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Engineered chimeric peptides with antimicrobial and titanium-binding functions to inhibit biofilm formation on Ti implants

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ABSTRACT

Titanium (Ti) implants have been commonly used in oral medicine. However, despite their widespread clinical application, these implants are susceptible to failure induced by microbial infection due to bacterial biofilm formation. Immobilization of chimeric peptides with antibacterial properties on the Ti surface may be a promising antimicrobial approach to inhibit biofilm formation. Here, chimeric peptides were designed by connecting three sequences (hBD-3-1/2/3) derived from human β-defensin-3 (hBD-3) with Ti-binding peptide-l (TBP-l: RKLPDAGPMHTW) via a triple glycine (G) linker to modify Ti surfaces. Using X-ray photoelectron spectroscopy (XPS), the properties of individual domains of the chimeric peptides were evaluated for their binding activity toward the Ti surface. The antimicrobial and anti-biofilm efficacy of the peptides against initial settlers, *Streptococcus oralis* (*S. oralis*), *Streptococcus gordonii* (*S. gordonii*) and *Streptococcus sanguinis* (*S. sanguinis*), was evaluated with confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). Transmission electron microscopy (TEM) and real-time quantitative PCR (qRT-PCR) were used to study cell membrane changes and the underlying antimicrobial mechanism. Compared with the other two peptides, TBP-1-GGG-hBD3-3 presented stronger antibacterial activity and remained stable in saliva and serum. Therefore, it was chosen as the best candidate to modify Ti surfaces in this study. This peptide inhibited the growth of initial streptococci and biofilm formation on Ti surfaces with no cytotoxicity to MC3T3-E1 cells. Disruption of the integrity of bacterial membranes and decreased expression of adhesion protein genes from *S. gordonii* revealed aspects of the antibacterial mechanism of TBP-1-GGG-hBD3-3. We conclude that engineered chimeric peptides with antimicrobial activity provide a potential solution for inhibiting biofilm formation on Ti surfaces to reduce or prevent the occurrence of peri-implant diseases.

1. Introduction

Titanium (Ti) and its alloys have become the key materials used for dental implants, primarily due to their biocompatibility with human tissues and excellent mechanical properties [1]. However, oral peri-implantitis, which is generally characterized by its effects on the surrounding soft and hard tissues, has become one of the most inflammatory reactions observed after implant placement [2]. It causes inflammation of the peri-implant mucosal tissue and progressive damage of supporting bone, leading to implant removal and, occasionally, to loosening and loss of the implant [2–5]. A study evaluating two cohorts (662 and 216 subjects) showed that the prevalence of peri-implantitis in affected patients was 28% and ≥56%, respectively, whereas it was 12% and 43% for individual implants [2]. A systematic review published in 2015 indicated that the prevalence of peri-implantitis may range from 1% to 47%, with a weighted mean prevalence

Abbreviations: Ti, titanium; hBD-3, human β-defensin-3; TBP-l, Ti-binding peptide-l; XPS, X-ray photoelectron spectroscopy; CLSM, confocal laser scanning microscopy; SEM, scanning electron microscopy; TEM, transmission electron microscopy; qRT-PCR, real-time quantitative PCR; *S. oralis*, *Streptococcus oralis*; *S. gordonii*, *Streptococcus gordonii*; *S. sanguinis*, *Streptococcus sanguinis*; AMPs, antimicrobial peptides; *F. nucleatum*, *Fusobacterium nucleatum*; *P. gingivali*, *Porphyromonas gingivali*; G, glycine; PBS, phosphate-buffered saline; AMC, 7-amino-4-methylcoumarin; CLSM, confocal laser scanning microscopy; CD, circular dichroism; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; OD, optical density; BHI, brain heart infusion

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rate of 22% [6]. Therefore, the occurrence of peri-implantitis is still an urgent problem that requires a solution.

Pathogenic bacterial adhesion to the implant surface is the first step in pathogenic biofilm formation, which is an important factor in the occurrence of peri-implantitis and implant failure [7]. In the early phases of oral biofilm formation, the predominant tooth colonizers have been found to be streptococci; in particular, *Streptococcus gordonii* (*S. gordonii*), *Streptococcus sanguinis* (*S. sanguinis*) and *Streptococcus oralis* (*S. oralis*) have been identified as initial colonizers that provide the foundation for subsequent bacterial adhesion, such as by middle colonizers (*Fusobacterium nucleatum*, *F. nucleatum*) and late colonizers (*Porphyromonas gingivali*, *P. gingivali*), which finally results in biofilm formation [8–10]. Therefore, one of the key factors for implant success is inhibition or elimination of early bacterial adhesion and biofilm formation on the surface of dental implants.

Currently, different approaches and types of coatings are used to modify Ti implant surfaces to improve their antimicrobial activity, including antibiotic loading [11], covalent immobilization of antimicrobial peptides (AMPs) [12–15], and polymeric functional modification [16,17]. The nanomaterials of varying size, surface structural and morphological properties, and physicochemical behavior are important in biomedical fields [18–21] and have been one of research hotspots. Through designing the desired nanoparticles with organic and inorganic material with special type, size, surface properties for target implication to realize the purposes of drug delivery, biosensors, antibacterial and antibiofilm activity coating [22–26]. The noble metal nanoparticles were also investigated to modify oral implant materials surfaces to achieve antimicrobial purpose [27]. Among these strategies, the use of engineered chimeric peptides with antimicrobial activity and binding capability to modify Ti surfaces is a simple approach [28]. AMPs have been described being as more effective against antibiotic-resistant strains, with less development of drug resistance [29–31], and have been shown to present satisfactory biocompatibility [15]. Human β-defensin-3 (hBD3) with 45 amino acid residues, is a molecule in the AMP family that has a wide range of antibacterial properties and has received much attention [32,33]. Recently, three fragment sequences derived from hBD3 have been demonstrated to exhibit cell- and skin-penetrating properties and anti-inflammatory activity [34]. However, it is necessary to investigate whether the three sequences can exert antibacterial activity and inhibit biofilm formation on dental implant surfaces. Recently, it was confirmed that TBP-1 (RKLPDAPGMHTW), a biomolecular linker, has high affinity to Ti biomaterials due to its electrostatic interaction with the amphoteric Ti surface, which is often covered by an oxide film composed of -Ti-OH + and/or -Ti-O − [35]. TBP-1 can thus serve as an approach for binding AMPs onto Ti implant surfaces.

In this study, three chimeric peptides with high-affinity Ti-binding capability and antibacterial activity, TBP-1-GGG-hBD3-1, TBP-1-GGG-hBD3-2 and TBP-1-GGG-hBD3-3, were synthesized in an attempt to develop alternative agents to load onto Ti surfaces. A triple glycine (G) peptide was used as a space linker between the sequences derived from hBD3 and TBP-1. We used three sequences derived from hBD3 to modify Ti surfaces via TBP-1-mediated linkage to inhibit initial colonizer adhesion and affect biofilm formation on Ti dental implant surfaces.

2. Experimental section

2.1. Peptide design and synthesis

The peptides TBP-1-GGG-hBD3-1, TBP-1-GGG-hBD3-2, and TBP-1-GGG- hBD3-3 (Table 1) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Peptides were labeled with 7-amino-4-methylcoumarin (AMC) for confocal laser scanning microscopy (CLSM). The final chimeric peptides were purified by HPLC up to > 95% purity and confirmed using mass spectrometry analysis. The stock solutions and subsequent dilutions of each peptide for the experiments were prepared using sterile phosphate-buffered saline (PBS) to dissolve the peptide powder and dilute the solutions. The secondary structures and basic properties of the peptides were predicted using PSIPRED software (http://bioinf.cs.ucl.ac.uk/psipred/) and the peptide property calculator (http://www.pepcalc.com/).

2.2. Preparation of titanium substrates

In this study, Ti substrates were prepared by evaporating pure Ti (99.998% purity) films with a thickness of 300 nm on 10 mm × 10 mm silicon wafers using electron beam evaporation (Sharon Vacuum, Brockton, MA, USA). All Ti substrates were ultrasonically cleaned with acetone and 70% (v/v) ethanol for 20 min, followed by sterile deionized water for 10 min. The samples were then dried in an oven at 80 °C for 15 min and sterilized in a steam autoclave at 120 °C and 102.9 kPa for 30 min.

2.3. Circular dichroism (CD) spectroscopy

Each peptide was first dissolved at 0.1 mg/mL in sterile PBS. The CD spectra were recorded with a CD spectrometer (BIO-LOGIC Corp. J-810, France) at room temperature and at 37 °C, using a 1.0 mm cuvette. CD spectra were acquired with solvent subtraction from 190 to 350 nm at a scanning speed of 60 nm/min and were averaged from 3 runs per peptide sample. The acquired CD spectra of peptides were analyzed using a SELCON3 algorithm to perform quantitative analysis of the secondary structure of TBP-1-GGG-hBD3-1, TBP-1-GGG-hBD3-2, and TBP-1-GGG-hBD3-3.

2.4. Raman spectroscopy

Samples of 5 mg were placed on a spotting plate. The samples were analyzed using a Raman spectrometer (Renishaw, Gloucestershire, United Kingdom) with 785 nm laser excitation (Coherent, Santa Clara, CA, USA) at an intensity of 2 mW and an exposure time of 10 s aimed directly at the sample. Scanning was performed at 1800–300 cm^{-1} with a pixel size of 16 × 16 μm. All experiments were repeated three times.

2.5. XPS analysis

One Ti plate was incubated in 320 μg of peptide solution (2 mL), and another Ti plate was cultured in 2 mL of sterile PBS for 2 h at room temperature. The peptide-treated and peptide-untreated substrates were rinsed extensively with sterile PBS and ultrapure deionized water and dried under argon gas. The chemical composition of the Ti substrates was determined via X-ray photoelectron spectroscopy (XPS), which was performed on an XPS spectrometer (ESCALAB 250, USA) with a monochromatized Al Kα X-ray source (1486.6 eV photons) under vacuum (10^{-8} Torr or lower) using an incidence angle of 45° at a power of 150 W. The C 1s peak (284.80 eV) was utilized as a standard to calibrate all other binding energies. The experiment was repeated three times.

2.6. Antibacterial assays

S. oralis (ATCC No. 9811), *S. gordonii* (ATCC No. 10558) and *S. sanguinis* (ATCC No. 10556) were obtained from the ATCC (American Type Culture Collection, VA, USA). *S. oralis* was cultured aerobically in a freshly prepared brain heart infusion (BHI) agar plate supplemented with 1% yeast extract for 24 h at 37 °C. *S. gordonii* and *S. sanguinis* were cultured separately in freshly prepared BHI agar plates supplemented with 5% sterile defibrinated sheep blood, 1% hemin, and 0.1% menadione in a chamber under anaerobic conditions of 80% N_2, 10% H_2 and 10% CO_2 for 48 h at 37 °C.