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Sequential release of vancomycin and BMP-2 from chitosan/nano-hydroxyapatite thermosensitive hydrogel for the treatment of chronic osteomyelitis

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Abstract

In this study, we developed scaffolds materials with microspheres to form a double sustained release system. Chitosan/nano-hydroxyapatite (CS-HA) was used as a drug carrier to construct a sustained-release system for Bone morphogenetic protein-2(BMP-2) and Vancomycin (VAN). Furthermore, VAN and BMP-2 loaded microspheres (Ms) were prepared by the emulsion ultrasonic method.The resultant composites were characterized by Scanning electron microscope (SEM), compressive strength, porosity, and biodegradation. The characterization results showed uniform porous and rough surface, enhanced thermal stability, and highest compressive strength ((1.912 ± 0.012) Kpa, the surface of the two microspheres was slightly folded and showed a regular spherical shape. The loading rate of BMP-2 was $(59.611 \times 10^{-4} \pm 0.023 \times 10^{-4})$% and the encapsulation rate was (6.022 ± 0.005)%. The release rate of vancomycin and BMP-2 was 57.194% and 12.968% respectively. Osteogenic differentiation of Bone marrow mesenchymal stem cells (BMSCs) was confirmed by alkaline phosphatase quantification. The deposition of late osteogenic markers (calcium phosphates) detected by Alizarin red, which indicated extracellular matrix mineralization. The results showed that BMP-2/VAN in CS-HA hydrogel successfully achieved the sequential release of the double drugs, which could benefit bone regeneration.

Keywords Chitosan, Nano-hydroxyapatite, Hydrogel, VAN, BMP-2

Introduction

Chronic osteomyelitis, mostly caused by Staphylococcus aureus infection can lead to very severe inflammation [1], bone destruction, and callus formation [2]. Existing materials such as Polymethacrylates(PMMA)beads need to be removed surgically, and patients will suffer not only the pain of reoperation but also increased susceptibility to secondary infection [3, 4].

A hydrogel is a network of cross-linked polymer chains that are hydrophilic, which is characterized by softness and flexibility [5]. The conditions for hydrogel formation are relatively mild as such they are analogous to naturally occurring living cells and tissues [6]. Smart materials have been used to build degradable hydrogels, which can be photosensitive [7], temperature-responsive, or pH-responsive [8–10]. Hydrogels can also be used to facilitate targeted delivery [11] and to enable controlled drug release [6]. Hydrogels can be introduced into the body in a fluid state, after which the coagulation or gelation can

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occur in vivo.Hence, hydrogels are favorable to achieve injectable implantation in a minimally invasive manner [12, 13].

As a natural polysaccharide, chitosan has such advantages as antibacterial function, anti-inflammatory property, and degradability. With a hydrophilic surface, it can promote the adhesion, proliferation, and differentiation of cells and enhance bone mineralization, maintaining and attracting liquids and cells to the defect site [14, 15]. Chitosan can be used to prepare microspheres for sustained drug release [16, 17], or smart hydrogels that allows for temperature-responsive drug delivery [18].

Pure chitosan material is not sufficient to provide strength support for bone tissue. Some bone-compatible materials such as silica or hydroxyapatite need to be incorporated to form a composite biomaterial [19]. Hydroxyapatite is a bioceramic material of which the chemical structure and composition match with human bones.because of its good biocompatibility, osteoconductivity, and osteoinductive activity, hydroxyapatite is widely used to build bone scaffolds in tissue engineering [20]. The combination of chitosan and hydroxyapatite has been shown to effectively increase Young's modulus and crystallinity, and significantly improve the adhesion and proliferation of MG-63 osteoblasts [21].

Bone morphogenetic protein-2 (BMP-2) as a growth factor has been widely used to induce bone formation. However, the clinical performance of growth factors is oftentimes limited by its poor stability and short half-life. Therefore, developing a new delivery system that can address these challenges for the controlled release of growth factors represents a promising research direction [22, 23].

Vancomycin is a broad-spectrum antibacterial drug with a good therapeutic effect in treating chronic osteomyelitis [24]. However, a high drug concentration of VAN in serum should be avoided due to its high ototoxicity and nephrotoxicity [25, 26].

The present work reports a more suitable smart gel that can enable a sustained release of vancomycin and BMP-2 for more than 30 days. The slow-release method can deliver the drug in situ, avoiding the rapid degradation of growth factor protein while avoiding the side effects of antibiotics. Moreover, the addition of hydroxyapatite can further increase the mechanical properties of the gel.Providing a promising therapeutic strategy for the treatment of chronic osteomyelitis.

Materials and methods

Materials

Chitosan (CS,90% deacetylation) phosphate-buffered saline (PBS) and was purchased from Shanghai Sangon Biological Co, poly(lactic-co-glycolic acid(PLGA) and β-sodium glycerophosphate(β-GP) were purchased from Shanghai Yuanye Biological Technology Co.,Ltd. Nano-hydroxyapatite(HA)was purchased from Shanghai Lianmai Biological Engineering Co.,Ltd.BMP-2 ELISA kit was purchased from Abcam. Vancomycin and dichloromethane(DMC) were purchased from Shanghai Myrell Chemical Technology Co, Ltd.Bone Morphogenetic Protein-2(BMP-2) was purchased from Perpotech.

Synthesis of CS/HA hydrogel

2 g of CS was dissolved in 100 ml of 0.1 mol/l hydrochloric acid, which was then stirred at room temperature until all were dissolved, and set aside; The HA powder and CS raw material were added into the CS solution at a ratio of 1:2.After ultrasonication for 3 h,500 μl of the β-GP solution was added dropwise to a certain amount of CS/HA solution under low-frequency ultrasound. A small amount of hydrogen phosphate was added after the pH of the sodium saturated was adjusted to the physiological level (PH=7). The final solution was quickly placed in a 37 °C water bath.

Synthesis of microsphere

Chitosan was dissolved in 2% acetic acid to make a 1% chitosan solution. Polyvinyl alcohol(PVA) was dissolved in deionized water to make a 3% PVA solution.9 μg BMP-2 was dissolved in 250 μl double distilled water as the internal water phase. 100 mg VAN was dissolved in 0.5 ml double distilled water while 250 mg PLGA copolymer was dissolved in 1 ml DMC as the oil phase. After that, the mixture of BMP-2 and PLGA was stirred at high speed for 2 min, then quickly dropped into the PVA solution, which was then stirred at high speed for 5 min to form colostrum; the CS solution and PVA solution was mixed at a ratio of 3:2. The stirred colostrum was then dropped into the CS/PVA mixture quickly and uniformly, which was placed on a magnetic stirrer and stirred in an ice bath at 1500r/min for 4 h. The resulting mixture was centrifuged at 12000r/min for 10 min in an ultrahigh-speed centrifuge at 4℃. The supernatant was discarded. The precipitate was collected and washed with refrigerated sterile deionized water for three times. The final product was frozen overnight at -80℃ and then freeze-dried for use.

Characterization of the scaffold and microsphere

The surface morphology of the scaffolds was examined using scanning electron microscopy (SEM). Scaffold samples were dried under vacuum, gold-coated, and examined with SEM(ZEISS Gemini SEM 300,Germany). To observe the internal cross-sectional structure of the microspheres, the microspheres were immersed in resin, embedded, and sliced after solidification, and the samples were observed under a transmission electron microscope(TEM)(Talos L120C, Thermo Fisher

Scientific). particle size is detected by particle size analyzer(Zetasizer Nano ZS laser particle size analyzer, Malvern).

Entrapment efficiency (EE%) and drug loading capacity (DLC%)

The amount of VAN in the microspheres was determined using a UV spectrophotometer(HITACHI Japan). Briefly,10 mg of VAN drug-loaded microspheres was dissolved in 1 ml of 2% acetic acid.After centrifugation at 10000r for 5 min, the supernatant was collected for use. The precipitate was dissolved 1 ml of acetonitrile, which was then centrifuged at 10000r for 5 min. The supernatant was mixed with the acetic acid to form a sample mixture. The mixture of pure acetic acid: acetonitrile=1:1 was used to adjust the zero of the UV spectrophotometer and configure the standard solution. The concentration of the VAN standard solution was 10,20,40,80,160 μg/ml. triplicate sample mixtures were used to determine the sample concentration; The amount of BMP-2 in the microspheres is analyzed using a BMP-2 ELISA kit (Abcam, UK).

$$\mathrm{DLC\%} = \frac{\text{weight of drug contained in microparticles}}{\text{total weight of microparticles}} \times 100\%$$

$$\mathrm{EE\%} = \frac{\text{the amount of drug encapsulated in the particle}}{\text{the total amount of encapsulated and unencapsulated drug in the particle}} \times 100\%$$

Porosity and water absorption of hydrogel

The material porosity was determined by the absolute ethanol substitution method [27]: The material was freeze-dried and cut into small pieces, and then immersed in a graduated cylinder filled with anhydrous ethanol.The volume of anhydrous ethanol is V_1. The material was placed in a vacuum for 5 min to allow for the pore formation. The internal air was evaporated to allow ethanol to enter the pores of the material until no bubbles overflow.At this time, the total volume was counted as V_2. The volume of the remaining absolute ethanol is V_3. The average value from triplicate samples was used.

$$\mathrm{Porosity}(\%) = (V1 - \mathrm{V3})/(V2 - \mathrm{V3}) \times 100\%$$

The water absorption was measured by immersion overnight. The material was freeze-dried and cut into small pieces. The weight was recorded as W_1,and it was immersed in deionized water for 24 h.The sample was taken out and the surface water was absorbed by filter paper. The weight was recorded as W_2. The average value from triplicate samples was used.

$$\text{Water absorption rate } (\%) = (W2 - \mathrm{W1})/W1 \times 100\%$$

Mechanical property test

The hydrogel liquid was injected into the mold and placed at 37 °C to form a hydrogel cylinder with a diameter of 7 mm. The sample was compressed to 60% of the original height by the texture analyzer(TAXT plus, UK) with a constant displacement rate of the plunger of 1 mm/S. The compressive strength obtained from the peak value is calculated by the formula P=mg/S.The average value from triplicate samples was used [28].

In vitro drug release and hydrogel degradation

To evaluate the in vitro VAN and BMP-2 release from the microspheres,10 mg of VAN microspheres and 20 mg BMP-2 microspheres were dispersed in 5 ml of the hydrogel, which was then incubated with 20 ml PBS (containing 10000U/l lysozyme) on a constant temperature shaker (ZWY111-C Benchtop Constant Temperature Shaker, China) at 30 rpm and 37 ° C. After shaking for 31days, the hydrogels before and after shaking were frozen overnight at -80℃,and weighed after lyophilization in vacuum to draw the degradation curve.

Release test of VAN: The hydrogel containing VAN microspheres were soaked in 20 ml PBS (containing 10000U/l lysozyme) for 31 days. 200 μl of the solution was diluted in the tube by 10 times and centrifuged at 2000r for 50s, after which the supernatant was collected for UV detection. Based on the UV absorbance, a standard curve line was drawn to determine the content. Then 200μL of sterile PBS containing lysozyme was injected into the tube for continued shaking.

Release test of BMP-2: The hydrogel containing BMP-2 microspheres was shaken in 20 ml PBS (containing 10000U/l lysozyme) for 31 days. 100 μl of the sample solution was supplemented with 100 μl of sterile lysozyme PBS.The amount of BMP-2 in the microspheres was analyzed using a BMP-2 ELISA kit (Abcam, UK).

Cell culture studies

Cell culture

To test the biological response to our hydrogels, Rat BMSCs were purchased from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences(Shanghai, China). The cells were cultured α-Minimum Essential Medium (MEM, GIBCO, USA) containing 1% penicillin-streptomycin and 10% fetal bovine serum (FBS, GIBCO, USA), placed in a 37℃,5% CO_2 incubator, and the medium was changed every 2 days.

Co-culture of cells and hydrogel

The BMSC cells cultured to the fourth generation were digested with 0.25% trypsin and spread into the lower chamber of the transwell plate at a density of 3×10^5/ml; the cells were divided into 3 groups: A: blank control group; B: blank microsphere hydrogel group; C.drug-loaded microsphere hydrogel group. After the cells adhered to the well, each group was treated as the following: the medium of Group A was replaced with BMSC cell osteogenic induction medium, after which the normal culture was continued; the medium of Group B was replaced with BMSC cell osteogenic induction medium, after which the transwell chamber was put into the plate.0.2 g of blank microsphere gel was added into the chamber together with a 1 ml medium; the medium of group C was replaced with BMSC cell osteogenic induction medium, after which the transwell chamber was put into the plate.0.2 g of drug-loaded microsphere gel was added into the chamber together with a 1 ml medium; afterward, the medium was changed every 3 days, and the microsphere gel in the transwell chamber was also replaced.

Cell viability

The extract of hydrogels was prepared following the sample preparation principles of ISO10993-12:2012. BMSC cells were seeded at a density of 5×10^3 cells/ml on 96-wells. After the cells adhered to the well, they were divided into three groups: A. Control group B.Blank microsphere hydrogel group C. Drug-loaded microsphere hydrogel group. The medium was replaced with hydrogel extract and the culture was continued for 24,48,72,96 h. Cell Counting Kit-8(CCK-8) solution (10%) was added to each well and incubated for 2 h, and optical densities (OD) were determined at 450 nm using a microplate reader (SpectraMax® i3X Platform, Dyna Max Biotech).

Alkaline phosphatase (ALP) activity

The cells were cultured for 7 and 14 days, after which the medium was removed, washed twice with PBS, and homogenized on ice with a hand-held electric homogenizer, centrifuge at 4000 g for 10 min at 4 °C, collect the supernatant into an EP tube, The ALP activities were determined by ALP detection kit(LEAGUE, China).

Alizarin red staining

Calcium deposition was stained with Alizarin Red to measure osteoblast differentiation. The BMSCs group culture on the 14th day was used to detect the degree of mineralization of cells and the medium was changed once in 2 days. The cultured cells were washed twice with PBS and fixed with 95% ethanol for 30 min. After the fixation, the 95% ethanol was discarded. After a little drying,0.3 ml of the prepared Alizarin Red S staining solution was added to each well for a 15 min stain.After the staining solution was removed, cells were rinsed with distilled water three times [29]. After conventional dehydration, the slides were mounted and observed under a light microscope.

Statistical analysis

All experimental data were obtained from triplicate samples, which were analyzed using GraphPad Prism 8 statistical software, and the results were expressed as mean±standard deviation (SD). The comparison between groups was performed by t-test, and $P<0.05$ was considered a significant difference.

Results

Hydrogel fabrication and characterization of scaffolds

The results of various indicators of three groups of materials showed that the addition of HA could greatly increase the compressive strength of the hydrogel, the porosity and water absorption to a certain extent. After the drug-loaded microspheres were added, a further slight improvement was observed in regarding to the porosity, water absorption and gel time of the hydrogel. Meanwhile, all groups have shown thermal stability. Once the hydrogel formed, it would not turn into a fluid again (Fig. 1).

SEM observation and porosity determination

The lyophilized hydrogels under SEM showed that the three groups of gels had different structures (Fig. 2A, B and C). Compared with the blank chitosan group (Fig. 2A), the surface of the hydrogel containing HA was much rougher, which could provide more adherent points to support cell growth, and their pores were also relatively uniform and sufficient (Fig. 2D). Notably, the pores of the hydrogel containing drug-loaded particles were more uniform and dense, presumably because the microspheres could provide fulcrums for the gel formation. Drug-loaded microspheres were locked in the sheet (Fig. 2C) and the surface of the frame was rough, suitable for cell adhesion and growth.

Microsphere morphology and structure

The microstructure of the studied drug-loaded microspheres was investigated using SEM imaging (Fig. 3A and B) and TEM imaging (Fig. 3C and D), it could be found that the VAN and BMP-2 microspheres were round in shape and have some small wrinkles on the surface. Through TEM, it can be found that the edges of the two microspheres are regular and clear. There are several small spheres inside, forming a ball-in-ball structure. There is no obvious difference in the shape of the two microspheres.

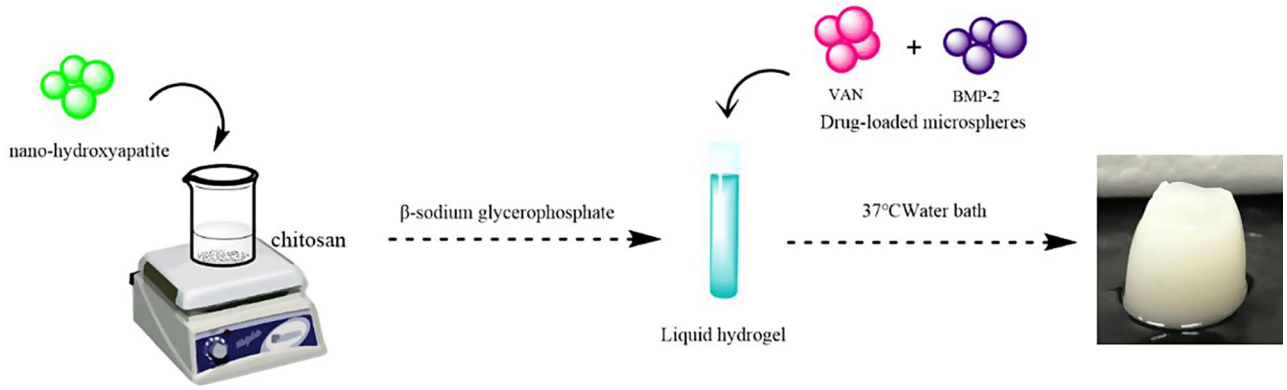

Fig. 1 Gel preparation process and result display

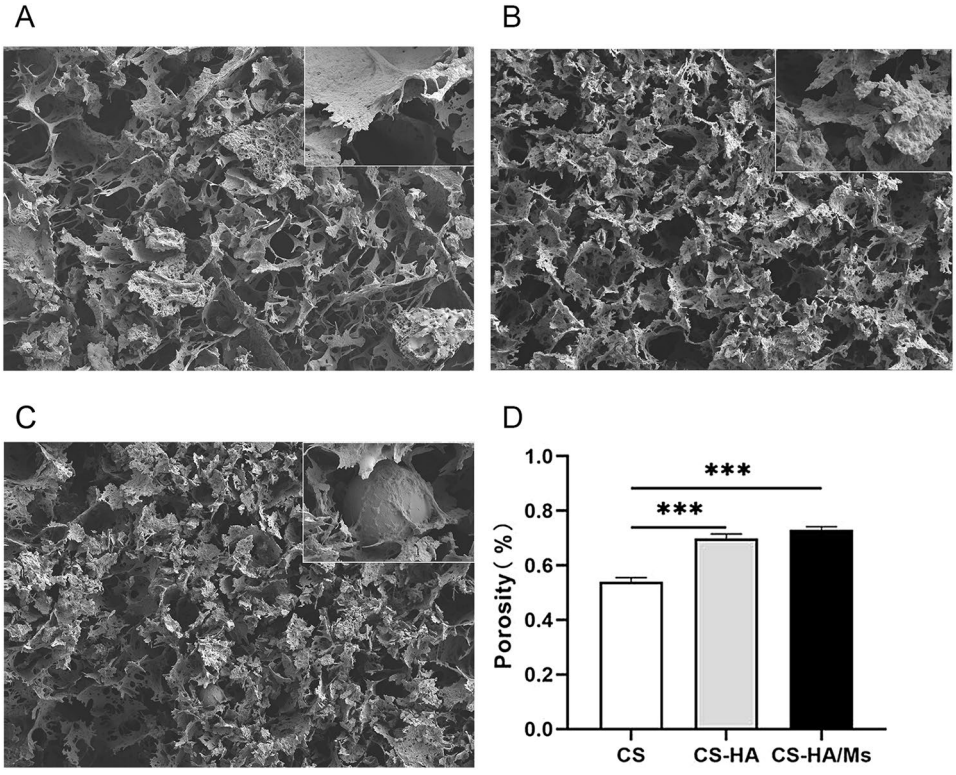

Fig. 2 Scanning electron microscopy images of hydrogels. (**A**) CS, (**B**)CS-HA, (**C**)CS-HA/Ms, and magnified image is in the top right corner.(**D**) Porosity analysis.The data were presented as mean ± S.D, $n = 3$.The CS-HA and CS-HA/Ms compared with the CS, ***$P < 0.001$

Mechanical property and water absorption of hydrogel

According to the results of the mechanical properties (Fig. 4A) and water absorption (Fig. 4B) test: the mechanical strength values are: 1.781±0.017, 1.898±0.013 and 1.912±0.012 (kPa) respectively. After adding HA, the strength can be increased by (6.59±0.62)%. After adding HA and Ms, the strength can be increased by (7.36±0.59)%;the water absorption rates are: 561.716±26.448, 827.184±32.883 and 968.329±44.041(%) respectively.

Addition of HA remarkably enhances the mechanical properties and water absorption of the gel. Additionally, adding Ms on this basis can also bring a slight improvement.

Drug encapsulation rate

The average mass of drug contained in the microspheres was 12.20 mg, and the loading rate of PLGA/CS-VAN composite drug-loaded microspheres was (1.20±0.01)% and the encapsulation rate was (12.20±0.57)%. The standard curve of VAN was obtained by UV spectrophotometric detection and fitted by linear fitting.The fitted formula was y=ax+b (a=0.0032,b=0.02),$R^2 = 0.99$.

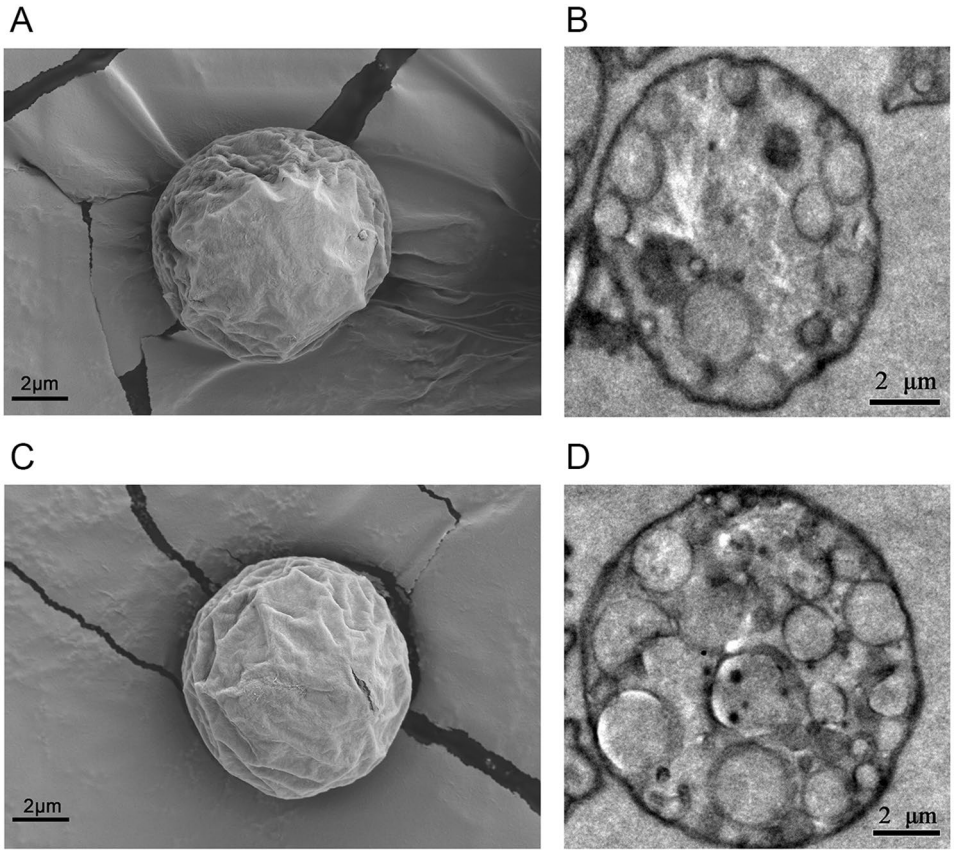

Fig. 3 Scanning electron microscopy images showing the VAN microspheres and BMP-2 microspheres (**A** and **B**); Transmission electron microscope internal images showing the VAN microspheres and BMP-2 microspheres(**C** and **D**)

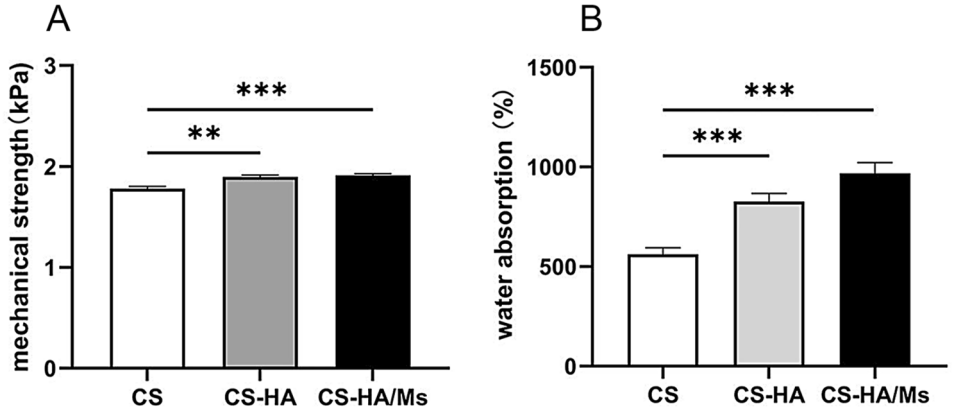

Fig. 4 (**A**) Mechanical strength of different scaffolds; (**B**) water absorption of different scaffolds

The original input of BMP-2 was 9 g. The average mass of drug contained in the microspheres was 0.54 g, and the drug loading rate of PLGA/CS-BMP-2 composite drug-loaded microspheres was $(59.6010^{-4}\pm0.0210^{-4})\%$ and the encapsulation rate was (6.02±0.01)%. The BMP-2 standard curve was obtained by ELISA and fitted by Sigmoidal Models. The fitting formula was y=a/ (1+b*exp(-cx)) (a=5255.06,b=31.63,c=6.59),R^2=0.99.

Release of VAN and BMP-2 in vitro and hydrogel biodegradation

According to the release curve (Fig. 5A and B) and the degradation curve of VAN and BMP-2,it can be seen that the drug release continued to rise. Due to the enveloping effect of hydrogels on drug-loaded microspheres, the release curve showed a uniform growth without obvious burst release. While the recovery rate of VAN reached 57.104%,the recovery rate of BMP-2 was only 13.570%. It

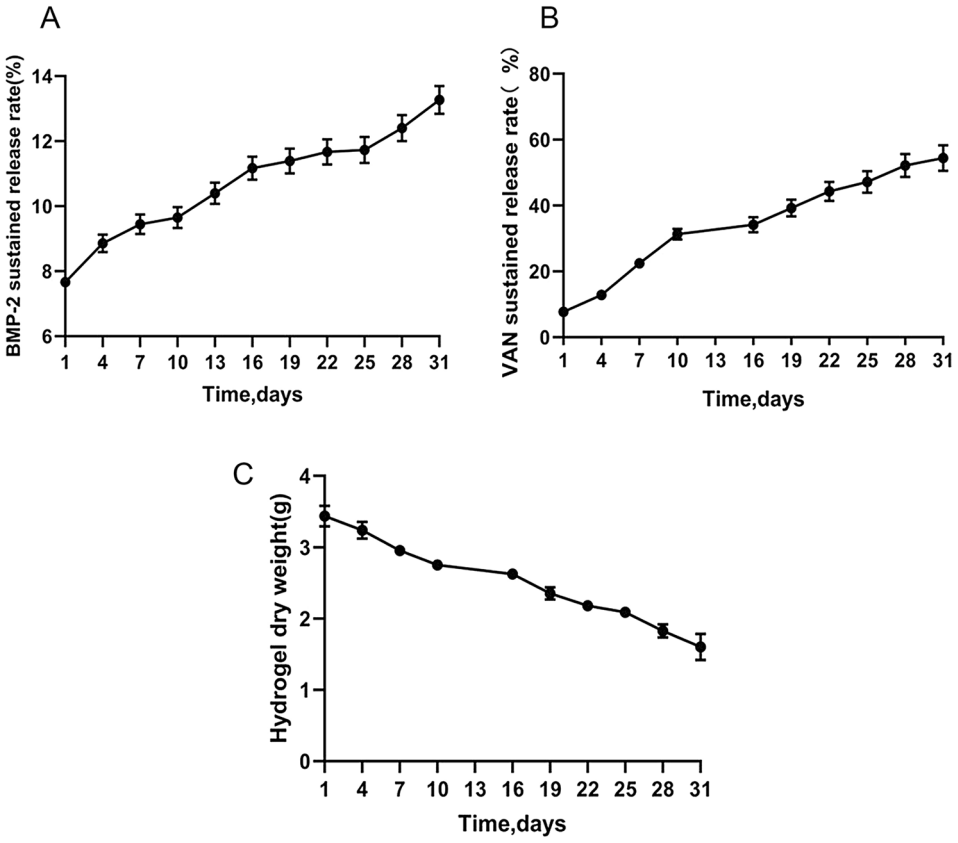

Fig. 5 Slow-release rate of drug-loaded microspheres and degradation of hydrogel.(**A**) BMP-2 sustained release for 31 days; (**B**) VAN sustained release for 31 days; (**C**) Hydrogel biodegradation

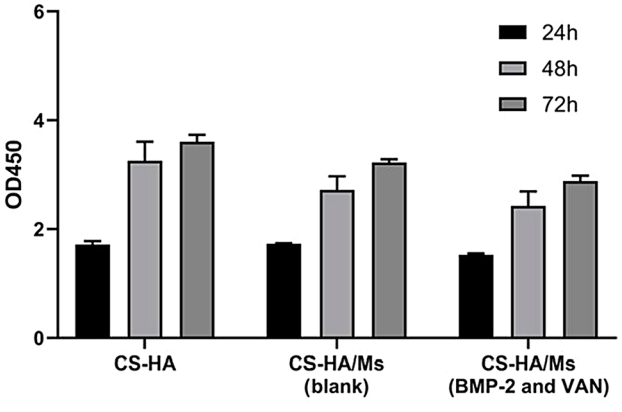

Fig. 6 The cell viability of BMSCs cultured on different scaffolds at each time point

is speculated that BMP-2,as a growth factor protein can be easily degraded. On the one hand, the detection time is as long as 31 days; on the other hand, the liquid containing lysozyme at 37 °C accelerated the degradation of BMP-2. Therefore, the recovery rate of BMP-2 is much lower than that of VAN as a compound.The composite gel is uniformly degraded within 31 days while a degradation rate of 55.051% (Fig. 5C).

Cytotoxicity of hydrogel

The analysis of CCK-8 revealed (Fig. 6) that both the CS-HA/Ms and the CS-HA/Ms (blank) inhibited cell proliferation at 48 h, mainly because the degradation of polymer PLGA led to the production acidic substances that mignt cause inflammatory reactions.Compared with the CS-HA, microspheres displayed inhibitory effect upon cell proliferation, which decreased with the time extending. Compared with the CS-HA/Ms(blank), the encapsulated drug did not cause obvious inhibition of cell proliferation, and it will promote the effect in a short time (24 h), and there is no big difference in a long time (72 h).

ALP activity

According to the results of ALP test (Fig. 7),the amount of ALP produced in the CS-HA/Ms(blank) and the CS-HA/Ms was higher than that of the control group at 7 days, while the difference between the blank group and the drug-loaded group was not significant ($P>0.05$). On the 14th day, the ALP of CS-HA/Ms was significantly higher than that of the other two groups, while the growth rate of the unloaded group was lower. This is probably because the acidic substances generated by

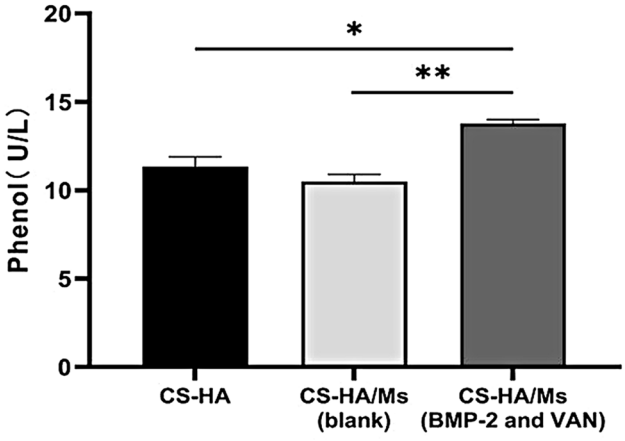

Fig. 7 Alkaline phosphatase (ALP) activity of BMSCs in different treatment groups on the 14th days after seeding

the degraded PLGA in the blank microspheres over time affect its induced cell activity.

Alizarin red staining to detect cell mineralization

The mineralization of the extracellular matrix (ECM) on the 14th day post to the co-cultivation with the hydrogel is shown in Fig. 8. Calcium deposits were positively stained in red color. The extracellular matrix (ECM) mineralization on the 14th day after co-cultivation with hydrogel (Fig. 8). Calcium deposition was positive red. After 14 days of incubation, the size of the red sediment area indicates the degree of ECM mineralization. CS-HA/Ms (Fig. 8C) has a significant effect on promoting calcium deposition.Compared with CS-HA/Ms (blank) (Fig. 8B) and CS -HA (Fig. 8A). Thus CS-HA/Ms could significantly promote the differentiation and mineralization BMSCs.

Discussion

The selection of high polymers such as PLGA and PVA combined with chitosan to prepare drug-loaded particles is mainly due to their ability to prolong the sustained-release time and good spheronization ability. During the particle preparation process, it often occurs that the weight of freeze-dried powder is very large, but the drug loading content is very small. Finally, microscopic observation reveals that there are a large number of crystalline substances in the powder, which is determined to be caused by incomplete dissolution or precipitation of high polymers. PLGA is extremely volatile when dissolved in dichloromethane, and the PVA solution is too viscous and not suitable for filtration. Therefore, the liquid preparation process should be continuously shaken under sealed conditions and stabilized overnight to avoid the situation where although there is no visible precipitation to the naked eye, there are fine crystals. Only in this way can it be ensured that the prepared microspheres are regular spheres with stable drug loading rate and encapsulation efficiency.

Thermosensitive gel materials are widely studied tissue engineering materials nowadays due to their advantages of degradability, drug loading capacity, and strong compatibility. The thermosensitive gel formed by CS/β-GP in this article is mainly due to the transfer of protons on the amino groups of chitosan molecules when the temperature is higher than the critical temperature, reducing the electrostatic repulsion between molecules. At the same time, the water molecules combined with it are captured by glycerol hydroxyl groups, leading to the aggregation of hydrophobic groups and the formation of intramolecular hydrogen bonds, thereby realizing gelation. This kind of gel material still has great defects in loading strength that need to be compensated. However, increasing the concentration of materials will reduce biocompatibility. Although adding bioceramics can enhance some mechanical strength, as seen in this experiment, its effect is still limited. In the future, cross-linking agents with lower toxicity or more stable cross-linking methods should be explored, such as involving bioceramics in the cross-linking process, to balance strength and biocompatibility.

Conclusion

In the multi-angle detection within 31 days, the hydrogel system could successfully encapsulate the drug-containing particles and effectively deliver growth factors and antibiotics. The degradation rate of hydrogels reached 55%,resulting in a desirable drug recovery rate. Moreover, the porous structure of composite materials in hydrogels could provide stress support to bone regeneration. The gel-forming speed was controlled at about two

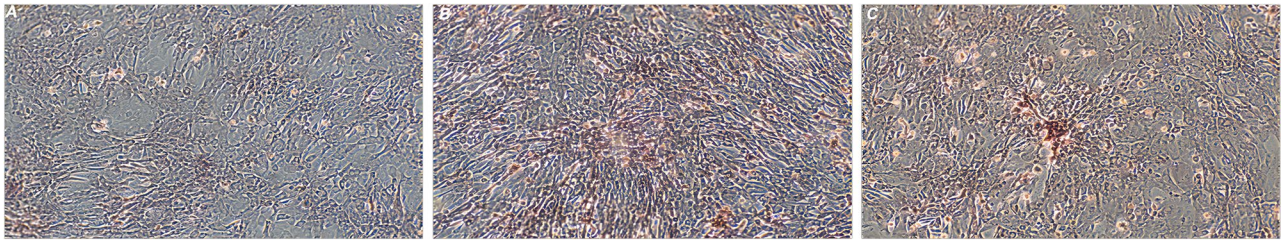

Fig. 8 Alizarin red stained images of (**A**) CS-HA; (**B**) CS-HA/Ms(blank); (**C**) CS-HA/Ms

minutes.The hydrogel showed thermal stability and did not reverse back to liquid again. It enabled the sustained release of antibiotics and growth factors for more than 30 days. The total period of sustained release is expected to reach 50 days. Over a treatment window of 72 h, the hydrogel system did not show any phenomenal negative effect on the cell viability. Further research will be carried out on this basis.

Author contributions
HYY contributed to experimental design, manipulation, data collection and analysis. She also drafted the manuscript and reviewed and edited the article. WYQ was involved in experimental operation, data processing and article writing. ZYL contributed to experimental design and supervision, and also reviewed and edited the article.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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