Effect of Pore Size on the Physicochemical Properties and Osteogenesis of Ti6Al4V Porous Scaffolds with Bionic Structure

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ABSTRACT: Ti6Al4V is widely used in implants in the fields of orthopedics and dentistry due to its high compressive strength and good biocompatibility. Nevertheless, Ti6Al4V has a certain degree of biological inertness and the elastic modulus of Ti6Al4V is much higher than the cortex and trabecular bone. In this study, we designed and printed a new type of pore size Ti6Al4V with like-trabecular structure scaffold (the pore size is 800/900/1000 μm, named P8/P9/P10, respectively) with electron beam melting (EBM). Its elastic modulus, compressive strength, and other physical and chemical properties, as well as cell adhesion, proliferation, and differentiation ability and in vitro biological properties were studied. The physical and chemical performance test results showed that as the pore size increased, the surface wettability increased and the elastic modulus decreased. As the pore size increased, F-actin and alkaline phosphatase (ALP) increased significantly, and osteogenesis-related genes including BMP2, OCN, RUNX2, and ALP were upregulated significantly. The reason may be that the components on the Ti6Al4V pore size may have an influence on intracellular signal conversion and then change the mode of cell proliferation and diffusion. In summary, the like-trabecular porous structure can effectively reduce the elastic modulus of metal materials, thereby avoiding stress concentration and promoting the adhesion and proliferation of osteoblasts. Porous materials with larger pores are more conducive to the proliferation and differentiation of osteoblasts. The irregular porous Ti6Al4V scaffold prepared by the EBM technology has good mechanical properties and the potential to promote adhesion, proliferation, and differentiation of osteoblasts, and has the possibility of application in the field of implantation.

1. INTRODUCTION

Titanium and its alloys are widely used in implants in the fields of orthopedics and dentistry due to their high compressive strength and good biocompatibility.1,2 Especially, Ti6Al4V is the first choice for repairing bone defects in clinics.3 However, Ti6Al4V has a certain degree of biological inertness, and the elastic modulus of Ti6Al4V is much higher than the cortex and trabecular bone.4 Moreover, the elastic modulus of Ti6Al4V (100–140 GPa) is much higher than cortex and trabecular bone (1–30 GPa).5−7 When the elastic modulus of the implant and bone tissue is different, the stress transmission between the two is not uniform, which is called stress shielding.8,9 In this case, the bone tissue around the implant will shrink and cause the implant to loosen or fracture.10 How to improve the osseointegration ability of the scaffold and reduce the elastic modulus of the scaffold simply and quickly has become the key and difficult point in material manufacturing.

To improve the binding ability of Ti6Al4V scaffold with bone tissue, surface modification is mainly used at present. Surface modification methods are mainly divided into chemical modification, biological modification, and physical modification. Chemical modification can add a layer of Ti oxide on the surface of Ti6Al4V, which can improve the bone-bonding ability of Ti6Al4V implant.11,12 However, the oxide coating is very thin and easy to peel off from the metal substrate, and the coating treatment time is long, so it is difficult to apply and promote in clinical practice.13 Biological modification involves particle release and other issues, which reduces the survival rate and long-term use of clinical implants.14 In recent years, the preparation of microstructural materials by physical modification has gradually shown significant advantages. The titanium alloy scaffold is changed from a dense structure to a porous interconnected structure, and the osseointegration ability of the scaffold is enhanced by changing the porosity and pore size, thereby guiding bone tissue to grow into the scaffold.15−17

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The mechanical properties including elastic modulus and yield stress are related to the porosity of porous materials.\textsuperscript{18,19} Hence, applying porous structures could be the solution to reduce the modulus difference between titanium alloy scaffold and human bones. Heinl constructed a porous scaffold with Ti6Al4V and found that when the pore size is 1.23 mm and the porosity is 81.1\%, the elastic modulus is only 1.6 GPa. When the pore size is 0.45 mm and the porosity is 59.5\%, its elastic modulus is only 12.9 GPa.\textsuperscript{20} Li prepared three porous materials with a pore size of about 0.6 mm using the same raw materials. The study found that when the pore size is 0.65 mm, the elastic modulus is 25.9 GPa, and when the pore size is 0.5 mm, the elastic modulus is 14.5 GPa, which is close to the elastic modulus of the adult cortical bone.\textsuperscript{21} The above research results indicated that the porous structure has a lower elastic modulus than the solid structure titanium alloy.

It has been reported that the porous structure with high porosity can not only avoid the stress shielding effect but also improve the bone ingrowth effect by introducing sub-millimeter-sized pores.\textsuperscript{22} Moreover, when the metal scaffold pore shape is similar to the trabecular bone, specifically an irregular three-dimensional structure, it can improve the mechanical properties of the material and facilitate the proliferation of osteoblasts.\textsuperscript{23} However, only a few studies reported the role of pore size in the osteogenic effect of porous structure under constant porosity.\textsuperscript{15} Especially in the case of constant high porosity, the effect of pore size on the osteogenic effect of porous scaffolds with bone-like trabecular structure has not been reported.

In this study, we designed and printed a new type of pore size Ti6Al4V with like-trabecular structure scaffold (the pore size is 800/900/1000 μm, named P8/P9/P10, respectively) with electron beam melting (EBM). Its elastic modulus, compressive strength, and other physical and chemical properties, as well as cell adhesion, proliferation, and differentiation ability and in vitro biological properties were studied. The purpose of this study was to explore the effect of pore size on the physical and chemical properties and biological properties of irregular porous Ti6Al4V scaffolds with low elastic modulus.

\section*{2. RESULTS AND DISCUSSION}

\subsection*{2.1. Surface Characterization}

\subsection*{2.1.1. Surface Morphology}

Figure 1 shows the like-trabecular porous Ti6Al4V scaffolds with different pore sizes. Scanning electron microscopy (SEM) was used to observe the surface morphology and local characteristics of Ti6Al4V scaffolds. On the surface of the titanium alloy scaffolds precisely printed by EBM, the complete interconnection structure can be seen clearly and there was no obvious sintering powder residue (Figure 2). In this study, three scaffolds with different pore sizes were printed layer-by-layer using the EBM technology. The entire printing process was controllable, repeatable, and accurate.\textsuperscript{24} The irregular pore size sample designed by this research was different from the traditional regular pore structure in three-dimensional space. In fact, it was an interconnected porous structure. This structure was similar to the natural bone trabecula structure of human body, and its geometric structure and mechanical properties were similar to the bone trabecula, which was bionic.\textsuperscript{25} Compared with the regular structure, this structure is conducive to cell adhesion and proliferation on its surface and improves the osseointegration ability of the sample.\textsuperscript{26,27}

The objectivity of experimental research lies in whether the shape and specification of materials are similar to those of design and whether the composition of internal chemical elements is similar to that of standards. In this study, EBM was used for printing. At present, the main manufacturing technologies of biomaterials are selective laser melting (SLM) and EBM. Compared with selective laser melting (SLM), the EBM process may lead to steps, resulting in higher surface roughness on dense solid samples.\textsuperscript{28} For biomimetic implants, the surface roughness will affect the adhesion performance of bone cells, and the number of adhesion cells would increase with the increase of surface roughness.\textsuperscript{29} On the other hand, in terms of mechanical properties, rough surfaces can cause stress concentration, thus reducing the fatigue life of porous materials manufactured by the additive manufacturing (AM) process.\textsuperscript{30} The adhesion and mechanical properties of bone cells need to be considered.

After printing, SEM was used for photographing. The surface area, volume, and porosity of the samples were analyzed and measured. Previous studies have shown that when performing three-dimensional (3D) printing, it is very easy to cause residual metal powder to adhere to the scaffold, which leads to a larger error and an increase in the surface area and volume of the sample compared to the design. Meanwhile, the residual powder surface of the scaffold is more likely to cause cell adhesion, which also easily affects the objectivity of in vitro experiments.\textsuperscript{31,32}

\subsection*{2.1.2. Surface Phase Composition and Chemical Composition}

The X-ray diffraction (XRD) patterns (Figure 3A) showed the characteristic peaks of Ti in Ti6Al4V. Figure 3B shows the typical Ti6Al4V chemical elements in the scaffolds, with obvious Ti, Al, and V peaks, and no other element peaks. The weight percentage of Ti, Al, and V elements was also close to that of standard Ti6Al4V alloy, indicating that no contamination of other elements occurred in the preparation process.

\subsection*{2.1.3. Contact Angle Test}

Contact angle values are the indicative of porous metal scaffolds wettability, in which wettable surfaces are often referred to the scaffolds having contact angle less than 90°. The average water contact angle for Ti6Al4V scaffolds gradually decreases with the increase of the pore size (Figure 4). The largest contact angle is P8 (62.3°), and the smallest is P10 (43.5°). It has been reported that the cell activity of any substance is a function of wettability. Wettability is the property of a material that affects the adsorption of protein on its surface, and the protein adsorption method depends on the surface wettability of the material. Therefore, the wettability of the material surface will affect the cell activity adhered to its surface. As the pore size of the scaffolds increases, the wettability increases and its surface cell activity may also increase.\textsuperscript{33,34}
2.2. Mechanical Property Test. In this study, the static compression test results of three kinds of scaffolds with different pore sizes were measured and analyzed, including the calculated elastic modulus and the representative stress–strain curve. Elastic modulus is a key parameter to evaluate the mechanical properties of porous biomaterials. As the pore size increases, the Ti6Al4V elastic modulus gradually decreases. In the Ti6Al4V scaffolds, the elastic modulus of P8 was the largest, which was $19.17 \pm 0.21$ GPa, and the elastic moduli of P9 and P10 were $13.53 \pm 0.12$ and $10.43 \pm 0.12$ GPa, respectively. The elastic modulus of Ti6Al4V with different pore sizes were all close to the cortical bone ($\sim 17$ GPa). P10 was closest to the elastic modulus ($\sim 4$ GPa) of cancellous bone. Therefore, we can speculate that the mechanical properties of the titanium alloy metal scaffolds designed in this study are conducive to the uniform distribution of stress around the implant, especially P10. The elastic modulus of P10 is very close to that of cancellous bone, which can prevent the phenomenon of “stress shielding”.

The stress–strain curve is a typical curve of porous biomaterials, including the linear increase of stress with strain and the stationary region of stress fluctuation (Figure 5). In addition, it is observed that the initial approximate parabola upward trend is observed at the beginning of loading. This may be due to the uneven contact interface between the indenter and the porous sample or the slight deformation of the scaffolds. As shown in Figure 5, in these scaffolds, the compressive strength of P8 was also the largest, which was $333.35 \pm 7.04$ MPa, and the compressive strengths of P9 and P10 were $235.32 \pm 9.99$ and $115.43 \pm 5.38$ MPa in this order. The relevant value of compressive strength in this study is slightly higher than that of natural bone (130–180 MPa). The results showed that, because the prefabricated sample can not only reduce the stress shielding effect but also provide the appropriate yield strength for the implant, avoiding permanent shape changes under physiological load, its mechanical properties were suitable for the repair of bone tissue defects. Although the quasi-static mechanical properties of Ti6Al4V scaffolds were close to the stiffness and strength of natural bone, the compatibility of compression mechanics and strain rate sensitivity within the physiological strain rate range under dynamic conditions still need to be studied in depth.

2.3. In Vitro Cell Experiment. During in vitro cell culture, cell behavior was affected by the surface properties of titanium alloy scaffolds, including the surface composition, pore size, and roughness of metal scaffolds. Cell loading on the surface of a material typically undergoes three consecutive stages: adhesion, proliferation, and differentiation. As shown in Figure 5, in these scaffolds, the compressive strength of P8 was also the largest, which was $333.35 \pm 7.04$ MPa, and the compressive strengths of P9 and P10 were $235.32 \pm 9.99$ and $115.43 \pm 5.38$ MPa in this order. The relevant value of compressive strength in this study is slightly higher than that of natural bone (130–180 MPa). The results showed that, because the prefabricated sample can not only reduce the stress shielding effect but also provide the appropriate yield strength for the implant, avoiding permanent shape changes under physiological load, its mechanical properties were suitable for the repair of bone tissue defects. Although the quasi-static mechanical properties of Ti6Al4V scaffolds were close to the stiffness and strength of natural bone, the compatibility of compression mechanics and strain rate sensitivity within the physiological strain rate range under dynamic conditions still need to be studied in depth.
and proliferation are closely related to the biocompatibility of the material. The relationship between cell adhesion and proliferation on the titanium alloy scaffold and the pore size of the scaffold is very complicated. In this study, cell adhesion, proliferation, and differentiation were divided into three parts to discuss.

2.3.1. Cell Viability. Bone marrow stromal cells (BMSCs) were loaded on Ti6Al4V samples, cultured in vitro for 3 and 7 days, respectively (Figure 6), and then cell’s LIVE/DEAD viability was detected. The results showed that most of the cells began to proliferate and diffuse, the adherent cells showed fusiform appearance, and a few of the suspension cells showed round point appearance. The number of BMSCs increased with the increase of culture time, which indicated that all kinds of titanium alloy scaffolds had good biocompatibility. With the increase of titanium alloy scaffold pore size, cell proliferation gradually increased (Figure 6), which was basically consistent with the conclusions drawn by Kapat, Liang, and Ran et al. The reason may be that as the pore size increased, it was more conducive to the transportation of oxygen and nutrients, and provided a good external environment for cell adhesion. However, Ita and Takahashi believed that the pore size does not affect cell adhesion but results in increased cell proliferation, because cell adhesion is only related to metal surface components and roughness.41,42

2.3.2. Cell Morphology. In Figure 7A,B, the morphology of the adherent BMSCs cells is visualized using phalloidin and DAPI staining, and the superimposed image of double staining is depicted, in which F-actin was red and the nucleus was blue. We can see the different shape and adhesion states of cells on the surface of scaffolds with different pore sizes. With the increase of culture time, no obvious contact inhibition was observed. The cells were firmly attached to the scaffolds and filled a part of the scaffolds.

In Figure 7A, after cells were cultured on the scaffold for 7 days, P10 actin fibers and nuclei were more than P8 and P9, indicating that P10 had more cell adhesion and stronger cell viability than P8 and P9. It was shown that with the increase of pore size, the adhesion of cells increases and the connection between cells becomes tighter. Meanwhile, the actin fiber on the 7th day was formed into one piece, which is denser than the fiber on the 3rd day. It was shown that on the Ti6Al4V scaffolds with the same pore size, the fiber bundle aggregation was more obvious, the fiber mesh was denser, and the cell viability was better with time. The reason may be that the components on the Ti6Al4V pore size may have an influence on intracellular signal conversion and then change the mode of cell proliferation and diffusion. This leads to an increase in actin filaments, and actin mediates various transmembrane signal transduction, activating the cascade response, differentiation, and mineralization involved in osteoblast proliferation.43

A typical image of the cells on the sample surface is shown in Figure 7B. On the 3rd day, the cells adhering to the surface of the sample were mainly spherical, with a small number of pseudopodia extending short and small. On the 7th day, the pseudopodia gradually grew, some even became one piece, and most cell adhesion was found on P10. Figure 7B showed that the cells mainly adhere to the metal particles on the surface of the scaffold. These particles are the residues produced by incomplete melting of the Ti6Al4V powder after EBM printing. The rough surface could support cell attachment and proliferation at the early stage through the provided physical binding sites for serum proteins.44,45 Therefore, the biocompatible surface characteristics of the scaffold determined that both types of samples have an acceptable surface property for cytoocompatibility.41,46

2.3.3. Cell Proliferation. As shown in Figure 8, the BMSCs loaded on the Ti6Al4V scaffolds showed an increasing trend

![Figure 6](image1.png)

**Figure 6.** Observation of cell viability on the surface of Ti6Al4V scaffold. Fluorescence micrographs representing the live (green) cells and dead (red) cells of osteoblasts cultured on Ti6Al4V alloy after 3 and 7 days.

![Figure 7](image2.png)

**Figure 7.** Observation of cell morphology on the surface of Ti6Al4V scaffold. (A) Confocal results of cells adhered on porous Ti6Al4V scaffolds after 3 and 7 days. (B) SEM micrographs to observe the adhesion of cells on the Ti6Al4V scaffolds after 3 and 7 days. The green arrow refers to the BMSCs adhering to the growth of the samples.
with the increase of time, and the number of cell proliferation was highest on the 7th day. From a single-day observation, as the pore size of the titanium alloy scaffolds increases, cell proliferation gradually increases. The number of cell proliferation was the highest in P10 on the 7th day. The number of cell proliferation in P10 was significantly higher than that in P9 (p < 0.05). The results were also consistent with the results reported by Sollazzo and Li. In this study, with the increase of pore size, the proliferation of osteoblasts increased. This view has been recognized by a large number of scholars at home and abroad. Sollazzo believed that the proliferation of osteoblasts was highly dependent on the pore size of the sample. If the pore size of the sample exceeded 300 μm, the cell proliferation rate increased with the increase of the pore size. Kapat believed that, due to adequate nutrition and oxygen supplementation, small pore size was conducive to cell adhesion and differentiation, while large pore size was conducive to cell proliferation. The reason may be that, as the pore size increased, the permeability coefficient of the porous scaffold also increased and the entire scaffold formed a highly interconnected structure, which avoided the obturator pore and was more suitable for cell maintenance and proliferation.

2.3.4. Osteogenic Gene Expression. BMP2, OCN, Runx2, and ALP are all key genes extracted from BMSCs RNA that can induce stem cells to differentiate into osteoblasts and participate in bone growth and development. Figure 9 shows that the stimulation of OCN and ALP in BMSCs is relatively high, especially ALP. All gene expressions were upregulated over time. In the Ti6Al4V sample, as the pore size increases, the expression of ALP also upregulated gradually. On the 7th and 14th days, the expression level of P10 in the ALP group was significantly higher than that in other pore size groups, and there was statistical difference (p < 0.05).

The results obtained in this study were also consistent with the results of Kapat. On the metal scaffolds with 297, 178, and 92 μm scaffolds, the detection of expression level of the col1 gene closely related to osteogenesis and showed that $S_{297} > S_{178} > S_{92}$. Kapat believed that the reason is that the larger the pore size of the metal scaffold, the denser the cells and the more contact between the cells, leading to more extracellular matrix secretion. Direct contact between cells may directly enhance osteoblast differentiation. Due to the large pore size, the distance between cells is increased so that the cells can be stretched as necessary to promote their own differentiation. Therefore, as the pore size increased, the gene expression level associated with osteogenesis upregulated significantly.

2.3.5. ALP Assay. The ALP activity of BMSC protein extracts on both metal scaffolds (shown in Figure 10) gradually increased from the 7th to the 14th day. The activity of ALP increases with the increase of pore size. On the 7th and 14th days, the ALP activity of P10 surface cells was significantly higher than that of other groups (p < 0.05), indicating that the P10 surface had good cytocompatibility and the ability to promote osteogenic differentiation. The reason may be that a larger pore size can provide more oxygen and nutrients for cells. The larger the pore size, the greater the surface tension of cells and greater the mechanical stimulation, thus enhancing the osteogenic differentiation of cells. More scholars have pointed out that the differentiation of osteoblasts was accompanied by cell proliferation.

3. CONCLUSIONS

In this study, irregular porous Ti6Al4V scaffolds with different pore sizes were constructed and successfully fabricated by the EBM technology. As a typical AM technology, the EBM technology has shown great potential in the preparation of irregular porous scaffolds. However, it also has some shortcomings, such as the accuracy of printing, unsintered metal powder residue, and so on. Ti6Al4V scaffolds with pore.
sizes of 800, 900, and 1000 μm all have satisfactory mechanical properties and good biocompatibility. Moreover, this study also found that as the pore size of the porous metal scaffold increased, the elastic modulus decreased. The elastic modulus of the porous Ti6Al4V scaffold with a pore size of 1000 μm was closer to that of human bone tissue, which can more effectively avoid the stress shielding effect. Preliminary in vitro experiments showed that as the pore size increased, the permeability coefficient increased, resulting in more nutrients and oxygen entering, which was more conducive to the adhesion, proliferation, and differentiation of osteoblasts.

In summary, this study showed that the irregular porous Ti6Al4V scaffold prepared by the EBM technology has good mechanical properties and the potential to promote adhesion, proliferation, and differentiation of osteoblasts. In our future work, we will further study the in vivo biocompatibility and bone formation mechanism of this irregular porous scaffold.

4. MATERIALS AND METHODS

4.1. Porous Titanium Alloy Samples Design and Manufacturing. Within the Medica software (Autodesk, CA), Trabecular algorithm plug-in was used for the design of irregular and disordered porous models. The model was set by Boolean operation with the Medica software. After setting the diameter of the ball (800, 900, and 1000 μm, respectively) and the thickness of the rod (300 μm), the ball was randomly put into our model. The center of the triangle formed by the center of the three adjacent balls was taken as the node, and the nodes were connected by the rods. The rest of the gap would be filled by a smaller or larger diameter sphere (Figure 11A). The diameter of the set ball was defined as the pore size of the scaffold (800, 900, 1000 μm) (Figure 11B).

The data of layered irregular porous samples were imported into printer equipment for printing. EBM (ARCAM A1, Sweden) was used to print Ti6Al4V powder (Institute of Metals, Chinese Academy of Sciences). The powder is spherical metal powder, and the particle size range is 45–100 μm. High-energy electron beam sintering powder according to the path of layered data planning, through layer-by-layer stacking, of the porous samples consistent with the design model was prepared. The thickness of the pore wall was determined by the diameter of the electron beam spot, and the minimum wall thickness was 300 μm. The surface was polished using the SiC paper with a particle size range of 10–57 μm and then polished using grinding and polishing equipment (Saphir 360, ATM, Mammelzen, Germany). The polished samples were then ultrasonically washed with acetone, deionized water, and absolute alcohol. After washing, the samples were dried in a 50 °C oven.

4.2. Surface Characterization. 4.2.1. Surface Morphology. The surface morphology and chemical composition of the prepared samples were characterized by field emission scanning electron microscope (SEM, Nexsa, Thermo Fisher Scientific).

4.2.2. Surface Phase Composition and Chemical Composition. The X-ray diffraction (XRD) patterns of the Ti6Al4V samples were determined using a glancing angle X-ray diffractometer (GAXRD; D/max2400, Rigaku, Japan) employing a Cu Kα radiation source with an accelerating voltage of 40 kV and a current of 250 mA.

4.2.3. Contact Angle Test. The wettability of the sample surface was measured by the sessile drop method. After setting the contact angle measuring instrument (DSA30, Kruss, Germany) program, the water in the syringe on the surface tension tester was dripped on the sample surface, and the image was captured after stabilization. The angle between the
BMSCs were inoculated into these plates with 1 pore sizes were placed in 24-well plates, respectively. Then, dark at 37 °C for 25 min. After incubation, the samples were washed three times with PBS for 5 min each time and then dehydrated with ethanol (30, 50, 70, 90, 95, 100%). After dehydration, the samples were dried, and then the compression stress was measured as a compression offset stress of 0.2%. The compressive stress is also recorded and measured.

4.4. In Vitro Cell Test. 4.4.1. Cell Culture and Seeding. Bone marrow stromal cells (BMSCs) were isolated from the femurs of 3-week-old male Sprague Dawley rats (Animal Experiment Center, Sun Yat-sen University, China) and subsequently cultured in DMEM-F12 (Gibco, Gaithersburg, MD) with 10% fetal bovine serum in an incubator with an atmosphere of 5% CO2 at 37 °C. After 48 h, unattached cells were rinsed away, and fresh culture medium was added. After that, the culture medium was changed every 2–3 days. When the cell coverage area reached 80–90% of the dish, the cells were subcultured.

4.4.2. Cell Viability. The samples of Ti6Al4V with different pore sizes were placed in 24-well plates, respectively. Then, BMSCs were inoculated into these plates with 1 × 10⁵ cells/well for 1 and 3 days, respectively, and the cell viability was measured by the LIVE/DEAD viability/cytotoxicity kit (calcein AM/PI, bestbio, China). The samples were washed two to three times with phosphate-buffered saline (PBS) and then placed in the prepared calcein AM and incubated in the dark at 37 °C for 25 min. After incubation, the samples were washed two to three times with PBS again. Images were captured by a laser scanning confocal microscope (LSM780, Zeiss, Germany). The viable cells (in green) and nonviable cells (in red) could be easily distinguished under the laser scanning confocal microscope.

4.4.3. Cell Morphology. BMSCs at a density of 5 × 10⁴ cells/mL were seeded on the materials placed in 24-well plates and incubated, as described in the preceding paragraph. After culturing for 1 and 3 days, the medium was removed, and then the scaffolds were fixed in 4% paraformaldehyde at 37 °C. Finally, samples were washed three times with PBS and then placed in the prepared calcein AM and incubated in the dark at 37 °C for 12 h. After fixation, the samples were dehydrated with ethanol (30, 50, 70, 90, 95, 100%). After dehydration, the samples were dried, and then the adhesion and spreading of cells on the sample surface were observed by SEM.

4.4.4. Cell Proliferation. BMSCs were added to a 24-well plate with samples at 5 × 10⁴ cells/well. Each sample was set up with three replicates. After being cultured in complete medium for 1, 3, 5, and 7 days, the culture was terminated and washed with PBS three times. Then, F12 containing 10% Cell Counting Kit-8 (CCK8) reagent (Dojingo, Japan) was added and incubated at 37 °C in the dark for 1 h. Then, the culture medium in the well plate was transferred to a 96-well plate (100 μL/well), six duplicate wells were set up, and the absorbance value of each well was set at 450 nm in a microplate reader (BioTek, Winooski, VT).

4.4.5. Osteogenic Gene Expression. BMSCs were added to a 24-well plate with samples at 1 × 10⁵ cells/well. Each sample was set up with six replicates. After being cultured in osteogenic induction fluid for 7 and 14 days, the culture was terminated and washed with PBS two times. The TRIZol method was used to extract total RNA; the RNA was converted into cDNA by the Prime Script RT Master Mix (Takara, Japan), and the cDNA concentration was adjusted to below 100 ng/μL. The reaction system was prepared in a 96-well plate using the instructions of the Takara kit: add 1 μL of gene-positive primer, 1 μL of gene reverse primer, 2 μL of template cDNA, 8.5 μL of enzyme-free water, and 5 μL of SYBR Green per well. The gene expression levels of alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP2), osteocalcin (OCN), and Runx-related transcription factor 2 (Runx2) were detected. The internal reference was GAPDH. The gene primer design is shown in Table 1.

4.4.6. ALP Assay. Cell inoculation and replicate settings were the same as the gene expression test experiments. The ALP experiment was also cultured with osteogenic induction fluid for 7 and 14 days after termination of culture. After the culture medium was absorbed, the well plates were placed on dry ice, RIPA (Keygen Biotechnology, Nanjing, China) was added for cell lysis, and 1% protease inhibitor mixture (Cwbio, Beijing, China) was added to extract the total protein. Bicinchoninic acid (BCA) protein assay kit (Cwbio) was used to measure the protein concentration.

4.5. Statistical Analysis. OriginPro 2018C software was used to perform statistical analysis on the physical and chemical test data of the material, and GraphPad Prism 8.0 software was used to perform statistical analysis on the biological test data of the material, and three replicates were performed for each experiment. All quantitative results in this study are expressed as mean standard deviation (SD). The statistical significance of the data is a comparative analysis by t test and one-way analysis of variance (ANOVA) for analyzing the difference between groups; p < 0.05 is considered significant.
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Notes

The authors declare no competing financial interest.

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