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Multilayered coating on titanium for controlled release of antimicrobial peptides for the prevention of implant-associated infections

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ABSTRACT

Prevention of bacterial colonization and formation of a bacterial biofilm on implant surfaces has been a challenge in orthopaedic surgery. The treatment of implant-associated infections with conventional antibiotics has become more complicated by the emergence of multi-drug resistant bacteria. Antimicrobial eluting coatings on implants is one of the most promising strategies that have been attempted. This study reports a controlled release of an antimicrobial peptide (AMP) from titanium surface through a non-cytotoxic multilayered coating. Three layers of vertically oriented TiO_2 nanotubes, a thin layer of calcium phosphate coating and a phospholipid (POPC) film were impregnated with a potent broad-spectrum AMP (HHC-36). The coating with controlled and sustained release of AMP was highly effective against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria. No cytotoxicity to osteoblast-like cells (MG-63) was observed. Moderate platelet activation and adhesion on the implant surface with no observable activation in solution, and very low red blood cell lysis was observed on the implant. This multi-layer assembly can be a potential approach to locally deliver AMPs to prevent peri-implant infection in orthopaedics without being toxic to host cells.

1. Introduction

Titanium and titanium alloys are frequently used in orthopaedic implants because of their good biocompatibility and reliable mechanical properties [1]. However, the formation of a bacterial surface biofilm and compromised immunity at the implant/tissue interface may lead to persistent infections on and around titanium implants. Pathogens such as *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *Pseudomonas aeruginosa* can be acquired shortly after the surgical installation of implants or at a later stage (e.g. via a haematogenous route) [2]. The resulting infection is usually difficult to treat and in most cases, replacement of a prosthesis is the only remedy [3]. Moreover, the emergence of multi-drug resistant bacterium like methicillin-resistant *S. aureus* (MRSA) has critically challenged the use of conventional antibiotics [4]. Systemic administration of antimicrobial agents have several drawbacks such as the relatively low drug concentration at the target site and potential toxicity [5]. Hence, localized delivery of antimicrobial agents with time-effective handling of infection, while potentially eliminating problems associated with systemic administration, is highly desirable [6,7].

The inhibition of organisms in a complex biofilm requires up to 1000-times the antibiotic dose necessary to combat bacteria in suspension [8]. An ideal local antibiotic release profiles should exhibit a high initial release rate within 6 h post implantation while the immune system is weakened/compromised leaving the implant susceptible to surface bacterial colonization, followed by a continuous 'prophylactic' slow release [8,9]. Conventional antibiotics like vancomycin, tobramycin, and gentamicin have been incorporated in controlled release devices [9]. A serious concern regarding the use of these antibiotics is that the release at levels below the minimal inhibitory concentration (MIC) is likely to evoke bacterial resistance [10]. High doses of antibiotics often generate cell toxicity and may impair osteogenic activity [11]. A promising alternative to conventional antibiotics is the short cationic antimicrobial peptides (AMPs) [12]. AMPs have broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, and are also known to be antifungal and antiviral [13]. Due to the complex

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mechanisms of AMPs bacteria are killed more rapidly than with conventional antibiotics and it is extremely difficult for bacteria to develop resistance [14,15].

Calcium phosphate coatings and vertically aligned titania nanotubes (NT) are two platforms used for delivering drugs from orthopaedic implants [16,17]. In our previous work, we successfully examined, *in vitro* and *in vivo*, the feasibility of using micro-porous CaP [18,19], and self-organized and vertically oriented TiO_2 nanotubes coatings [20] on Ti surfaces as carriers to deliver peptide HHC-36, one of the most potent broad-spectrum AMPs. Both strategies led to an initial burst release of HHC-36. And HHC-36 onto loaded CaP coated Ti showed no bone growth inhibition. However, the release rate in both systems was too fast, limiting the antimicrobial effects to early stage peri-implant infection [18–20]. The main objective of this study was to develop a layer-by-layer assembly of multi-layer thin films in order to encourage prolonged AMP release on Ti implants. To create a coating that had dual beneficial effects, i.e. antimicrobial and osteoconductive, thin layers of titania NT and CaP coatings were impregnated with AMPs. These films were topped with a thin phospholipid (POPC, palmitoyl-oleoyl phosphatidyl-choline) film to control the release of AMP based on a bio-inspired cell membrane [21,22]. POPC is found naturally in eukaryotic cell membranes and offers the least support for bacteria growth (81% reduction), and the most suitable platform for bone cell attachment [23]. POPC has also been shown to exhibit clinically acceptable osteointegration [24].

Testing of the biocompatibility of coatings has generally been performed through *in vitro* assessment of the interaction of the coatings with recognized cell culture lines. However, this does not adequately address the acceptability of these materials in the blood interfacing environment, which consists of a fibrin film containing platelets and red blood cells and plays a significant role in osteogenesis [25,26]. In this regard, platelet adhesion and activation on an implant surface and the surrounding fluid are critical steps in initiating osteoconduction [27,28]. Therefore, it was also the purpose of this study to address the hemocompatibility of the multi-layer coating systems, using platelet adhesion, activation, and haemolysis studies.

2. Materials and methods

2.1. Fabrication of TiO_2 nanotubes on titanium surface

The commercially pure Ti foils (0.1 mm, 99.6% purity, Goodfellow) were consecutively sonicated in acetone, ethanol, and distilled water and then air dried. Titania nanotubes were prepared using anodization technique, in which Ti was used as the working electrode (anode), and platinum as the cathode. The TiO_2 nanotubes were prepared in 75% glycerol ($C_3H_8O_3$, Fisher Scientific, Canada) solution containing 0.27 M ammonium fluoride (NH_4F, Fisher Scientific, Canada) at 30 V (DC power supply, Matsusada R4K-80 Series) for 6 h at room temperature. After anodizing, the samples were rinsed with water, and sequentially soaked in absolute ethanol and distilled water overnight and air dried. The nanotube samples were then annealed at 400 °C (5 °C/min) for 3 h and then gradually cooled down in the furnace to crystallize the amorphous TiO_2 nanotubes into the anatase structure, following the protocol of Macak et al. [29].

The antimicrobial peptide HHC-36 (KRWWKWWRR-NH_2) (CPC Scientific, Sunnyvale, CA), was used in our study [30]. To load the HHC-36 into NT specimens (1 × 1 cm), the AMP was dissolved in low surface tension solvent (ethanol), and forced into NT using vacuum-assisted physical adsorption method. Fifty microlitre of a 672 μM HHC-36 solution was pipetted onto the nanotube surfaces, gently spread, and allowed to dry under vacuum desiccator at room temperature for 30 min. The loading process was repeated three times.

2.2. Processing of CaP on TiO_2 nanotubes

Calcium phosphate coating was prepared on titania nanotubes using the drop-and-dry technique, a modified approach of the evaporation-induced surface crystallization technique developed by Duan et al. [31]. The supersaturated calcium phosphate (SSCP) containing 2.32 mM $NH_4H_2PO_4$ (Fluka), 3.87 mM $CaCl_2$ (Fluka), 150 mM NaCl (Fluka), 40 mM HCl (Fisher), and 50 mM tris(hydroxymethyl) aminomethane (Tris) (Fluka) was adjusted to pH 7.30 at room temperature with NaOH (Fisher). Fifty μL of SSCP solution was pipetted onto the specimen surfaces, gently spread, and dried in air at room temperature for 3 h. After repeating the drop-and-dry treatment four times, the samples were each rinsed with PBS and allowed to dry in air. The CaP coated specimens were loaded with AMP by pipetting fifty microlitre of 672 μM HHC-36 solution in ethanol onto the CaP surfaces, and air dried. The loading process was repeated three times.

2.3. Phospholipid coating on CaP

POPC (1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) (Genzyme Pharmaceuticals) and AMP were dissolved in an appropriate volume of ethanol by sonication to give final concentrations of 26 mM POPC, including 672 μM HHC-36. Fifty μl of this solution (POPC-20) were pipetted onto the specimen surfaces, and dried in air at room temperature. The samples were kept at 4 °C for future experiments.

2.4. Surface characterization

Surface morphologies of the coatings were studied, after being sputter coated with a thin layer of gold, with a field emission scanning electron microscope (FE-SEM Zeis Sigma) equipped with an energy dispersive X-ray analysis unit (EDS). A focused ion beam (FIB-SEM) analysis was performed using a FEI Helios 650 dual beam microscope to investigate the cross section of specimens. The analysis was carried out at low beam current of 0.20 nA, while the energy of ions was 3 kV. Chemical compositions of coatings were evaluated using EDS, and attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR Perkin Elmer Spectrum 100) in the range from 4000 to 400 cm^{-1}. Chemical binding between POPC and the peptide in the POPC-20 solution was investigated using liquid chromatography-mass spectrometry (BrukerEsquire-LC/MS). The POPC-20 solution was diluted by ten-fold (HHC-36/POPC:67 μM/2.6 mM in ethanol) and injected at 10 μL/min infusion rate. The *m/z* values were obtained from a mass spectrum average. The mass spectrometer was calibrated using pure POPC and HHC-36 standards.

The wettability of surfaces was characterized by measuring contact angles by the sessile drop method in 1 μL distilled water at room temperature. Images were captured and analysed using Northern Eclipse software at 10× magnification.

2.5. Release profile of AMP

The *in vitro* AMP release kinetics of the samples were measured using ultraviolet–visible spectroscopy (UV/Vis) by recording the absorption peak at 280 nm, which is the characteristic excitation wavelength for tryptophan [32]. Three specimens, nanotube coated Ti loaded with AMP (NT), CaP coated nanotubes loaded with AMP (CaP), and POPC-20 were gently rinsed with PBS and dried at room temperature. The specimens, assessed in triplicate, were then immersed in 1 mL of PBS (pH 7.4) in a glass vial while rotating at 37 °C. All samples were rinsed with PBS. After 30 min, 90 min, 150 min, 270 min, 1 day, 2 day, 3 day, 5 day and 7 day, 500 μL of solution was sampled and fresh PBS was replenished each time samples were taken. The samples were stored at −20 °C, and AMP content was analysed using a UV/Vis to assess the AMP cumulative release ratio. A series of standards in the concentration range of 2–100 μg/mL of HHC-36 in PBS were prepared in triplicate to calibrate the system. AMP quantification was then calculated based on the external standard method. Degradation of POPC-20 samples in 1 mL of PBS (pH 7.4) in a glass vial while rotating at 37 °C were studied in triplicate for up to 7 days. After each day, the samples were fixed in 2.5% glutaraldehyde in PBS for 2 h at 4 °C, dehydrated with graded ethanol (50, 70, 80, 90, 95, and 100%, 15 min each), and examined using an FE-SEM.

2.6. Antimicrobial activity

The assessment of antibacterial activity against both Gram-positive (*S. aureus* ATCC 25293) and Gram-negative (*P. aeruginosa* H1001: lux-CDABE) bacteria was performed by the disk-diffusion assay (Kirby–Bauer). To obtain bacteria in the mid logarithmic phase of growth, 100 μL of an overnight culture of bacteria was transferred into sterile tubes containing 5 mL of MHB and incubated at 37 °C for 1 h. *P. aeruginosa* and *S. aureus* bacterial suspensions were then re-suspended in MHB, to provide a final density of $\sim 10^6$ CFU mL^{-1}. The bacterial suspension (1 mL) was applied uniformly to the surface of a nutrient MH agar plate before placing the disks on the plate. The inoculated agar plates were allowed to dry for 10 min, and then the round disks (1 cm in diameter) of specimens and Ti as negative control, were placed on the inoculated agar, with the coated side touching the inoculated agar. The agar plates were then incubated at 37 °C for 24 h.

The antimicrobial activity of POPC samples was also confirmed by SEM imaging of samples incubated with $\sim 10^6$ CFU mL^{-1} of *S. aureus* or *P. aeruginosa* overnight at 37 °C. The nanotube sample without AMP treatment was used as control.

2.7. Cell study

Commercially available MG-63 human osteoblast-like cells (ATCC CRL-1427, USA) were cultured in a medium consisting of Dulbecco's Modified Eagle's