

Preparation, release profiles and antibacterial properties of vancomycin-loaded Ca–P coating titanium alloy plate

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Received: 25 September 2010 / Accepted: 28 February 2011 / Published online: 19 March 2011
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Abstract The aim of the study was to explore the feasibility of the Ca–P coating titanium alloy plate to be used as the vancomycin drug-delivery system by biomimetic coating technology. Through the X-ray diffraction study, the main components of the coatings were identified as octocalcium phosphate. The in vitro vancomycin release, bacteriostasis activity to *Staphylococcus aureus* (*S. aureus*), the scanning electron microscope (SEM) image and osteoblast adhesion and proliferation test of vancomycin-loaded Ca–P coating plate were evaluated. The bacteriostatic activity of the vancomycin-loaded Ca–P coating plate showed a continuous drug release and had an inhibitory effect on the growth of the *S. aureus*. In vitro osteoblast

culture results showed that the Ca–P coating plate loaded with or without the vancomycin both obviously promoted the osteoblast attachment. It was suggested that the vancomycin-loaded Ca–P coating may be compounded in the surface of the internal fixators to reduce the incidence of the implant-associated infection.

1 Introduction

According to the report of Darouiche [1], there are about two million cases of nosocomial infections per year in the United States, about 50% of which is related to the internal implant device. At the same time, the implant-associated infection may also bring about many serious adverse consequences, such as overuse of antibiotics, repeated debridement, prolonged hospitalization, increased morbidity and mortality, and serious economic burden [2]. Therefore, the implant-associated infection has become one of the focal points in orthopedics, microorganisms, biomaterials and even new drug synthesis.

We have reported the in vitro and in vivo researches concerning the release kinetics and bacterial properties of the vancomycin from poly (D, L-lactide) (PDLLA) coated plate [3–5]. However, the PLLA has no bone bioactivity. Combining the osteoconductive properties of the bioactive materials with a sustained antibiotic release could produce a two-fold beneficial effect. Thus, attention should also be paid to the use of biomimetic coating as one of drug delivery systems. Compared with other coating techniques, the biomimetic coating was conducted at a temperature closer to body temperature (37°C) and at a better pH value (7.4) [6], which facilitated that the biologically active molecules, such as growth factors, could be co-precipitated with Ca–P crystals onto the surface of the metal implants

Jun Fei and Guo-dong Liu shared the same duties in this study.

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without compromising the bioactivity of the osteogenic agents due to mild conditions employed during the coating process [7]. It has been reported that the Ca–P coatings incorporated with the bioactive molecules can substantially improve the bioactivity [7, 8] and enhance the osteogenic activity of the implant [9–11], especially in the setting of cementless arthroplasty, as was helpful for a success of cementless total hip replacement. Under these conditions, it was feasible that the ceramic coatings could be used as a way of fixation or drug carriers.

In this study, with the calcium phosphate as drug carrier, the vancomycin-loaded Ca–P coatings were fabricated by the biomimetic method on the surface of the titanium alloy plate for local continuous release of vancomycin. The bacteriostatic activity to the *S. aureus* and the drug-release profile of the vancomycin-loaded Ca–P titanium alloy plates were evaluated in vitro. The osteoblast culture was conducted with the vancomycin-loaded Ca–P titanium alloy plate prepared by adding vancomycin into the coating solution (at a concentration of 600 mg/l). It was expected that the vancomycin-loaded Ca–P titanium alloy plate could be used as vancomycin drug-delivery system, for it could locally release high dose of vancomycin to effectively kill the bacteria during fracture fixation or cementless arthroplasty. In the meantime, the vancomycin-loaded Ca–P titanium alloy plate could prolong the duration of the action, reduce the risk of drug-resistant strains and decrease the high concentration of the vancomycin in blood.

2 Materials and methods

2.1 Preparation of coating

The biomimetic method was used to produce the drug-loaded titanium alloy plate (Ti-6Al-4 V). The commercially available titanium alloy square plate (10 mm × 10 mm × 0.5 mm, Kanghui Medical Device Company, China) were used as the substrates in this study. These plates were roughened by #400, #600 and #800 sandpapers, sonicated in double-distilled water, acetone, 70% ethanol and ultrapure water for 5, 15, 15 and 10 min, respectively. After dried in highly compressed air, the cleaned plate was placed in the mixture with 48% sulfuric acid:18% hydrochloric acid (1:1, V/V) for 10 min in the boiling water. After drying, the plate was washed by plenty of the distilled water and dried, followed by an alkaline treatment using 2.5 M NaOH at 80°C for 12 h in a water bath. Then, the plate was put into 0.1 M CaCl₂ and distribution droplets of isovolumic 0.1 M K₂HPO₄ slowly, when the solution was stirred continuously and then preserved for 1 h. The plate was thoroughly washed with the deionized water and immersed in a supersaturated calcification solution

(SCS). The SCS solution was made by dissolving 8.035 g NaCl, 0.24 g K₂HPO₄ and 0.438 g CaCl₂ in the ultrapure water, and was adjusted by 1.0 M hydrochloric acid and 1.0 M (CH₂OH)₃CNH₂ to a liter solution (pH 7.4) at room temperature. The vancomycin (purchased from ELI Lilly K.K Seishin Laboratories of Japan) was added into the above solution to prepare the concentration of 100, 200, 400, 600, 800 and 1,000 mg/l SCS containing the vancomycin. Seven sets of plates were incubated, one in SCS and the others in 100, 200, 400, 600, 800 and 1,000 mg/l SCS containing the vancomycin solutions. Each set of plates was immersed in the shaking water bath at 37°C for 48 h and replaced by fresh SCS or SCS + vancomycin solutions in 48 h to ensure the concentration of ions in SCS and vancomycin. After air drying overnight in the well-ventilated place, the plate was sealed for preservation at 4°C. The above operations were all carried out under sterile conditions.

2.2 Characterization of the coating

The vancomycin-loaded Ca–P titanium alloy plates prepared by adding the vancomycin into the coating solution (at a concentration of 600 mg/l) were examined using an X-ray diffractometer (D/Max 2500PC, Rigaku Company, Japan) with a copper target, with the voltage and current settings for 40 kV and 200 mA, respectively and the scanning range for 10–60°.

2.3 Release profile of norvancomycin in vitro

The Ca–P coatings containing the vancomycin was directly prepared on the titanium alloy plate according to the method described in the above-mentioned section. The Ca–P coating plates without vancomycin were served as control. The release of the vancomycin was performed by immersing one vancomycin-loaded Ca–P titanium alloy plate in a glass vial containing 10 ml of PBS (pH 7.4) as a releasing medium. The sealed vials were placed in a shaking water bath at 37°C and centrifuged at a frequency of 60 rpm. After 1, 2, 4, 8, 12, 24 h, 1 ml PBS solution was collected and 1 ml refreshed PBS was added into the solution. The drug concentration in 1 ml PBS was assayed according to the method previously described.

2.4 Source of *S. aureus*

The pathogen applied was *S. aureus* (ATCC 25923 standard strain, purchased from the Chinese Ministry of Health). This strain was sensitive to the vancomycin. It was incubated for 24 h in 80 ml tryptone soya broth (TSB) at 37°C in a water bath. By means of the McFarland densitometric germ count method (1907), the suspension of the

S. aureus was deliquated to 10^2 , 10^3 , 10^4 , 10^5 , 10^6 and 10^7 CFU/ml, respectively.

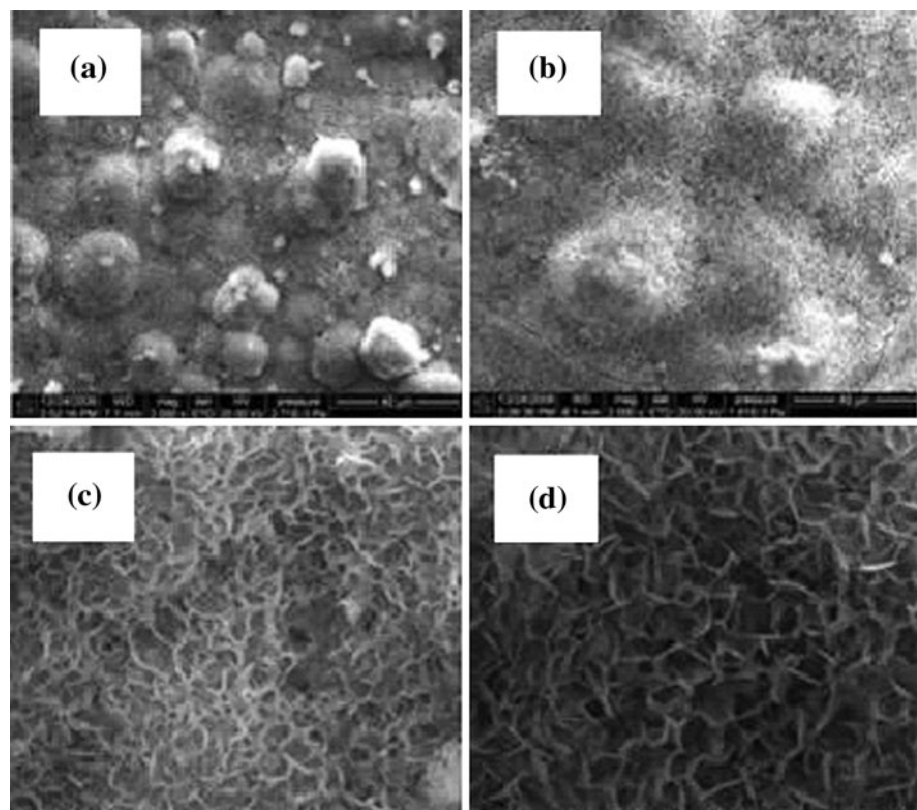
2.5 Antibacterial experiment of plate eluent [12]

The sterile vancomycin-loaded Ca–P titanium alloy plates, Ca–P titanium alloy plates without antibiotic and uncoated titanium alloy plates were incubated and continuously shaken in 5 ml TSB for 1 h separately. After the plates were taken out of the TSB, the TSB was incubated again in a mixture with 1 ml TSB and bacteria at different concentrations of 10^2 , 10^3 , 10^4 , 10^5 , 10^6 and 10^7 CFU/ml for 3 h at 37°C in a water bath. Portion for 100 μl was spread on the blood agar plates and incubated for 24 h in the incubator at 37°C . The *S. aureus* colonies were counted on the blood agar plates for a quantitative analysis.

2.6 Bacterial adhesion experiment of the plate

The *S. aureus* (ATCC 25923) was cultured in 80 ml TSB (tryptone soy broth) at 37°C for 24 h. The sterile Ca–P titanium alloy plates loaded with or without the vancomycin were incubated in 10^7 CFU/ml for 1 h and cleaned twice with PBS after being taken out to remove the un-adhering bacteria. Then, the plates were washed with 5% formaldehyde and PBS and fixed with 2% glutaraldehyde for scanning electron microscope (SEM) scanning.

Fig. 1 The titanium alloy surface after coating showed by SEM (a, c). The vancomycin-loaded Ca–P coating (600 mg/l) $\times 2000$, $\times 10,000$ (b, d). Ca–P coating $\times 2000$, $\times 10,000$



2.7 Osteoblast adhesion and proliferation of the plate

Six born fetus rats were sacrificed and placed in 75% alcohol for immersion disinfection. Under sterile environment, the skull was removed for primary culture and purified by repeated adherence. The cells cultured for 2 weeks were used to detect the statutory nature of the alkaline phosphatase (ALP) by means of ALP Gomori. While the cells cultured for 30 days were used for detecting the calcium deposition by using Von Kossa staining so as to identify the osteocytes. The third generation of the osteocytes were first digested in 0.25% trypsin to make cell suspension by using DMEM cell culture medium (containing 10% fetal bovine serum). Then, the cell suspension was inoculated to a 24-well plate containing the samples with different surfaces (vancomycin-loaded Ca–P titanium alloy plate group at concentration of 600 mg/l, Ca–P coating group, uncoated titanium blank group), with 5×10^4 cells per well. After inoculation, the cultured vancomycin-loaded Ca–P titanium alloy plates were removed at 3, 6, 12 and 24 h and digested with trypsin for cell counting. For the remaining vancomycin-loaded Ca–P titanium alloy plates, the culture medium was replaced once every either day after inoculation. The cell culture was terminated at time points of 2, 4, 6 and 8 days for cell counting with the MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazoliumbromide test) (Promega, Milan, Italy) method.

2.8 Statistical analysis

Data were expressed as mean \pm standard deviation (Mean \pm SD). Paired *t* test between different time points was performed with SPSS software version 10.0 (SPSS Inc, Chicago, Illinois). A *P* value < 0.05 was considered significant and < 0.01 was considered remarkable.

3 Results

3.1 Surface topography of coating plate

The coating morphology was evaluated by SEM (TESCAN VEGAII LMU, TESCAN, Czech) at 20 kV. After the coating procedure, a stable and regular Ca–P coating could be observed through SEM. The titanium alloy plate surface was covered by plate-shaped crystals (Fig. 1a, b), which was well distributed in the enlarged drawing to form the needle-shaped crystals (Fig. 1c, d). A lot of white depositions of Ca–P was detected on the surface topography of the vancomycin-loaded Ca–P titanium alloy plate (Fig. 1a) compared to the Ca–P coating plate free from the vancomycin (Fig. 1b). The uniform and condensed coating was obtained in the enlarged drawing, with no statistical difference between the minute structures (Fig. 1c, d). It was suggested that the vancomycin exerted effect on the concentration of ions in SCS that resulted a portion of direct sediment of the Ca–P.

3.2 Characterization of the coating and release profile of vancomycin in vitro

In addition to the diffraction peaks of the titanium substrate and the hydroxyapatite, there appeared diffraction peaks of the octacalcium phosphate (OCP, $\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$). The coating should be the mixture with hydroxyapatite as main components; Ab. The X-ray diffraction (XRD) patterns of the Ca–P without vancomycin coating. In addition to the diffraction peaks of the titanium substrate, the coatings were identified as the hydroxyapatite, which exhibits a unique diffraction peak at $2\theta = 26^\circ$ in response to the (002) plane of hydroxyapatite. Other peaks appeared at 31° , 32° and 54° corresponding to the (211), (112) and (004) plane of hydroxyapatite in the XRD, consistent with the JCPDS09-HA diffraction peaks. See Fig. 2a.

In vitro release test was conducted with the vancomycin-loaded Ca–P titanium alloy plate prepared by adding the vancomycin into the coating solution at a concentration of 600 mg/l. The concentration of the norvancomycin in the tissue reached the highest at day 1 and decreased gradually, with gradual decrease of the release speed. The concentration of the norvancomycin in the surrounding tissues of

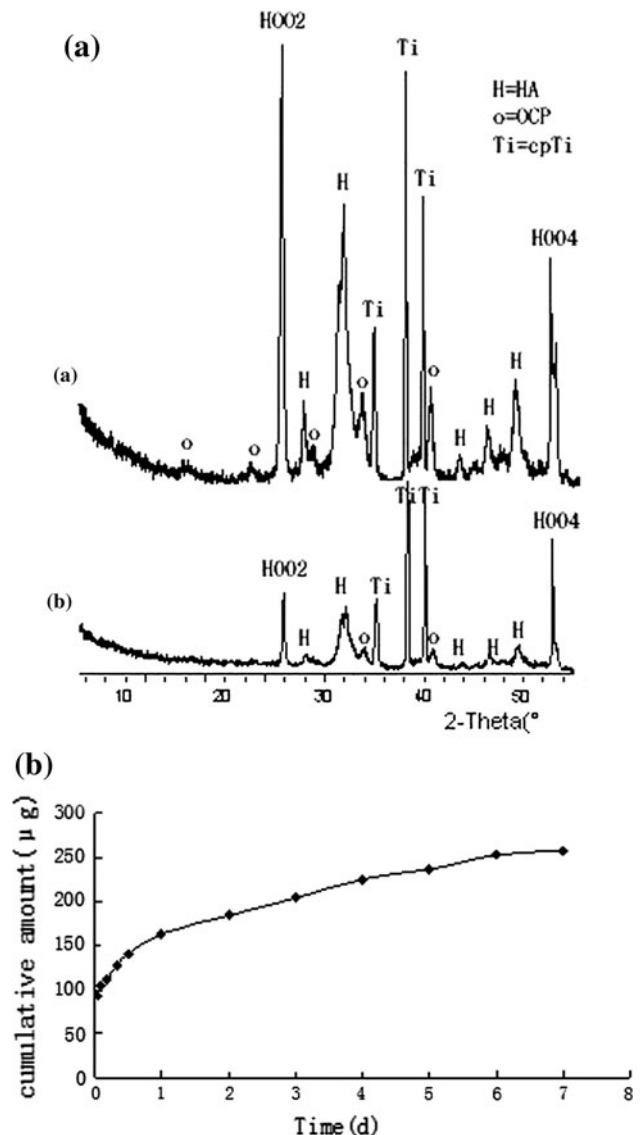


Fig. 2 XRD spectrum of the Ca–P coating on the titanium alloy surface and the release curves of the vancomycin in vitro. **a** XRD spectrum of the vancomycin-loaded Ca–P coating; **b** XRD spectrum of the Ca–P without vancomycin coating. **b** The release curves of the vancomycin in vitro

the vancomycin-loaded Ca–P titanium alloy plate still met the MIC of the norvancomycin at day 7 (see Fig. 2b).

3.3 Bacteriostasis experiments

In the culture capsule of the uncoated titanium alloy plate, there detected a large amount of the *S. aureus* colonies. With the increase of the concentration of SA (102–107 CFU/ml), the colonies were also increased significantly. But under the condition of the same concentration of the *S. aureus*, the colonies of the *S. aureus* were less significantly in the culture capsule of the vancomycin-loaded Ca–P titanium alloy plate. The colonies were

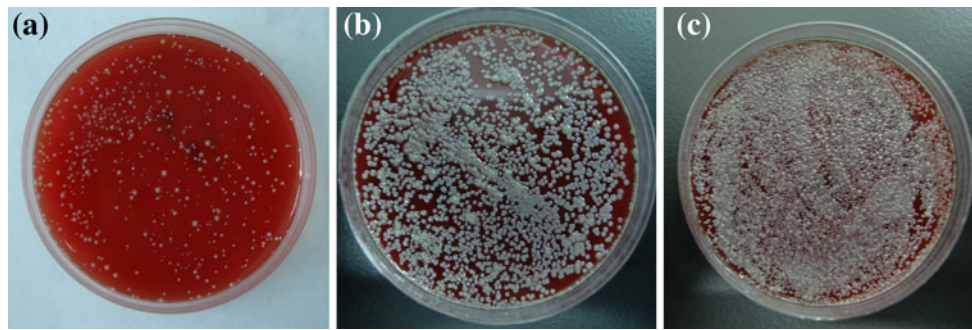
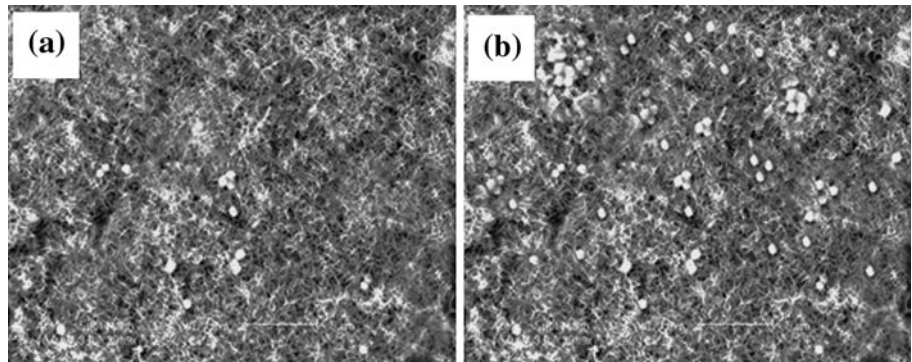


Fig. 3 Inhibitive effect of the eluent of the specimen and the control at a bacterial concentration of 105 CFU. **a** Vancomycin-loaded Ca-P titanium alloy plate; **b** Titanium alloy plate with Ca-P coating; **c** Non-coating titanium alloy plate

Fig. 4 The bacterial adhesion to the Ca-P/vancomycin plate showed by SEM and the control. **a** Vancomycin-loaded Ca-P coating; **b** Ca-P coating



increased significantly with the increase of the concentration of the *S. aureus*. The experiment showed that the vancomycin-loaded Ca-P titanium alloy plate had a specific inhibitory effect on the growth of the *S. aureus* (Fig. 3).

3.4 Comparison of SEM image

The SEM image of the Ca-P coating plates after incubation in 107 CFU/ml *S. aureus* suspension showed that the *S. aureus* was gathered in clusters of high density (Fig. 4a). The SEM image of the vancomycin-loaded Ca-P titanium alloy plate showed that the *S. aureus* was scattered, indicating that the growth of the *S. aureus* on the plate surface was obviously inhibited (Fig. 4b).

3.5 Effect of specimens on osteoblast adhesion and proliferation

As the sediment containing the vancomycin from the vancomycin-loaded Ca-P titanium alloy plate was directly related to human bone contact, it was necessary to explore the role of the vancomycin in the osteocyte growth. Inoculation of three different specimens at different times of osteoblast cells of the adhesion indicated that the test cell adhesion rate in the vancomycin-loaded Ca-P titanium alloy plate was higher than that in the Ti alloy plates ($P < 0.05$) within 6 h (Fig. 5). Then, the cell adhesion rate

was slowed down and reached the maximum after 6 h. The cell adhesion rates of the Ca-P coating plate and the vancomycin-loaded Ca-P titanium alloy plate were still higher than that the Ti alloy plates ($P < 0.05$). There showed no statistical difference upon cell adhesion rate between the vancomycin-loaded Ca-P titanium alloy plate and the Ca-P coating plates ($P > 0.05$). Addition of the vancomycin into the Ca-P coating plate exerted no significant effect on the osteoblast adhesion to surface.

Cells of the vancomycin-loaded Ca-P titanium alloy plate, the Ca-P coating plate and the titanium control plate

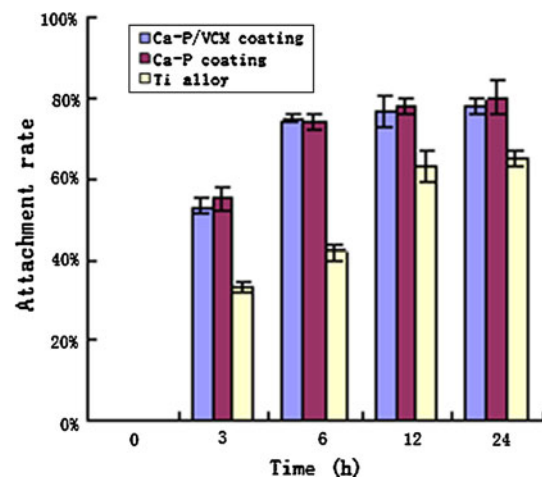


Fig. 5 Attachment rate of cells on the sample and control in 24 h

Table 1 Cell numbers of the vancomycin-loaded Ca–P titanium alloy plate and the Ti alloy control plate at different time points ($\times 10^4$)

Specimens	Time points (days)			
	2	4	6	8
Ca–P/vancomycin coating	8.06 \pm 0.72	18.9 \pm 0.26	23.65 \pm 0.67	32.82 \pm 1.25
Ca–P coating	8.12 \pm 0.65	18.7 \pm 0.37	23.85 \pm 0.59	33.12 \pm 0.88
Ti alloy	7.56 \pm 0.65	16.92 \pm 0.66	22.97 \pm 1.17	29.6 \pm 0.97

at different times were shown in Table 1. Cell proliferation rate of three kinds of materials at days 2, 4, 6 and 8 was not significantly different ($P > 0.05$), indicating an insignificant effect of three kinds of materials on the early osteoblast attachment and late proliferation.

4 Discussion

In modern orthopedic surgery, foreign body materials are frequently implanted. Deep infection occurs in approximately 1–2% of all primary total hip arthroplasty (THA) and total knee arthroplasty (TKA). It is well known that the revision surgery appears to induce 2–3 times the risk of infection in comparison with primary THA and TKA. In fact, further revisions and long-term antibiotic therapy may be frequently required due to difficulty in treatment.

Perioperative administration of the antibiotics would encounter bacteria within the tissues exposed to possible contamination during surgery. In most surgical procedures involving incorporation of the implants, the interface between the tissues and the implants was especially endangered to be contaminated. A local delivery system for the antibiotics based on a polymer implant coating has been developed to optimize the prophylaxis [13–15]. But such materials were not bone-bioactive, especially in the arthroplasty. The Ca–P coating produced by the biomimetic method was demonstrated to be able to enhance the early stage bone apposition and long-term fixation of the titanium prostheses [16]. The release behavior of the incorporating biomolecules onto/into the biomimetic coating has been investigated [17, 18]. It was found that if the biomolecules were simply adsorbed onto the surface of the coating, a “burst” release of the proteins would occur [19].

So, the aim of this study was to formulate an effective carrier system for local delivery of the vancomycin through the ceramic-coated titanium alloy substrates so as to facilitate the internal fixation and joint revision surgery. Drug loading by soaking the biomaterials in a simulated body fluid (SBF) containing the vancomycin was the focus of the majority of the experiments. In this study, the vancomycin was added to the SBF solution and co-precipitated with the Ca–P coating onto the titanium alloy plate. In this

manner, a more sustained release was achieved, which may facilitate one-stage revision of the infected artificial joint replacement.

In this study, the titanium alloy plate was first treated with the mixed acid in the boiling water for 10 min so that large amounts of acid pits and grooves could be formed in the titanium alloy substrate surface by corrosion, which increased the surface area and surface roughness. Then, the plate was placed in the NaOH solution, which induced production of a large number of sub-micron pores on the surface of the titanium alloy substrate and a dense small-diameter porous cross-linked network. This surface structure was conducive to non-homogeneous nucleation and to the apatite formation.

In this study, 2.5 mol/l of NaOH solution was used to treat the titanium alloy substrate, which could not only fully provide alkali concentration, but also avoid destroy of the mechanical properties of the substrate. On the other hand, the SCS ionic species and concentration also exerted certain effect on the apatite deposition. According to Ostwald’s nucleation theory [20], the nucleation free energy and (S), the nucleation network interface energy (δ), temperature (T) and the powder surface area (A) had the following relationship: $\Delta G = -RT \ln S + \delta A$, as showed that the increase of the solution supersaturation S would reduce the nucleation free energy (ΔG). In this study, the use of higher concentration of Ca–P saturation degree of SCS speeded up the deposition of the apatite in the titanium alloy substrate.

The in vitro release tests with the vancomycin-loaded Ca–P titanium alloy plate demonstrated sizeable drug elution at 2 h with rapid release. This ‘burst effect’ was very similar to the pattern of drug release seen in other studies utilizing calcium phosphate ceramics as drug carriers. It was suggested that the vancomycin was adsorbed to the coating during immersion, with drug solution penetrating the pores of the ceramic and accessing a large part of the surface area. By varying the adsorption conditions, we found that the adsorption was in a concentration-dependent manner but apparently was independent from the immersion time, as was also consistent with a rapid adsorption mechanism.

In this study, the implants were coated with the calcium phosphate by a biomimetic coating technique and the

vancomycin was delivered from a calcium phosphate coating on the titanium alloy plates. The antibacterial effect was quantified by the antibacterial experiment of the plate eluent and bacterial adhesion experiment by SEM. As illustrated in Fig. 4, the vancomycin incorporated in the Ca–P coating plate could inhibit the growth of the *S. aureus* in vitro. The quantity of the vancomycin released from the coating, therefore, exceeded the minimal inhibitory concentration (MIC) of the *S. aureus*. As shown in Fig. 4, after 1 h in the broth culture of the control group, SEM showed a large number of bacterial adhesion, but the majority of the bacteria gathered on the surface and were easy to form a biofilm, while surface adhesion of bacteria in the experimental group was significantly less than that in the control group.

The cells in the matrix were attached to the first step on the serum protein adsorption in the substrate containing the vancomycin-loaded Ca–P titanium alloy plate and in the surface of Ca–P coating plate due to formation of the bone-hydroxyapatite. The Ca^{2+} and PO_4^{3-} could be used as cell ligands adsorbed protein. So, the surface could absorb more proteins and increase the rate of cell attachment. But there was no significant difference on the osteoblast proliferation behavior in the titanium surface. It was shown that the apatite layer for bone formation did not affect the cell proliferation. However, more experiments are needed to explore the in vitro effect of the biomimetic hydroxyapatite and the bone mineralization on the osteoblast responses and bone integration.

5 Conclusions

The vancomycin-loaded Ca–P titanium alloy plate by using the biomimetic method can stably release the vancomycin at a low level in vitro and has a specific inhibitory effect on the growth of the *S. aureus*. Both the vancomycin-loaded Ca–P titanium alloy plate and Ca–P coating plates can obviously promote the osteoblast attachment in the early period, but did not significantly affect the proliferation of the osteoblast. Hence, the vancomycin-loaded Ca–P titanium alloy plate can be an alternative as vancomycin drug-delivery system.

Acknowledgments This study was supported by Grants from the National Natural Science Foundation of China (Nos. 30700177, 81071459), Tackle Key Problems in Technology Foundation of Chongqing (No. CSTC, 2009AC5022) and Chinese Postdoctoral Science Foundation (Nos. 20090460108, 201003775). We thank Chongqing University Bioengineering Institute for providing technical support in the vancomycin-loaded Ca–P titanium alloy plate.

Conflict of interest There is no any conflict of interests to disclose.

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