed first, and then merged into a layer(49). After 2 hours, there was a 20 um-thick layer formed on the surface of the enamel. The acquired pellicle has multiple functions. It serves as a lubricant to facilitate speech and mastication. Some components of the pellicle, especially acidic proline-rich protein 1 (aPRP1), mucin, and statherin(51), could effectively protect the enamel and dentin against toothbrush abrasion(52). Moreover, acquired pellicle could regulate mineral homeostasis. Histatin(53) and statherin(54) prevent against acid-induced demineralization. The acquired pellicle also promotes remineralization. Its porous structure permits the diffusion of the minerals(47), and the peptides under the basal layer of the pellicle may serve as a scaffold for mineralization(55). The acquired pellicle also participates in host defense. Several proteins associated with immune defense have been identified in the pellicle, such as immunoglobulins, cystatin, and histatins(56). Additionally, the acquired pellicle interacts with microbials and promotes their attachment via specific receptors or nonspecific mechanisms. It determines the components of early colonizers by specific bacterial attachment(53), affecting the pattern of subsequent plaque formation(46).

After formation of the acquired pellicle, bacteria start to colonize on the surfaces of the tooth or implant. Initially, bacteria are adsorbed on the pellicle via a weak, reversible physio-chemical force(57). Then, specific bacterial proteins bind to their cognate ligands on the surface of the pellicle, leading to a stronger attachment. *Streptococci* are the first

colonizers and attach through binding via specific receptors known as adhesins. These include the antigen I/II family, lipoprotein receptor antigen (LraI) family, surface lactins, amylase-binding proteins, and so on(58). *Actinomyces sp.* are also common early colonizers following *Streptococci*. They bind to proline-rich proteins and statherin on the pellicle via type 1 fimbriae-associated protein. *Veillonella sp.* are non-pathogenic gram negative early colonizers(59). They utilize the lactate produced by acidogenic bacteria and clear the local microenvironment for other colonizers(60). Moreover, *Veillonella sp.* play a critical role in metabolic communication among the microbes of plaque(59). A study by Periasamy, *et al*. demonstrated that without *Veillonella sp.*, later colonizers of dental plaque, such as *Fusobacterium nucleatum (F. nucleatum)*, *Aggregatibacter actinomycetemcomitans* (*A.a*), and *Porphyromonas gingivalis* (*P. gingivalis*), could not grow with *Streptococci*(59).

As early colonizers proliferate, later colonizers attach and multiply, leading to increased microbial diversity and maturation of plaque. The critical species in this coaggregation process are *Fusobacterium sp*. They are able to bind to the acquired pellicle and most of the bacterial species and serve as a bridge between the early and later colonizers(61). Most periodontal pathogens, including *P. gingivalis, A.a*, *Prevotella intermedia* (*P. intermedia*), and *Spirochetes*, are later colonizing species. In the last phase of plaque formation, after maturation, the bacteria detach and disperse to new colonies.

Microbes in dental plaque are more stable and resistant to antibiotics than their planktonic forms. Several mechanisms are proposed: 1. Neutralizing enzymes and matrix in dental plaque prevent the diffusion of antibiotics(62); 2. Bacterial cells can obtain antibiotic resistance by gene transfer or mutation; 3. Proliferation rates of cells in plaque slow,

leading to less sensitivity to antibiotics(63). Additionally, the microbial community could expand the habitat for bacteria, increase the diversity of metabolism, and enhance their pathogenic capacities(64, 65).

Pathogens associated with peri-implantitis

Multiple species of bacteria have been identified at peri-implantitis sites. Persson and Renvert analyzed bacteria samples harvested from peri-implantitis sites using DNA-DNA checkerboard hybridization and found that the levels of seven species were significantly increased when compared with healthy implants, including *P. gingivalis*, *Tennerella forsythia (T. forsythia), Treponema socranskii (T. socranskii), Staphylococcus aureus (Staph. aureus), Staphylococcus anaerobius (Staph. anaerobius), Streptococcus intermedius (Strep. intermedius),* and *Streptococcus mitis (Strep. mitis)*(66). A case-control study by De Waal *et al.* reported that four periodontal pathogens, *P. gingivalis, P. intermedia, T. forsythia,* and *F. nucleatum*, were associated with peri-implantitis, but not *A.a* and *Staphylococcus*(67). However, in a systematic review by Rakic *et al*., it was shown that *P. gingivalis*, *A.a, P. intermedia,* and *Prevotella nigrecens (P. nigrecens)* were detected in 60% of peri-implantitis sites, and that *A.a* was most significantly associated with peri-implantitis(68). Another study reported that *Treponema denticola* (*T. denticola*) was the strongest diagnostic marker for peri-implantitis, followed by *T. forsythia* and *P. gingivalis(69)*. A recent systematic review by Lafaurie *et al*. summarized selected articles and found that *P. intermedia* and *P. nigrescens* were more associated with peri-implantitis than red complex microorganisms like *P. gingivalis(70)*. Moreover, other non-periodontal pathogens, such as *Escherichia coli (E. coli)*, were also identified at peri-implantitis sites(71). Additionally, increased levels of viruses are also detected in peri-implantitis,

including human cytomegalovirus and Epstein-Barr virus(72), indicating the potential role of these viruses in the pathogenesis of peri-implantitis. In summary, *P. gingivalis, P. intermedia/nigrescens,* and *T. forsythia* are the most common pathogens associated with peri-implantitis.

Treatment of peri-implantitis

Currently, treatment of peri-implantitis has gained increasing attention and importance. There are two categories of treatment, nonsurgical and surgical; both are focused on the removal of dental plaque.

In the nonsurgical category, mechanical debridement with curettes has been investigated intensively. Since traditional steel curettes are harder than titanium, they might scratch the surface of the implant(72). Therefore, different materials have been evaluated as curettes, including carbon-fiber(73), plastics(74), titanium(75, 76), and Teflon(77). An ultrasonic system is another commonly used instrument for debridement. And similar to curettes, carbon-fiber(78) or titanium(75) tips were suggested to prevent the damage of the implant surface with limited evidence. Moreover, the air-abrasive system is also used for the debridement of implants(79). Low-abrasive powders used in the system had been suggested not to damage the implant and injure the tissues(80). Additionally, the nozzle, with horizontal exit of air-powder mixture, might improve the removal of biofilm in the threads of implant and prevents the formation of emphysema in surrounding soft tissue(81). Recently, lasers have been used to debride the implant surface, due to their anti-infective effects even though the evidence to support their use is inconsistent and insufficient(82). The use of antibiotics adjunctive to mechanical debridement can further reduce the bacterial load and improve the outcome of nonsurgical treatments(83). However, these

nonsurgical protocols are often not satisfactory and unpredictable in terms of improvement in clinical and microbiological parameters(83), and advanced therapies, like surgical interventions, are usually required.

Surgical treatments of peri-implantitis include access flap surgery, apically positioned flap, and regenerative surgery. These not only provide better access for debridement, but also attempt to recontour the soft and hard peri-implant tissues(73). In clinic, cumulative interceptive supportive therapy (CIST) is a widely used guideline for treatment of peri-implantitis(84). This decision tree demonstrates the indications of nonsurgical and surgical treatments and guides the clinician to select the proper therapy(85) (see Table 4).

Table 4

Cumulative interceptive supportive therapy (CIST)(85)		
Probing depth	Plaque, BOP & Bone loss (BL)	Treatment
PD ≤ 3 mm	Plaque (-); BOP (-)	No treatment
	Plaque (+); BOP (+)	Mechanical debridement + polishing
PD 4~5 mm		Mechanical debridement + polishing + antiseptics (chlorhexidine (CHX))
PD ≥ 5 mm (radiographs)	BOP (+); BL (-)	Mechanical debridement + polishing + antiseptics (CHX)
	BOP (+); ≤ 2 mm BL	Mechanical debridement + polishing + antiseptics (CHX) + antibiotics (local or systemic); Regenerative surgeries + systemic antibiotics
	BOP (+); ≥ 2 mm BL	Regenerative surgical interventions + systemic antibiotics

Surface modification of dental implant

Although surgical interventions show better effectiveness than nonsurgical approaches, the overall outcome of peri-implantitis therapies is unpredictable. The main reason is that the rough surfaces and threads of implants impede the removal of dental plaque even with advanced treatment modalities. As a proverb says, “an ounce of prevention is worth a pound of cure”. Currently, numerous studies have been focusing on the prevention of peri-implantitis by inhibiting plaque formation. Modification of the implant surface, one of the most promising methods, has been intensively investigated.

Antibiotic coatings

Antibiotics are the first-line medications for infection. Local delivery of antibiotics has been used adjunctively to treat peri-implantitis with improved outcomes(86, 87). Subsequently, various antibiotics were studied to coat implant surfaces to prevent the formation of the biofilm, among which gentamicin is the most commonly used(88). Gentamicin is a broad-spectrum antibiotic with bactericidal activity, especially for gram negative bacteria. Due to its thermostability, it has been widely used for dental/bone implant coating(89). Guillaume and colleagues reported that a dual drug coating, ofloxacin and rifampicin, significantly reduced bacterial adhesion and growth(90). Additionally, some other antibiotics, such as tigecycline(91), cefotaxime(92), and vancomycin(93), have also shown promising outcomes. With regard to the antibiotic delivery vehicles, different materials have been used, including biodegradable polymers(91), calcium phosphates, and hydrogel(89). However, the antibiotic coating has some major drawbacks limiting its clinical applications. First, release of coated antibiotics is unsustainable, resulting in unmaintainable antimicrobial effects. An alternative, covalent grafting was used to

stabilize antibiotics on the surface of implants. With this approach, antibiotics are attached to the titanium surface permanently, rather than being released, extending activity(94). Nonetheless, the long-term antimicrobial effects are limited due to finite drug release(95). Second, the release of antibiotics is usually high at the early stages of the implant lifespan, which might be detrimental to the peri-implant supporting tissue(96). Additionally, the high concentration of antibiotics may lead to the development of persister cells in bacteria which are high resistant to antibiotic(96). When the concentration of antibiotics reduced, the surviving persister cells will contribute to the relapse of the infection(97). Finally, continuous release of antibiotics at the subtherapeutic levels could promote the selection of antibiotic-resistance bacteria(96).

Nanoparticle coating

Nanoparticles, defined as particles with a diameter ranging from 1 to 100 nm, are used for implant surface coatings due to their unique advantages. They modify the surface chemical, physical, and optical properties by incorporating nano-sized metals, which could improve integration with soft tissues(98) and osseointegration of implants(99). Moreover, various nanoparticle coatings were demonstrated to prevent the formation of the dental biofilm. Silver nanoparticles have been reported to be effective nanomaterials(100). Silver inhibits the respiratory process of bacteria by binding to the thiol group of the respiratory enzyme(101). Silver nanoparticles not only significantly increase contact area with bacteria, but also penetrate into bacterial cells, which improves bactericidal activities(100). Besides improved antimicrobial effects compared to silver nanoparticles, zinc oxide (ZnO) nanoparticles also have good biocompatibility. They attach to bacterial surfaces by electrostatic forces and generate hydrogen peroxide, leading to bacterial death(98). Other

metal nanoparticles(89), including copper, magnesium, and gold, and antiseptic nanoparticles, such as chlorhexidine (CHX)(102), also display enhanced antimicrobial effects. Titanium dioxide (TiO_2) nanoparticle coating shows both antimicrobial and osteogenic activities(98). It not only has anticandidal effects(99), but also promotes the proliferation of osteoblast cells inducing bone formation(103). However, there are some important drawbacks for the use of nanoparticles, as well. Silver nanoparticles might have cytotoxic effects on osteoblasts and osteoclasts(104). Moreover, due to their ultrafine size, nanoparticles may be hazardous to the environment(100). In addition, nanoparticle coating may be useful in the sites with low bacterial load, such as after the implant surgery. However, in challenging situations with high bacterial load, such as implant exposed to the oral cavity, their effects are usually insufficient to prevent the infection(96).

Superhydrophobic modification

Superhydrophobic surfaces maintain a layer of air, preventing protein binding to material surfaces, which is the first step of biofouling(105). An *in vitro* study by Tang and colleagues reported that a hydrophobic film fabricated onto the surface of titanium could inhibit the attachment of *Staph. aureus(106)*. Crick *et al*. prepared a hydrophobic silicone elastomer surface which could prevent the adhesion of *E. coli(107)*. However, the limitations of the superhydrophobic modification are obvious. First, the effects of superhydrophobic modification are dependent on the bacterial strains. Li and colleague created a novel hydrophobic liquid-infused porous poly(butyl methacrylate-co-ethylene dimethacrylate) surface and tested the biofilm formation with different strains of *Pseudomonas aeruginosa*(108). Their results showed that this hydrophobic surface could only prevent certain bacterial strain in high nutrition environment. Moreover,

superhydrophobic surface modifications can inhibit not only the bacterial adhesion, but also the host cells attachment. Studies reported that the air layer on the material surface impairs adhesion and growth of host cells(109). Therefore, this technique is not ideal for the materials which requires tissue ingrowth.

Antimicrobial peptide coating

Antimicrobial peptides (AMPs), a group of short positively charged peptides found in all classes of life, play an important role in innate immune defense(110). They have broad-spectrum antibiotic activities against both gram positive and negative bacteria, fungi, and viruses(111). AMPs not only inhibit adhesion and proliferation of bacteria directly, but also modulate the host immune response, leading to increased bacterial clearance(110). Several mechanisms underlying their anti-adhesive and antimicrobial effects have been proposed, including interaction with the bacterial membrane(110), membrane disruption, intracellular targets of bacterial cells, or immunomodulatory activities(112). Due to these properties and benefits, they have been used for implant coating to prevent infection. A study by Godoy-Gallardo *et al*. demonstrated that human lactoferrin-derived peptide (hLF1-11), an antimicrobial peptide, significantly reduced the proliferation of *Streptococcus sanguinis* and *Lactobacillus salivarius(113).* AMPs display many advantages, including long-term effectiveness, minimal adverse effects, and less induction of bacterial resistance. Moreover, they are effective against antibiotic resistant bacterial strains, such as *E. coli(114)* and methicillin-resistant *S.aureus(115).* Unlike antibiotics, AMPs may only target pathogenic micro-organisms without affecting normal microbials(116). However, AMPs also have some limitations(117). First, some AMPs have systemic and local toxicity. For example, gramicidin S shows an effective activity against both gram-positive and -negative bacteria,

however, it also has a hemolytic activity(118). Second, AMPs are susceptible to proteolytic degradation. They might be degraded by the enzymes or deactivated by protein binding(119). Another concern is the long term or repeated use of AMPs might elicit the allergic reaction(119). Finally, although AMPs show less bacterial resistance induction, some bacterial strains can develop resistance which compromises the clinical applications. Groisman and colleagues found that *Salmonella typhimurium* show the resistance to cationic peptides by developing a defective lipopolysaccharide. Salmonella also have a gene mutation encoding a peptidase against the AMPs(120).

Recently, two novel peptides that prevent bacterial attachment to surfaces were reported by Reches *et al.(114)*. These short peptides are comprised of only six amino acids (hexapeptide): i) two fluorinated phenylalanine residues (4F-Phe), which promote self-assembly into a hydrophobic coating and prevent adhesion of bacteria to the surface of the implant, ii) an Arginine-Glycine-Aspartic acid (RGD) motif, to promote attachment of host cells through integrins, and iii) the amino acid 3,4-dihydroxyphenylalanine (DOPA), to attach the peptide to the implant surface.

Li and colleagues reported that D-phenylalanine could prevent bacterial adhesion and inhibit biofilm formation on stainless metal surfaces without affecting cell growth(121). Maity and colleagues designed a tripeptide which contains two fluorinated phenylalanine and one L-DOPA. The results demonstrated that the attachment of *E. coli* was reduced by 74%, and the attachment of *Pseudomonas aeruginosa* was reduced by 93%(122). It was reported that fluorinated phenylalanine residues are able to form a well-organized structure(122), such as peptide nanotubes(123). These structures promote the molecular recognition and self-assemble the peptides into a film(122). And the carbon−fluorine bond

of the aromatic ring of phenylalanine with the fluorinated residues could alter the surface properties by creating a "Teflon-like" characteristic, and then prevent the biofouling of the bacteria(124). Additionally, a previous study(114) compared a peptide which only included DOPA and RGD, and a peptide with DOPA and fluorinated phenylalanine. The results showed a significantly greater reduction in *E. coli* attachment on titanium surfaces using the peptide with fluorinated phenylalanine when compared with the other peptide (66% versus 48%), indicating the effects of fluorinated phenylalanine on inhibition of biofilm formation(114).

RGD is a three-amino acid cell-adhesion peptide which is commonly found in extracellular matrix proteins, such as fibronectin, vitronectin, collagen, and osteopontin(125, 126). This sequence is able to recognize and bind to the focal adhesion sites of host cells and promote cell attachment(127). RGD has been intensively investigated to coat various biomaterial surfaces to promote the adhesion and proliferation of host cells(114, 127-130). A study by Li and colleagues reported a triple-layer surface modification on various substances, including a cell-adhesive peptide layer (RGD), an infectious-environment-responsive peptide, and an antifouling layer(129). This surface modification significantly improved host adhesion and promoted recovery from injury after surgical implantation. The study by Maddikeri *et al.* demonstrated that RGD functionalized poly(L-lysine)-grafted-poly(ethylene glycol) (PLL-g-PEG) polymer promoted the attachment of fibroblasts and osteoblasts on the titanium dioxide surface without increasing bacterial adhesion(127). The novel hexapeptides created by Reches and colleagues contain the RGD motif and were shown to increase the attachment and proliferation of Chinese Hamster Ovary cells by ~35%, while peptides without the RGD motif resulted in only a 9% increase(114). DOPA,

as one of the main constitutes of mussel adhesive proteins, plays a critical role in the attachment of mussels to the surfaces in the sea(131). DOPA not only self-chemosorbs to the surface by metal bidentate coordination or hydrogen bonding, but also easily oxidizes to DOPA-quinone, a covalent cross-linking unit, contributing to adhesion. DOPA is incorporated into the novel hexapeptides to enhance their adhesion to titanium surfaces. With these six amino acids, it was hypothesized that the hexapeptides could form a coating layer on the implant surface, prevent bacterial biofouling, and improve adhesion of mammalian cells.

A previous study demonstrated that these novel hexapeptides significantly inhibited attachment of *E. coli* to the surface of implants and promoted proliferation of mammalian cells(114). However, *E. coli* is not a common pathogen associated with peri-implantitis. Therefore, our study was to investigate whether these two hexapeptide coatings could prevent the adhesion of *P. gingivalis,* which is one of the key pathogens associated with peri-implantitis.

Hypothesis, Objective, and Aims

We hypothesized that the hexapeptide coating will have an inhibitory effect on *P. gingivalis* attachment to the surface of titanium implants *in vitro*. The objective of this study was to investigate whether these two hexapeptide coatings could prevent the adhesion of *P. gingivalis*. The first aim was to compare the effect of the two hexapeptide coatings on the attachment of *P. gingivalis* as a single bacteria biofilm. The second aim was to investigate whether the peptide coating affected the formation of the salivary acquired pellicle and the attachment of *P. gingivalis* incorporated into a multi-bacterial biofilm. Additionally, the

preliminary studies optimized the bacterial seeding density and verified the antimicrobial effects of the hexapeptides.

Chapter 2 Materials and Methods

Chemicals and Supplies

Unless specified, general chemicals, plasticware and supplies were from Fisher Scientific.

Titanium discs

Titanium discs with a diameter of 9 mm and a thickness of 1 mm were provided specifically for our study by Adin Dental Implant Systems LTD (Alon Tavor, Israel). The surfaces of the discs were large-grit sandblasted (alumina oxide) and acid-etched (SLA) treated before the peptide coating process (Figure 1).

Figure 1: Titanium disc with SLA treatment.

Hexapeptide synthesis

The hexapeptides, NH_2-DOPA-Phe(4-F)-Phe(4-F)-Arg-Gly-Asp-$CONH_2$ (hexapeptide 1) and NH_2 -Phe(4-F)-Phe(4-F)DOPA-Arg-Gly-Asp-$CONH_2$ (hexapeptide 2), were synthesized using solid state peptide synthesis as described previously(114).

Surface modification

Titanium discs were treated with cold oxygen plasma for 5 minutes and desiccated at least 24 hours before the coating process. The discs were sonicated (Sonic Dismembrator Model 120, Fisher Scientific, MA, USA) for 15 minutes in 95% ethanol, washed with sterile distilled water, and then dried with airflow. The fresh peptide solution (1 mg/ml, 1.1 mM) was prepared by dissolving the hexapeptide in filtered 10 mM Tris pH 8.5, 154 mM NaCl. The discs were immersed in the peptide solution overnight at room temperature. The discs were rinsed with 1 ml sterile distilled water three times and dried with air-flow. The

concentration of the peptide solution was optimized, and the surface modification was confirmed with X-ray photoelectron spectroscopy, scanning electron microscopy, and atom force microscopy in previous study(114).

Part I: Preliminary studies

Bacterial culture

P. gingivalis (33277^{TM}, American Type Culture Collection (ATCC), Manassas, VA, USA) were grown in BBL^{TM} Schaedler Broth (SB, Table 5) supplemented with 1% Vitamin K1-Hemin (Becton Dickinson, NJ, USA) at 37°C in a GasPak anaerobic jar (Becton Dickinson, NJ, USA).

Optimization of bacterial seeding density

P. gingivalis were harvested and seeded on titanium discs at different densities: 10^3/ml, 10^4/ml, 10^5/ml, and 10^6/ml, in a 24-well plate (n=12). After incubation at 37°C in the GasPak anaerobic jar overnight, the contents of each well was harvested and the concentration of *P. gingivalis* quantified by spectrophotometer reading (The Synergy™ HT, BioTek instruments, VT, USA) at 620nm wavelength. (*This part was done by Raisa Catunda and Ji Min Lim.)*

Antimicrobial effects of hexapeptides

Titanium discs with or without hexapeptide 1 or 2 coating were placed on top of 1.5% BBL^{TM} SB with Vitamin K1-Hemin agar plates (100mm, 211849, Becton Dickinson, NJ, USA) and then overlayed with 5ml of 0.7% agar containing 50µl *P. gingivalis* at a concentration of $6.67x10^8$/ml (n=12). Plates with filter-paper discs (Cat.#88600, ThermoFisher, MA, USA) soaked in 300 units penicillin and 300 µg of streptomycin (Cat. #15070-630, Gibco, Life Technologies, MD, USA) and plates without *P. gingivalis* were

included as positive and negative controls, respectively. After 48 hours of incubation at 37°C in the GasPak anaerobic jar, the zone of inhibition around the discs was observed and measured. Afterwards, the discs were removed. The agar contacting the coating surfaces of the discs was harvested and cultured in BBL^{TM} SB supplemented with Vitamin K1-Hemin at 37°C in a GasPak anaerobic jar. Agar not in contact with the discs was used as negative control. After 24 hours of incubation, the contents of each well was harvested and the concentration of *P. gingivalis* was quantified by spectrophotometer reading at 620nm wavelength.

(*This part was done by Raisa Catunda and Ji Min Lim.)*

Inhibitory effects of hexapeptides on bacterial attachment: single-bacterial biofilm

P. gingivalis were harvested and seeded on implant discs, with or without hexapeptide coating, at a density of 10^5/ml (n=9 for each group). Discs without bacteria were used as a negative control. After incubation at 37°C in the GasPak anaerobic jar overnight, unattached bacteria were washed away, and attached bacteria were labeled with the red fluorescent dye, SYTO 17 (Molecular Probes, Cat. # S7579, Invitrogen, Oregon, USA). Briefly, SYTO 17 was diluted in Schaedler Broth and incubated with bacteria on the discs for 30 minutes at 37°C. After three times washing with phosphate buffered saline (PBS), the discs were imaged under a fluorescent microscope (EVOS M5000 Imaging System, ThermoFisher, MA, USA). The bacteria were semi-quantified by fluorescence intensity, calculated from at least 5 fields/disc at 100X magnification with Image J software (National Institutes of Health, USA).

Table 5: Schaedler Broth Recipe

Reagents	
Casein	8.1 g
Peptic Digest of Animal Tissue	2.5 g
Papaic Digest of Soybean Meal	1.0 g
Dextrose	5.82 g
Yeast Extract	5.0 g
Sodium Chloride	1.7 g
Dipotassium Phosphate	0.82 g
Hemin	0.01 g
L-Cystine	0.4 g
TRIS aminomethane	3.0 g
Milli Q Water	Up to 1000 ml
Autoclaved 121°C for 15 minutes	

Part II: Inhibitory effects of hexapeptides on bacterial attachment: multispecies biofilm

Salivary pellicle formation

Whole-mouth saliva was collected from a healthy, non-smoking volunteer after obtaining informed consent (study ID: MS1_Pro00083117; human research ethics board of the University of Alberta). The volunteer consumed no food in the 2 hours prior. Saliva (50 ml) was collected into a sterile 50 ml conical tube on ice and dithiothreitol (DTT, Cat. # R0861, ThermoFisher, MA, USA) was added to a final concentration of 2.5 mM. The saliva was incubated on ice for 10 minutes with gentle shaking, and then centrifuged at 4°C, 14,000xg for 30 minutes. The pellet was discarded, and the supernatant was centrifuged at 14,000xg for another 30 minutes. The supernatant was sterilized by sequential filtration using 0.8 μm and 0.22 μm filters, aliquoted and stored at -20°C.

Titanium discs with/without the hexapeptide coating were sterilized in 70% ethanol, followed by rinsing three times in sterile PBS. Each disc was incubated in 1 ml saliva with gentle agitation at room temperature for 4 hours based on our preliminary study, and then washed with sterile distilled water to remove the excess saliva and unbound proteins. Titanium discs were air dried overnight.

Characterization of the salivary pellicle

Salivary pellicle formation was confirmed by confocal microscopy. Briefly, saliva-coated and control discs were fixed in 10% formalin for 15 minutes, and then blocked in sterile 1% bovine serum albumin in PBS for 1 hour at room temperature. Two antibodies were used to validate pellicle formation: goat anti-human immunoglobulin G, A, and M (Ig GAM) conjugated to tetramethylrhodamine (TRITC) (1:250 dilution in PBS, Cat.# A24499,

ThermoFisher, MA, USA) and rabbit anti-human fibrinogen (1:100 dilution in PBS, Cat.# PA5-16599, ThermoFisher, MA, USA). Sterile PBS served as the negative control. Discs (n=6 for each antibody) were incubated with antibodies or PBS (n=3) for 2 hours at room temperature, and then washed three times with PBS. In the case of the anti-human fibrinogen antibody, the discs were next incubated with secondary antibody (goat anti-rabbit IgG 488 conjugated, Cat.# 35552, ThermoFisher, MA, USA) for 1 hour at room temperature, and then washed three times with sterile PBS. Immunofluorescent images were taken using a confocal microscope and Volocity software (Olympus IX-81 with Yokagawa CSU 10 confocal scan-head). The thickness of the salivary pellicle was measured based on the thickness of the immunofluorescent signals, and the average thickness was calculated for at least 5 randomly selected fields per discs under 200X magnification. Five spots (four corners and center point) were selected for thickness measurement in each field (Figure 2).

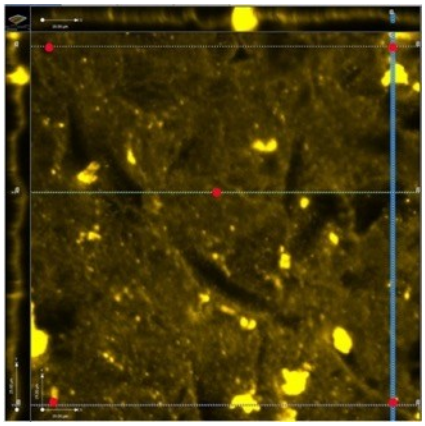

Figure 2: Measurement spots for thickness measurement (Red dots).

Bacterial culture and seeding

Veillonella parvula (*V. parvula,* ATCC® 17745™, Manassas, VA, USA) and *P. gingivalis* (ATCC® 33277™, Manassas, VA, USA) were grown in ATCC® medium 188 (Table 6) and BBL™ Schaedler Broth (Table 5) supplemented with Vitamin K_1-Hemin, respectively,

at 37°C in an anaerobic chamber (Bactron Anaerobic/Environmental Chamber). *Streptococcus sobrinus (S. sobrinus*, ATCC® 33478™, Manassas, VA, USA) was cultured in ATCC® Medium 44 (Brain Heart Infusion Broth 3.7%, Cat. #237500, Becton Dickinson, NJ, USA) at 37°C in an aerobic incubator (Innova®42, New Brunswick Scientific) with shaking.

Table 6: ATCC® medium 188 preparation

Reagents	
Tryptone	5.0 g
Yeast extract	3.0 g
Sodium lactate (60% solution)	19.5 ml
Sodium Thioglycollate	0.75 g
Tween 80	1.0 ml
Dextrose	1.0 g
Milli Q Water	Up to 1000 ml
Adjust the pH to 7.5 with K_2CO_3, and autoclaved 121°C for 15 minutes	

Early colonizers of the dental biofilm, *V. parvula* and *S. sobrinus*, were mixed in a 1:1 ratio in ATCC® medium 188 and seeded on the discs at a density of 10^5/ml (n=12 for each group). Discs without bacteria were used as a negative control (n=3). After incubation at 37°C in an anaerobic chamber overnight, the unattached bacteria were washed away, and the attached bacteria were labeled with the green fluorescent dye, SYTO 9 (Molecular Probes, Cat. #S34854, Invitrogen, Oregon, USA). Briefly, SYTO 9 was diluted in Schaedler Broth supplemented with Vitamin K_1-Hemin and incubated with bacteria on the discs for 30

minutes at 37°C. Meanwhile, *P. gingivalis* were labeled with SYTO 17 (Molecular Probes, Cat. #S7579, Invitrogen, Oregon, USA). After washing the discs with PBS three times, the red-labeled *P. gingivalis* were seeded on the discs with the early colonizers at a density of 10^8/ml and incubated for 2 hours at 37°C in the anaerobic chamber. After washing three times with PBS, the discs were imaged under a fluorescent microscope (EVOS M5000 Imaging System, ThermoFisher, MA, USA). The bacteria were semi-quantified by calculating the fluorescence intensity from at least 5 randomly selected fields/disc at 100X magnification, using Image J software. Images of the bacterial biofilm structure were taken using confocal microscopy at 200X magnification and analyzed with Volocity software.

Statistical analysis

Statistical analyses were performed with SPSS version 23. For the preliminary study in Part I, one-way ANOVA with a *post-hoc* test was used to determine the differences in the fluorescent intensity of bacteria amongst the groups with/without peptide coatings. For Part II, since a Kolmogorov-Smirnov test of normality showed that the data were not normally distributed, we used the Mann-Whitney *U* test to determine if there were differences in the thickness of the salivary pellicle and fluorescent intensity of bacteria amongst the groups with/without peptide coating. Statistical significance was defined as $p < 0.05$.

Chapter 3 Results

Part I: Preliminary studies

Optimization of bacterial seeding density

The purpose of this study was to investigate the effects of the hexapeptide coating on the bacterial attachment in a high bacterial load environment. In order to achieve high bacterial load, the seeding density was optimized. The results showed that wells with 10^5/ml of *P. gingivalis* had a significantly higher bacterial load after overnight incubation when compared with wells seeded with 10^3/ml and 10^4/ml of *P. gingivalis*. However, there was comparable bacterial load when seeding at 10^6/ ml (Figure 3). Therefore, 10^5/ml was considered the optimal seeding density and was used in the subsequent experiments.

(*This part was done by Raisa Catunda and Ji Min Lim.)*

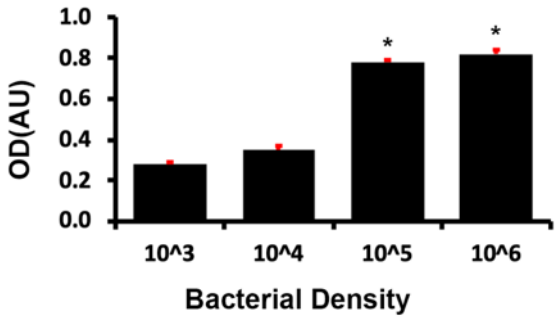

Figure 3: Optimization of *P. gingivalis* seeding density. *$p<0.05$ compared to 10^3/ml group (one-way ANOVA). OD: Optical density. AU: Arbitrary unit

Hexapeptides do not have antimicrobial effects

In published work, hexapeptides were shown not to have antimicrobial effects against *E. coli.* In the next experiment, we sought to determine if hexapeptides had antimicrobial effects against *P. gingivalis.* Titanium discs with or without hexapeptides were placed on the surface of agar seeded with *P. gingivalis* and incubated for 48 hours. As a control, a filter-paper disc of the same size was soaked with penicillin and streptomycin. As shown in Figure 4 (red arrow), the antibiotics resulted in a clear bacteria-free zone, named zone

of inhibition. However, no zone of inhibition was observed around the titanium discs with/without hexapeptide coatings (Figure 4), indicating the hexapeptide coatings do not show antimicrobial effects against *P. gingivalis*.

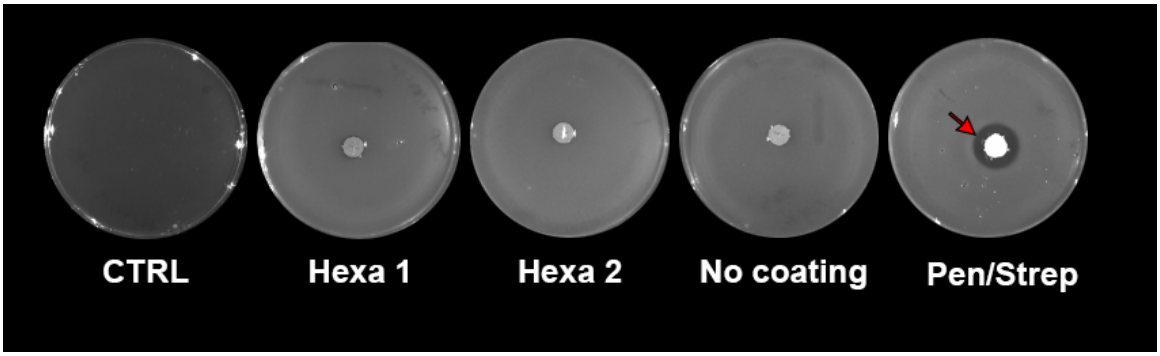

Figure 4: Zone of inhibition after 48 hours incubation. Red arrow shows the zone of inhibition. CTRL: Control group without *P. gingivalis;* Hexa: Hexapeptide; Pen/Strep: Penicillin and streptomycin.

Afterwards, the agar in contact with the discs was harvested and incubated in SB medium for 24 hours. The results showed that no bacteria grew out from the antibiotics group, while the titanium discs with/without hexapeptide coatings had comparable bacterial load as the control which had only bacteria growing (Figure 5). This confirms that the hexapeptide coatings do not have antimicrobial effects against *P. gingivalis*.

(*This part was done by Raisa Catunda and Ji Min Lim.)*

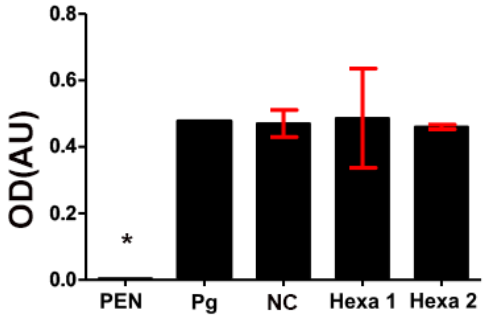

Figure 5 Antimicrobial effects of the hexapeptides. The titanium discs with/without hexapeptide coatings had comparable bacterial load with the control ("Pg" group, $p>0.05$ with one-way ANOVA). *$p<0.05$ compared to "Pg" group. NC: No coating group*;* Hexa: Hexapeptide; PEN: Penicillin; Pg: the control which had solely *P. gingivalis* growing. AU: Arbitrary unit; OD: Optical density.

Effects of the hexapeptide coating on the attachment of *Porphyromonas gingivalis* on the surface of implant discs: A preliminary study with single bacterial biofilm

In order to compare the effects of hexapeptides 1 and 2 on prevention of bacterial attachment, *P. gingivalis* were seeded on the surfaces of titanium discs with/without hexapeptide coatings, and incubated under anaerobic conditions overnight. The results demonstrated that a biofilm of *P. gingivalis* formed on the surface of dental implant without hexapeptide coating (Figure 6 "control"). Hexapeptide 1 coating resulted in a significant reduction in the attachment of bacteria when compared with the control and hexapeptide 2 groups ($p<0.05$, Figures 6 &7 "Hexa 1"). There was no statistically significant difference between hexapeptide 2 and the control group ($p>0.05$, Figures 6 &7 "Hexa 2"). We concluded that hexapeptide 1 coating, not hexapeptide 2 coating, significantly reduced the attachment of *P. gingivalis*.

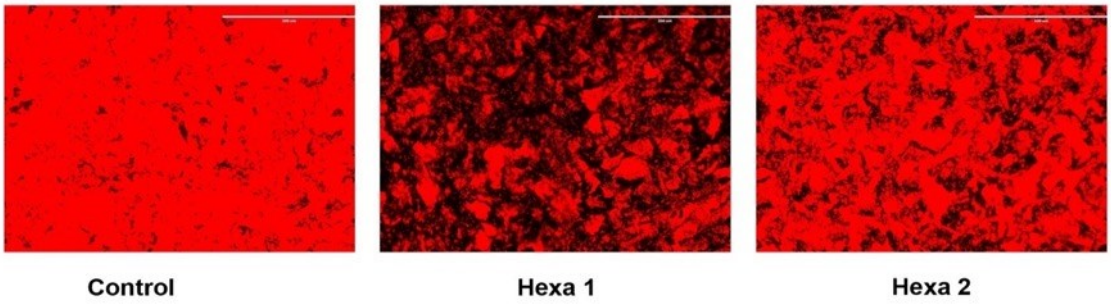

Figure 6: Effects of hexapeptide coatings on *P. gingivalis* attachment. *P. gingivalis* were labelled with SYTO 17 (red fluorescent dye)

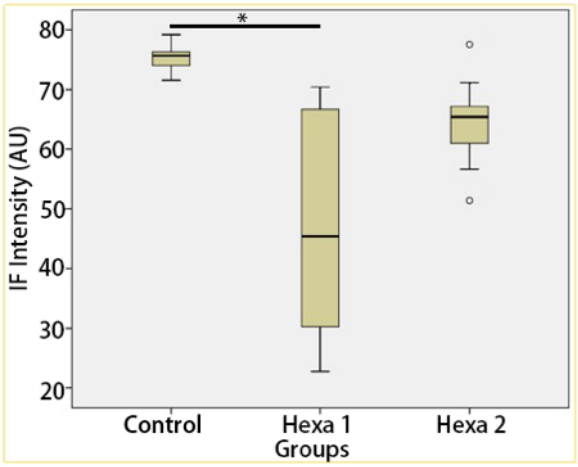

Figure 7: Semi-quantification of *P. gingivalis* on the surfaces of discs. The amount of *P. gingivalis* was semi-quantified by calculating the fluorescence intensity from at least 5 fields/disc at 100X magnification using Image J software. *$p<0.05$ (one-way ANOVA). AU: Arbitrary unit; Hexa: Hexapeptide coating; IF: Immunofluorescence

Part II: Effects of hexapeptide coating on attachment of *Porphyromonas gingivalis* incorporated into a multi-bacterial biofilm

In the oral cavity, *P. gingivalis* attach to the implant surface in an organized way, as a component of dental plaque. To mimic this process, a multispecies biofilm was created, including salivary pellicle, two early colonizers, and *P. gingivalis*. Then, the effects of the hexapeptide coating on the formation of dental plaque were investigated. Since Part I of the study showed that hexapeptide 1 had a significant inhibitory effect on *P. gingivalis* attachment, we focused only on this novel hexapeptide in Part II of the study.

Characterization of salivary pellicle

Confocal microscopy was used to visualize the salivary pellicle on the surface of titanium discs, using fluorescent antibodies against two markers: fibrinogen and Ig GAM. The results showed that the salivary pellicle was homogenous and consistent on the discs (Figure 8 a-e). The thickness of the salivary pellicle was measured using Z-stack analysis (Figure 8 f), and the average thickness was found to be 3.84 ± 0.32 μm. To investigate whether the hexapeptide coating affected the formation of the salivary pellicle, we also compared the salivary pellicle created on coated discs. We found that, similar to the uncoated discs (Figure 8 b&e), the hexapeptide coated discs (Figure 8 a&c) had an even and homogenous salivary pellicle with no significant difference in thickness (Figure 8 g, $p < 0.05$). These results show that the hexapeptide coating does not disturb the normal formation of the salivary pellicle.

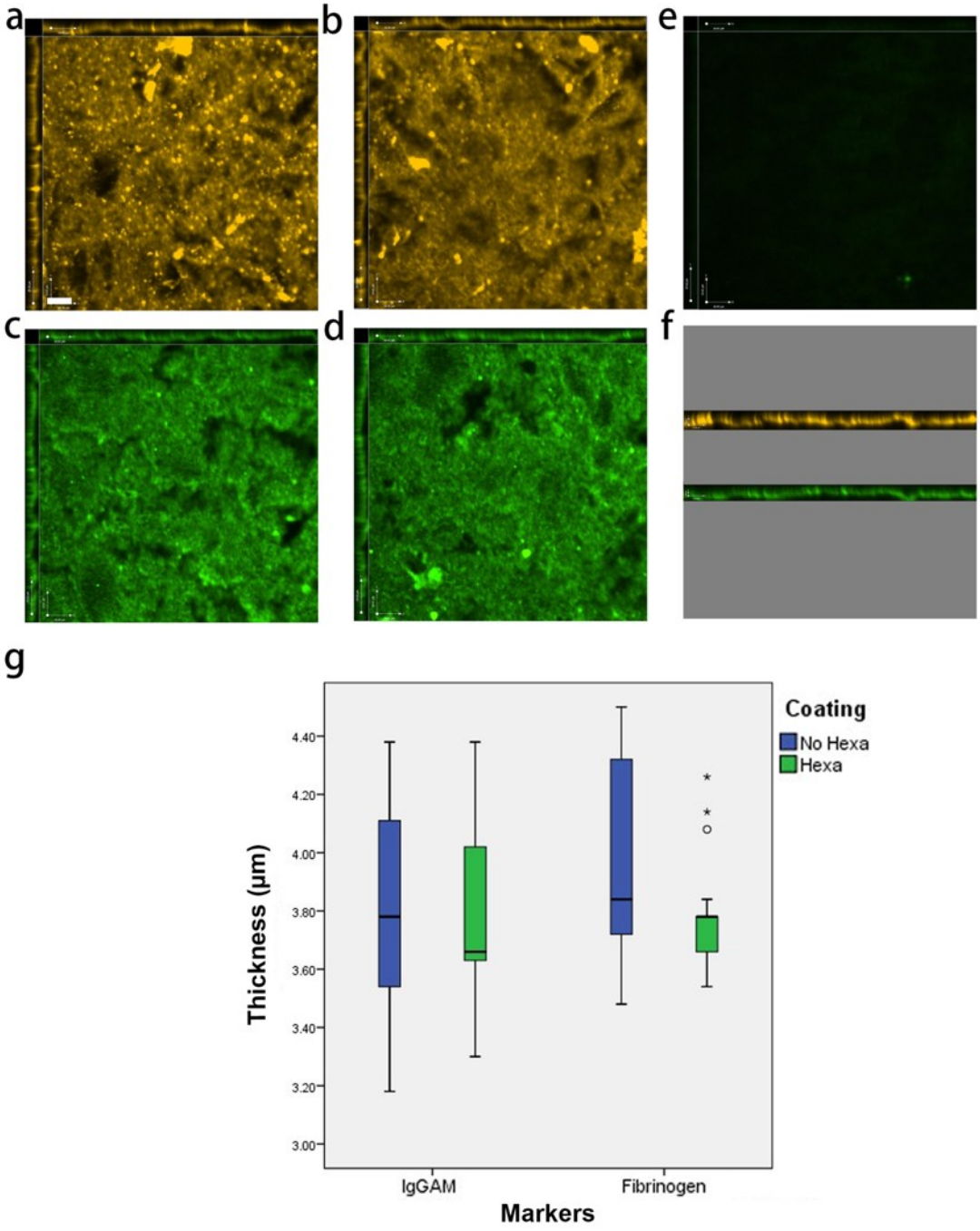

Figure 8: Formation and characterization of salivary pellicle. Salivary pellicle formation was visualized with anti-human Ig GAM and fibrinogen. (a) Ig GAM on implant discs without the hexapeptide coating; (b) Ig GAM on the hexapeptide coated discs; (c) fibrinogen on implant discs without the hexapeptide coating; (d) fibrinogen on the hexapeptide coated discs; PBS served as the negative control (e). The thickness of the salivary pellicle was measured using Z-stack analysis (f) and compared between coated and non-coated discs with one-way ANOVA test (g). Scale bar = 19 µm. Hexa: Hexapeptide coating. IgGAM: Immunoglobulin G, A, and M.

Hexapeptide coating prevents bacterial biofilm formation

To resemble the multistep development and formation of the dental biofilm in the oral cavity, several species of bacteria were seeded on the salivary pellicle, including two early colonizers, *V. parvula* and *S. sobrinus*, and then a later colonizer, *P. gingivalis*. The early and later colonizers were labeled with green and red colors, respectively, using vital dyes that do not interfere with bacterial proliferation or viability. Confocal microscopy was then utilized to visualize the structure of the biofilm. The results showed that after overnight incubation, *V. parvula* and *S. sobrinus* formed colonies on the pellicle-coated surfaces of the discs. Within 2 hours, *P. gingivalis* were able to also colonize the discs (Figure 9). Interestingly, most *P. gingivalis* colonies were attached on top of the early colonizers, as would be expected in the oral cavity.

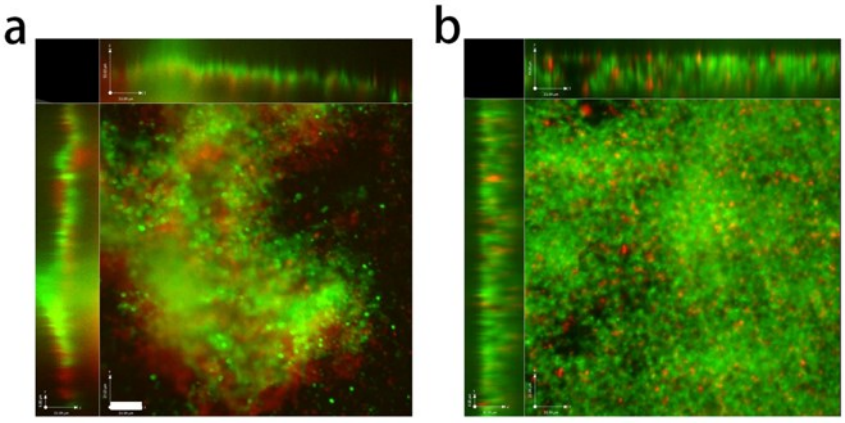

Figure 9. Structure of the multi-species biofilm. Confocal microscopy was used to visualize the structure of the bacterial biofilm. The early colonizers, *V. parvula* and *S. sobrinus*, were labeled with green fluorescent signals and the later colonizer, *P. gingivalis*, was labeled with red (a&b). Scale bar = 21 μm.

The amount of bacteria on the surface of the discs was semi-quantified by intensity of fluorescence. The results showed that the discs with hexapeptide coating had an approximate 25% reduction in the attachment of *V. parvula* and *S. sobrinus,* and 50% reduction in *P. gingivalis* when compared to discs without coating ($p<0.05$, Figure 10). These data suggest that the hexapeptide coating was able to inhibit the adhesion of a specific human pathogen associated with peri-implantitis.

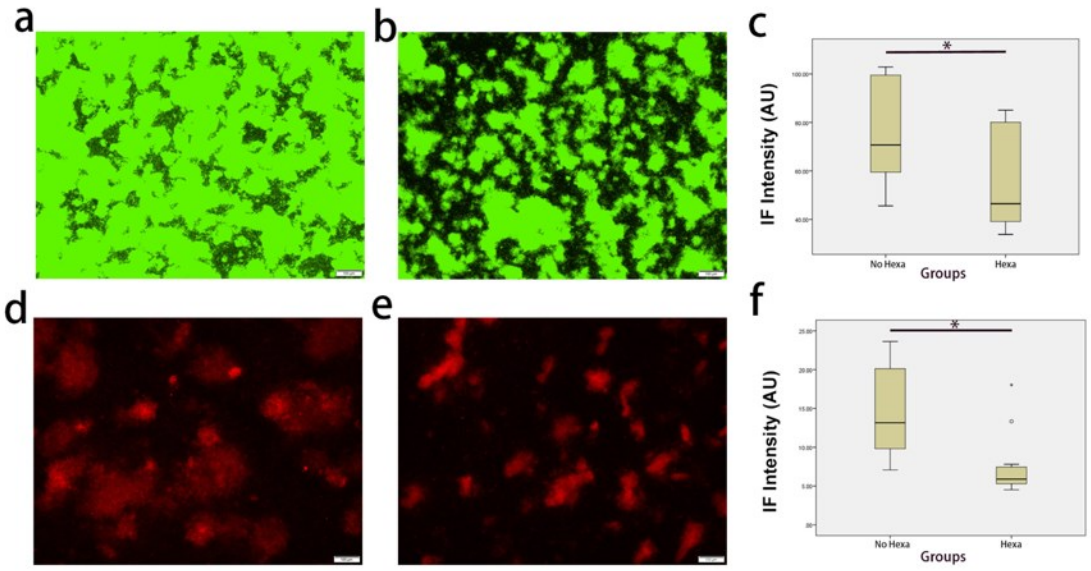

Figure 10 Hexapeptide coating prevents bacterial biofilm formation. The early colonizers, *V. parvula* and *S. sobrinus*, were labeled with green fluorescent dye on discs without the hexapeptide coating (a) and coated discs (b). The early colonizers were semi-quantified by calculating the fluorescence intensity from at least 5 fields/disc at 100X magnification, using Image J software (c). The later colonizer, *P. gingivalis*, was labeled with red fluorescent dye on discs without the hexapeptide coating (d) and coated discs (e), and semi-quantified by calculating the fluorescence intensity with Image J software (f). Scale bar=100 μm. *$p < 0.05$ (Mann-Whitney *U* test). AU: Arbitrary unit; Hexa: Hexapeptide coating; IF: Immunofluorescence.

Chapter 4 Discussion

The main findings of this study were: 1) hexapeptide 1 coating, not hexapeptide 2 coating, significantly reduced the attachment of *P. gingivalis* with a single-bacterial biofilm. 2) hexapeptide 1 coating does not affect the formation of the salivary pellicle, and 3) hexapeptide 1 coating significantly reduces the adhesion of bacteria and prevents the formation of the dental biofilm.

In order to investigate the inhibitory effects of the hexapeptide coatings on bacterial attachment, an environment with high bacterial load is preferable. The first step of this project was to determine the optimal seeding density of bacteria to establish this harsh environment. Various seeding densities were tested from 10^3/ml to 10^6/ml. With increasing seeding density, the bacterial load after incubation increased and reached a peak level at 10^5/ml. Therefore, 10^5/ml was considered the optimal seeding density and was used in the subsequent experiments. The antimicrobial effect of the hexapeptides against *P. gingivalis* was also investigated in our preliminary studies. The discs were placed in agar seeded with *P. gingivalis* and the results showed that the hexapeptides did not kill the bacteria, suggesting that the bacterial reduction on the hexapetide coated surfaces was attributed to the prevention of attachment, rather than direct antimicrobial effects.

Our collaborators characterized several hexapeptides(114) and provided us two of them with the best inhibitory effects on bacterial attachment. In order to preliminarily select the best one for further research, Part I of the current study used a simple single-bacterial biofilm to compare their inhibitory effects on bacterial attachment. This experiment demonstrated hexapeptide 1 coating significantly reduced the amount of *P. gingivalis* attachment, while discs with hexapeptide 2 coating had comparable amount of *P. gingivalis*

attachment to the control group. These results are consistent with previous work, which also demonstrated that hexapeptide 1 coating had the best effectiveness in prevention of *E. coli* colonization on titanium surfaces(114). Thus, we focused on hexapeptide 1 coating in Part II of the study. To resemble the actual local environment in the oral cavity, a multispecies biofilm was created, including salivary pellicle, two early colonizers, and *P. gingivalis*, for studies in Part II.

The salivary pellicle is a film composed of specific proteins from saliva. It protects the enamel from abrasion(132) and regulates the formation of the dental biofilm(133). Many proteins have been identified in the pellicle(48), including immunoglobulins and fibrinogen, which are found in > 80% of pellicles(134). Therefore, our study used these two proteins as markers to detect and characterize the salivary pellicle. The thickness of the pellicle reported in *in vitro* studies varied from 0.1 μm to 90 μm, depending on the rates and amount of proteins adsorbed on the enamel(49). Salivary pellicles created in this study had an average thickness of 3.84 μm, which is consistent with previous studies. Salivary mucins, key components of pellicle formation, have higher affinity for hydrophobic surfaces(135). Since the hexapeptide coating is hydrophobic, we considered that it could lead to greater pellicle formation. Interestingly, our results showed that the coating did not affect pellicle formation.

After pellicle formation, we created a biofilm with two early colonizers, *S. sobrinus* and *V. parvula*, and one later colonizer, *P. gingivalis*. *S. sobrinus* is a member of mutans streptococci, which attach to the salivary pellicle at an early stage in biofilm formation. It is also one of the primary cariogenic pathogens(60) with high acidogenic activity(136). Moreover, *S. sobrinus* suppresses the host immune response(137), and adheres to and

invades gingival fibroblasts, contributing to periodontal diseases(138). *V. parvula* is an anaerobic gram negative early colonizer of dental plaque with non-pathogenic activity(137, 139). Since its growth depends on organic acids, such as lactate, as its carbon source instead of carbohydrates, *V. parvula* is associated with acidogenic species, including *S. sobrinus*(60). Additionally, it has been reported that without *V. parvula*, some later colonizers, such as *A. a, P. gingivalis*, and *F. nucleatum*, would not be able to grow in the presence of Streptococci(59). *P. gingivalis* is one of the most common human pathogens found in peri-implantitis. As a later colonizer, *P. gingivalis* adheres to early colonizers and their products, such as glucans, via multiple adhesion molecules, including fimbriae and hemagglutinins(140). Proteinases produced by *P. gingivalis* modulate the host immune response, lead to the formation of bradykinin, alter the function of clotting factors and degrade collagens, resulting in the destruction of periodontal tissues(141).

We designed this experiment to resemble *in vivo* dental biofilm formation, with a focus on the effect of the hexapeptide coating on the attachment of the peri-implantitis pathogen, *P. gingivalis*. Early colonizers were first seeded on pellicle-containing hexapeptide coated or uncoated discs. After overnight incubation, these bacteria were labeled with SYTO 9 (green fluorescence). *P. gingivalis*, labeled with SYTO 17 (red fluorescence) at a concentration of 10^8/ml, were seeded next, based on the study of Zheng *et al*(142). They showed that approximately 10% of *P. gingivalis* attached to saliva-coated surfaces within a 2-hour incubation period(142). Therefore, *P. gingivalis* were seeded at high density and incubated for only 2 hours in our study. SYTO 9 and 17 are fluorescent nucleic acid stains and have been commonly used for bacterial tracking, including *P. gingivalis* (143, 144). Our preliminary study also showed that both could label the bacteria with minimal false positive

background (Figure 11). The effectiveness of washing was verified by monitoring for bacteria in the wash solution under the immunofluorescent microscope. The number of attached bacteria was comparable after three and five times washing, and there was minimal further bacteria loss after 3 washes, which indicated that three times washing was able to effectively remove the unattached bacteria (data not shown).

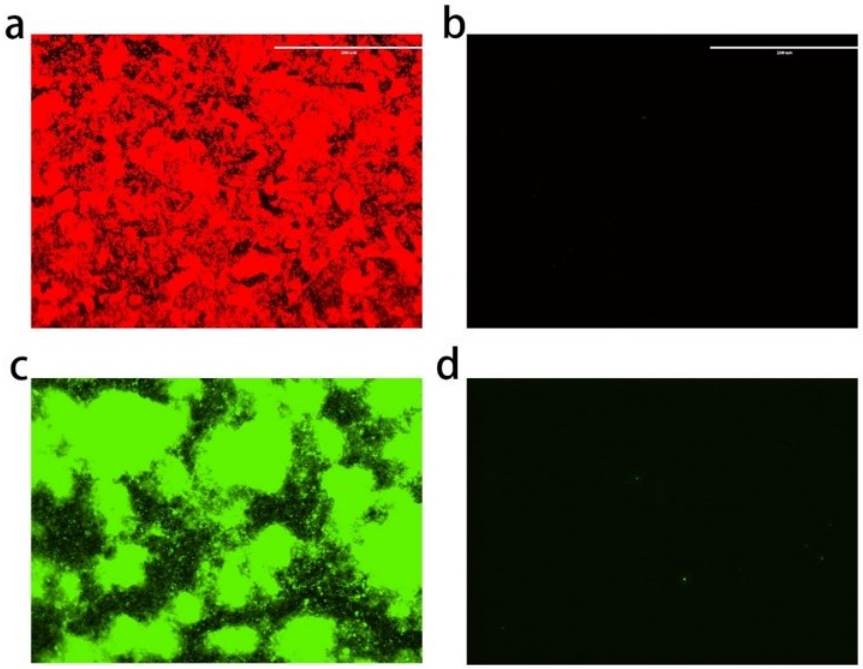

Figure 11 Labelling of bacteria with SYTO 9 and 17. (a) *P. gingivalis* was labeled with SYTO 17. (b) SYTO 17 incubation on the discs without *P. gingivalis*. (c) First colonizers, *V. parvula* and *S. sobrinus*, were labeled with SYTO 9. (d) SYTO 9 incubation on the discs without first colonizers. Scale bar = 200 μm.

A previous study used *E. coli* to create a biofilm to test the inhibitory effects of the novel hexapeptides(114). However, *E. coli* is not a common pathogen associated with peri-implantitis. The current study design improves upon the previous work by mimicking a pellicle containing dental biofilm and incorporating multiple bacterial species more appropriate to the oral cavity. Our results showed that this novel hexapeptide coating could inhibit the attachment of both early and later colonizers of the dental biofilm, and provides important support for continued research.

Regarding the underlying mechanism, we hypothesize that the inhibition of bacterial binding may be attributed to the carbon−fluorine bond of the fluorinated aromatic ring.

Fluorinated phenylalanine residues could promote the self-assembly of the peptides on the titanium surfaces and prevent the biofouling of the bacteria(124). Another potential mechanism for the inhibition of bacterial attachment observed may be that the peptide coating modifies the surface properties of the titanium discs. It has been well documented that the hydrophobicity of materials could affect attachment of bacteria. Cells tend to attach to hydrophilic materials when the surface energy of cells is greater than that of liquid, but bacterial surface energy is generally lower and they usually prefer hydrophobic materials(145). However, some studies suggest that bacteria are easier to remove from materials with hydrophobic surfaces(146-148). Pereni and colleagues compared the surface free energy of five coatings on stainless steel and found that materials with low surface free energy, such as silicone, had less attachment of *Pseudomonas aeruginosa,* while materials with high surface free energy, like Nickel–1% Aluminum alloy, had more bacterial attachment(148). Hydrophobic materials have relatively low surface free energy when compared to hydrophilic materials, therefore, less bacteria attachment can be expected. Our hexapeptide could change the hydrophilic surface of the titanium discs to a hydrophobic surface(114). The hydrophobicity of the titanium discs was determined previously by our colleagues by the contact angle measurement, a commonly used method to measure the wettability of the solid surface. The higher the contact angle, the more hydrophobic the surface is. Hexapeptide coated discs had a higher contact angle than the bare titanium discs, 57° versus 25°. This hydrophobic character might contribute to the inhibition of bacterial attachment. However, the exact mechanism is still poorly understood and requires further investigation.

Our study demonstrated that this novel hexapeptide coating for dental implants could

inhibit the attachment of bacteria and prevent the formation of biofilm. Since bacteria underlie the major etiology for peri-implantitis, the hexapeptide coating might have the potential to impede the development of peri-implantitis. The rough surface modifications of dental implants improve osseointegration, promote osteoblast differentiation and proliferation, and increase the survival rate of the implant(149) when compared to machined surface implants. However, these modifications decrease detachment of bacteria(148) and also make it more difficult to mechanically remove plaque. The hydrophobic character of the hexapeptide coating could facilitate the detachment of bacteria(146, 147), aid in the debridement of the implant surface, and improve the treatment of peri-implantitis. Moreover, this novel hexapeptide contains RGD, a motif that could improve the attachment and proliferation of mammalian cells. Therefore, we speculate that the hexapeptide coating would also promote cell attachment on the surface of the implant inserted into the bone and improve osseointegration, leading to an increased survival rate.

Admittedly, there are limitations to our study. Since this *in vitro* study is the initial stage of the project, some questions remain to be addressed in follow-up experiments, including the chemical and mechanical stability of the hexapeptide coating, and the *in vivo* effects of the coating on peri-implantitis. Moreover, the reason why the hexapeptide coating does not affect pellicle formation and how the hexapeptide coating interacts with salivary proteins are not fully understood and need further investigation. Protein components of saliva vary among individuals due to systemic conditions, food, etc., and they might affect the formation of the salivary pellicle. Saliva was collected from only one participant for this study. Saliva from multiple individuals is required to verify the variation of salivary pellicle formation and its influence on bacterial attachment. Furthermore, saliva was collected prior

to the experiments, stored at -20°C and thawed before the experiment; there is the risk of protein degradation during the freeze-thaw cycle. In our study, saliva was aliquoted into small volumes and went through only one cycle of freeze-thaw to minimize potential protein degradation. While this study demonstrated that the hexapeptide coating did not affect the thickness of the salivary pellicle, whether the coating affected the components of the salivary pellicle remains unknown. Mass spectrometric analysis could be conducted to compare the protein components of salivary pellicles on the discs with/without hexapeptide coating.

Dental plaque is a well-organized microbial community composed of more than 1000 species of bacteria(150). Currently, the human oral microbiome database identified 771 microbial species, of which 57% are named, 13% unnamed and cultivated, and 30% uncultivated(151). Only three bacteria were used in this study to establish a biofilm, and some other common pathogens were missing in this *in vitro* model, such as *T. forsythia, T. denticola,* and *F. nucleatum*. Follow-up experiments should include these species to establish a more complex biofilm. Last but not least, this study investigated the inhibitory effects of the hexapeptide coating on bacterial attachment. The labeled *P. gingivalis* were seeded on the first colonizers and incubated for 2 hours only. Longer incubation of *P. gingivalis* is required in subsequent experiments.

This study lays the foundation for follow-up experiments. In the future, we would like to investigate the mechanisms underlying the inhibitory effects of the novel hexapeptide coating. Implant requires certain torque to get primary stability in the placement surgery which might damage the surface coating of implant. Moreover, mechanical toothbrushing and food could also influence the effects of the coating when the implant is exposed to the

oral cavity. Therefore, mechanical and chemical stability of the hexapeptide coating should be tested. In the current study, we used freshly prepared hexapeptide-coated discs. However, there could be a long period from the implant fabrication to the clinical application. Long-term storage stability should be investigated. Further down the line, an *in vivo* study should be performed to investigate the influence of the coating on osseointegration and bone-implant contact, and verify whether the hexapeptide coating could reduce the prevalence of peri-implantitis.

Chapter 5 Conclusion

Both hexapeptide 1 and 2 coatings do not have antimicrobial effects against *P. gingivalis.* In a single-bacterial biofilm model, hexapeptide 1 coating, not hexapeptide 2 coating, significantly reduced the attachment of *P. gingivalis* on the surface of titanium discs. In a multi-bacterial biofilm model, hexapeptide 1 coating significantly reduces the adhesion of early and later colonizers without affecting the formation of the salivary pellicle. This hexapeptide coating may have the potential to prevent peri-implantitis and improve the success rate of dental implants.

Our future experiments will focus on the mechanisms underlying the effects of hexapeptide coating and its mechanical, chemical, and long-term storage stabilities. Additionally, effects of hexapeptide coating will be tested in a *in vivo* study.

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Appendix:

PARTICIPANT CONSENT FORM

Title of Study: **Effect of peptide coating of dental implant on the formation of the dental plaque**

Principal Investigator: Dr. Liran Levin (780-407-5562)

Research/Study Coordinator: Dongdong Fang

Why am I being asked to take part in this research study?

You are being asked to be in this study because we are trying to learn about the new surface modification method to prevent the infection around the dental implant. We would like to collect around 10 ml saliva from your mouth.

Before you make a decision one of the researchers will go over this form with you. You are encouraged to ask questions if you feel anything needs to be made clearer. You will be given a copy of this form for your records.

What is the reason for doing the study?

Dental implant has been widely used to replace the missing tooth since it was introduced more than five decades ago. It is able to improve the esthetics and the masticatory function of patients and protect the other teeth. However, It also has some complications. Infection of the gum surrounding the implant is one of the most prominent complications which could lead to the failure of the dental implant. The major cause of this disease is the bacteria. Our study is to test a new method to modify the surface of the implant to prevent the attachment of bacteria and then increase the success rate of the dental implant.

What will happen in the study?

We would like to collect around 10 ml saliva from your mouth. We ask you not to eat any food 2 hours before the collection. You can drink more water to increase the saliva secretion during that time. You will not feel any discomfort after the procedure.

What are the risks and discomforts?

Placing a sterile tube in your mouth to collect the saliva causes no discomfort and has no risks.

What are the benefits to me?

There are no specific benefits to you. However, by participating, you are contributing to our study, and you may consider that this may help us prevent the gum infection around the dental implant.

What will I need to do while I am in the study?

We ask you not to eat any food 2 hours before the saliva collection and drink more water to increase the saliva secretion.

Do I have to take part in the study?

You are under no obligation to participate in this study. The participation is completely voluntary. There will be no penalty if you choose not to participate. After the saliva has been collected you will be unable to withdraw the information as the samples are anonymous and pooled.

What will it cost me to participate?

There is no additional cost to participate in our study.

Will I be paid to be in the research?

Participants will not get paid from this project.

Will my information be kept private?

The samples will be pooled, and no personal information will be identifiable. All data will be confidential and used by the researcher, supervisor and those involved in the statistical analysis. We may use the data we get from this study in future research, but if we do this, it will have to be approved by a Research Ethics Board.

What if I have questions?

If you have any questions about the research now or later, please contact Dongdong Fang (438-929-2566).
If you have any questions regarding your rights as a research participant, you may contact the Health Research Ethics Board at 780-492-2615. This office is independent of the study investigators.

CONSENT

Title of Study: **Effect of peptide coating of dental implant on the formation of the dental plaque**

Principal Investigator(s): Dr. Liran Levin
Phone Number(s): 780-407-5562
Study Coordinator: Dongdong Fang
Phone Number(s): 438-929-2566

Yes	No
Do you understand that you have been asked to be in a research study?	
☐	☐
Have you read and received a copy of the attached Information Sheet?	
☐	☐
Do you understand the benefits and risks involved in taking part in this research study?	
☐	☐
Have you had an opportunity to ask questions and discuss this study?	
☐	☐
Do you understand that you are free to leave the study at any time?	
☐	☐
Has the issue of confidentiality been explained to you?	
☐	☐

Who explained this study to you?
_____

I agree to take part in this study:

Signature of Research Participant
_____

(Printed Name)
_____

Date:_____

Signature of Witness

_____

I believe that the person signing this form understands what is involved in the study and voluntarily agrees to participate.

Signature of Investigator or Designee _____

Date _____

THE INFORMATION SHEET MUST BE ATTACHED TO THIS CONSENT FORM AND A SIGNED COPY GIVEN TO THE RESEARCH PARTICIPANT

Health Research Ethics Board

308 Campus Tower
University of Alberta, Edmonton, AB T6G 1K8
p. 780.492.9724 (Biomedical Panel)
p. 780.492.0302 (Health Panel)
p. 780.492.0459

Health Approval

Date:	July 25, 2018
Study ID:	Pro00083117
Principal Investigator:	Liran Levin
Study Title:	Effect of peptide coating of dental implant on the formation of the dental plaque
Approval Expiry Date:	July 24, 2019
Approved Consent Form:	Approval Date: 7/25/2018 — Approved Document: consent letter_Dongdong Fang_Dentistry
Sponsor/Funding Agency:	funding from Adin dental implant system LTD

Thank you for submitting the above study to the Health Research Ethics Board - Health Panel. Your application has received a delegated review and has been approved on behalf of the committee.

The Health Research Ethics Board assessed all matters required by section 50(1)(a) of the Health Information Act. Subject consent for access to identifiable health information is required for the research described in the ethics application, and appropriate procedures for such consent have been approved by the HREB Health Panel. In order to comply with the Health Information Act, a copy of the approval form is being sent to the Office of the Information and Privacy Commissioner.

A renewal report must be submitted next year prior to the expiry of this approval if your study still requires ethics approval. If you do not renew on or before the renewal expiry date (Wednesday, July 24, 2019), you will have to re-submit an ethics application.

Approval by the Health Research Ethics Board does not encompass authorization to access the patients, staff or resources of Alberta Health Services or other local health care institutions for the purposes of the research. Enquiries regarding Alberta Health approval should be directed to (780) 407-6041. Enquiries regarding Covenant Health approvals should be directed to (780) 735-2274.

Sincerely,

Glen J. Pearson, BSc, BScPhm, PharmD, FCSHP, FCCS
Associate Chair, Health Research Ethics Board – Health Panel

Note: This correspondence includes an electronic signature (validation and approval via an online system).