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Antibiofilm peptides against biofilms on titanium and hydroxyapatite surfaces

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ABSTRACT

Biofilms are the main challenges in the treatment of common oral diseases such as caries, gingival and endodontic infection and periimplantitis. Oral plaque is the origin of microbes colonizing in the form of biofilms on hydroxyapatite (tooth) and titanium (dental implant) surfaces. In this study, hydroxyapatite (HA) and titanium (Ti) disks were introduced, and their surface morphology was both qualitatively and quantitatively analyzed by a scanning electron microscope (SEM) and atomic force microscope (AFM). The average roughness of Ti disks (77.6 ± 18.3 nm) was less than that of HA (146.1 ± 38.5 nm) ($p < 0.05$). Oral multispecies biofilms which were cultured on Ti and HA disks for 6 h and three weeks were visualized by SEM. We investigated the ability of two new antibiofilm peptides, DJK-5 and 1018, to induce killing of bacteria in oral multispecies biofilms on Ti and HA disks. A 6-h treatment by DJK-5 and 1018 (2 or 10 μg/mL) significantly reduced biomass of the multispecies biofilms on both Ti and HA disks. DJK-5 was able to kill more bacteria (40.4–75.9%) than 1018 (30.4–67.0%) on both surfaces ($p < 0.05$). DJK-5 also led to a more effective killing of microbes after a 3-min treatment of 3-day-old and 3-week-old biofilms on Ti and HA surfaces, compared to peptide 1018 and chlorhexidine ($p < 0.05$). No significant difference was found in the amount of biofilm killing between Ti and HA surfaces. Both peptide DJK-5 and 1018 may potentially be used as effective antibiofilm agents in clinical dentistry.

1. Introduction

Most microorganisms in nature live in a biofilm state, as aggregates with a complex construction on different surfaces. In the oral cavity, biofilms on tooth surfaces (hydroxyapatite) form the “bacterial plaque”, which lead to caries [1], gingival infection and periodontitis [2]. Titanium has been widely employed as a dental implant material for several decades owing to its excellent properties of biocompatibility, low allergenicity, osseointegration, and resistance to corrosion [3,4]. Ti implants have high success in the oral cavity [5]. However, biofilm associated infections (including periimplant mucositis and periimplantitis) are the leading causes of Ti implant failures [6,7]. Once Ti implants or abutments are installed in the oral environment, bacteria start to immediately colonize the Ti interfaces [8] providing target for new planktonic bacteria to adhere, proliferate and develop into a complex biofilm structure.

The scaffolds of biofilms are composed of extracellular polymeric matrix [9] and DNA of microbial origin [10], which can protect bacteria in biofilms against disinfecting solutions [11]. Therefore, microbes in the biofilm are more resistant than planktonic bacteria to most antimicrobial agents [12,13]. Various antibiofilm substances and strategies have been explored and reported in the past decades. However, over time many bacteria have developed resistance to commonly used antibiotics [14]. Hence, more efficient antibiofilm agents need to be developed to overcome this challenge and ensure success in treating infections in the oral cavity.

Antimicrobial peptides (AMPs) have drawn researchers' attentions due to their promising antimicrobial effect on biofilms related to infections over the past several years. AMPs can be either natural or synthetic peptides with antimicrobial properties against most bacterial pathogens [15]. Most AMPs are positively charged amphipathic peptides which interact with the negative charged groups on the bacterial cell membrane destroying its integrity [16]. Recently, peptides DJK-5 and 1018 have been developed with a special broad-spectrum

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antibiofilm activity against both Gram-positive and Gram-negative bacteria. The two peptides prevent the intracellular ppGpp accumulation which plays a critical role in biofilm development [17,18].

Because artificial implants (titanium) and natural teeth (hydroxyapatite) are in a similar ecological environment (the oral cavity), biofilms on the tooth surface may have similar formations and compositions [19]. However, some research on implant abutment surfaces implies that biofilm activities on biofilm surfaces may have characteristics (such as material, roughness and so on) different from tooth surface biofilms [20]. Therefore, the purpose of this study was to 1) analyze the influence of the surface morphology of Ti and HA disks on the development of biofilms; 2) evaluate the antimicrobial effects of two new peptides, DJK-5 and 1018 against biofilms on both Ti and HA surfaces.

2. Materials and methods

2.1. Ti and HA disks preparation

Commercially available pure titanium disks (ASTM Grade 1) were processed from a rod into a disk with a diameter of 12 mm and a thickness of 2 mm. They were then wet-polished with silicon carbide abrasive papers in sequence (400, 800 and 1200 grit). Afterwards, ultrasonic bathing was executed in distilled water, acetone, 75% ethanol and distilled water.

HA disks (Clarkson Chromatography Products, Williamsport, PA, USA) were autoclave sterilized (121 °C for 20 min), while titanium (Ti) disks were sterilized by ultraviolet radiation overnight.

2.2. Surface characteristics by atomic force microscope (AFM) and scanning electron microscopy (SEM)

Ti and HA disk surfaces were detected and analyzed with the SPM-9600 AFM system (Shimadzu, Kyoto, Japan). The scanning was processed in air under ambient conditions with a silicon nitride tip of NSG01 (NT-MDT, Moscow, Russia) in phase mode and a scanning rate of 1 Hz. For both kinds of surfaces (Ti and HA), three samples of each were analyzed. Five areas of 10 μm × 10 μm were randomly chosen in each sample and scanned. A two-dimensional picture was captured and a three-dimensional reconstruction was obtained for each area. The mean roughness of Ti and HA disk surfaces was calculated by the Ra values of both materials.

The surface morphologies were also observed using SEM. Briefly, HA disks were coated with gold palladium sputter (Hummer VI; Technic Inc, Anaheim, CA, USA), and both Ti and HA disks were surveyed by SEM (Hitachi SU3500 VPSEM; Hitachi High-Technologies Canada Inc, Toronto, Canada) at 3 kV and at a magnification of 1000 × and 3000×.

2.3. Biofilm model

Multispecies biofilm was grown using subgingival plaque from the second upper molar from one healthy adult volunteer. The subgingival plaque was suspended in brain heart infusion broth (BHI) (Becton Dickinson, Sparks, MD, USA) and incubated anaerobically at 37 °C overnight. The present study was approved by the University of British Columbia Clinical Research Ethics committee review boards (certificate H12-02430) and written informed consent was obtained from the volunteer.

The dispersed plaque suspension was measured in a microplate reader (ELx808 Absorbance Reader, BioTek Instruments, Inc, Winooski, VT, USA) in 96-well plates with 150 μL per well at 405 nm, and an optical density (OD) of 0.1 was used as the standardized density of the bacterial solution.

2.4. Coating the Ti and HA disks

Fresh saliva was collected in polypropylene tubes (Corning, NY, USA) from a healthy volunteer at least 2 h after meals and filtered using sterilized 0.22 μm syringe filters (Pall corporation, Ann Arbor, MI, USA). Ti and HA disks were precoated with filter sterilized saliva for 4 h at room temperature before use and gently rinsed with phosphate buffer saline (PBS, pH = 7.0) (Sigma-Aldrich, St Louis, MO, USA).

2.5. SEM examination of biofilms at different stages of development

After biofilm growth on Ti and HA disks for 6 h (initial adhesion stage) and three weeks (mature biofilm stage), the specimens were washed with PBS for 5 min. Fixation was performed by adding 2.5% glutaraldehyde for 10 min and 1% osmium tetroxide for 1 h. The specimens were dehydrated by increasing concentrations of ethanol, dried by using a critical point drier (Samdri-795; Tousimis Research Corporation, Rockville, MD, USA), and sputter-coated with gold-palladium in a vacuum evaporator (Hummer VI; Technics West Inc, Anaheim, CA, USA). SEM observation was executed at 3 kV, under a low (1000 ×) and a high magnification (5000 × or 6000 ×).

2.6. Preparation of antimicrobial agents

Peptides 1018 and DJK-5 were synthesized by CPC Scientific (Sunnyvale, CA, USA) using solid-phase 9-fluorenylmethoxy carbonyl (Fmoc) chemistry and purified to a purity of > 95% using reverse-phase high-performance liquid chromatography as previously described [21]. For the experiments the peptide was obtained from peptide stocks in deionized water.

Two percentages and 0.2% chlorhexidine digluconate (CHX) was freshly prepared by diluting from a 20% solution (Sigma Chemical Co.).

2.7. Long-term antibiofilm effect of the peptides on pre-formed biofilms on Ti and HA disks

For long term exposure experiment, 2 mL of the above mentioned plaque bacteria suspension was cultured anaerobically at 37 °C for 6 and 24 h or 7 days. Fresh BHI containing DJK-5 or 1018 peptide (2 or 10 μg/mL) was supplied to wells holding Ti and HA disks. Control groups for biofilms on both Ti and HA disks were only submerged BHI and sterilized water for the same times as described above. All samples were further incubated for 6 h.

2.8. Short-term antibiofilm effect of the peptides on pre-formed biofilms on Ti and HA surfaces

For short term exposure experiment (eradication), 0.2 mL of the above mentioned plaque bacteria suspension was added to 1.8 mL fresh BHI to saliva-coated Ti or HA disks and incubated anaerobically at 37 °C for either three days or three weeks. Fresh BHI was replaced once every week. At the end of incubation period, each sample was gently rinsed with 2 mL PBS in a well for 1 min and exposed to the peptide DJK-5 (2 and 10 μg/mL), 1018 (2 and 10 μg/mL) or CHX (0.2% and 2%) for 3 min.

2.9. Confocal laser scanning microscopy (CLSM) examination of biofilms on Ti and HA surfaces

Following the exposure to the above solutions, all specimens were rinsed gently in 0.85% physiological saline and then stained with a 1:1 mixture of SYTO 9 and propidium iodide (BacLight LIVE/DEAD Bacterial Viability kit, Molecular Probes, Eugene, OR) following the manufacturer's instructions. Images of the stained samples were taken by a CSLM (FV10i-LIV, Olympus, Canada) at 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide, respectively. Five random areas