Antimicrobial Effects of GL13K Peptide Coatings on *S. mutans* and *L. casei*

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Abstract

Background: Enamel breakdown around orthodontic brackets, so-called "white spot lesions", is the most common complication of orthodontic treatment. White spot lesions are caused by bacteria such as *Streptococci* and *Lactobacilli*, whose acidic byproducts cause demineralization of enamel crystals. **Aims:** The aim of this project was to develop an antimicrobial peptide coating for titanium alloy that is capable of killing acidogenic bacteria, specifically *Streptococcus mutans* and *Lactobacillus casei*. The long-term goal is to create an antimicrobial-coated orthodontic bracket with the ability to reduce or prevent the formation of white spot lesions in orthodontic patients thereby improving clinical outcomes. **Methods:** First, an alkaline etching method with NaOH was established to allow effective coating of titanium discs with GL13K, an antimicrobial peptide derived from human saliva. Coatings were verified by contact angle measures, and treated discs were characterized using scanning electron microscopy. Secondly, GL13K coatings were tested against hydrolytic, proteolytic and mechanical challenges to ensure robust coatings. Third, a series of qualitative and quantitative microbiology experiments were performed to determine the effects of GL13K–L and GL13K–D on *S. mutans* and *L. casei*, both in solution and coated on titanium. **Results:** GL13K-coated discs were stable after two weeks of challenges. GL13K–D was effective at killing *S. mutans in vitro* at low doses. GL13K–D also demonstrated a bactericidal effect on *L. casei*, however, in contrast to *S. mutans*, the effect on *L. casei* was not statistically significant. **Conclusion:** GL13K–D is a promising candidate for antimicrobial therapy with possible applications for prevention of white spot lesions in orthodontics.

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Introduction

White spot lesions (WSLs) are localized areas of enamel demineralization, which are among the most prevalent adverse outcomes of orthodontic treatment (Melo *et al.*, 2009). WSLs are caused by acid secreted from oral bacteria such as *Mutans streptococci* and *Lactobacilli sp.*, which demineralizes enamel crystals (Rosembloom *et al.*, 1999; Lombardo *et al.*, 2013). The literature reports an incidence of WSLs among orthodontic patients with fixed appliances ranging from 50-97% (Gorelick *et al.*, 1982; Heymann *et al.*, 2013, Lucchese *et al.*, 2013, Zabokova-Bilbilova *et al.,* 2014).

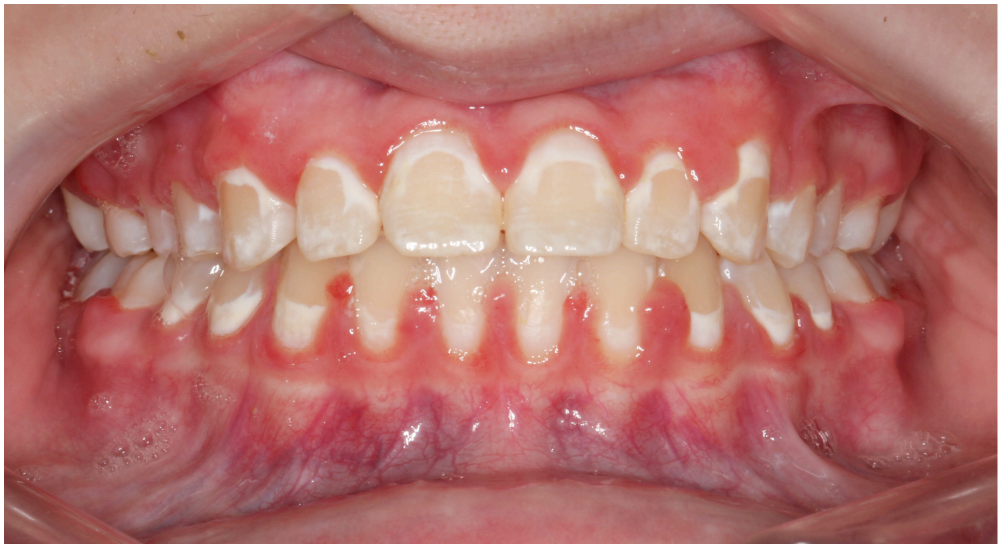

Figure 1. White spot lesions resultant from poor oral hygiene during orthodontic treatment with fixed appliances. (Courtesy of Thorsten Grünheid)

As a part of the informed consent process, the patient is alerted to the risk of WSL formation during orthodontic treatment if oral hygiene is inadequate (Figure 1). It is the responsibility of the patient to remove plaque from orthodontic appliances and teeth on a daily basis, and patients are asked to follow diligent oral hygiene regimens, including the

use of fluoride toothpaste and mouthwash, in an effort to prevent enamel demineralization (Pratt *et al.,* 2010; Maxfield *et al*., 2012; Lucchese *et al*., 2013). Unfortunately, compliance with oral hygiene regimens is poor and has been found to be less than 15% among orthodontic patients (Geiger *et al*., 1992). If oral hygiene is poor and there is evidence of forming WSLs, the orthodontist may elect to terminate treatment and remove the appliances as the risk for caries may outweigh the benefit of continuing orthodontic therapy.

Currently, there is no reliable non-patient dependent method to prevent the formation of WSLs during orthodontic treatment (Heymann *et al.*, 2013; Lucchese *et al.*, 2013). Attempts have been made in the past to use various remineralizing agents such as fluoride mouth rinse, fluoride varnish, fluoride chewing sticks, and casesin phosphopeptide-amorphous calcium [fluoride] phosphate. However, these methods are dependent on patient compliance and there is lack of reliable evidence to support their efficacy for reducing WSLs in post-orthodontic patients (Chen *et al.*, 2013; Kalha *et al.,* 2013; Melo *et al*., 2014). Glass ionomer cements held promise for use as orthodontic bonding agents due to their fluoride-releasing and enamel-remineralizing properties (Sharma *et al*, 2013). Unfortunately, orthodontic brackets bonded with glass ionomer cements have unreliable bonding strengths with failure rates ranging from 3.2-50% (Summers *et al.*, 2004). An alternative method to prevent WSLs may be targeting the oral bacteria that cause enamel demineralization.

Review of the Literature

Streptococcus mutans is an acidogenic bacterium and widely implicated as the major cariogenic microbe. It has been reported that *S. mutans* is present in 100% of orthodontic patients (Nelson-Filho *et al.*, 2011), and that salivary levels of *S. mutans* are elevated in orthodontic patients with fixed appliances (Rosenbloom *et al*., 1999). *Lactobacilli* are also highly acidogenic and cariogenic microorganisms. In a review of *Lactobacilli* in the oral cavity, Badet and Thebaud (2008) report that *Lactobacillus casei* is the most frequent species of *Lactobacilli* in dental plaque. *L. casei* is a bacterium used in everyday commercial foods and a strain to which the public may be commonly exposed. Both *S. mutans* and *L. casei* are present in cariogenic biofilm on fixed orthodontic brackets (Andrucioli *et al*., 2012). It has been shown that the type of orthodontic bracket does not influence the bacterial counts of *S. mutans* and *L. acidophilus in vivo* (Anhoury *et al.*, 2002).

At present, very few scientific groups have reported attempts to create antimicrobial orthodontic brackets. One group designed a polytetrafluroethylene-coated orthodontic bracket that reduced biofilm adhesion by 18% on average (Demling *et al.*, 2010). These brackets prevent adhesion of bacteria in general and do not specifically target the bacteria that cause WSLs. A different group developed a nitrogen-doped titanium dioxide bracket coating, which prevents adherence of *S. mutans* by generation of free radicals (Cao *et al.*, 2013). Introducing free-radical generating brackets into the human oral cavity may pose concern with regard to biocompatibility. Yet another group developed a silver-platinum alloy coating for stainless steel orthodontic bracket, which

demonstrated antimicrobial effects on *S. mutans* and was biocompatible with human gingival fibroblasts (Ryu *et al.,* 2012). While effective, the precious metals required for coating are expensive and may corrode.

The use of antimicrobial peptides on orthodontic brackets has not yet been reported in the literature. Antimicrobial peptides are short sequences of amino acids ranging from 12-100 amino acids in length, and have a net positive charge ranging from +2 to +9 (Wang *et al*., 2004; Jenssen *et al.*, 2006). Antimicrobial peptides are produced in a wide variety of organisms, including single-celled organisms, insects, plants, fish, birds and mammals, including humans. Antimicrobial peptides are involved in innate immunity and inflammatory responses, and function by disrupting and permeabilizing bacterial membranes, leading to bacterial cell death (Jenssen *et al.*, 2006; Gorr *et al.*, 2009; Melo *et al*., 2009). To date, over 1000 antimicrobial peptides have been identified (Gorr *et al.*, 2009) and therapeutic applications for antimicrobial peptides in the oral cavity are becoming more widely studied (Devine, 2003). For instance, several studies on the use of antimicrobial peptides for oral therapy suggest that the use of antimicrobial peptides as a tool for controlling microbial growth is of increasing importance due to its mechanism of action and low rates of bacterial resistance (Ouhara *et al.,* 2005; Ji *et al*., 2007; da Silva *et al*., 2012).

Recent studies conducted at the University of Minnesota have successfully anchored an antimicrobial peptide know as GL13K to the etched surfaces of titanium, and demonstrated the peptide-coated metal's sustained antimicrobial activity against *S. gordonii* in a peri-implantitis model (Chen *et al.*, 2013; 2014). GL13K is a synthetic

peptide (Figure 2) derived from the human parotid secretory protein, which has demonstrated biocompatibility with both osteoblasts and human gingival fibroblasts (Gorr *et al.,* 2011; Hirt and Gorr, 2013; Holmberg *et al*., 2013). In very small quantities, GL13K has demonstrated a dose-dependent antimicrobial effect on both gram-positive and gram-negative oral microbial pathogens (Gorr *et al.*, 2009; Gorr *et al*., 2011; Hirt and Gorr, 2013). GL13K reportedly shows specificity for bacterial membranes over eukaryotic membranes and disrupts bacterial membranes by micellization (Balhara *et al.,* 2013; Hirt and Gorr, 2013). These combined features make GL13K a promising peptide candidate for human oral antimicrobial therapy.

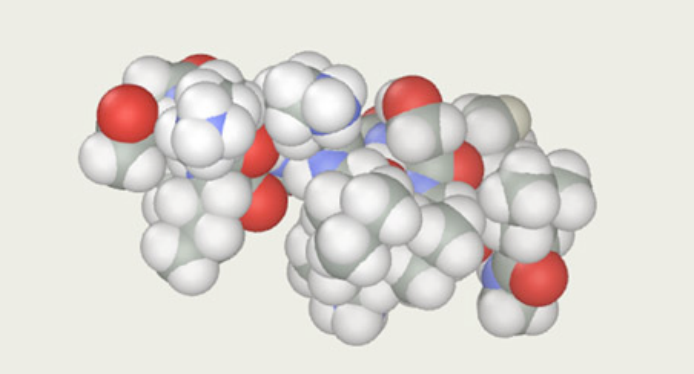

Figure 2. Molecular model of GL13K peptide.

GL13K can exist in two enantiomeric forms: GL13K–L, the levorotatory or left-handed version, and GL13K–D, the dextrorotatory or right-handed version. Similar to amino acids, the –L form is more commonly found in nature. Previously published research on GL13K is all in regard to GL13K–L (Chen *et al.*, 2013; Hirt and Gorr, 2013; Holmberg *et al*., 2013). It would be interesting to see if GL13K–D holds different or possibly stronger antimicrobial properties than the more common –L form.

Aims and Hypotheses

The overall aim of this project is to develop a targeted, bioinspired antimicrobial-coated metal, specifically designed to kill the bacteria responsible for the formation of WSLs in orthodontic patients.

Specific Aim #1: Develop a reliable method for immobilizing GL13K peptides on titanium alloy and characterize the surfaces at each stage of preparation.

Specific Aim #2: Evaluate the resistance of GL13K-coatings to degradation by mechanical, hydrolytic, and proteolytic challenges.

Specific Aim #3: Evaluate the antimicrobial effect of GL13K, both in solution and coated on titanium alloy, on *Streptococcus mutans* and *Lactobacillus casei in vitro*.

The following hypotheses were tested:

(1) Alkaline-etching of titanium discs with 5M NaOH is a reliable method for creating a surface capable of immobilizing GL13K peptides on titanium alloy;

(2) GL13K coatings will not degrade when exposed to mechanical, hydrolytic, and proteolytic challenges;

(3) GL13K–L and GL13K–D peptides are antimicrobial against *S. mutans* and *L. casei* both in solution and immobilized on titanium discs.

Materials and Methods

Fabrication and Characterization of GL13K Coatings on Titanium

Commercially pure Titanium Grade 5 (Ti6Al4V) metal sheets (McMaster-Carr, Robinsville, NJ) were cut into uniform discs of ¼" diameter and $^{1}/_{32}$" thickness. In preparation for surface polish, the discs were placed on a mounting jig in groups of 6 discs by fixation into epoxy resin (EPO Thin 2 Resin and EPO Thin 2 Hardener; Buehler, Lake Bluff, IL) inside of 1.25" cups (SamplKups; Buehler, Lake Bluff, IL), which was allowed to harden overnight. The mounting jigs with discs were removed from the cups using a releasing agent (Releasing Agent; Buehler, Lake Bluff, IL). The use of mounting jigs helped ensure even surface contact of all discs with the polishing wheel.

The mounted discs were placed on a polishing wheel with power head (Ecomet 3 and Automet 2; Buehler, Lake Bluff, IL) and sequentially polished with 320-, 600-, 800- and 1200- grit SiC paper and thereafter micro-polished with cotton microfiber using 1.0 μm, 0.3 μm and 0.05 μm alumina suspensions (TED Pella, Redding, CA). The polished discs were removed from the epoxy resin with a thin sectioning wheel (IsoMet; Buehler, Lake Bluff, IL), cleaned with acetone, and allowed to dry. Insufficiently polished discs were discarded. In preparation for surface coating, the polished discs were activated by an alkaline etching process. More specifically, the polished discs were placed in 10 ml of 5M NaOH overnight at 60ºC, rinsed with deionized water 3x and dried with N_2 gas flow.

Lyophilized forms of the non-antimicrobial peptide GK7-NH2 (GQIINLK-CONH2, MW = 784 g mol^{-1}), the antimicrobial peptide GL13K in both –L

(GKIIKLKASLKLL, MW = 1424.89 g mol^{-1}) and –D forms (GKIIKLKASLKLL, MW = 1424.88 g mol^{-1}), and the fluorescently tagged GL13K-5FAM (GKIIKLKASLKLLK-5FAM, MW = 1911.39 g mol^{-1}) were obtained at > 95% purity (AAPPTec, Louisville, KY and A+ Peptide, Shanghai, China). 10 mg ml^{-1} stock solutions of GK7, GL13K-D, GL13K-L, and GL13K-FAM were prepared in 4 ml of Na_2CO_3 (0.5 mg ml^{-1}, pH 9.5). The discs were coated with 28 µl of stock peptide solution using a micropipette, covered with tin foil, and incubated at room temperature overnight in a desiccator. The coated discs were then washed 2x with deionized water, dried under nitrogen flow, and washed with acetone to encourage active residue exposure.

Sessile drop contact angles (θ_c) of the coated Ti surfaces were measured using a drop master macro contact angle meter (DM-CE1; Kyowa Interface Science, Niiza, Japan) and contact angle analyzer (FAMAS; Kyowa Interface Science, Niiza, Japan) to verify that proper etching and peptide coatings were achieved at each step of fabrication. Deionized water was used as the wetting liquid with a drop volume of 2 µl. Figure 3 demonstrates visualization of contact angles for the 4 different samples measured.

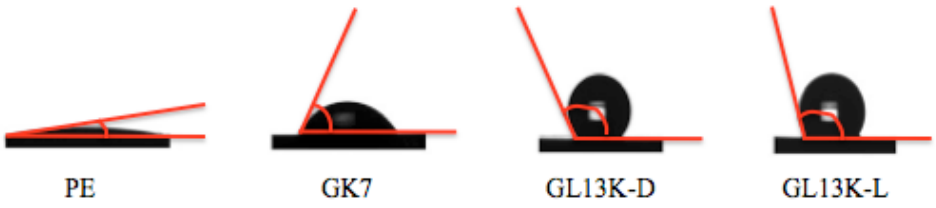

Figure 3: Visualization of contact angle measurements

After verification of peptide coatings, the discs were then transferred to sterilized, non-coated 24-well plates (Nunc Thermo Scientific, Waltham, MA) and exposed to UV light for 10 min prior to use for subsequent experimentation.

Stability Tests for GL13K Coatings

To test the stability of the GL13K peptide on etched Ti, discs were coated with fluorescently labeled GL13K-FAM, and subsequently exposed to hydrolytic, proteolytic and mechanical challenges simulating the environment of the oral cavity, as detailed below. Polished discs without surface activation were used as negative controls. All experiments conducted with GL13K-FAM were performed in the dark. For each of the 3 stability tests the following groups were used: Polished only (P) as a negative control and polished + etched (PE) as the experimental method for surface activation. Each of the 3 challenges was performed with a sample size of 5 per group.

For the hydrolytic challenge, GL13K-FAM discs were placed into individual wells in 24-well plates. 1.0 ml of phosphate buffered saline (PBS) treated with 0.02% sodium azide was added to each well to prevent unwanted antimicrobial growth. The plates were wrapped in tin foil and placed on a shaker at 20 rpm at 37°C. PBS was refreshed after 7 days. Images of the fluorescent peptide coatings were acquired at 492-517 nm using a light microscope (Eclipse E800; Nikon, Tokyo, Japan) at time points Day 0, Day 7, and Day 14 of the hydrolytic challenge. At imaging time points, samples were removed from PBS, washed 3x with sterile deionized water and dried with gentle N_2 flow.

For the proteolytic challenge, 5 ml of whole saliva was collected from a healthy volunteer and spun down for 10 min at 10,000 rpm. Supernatant was collected and diluted 1:1 with Gibbon's buffer. This dilution was then filtered with 0.22 μm filters (Millex-GP; Millipore, Billerica, MA) and stored on ice. After initial imaging, GL13K-FAM discs were placed into individual wells in 24-well plates. 1.0 ml of filtered saliva

treated with 0.02% sodium azide was added to each well. The plates were wrapped in tin foil and incubated at 37°C. The filtered saliva was refreshed every 2 days. Images of the fluorescent peptide coatings were acquired at 492-517 nm at time points Day 0, Day 7, and Day 14 of the proteolytic challenge. The samples were removed from the saliva, washed 3x with sterile deionized water and dried with light N_2 flow prior to imaging.

For the mechanical challenge, samples were placed in individual vials wrapped with tin foil, which were then placed in an ultrasonic water bath at room temperature for 1 hour. This process simulated approximately 2 weeks of continuous brushing (4 min brushing/day * 15 days = 60 min). Images were acquired at 0 min and 60 min as detailed above.

Microbiology Experiments

Streptococcus mutans (ATCC 700610) and *Lactobacillus casei* (ATCC 393) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and stored at -80°C. The following broth and media were used for *S. mutans* cell culture: 38.0 g Brain Heart Infusion (BHI) broth (BD Biosciences, Franklin Lakes, NJ) mixed with 1.0 l deionized water and autoclaved to 121°C; 52 g BHI agar (BD Biosciences, Franklin Lakes, NJ) mixed with 1.0 l deionized water, autoclaved at 121°C for 15 min, cooled to 50-55°C and poured onto sterile 100 x 15 mm petri dishes (Fisher Scientific, Waltham, MA); and 90 g Mitis Salivarius Sucrose Bacitracin (MSSB) agar (BD Biosciences, Franklin Lakes, NJ) dissolved with 1.0 l deionized water, fortified with 20% sucrose, autoclaved at 121°C for 15 min, cooled to 50-55°C, supplemented with 1% potassium tellurite solution and 200 units/l Bacitracin (Sigma-Aldrich, Saint Louis, MO), and poured onto sterile 100 x 15 mm petri dishes (Fisher Scientific, Waltham, MA).

The following broth and media were used for *L. casei* cell culture: 55.0 g Lactobacilli MRS broth (BD Biosciences, Franklin Lakes, NJ) mixed with 1.0 l deionized water, autoclaved at 121°C for 15 min; and 70 g Lactobacilli MRS agar (BD Biosciences, Franklin Lakes, NJ) mixed with 1.0 l deionized water, autoclaved at 121°C for 15 min, cooled to 50-55°C, and poured onto sterile 100 x 15 mm petri dishes (Fisher Scientific, Waltham, MA).

Pure bacterial cultures of *S. mutans* and *L. casei* were obtained from overnight cultures by streaking a loop of each original culture onto MSSB or BHI agar plates for *S. mutans*, or MRS plates for *L. casei*. Culture plates were incubated overnight at 37°C in

an anaerobic chamber with manual air lock (Coy Laboratory Products Inc, Ann Arbor, MI). A single colony of *S. mutans* was inoculated into 5.0 ml of BHI broth medium and a single colony of *L. casei* was inoculated into 5.0 ml of MRS broth medium. Inoculated media were incubated in an anaerobic chamber overnight at 37°C on a shaker at 20 rpm.

Optical density (OD) was measured at 600 nm on a microplate reader (Synergy HT; Biotek Instruments, Winooski, VT) and subsequently diluted with cell culture media to OD_{600} = 0.2. Optimal concentrations of *S. mutans* and *L. casei* were determined to be 2.15 x 10^6 CFU/ml and 1.67 x =10^7 CFU/ml, respectively, at OD_{600} = 0.2. *S. mutans* was further diluted at a 1:25 ratio and *L. casei* at a 1:50 ratio in order to achieve optimal growth. 1.0 ml of diluted bacterial culture was then added to each well of the 24-well plates containing treated or untreated Ti discs. For all experiments, *S. mutans* was incubated for 72 h, and *L. casei* was incubated for 24 h at 37°C in an anaerobic chamber on a shaker at 20 rpm.

To determine the minimum inhibitory concentration (MIC) of GL13K–L and GL13K–D with *S. mutans* and *L. casei* in cell culture solution, the bacteria were plated in polypropylene 96-well plates (Thermo Scientific, Waltham, MA) with the following serial dilutions from a 10 mg ml^{-1} stock solution of GL13K–L and GL13K–D: 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 μg ml^{-1}. Sterile 0.01% acetic acid was used as the dilutant. A medium-only solution served as a negative control. Caution was used not to use polystyrene plates because their negative charge attracts and potentially bonds the peptide to the plastic. Experiments were performed in triplicate with a samples size of 3 per group. OD_{600} was read and graphed to determine the MICs of bacteria with GL13K in

solution.

For the bactericidal assays, *S. mutans* and *L. casei* were pre-treated for 24 h with varying concentrations of GL13K–L or GL13K–D in serial dilution as follows: 512, 256, 128, 64, 32, 16, 8, and 4 μg ml^{-1} (10 mg ml^{-1} stock solution). Samples were subsequently plated onto their corresponding agars. *S. mutans* treated with GL13K–L or GL13K–D were plated onto both BHI agar (pale yellow) and MSSB agar (blue), an alternative media for culturing this strain. *S. mutans* on both BHI and MSSB plates were cultured for 72 h at 37°C in an anaerobic chamber *L. casei* pre-treated with –L and –D forms of GL13K were plated onto MRS agar and cultured for 24 h at 37°C in an anaerobic chamber. For each group, untreated bacteria were used as a positive control and a blank agar plate was used as a negative control. Photos were acquired using a digital camera (PowerShot SX50 HS; Canon, Melville, NY) at the end of the assay to visualize differences in bacterial growth among groups. All bactericidal assays were performed in duplicate.

To determine the remaining biofilm after each treatment, crystal violet assays were performed. GL13K-coated discs were transferred into wells of a 24-well plate and washed 3x with 1 ml deionized water. 0.5 ml of 0.1% crystal violet solution (Sigma-Aldrich, Saint Louis, MO) was added to each well. Cells were stained for 15 min at room temperature on a shaker at 20 rpm. Each disc was washed 2x with 1.0 ml deionized water until all unbound crystal violet stain was removed. Photos were acquired to visualize the biofilm. Subsequently, each disc was placed into a 1.5 ml microcentrifuge tube. 0.25 ml of 30% acetic acid was added to each tube and incubated 15 min at room

temperature on a shaker at 20 rpm to allow the crystal violet dye to solubilize. 200 µl of each crystal violet/acetic acid solution was added to a well on a clear, flat-bottom 96-well plate. OD_{600} was measured on a microplate reader to calculate the biomass for each sample.

To determine the presence of live and dead bacteria on the treated and untreated Ti disc surfaces, a viability test (Filmtracer™ LIVE/DEAD Biofilm Viability Kit; Thermo Fisher Scientific, Waltham, MA) was used according to the manufacturer's instruction. In brief, SYTO®9 stain and propidium iodide stains were mixed in a 1:1 ratio. 200 µl of the mixed stain was added to each sample disc, which was then covered in tin foil and incubated for 30 min at room temperature. The discs were then transferred to slides with a cover slip and visualized at 40x magnification under a light microscope (Eclipse E800; Nikon, Tokyo, Japan). Images of live (green) and dead (red) bacteria were acquired and ImageJ software 1.48v (National Institutes of Health, Bethesda, MD) was used to merge "live" and "dead" images. All viability tests were performed in duplicate with a samples size of 3 per group.

To determine the number of colony forming units (CFU) of bacteria grown on GL13K coated discs, the Miles and Misra (drop-plate) method was used. Bacteria from 15 discs per group were collected in 1.0 ml 0.9% NaCl via ultrasonication in a warm water bath for 10 min. 5 discs of the 15 discs per sample were pooled into one sample, resulting in a total of 3 samples per group. 100 µl of each sample was serially diluted x5 in 0.9 ml of 0.9% NaCl. 10 µl of each dilution in series from 1-5 was pipetted in a row on the corresponding BHI, MSSB, or MRS agar, and plates were tilted to allow the

dilutions to streak the plate. Plates with *S. mutans* and *L. casei* were cultured for 72 h and 24 h, respectively, in a 37ºC anaerobic chamber. Colonies present on each plate were recorded using a mechanical counter, and CFUs were calculated from dilutions with 1-100 colonies using the following equation: Unknown Starting CFU/ml = (colonies on the plate) x $10^{\text{Tube\# }+2}$. All CFU experiments were performed in quadruplicate with a sample size of 3 per group.

In preparation for scanning electron microscopy of *S. mutans* and *L. casei* on GL13K-coated discs, all samples were fixed for 60 min at room temperature with 2% glutaraldehyde (Sigma-Aldrich, Saint Louis, MO) and 0.15% alcian blue (Sigma-Aldrich, Saint Louis, MO). The discs were either stored at 4ºC for later processing, or processed immediately as follows. The discs were washed with 0.1 M sodium cacodylate buffer (Sigma-Aldrich, Saint Louis, MO) for 5 min and underwent secondary fixation in 1% osmium tetroxide (OsO_4) (Sigma-Aldrich, Saint Louis, MO) and 0.1 M sodium cacodylate buffer for 60 min. The discs were then washed again for 5 min with 0.1 M sodium cacodylate buffer. The samples were dehydrated in a sterile beaker using increasing concentrations of ethanol each for 5 min: 50%, 70%, 80%, 95% and 100%.

A Samdri-780 Critical Point Dryer (Tousimis, Rockville, MD) was used for critical point drying with CO_2. The samples were finally coated with platinum for 30 sec using a DV502A Au/Pd sputter coating unit and mounted with adhesive carbon tape onto a SEM carrier platform. All SEM images were acquired using a JEOL 6700 Field Emission Gun-Scanning Electron Microscope (JEOL Ltd., Peabody, MA) at the University of Minnesota Characterization Facility, Shepherd Laboratory.

Statistical Analysis

For contact angle measures and biofilm assay, mean values and standard deviations were calculated to show differences between uncoated (PE) and coated (GK7 and GL13K) groups. For the CFU data, first boxplots were generated to technically identify outliers. Because the overall CFU data was scattered, extreme outliers were considered anomalies of the general behavior and therefore removed from subsequent analysis. Boxplots were also used to visualize differences among groups. Levene's test for homogeneity of variances was performed. A univariate general linear model was used to compare the fixed groups versus random replicates. A post-hoc Tukey test was used to determine statistical significance with multiple comparisons between the groups. All statistical tests were performed using SPSS software (IBM, Armonk, NY) with $p < 0.05$ considered statistically significant.

Results

Fabrication and Characterization of GL13K Coatings on Titanium

The microscopic appearance of titanium disc surfaces at each stage of preparation is shown in Figure 4. The surface of etched titanium is highly nanoporous.

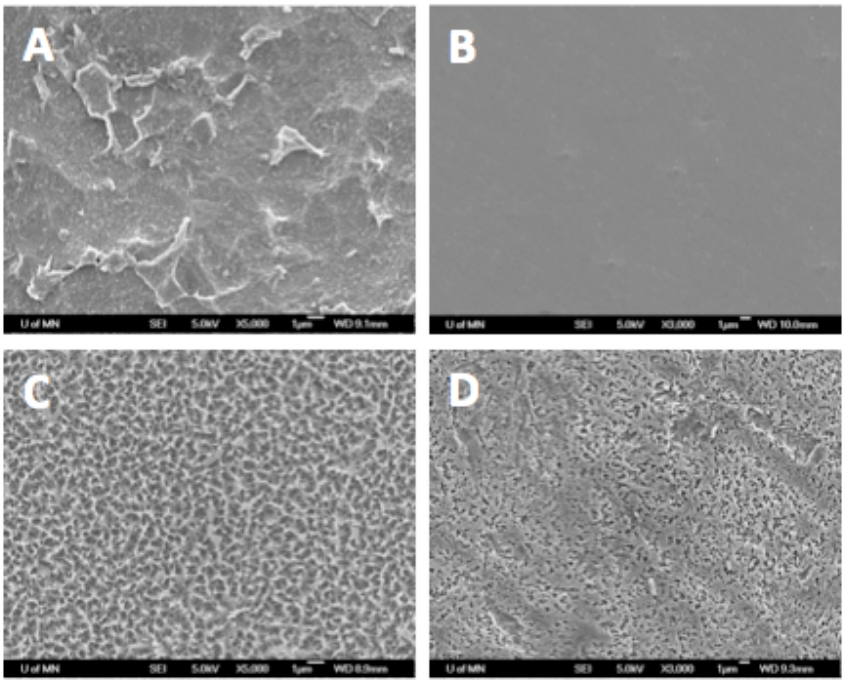

Figure 4. Scanning electron microscopy imaging of titanium surface at each stage of preparation for peptide coating. (A) Unpolished Ti at x5000 magnification, (B) Polished Ti at x3000 magnification, (C) Polished and etched Ti (PE) at x5000 magnification, and (D) PE + GL13K–D antimicrobial peptide coating at x3000 magnification.

The mean contact angles from treated and untreated groups were: θ_c(PE) = 10.4 ± 2.2º, || θ_c(GK7) = 95.4 ± 1.2º, ||θ_c(GL13K–D) = 109.7 ± 7.5º, ||and θ_c(GL13K–L) = 101.0 ± 2.9º.

Stability Tests for GL13K Coatings

At Day 0, a strong presence of GL13K-FAM was detected on PE samples. A very small amount of GL13K-FAM was retained on the surface of the polished-only discs, which were used as negative controls. The fluorescent signal on PE dics after each challenge was comparable to that of the initial fluorescent signal on PE discs (Figure 5).

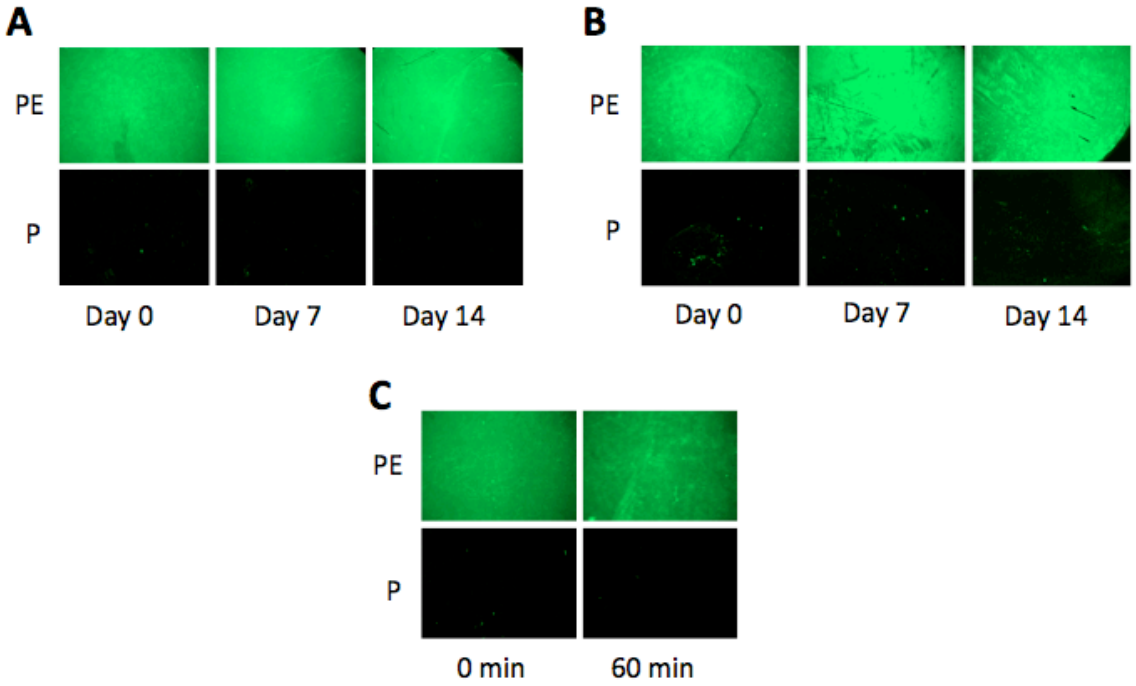

Figure 5. Fluorescent GL13K coatings after stability tests. (A) Hydrolytic challenge of PBS for 14 days; (B) Proteolytic challenge of human saliva for 14 days; (C) Mechanical challenge by ultrasonication for 60 minutes. Each image represents one of 5 samples.

Microbiology Experiments

Optical density as a function of GL13K concentration is shown in Figure 6. The MIC for *S. mutans* with GL13K–L was 64 µg/ml, and 8 µg/ml with GL13K–D. The MIC for *L. casei* with GL13K was 64 µg/ml for both the –L and –D forms of GL13K.

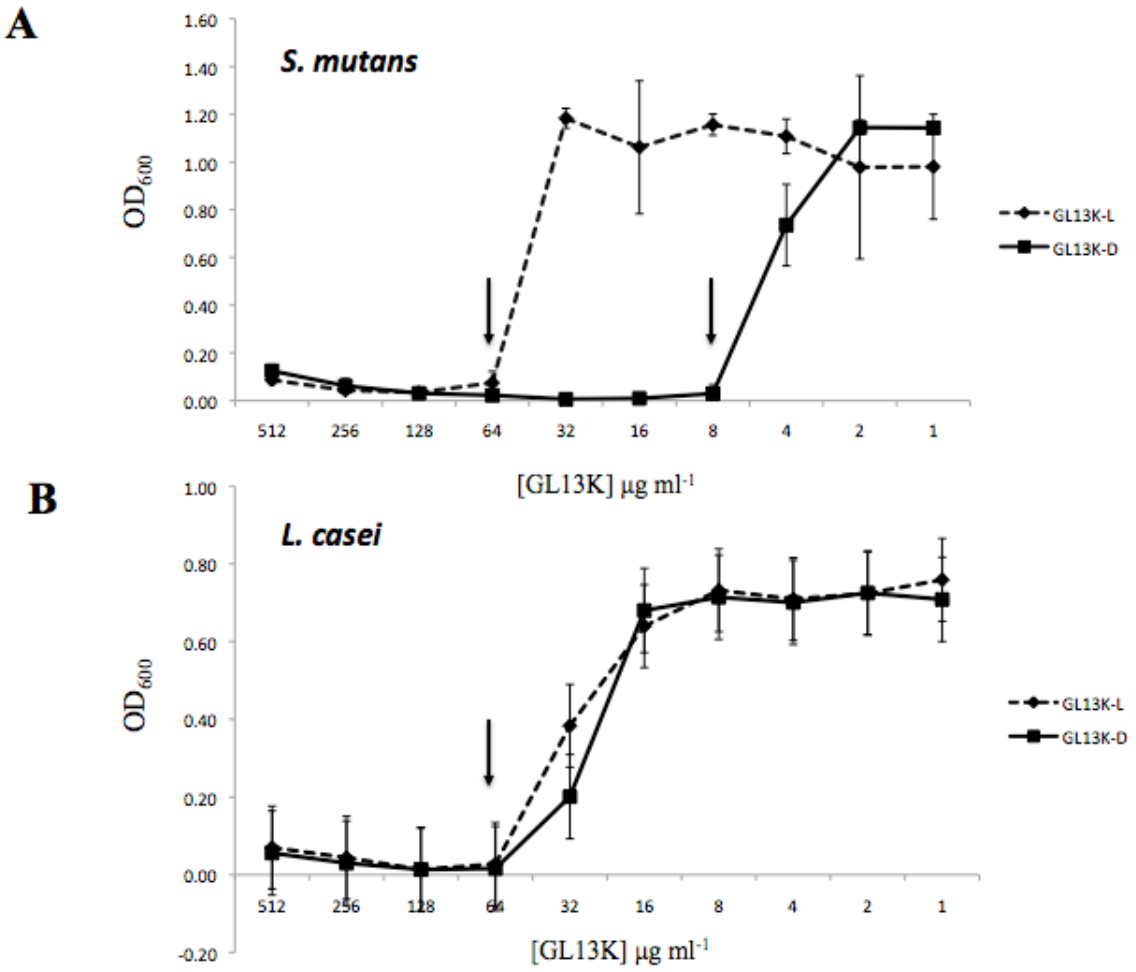

Figure 6. Minimum inhibitory concentration (MIC) for GL13K in solution with *S. mutans* and *L. casei*. MICs for GL13K on (A) *S. mutans* and (B) *L. casei*. Error bars indicate standard deviation. Arrows indicate MIC.

Data for the bactericidal effect of GL13K on *S. mutans* is presented below on two types of culture media: BHI (pale yellow, Figure 7) and MBBS (blue, Figure 8). GL13K–L at a concentration of 512 µg/ml had a partial bactericidal effect on *S. mutans* grown on BHI agar. *S. mutans* survived cell culture with GL13K–L in solution even at the highest concentration used. GL13K–D demonstrated a near complete bactericidal effect on *S. mutans* at 128 µg/ml, and a complete bactericidal effect on *S. mutans* at ≥ 256 µg/ml, when cultured on BHI agar (Figure 7).

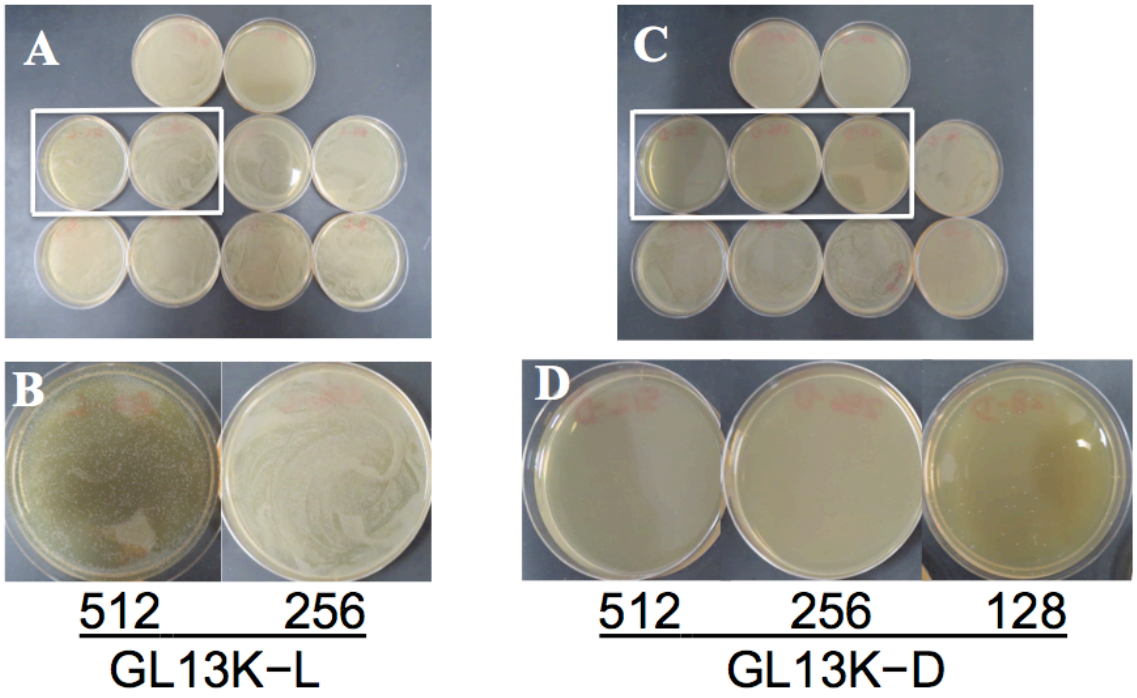

Figure 7. Bactericidal effect of GL13K on *S. mutans* cultured on BHI agar. (A) From right to left, top to bottom: + control, –control, 512, 256, 128, 64, 32, 16, 8, and 4 µg ml^{-1} GL13K–L treatment. (B) Enlargement of insert from A: *S. mutants* treated with 512, and 256 µg ml^{-1} GL13K–L. (C) From right to left, top to bottom: + control, –control, 512, 256, 128, 64, 32, 16, 8, and 4 µg ml^{-1} GL13K–D. (D) Enlargement of insert from C: *S. mutants* treated with 512, 256, and 128 µg ml^{-1} GL13K–D.

When *S. mutans* was cultured on MSSB agar, bacterial confluence existed with GL13K–L treatment even at the highest concentrations tested. This result is similar to that observed when *S. mutans* was cultured on BHI agar after GL13K–L peptide treatment. On the other hand, GL13K–D treatment demonstrated a near complete bactericidal effect on *S. mutans* at 128 μg/ml, and a complete bactericidal effect on *S. mutans* at concentrations ≥ 256 μg/ml as shown in Figure 8 below.

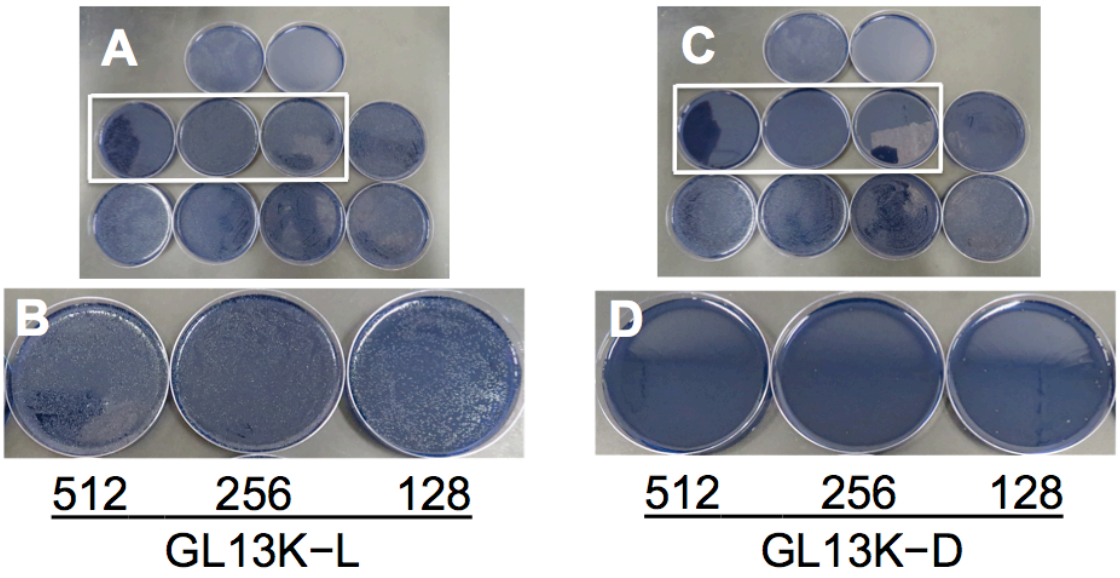

Figure 8. Bactericidal effect of GL13K on *S. mutans* cultured on MSSB agar. (A) From right to left, top to bottom: + control, - control, 512, 256, 128, 64, 32, 16, 8, and 4 μg ml^{-1} GL13K–L treatment. (B) Enlargement of insert from A: *S. mutants* treated with 512, 256, and 128 μg ml^{-1} GL13K–L. (C) From right to left, top to bottom: + control, - control, 512, 256, 128, 64, 32, 16, 8, and 4 μg ml^{-1} GL13K–D. (D) Enlargement of insert from C: *S. mutants* treated with 512, 256, and 128 μg ml^{-1} GL13K–D.

The bactericidal effect of GL13K on *L. casei* is presented below. GL13K–D had a complete bactericidal effect on *L. casei* at 512 and 256 µg/ml, and a partial bactericidal effect at 128 µg/ml (Figure 9). In contrast, GL13K–L partially inhibited growth of *L. casei* at the strongest concentration, 512 µg/ml.

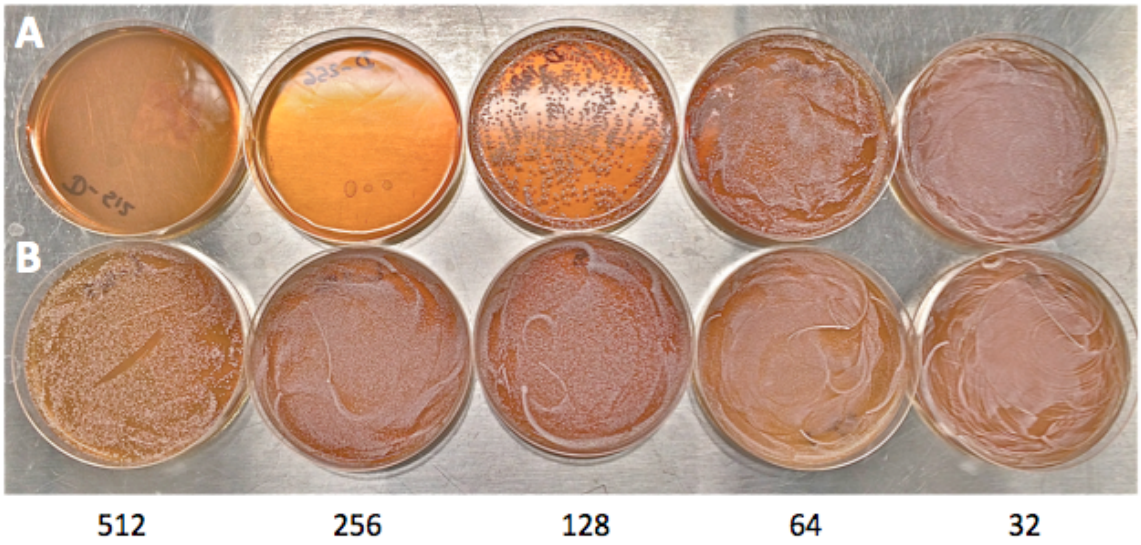

Figure 9. Bactericidal effect of GL13K on *L. casei* cultured on MRS agar. (A) GL13K–D treatment and (B) GL13K–L treatment. Decreasing concentrations of peptide from right to left: 512, 256, 128, 64, and 32 µg ml^{-1}.

Data on the remaining biofilm after each treatment are presented below. The qualitative crystal violet biofilm assay and quantitative biomass assay were comparable for each of the treatment and control groups (Figure 10).

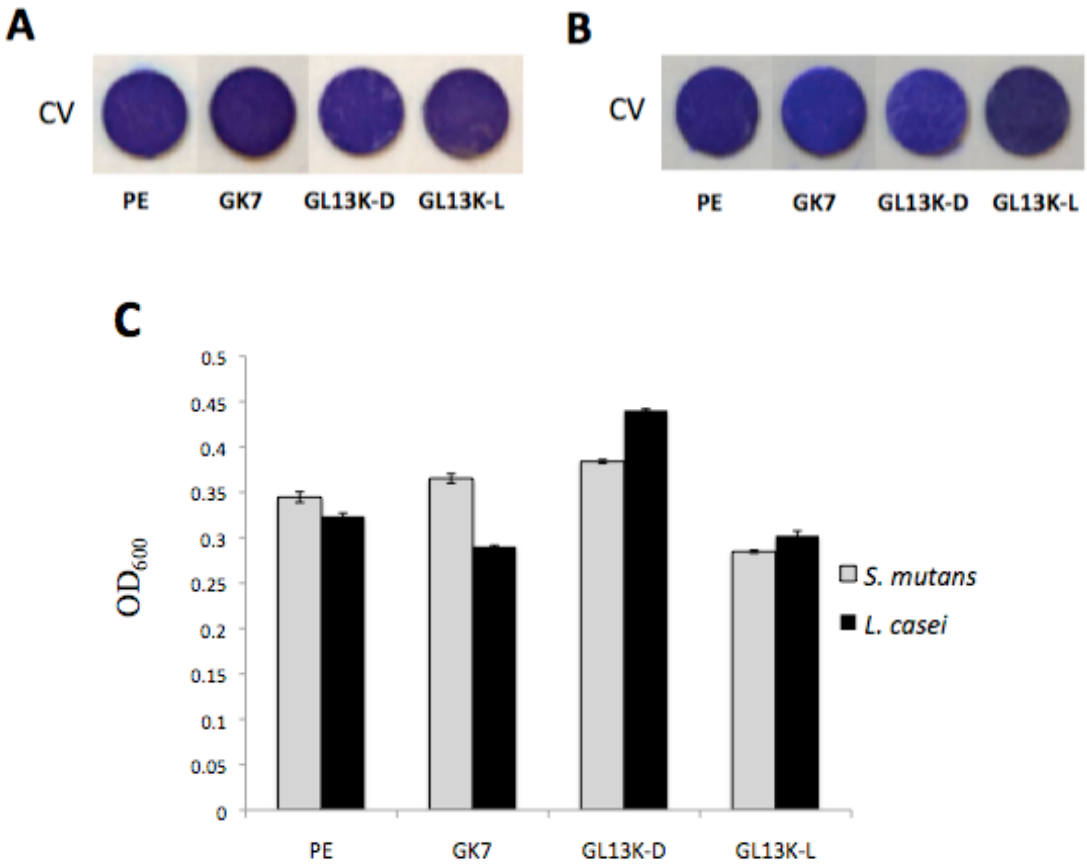

Figure 10. Quantification of biofilm and biomass on GL13K-coated discs. (A) *S. mutans* and (B) *L. casei* on sample discs stained with crystal violet to verify presence of biofilm; (C) Quantification of biomass on sample discs for *S. mutans* and *L. casei*. Biomass was quantified at an optical density of 600 nm. No statistically significant differences between groups.

Viability testing of *S. mutans* and *L. casei* cultured on GL13K–coated discs showed bacteria present in the treated and untreated disc biofilm. Figure 11 shows that more *S. mutans* was killed (red cells) by GL13K–D compared to both the GL13K–L peptide, and the PE and GK7 negative controls. The results of the viability assay with *L. casei* show that live bacteria (green) are strongly present on all samples except for the GL13K–D coated sample.

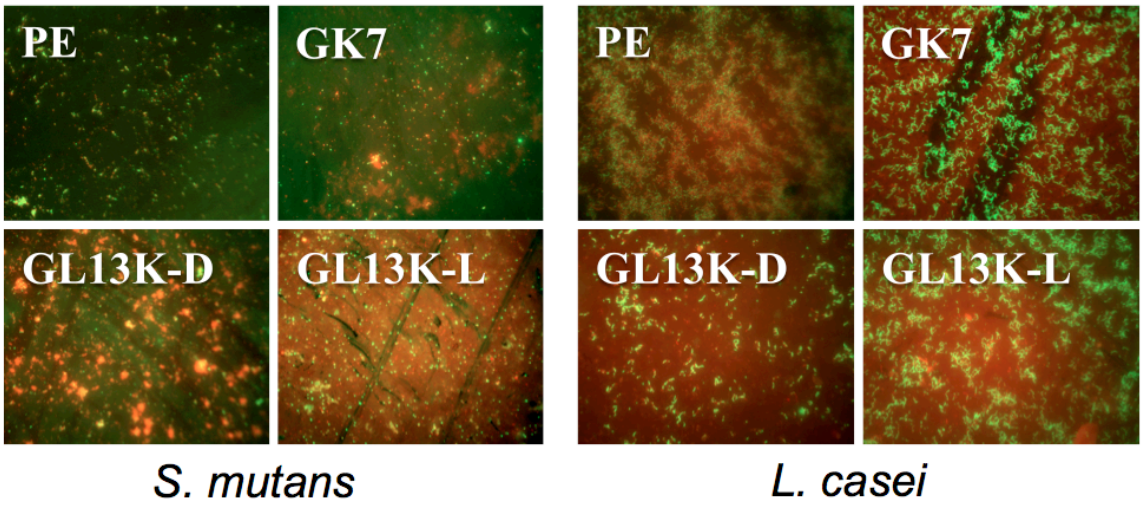

Figure 11. Merged LIVE/DEAD images of *S. mutans* and *L. casei* cultured on GL13K-coated discs. Live (green) and red (dead) bacteria. Images shown are representative of 6 samples from duplicate experiments. Images were acquired at x40 magnification.

The numbers of colony forming units of *S. mutans* collected from culture on GL13K–coated discs and plated onto BHI agar are shown in Figure 12. GL13K–D significantly reduced the number of CFUs for *S. mutans* ($p = 0.029$). No significant effect of GL13K–L on *S. mutans* was noted ($p > 0.05$).

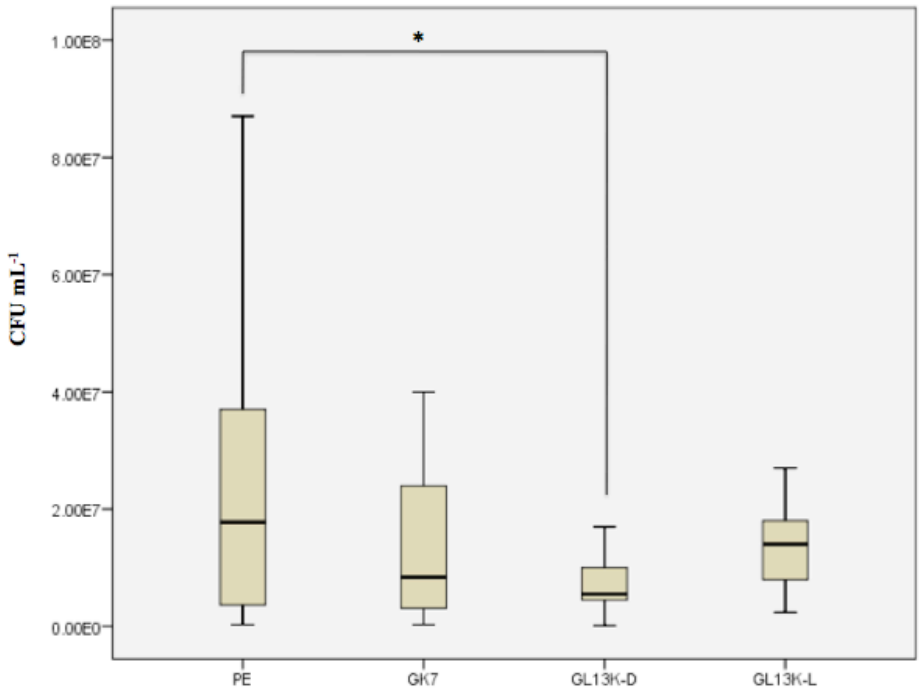

Figure 12. GL13K–D coatings reduce colony forming units of *S. mutans* on BHI agar. Data are representative of 4 repeat experiments with 3 samples per treatment group. (*) Indicates a statistically significant difference.

The numbers of colony forming units of *S. mutans* collected from culture on GL13K–coated discs and plated onto MSSB agar are shown in Figure 13. GL13K–D coatings significantly reduced the number of *S. mutans* CFUs when plated on MSSB agar ($p = 0.004$). No significant effect of GL13K–L on *S. mutans* was noted ($p > 0.05$).

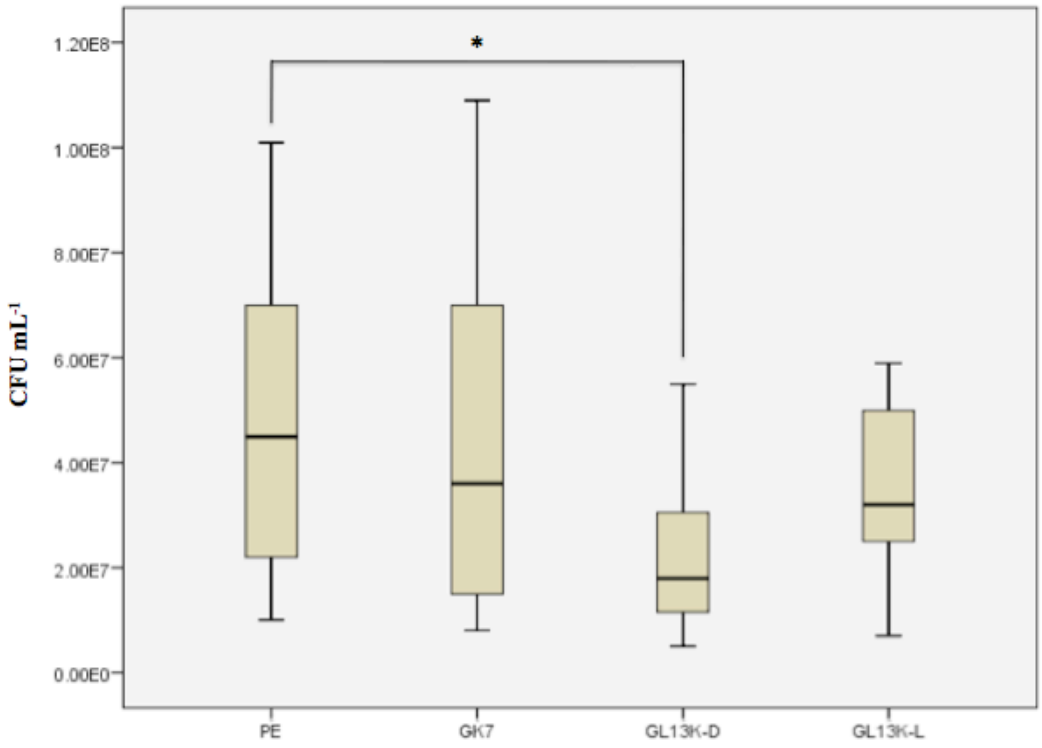

Figure 13. GL13K-D coatings reduce colony forming units of *S. mutans* on MSSB agar. Data are representative of 4 repeat experiments with 3 samples per treatment group. (*) Indicates a statistically significant difference.

The results of the colony forming units of *L. casei* collected from GL13K-coated discs and plated on MRS agar show no statistically significant differences between groups (Figure 14; $p > 0.05$).

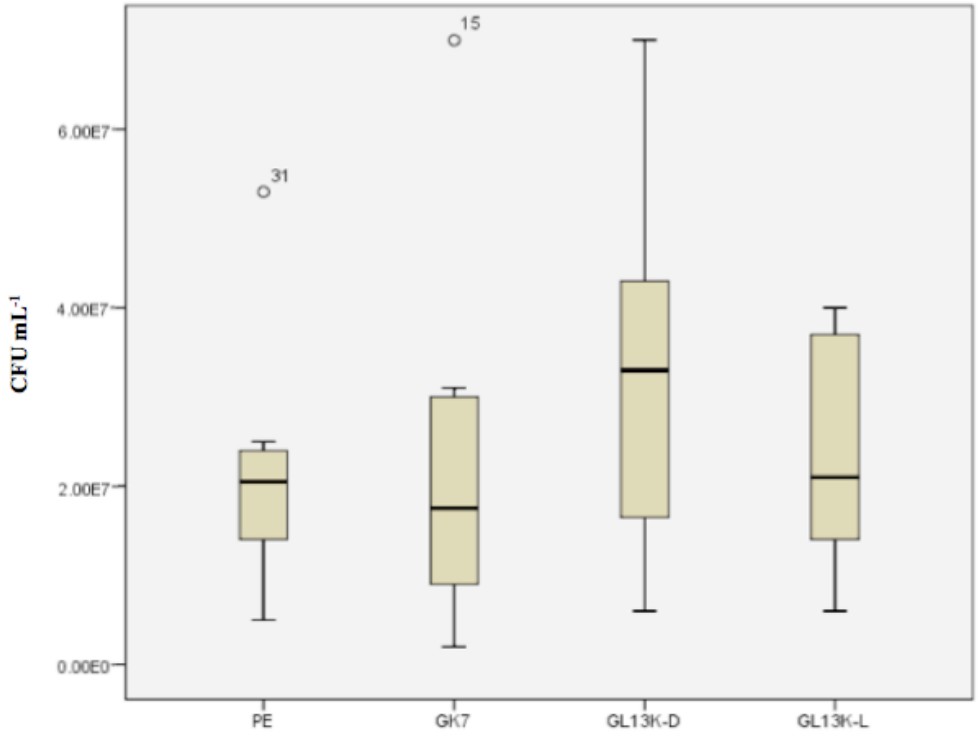

Figure 14. MRS agar colony forming units of *L. casei* cultured on GL13K-coated discs. Data are representative of 4 repeat experiments with 3 samples per group. °# indicates moderate outliers included in statistical analysis.

Scanning electron microscopy revealed cell membrane disruption and cell lysis with *S. mutans* treated with either GL13K–L or GL13K–D (Figure 15). This phenomenon was observed only with *S. mutans* treated with GL13K–L or GL13K–D. No similar observations were made with *L. casei* cultured on GL13K-coated Ti discs.

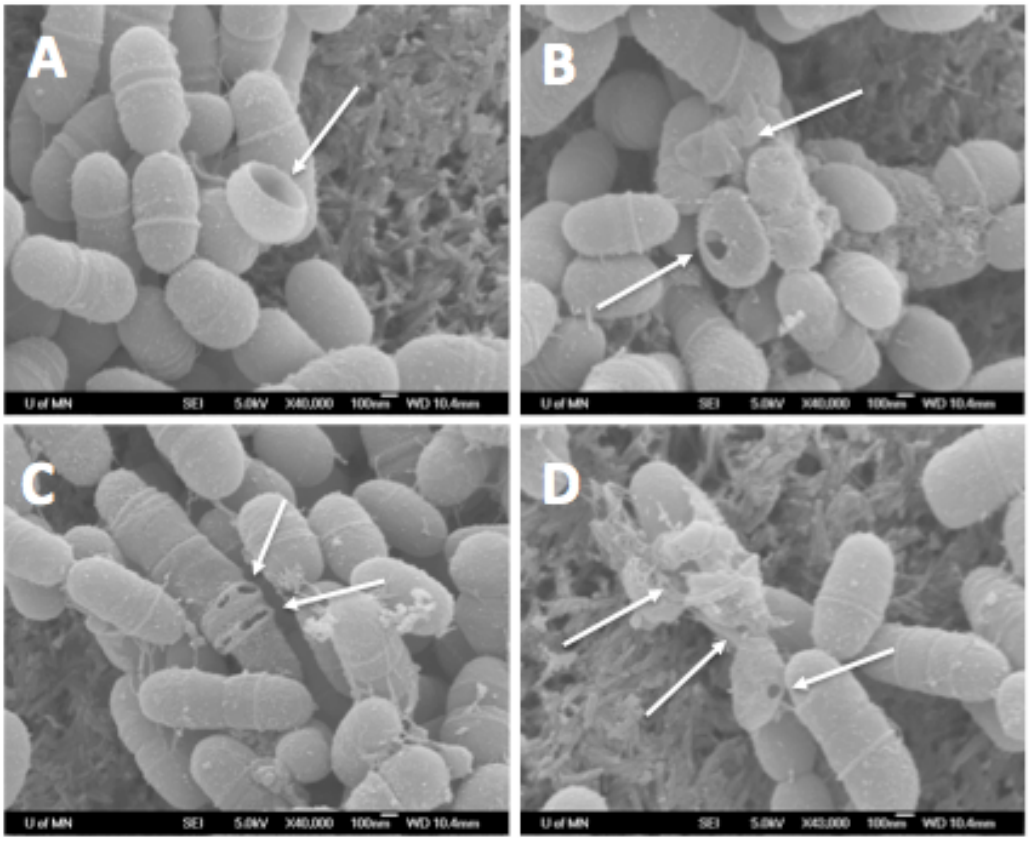

Figure 15. Cell membrane disruption of *S. mutans* treated with GL13K–D or GL13K–L. *S. mutans* on (A-C) GL13K–L and (D) GL13K–D coated discs. White arrows indicate areas of cell membrane disruption. Images acquired at x40,000 magnification using a JEOL 6700 Field Emission Gun-Scanning Electron Microscope.

Discussion

WSLs are the most prevalent adverse outcome of orthodontic treatment with fixed appliances (Heymann *et al*., 2013). Previous attempts at WSL prevention include the use of adjunctive treatments such as fluoride-containing toothpastes, mouthwashes, or varnishes; or chlorohexidine gluconate prescription mouthwash to help control bacterial growth around orthodontic appliances. However, these adjuncts require patient compliance, which is unlikely given a patient with poor oral hygiene (Geiger *et al*., 1992). Glass ionomer cements have been used as bonding agents with some success, yet they also result in decreased shear bond strength of orthodontic brackets, lending to increased bracket failure during treatment (Wiltshire, 1994; Marcusson *et al*., 1997). Elastomeres impregnated with fluoride have been developed and used in orthodontic patients with success (Storie *et al.,* 1994; Banks *et al*., 2000; Mattick *et al*., 2001). However, there are notable concerns with leaching of fluoride into the oral cavity and the possible adverse effects of systemic fluoride from elastomeres (Benson *et al.* 2004).

The present project aimed at developing a novel bioactive peptide coating on titanium alloy capable of killing the acidogenic oral bacteria *S. mutans* and *L. casei* in an attempt to develop an antimicrobial peptide-coated orthodontic bracket for the purpose of preventing the formation of WSLs. As performed in this study, SEM imaging of discs at each stage of preparation showed a highly nanoporous structure created by the alkaline etching process. The nanopores allow for reservoirs of GL13K to be retained in the metal surface. In addition, the negative charge of titanium etched with NaOH provides a strong electrostatic attraction to the positively charged GL13K peptide, which immobilizes the

peptides on the activated metal surfaces. As a consequence of their identical amino acid sequences, there was no difference between the appearance of the GL13K–D and GL13K–L peptides in the SEM images.

The low contact angle measures of PE discs suggest that etched titanium is hydrophilic. The higher contact angles of the peptide coatings on discs demonstrate hydrophobicity and serve to indirectly verify the presence of peptide coatings. These findings of contact angle measures by sessile drop are comparable to the results published by Holmberg *et al.* (2013) who reported highly hydrophilic etched titanium surfaces and contact angles of 125 ± 1.35° for GL13K–L coated titanium. On average, the GL13K–L and GL13K–D contact angles determined in the present study were 16-24° less than those reported by Holmberg *et al.* This is likely due to the use of different batches of peptides from different companies.

The stability tests revealed that the alkaline etching method is an effective method of preparation for coating the peptide onto the Ti surface—only the discs pretreated with the alkaline etching method showed a strong signal when coated with the fluorescent-labeled peptide. For each of the 3 challenges, hydrolytic, proteolytic, and mechanical, the fluorescent signal was still robust indicating that GL13K-coatings on etched titanium are able to withstand physical, chemical, and mechanical challenges representative of those in the oral environment.

The GL13K peptide coatings are highly hydrophobic, which should hinder the interaction of the peptides with aqueous solutions such as water, PBS, and/or saliva. For this reason, it can be assumed that GL13K coatings were readily able to sustain the

hydrolytic and proteolytic challenges. Of the types of challenges tested, the mechanical challenge may pose the greatest threat to peptide stability, as surface scratches were observed in multiple samples. It would be valuable for future studies to perform the stability tests for extended durations of time, as orthodontic brackets typically are in the oral cavity for an average of 2 years. Additionally, it is suggested that future studies use a mechanical challenge more representative of abrasive forces from eating and tooth brushing.

The results of the microbiology experiments suggest that GL13K–D, both in solution and immobilized on titanium alloy discs, is bactericidal against *S. mutans*. The results of the MIC experiments demonstrate that the –D form of GL13K is about 8 times more potent against *S. mutans* than the –L form. While *S. mutans* was killed by GL13K–L in solution at 64 μg/ml, and similarly *L. casei* was killed by GL13K–D and GL13K–L in solution at 64 μg/ml, this concentration is on the moderate to higher end of what is typically required for an effective antimicrobial peptide MIC. Some of the most effective antimicrobial peptides, such as adrenomedullin, have MICs on the order of magnitude of 10^{-4} μg/ml (Gorr, 2009).

The results of the bactericidal assays demonstrate that *S. mutans* is susceptible to bactericidal effects of GL13K–D, and minimally susceptible to GL13K–L. The observations of increased bacterial susceptibility with the –D peptide were consistent for experiments conducted on both BHI agar and MSSB agar. The slight variations in *S. mutans* survival observed for the –L peptide may result from the two different types of cell culture growth medium, or possibly due to inherent experimental variation. The

results of the bactericidal assay with *L. casei* also demonstrate the superior bactericidal effect of the GL13K–D peptide when compared to the –L peptide. It is interesting to note that the concentrations of GL13K required for a bactericidal effect on *S. mutans* and *L. casei* are several folds of magnitude greater than the MICs that were determined for the peptides in solution. These variations are likely a result of differences in methodology used for the two experiments (i.e., bacteria cultured in solution with GL13K for the MIC tests vs. GL13K pretreatment for the bactericidal assays). In other words, the antimicrobial effect may be greater when the bacteria are cultured directly with the peptide in solution, giving the peptide additional time to interact with and disrupt the cell membrane integrity.

The results of the crystal violet and biomass assays confirm the presence of biofilm and suggest that while GL13K may have a bactericidal effect on *S. mutans* and *L. casei*, it does not reduce the amount of biofilm on GL13K-coated discs. It should be emphasized that the crystal violet and biomass assays do not distinguish between live and dead bacteria present on the discs. Therefore, we elected to examine more closely if there were any differences in the amount of live and dead bacteria between treatment and control groups with the viability assay.

The findings from the LIVE/DEAD viability assay for both *S. mutans* and *L. casei* are consistent with the notion that the GL13K–D peptide demonstrates a more potent bactericidal effect than GL13K–L. The results of the LIVE/DEAD experiments, when considered in conjunction with the results of the crystal violet and biomass assays, allow the conclusion that while GL13K may not reduce the amount of biofilm present on the

coated discs, there are more dead and fewer live bacteria present within *S. mutans* or *L. casei* biofilm on the GL13K–D coated discs, respectively.

The CFU experiments demonstrated a statistically significant antimicrobial effect of the GL13K–D disc coatings on *S. mutans*. Similar to the MIC tests, bactericidal assays and LIVE/DEAD viability tests, these finding suggest that GL13K–D has a potent antimicrobial effect on *S. mutans*. To the best of our knowledge, this is the first time an antimicrobial effect of GL13K–D was observed on *S. mutans in vitro*, both in solution and on coated metal surfaces. The difference in the results of the MICs, bactericidal assays, and CFU assays between the two forms of GL13K warrants further research. It is possible that the function of the GL13K peptide is altered when bound to the metallic surface, or that one form is superior to the other in its antimicrobial effects. Since it is currently not known exactly how GL13K exerts its antimicrobial effects or what the active site is on the GL13K peptide, it is possible that the molecular interactions which electrostatically bind the positively charged GL13K to the negatively charged etched titanium surface could alter the bound molecular conformation enough to effect the active site and, in turn, effect the antimicrobial activity of the peptide.

Furthermore, the results of these experiments are dependent on how much of the peptide placed onto the surface for coating is actually retained. The amount of peptide retained on the surface mainly depends on the total surface area obtained after etching. The greater the surface area, the more negative charges are available for the cationic antimicrobial GL13K peptide to bond. Since it is not possible to calculate the total

surface area after etching due to its nanoporous nature, is it not possible to make predictions of the number of peptides bound to the surface.

Direct measurement of peptides on the surface would require surface plasmon resonance equipment, which was not available at the time of the study. It is possible to roughly quantify the amount of peptides on the surface indirectly, as performed by Chen *et al.* (2013) with a protein-based colorimetric or fluorometric test, such as the Bradford test. Chen *et al.* (2013) determined the concentration of GL13K on the surface in the order of a few micrograms per square centimeter, when bound covalently via silanization. This is a very high concentration in comparison to what other groups have published for peptide coatings, which is on the order of a few dozens of nanograms per square centimeter (Stravalaci *et al*., 2011). While the fluorescent images from the stability tests performed in this study do not quantify the amount of peptides, they do suggest that the amount of peptide retained was significant and homogeneously distributed.

The results of the SEM imaging are somewhat inconclusive due to the overall low numbers of bacteria present on the surfaces. One possible explanation for the low bacterial presence is that bacteria may have been lost during sample preparation. One of the steps in SEM sample preparation involves platinum plating under a vacuum. It is possible that bacteria from the surfaces were lost in the low-pressure environment.

Low cell numbers aside, there were a few interesting SEM imaging findings of note. We observed under high magnification the appearance of the nanoporous etched titanium surface. We also observed that disc surfaces on which *L. casei* was cultured were covered with what appeared to be non-cellular biological material. Perhaps the

material is an extracellular protein secretion by *L. casei* to help colonize the biologically foreign Ti surface. The strong presence of this secretion on the SEM samples but the minimal presence of *L. casei* further supports that bacteria may have been lost in SEM sample preparation.

The most interesting finding from the SEM imaging was the observation of damaged or compromised cell membranes of *S. mutans* treated with the GL13K–L or GL13K–D peptide. With both –L and –D forms of GL13K, we observed perforations in the cell membrane of *S. mutans*. To the best of our knowledge, this is the first time cell membrane disruption of *S. mutans* was observed, resultant from treatment with an antimicrobial GL13K peptide coating on titanium alloy discs, a pilot model for antimicrobial orthodontic brackets.

The method of action of antimicrobial peptides is by disrupting and permeabalizing cell membranes. The internal pressure of gram-positive bacterial cells is roughly 15–25 atmospheres and any disruption in the wall's integrity will result in the extrusion of the cytoplasmic membrane and ultimately hypotonic lysis (Fischetti, 2011). The images of cell membrane disruption presented here are similar to the observations made when *S. gordonii* was exposed to GL13K in a peri-implantitis model (Chen *et al*., 2014). This supports our finding that GL13K has significant antimicrobial activity against *S. mutans*.

Future studies may benefit from examining the feasibility of GL13K-coatings on stainless steel, the most common metal used in fabrication of orthodontic brackets. The alkaline etching method, which proved effective with titanium, may cause embrittlement

with stainless steel, which properties different from titanium. Charging a stainless steel surface with negatively charged O_2 by plasma cleaning may be an alternative method of surface activation. Future studies may also benefit from utilizing multiple antimicrobial peptides to coat a single surface, thereby increasing the bacterial susceptibility to cell lysis. Successful methods for co-immobilization of oligopeptides have been described in the literature and involve a silanization process (Chen *et al*., 2013). This approach may allow for redundancy in killing multiple species of acidogenic oral bacteria.

While the results of the experiments with GL13K and *L. casei* remain conflicting and inconclusive, our data support that GL13K–D is an antimicrobial peptide effective against *S. mutans in vitro* and holds promise for oral therapeutic applications in humans. Additional research is suggested to further characterize the effects of GL13K–L and –D on other acidogenic oral bacteria such as *S. sobrinus* and *L. acidophilus*. Future studies may also seek to explore the effects of GL13K on intermediate biofilm colonizers such as *F. nucleatum*. The durability, biocompatibility, and antimicrobial efficacy of the GL13K-coatings make it an exciting and promising candidate for orthodontic application.

Conclusions

• Alkaline etching of Ti6Al4V with NaOH is an effective and reliable method for activating the Ti surface to subsequently anchor GL13K peptide coatings.

• GL13K coated discs are able to withstand approximately 2 weeks of hydrolytic, proteolytic, and mechanical challenge simulating the conditions of the oral environment.

• GL13K–D and –L Ti coatings can disrupt the cell membrane of *S. mutans*.

• GL13K–D coatings have a significant antimicrobial effect against *S. mutans* and hold promise for the prevention of white spot lesions in orthodontic patients.

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