Effects of Fiber Cross-Angle Structures on the Mechanical Property of 3D Printed Scaffolds and Performance of Seeded MC3T3-E1 Cells

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ABSTRACT: The three-dimensional (3D) printing technology combined with bone tissue engineering has become one of the major methods for mandibular reconstruction. However, the key factor retarding mandible reconstruction is the barrier of understanding and achieving the complex 3D gridwork formed by the trabeculae. This study innovatively constructed a low-temperature 3D printing silk fibroin/collagen/hydroxyapatite (SF/COL/HA) composite scaffold with a stable structure and remarkable biocompatibility. We designed three kinds of six-layer scaffolds with mixed fiber cross-angle structures (FCAS) of [0°/90°/45°/90°/0°/90°], [0°/45°/90°/135°/180°/225°] and [0°/30°/60°/90°/120°/150°]. Material properties of these scaffolds such as porosity, water absorption rate, X-ray diffraction, Fourier transform infrared spectroscopy, and compression performance were detected. Then, the MC3T3-E1 cells were seeded on these scaffolds and the adhesion, proliferation, and differentiation were investigated. To be more convincing, the same experiments were performed on another polycaprolactone/hydroxyapatite scaffold. The results suggested that the changes of FCAS affected the mechanical properties of 3D printed scaffolds and performance of seeded cells. Besides, the 90° FCAS significantly enhanced the compressive modulus in two groups and were more conducive to the cell proliferation and osteogenesis, which provided evidence for exploring the influence of FCAS on the properties of scaffolds and the application of two composite scaffolds in tissue regeneration.

1. INTRODUCTION

The reconstruction of large segmental mandibular defects, caused by trauma, osteomyelitis, or tumor resection, remains a clinical concern. Irreparable defects led to severe maxillofacial deformity and masticatory function loss, which reduced the patients’ quality of life. However, the mandible is the only movable load-bearing bone of the skull that needs to withstand the force in function. It also needs adequate bone mass to support dental implants. This makes the mandibular reconstruction more changeable.1 The ideal solution, which can replace the function and formation of the mandible, has never been found. One promising approach for meeting these clinical needs could be the implementation of a three-dimensional (3D) additive manufacturing customized tissue engineering (TE) scaffold.

The construction of scaffolds for medical implantation was a mainstream application of 3D printing in biomedicine. TE is the combination of materials engineering and life science, which can create artificial structures for the tissue regeneration. Because the human body is a complex biological and sensitive system, the requirements of TE scaffold are extremely manifold and challenging.2 Biomaterials for 3D printing TE scaffold must be available for printability, biocompatibility, sufficient mechanical properties, sterilizability, and degradability.3 A further requirement for a scaffold particularly in bone engineering is a controllable interconnected porosity to allow for the cells to grow into the desired physical form and to support vascularization of the ingrown tissues.4 3D printing-based technologies can offer great precision to control internal architecture of scaffolds and print complicated structures based on the deflection.

Biomaterials for TE can be broadly divided into synthetic and natural materials. The natural materials, such as alginate,5 hyaluronic acid,6 collagen,7,8 fibrin,9 and so on, have excellent biocompatibility and biodegradability. The synthetic polymers, such as polycaprolactone, bioactive glass,10 and polyactic acid (PLA),11 can promote the scaffold stability and stabilize their interaction with tissues. In addition, hydroxyapatite is the main inorganic component of bone and its addition can obviously

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improve the mechanical properties of the material. All these materials can simulate the structures of natural bones and promote bone regeneration, making them to be the ideal scaffolds for bone TE. Early studies pointed out that collagen−HA composite scaffolds supported the osteogenic differentiation of bone marrow stromal cells (BMSCs) both in vitro and in vivo as demonstrated. It was confirmed that apatite-coated silk fibroin scaffolds could be successfully applied to repair mandibular critical size border defects. The collagen−silk fibroin/hydroxyapatite bi-template-induced...
Biomimetic bone substitutes were prepared and found to be able to support the attachment and proliferation of BMSCs better than the single-template materials. In a recent study, a pretreated polycaprolactone/hydroxyapatite (PCL/HA) scaffold was constructed and implanted into the subcritical cranial defect of rats and resulted in excellent osteogenesis. Bioactive synthetic bone implants may contribute to reconstruction and function of bone defects by serving as a scaffold for bone growth, thus promoting the healing process. These previous studies provided considerable cues for our selection of materials.

One clinical challenge of mandible reconstruction maybe the difficulty in understanding and achieving the complex 3D gridwork formed by the trabeculae. Therefore, in addition to choosing suitable scaffold materials, we also need to evaluate the spatial structure of the scaffold. The spatial geometry of the pores in the scaffold constitutes material transport properties, which in turn determine local nutrient supply, endovascular, and host–graft interactions. The change of microstructure affects the mechanical properties of the scaffold. Studies have revealed that the 0°/45°/90° scaffold exhibited the highest tensile strength in a mixed isotropic carbon fiber 3D-printed composite. Therefore, it is necessary to apply the spatial structure design to the construction of bone TE scaffolds. These scaffolds can serve as templates for the formation of bone-like tissue and allow for the controlled engineering of different bone-like structures originated from a single scaffold.

In this study, we aimed to design three kinds of simple, stable, and repeatable structures. We rotated the fiber counterclockwise at 90, 45, 30° and printed six layers because when we multiplied them by a certain integer multiple, it makes 360°, which makes the structure more regular. In this way, we prepared scaffolds with FCASs of [0°/90°/0°/90°/0°/90°], [0°/45°/90°/135°/180°/225°] and [0°/30°/60°/90°/120°/150°]. Two porous bioreabsorbable nanocomposite bone scaffolds including SF/COL/HA and PCL/HA were investigated by low-temperature deposition 3D printing technology for the effect of FCASs on the TE scaffold. X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM) were utilized to characterize the composites. The mechanical properties, proliferation, and osteogenesis properties of MC3T3-E1 cells in scaffolds with mixed FCASs were further investigated.

2. RESULTS
2.1. Establishment of the 3D Printed Scaffolds. From the top views of three-dimensional digital models of composite scaffolds with three kinds of FCASs (Figure 2A), the fiber-
cross angle on model surfaces were uniform and stable (Figure 2B). All these freeze-dried scaffolds were oyster white with a stable structure. The interconnected channels seen on the surface were actually created by a 3D printing technique to provide a pathway for the transport of essential nutrients and metabolic wastes into the central regions of the cell-loaded scaffolds. The shape of pore in 90° scaffold was square while those in 45° and 30° scaffolds were rhombus. These fibers in the 30° scaffold stretched out in more directions, so the surface seemed more denser when stacked (Figure 2C–E).

### 2.2. 3D Printed Scaffolds had Interlinked Microscopic Pores.

The composite scaffolds were examined by SEM to evaluate their ultrastructure. All scaffolds with FCAS had regular structures and interconnected pores. There were many

| Fiber cross angle | Pore shape | Fiber directions | Surface density |
|------------------|------------|-----------------|----------------|
| 90°              | Square     | 4               | Low            |
| 45°              | Rhombus    | 8               | Medium         |
| 30°              | Rhombus    | 12              | High           |

**Table 1.** Parameters of different composite scaffolds.
irregular round micropores on the surface of SF/COL/HA scaffold and many irregular strips micropores on the surface of PCL/HA scaffold. These micropores were conducive to cell attachment and proliferation. On the surfaces of these scaffolds, an integrated network of pores could be seen with high interconnectivity (Figure 3).

Figure 5. Biocompatibility of 3D printed scaffolds. (A) Fluorescence imaging of calcein-AM/PI staining in SF/COL/HA and PCL/HA scaffolds. Green and red fluorescence represent living and dead cells, respectively. (B,C) Cellular state in SF/COL/HA and PCL/HA scaffolds, respectively. (D) SEM images of blank SF/COL/HA scaffold, (E) SEM images of SF/COL/HA scaffold with MC3T3-E1 cells. (F) SEM images of blank PCL/HA scaffold, (G) SEM images of PCL/HA scaffold with MC3T3-E1 cells. The blue arrows are the MC3T3-E1 cells. (**P < 0.001).
2.3. Parameter of 3D Printed Scaffolds met TE Scaffold Requirements. Both kinds of composite scaffolds had suitable porosity and water absorption (Figure 4A). The water absorption performance and porosity in the SF/COL/HA scaffold were better ($p < 0.01$). However, there were no statistical difference in scaffolds with mixed FCAS (Figure 4J). Therefore, subsequent studies on the scaffolds with mixed FCASs could exclude the influence of porosity changes. It could be seen that in the SF/COL/HA and PCL/HA scaffolds, the average diameter of pores was 0.47 and 0.45 mm, respectively. This might be due to the collapse of the SF/COL/HA composite which increased the fiber diameter and decreased the aperture.

2.4. Composition of Composite Materials. The phase composition of composite scaffolds was characterized by XRD. The results suggested that the characteristic diffraction peaks of PCL, HA, SF, and COL were at 21−24°, 31−35°, and 11−17°, respectively, indicating a much higher crystallinity at these places (Figure 4C,F).

The PCL related to stretching modes was observed in PCL/HA scaffolds, including 2946 cm$^{-1}$, 2868 cm$^{-1}$ (CH$\_2$ stretching), 1727 cm$^{-1}$ (carbonyl stretching), and 1293 cm$^{-1}$ (C−O and C−C stretching in the crystalline phase). Typical proteins related to bonds were found at 1652 cm$^{-1}$ (amide I) and 1549 cm$^{-1}$ (amide II), corresponding to C=O stretching vibration, and coupling of bending of N−H bond and stretching of C−N bond. The characteristic absorption peaks of HA were found at 556, 599, 1030, 1163 cm$^{-1}$ (different spatial structures PO$_3^{\_2}$ stretching), 1400−1450, 1631 cm$^{-1}$ (CO$_3^{\_2}$ stretching), and 3547, 3291 cm$^{-1}$ (OH$^-$ stretching), respectively. Collagen and silk fibroin amide I and II were overlapped completely at 1666 and 1519 cm$^{-1}$, forming sharp and narrow absorption peaks. 3069 and 3291 cm$^{-1}$ were characteristic peaks of collagen amide A and B. At 1631 cm$^{-1}$, the β-pleated sheet of silk fibroin was coincided with CO$_3^{\_2}$ stretching of HA (Figure 4D,G).

2.5. 90° FCAS had a Better Mechanical Property. Representative stress−strain curves of the 3D printed scaffolds with mixed FCASs were displayed. The compression stress−strain test results showed that the scaffold materials were viscoelastic. All scaffolds were able to automatically return to their original shapes after a certain range of external forces. Below 10% strain, the figure line was approximately linear, so we chose the slope of the stress−strain curve when the strain value was less than 10%, part of a straight line, as the elastic modulus (Figure 4B,E). It was indicated that the stress−strain changed with the varying FCAS. The elastic modulus of 90, 45, and 30° in the SF/COL/HA scaffold was (39.89 ± 3.09) kPa, (29.7 ± 2.21) kPa, and (12.9 ± 1.44) kPa, respectively (Figure 4H). The elastic modulus of 90, 45, and 30° in the PCL/HA scaffold was (2.0 ± 0.15) MPa, (0.9 ± 0.04) Mpa, and (0.45 ± 0.07) MPa, respectively (Figure 4I). The 90° scaffold showed the maximum elastic modulus and the 30° showed the minimum elastic modulus. The tendency of the results on SF/COL/HA and PCL/HA scaffolds was consistent. Overall, the 90° FCAS significantly increased the compressive modulus compared with the 30 and 45° FCASs in all groups ($p < 0.01$).

2.6. 3D Printed Scaffolds had No Significant Inhibitory or Toxic Effects on MC3T3-E1 Cells. By calcein-AM/PI double staining and cell counting, it was found that both SF/COL/HA and PCL/HA scaffolds had significantly more living cells (green fluorescence) than dead...
Figure 7. Proliferation and osteogenesis properties of MC3T3-E1 cells in mixed fiber angle scaffolds. (A,D) Cell counting kit-8 (CCK-8) assay was used to detect the proliferation activity of MC3TC-E1 cells in SF/COL/HA and PCL/HA scaffolds, respectively. (B,E) ALP assay was used to detect the osteogenesis activity of MC3TC-E1 cells in SF/COL/HA and PCL/HA scaffolds, respectively. (C,F) Expression of proliferation- and osteogenesis-related mRNA in SF/COL/HA and PCL/HA scaffolds, respectively. (**P < 0.01 and ***P < 0.001).

cells (red fluorescence) (p < 0.001) (Figure 5A–C). The results showed that the liquid extract of these scaffolds had no cytotoxicity and could be used for MC3T3-E1 cell culture.

SEM showed that cells were attached to the surface of SF/COL/HA and PCL/HA scaffolds. The cells on both types of scaffolds were spindle-shaped or star-shaped, indicating a normal expansion. The cells showed a large number of filopodia, which were firmly attached to the surfaces of the materials. It facilitated the cells on the scaffold to furtherly spread and contact with each other, forming a thin layer. Some of the cells remained unicellular and scattered, leaving efficient room for further proliferation (Figure 5D–G).

At 14 days after culture, MC3T3-E1 cells were found not only on the surface but also inside the scaffolds. By HE staining, the cells were found to grow into the microporous structure of the scaffolds. Cells adhered to the fibers on the parts of the scaffolds, which left efficient space for the proliferation and the formation of a typical spindle shape. At the turning point, the cells grew more densely on all scaffolds and mixed FCASs (Figure 6). The toughness of the PCL/HA scaffolds was too high to perform the cutting.

2.7. 90° FCAS Promoted the Proliferation of MC3T3-E1 Cells. It could be found that during the seven days, the OD value of each group increased with time. In Figure 7A, the OD value on day 1 in the 90° SF/COL/HA scaffold was lower than that in the 45° and 30° scaffolds (p < 0.01). On day 3, it showed a similar trend with a maximum in the 45° scaffold (p < 0.01). Interestingly, the value in the 90° scaffold was reversed with time and higher than that in the 30° scaffold on day 7 (p < 0.05). A similar trend occurred to the PCL/HA scaffold on day 1. However, the highest value was observed after the cells were cultured in the 90° scaffold for five and seven days (p < 0.01) and the value in the 45° scaffold was higher than that in the 30° scaffold (p < 0.05) (Figure 7D). The results indicated that MC3T3-E1 cells proliferated in all scaffolds had a good condition and the proliferation performance was changed with the varying FCAS.

Real-time polymerase chain reaction (RT-PCR) analysis showed that in the SF/COL/HA scaffold on day 7, the relative expression levels of cell proliferation-related transcription factors (PCNA) in the 90 and 45° scaffolds were higher than those in the 30° scaffold (p < 0.01) (Figure 7C). In the PCL/HA scaffold, the relative expression levels of PCNA in the 90° scaffold was higher than these in the 45 and 30° scaffolds (p < 0.05) (Figure 7F). All in all, the cells showed a stronger proliferation performance in 90° scaffold.

2.8. FCAS Affected Osteogenesis Differentiation Viability of MC3T3-E1 Cell. According to the detection of alkaline phosphatase (ALP) activity, there was no statistical difference in the SF/COL/HA scaffold with mixed FCASs (Figure 7B). However, the PCL/HA scaffold with 90° FCAS exhibited the strongest ALP activity (p < 0.01) (Figure 7E). RT-PCR analysis showed that in the SF/COL/HA scaffold on day 7, the expression levels of ALP and COL-1 in scaffolds with mixed FCASs tended to be consistent, but they had the highest expression levels of OPN in the 90° scaffold and RUNX2 was more highly expressed in the 90 and 30° scaffolds than in the 45° scaffold (p < 0.01) (Figure 7C). In the PCL/HA scaffold on day 7, the expression levels of ALP and COL-1 in the 90° scaffold were the highest (p < 0.01). OPN and RUNX2 were more highly expressed in the 90 and 45° scaffolds than that in the 30° scaffold. These results suggested that the 90° scaffold showed a remarkable osteogenic property.
and the difference was more pronounced in the PCL/HA scaffold (Figure 7F).

3. DISCUSSION

The mandible is a crucial organ in the stomatognathic system with a complex function. When the mandible is reconstructed, it needs to withstand the forces transmitted during mastication. The bone mass and activity in the trabecular bone vary between bones with different functions. As the function is variable in the regions of incisor, premolar, and molar in the mandible and dependent on the state of dentition, a variation in bone structure in the cancellous bone within the single mandible would be expected.15 The elastic properties of cancellous bone could be related to mineral contents as well as to density and architecture.16 Although the precise mechanism of adaptation is uncertain, it is generally believed that the architecture of the trabecular bone corresponds to mechanical loading. S. TENG17 explored trabecular-plate thickness, separation, and trabecular number, showing a significant linear relation between connectivity, trabecular-plate thickness, and bone-volume fraction based on the stereological principle. The structural density and the ratio of trabecular thickness to length or separation were correlated with Young’s modulus and bone strength. These studies provided the inspiration for our experiment.

With advances in three-dimensional manufacturing, TE scaffold can be spatially structured not only to mimic natural organs but also to allow for the transplantation of cells in large quantities. Layered design of the topographic and spatial features of scaffolds was a key step in facilitating TE and cell therapy.18 Each physicochemical characteristic of the biomaterial surface (such as topographical features, stiffness, functional groups, and interfacial free energy) could profoundly affect biochemical mechanisms.19 The potential for nacre topography to be used for regulating osteogenic differentiation and the controlled cell differentiation strategies aided in the translation of cell therapies to the clinic.20 The thick fibers and large pores of electrospun poly markedly were found to enhance cell infiltration and extracellular matrix (ECM) secretion.21 Rapid prototyping of thermoplastic materials such as PCL, PLA, and poly (lactic-co-glycolic acid) using fused deposition modeling (FDM) is an accurate, repeatable, and sterilized method for obtaining 3D models. However, due to the relatively high temperature of the operation, FDM does not support simultaneous printing of cells or temperature-sensitive biomaterials.22 Low-temperature 3D printing can preserve the biological activity of the material. Compared with scaffolds printed by the FDM technology, low-temperature deposition modeling printed sponges enhanced adhesion, proliferation, and migration of MSCs. Further functional analysis suggested that focal adhesion kinase, downstream AKT, and yes-associated protein signaling were involved in the required mechanical transduction pathways, through which the multilayer porous structure stimulated the paracrine effect of MSCs.23 In this study, we innovatively constructed a low-temperature 3D printing SF/COL/HA scaffold. The biological activity of the material protein could be retained by low-temperature 3D printing. Collagen is the main component of organic matter in natural bone tissue and the ECM secreted by osteoblasts during osteogenesis. It is widely used in bone tissue engineering.26 Our research found that, the SF/COL/HA composite scaffold restored good material stability and cell compatibility, and MC3T3-E1 cells exhibited proliferation and osteogenic differentiation on it. It showed a spongy structure with interwoven microcolumns, which was similar to those of natural trabecular bones.

On the basis of the existing traditional 90° scaffold morphology, we innovatively proposed two new scaffold structures. The three kinds of six-layer scaffolds were designed with mixed FCASs of [0°/90°/0°/90°/0°/90°], [0°/45°/90°/135°/180°/225°] and [0°/30°/60°/90°/120°/150°], respectively. By comparing their mechanical properties and the proliferation and osteogenesis properties of MC3T3-E1 cells cultured on them, it was suggested that there was a significantly increased compressive modulus in the 90° fiber structure compared with 30 and 45° scaffolds. The reason might be the change of FCAS in the contact area and method between the adjacent fibers, which could change the overall mechanical properties of the support. Therefore, under the same volume and porosity, three kinds of different mechanical properties scaffolds could be obtained using the same composition. Our experiment confirmed that the performance of scaffold could be changed by the regularly changing of FCAS based on unchanged scaffold materials. This difference can be utilized in the subsequent construction of the complex mandible scaffolds.

The existing studies on changing the FCAS of scaffolds focused more on mechanical properties than on cells14 and the mechanical properties of ECM could influence the properties of cells.24 We designed the scaffolds to further characterize the performance of seeded cells. In this study, when the FCAS was demonstrated to modulate the mechanical properties, the MC3T3-E1 cells were seeded on these scaffolds and the properties of cells were detected by SEM, HE, CCK, ALP, and RT-PCR. In order to make our results more convincing and avoid the contingency of our materials, the three forms of combinations of PCL/HA with higher hardness were selected to repeat the above experiments.25 Both kinds of composite scaffolds had suitable porosity and water absorption, which facilitated medium absorption and infiltration and satisfied the basic characteristics of TE materials.

The structure of scaffolds was treated as a priority for cellular colonization in tissue regeneration, which would induce the interaction of cells with the scaffolds. The initial adhesion efficiency of MC3T3-E1 cells to the 90° scaffold was unsatisfactory. It might be related to the lower surface density and higher pore connectivity in the 90° scaffold. Over time, the cells cultured in the 90° scaffold performed an outstanding proliferation. RT-PCR analysis for osteogenic differentiation-related transcription factors and assay for ALP activity found that in SF/COL/HA scaffolds, the expression of OPN and RUNX2 in the 90° scaffold were outstanding and there were increased COL-1, OPN, and RUNX2 in the 90° PCL/HA scaffold with enhanced ALP activity. This might suggest that FCASs affected cell proliferation and osteogenic properties, and the 90° structure was superior inside. Most results of two composite materials shared the same general trend. Without changing the materials to avoid complicated effects, the shape and structure can be changed by changing the FCAS to realize it. It provided feasibility to design gradient bone scaffolds, some parts will be stiffness and others will be softness. In future research, we shall superimpose mixed FCASs together and print them in combination according to needs. Furthermore, it can be combined with finite element analysis, which provides a new idea for the simulation design of
complex bone scaffolds. Few studies have been conducted on the effects of FCASs.

4. CONCLUSIONS

The SF/COL/HA and PCL/HA scaffolds relied on low-temperature deposition 3D printing were two high-performance bioengineering materials. The pore size, porosity, water absorption, and elastic modulus of the composite scaffolds, all met the requirements of constructing tissue engineered bones. Changing of the FCAS in scaffolds determines varying degrees of effects on the mechanical properties, as well as the adhesion, proliferation, and osteogenic differentiation of MC3T3-E1 cells. Among them, the 90° FCAS significantly increased the compressive modulus in two groups and more conducive to the MC3T3-E1 cell proliferation and osteogenesis. This study provided the preliminary basis for the influence of FCAS on the properties of composite scaffolds and research of SF/COL/HA and PCL/HA scaffolds in mandibular reconstruction.

5. MATERIALS AND METHODS

5.1. Preparation of SF/COL/HA Composite Biomaterials. The natural silk was boiled in an aqueous solution of 0.5% Na2CO3, three times for 30 min. After dialysis, filtration, concentration, and centrifugation, a certain concentration of silk fibroin protein solution was obtained. Collagen was prepared by a salting out method by adding fresh bovine tendon to acetic acid solution with pepsin. Silk fibroin was mixed with the collagen and HA (Emperor, Nanjing) at a mass ratio of 5:4:1 and stirred for 4 h. A moderate amount of acetic acid could be added to aid mixing (Figure 1).

5.2. Preparation of PCL/HA Composite Biomaterials. PCL (average Mw = 80 000) and acetic acid (purity 99.8%) were purchased from Sigma-Aldrich (USA) and Pronolab (Lisboa, Portugal), respectively. PCL was dissolved in acetic acid to produce a solution with a concentration of 15% w/v. HA was added to PCL solution at a mass ratio of 1:4 and stirred for 24 h to promote a complete dissolution (Figure 1).

5.3. Model Specification. Three kinds of microstructural scaffold models with FCASs of [0°/90°/0°/90°/0°/90°], [0°/45°/90°/135°/180°/225°] and [0°/30°/60°/90°/120°/150°] were designed using Abaqus CAE software. In between, the positive direction of X and Y axis was 0° and 90°, respectively, and the Z axis was the height adjustment direction. The crossing angle in fiber between two adjacent floors were 90, 45, and 30°, respectively. (The following references were described with these) (Figure 2). The model data were converted to STL and imported into a 3D printer (Organ P 1800).

5.4. Low-Temperature Deposition 3D Printing. These prepared composite materials were loaded into the needle tubing. In the printing process, the printing parameters and floor temperature were set up to ensure the molding. The samples were then freeze-dried using a freeze dryer (EYELA FDU-1200) at low temperature and cross-linked under absolute alcohol at room temperature for 24 h, respectively. After cross-linked, the scaffolds were treated with NaOH (PH = 10), washed with deionized water, sterilized with CO2 and saved in ~20 °C.

5.5. Compression Test. The scaffolds with 1 cm² were compressed to a 30% strain of total thickness with a 0.5%/s compression speed. We performed a compression test with a care electromagnetic dynamic mechanics test system M-100T apparatus (Care M-100T) and non-contact image acquisition system. After that, the stress–strain diagram of each scaffold was obtained. We chose the slope of the stress–strain curve when the strain value was less than 10%, part of a straight line, as the elastic modulus. The test was repeated for three times.

5.6. X-Ray Diffraction. Crystallinity of the SF/COL/HA and PCL/HA composites was studied using the XRD. This experiment was conducted by a Bruker D8 ADVANCE apparatus, Cu target, at room temperature. Spectra were acquired to qualitatively assess phase composition of the composite materials. Spectra were acquired from 2θ range of 10–80° with a step size of 0.02°.

5.7. Fourier Transform Infrared Spectroscopy. The FTIR experiment was carried out with a FT6000 Fourir transform infrared spectrometer (Bio-rad). The tested sample and potassium bromide were mixed and pressed to prepare the sample. A wavelength of 11 000−400 cm⁻¹ and a resolution 0.075 cm⁻¹. The chemical composition of the sample was determined by comparing with the standard peak spectral.

5.8. Porosity Estimation. An improved fluid transfer method was used to measure porosity. The scaffold was immersed in graduated cylinder with a certain volume (v1) of ethanol. The total volume of ethanol containing the saturated scaffold was v2. When the saturated scaffold was removed from the cylinder, the residue ethanol volume was recorded as v3. Porosity = (v1 − v3)/(v2 − v3) ×100%. All experiments were repeated for three times.

5.9. Water Absorption Ratio. The over dried scaffolds were weighed at a weight of m1. The scaffolds were then immersed in a phosphate-buffered saline (PBS; pH = 7.4) for 24 h at 37 °C. At sampling point, they were removed from the PBS solution and weighed for a weight of m2. Water absorption ratio = (m2 − m1)/m1 × 100%. All experiments were repeated for three times.

5.10. Cell Culture. The pre-osteoblast cell line MC3T3-E1 was purchased from Bmcr (Chinese Academy of Medical Sciences). The cells were cultured in culture flasks with α-MEM (Gibco, USA) supplemented with 10% FBS and 1% penicillin/streptomycin solution (Solarbio, China) at 37 °C and 5% CO2. When reached 80−90% confluence, cells were passaged.

5.11. Calcein-AM/PI Staining. The preparation of the liquid extracts from the scaffolds follows the standard of liquid extract (GB/T1688 and ISO10993). The ratio of scaffold surface area to medium volume was 1 cm²/mL. After the scaffolds were stored in complete medium at 4 °C for 48 h, the corresponding liquid extracts from the scaffolds were obtained for being used in cell cultures. The staining was performed according to the instructions of calcein-AM/PI living/dead cell double staining kit to observe the survival of the cells.

5.12. Scanning Electron Microscopy. The cells were seeded on each scaffold at a density of 1 × 10⁵/cm² and cultured for seven days. The cell–scaffold complexes were then fixed with 2.5% glutaraldehyde, dehydrated by gradually increasing grade of alcohol. They were then coated by gold and observed by a SU8100 scanning electron microscope (Hitachi, Japan).

5.13. HE Staining. The cells were seeded on each scaffold at a density of 1 × 10⁵/cm² and cultured for 14 days. The medium was replaced every two days. At sampling point, the cell–scaffold complexes were removed from the medium and fixed in 10% neutral buffered formalin for 4 h. After dehydration, paraffin embedding and sectioning, the samples
were stained with HE to observe cell distribution in the scaffolds.

5.14. CCK-8 Assay. The cells were seeded on each 5 mm² scaffold at a density of 1 × 10⁵/cm² in a 24-well plate. The next day, the cell–scaffold complexes were transferred to a new plate to prevent the proliferation of unattached cells on the plate. At 1, 3, 5, and 7d of culture, the medium in each well was replaced by 500 μL α-MEM, then 50 μL CCK-8 solution (Solarbio, China) was added to each well. They were then incubated in darkness at 37 °C for 4 h. The absorbance value (OD) was measured at 450 nm with a multi-function enzyme labeling instrument (Tecan, Switzerland).

5.15. ALP Activity. The cells were seeded on the scaffolds at a density of 1 × 10⁵/cm² and cultured for seven days. After rinsing the scaffolds with PBS, they were removed into the EP tube with 500 μL of lysate followed by centrifuged at 12 000 rpm for 5 min for the supernatant. The ALP activity was detected and calculated according to the instruction of ALP Assay Kit (Beiyotime, China).

5.16. Real-Time PCR. We took out the cell–scaffold complexes at day 7 and added it into the EP tube, fully sheared the scaffold into pieces. Total RNA in cells loaded on the scaffolds was extracted using a Mini BEST Universal RNA Extraction Kit (TaKaRa, Japan). Reverse transcription was performed using a Prime Script RT reagent Kit (TaKaRa, Japan). The cDNA template was amplified by RT-PCR using a TB Green Premix Ex Taq II (TaKaRa, Japan). GAPDH was used as an internal control. RT-PCR was performed using a Light Cycler/Light Cycler 480 System (Roche, Switzerland). The expression of mRNA was calculated by 2−ΔΔCt. All experiments were repeated for three times. All primers were purchased from Sangon Biotech (China). GAPDH: Forward 5′-AGCAGAACCCACTCCACTCTTC-3′; Reverse 5′-TTGAGCCGATTTACATCTGC-3′; PCNA: Forward 5′-TTTAGGGACGCCCTGATCC-3′; Reverse 5′-GGA-GACGTGAGACGAGTCCAT-3′; ALP: Forward 5′-TGACCTTCTCTCTCCATCC-3′; Reverse 5′-CTTTCCTGGAGCTCTCATCCT-3′; OPN: Forward 5′-AACCAGCCAAGTAAAGACTG-3′; Reverse 5′-AGTTAGCTCAGAATTAGCCG-3′; Runt2: Forward 5′-CCGAAAATGCTCATCCTTGTTAG-3′; Reverse 5′-GGATTTGGAACTGTATATGTG-3′; COL-1: Forward 5′-CAGTGGGCGGTATGACTT-3′; Reverse 5′-CTGGGAGTTTCTCAATTCT-3′.

5.17. Statistical Analysis. Statistical analysis was performed using GraphPad Prism 5.0 software (San Diego, USA). All data were expressed as the mean ± the standard deviation. Statistical difference between two groups was assessed by a two-tailed student t-test. One-way ANOVA was used to the comparison of data among more than two groups. P < 0.05 was considered statistically significant.

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### Notes

The authors declare no competing financial interest.

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