Biomaterials 31 (2010) 9519–9526

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Antimicrobial peptides on calcium phosphate-coated titanium for the prevention of implant-associated infections

Mehdi Kazemzadeh-Narbat [a], Jason Kindrachuk [b], Ke Duan [a], Håvard Jenssen [b], Robert E.W. Hancock [b], Rizhi Wang [a,*]

[a] Department of Materials Engineering, University of British Columbia, 309 - 6350 Stores Road, Vancouver, BC, V6T 1Z4, Canada
[b] Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada

ARTICLE INFO

Article history:
Received 8 June 2010
Accepted 18 August 2010
Available online 5 November 2010

Keywords:
Calcium phosphate coating
Electrolytic deposition
Antimicrobial peptide
Titanium
Infection
Orthopaedic implants

ABSTRACT

Prevention of implant-associated infections has been one of the main challenges in orthopaedic surgery. This challenge is further complicated by the concern over the development of antibiotic resistance as a result of using traditional antibiotics for infection prophylaxis. The objective of this study was to develop a technique that enables the loading and local delivery of a unique group of cationic antimicrobial peptides (AMP) through implant surfaces. A thin layer of micro-porous calcium phosphate (CaP) coating was processed by electrolytic deposition onto the surface of titanium as the drug carrier. The broad spectrum AMP Tet213 (KRWWKWWRRC) was selected and loaded onto the CaP coating. SEM, XRD and FTIR analyses confirmed the CaP coating to be micro-porous octacalcium phosphate. By using a luminescence spectrometer technique, it was demonstrated that a 7 μm thick porous CaP coating could load up to 9 μg of AMP/cm^2 using a simple soaking technique. The drug-loaded CaP coating (CaP-Tet213) was not cytotoxic for MG-63 osteoblast-like cells. The CaP-Tet213 implants had antimicrobial activity against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria with 10^6-fold reductions of both bacterial strains within 30 min as assessed by measuring colony-forming units (CFU). Repeated CFU assays on the same CaP-Tet213 specimen demonstrated retention of antimicrobial activity by the CaP-Tet213 surfaces through four test cycles. The susceptibility of bacteria to the CaP-Tet213 surfaces was also evaluated by assessing the inhibition of luminescence of *P. aeruginosa* containing a *luxCDABE* cassette at 4 h and 24 h with ~92% and ~77% inhibition of luminescence, respectively. It was demonstrated that CaP-Tet213 was a more efficient antimicrobial coating than CaP-MX226, CaP-hLF1-11 or CaP-tobramycin following incubation of CaP implants with equimolar concentrations of Tet213, the commercially developed antimicrobial peptide MX-226, hLF1-11 or tobramycin. A device coated with CaP-Tet213 could be a potential solution for the prevention of the peri-implant infection in orthopaedics.

1. Introduction

Implant-associated infections are one of the most serious complications in orthopaedic surgery. The overall infection rates associated with such surgeries are approximately 5% for fracture-fixation devices, 2% for primary joint replacements and at least 14% of the total hip and knee revisions are due to infection [1–7]. The cost to treat such infections is tremendous, estimated to be least $50,000 per patient and $250 million per year in the USA [8] and with the increasing use of orthopaedic devices the number of infected implants will continue to rise [9]. Despite the tremendous morbidity and economic burden associated with implant-associated infections there has been a paucity of preventative solutions. These have been limited to the use of ultra-clean surgical technique and prophylactic antibiotics.

Orthopaedic implant surfaces provide the bacterial growth with ideal substrates. Common pathogens such as the *Staphylococcus* species, *S. aureus* and *S. epidermidis*, and *Pseudomonas aeruginosa*, can be acquired at the time of surgery or at a later stage (via a haematogenous route) and can attach to the surfaces of the implants [10–12] to nucleate infections. This can potentiate the formation of complex biofilms resulting in up to 1000-fold decrease in susceptibility to antimicrobial agents, largely due to poor antibiotic penetration into the biofilm and the stationary phase of growth of the bacteria underlying the surface layer [10,13]. The presence of biofilms and the poor vascularization of the bone/

* Corresponding author. Tel.: +1 604 822 9752; fax: +1 604 822 3619.
E-mail address: rzwang@interchange.ubc.ca (R. Wang).

0142-9612/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2010.08.035

implant interface, make prosthetic joint infections extremely difficult to treat. For example, the current treatment solutions for the deep infection in total joint arthroplasty include two-stage radical debridement with implant removal, antibiotic therapy, and delayed reimplantation [12,14–16].

An ideal strategy to combat implant-associated infections would be prevention of infection at the site of the implant. Among the techniques explored, local delivery of antibiotics such as gentamicin, tobramycin and vancomycin through implant surfaces has received much attention recently particularly due to their potential and low systemic side effects of this approach [17–23].

There are two challenges facing the local delivery of antibiotics through orthopaedic implants. The first one is avoiding antibiotic resistance; the second one is achieving antimicrobial activity without impairing peri-implant bone growth (i.e. osseointegration). A serious concern regarding the use of conventional antibiotics in such an approach is the potential development and spread of antibiotic-resistant pathogens such as the methicillin-resistant *Staphylococcus aureus* (MRSA) [24–28]. The development of antibiotic-resistant infections can lead to devastating effects in the absence of any valid medical treatment to control the infection, and has become a serious public health problem. Therefore, an intriguing alternative would be the use of non-conventional antibiotics for local delivery so as not to compromise the treatment of infections. A preferred solution would be to employ non-conventional antimicrobial drugs that do not potentiate the development of resistant phenotypes. One example of such non-classical drugs is the cationic antimicrobial peptides (AMP) [29]. In Nature, AMPs form a central component in the defense mechanisms of all species of life [30,31]. They are short (12–50 amino acids in length), cationic (due to Lys and Arg residues), and hydrophobic (containing $\geq$ 50% hydrophobic amino acids) [32]. Many have very broad bactericidal activity spectra against both Gram-negative and Gram-positive bacteria including clinically relevant bacteria [29]. These agents do not readily lead to the selection of resistant mutants [29,33] and therefore are ideal candidates for infection prevention through local delivery. Recently, it was demonstrated that the sequence of native AMPs can serve as templates for the design of synthetic AMPs with improved antimicrobial activity [34–37]. By robotic spot synthesis of arrays of variant peptides, large numbers of short cationic peptides were screened by Hilpert et al. for antimicrobial activity and together with QSAR modeling and rapid screening approaches led to the identification of a group of highly active small broad spectrum AMPs [38].

The main objective of this study was to develop techniques for delivering AMPs on the surface of metallic orthopaedic implants. Despite active research on immobilization of peptides (e.g. growth factors) onto implant surfaces to enhance cell adhesion and bone growth [39–41], reports regarding the delivery of antimicrobial peptides on a solid surface have been very limited [42]. In this study, we examined the feasibility of using micro-porous CaP coatings on titanium surfaces as a carrier to deliver the candidate AMP. Since it is well-known that CaP coating can enhance bone growth onto orthopaedic implants [43], such a combination of CaP and the short cationic peptides may achieve antimicrobial activity and osteoconductivity at the same time. As a first step, here we report the results on processing of the coating, peptide loading, and *in vitro* cell culture with both bacteria and osteoblast-like cells.

2. Materials and methods

2.1. Processing of CaP coating on titanium surface

Calcium phosphate (CaP) coating onto Ti surfaces was performed using the electrolytic deposition ELD technique reported earlier [44,45]. Commercially pure titanium plates (Goodfellow, USA) of 10 × 10 × 0.5 mm dimensions were used as the working electrode (cathode), while a platinum plate served as the anode. The distance between the working electrode and the anode was set to 3 cm. The electrolyte solution used for the ELD consisted of 5.25 mM of $Ca(NO_3)_2$ (Sigma Aldrich), 10.5 mM of $NH_4H_2PO_4$ (Fisher Scientific), and 150 mM of NaCl (Fisher Scientific). The pH of the solution was adjusted to 5.30 by adding the NaOH (Fisher Scientific) and the coating process was conducted with a DC power source operated at 2.5 V at room temperature for 3 h. Before ELD processing, the Ti specimens were ground with 320 grit sandpaper and cleaned with alkaline detergent (Fisher Scientific SF105), acetone, 70% ethanol and distilled water. The specimens were then etched in 2% HF (Fisher Scientific) for 1 min at room temperature. They were ultrasonically cleaned in distilled water and air-dried.

Surface morphology and thickness of the CaP coating were analyzed by a scanning electron microscope (SEM Hitachi S3000N) after being sputter-coated with a thin layer of Au–Pd. For coating thickness measurement, three samples were embedded into the epoxy resin (Buehler 208130), mechanically ground using sandpapers and polished using diamond suspensions and silica colloidal suspension (0.05 μm). Chemical compositions were also analyzed with the Energy Dispersive Spectrometer equipped in the SEM. To examine the crystal structure, CaP coating was scratched off the Ti plates, and analyzed with Fourier transform infrared spectroscopy (FTIR Nexus 870) and powder X-ray diffraction (XRD Bruker D8, Cu Kα, 40 kV and 40 mA, step scanning).

2.2. AMP loading on titanium

One of the most potent short (9 amino acids), broad spectrum AMPs identified in our recent large QSAR study was HHC36 [37,46]. Further testing with surface-immobilized HHC36 (C-terminally modified and renamed Tet213) demonstrated that it retained antimicrobial activity [37,46]. Tet213 with a C-terminal Cys residue (KRWWKWWRRC) was therefore selected for the current study. To load Tet213 onto the titanium samples, a buffer solution of 50 mM of Na_2HPO_4 (Calbiochem) in distilled water was prepared, and the pH was adjusted to 7.4 by dropping 0.1 M NaOH. 1 mL of this buffer was transferred into a vial containing 1 mg of peptide. The Ti sample was immersed into the vial and kept for 1 h at room temperature under constant, gentle shaking. The plate was then rinsed with distilled water for 10 times, 1 min each, and kept in a vial after being dried in slow air stream.

2.3. Immobilized AMP detection

The amount of AMP immobilized on the Ti surface was determined by fluorometry technique [40]. After dissolving the coating in 1 mL of 0.1 M HCl (Fisher Scientific) for 1 h in ultrasound bath, 3 mL of 3.23 mM PHQ (9,10- Phenanthrenequinone) solution (Fluka Chemika) was added into the vial. Subsequently, 0.5 mL of 2 M NaOH solution was added. The vial was then placed in 30 °C water bath for 3 h to allow the reagent PHQ react with Arg residues within the AMP. The reaction product was a compound with fluorescence that could be used to quantify sub-μg amounts of Arg. To stop the reaction, acidification was performed by adding 2.25 mL of 2.4 M HCl into the vial. The amount of AMP, based on arginine concentration, was measured using a Luminometer (Perkin Elmer Ltd UK LS-50B) [47], calibrated with six standard solutions of known AMP concentrations. Four groups of samples, six in each group, were tested to study the efficiency of AMP loading onto the CaP coating. Group one (CaP) was the CaP-coated Ti without AMP as a control. Group two (CaP-AMP) was the CaP-coated Ti, immobilized with AMP and rinsed with distilled water ten times. Group three (CaP-AMP-PBS) was the coated Ti, immobilized with AMP. Instead of using water, this group was rinsed with PBS and immersed in PBS for 30 min before the test. Group four (Ti–AMP) was Ti without CaP coating, but loaded with AMP in the same way as Group two.

2.4. Antimicrobial activity testing

Antimicrobial activity of the specimens was tested against both Gram-positive (*S. aureus*) and Gram-negative (*P. aeruginosa*) bacteria. Both bacteria strains were cultured overnight. One hundred μl of each solution was transferred into sterile tubes containing 5 mL of Mueller Hinton Broth (MHB) and incubated at 37 °C for 1 h to obtain bacteria in the mid logarithmic phase of growth. *P. aeruginosa* and *S. aureus* bacterial suspensions were then re-suspended using Basal Medium 2 (BM2) or MHB, respectively, to dilute the solution and provide a final density of $\sim 10^6$ cells/mL. To study the capability of specimens in killing bacteria, a survival assay was performed. Four hundred μl of the *P. aeruginosa* or *S. aureus* bacterial suspensions were separately dripped onto groups of three bare Ti plates and three CaP-coated Ti plates, which had been treated with AMP and rinsed with distilled water, as described above. After 30, 90, 150 and 270 min incubation with *P. aeruginosa* (H1001: *luxCDABE*) or *S. aureus* (ATCC 25293) bacterial suspensions the residual bacteria were plated on nutrient agar and incubated overnight at 37 °C and bacterial survival assessed by counting the number of colony-forming units (CFU). The susceptibility of bacteria to the peptide from six CaP-AMP specimens was also evaluated by the inhibition of *P. aeruginosa* [48] that constitutively expresses a luciferase gene cassette. Samples were incubated at 37 °C for 4 h and 24 h and the inhibition of *P. aeruginosa* was measured by the decrease in bacterial luminescence (which is

dependent on bacterial energization). The result was reported as percent inhibition relative to average luminescence value of the untreated *P. aeruginosa* H1001.

Since the direct measurement of peptide release from CaP-AMP samples over time was a substantial challenge, the antimicrobial test was used to indirectly study the release of AMP and to evaluate the antibacterial efficacy after different antimicrobial test cycles. Six CaP-AMP samples were incubated with *P. aeruginosa*. After 30 min, the bacteria solution was pipetted and the samples were rinsed three times with PBS. The samples were then air-dried, and treated with equal amounts of bacteria for another 30 min after which remaining CFU were determined as above. This antimicrobial activity cycle was performed four times consecutively.

To compare the efficacy of AMPs with conventional antibiotics on CaP coating, CaP samples, were incubated with equimolar concentrations of (0.63 mM) Tet213, two commercially developed antimicrobial peptides MX226, and hLF1-11 or Tobramycin. The specimens were rinsed with distilled water and air-dried. The prepared CaP-Tet213, CaP-MX226, CaP-hLF1-11 and CaP-Tobramycin plates were incubated with *P. aeruginosa* solution for 1 h before measuring bacterial survival.

2.5. Cytotoxicity assay

MG-63 osteoblast-like cells from human osteosarcoma (ATCC® CRL-1427™, USA), were cultured in standard culture medium Dulbecco's Modified Eagle Medium (DMEM, GIBCO), which consisted of a minimal essential medium, supplemented with 10% fetal bovine serum, and 1% non-essential amino acids (GIBCO). The medium was renewed every 2 days. Cultures were maintained in a humidified atmosphere with 5% CO_2, at 37 °C. The confluent osteoblast cultures (passage nine) were detached from the culture flask by incubation with 0.1% trypsin and 0.1% ethylenediaminetetraacetic acid (EDTA) for 5 min. The Osteoblast solution was centrifuged at 400 g for 10 min, and re-suspended in the medium. The growth and viability of cells colonizing the samples were evaluated by measuring the mitochondrial dehydrogenase activity using a modified MTT (3-(4,5-dimetyl-2-tiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Biotium Inc., USA) reduction assay. To determine the cytotoxicity of CaP-AMP samples cells, MG-63 cells were seeded in 12-well plates at 10^4 cells/mL in a humidified 5% CO_2 atmosphere. Three types of samples were studied in triplicate: (1) bare titanium (as control) (2) CaP-coated Ti (3) CaP-coated Ti soaked with Tet213. Negative controls in this experiment were cells cultured under normal conditions without AMP. After 24 h of incubation, MTT solution in 1 mL serum free medium was added and the plate was incubated for 4 h at 37 °C in a humidified 5% CO_2 atmosphere. The solution was then removed, dimethyl sulfoxide (DMSO) added, and the plate was shaken for 15 min before measuring adsorbance at 570 nm (the reference value was 690 nm) on an ELISA microplate reader (Bio-Tek Instruments). The MTT assay was performed after 1, 2, 3, 7 and 10 days.

2.6. Statistical analyses

The differences between all values were analyzed using an independent *t*-test, and a *p* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Calcium phosphate coating on titanium surface

The CaP coating on Ti was micro-porous and consisted of plate-like crystals (Fig. 1a). The cross-sectional image of the coating showed an average thickness of ~7 μm, with increased pore and crystal sizes from the bottom to the surface (Fig. 1b). The coating adhered to the Ti substrate without cracks.

An XRD spectrum of the CaP coating matched that of octacalcium phosphate (OCP). The 2θ at 4.722 in Fig. 2a is the characteristic peak of OCP (010) plane. However, due to the peak overlapping between hydroxyapatite (HA) and OCP, the presence of small amount of HA could not be excluded [43,49,50]. Electron Dispersive Spectroscopy under the SEM measured the average Ca/P ratio of the CaP coating to be 1.3, which is close to OCP.

FTIR spectroscopy (Fig. 2b) was used to compare the CaP coating with HA (Acros Organics). A clear distinction could be observed between HA and the ELD CaP coating in the phosphate ν4 vibration mode in the 500–650 cm^{-1} range. The wave numbers attributed to HA were 566 cm^{-1}, 603 cm^{-1} and a shoulder at 630 cm^{-1} from the OH group. In contrast, the two peaks for the CaP coating were at 601 cm^{-1} and 561 cm^{-1}, which could be attributed to typical OCP absorption. Both spectra exhibited obvious strong bands attributed to PO_4^{3-} groups. The bands at about 1100 cm^{-1} and about 1040 cm^{-1} are assigned to the components of the triply degenerated ν3 antisymmetric P–O stretching mode. The ~962 cm^{-1} band is assigned to νl, the non-degenerate P–O symmetric stretching mode [44,51,52].

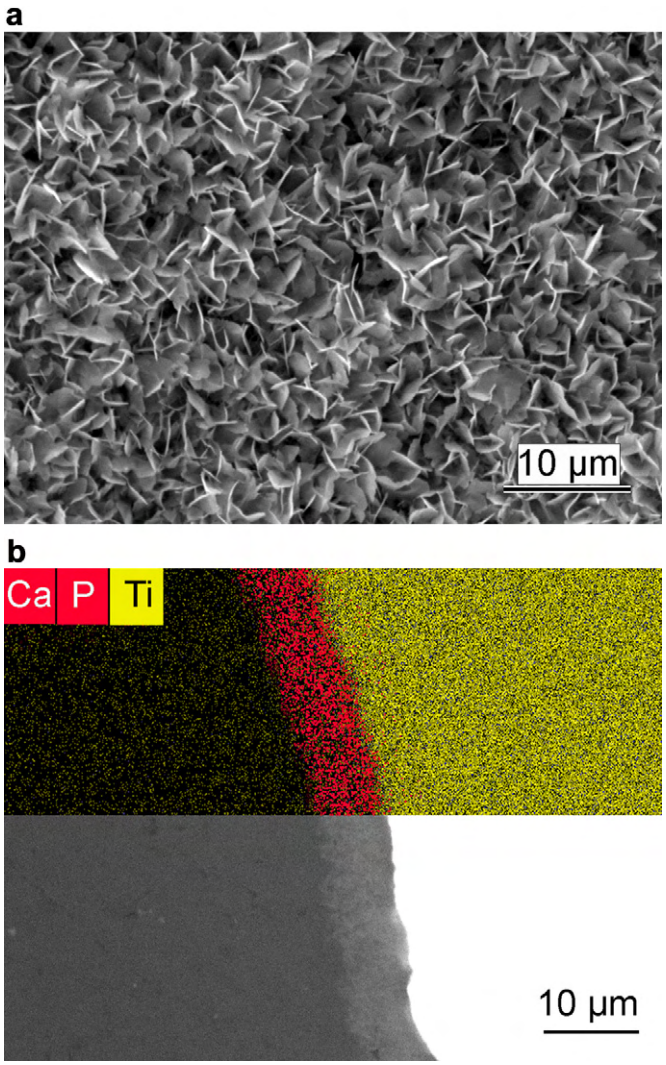

Fig. 1. Calcium phosphate (CaP) coating on Ti surfaces (a) SEM image showing micro-porous plate-like CaP crystals, (b) A back scattered electron image (*lower half*) and EDS mapping of the CaP coating cross-section showing an average thickness of ~7 μm.

3.2. Antimicrobial peptide loading onto CaP coatings

The amount of AMP Tet213 absorbed to the CaP surface in the four sample groups is shown in Fig. 3. The CaP group that was not treated with peptide served as a negative control. Among the three other groups that were treated with peptide, the CaP-Tet213 group, i.e. CaP coating loaded with peptide and rinsed in water, had the highest peptide concentration of ~9 μg/cm^2. Interestingly, the CaP-Tet213-PBS group, which was soaked in the PBS for 30 min after peptide loading, still showed an average peptide concentration of ~8.5 μg/cm^2. No statistical difference was found between the two groups. The Ti-Tet213 samples that had no CaP coatings, showed no significant AMP loading (less than 0.1 μg/cm^2).

3.3. Antimicrobial activity

In assessing the antimicrobial effect against *P. aeruginosa* (Fig. 4a), the Ti-Tet213 group showed no antibacterial activity and

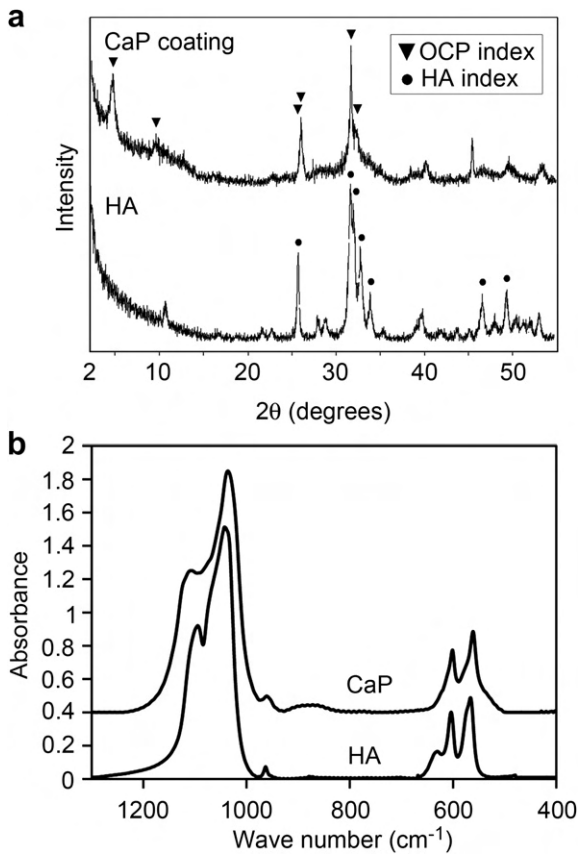

Fig. 2. (a) X-ray diffraction patterns of the HA powder and CaP from the coating. The 2θ at 4.722° is the characteristic peak of OCP. (b) FTIR spectra of HA and CaP coating. The two peaks for the CaP coating at 601 cm^{-1} and 561 cm^{-1} could be attributed to OCP absorption.

there was no change in bacterial growth compared to the negative control group. The CaP-Tet213 group killed all bacteria within 30 min. The same results were observed for the antimicrobial effects against *S. aureus* (Fig. 4b), demonstrating the ability of CaP-coated Ti to deliver active antimicrobial peptides with bactericidal

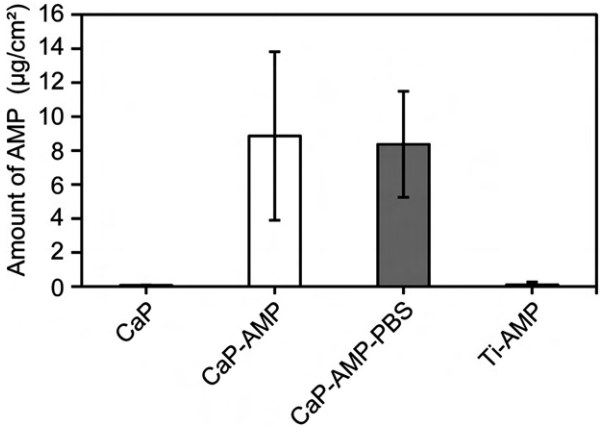

Fig. 3. The amount of peptide immobilized on sample surfaces. CaP: CaP-coated Ti without AMP, CaP-AMP: CaP-coated Ti immobilized with Tet213 and rinsed with distilled water, CaP-AMP-PBS: CaP-coated Ti immobilized with Tet213 rinsed and preserved in PBS for 30 min, Ti-AMP: Ti without CaPbcoating immobilized with Tet213. The Ca-AMP and Ca-AMP-PBS groups showed an average peptide concentration of ~9 and ~8.5 μg/cm^2, respectively.

effectiveness vs. a Gram-positive pathogen strain. When examined over longer periods (4 and 24 h), using the *lux* assay *P. aeruginosa* bacteria were still efficiently inhibited by CaP-Tet213 by 92% and 77% respectively.

To examine if the Tet213 loaded CaP surface was able to repetitively kill bacteria when challenged, four consecutive killing assays were performed in which the *P. aeruginosa* was added to the surface for 30 min, bacterial killing assessed and then the surface washed before a second, third and fourth addition of bacteria. In the first round 100% of bacteria were killed. In subsequent rounds killing was somewhat diminished but efficiently inhibited the bacteria growth relative to control bacteria incubated with negative control CaP surfaces (Fig. 5).

To compare the activity of the CaP-Tet213 samples with CaP surfaces coated with a potent conventional antibiotic, CaP surfaces were incubated with equimolar concentrations of MX226, hLF1-11, Tobramycin or Tet213. The CaP-Tet213 samples clearly demonstrated more effective antimicrobial activity against *P. aeruginosa* than did CaP-MX226, CaP-hLF1-11 and CaP–Tobramycin (Fig. 6). The CaP–Tobramycin samples reduced the total bacterial inoculum by 3 log orders over 4 h while 100% (6 log orders) of the inoculum was killed by CaP-Tet213 (Fig. 6b). Moreover, as shown in Fig. 4a the CaP-Tet213 was capable of killing 100% of the same number of bacteria in less than 30 min.

3.4. Cytotoxicity on osteoblast-like cells

The peptide-loaded samples (soaking the samples in 1 mg/mL Tet213 buffer solution), (Fig. 7) showed no statistical difference in cell activity compared to the CaP-coated and non-coated Ti controls ($P > 0.05$). After 7 days in culture the cells stopped growing as judged in the MTT assay but there was no difference in any treatment condition. Thus, the amount of peptide loaded on the CaP-coated Ti surface is thus not toxic to the osteoblast-like cells.

4. Discussion

We have demonstrated that cationic antimicrobial peptides can be successfully loaded to the CaP-coated Ti substrates. The peptide-loaded titanium samples had strong bactericidal effect against both Gram-positive and Gram-negative bacteria and were also biocompatible with osteoblast-like cells. A combinational device based on this system could be a potential solution for the peri-implant infection in orthopaedic surgery. In the current study, we used simple soaking technique to achieve efficient peptide loading and effective antimicrobial activity. The advantage of this technique is its clinical feasibility. Both the implants and the drugs can be developed and packed independently. The drug loading can be done in the operating room. This will simplify the regulation process and prolong the shelf-time.

4.1. Cationic antimicrobial peptides

AMPs have been actively researched in the past three decades, mainly due to their potent bactericidal capability and the low risk of developing antibiotic-resistant pathogens. It has been previously demonstrated that AMPs have an increased affinity for the negatively charged membranes of bacteria and act through either permeabilization of the bacterial membrane or translocate across the bacterial membrane to attack cytoplasmic targets [46]. The initial step of this mechanism is the affinity of AMPs for the bacterial membrane as a result of the electrostatic interaction between the negatively charged outer layer of the bacterium and the positively charged AMP. This attraction would cause the microbes to more strongly associate with these surfaces leading to enhanced killing.

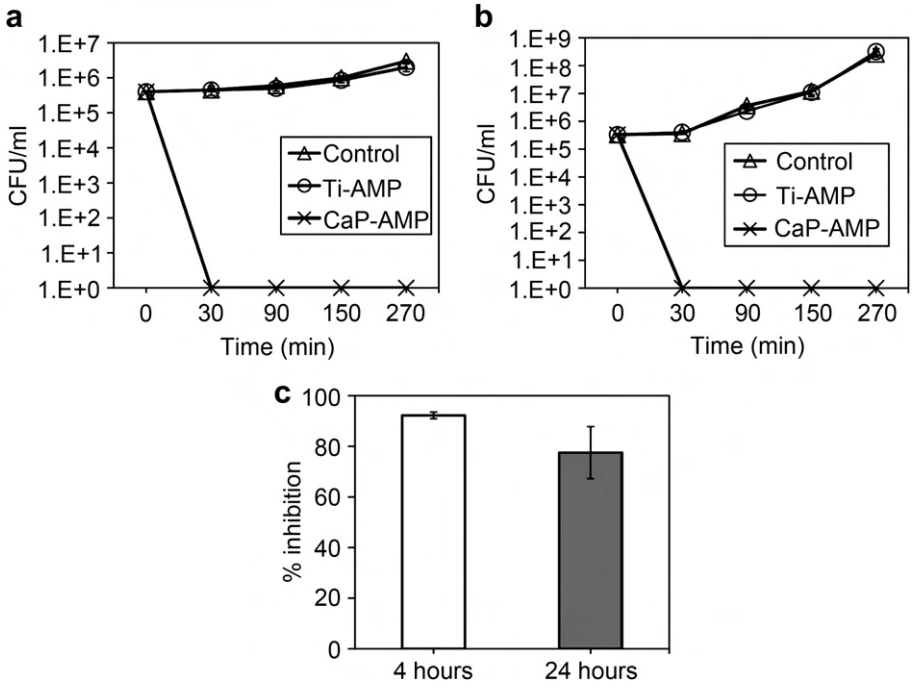

Fig. 4. (a) Antimicrobial activity of Ti-Tet213 and CaP-Tet213 samples against *P. aeruginosa*. (b) Antimicrobial activity of Ti-Tet213 and CaP-Tet213 samples against *S. aureus*. The CaP-Tet213 group killed all bacteria of both strains within 30 min, while the Ti-Tet213 group showed no antibacterial activity. (c) *lux* assay showing *P. aeruginosa* bacteria efficiently inhibited by CaP-Tet213 by 92 and 77% at 4 and 24 h.

Subsequently, the high local concentration of the AMP would result in the displacement of positively charged counterions attached to the outer surface layers, and could thus induce a dramatic change in bacterial surface electrostatics which results in lysis [46,53,54]. Recently, AMPs have also been shown to have immunomodulatory activities that include angiogenesis, modulation of cytokine/chemokine expression, wound healing, and reduction of LPS-mediated pro-inflammatory responses [53,55]. These unique properties make cationic antimicrobial peptides ideal candidates for the local delivery of novel anti-infective therapeutics on orthopaedic implants. The drug-loaded implants will protect the surfaces from colonization by microbes without incurring the problem of resistance against common antibiotics.

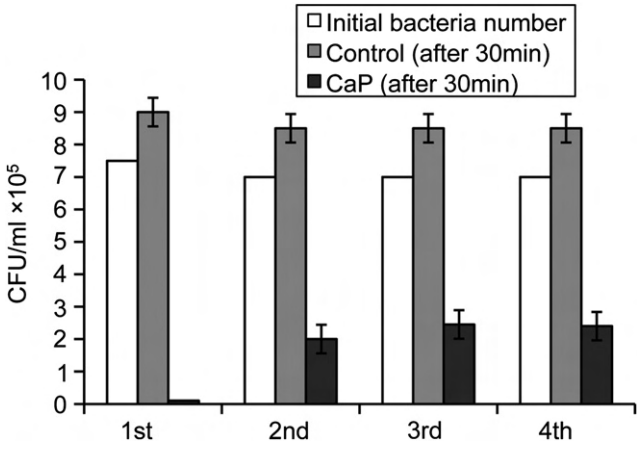

Fig. 5. Antimicrobial activity of CaP-Tet213 specimens against *P. aeruginosa* for four 30 min cycles. After each 30 min, the samples were rinsed with PBS and treated with equal amounts of bacteria for another 30 min after which remaining CFU were determined. In the first cycle (1st) 100% of bacteria were killed. In subsequent cycles killing was diminished but efficiently inhibited the bacteria growth.

The antimicrobial peptide used in this study is a cysteinylated version of HHC-36, one of the most potent peptides identified by the high-throughput peptide synthesis (peptide arrays on cellulose and rapid screening technologies) in combination with quantitative structure activity relationship (QSAR) modeling [36]. It was effective against a broad array of multi-drug resistant "Superbugs" including MRSA. It has also been shown that this peptide caused minimal red blood cell lysis for concentrations up to 251 μM [36]. In the current study the results confirmed that the peptide has low cytotoxicity and is pathogen-specific. The selection of carriers for local delivery of antibiotics through orthopaedic implants has to meet two goals. The carrier should be able to deliver adequate amount of antibiotics to achieve the antimicrobial activity. At the same time, it should not impair peri-implant bone growth. Ideally, the carrier should also enhance bone growth onto the implants. For these reasons, we chose a calcium phosphate coating as the delivery carrier in this study since calcium phosphates are known for their osteoconductivity [56]. With a simple soaking technique, we demonstrated that high amount of peptide (9 μg/cm^2 on the 7 μm coating) can be loaded onto the CaP coating. The peptide-loaded samples did not have significant effect on the activity of the osteoblast-like cells (Fig. 7), but could effectively kill both *S. aureus* and *P. aeruginosa* within 30 min (Fig. 4). Interestingly, the antimicrobial activity of CaP-Tet213 was maintained following extended incubation (24 h) with *P. aeruginosa*. Since MX226 peptide has been reported as the most advanced AMP to show statistically significant clinical effects to date [55] and due to a few study performed on delivery of hLF1-11 AMP from CaP coatings [57–59], these two AMPs were selected to be compared with Tet213. In this study we also demonstrated that this CaP coating was a better carrier for the Tet213 antimicrobial peptide than for MX226, hLF1-11 or tobramycin in terms of bactericidal efficiency (Fig. 6). One limitation of current study was the relatively short term *in vitro* tests. Longer

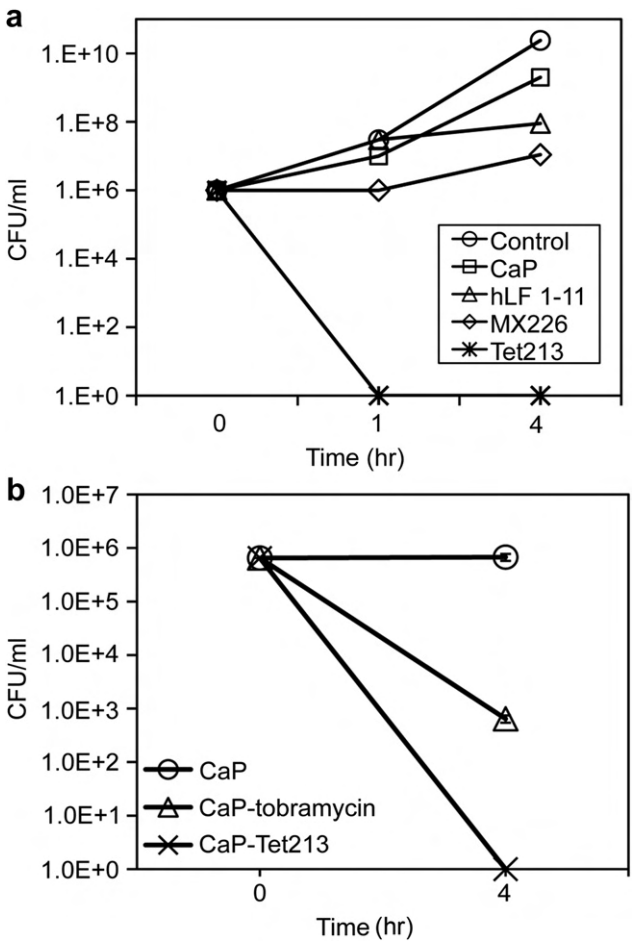

Fig. 6. (a) Antimicrobial activity of the CaP-Tet213 sample as compared with CaP-MX226 and CaP- hLF1-11 AMPs. CaP-Tet213 clearly demonstrated more effective antimicrobial activity against *P. aeruginosa*. (b) Antimicrobial activity of the CaP-Tet213 as compared with CaP–Tobramycin. The CaP–Tobramycin samples reduced the total bacterial inoculum by 3 log orders over 4 h while 100% (6 log orders) of the inoculum was killed by CaP-Tet213. Since CaP–Tobramycin showed antimicrobial activity, it was compared to CaP-Tet213 in a separate test.

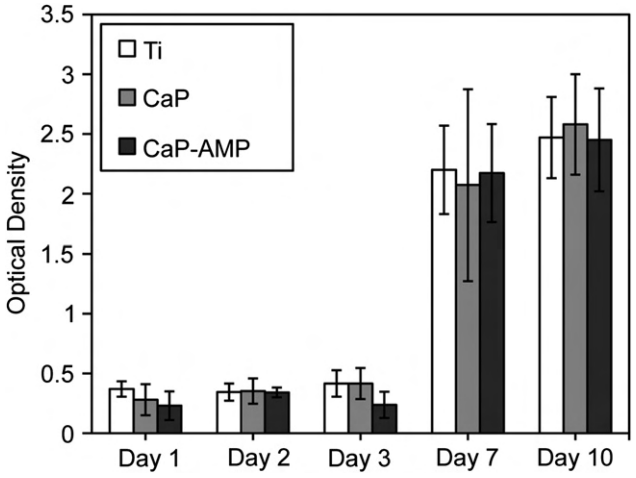

Fig. 7. MTT assay performed to evaluate the cytotoxicity of CaP-Tet213 with MG-63 osteoblast-like cell in the solution. No statistical difference in cell activity between the peptide-loaded (CaP-AMP) and the two controls (CaP coating and Ti) after 10 days incubation.

duration of *in vitro* tests and especially *in vivo* animal study would be needed to verify such an antimicrobial effect.

Calcium phosphates have been extensively studied for delivering growth factors and drugs for the purpose of enhancing bone growth [45,60,61]. Delivering traditional antibiotics through calcium phosphate-coated implants has also been actively studied [62–64]. However, there are few reports if any on the delivery of cationic antimicrobial peptides through calcium phosphate coatings. Relevant studies were done by Stallmann et al., who loaded the 11-mer antimicrobial peptide hLF1-11 (GRRRRSVQWCA, which consists of N-terminal amino acids 1–11 of human lactoferrin) onto calcium phosphate bone cements for potential application in osteomyelitis [57–59]. When mixed into the calcium cements, the peptide showed slow release both *in vitro* and *in vivo* [57,59]. When loaded onto CaP granules through soaking, it was commented that the peptide had burst release only [58], indicating a weak interaction between the peptide and the CaP granules. In our current study, the high amount of peptide was measured after 10 min of water rinse. The samples killed all the *P. aeruginosa* in 30 min. Subsequent wash and repeated testing of the same samples showed mild bactericidal properties (Fig. 5). Our results indicated a relatively strong interaction between the studied peptide (Tet213) and CaP coating. Both the morphological factor and the chemical factor of the coating could contribute to the efficient loading of the peptide. Firstly, the CaP coating processed by the electrolytic deposition technique is microscopically porous (Fig. 1). This created large surface area for peptide-CaP interaction. Secondly, the antimicrobial peptide (Tet213) has an isoelectric point of 11.72 with five positively charged residues (Arg and Lys), which makes it a highly positive peptide at working pH (7.4). The positively charged side groups in the cationic antimicrobial peptide may electrostatically interact with the negatively charged phosphate group in the CaP crystals. The AMP in this study has two adjacent Arg residues in its sequence. This may further enhance the electrostatic interaction. In protein–protein interaction, it has been reported that the electrostatic interactions between the guanidinium groups in a basic epitope containing adjacent Arg residues and the phosphate groups in acidic epitopes can possess “covalent-like” stability [65]. It has also been well known that some short peptides could selectively bind to solid surfaces [66]. One of the identified short peptide sequence (SVSVGMKPSPRPGGGK) reported by Weiger et al. for HA is also cationic [67]. It is not clear whether similar interaction mechanisms are also involved in our systems.

5. Conclusions

Calcium phosphate coating was successfully formed on titanium substrate by electrolytic deposition. The as-processed CaP coating was mainly octacalcium phosphate. This micro-porous CaP coating has high drug loading efficiency for the antimicrobial peptide, with 9 $\mu g/cm^2$ of peptide on a 7 μm thick coating. The peptide-loaded CaP coating on titanium surface has no cytotoxicity with osteoblast-like cells. It could kill both Gram-positive (*S. aureus*) and Gram-negative (*P. aeruginosa*) bacteria within 30 min *in vitro*. The local delivery of antimicrobial peptides through implant surfaces could be a potential solution for early stage peri-implant infection.

Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council of Canada and the Canadian Institutes of Health Research. R.W. is incumbent of the Canada Research Chair in Biomaterials while REWH has a Canada Research Chair in Health and Genomics. The authors wish to thank Dr. Paul Clarkson for kindly providing the osteoblast-like cell line used in this study,

Dr. Guangzheng Gao for assisting in FTIR testing, and Ms. Shanshan Lu for assisting in peptide detection. We are also grateful for the discussions with Dr. Clive Duncan, Dr. Donald Garbuz, Dr. Bassam Masri, and Dr. Jayachandran N Kizhakkedathu.

Appendix

Figures with essential colour discrimination. Certain figures in this article, particularly Fig. 1 is difficult to interpret in black and white. The full colour image can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.08.035.

References

[1] Darouiche RO. Treatment of infections associated with surgical implants. N Engl J Med 2004;350(14):1422–9.
[2] Total hip and total knee replacements in canada. CJRR annual report. Can Institute for Health Information; 2005.
[3] Sia IG, Berbari EF, Karchmer AW. Prosthetic joint infections. Infect Dis Clin North Am 2005;19(4):885–914.
[4] Roy M, Bandyopadhyay A, Bose S. In vitro antimicrobial and biological properties of laser assisted tricalcium phosphate coating on titanium for load bearing implant. Mater Sci Eng C 2009;29(6):1965–8.
[5] Chen W, Liu Y, Courtney HS, Bettenga M, Agrawal CM, Bumgardner JD, et al. In vitro anti-bacterial and biological properties of magnetron co-sputtered silver-containing hydroxyapatite coating. Biomaterials 2006;27(32):5512–7.
[6] Rohde H, Burandt EC, Siemssen N, Frommelt L, Burdelski C, Wurster S, et al. Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. Biomaterials 2007;28(9):1711–20.
[7] Ewald A, Gluckermann S, Thull R, Gbureck U. Antimicrobial titanium/silver PVD coatings on titanium. Biomed Eng Online 2006;5(1):22.
[8] Tp S. The economic impact of infected joint arthroplasty. Orthopedics 1995;18:871–3.
[9] Bullough PG. Metallosis. J Bone Jt Surg Br 1994;76B(5):687–8.
[10] Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999;284(5418):1318–22.
[11] Rogers SS, van dW, Waigh TA. Microrheology of bacterial biofilms in vitro: *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Langmuir 2008;24 (23):13549–55.
[12] Antoci Jr V, Adams CS, Parvizi J, Davidson HM, Composto RJ, Freeman TA, et al. The inhibition of *Staphylococcus epidermidis* biofilm formation by vancomycin-modified titanium alloy and implications for the treatment of periprosthetic infection. Biomaterials 2008;29(35):4684–90.
[13] Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The Calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol 1999;37(6):1771–6.
[14] Toms AD, Davidson D, Masri BA, Duncan CP. The management of peri-prosthetic infection in total joint arthroplasty. J Bone Joint Surg Br 2006;88-B (2):149–55.
[15] Arica MY, Tuglu D, Basar MM, Kilic D, Bayramoglu G, Batislam E. Preparation and characterization of infection-resistant antibiotics-releasing hydrogels rods of poly[hydroxyethyl methacrylate-co- poly(ethylene glycol)-methacrylate]: biomedical application in a novel rabbit penile prosthesis model. J Biomed Mater Res B Appl Biomater 2008;86(1):18–28.
[16] Lombardi Jr AV, Karnes JM, Berend KR. A motion maintaining antibiotic delivery system. J Arthroplasty 2007;22(4):50–5.
[17] Zhao L, Chu PK, Zhang Y, Wu Z. Antibacterial coatings on titanium implants. J Biomed Mater Res B Appl Biomater 2009;91B(1):470–80.
[18] Moojen DJF, Vogely HC, Fleer A, Nikkels PGJ, Higham PA, Verbout AJ, et al. Prophylaxis of infection and effects on osseointegration using a Tobramycin-Periapatite coating on titanium implants-an experimental study in the rabbit. J Orthop Res 2009;27(6):710–6.
[19] Tamai K, Kawate K, Kawahara I, Takakura Y, Sakaki K. Inorganic antimicrobial coating for titanium alloy and its effect on bacteria. J Orthop Sci 2009;14 (2):204–9.
[20] Moskowitz JS, Blaisse MR, Samuel RE, Hsu HP, Harris MB, Martin SD, et al. The effectiveness of the controlled release of gentamicin from polyelectrolyte multilayers in the treatment of Staphylococcus aureus infection in a rabbit bone model. Biomaterials 2010;31(23):6019–30.
[21] Zilberman M, Elsner JJ. Antibiotic-eluting medical devices for various applications. J Control Release 2008;130(3):202–15.
[22] P.A.N. Jr , Bumgardner JD. Biomaterial and antibiotic strategies for peri-implantitis: a review. J Biomed Mater Res B Appl Biomater 2009;88B (2):530–43.
[23] Basak P, Adhikari B, Banerjee I, Maiti TK. Sustained release of antibiotic from polyurethane coated implant materials. J Mater Sci Mater Med 2009;20 (1):213–21.
[24] Campoccia D, Montanaro L, Speziale P, Arciola CR. Antibiotic-loaded biomaterials and the risks for the spread of antibiotic resistance following their prophylactic and therapeutic clinical use. Biomaterials 2010;31(25): 6363–77.
[25] Campoccia D, Montanaro L, Arciola CR. The significance of infection related to orthopedic devices and issues of antibiotic resistance. Biomaterials 2006;27 (11):2331–9.
[26] Outline for healthcare-associated infections surveillance: National Nosocomial Infections Surveillance (NNIS) System and the National Healthcare Safety Network (NHSN). 2006.
[27] Smith TL, Pearson ML, Wilcox KR, Cruz C, Lancaster MV, Robinson-Dunn B, et al. Emergence of Vancomycin resistance in Staphylococcus aureus. N Engl J Med 1999;340(7):493–501.
[28] Trampuz A, Zimmerli W. Antimicrobial agents in orthopaedic surgery: prophylaxis and treatment. Drugs 2006;66(8):1089.
[29] Hancock REW, Patrzykat A. Clinical development of cationic antimicrobial peptides: from natural to novel antibiotics. Curr Drug Targets Infect Disord 2002;2:79–83.
[30] Hancock REW. Cationic peptides: effectors in innate immunity and novel antimicrobials. Lancet Infect Dis 2001;1(3):156–64.
[31] Hancock REW, Brown KL, Mookherjee N. Host defence peptides from invertebrates - emerging antimicrobial strategies. Immunobiology 2006;211 (4):315–22.
[32] Jenssen H, Hamill P, Hancock REW. Peptide antimicrobial agents. Clin Microbiol Rev 2006;19(3):491–511.
[33] Steinberg DA, Hurst MA, Fujii CA, Kung AHC, Ho JF, Cheng FC, et al. Protegrin-1: a broad-spectrum, rapidly microbicidal peptide with in vivo activity. Antimicrobial Agents Chemother 1997;41(8):1738–42.
[34] Ingham AB, Moore RJ. Recombinant production of antimicrobial peptides in heterologous microbial systems. Biotechnol Appl Biochem 2007;47(1):1–9.
[35] Hilpert K, Elliott MR, Volkmer-Engert R, Henklein P, Donini O, Zhou Q, et al. Sequence requirements and an optimization strategy for short antimicrobial peptides. Chem Biol 2006;13(10):1101–7.
[36] Cherkasov A, Hilpert K, Jenssen H, Fjell CD, Waldbrook M, Mullaly SC, et al. Use of artificial intelligence in the design of small peptide antibiotics effective against a broad spectrum of highly antibiotic-resistant superbugs. ACS Chem Biol 2008;4(1):65–74.
[37] Fjell CD, Jenssen H, Hilpert K, Cheung WA, Panté N, Hancock REW, et al. Identification of novel antibacterial peptides by chemoinformatics and machine learning. J Med Chem 2009;52(7):2006–15.
[38] Hilpert K, Volkmer-Engert R, Walter T, Hancock REW. High-throughput generation of small antibacterial peptides with improved activity. Nat Biotechnol 2005;23(8):1008–12.
[39] Gawalt ES, Avaltroni MJ, Danahy MP, Silverman BM, Hanson EL, Midwood KS, et al. Bonding organics to Ti alloys: Facilitating human osteoblast attachment and spreading on surgical implant materials. Langmuir 2002;19(1):200–4.
[40] Xiao SJ, Textor M, Spencer ND, Wieland M, Keller B, Sigrist H. Immobilization of the cell-adhesive peptide Arg–Gly–Asp–Cys (RGDC) on titanium surfaces by covalent chemical attachment. J Mater Sci Mater Med 1997;8(12):867–72.
[41] Nanci A, Wuest JD, Peru L, Brunet P, Sharma V, Zalzal S, et al. Chemical modification of titanium surfaces for covalent attachment of biological molecules. J Biomed Mater Res 1998;40(2):324–35.
[42] Haynie SL, Crum GA, Doele BA. Antimicrobial activities of amphiphilic peptides covalently bonded to a water-insoluble resin. Antimicrobial Agents Chemother 1995;39(2):301–7.
[43] Betty Leon JAJ. Thin calcium phosphate coatings for medical implants. Springer Science; 2009.
[44] Fan Y, Duan K, Wang R. A composite coating by electrolysis-induced collagen self-assembly and calcium phosphate mineralization. Biomaterials 2005;26 (14):1623–32.
[45] Garbuz DS, Hu Y, Kim WY, Duan K, Masri BA, Oxland TR, et al. Enhanced gap filling and osteoconduction associated with alendronate-calcium phosphate-coated porous tantalum. J Bone Jt Surg Am 2008;90(5):1090–100.
[46] Hilpert K, Elliott M, Jenssen H, Kindrachuk J, Fjell CD, Körner J, et al. Screening and characterization of surface-tethered cationic peptides for antimicrobial activity. Chem Biol 2009;16(1):58–69.
[47] Smith RE, MacQuarrie R. A sensitive fluorometric method for the determination of arginine using 9,10-phenanthrenequinone. Anal Biochem 1978;90 (1):246–55.
[48] Wu M, Hancock REW. Improved derivatives of Bactenecin, a cyclic Dodecameric antimicrobial cationic peptide. Antimicrobial Agents Chemother 1999;43(5):1274–6.
[49] Arellano-Jiménez MJ, García-García R, Reyes-Gasga J. Synthesis and hydrolysis of octacalcium phosphate and its characterization by electron microscopy and X-ray diffraction. J Phys Chem Solids 2009;70(2):390–5.
[50] Wang RZ, Cui FZ, Lu HB, Wen HB, Ma CL, Li HD. Synthesis of nanophase hydroxyapatite/collagen composite. J Mater Sci Lett 1995;14(7):490–2.
[51] Slósarczyk A, Paluszkiewicz C, Gawlicki M, Paszkiewicz Z. The FTIR spectroscopy and QXRD studies of calcium phosphate based materials produced from the powder precursors with different Ca/P ratios. Ceram Int 1997;23(4):297–304.
[52] Wang J, Layrolle P, Stigter M, de Groot K. Biomimetic and electrolytic calcium phosphate coatings on titanium alloy: physicochemical characteristics and cell attachment. Biomaterials 2004;25(4):583–92.
[53] Hale JDF, Hancock REW. Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. Expert Rev Anti Infect Ther 2007;5(6):951–9.
[54] Brown KL, Hancock REW. Cationic host defense (antimicrobial) peptides. Curr Opin Immunol 2006;18(1):24–30.

[55] Jenssen H, Hancock REW. Therapeutic potential of HDPs as immunomodulatory agents. Antimicrob Peptides: Methods Protoc; 2010:329–47.
[56] Lu X, Zhao Z, Leng Y. Calcium phosphate crystal growth under controlled atmosphere in electrochemical deposition. J Cryst Growth 2005;284(3–4):506–16.
[57] Stallmann HP, de Roo R, Faber C, Amerongen AVN, Wuisman P. In vivo release of the antimicrobial peptide hLF1-11 from calcium phosphate cement. J Orthop Res 2008;26(4):531–8.
[58] Stallmann HP, Faber C, Bronckers ALJJ, Nieuw Amerongen AV, Wuisman PIJM. Osteomyelitis prevention in rabbits using antimicrobial peptide hLF1-11- or gentamicin-containing calcium phosphate cement. J Antimicrob Chemother 2004;54(2):472–6.
[59] Stallmann HP, Faber C, Slotema ET, Lyaruu DM, Bronckers ALJJ, Amerongen AVN, et al. Continuous-release or burst-release of the antimicrobial peptide human lactoferrin 1-11 (hLF1-11) from calcium phosphate bone substitutes. J Antimicrob Chemother 2003;52(5):853–5.
[60] Duan K, Wang RZ. Surface modifications of bone implants through wet chemistry. J Mater Chem 2006;16(24):2309–21.
[61] Ginebra MP, Traykova T, Planell JA. Calcium phosphate cements as bone drug delivery systems: a review. J Control Release 2006;113(2):102–10.
[62] Stigter M, Bezemer J, de Groot K, Layrolle P. Incorporation of different antibiotics into carbonated hydroxyapatite coatings on titanium implants, release and antibiotic efficacy. J Control Release 2004;99(1):127–37.
[63] Stigter M, de Groot K, Layrolle P. Incorporation of tobramycin into biomimetic hydroxyapatite coating on titanium. Biomaterials 2002;23(20):4143–53.
[64] Radin S, Campbell JT, Ducheyne P, Cuckler JM. Calcium phosphate ceramic coatings as carriers of vancomycin. Biomaterials 1997;18(11):777–82.
[65] Woods AS, Ferré S. Amazing stability of the Arginine–phosphate electrostatic interaction. J Proteome Res 2005;4(4):1397–402.
[66] Tamerler C, Sarikaya M. Molecular biomimetics: nanotechnology and bionanotechnology using genetically engineered peptides. Phil Trans R Soc A 1894;2009(367):1705–26.
[67] Weiger M, Park JJ, Roy M, Stafford C, Karim A, Becker M. Quantification of the binding affinity of a specific hydroxyapatite binding peptide. Biomaterials 31 (11):2955–2963.