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Bio-inspired stable antimicrobial peptide coatings for dental applications

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ABSTRACT

We developed a novel titanium coating that has applications for preventing infection-related implant failures in dentistry and orthopedics. The coating incorporates an antimicrobial peptide, GL13K, derived from parotid secretory protein, which has been previously shown to be bactericidal and bacteriostatic in solution. We characterized the resulting physicochemical properties, resistance to degradation, activity against *Porphyromonas gingivalis* and in vitro cytocompatibility. *Porphyromonas gingivalis* is a pathogen associated with dental peri-implantitis, an inflammatory response to bacteria resulting in bone loss and implant failure. Our surface modifications obtained a homogeneous, highly hydrophobic and strongly anchored GL13K coating that was resistant to mechanical, thermochemical and enzymatic degradation. The GL13K coatings had a bactericidal effect and thus significantly reduced the number of viable bacteria compared to control surfaces. Finally, adequate proliferation of osteoblasts and human gingival fibroblasts demonstrated the GL13K coating's cytocompatibility. The robustness, antimicrobial activity and cytocompatibility of GL13K-biofunctionalized titanium make it a promising candidate for sustained inhibition of bacterial biofilm growth. This surface chemistry provides a basis for development of multifunctional bioactive surfaces to reduce patient morbidities and improve long-term clinical efficacy of metallic dental and orthopedic implants.

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1. Introduction

Dental implants machined from commercially pure titanium (Ti) are increasingly becoming the treatment of choice for replacing missing teeth, with 10–15 year survival rates of 89+% [1]. Despite recent improvements in implant survival rates, there remains a significant demand for improving osseointegration and maintaining a perimucosal seal. In particular, the long-term clinical efficacy of titanium dental implants is influenced by peri-implantitis, an inflammatory response resulting in bone loss and implant failure [2]. Peri-implantitis or infection can affect up to 14% of implants after 5 years, although the incidence may be higher due to poor clinical diagnosis, and the limited data and short duration of the clinical studies reported [1,3,4]. The transmucosal placement of dental implants presents unique challenges for designing surface modifications capable of decreasing the formation of bacterial biofilm. The coronal implant surface is exposed to the mucosal sulcus, capable of harboring biofilms. Similar to periodontitis, in peri-implantitis local and host factors cause an ecologic shift toward anaerobic, Gram-negative and Gram-positive bacteria associated with inflammation and bone loss, including *Porphyromonas gingivalis*, *Eikenella corrodens*, *Fusobacterium nucleatum* and *Peptostreptococcus micros* [5], as well as microorganisms not commonly associated with periodontitis, such as *Staphylococcus* spp., enterics and *Candida* spp. [6]. Noninvasive strains of *P. gingivalis*, e.g. ATCC-33277, feature hydrophobic fimbriae, which make the cell surface highly hydrophobic [7] and mediate binding to gingival epithelium and implant surfaces [8].

Cationic and histatin-derived antimicrobial peptides (AMPs) adsorbed to Ti have been shown to prevent biofilm formation [9,10]. The physical driving forces behind the antibacterial activity include positive charge, hydrophobicity and flexibility [11]. AMPs are hypothesized to bind to the bacterial cell membrane and disrupt its integrity by displacing positively charged counterions and inducing a change in the membrane electrochemical potential, resulting in activation of autolytic enzymes [12]. AMPs have also been shown to modulate inflammatory responses of host cells [11].

The antimicrobial peptide GL13K features a modified 13-amino-acid sequence based on the sequence of parotid secretory protein (PSP; BPIFA2) [13], a potential dual-function host defense salivary protein with agglutination and anti-inflammatory activity [14]. The GL13K peptide exhibits bactericidal activity in vitro and anti-endotoxin activity in a mouse model. GL13K is bactericidal in solution against *Pseudomonas aeruginosa*, *Escherichia coli* and

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Streptococcus gordonii, with a minimum inhibitory concentration of 5–10 μg ml^{-1} against *P. aeruginosa* and *E. coli* and 64 μg ml^{-1} against *S. gordonii*. GL13K also kills *P. aeruginosa* in biofilm and inhibits the lipopolysaccharide-stimulated secretion of TNFα from macrophages by 80% [14]. In contrast, GL13K is not effective in killing three strains of *P. gingivalis* (53977, W50 and DPG3), presumably due to gingipain proteases secreted by these bacteria [15]. These data support GL13K as a promising novel and efficacious antimicrobial agent for dental implants and restorative dentistry. While the potential for antimicrobial peptide surfaces has been demonstrated [16,17], an optimal surface for dental implants must retain its antimicrobial activity in a chronically microbially challenged environment. Most antimicrobial Ti surfaces have only been studied in vitro for 24–48 h, and existing approaches for surface modification of dental implants to reduce bacterial biofilm are not yet used clinically [17–19]. The only available treatments for peri-implantitis include mechanical debridement, surgical therapy and non-surgical local or systemic antibiotic therapy [20,21], but there is no reliable evidence suggesting which is the most effective intervention [22].

We present surface chemistry for stable immobilization of GL13K to Ti as a model antimicrobial bioactive Ti surface for applications in dentistry and orthopedics to reduce implant-associated infections and failure. We aim to fabricate a novel antimicrobial coating by covalently anchoring the cationic antimicrobial peptide, GL13K, to a Ti surface using a silane chemical linker. We investigate the mechanical and thermochemical stability, antimicrobial activity and cytocompatibility of the antimicrobial coatings.

2. Materials and methods

2.1. Materials

The antimicrobial GL13K (GKIIKLKASLKLL-CONH2, MW = 1424 g mol^{-1}) and non-antimicrobial GK7-NH2 (GQIINLK-CONH2, MW = 784 g mol^{-1}) peptides were obtained at >95% purity from the BioMedical Genomics Center-University of Minnesota. GL13K consists of a 13-amino-acid sequence of PSP (141–153) substituted with three lysine residues in positions 2, 5 and 11 [15]. The negative-control peptide GK7-NH2 consists of the N-terminal seven amino acids of GL13K without the lysine substitutions [13].

Ti disks (10 mm diameter) were punched from sheets of commercially pure Grade II Ti (McMaster-Carr; Robbinsville, NJ). 3-(Chloropropyl)-triethoxysilane (CPTES, 95%), other chemical reagents and solvents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Sample preparation

Ti disks were ground with 600-grit SiC disks and polished using 0.5 μm alumina suspensions (Buehler, Lake Bluff, IL). Samples were soaked in acetone overnight, then ultrasonicated in a series of solvents and dried with N_2 gas. Ti samples were etched (eTi) in 10 ml of 5 M NaOH overnight at 60 °C, further cleaned with deionized (DI) water for 30 min (2×) and dried with N_2 gas. A set of non-etched samples was instead treated with O_2 plasma (pTi; PDC-32G, Harrick Plasma, Ithaca, NY, USA) for 5 min for depth-profiling X-ray photoelectron spectroscopy (XPS) analysis.

2.3. Silanization

Activated samples (pTi or eTi) were silanized by immersion in a solution of 7 ml of anhydrous pentane, 1.2 ml of 3-(chloropropyl)-triethoxysilane (CPTES) and 0.6 ml of diisopropylethylamine (DIPEA) under a saturated N_2 atmosphere for 1 h. After silanization, the samples were washed and dried with N_2 gas.

2.4. Peptide immobilization

Solutions of GL13K (0.1 mM) or GK7-NH2 (0.25 mM) were prepared in 8 ml of Na_2CO_3 (0.5 mg ml^{-1}). The number of immobilization sites (free amines) was kept constant between the peptide solutions. Silanized eTi (eTi-Sil) and non-silanized eTi samples (eTi) were immersed in the appropriate peptide solutions overnight under argon in a desiccator and then rinsed with DI water to produce eTi-Sil-GL13K (cov-GL13K), eTi-Sil-GK7-NH2 (cov-GK7-NH2), and eTiGL13K (phys-GL13K) samples.

2.5. Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS)

Surfaces were characterized with DRIFTS (Nicolet Series II Magna-IR System 750 FTIR, OMNIC software) following ultrasonication for 2 h in DI water to detect amide bonds. Thirty-two scans per sample (2–4 cm^{-1} resolution) were averaged across replicates.

2.6. X-ray photoelectron spectroscopy

XPS was performed (SSX-100, Al K_α X-rays, 1 mm spot size, 35° take-off angle) to characterize the atomic composition of the surface. Samples were ultrasonicated for 5 min, washed and dried with N_2 gas prior to measurements. Survey scans were done at 1 eV step size for 3–4 scans per sample. High-resolution scans of C1*s*, N1*s* and O1*s* were taken at 0.1 eV step size. The chemical shifts of the high-resolution scans were based on C1*s* at 285.0 eV. Depth profiling of pTi samples used Ar^+ sputtering at 4 kV, 25 mA and 5 mPa (2 nm min^{-1} SiO_2) for 0–12 s (2 s intervals) with high-resolution O1*s* scans. The peak fittings and quantification of the surface chemical composition were conducted using ESCA 2005 software provided with the XPS system.

2.7. Water contact angles

Advancing DI water contact angles (θ_c) were measured with the sessile drop (3 μl) method (DM-CE1 with FAMAS software, Kyowa Interface Science Co., Niiza, Japan). θ_c were determined at 1 s intervals for 35 s. θ_c were calculated by averaging the values of the last 5 s for each experiment. Samples were tested before and after ultrasonication in DI water for 2 h, as well as after 5 and 8 days of immersion in phosphate-buffered saline (PBS, pH 7.4, 37 °C) following ultrasonication to determine the mechanical and thermochemical stability of the coatings, respectively.

2.8. Biofilm formation

A 48 h culture of *P. gingivalis* ATCC 33277 was diluted to 1×10^6 cells ml^{-1} in Todd Hewitt base (THB) broth with 4% heat-inactivated fetal bovin serum, 5.0 μg ml^{-1} Hemin and 0.5 μg ml^{-1} Menodine. The Ti disks were cleaned with ethanol and placed in culture plates, and 1 ml of diluted *P. gingivalis* was added to each well. The culture was incubated under anaerobic conditions at 37 °C for 8 days. After the incubation medium had been removed from the well, the Ti disks were removed to a new culture plate and washed with PBS. Discs were transferred to microcentrifuge tubes with 300 μl of pre-reduced oxygen PBS and sonicated for 60 s. Next, 100 μl volumes of the obtained solutions were transferred to an opaque wall 96-well plate, and the amount of ATP was quantified (Promega BacTiter-Glo™ Microbial Cell Viability Assay, Promega, Madison, WI). The obtained solutions were also diluted 100-fold and plated on THB–blood agar plates. The surviving CFUs were enumerated after 7 days of anaerobic incubation at 37 °C. To assess whether all bacteria had been removed from the substrates, following the removal of PBS after sonication we added

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