Abstract: In this paper, the micro-structured hydroxyapatite (HA)/TiO$_2$ coating was firstly produced on titanium surface by one-step micro-arc oxidation (MAO), and then bone morphogenetic protein-2 (BMP-2)-encapsulated chitosan (CS) coating was prepared on the HA/TiO$_2$ surface by dip-coating method to endow the bioactive HA/TiO$_2$ coating with good antibacterial property and improved biological properties. The bonding strength between coatings was studied by scratch method. The degradability of CS, BMP-2 release behavior, bioactivity, biocompatibility and antibacterial property of the BMP-2-CS/HA/TiO$_2$ composite coating were examined by in vitro tests. The results showed that, after loading BMP-2/CS on HA/TiO$_2$ surface, the surface roughness and wettability decreased but the bonding strength between the HA coating and titanium substrate was improved. CS, as a suitable carrier for sustained release of BMP-2, can control the release of BMP-2 continuously for 4 weeks long in a simulated human serum, and in PBS solution BMP-2 can be released more slowly. In addition, CS endowed the BMP-2/CS/HA/TiO$_2$ composite coating with good antibacterial activity. The BMP-2-CS/HA/TiO$_2$ showed good bioactivity, and it was also beneficial for cell adhesion, spreading and proliferation. Based on the above results, BMP-2-CS/HA/TiO$_2$ has great potential to be applied in clinic.

Key words: Hydroxyapatite, Chitosan, BMP-2, Antibacterial property, Biological property

Introduction

Titanium (Ti) is most widely used as an implant material for bone repair because of its excellent mechanical property, chemical stability and biocompatibility$^{1,2}$. But Ti-based implant is bio-inert, it cannot form strong bonding with bone tissues and it is easy to become loose after a long-term usage, consequently losing the implant effect. To improve the biological properties of Ti implant and achieve firm combination with bone tissues, HA which is the main inorganic component of natural bone tissues, is frequently deposited on Ti surface. Among the many methods that prepare HA coatings, micro-arc oxidation (MAO) is a convenient, economical and effective technique. By MAO, a HA/TiO$_2$ composite coating can be prepared rapidly on Ti surface. The binding force between HA coating and Ti matrix can be improved significantly due to the existence of the interfacial layer of TiO$_2$.$^{3}$ However, the HA/TiO$_2$ coating is still lacking in antibacterial activity, which may lead to the failed operation or postoperative infection. Therefore, it is necessary to modify the HA-coated implant to have good antibacterial property.

Based on the literature, antibacterial material could be introduced to the implant surface to decrease the risk of postoperative infection by preventing the bacterial adhesion and proliferation. Compared with other antibacterial materials, chitosan (CS) shows more advantages due to its suitable degradation, nontoxicity and biocompatibility$^{4,5}$. Some researchers also explored the combination of HA and CS to achieve good bioactivity and antibacterial property$^{6-9}$. And their results showed that, although the antibacterial property was enhanced by CS/HA composite, its biological property was still not satisfying, especially for serious diseases that require rapid wound healing, it cannot meet the clinical needs. Bone morphogenetic protein-2 (BMP-2) is one of the most widely used growth factors because of its essential role in bone regeneration, which can enhance the interfacial interaction between implant and tissue. But BMP-2 exhibits short-range diffusion and acts locally due to its short half-lives and slow diffusion, so it is necessary that BMP-2 must be carried and released in a controlled and sustained manner when applied. Ca-P composite has been used as a carrier of BMP-2, however, it shows burst release instead of sustained release, which fails to give full play of the osteoinductive effect of BMP-2$^{10}$. It has been reported that biodegradable polymers could be an excellent carrier for growth factors$^{11,12}$, therefore, CS may be a promising carrier for BMP-2 due to its degradable and biocompatible properties$^{13}$. After implantation, CS can control the release rate of BMP-2 by adjusting the degradation rate and assist the BMP-2 to function in surgical prostheses. Till now, there have been some reports that the HA/CS/BMP-2 scaffolds or microspheres were prepared to accelerate bone healing$^{13-16}$, but those materials often have poor mechanical properties, and their application is limited, especially applications for reparation of load-bearing bone tissues. Up to now, there has been no reports of preparing BMP-2/CS/HA/TiO$_2$ coatings on the surface of Ti implant.

In this work, micro-structured HA/TiO$_2$ coatings were firstly prepared on Ti surface by MAO technology, and then BMP-2 encapsulated CS was loaded on the surface. It was expected that the BMP-2/CS/HA
coating would show improved bonding strength, biological and antibacterial performance. The release kinetics of BMP-2 from CS/HA/TiO2 and HA/TiO2 were compared. The biological properties were investigated by SBF soaking test and MC3T3-E1 cell co-culture experiment. The antibacterial effect against E. coli was evaluated by the optical technique and bacterial counting method.

Materials and Methods

Materials preparation

Commercially pure Ti plates (TA1, Tianjin, China) with the diameter of 10 mm and the thickness of 1 mm were firstly polished with abrasive paper (800#, 1000#, 1500# and 2000#), washed with distilled water, and then used as the substrates for MAO treatment to obtain HA/TiO2 composite on Ti surface. During MAO process, Ti was used as anode, platinum plate as cathode, and the distance between the two electrodes was 5 cm. The voltage, electrolyte concentration of Ca(COOH)2/NaH2PO4, oxidation time, frequency and duty ratio were separately set at 360 V, 0.2/0.1 mol/l, 5 min, 100 Hz and 50%. To prepare the pure CS-coated HA/TiO2 (CS/HA/TiO2), BMP-2-immobilized HA/TiO2 (BMP-2/HA/TiO2) and BMP-2-encapsulated CS-coated HA/TiO2 (BMP-2/CS/HA/TiO2), the as-obtained HA/TiO2 coatings were dipped in 3 ml of the following solutions for 3 min and then lifted out in a rate of 150 mm/min. The applied solutions were showed as following: (a) a CS solution (1 mg/ml) which was prepared by dissolving pure CS in 2 vol% glacial acetic acid; (b) a BMP-2 solution (500 ng/3 ml) which was diluted from a BMP-2 stock solution with phosphate buffer solution (PBS), and the BMP-2 stock solution was prepared by dissolving BMP-2 in 2 vol% glacial acetic acid to form a stock solution (10 μg/ml); (c) a BMP-2/CS solution which was prepared by mixing 50 μl of BMP-2 stock solution with 2,950 μl of sterile-filtered CS solution. All of the samples were allowed to dry in oven at 60°C for 24 hours and then preserved in sterile conditions.

Surface characterization

The XRD patterns of each sample were obtained at room temperature using an analytical XPERTPRO powder diffractometer (Cu Kα radiation) operating at a voltage of 40 kV. The surface morphology and cross-sections of the samples were examined using scanning electron microscopy (SEM) with energy dispersive X-ray (EDX) analyzer attached for elemental analysis. The adhesive force was tested by a WS-92 scratch tester. The loading rate was 50 N/min with the final load of 50 N. The scratch length was 5 mm. Atomic force microscopy (AFM, Agilent 5500) was used to obtain topographic images and roughness of sample surfaces. Static contact angles (CAs) were measured with the deionized water at room temperature to evaluate the surface hydrophilicity.

Degradation of the CS containing composite coatings

There are 8 samples for one group and 3 groups were weighted, to calculate the CS load capacity of per unit area. The degradation of the bio-composite was studied by soaking in PBS - lysozyme solution, whose concentration (25 mg/l) was chosen corresponding to the concentration in human serum (20.40 ± 2.70 mg/l)16. The CS degradation amount was measured by weighing method. The samples were kept in the solution at 37°C for various time periods up to 28 days. Per 2 or 3 days the PBS - lysozyme solution was replaced by fresh solution. At the end of each time point, the samples were taken out, rinsed with distilled water, and vacuum dried at 60°C to constant weight.

In vitro BMP-2 release study

To evaluate the release kinetics of BMP-2 immobilized on BMP-2/CS/HA/TiO2 and BMP-2/HA/TiO2, the two group of samples were incubated in 5 ml of PBS and PBS-lysozyme (25 mg/l) solution at 37°C. At the time intervals of 2, 4, 8, 12, 15, 18, 22, 25 and 28 days, the solution was collected and replaced with fresh solution. The concentration of BMP-2 released to the collected extractions was detected using a mouse BMP-2 ELISA kit (EK0311, Boster, China). The total amount of BMP-2 released over a certain period and the total loaded amount were accumulated, and then an accumulative release rate was expressed.

Bioactivity evaluation

Bioactivity of HA/TiO2 and BMP-2/CS/HA/TiO2 was evaluated by investigating their influence on HA formation after soaking in a 1.5 SBF solution. A standard 1.5 SBF solution consisted of the following analysis-grade chemical reagents in deionized water: 9.8206 g/l NaCl, 3.4020 g/l NaHCO3, 0.5596 g/l KCl, 0.402 g/l Na2HPO4·7H2O, 0.4576 g/l MgCl2·6H2O, 0.552 g/l CaCl2·2H2O, 0.1066 g/l Na2SO4 and 9.0856 g/l Tris. The PH of 1.5 SBF solution was adjusted to 7.4. HA/TiO2 and BMP-2/CS/HA/TiO2 were immersed in the concentrated SBF at 37°C for 14 days with refreshing per 2 days.

Antibacterial activity evaluation

The antibacterial activity of the samples was evaluated by the optical technique and bacterial counting method. The microorganism of E. coli was used during the evaluation due to that it is one of the major causes of all infections. The samples (3 pieces for each sample) with total coating area of 2.4 cm2 were immersed in 20 ml nutrient solution that containing E.coli at a concentration of 10⁸ CFU/ml and incubated at 37°C for 24 h. For the optical technique, the samples were incubated in bacteria suspension for 40 h, and then the viable bacteria in the suspension was evaluated by ultraviolet spectrophotometry. While for bacterial counting method, after incubated for 24 h, 50 μl sonicated solutions were diluted 10⁴ by pure nutrient culture solution, and then 100 μl sonicated solutions were inoculated onto a standard agar culture medium. After incubation at 37°C for 24 h, the active bacteria were counted in accordance with the National Standard of China GB/T4789.2 protocol.

Biocompatibility evaluation

The mouse osteoblastic cell line MC3T3-E1 was purchased from Cell Resource Center (IBMS, CAMS/PUMC, China). The cells were seeded on the coating surfaces at a density of 7×10⁴ cells/cm², and then cultured in alpha minimal essential medium (α-MEM) supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C. After cultured for 1, 4 and 7 days, the cell viability and proliferation were determined utilizing Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) according to the protocol.

The cells were also fixed with 2.5% glutaraldehyde (Nanjing institute of biological engineering, China) at 4°C for 12 h, and then dehydrated by successive immersion in 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95% and 100% ethanol, each for 15 min and each gradient dehydrated twice. The cells were finally dried in hexamethyl-disilazane (HMDS) for 15 min, and observed by SEM.

Statistical analysis

Experiments were run in triplicate per sample. Standard deviations are plotted as error bars for the data points on all figures. Statistically significant differences were assessed by SAS. Difference with p values ≤ 0.05 was considered to be significant.
Figure 1. XRD patterns of different composites (a) and surface morphology of HA/TiO$_2$ (b), BMP-2/HA/TiO$_2$ (c), CS/HA/TiO$_2$ (d) and BMP-2/CS/HA/TiO$_2$ (e).

Figure 2. Cross-section and the magnification of HA/TiO$_2$ (a) and BMP-2/CS/HA/TiO$_2$ (b).
Figure 3. Nick curves, the scratch’s microscopy and magnification of HA/TiO$_2$ (a, b) and BMP-2/CS/HA/TiO$_2$ (c, d) marked with A, B, C.
Results

Structure of BMP-2/CS/HA/TiO\(_2\) composite coatings

Fig. 1a showed the XRD patterns of different coating surfaces. In the spectrum of BMP-2 immobilized MAO sample, peaks at around 26°, 32°, 34°, 47° and 50° respectively represent the lattice planes of (002), (211), (300), (222) and (213) in HA, peaks at 25° and 53° represent the existence of TiO\(_2\) phase\(^{17,18}\), illustrating the successful synthesis of the HA/TiO\(_2\) composite coating on Ti surface. For BMP-2/CS loaded MAO surface, excepted for the HA and TiO\(_2\) peaks, there also appeared the peaks of CS at around 11.5°\(^{19}\), suggesting that CS was successfully loaded on the MAO coatings by dip-coating method. However, the peaks of HA became a little weaker after loading BMP-2/CS, which may result from a coverage effect of BMP-2/CS on HA layer. The peaks representing BMP-2 were not obvious in both of the spectra, because the amount is too small to be detected. Fig.1b-e showed the morphology of the different coating surfaces. For the HA/TiO\(_2\) surface, petal sheets with a density of about 25-40 petals/100 \(\mu\)m\(^2\) were observed (in Fig. 1b).

| Sample          | Ti  | O    | Ca   | P    | C    | N   |
|-----------------|-----|------|------|------|------|-----|
| HA/TiO\(_2\)   | 2.47| 65.15| 18.40| 11.13| 2.86 | 0   |
| BMP-2/HA/TiO\(_2\) | 1.84| 65.38| 18.57| 11.20| 3.01 | 0   |
| CS/HA/TiO\(_2\) | 2.06| 60.49| 17.02| 10.01| 8.68 | 1.74|
| BMP-2/CS/HA/TiO\(_2\) | 1.73| 61.31| 16.91| 9.82 | 8.54 | 1.69|

Figure 4. Three-dimensional (3D) topography and the roughness of HA/TiO\(_2\) (a), BMP-2/HA/TiO\(_2\) (b), CS/HA/TiO\(_2\) (c) and BMP-2/CS/HA/TiO\(_2\) (d).

Figure 5. Contact angles of HA/TiO\(_2\) (a), BMP-2/HA/TiO\(_2\) (b), CS/HA/TiO\(_2\) (c) and BMP-2/CS/HA/TiO\(_2\) (d).
The petal sheet was 4-5 μm in length, 2-3 μm in width, and the average space between petal sheets was about 2-3 μm. It was proved that the petal sheets were HA formed during the MAO process. There were also some micro pores in HA/TiO₂ surface, which were the results of electric breakdown. There was little difference in surface morphology between BMP-2/HA/TiO₂ and HA/TiO₂, indicating that trace BMP-2 had no influence on the original morphology (Fig. 1c). It can be seen in Fig. 1d and e that CS and BMP-2/CS leaked into the gaps of the petals and the oxide layer, however the surface still retained the original porous and petaling topography. The elemental compositions of different samples were investigated by EDS spectra and summarized in Table 1. It can be found that Ca and P were detected in MAO treated surface and the Ca/P value (atomic ratio) was about 1.73, closed to that of HA (1.67), confirming that the petals were made up of HA. The C element existing in
the MAO coatings was due to the replacement of \( \text{PO}_4^{3-} \) by \( \text{CO}_3^{2-} \) from CO\(_2\) atmosphere during MAO treatment, which was in good accordance with the literature\(^3\). After loading BMP-2/CS and CS, the new element of N appeared in the composite coatings and the content of C increased. Considering that C and N are the main elements in CS molecules, the EDS results indicated that CS was successfully loaded on the oxide layer by dip-coating method. The surface composition of BMP-2/CS/HA/TiO\(_2\) was similar to that of CS/HA/TiO\(_2\), and the composition of BMP-2/HA/TiO\(_2\) was similar to the original HA/TiO\(_2\) surface, suggesting that trace BMP-2 did not affect the chemical composition of the coatings. The Ca/P value (atomic ratio) of the obtained coatings was similar to the Ca/P value of HA, indicating the formation of HA during MAO. The EDS results were in good agreement with the XRD spectra.

Fig. 2a illustrated that after MAO treatment, HA coating was about 26 \( \mu \text{m} \) in thickness and vertically oriented and randomly distributed on the multi-pore coating surface with a thickness of 14 \( \mu \text{m} \). And it was clear that the HA/TiO\(_2\) had a relatively loose structure and there was a certain porosity in it, after loading BMP-2/CS, the entire layer became tight, the porosity decreased and some gaps disappeared (Fig. 2b), suggesting that the BMP-2/CS solution can leak into the space of petals, making the petal coating denser.

**Bonding strength between the composite coatings**

The adhesive force of the HA coating on Ti surface of HA/TiO\(_2\) and BMP-2/CS/HA/TiO\(_2\) samples were evaluated by scratch testing and SEM observation, and the results were shown in Fig. 3. Fig. 3a and c showed the scratch testing curves, Fig. 3b and d showed the morphology of the scratch. The morphology and high magnification images indicated the trace that diamond indenter moved over through the composite layers. As the acoustic and frictional signals fluctuate, it is determined to be the location of the critical load. First, the load and friction were low when the acoustic signals were steady. During the process that diamond indenter moved over the petal layers, the coating became dense, thinner and deformed gradually, as seen in Fig. 3b. When the load was increased, at where the scratch trace reached 1.4 and 1.8 mm, the petal layers were peeled away from the porous layer and meanwhile an obvious change in the friction and acoustic signals appeared. At this moment, the critical loads followed by the bonding strength between the petal layer and porous layer were clear. As shown in Fig. 3a and c, the bonding strength was recorded as 18.9 N and 23.2 N respectively, indicating that the bonding strength between the HA and TiO\(_2\) was improved after loading BMP-2/CS on surface.

**Surface roughness**

Fig. 4 showed the surface roughness and 3D morphology of the corresponding samples. The surface roughness of BMP-2/CS/HA/TiO\(_2\) and CS/HA/TiO\(_2\) surfaces both ranged in 0.60 - 0.65 \( \mu \text{m} \), smaller than that of HA/TiO\(_2\) and BMP-2/HA/TiO\(_2\), whose roughness was around 0.77 \( \mu \text{m} \). As there were still many pores and spaces between petals which were not covered by CS, the latter coating surfaces still kept a high degree of roughness.

**Contact angle**

Hydrophilicity is also an important factor which can affect cells adhesion, spreading and proliferation on the surface\(^20\). Fig. 5 showed the water contact angles of the corresponding samples. It could be observed that on BMP-2/HA/TiO\(_2\) and HA/TiO\(_2\) surfaces, water contact angle was about 17°. BMP-2/CS/HA/TiO\(_2\) and CS/HA/TiO\(_2\) showed decreased water contact angles of about 22°, suggesting good wettability.

**Degradation of the CS containing composite coatings**

Fig. 6a-d showed the morphology of BMP-2/CS/HA/TiO\(_2\) composite layer degraded in PBS-lysozyme solution for different days. It can be seen that after 14 days of degradation, the amount of CS in petal gaps decreased, the petal contours of BMP-2/CS/HA/TiO\(_2\) was deepened and the exposed HA petals was increased. After 22 days of degradation, there were little CS residues in the gap between HA petals, the contour of HA petals became clearer, and the surface morphology was similar to that of HA/TiO\(_2\). After 28 days of degradation, CS disappeared completely from HA petals and the surface has no difference with that of HA/TiO\(_2\). The CS amount loaded on the petal-like structured surface, which was calculated by weighing method, was about 3.3 ± 0.4 mg/cm\(^2\). The degradation rate was shown in Fig. 6e. It was found that CS degradation could last as long as for 4 weeks in a human environment-like environment.

**In vitro BMP-2 release study**

The release rate of BMP-2 from two types of BMP-2-immobilized surfaces was compared and found to exhibit different release behaviors (Fig. 7). Direct immobilization of BMP-2 on HA/TiO\(_2\) surfaces (BMP-2/HA/TiO\(_2\)) showed burst release during the initial stage (about 95% of the BMP-2 was released within 2 days). BMP-2 amount loaded on BMP-2/HA/TiO\(_2\) was about 63.83±9.15 ng/cm\(^2\). While, the BMP-2 amount on BMP-2/CS/HA/TiO\(_2\) was much larger, about 112.06±16.74 ng/cm\(^2\). For BMP-2/CS/HA/TiO\(_2\), BMP-2 is encapsulated in CS coatings, exhibiting sustained release with a biphasic release pattern, characterized by a fast release rate at the first week, and then followed by a slow release rate in the next 3 weeks. During the first week, the amount of BMP-2 released from BMP-2/CS/HA/TiO\(_2\) was approximately 50% in PBS-lysozyme solution and 40% in PBS solution. After two more weeks of soaking in PBS-lysozyme, 80% of BMP-2 was released, while soaking in PBS solution, 80% of BMP-2 was released after three more weeks. The results showed that BMP-2 in BMP-2/CS/HA/TiO\(_2\) can be released for 4 weeks long in a human-like environment and it could last even longer in PBS solution.
Figure 8. SEM images of HA/TiO$_2$ (a) (b), BMP-2/CS/ HA/TiO$_2$ (c) (d) and XRD spectra of the both samples before and after immersed in 1.5 SBF for 14 days (e).

Table 2. EDS profiles of different samples after immersion in 1.5 SBF for 14 days (At. %)

| Sample          | C  | N  | O   | Ca   | P   |
|-----------------|----|----|-----|------|-----|
| HA/TiO$_2$      | 4.16 | 0  | 58.35 | 23.35 | 14.24 |
| BMP-2/CS/ HA/TiO$_2$ | 2.67 | 0.64 | 61.53 | 22.12 | 13.04 |
Bioactivity evaluation

Fig. 8a-d presented the surface morphology of HA/TiO₂ and BMP-2/CS/HA/TiO₂ after immersion in 1.5 SBF for 2 weeks. It can be seen that new substance grew entirely on both of the surfaces. The new layers were proved to be bone-like apatite (Fig. 8e). These results showed that the BMP-2/CS/HA/TiO₂ had the ability to induce apatite formation in SBF solution, showing good bioactivity. EDS analysis (Table 2.) also confirmed that the Ca/P value was close to 1.67, which indicated that HA was mineralized on the coating surfaces. After immersion, the contents of C and N reduced and the peak of CS in XRD pattern became weak and widen, suggesting a degradation of CS during immersion process.

Antibacterial activity evaluation

The antibacterial effect was evaluated by bacterial counting method and optical technique, as shown in Fig. 9. It can be seen that the percent reduction of E. coli seeded on CS/HA/TiO₂ was approximately 70% after 24 h incubation (Fig. 9c) and the calculated antibacterial rate of per unit area was about 30%, indicating the strong antibacterial activity. In contrast, the growth of E. coli was scarcely inhibited on HA/TiO₂ coating (Fig. 9a and b). The antibacterial effect of CS/HA/TiO₂ was also confirmed by the optical technique. The absorbance value of the vertical
axis represented the number of bacterial colonies. The absorbance value of HA/TiO2 coating was the highest. With the introduction of CS, the absorbance value decreased, suggesting the improved antibacterial ability. The results of the two experiments were consistent.

Biocompatibility evaluation

Cell proliferation on the samples was assessed by CCK-8, and the results were shown in Fig. 10. The higher OD value means a higher cell proliferation on sample surface. During the experimental period, the cell number on all sample surfaces increased. Obviously, the cells proliferated much faster on the BMP-2 immobilized coatings than on those without BMP-2, suggesting a beneficial effect of BMP-2 on cell behaviors. However, cells proliferation on BMP-2/HA/TiO2 surfaces was greater than those on BMP-2/CS/HA/TiO2 surfaces after culturing for 1 and 4 days, while lower after 7 days culture, which might be due to the sustained released of BMP-2 from BMP-2/CS/HA/TiO2. It is worth pointing out that there was no significant difference in MC3T3-E1 cells adhesion and proliferation on both CS/HA/TiO2 and HA/TiO2 coating specimens over the experiment time, indicating that CS had no adverse effect on MC3T3-E1 cells proliferation. The cell culture experiment proved that, BMP-2/CS/HA/TiO2 was most beneficial to cells proliferation promotion performance.

Discussion

An idea implant should have good bioactivity, biocompatibility and antibacterial property, it also should maintain stable when used in vivo. In the present study, to achieve the idea implant material, the bioactive HA, biocompatible BMP-2 and antibacterial CS were combined together to construct the BMP-2/CS/HA/TiO2 composite coating on Ti surface. The HA/TiO2 composite coating was firstly synthesized by MAO on Ti surface. During the process of MAO, oxidation and the electric breakdown phenomenon initially occurred on Ti surface, which lead to the formation of a porous TiO2 layer, and then with the rising of voltage and oxidation time, chemical reactions take place to form a petal-structured HA layer in the electrolyte solution containing calcium phosphate salts. The formation mechanism of the HA/TiO2 composite coating has been described in detail in the literature3). The micron-sized pores and HA can accelerate the formation of bone-like apatite in SBF, and facilitate the growth and proliferation of cells on the material surface18, 19). After immobilization of the BMP-2-encapsulated CS coating on the HA/TiO2 surface by dip-coating method, the antibacterial property can be endowed, meanwhile, the biological property will be further improved, in good agreement with the literature. In addition, the introduction of BMP-2/CS onto the surface can also enhance the bonding strength between HA petals and porous Ti substrate. The improved bonding strength is due to that CS is a polymer material with a degree of elastici-
ty and viscosity, which can help the petal-like HA layer resist deformation and magnify the binding area. While the contents of BMP-2 were too little to affect the bonding strength between coatings.

After soaking in SBF for 2 weeks, a large amount of apatite was formed on the surface of the BMP-2/CS/HTiO2 composite coating, confirming the good bioactivity. This finding was attributed to that the surface still remains large area of HA exposed outside. And, it was also widely reported that CS itself has good bioactivity. N atom in CS molecule is a nucleophilic reagent with two unpaired electrons that can absorb cations, thus effectively promote the deposition of apatite in SBF. Moreover, a large number of -OH and -CONH2 groups in CS have a strong interaction with Ca2+ and the protonated -NH3+ in CS can also induce PO43- depositing on the surface and consequently locate Ca2+, leading to the formation of HA crystal nucleus on CS film. CCK-8 result and SEM images showed that MC3T3-E1 cells adhered well on all surfaces, with pseudofoot sticking out, and the introduce of BMP-2 significantly promotes the proliferation and adhesion of MC3T3-E1 cells on the surface. The loading of CS has no negative effect on the original surface morphology and biological property of HA/TiO2, which may be related to the fact that the BMP-2/CS/HA/TiO2 surface still maintains good wettability, suitable surface roughness and large exposed area of HA, which are all important factors that can facilitate the growth and proliferation of the cells on surface.

CS can not only endow the coating with good antibacterial property but also controls the release rate of BMP-2 and the BMP-2 loading capacity on Ti surface. The antibacterial mechanism of CS is due to the positive charge on CS surface, which can easily adsorb the negatively charged E. coli to its surface, and then form a dense polymer membrane on the cell surface, preventing the transport of nutrients to cells and the discharge of physiological metabolic wastes, causing the metabolic disorders of bacteria and affecting the growth and reproduction of bacteria. Except for contact mode, CS can also play an antibacterial role by degrading antibacterial ions, that is -NH3+, which was got by dissolving in an acid solution. CS benefits the control of loading amount and release rate of BMP-2, that is because the CS is a polymer material which can form covalent bond with BMP-2, so that BMP-2 can only be released gradually from the composite layer with CS degradation.

In conclusion, the results implied that BMP-2/CS/HA/TiO2 had a more favorable comprehensive performance than the other samples, including good bioactivity, biocompatibility and antibacterial property, which made BMP-2/CS/HA/TiO2 promising to be applied in hard tissues replacement.

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Conflict of Interest

The authors have declared that no COI exists.

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