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Cryogenic 3D printing for producing hierarchical porous and rhBMP-2-loaded Ca-P/PLLA nanocomposite scaffolds for bone tissue engineering

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Abstract

The performance of bone tissue engineering scaffolds can be assessed through cell responses to scaffolds, including cell attachment, infiltration, morphogenesis, proliferation, differentiation, etc, which are determined or heavily influenced by the composition, structure, mechanical properties, and biological properties (e.g. osteoconductivity and osteoinductivity) of scaffolds. Although some promising 3D printing techniques such as fused deposition modeling and selective laser sintering could be employed to produce biodegradable bone tissue engineering scaffolds with customized shapes and tailored interconnected pores, effective methods for fabricating scaffolds with well-designed hierarchical porous structure (both interconnected macropores and surface micropores) and tunable osteoconductivity/osteoinductivity still need to be developed. In this investigation, a novel cryogenic 3D printing technique was investigated and developed for producing hierarchical porous and recombinant human bone morphogenetic protein-2 (rhBMP-2)-loaded calcium phosphate (Ca-P) nanoparticle/poly(L-lactic acid) nanocomposite scaffolds, in which the Ca-P nanoparticle-incorporated scaffold layer and rhBMP-2-encapsulated scaffold layer were deposited alternatingly using different types of emulsions as printing inks. The mechanical properties of the as-printed scaffolds were comparable to those of human cancellous bone. Sustained releases of Ca2+ ions and rhBMP-2 were achieved and the biological activity of rhBMP-2 was well-preserved. Scaffolds with a desirable hierarchical porous structure and dual delivery of Ca2+ ions and rhBMP-2 exhibited superior performance in directing the behaviors of human bone marrow-derived mesenchymal stem cells and caused improved cell viability, attachment, proliferation, and osteogenic differentiation, which has suggested their great potential for bone tissue engineering.

1. Introduction

Scaffold-based tissue engineering can provide an appropriate platform for supporting cell growth and inducing human tissue regeneration [1, 2]. Scaffolds with 3D porous structures and desirable biocompatibility, which mimic the native extracellular matrix (ECM) of human tissues, can act as suitable substrates for cell anchoring and for stimulating tissue formation in vivo [3–5]. Also, the shape, structure, and mechanical properties of scaffolds should ideally be comparable to the defects and anatomy of host tissues [6]. Different from scaffolds made by conventional manufacturing techniques such as electrospinning, salt leaching, and emulsion freezing/freeze-drying, scaffolds made by novel additive manufacturing techniques (also termed ‘3D printing’) such as fused deposition modeling (FDM) [7], stereolithography [8], and selective laser sintering (SLS) [9] have fully controlled shape, tailored interconnectivity, and sufficient mechanical strength, showing great potential for clinical use.

For scaffolds developed for promoting bone regeneration, not only the customized shape and
interconnected pores but the hierarchical porous structure (with porous surface structure of scaffold struts) and biological properties (both osteoconductivity and osteoinductivity) of scaffolds are critical factors affecting cell behavior. Compared to scaffolds having dense struts with a smooth surface, scaffolds having struts with appropriate wettability and a porous surface structure were found to be beneficial for cell attachment and cell infiltration [10]. Besides, as synthetic calcium phosphates (Ca-P) are bioactive and osteoconductive [11] and bone morphogenetic proteins (BMPs), particularly BMP-2, are capable of inducing the osteogenic differentiation of mesenchymal stem cells (MSCs), showing osteoinductivity [12, 13], the inclusion of Ca-P nanoparticles and/or BMP-2 within the scaffolds could accelerate bone tissue regeneration [14]. However, for scaffolds made by existing 3D printing techniques, the control over the surface morphology of the scaffold struts appears difficult [15] and the in situ delivery of bioactive agents, particularly BMP-2, with well-preserved biological activity, are highly challenging since the manufacturing processes usually involve high temperature or laser sintering, which would cause damage to the incorporated bioactive molecules [16].

In this investigation, a novel technique combining extrusion-based 3D printing and emulsion freezing/cryodrying was investigated for producing hierarchically porous structured bone tissue engineering scaffolds with tunable osteoconductivity and osteoinductivity. Water-in-oil (water/oil) emulsions prepared from a poly(L-lactic acid) (PLLA) polymer solution (the ‘oil phase’) and recombinant human bone morphogenetic protein-2 (rhBMP-2) containing deionized (DI) water (the ‘water phase’) with/without the incorporation of Ca-P nanoparticles were used as printing inks and extruded on a cryogenic platform through the nozzle of a modified commercial 3D printer in a programmed way. After a computer-aided layer-by-layer deposition process and subsequent solvent evaporation via cryodrying, 3D scaffolds with pre-designed interconnected porous structure were fabricated. Also, micropores were formed in the struts of the scaffolds, resulting in scaffolds with desirable hierarchical porous structure. The mechanical properties of the scaffolds were studied through compression tests. The scaffold degradability was investigated in an 8 week period. The in vitro release behaviors of Ca\(^{2+}\) ions and rhBMP-2 from the scaffolds, as well as the biological activity of released rhBMP-2, were examined. Human bone marrow-derived mesenchymal stem cells (hBMSCs) were cultured on 3D-printed scaffolds and the viability, formation of adhesion plaques, and the proliferation of hBMSCs after different cell culture times were evaluated. The osteogenic differentiation of the hBMSCs on the scaffolds was assessed through alkaline phosphatase (ALP) staining and cell mineralization staining.

2. Materials and methods

2.1. Materials

PLLA (inherent viscosity: 1.6 dL g\(^{-1}\)) was purchased from Lakeshore Biomaterials, USA. Amorphous Ca-P nanoparticles with an average diameter of 45 ± 8 nm were produced in-house by rapidly mixing an acetone solution of Ca(NO\(_3\))\(_2\) · 4H\(_2\)O with an aqueous solution of (NH\(_4\))\(_2\)HPO\(_4\) at a molar ratio of Ca:P = 1.5:1 using a magnetic stirrer, followed by stirring, centrifugation, and washing [9]. The slurry of nanoprecipitates was freeze-dried to obtain dry powders and no post-synthesis sintering was conducted. DI water for all experiments was obtained using a DI water producer (Model D12681, Barnstead International, USA). Tween 20, phosphate buffered saline (PBS) tablets and bovine serum albumin (BSA) were Sigma-Aldrich products (USA). Dichloromethane (DCM) was supplied by Uni Chem Co., Korea. rhBMP-2 was supplied by Shanghai Rebone Biomaterials, China.

2.2. Formulation of water/oil emulsion inks

Emulsions which are fluidic at room temperature and solid upon freezing were formulated as inks for 3D printing. In a typical experiment, 1 ml of DI water was blended with 10 ml of PLLA/DCM solution (20%, w/v) and 50 μl of Tween 20. After 5 min ultra-sonication, water/oil emulsion inks were successfully formed and used for cryogenic 3D printing. As-printed pure polymer scaffolds were used as the control and designated as ‘PLLA’. Also, an emulsion prepared using a PLLA solution with a PLLA concentration of 5% w/v at the same water/oil volume ratio were subjected to cryogenic 3D printing, resulting in scaffolds designated as ‘PLLA-1’, which helped us to study the effect of polymer solution concentration on the printability of emulsion inks with different viscosities. In addition, an emulsion formulated with 10 ml of PLLA/DCM solution (20%, w/v) and 2.5 ml of DI water was used for cryogenic 3D printing, resulting in scaffolds designated as ‘PLLA+-’ for the study of the effect of water volume on the surface porous structure of the scaffold struts. The viscosity of emulsions was measured using a digital viscometer (NDJ-4S, Rinch Industrial Co., Limited, Shanghai, China).

2.3. Cryogenic 3D printer

A desktop FDM printer (Replicator® 2X, Makerbot®, USA) was modified to become a cryogenic 3D printer. A custom-made cryogenic substrate (−30 °C) was mounted on the original printing stage inside the 3D printer to provide a low-temperature environment (figures 1(a) and (b)). Emulsion inks were filled in the reservoir of the nozzle with an outer diameter of 0.4 mm and the feeding rate of emulsion inks was maintained at 0.3 ml min\(^{-1}\) using an air pump. The printing speed was set at 4 mm s\(^{-1}\) and each as-printed strut could be solidified within 2 s.
2.4. Fabrication of scaffolds through cryogenic 3D printing

The schematic of scaffold fabrication through cryogenic 3D printing is shown in figure 1 (e). By providing suitable emulsion inks and using a designed CAD file (figures 1(c) and (d)) (SolidWorks, MA, USA), scaffolds with pre-designed patterns could be constructed. In a typical process, 50 μl of DI water containing 100 μg of rhBMP-2 and 5 mg of BSA were blended with the emulsion consisting of 1 ml of DI water and 10 ml of PLLA solution to form BPLLA inks. Similarly, 353 mg of Ca-P nanoparticles (15 wt%) were blended with 1 ml of DI water and 10 ml of PLLA solution to form CPLLA ink via a 5 min ultra-sonication treatment. According to the CAD model (each scaffold had a 4-layer structure and each layer consisted of five parallel rods with a length of 6.5 mm and a diameter of 0.5 mm; the distance between two parallelled rods was 1 mm; rods in adjacent layers had a cross-angle of 90°), osteoinductive BPLLA scaffolds and osteoconductive CPLLA scaffolds were constructed in the cryogenic 3D printer. Scaffolds with a higher Ca-P loading (30 wt%) were also fabricated (designated as CPLLA+). By using two ink sources in the same fabrication process (i.e. the printer could be paused at any time for the reservoir replacement), the BPLLA and CPLLA layers could be printed alternatingly to form 4-layer bicomponent scaffolds (designated as BCPLLA). The composition and viscosity of different emulsion inks are shown in table 1. After cryogenic 3D printing, the ‘frozen’ scaffolds (still on the cryogenic platform) were cryodried in a fume hood for 1 h to remove organic solvents.

2.5. Physical characterizations of scaffolds

The microstructure of the 3D-printed scaffolds was observed using SEM (Leo 1530 Gemini, Zeiss, Oberkochen, Germany), in which scaffold samples were coated with a thin layer of gold. Compression tests were conducted both on the as-fabricated scaffolds (dry condition) and degraded scaffolds (wet condition). Scafolds samples with a 6-layer structure (thickness: 3.0 mm; width: 3.5 mm; three paralleled struts/layer; strut diameter: 0.5 mm; struts gap: 1.0 mm) were fabricated for compression tests. Five samples were tested for each type of scaffold. For testing the degraded scaffolds, the samples were immersed in PBS (pH 7.4) with 0.05 wt% sodium azide in centrifugation tubes. The tubes were sealed and placed in a shaking water bath (SW22, Julabo, Seelbach, Germany), which was maintained at 37 °C and shaken horizontally at 30 rpm for up to 2 weeks. Afterwards, the samples
were removed from the tubes for compression tests in the wet condition.

2.6. In vitro degradation of scaffolds

In vitro degradation was investigated by monitoring the remaining weight (%) of the scaffolds in an 8 week period. Pre-weighed samples were immersed in PBS (pH 7.4) with 0.05 wt% sodium azide in centrifugation tubes. The tubes were sealed and placed in a shaking water bath, which was maintained at 37 °C and shaken horizontally at 30 rpm. At each time point (2, 4, 6, and 8 weeks), a group of test samples were taken out from the centrifugation tubes and rinsed in DI water five times to remove salts, followed by a 48 h drying. The ratio between the remaining weight and initial weight of each sample was calculated, showing the percentage of the remaining weight. The morphological changes in the scaffolds after an 8 week in vitro degradation were observed using SEM.

2.7. In vitro release behaviors of Ca\(^{2+}\) ions and rhBMP-2

To investigate in vitro releases of Ca\(^{2+}\) ions and rhBMP-2, pre-weighed scaffold samples were put into test tubes filled with PBS solutions supplemented with additives (0.02% sodium azide for Ca\(^{2+}\) ion tests; 0.02% sodium azide, 0.1% BSA, 0.05% EDTA, 0.1% heparin for rhBMP-2 release tests). The test tubes were put into a shaking water bath at 37 °C. At predetermined time intervals, the test liquid was taken out and the concentration of rhBMP-2 and Ca\(^{2+}\) ions was measured using human BMP-2 ELISA Kit (Peprotech Inc., USA) and Calcium Assay Kit (US Biological, USA), respectively.

2.8. Cell culture

hBMSCs were cultured with Dulbecco’s modified Eagle’s medium, (DMEM, Gibco, USA), which was supplemented with 10% fetal bovine serum (Biowest, France), 100 U ml\(^{-1}\) penicillin-streptomycin and 2 mM L-glutamine (Invitrogen, USA) and maintained in a humidified incubator at 37 °C with 5% CO\(_2\). The medium was changed every 2 d. Four-layer scaffold samples with dimensions of 6.5 × 6.5 × 2.0 mm were used in cell culture experiments, which were sterilized by \(^{60}\)Co \(\gamma\)-irradiation at a dosage of 5 kGy for 30 min. Cell seeding was conducted by dripping the cell suspension of 1 × 10\(^5\) cells/10 \(\mu\)L onto the sterilized scaffolds in a 48-well plate with an addition of 400 \(\mu\)L of culture medium for completely immersing the cell-scaffold constructs. To induce the osteogenic differentiation of hBMSCs, the culture medium was replaced by an osteogenic medium, which was based on a basal medium supplemented with 100 nM dexamethasone, 0.05 mM L-ascorbic acid, and 0.01 mM \(\beta\)-glycerophosphate (Sigma-Aldrich, USA).

2.9. Cell viability

A live and dead viability kit (Molecular Probes, USA) was used to stain cells after 1, 4, and 7 d of cell culture for cell viability study. For cell staining, the cell-scaffold constructs were washed and incubated in DMEM containing 4 \(\mu\)M EthD-1 and 2 \(\mu\)M calcein AM in a humidified incubator (37 °C, 5% CO\(_2\)) for 15 min, where live cells and dead cells were stained a green and red color, respectively. Fluorescence images of the cell-scaffold constructs were obtained using a fluorescence microscope (Nikon Eclipse TE2000-U inverted microscope, Japan). The ratio between live and dead cells at each time point (1, 4, and 7 d) was calculated.

2.10. Cell morphology

After 7 d culture, the cell-scaffold constructs were washed with PBS and subsequently fixed with 2.5% GTA at 4 °C for 4 h. After washing with cacodylate buffer containing 0.1 M sucrose, they were dehydrated through ethanol gradient dehydration and dried for 24 h through the critical point drying. The samples were then sputter-coated with a thin layer of gold for observations under the SEM (Leo 1530 Gemini, Zeiss, Oberkochen, Germany).

2.11. Immunofluorescence staining of cytoskeletons and adhesion plaques

After 1 d culture, hBMSCs grown on different types of scaffolds were subjected to nucleus, F-actin, and vinculin staining. The cell-scaffold constructs were

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Table 1. Formulations and viscosity of emulsions for producing tissue engineering scaffolds through cryogenic 3D printing.

<table>
<thead>
<tr>
<th>Scaffold designation</th>
<th>Polymer mass (g)</th>
<th>Oil phase 'DCM' (mL)(^a)</th>
<th>Water phase 'DI water' (mL)</th>
<th>rhBMP-2 (2 μg)(^b)</th>
<th>Ca-P mass (mg)</th>
<th>Viscosity (mPa · s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLLA−</td>
<td>0.5</td>
<td>10</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>4,800</td>
</tr>
<tr>
<td>PLLA</td>
<td>2.0</td>
<td>10</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>45,000</td>
</tr>
<tr>
<td>PLLA+</td>
<td>2.0</td>
<td>10</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
<td>58,000</td>
</tr>
<tr>
<td>CPLLA</td>
<td>2.0</td>
<td>10</td>
<td>1.0</td>
<td>0</td>
<td>353</td>
<td>123,850</td>
</tr>
<tr>
<td>CPLLA+</td>
<td>2.0</td>
<td>10</td>
<td>1.0</td>
<td>0</td>
<td>857</td>
<td>382,560</td>
</tr>
<tr>
<td>BCPLLA</td>
<td>2.0</td>
<td>10</td>
<td>1.0</td>
<td>100</td>
<td>0</td>
<td>45,830</td>
</tr>
</tbody>
</table>

\(^a\) 10 mL of DCM was supplemented with 50 μL of Tween 20;

\(^b\) 100 μg of rhBMP-2 was supplemented with 5 mg of BSA.
first fixed with 0.2% glutaraldehyde (GTA). After washing with PBS, the cells were permeabilized in 0.2% Triton X-100 solution and incubated in (1% w/v) BSA block solution, followed by incubation in vinculin monoclonal antibody containing blocking solution (Rabbit [clone 42H89L44], A6finity™ Recombinant, Life Technologies) for 1 h. After washing, the cell-scaffold constructs were incubated in aqueous solution of Alexa Fluor(r) 564 Goat Anti-Rabbit IgG (H + L) (Life technologies, USA) for 1 h. FITC phalloidin (Alexa Fluor 488 phalloidin, Molecular Probes, USA) was simultaneously added to the solution for the F-actin staining. With another washing, the 4',6-diamidino-2-phenylindole (DAPI, Life Technologies) solution was added to the samples and they were used for observations of the nucleus, F-actin filaments, and vinculin adhesion plaques under a confocal laser scanning microscope (CLSM, LSM 710 Meta, Carl Zeiss, Germany).

2.12. MTT assay
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to investigate cell proliferation. After washing with PBS, 400 µl culture medium and 40 µl MTT solution (5 mg ml⁻¹ in PBS) were added to each cell-scaffold construct, followed by incubation for 4 h at 37 °C. After removal of the culture medium, the formed formazan crystals were dissolved in 500 µl DMSO. The solution (100 µl) was then transferred to a 96-well plate and the optical density of the solution was measured using a microplate reader (UVM 340, Asys HiTech GmbH, Austria) at a wavelength of 570 nm.

2.13. Biological activity of released rhBMP-2
To investigate the biological activity retention of rhBMP-2 released from the scaffolds, hBMSCs were cultured in an osteogenic medium, supplemented with 100 ng ml⁻¹ of directly used rhBMP-2, 100 ng ml⁻¹ of rhBMP-2 released from B PLLA scaffold (up to 7 d) or nothing. After 7 d culture, the ALP activity of cells under different culture conditions was analyzed and compared.

2.14. ALP staining and ALP activity
After 7 d osteogenic induction, the cell-scaffold constructs were fixed and frozen sectioned. ALP staining was performed by using the ALP leukocyte kit (Sigma, USA). The stained cells were observed under an inverted microscope (Nikon Eclipse TE2000-U, Japan). ALP activity was measured after 4, 7, and 14 d induction medium and the scaffolds were separately placed on top of the cell layer. After 21 d induction, the scaffolds were removed and the cells were fixed with 2.5% GTA for 4 h. After thorough washing with PBS, 2% Alizarin Red S working solution (pH 4.1–4.3) was added for a 30 min staining at room temperature. With further washing, the stained cells were observed under an inverted microscope (Nikon Eclipse TE2000-U, Japan).

2.15. Cell mineralization
Alizarin Red S staining was used to visualize cell mineralization. hBMSCs were cultured in the wells of a 48-well plate. Once hBMSCs achieved 80% confluence, the culture medium was replaced by the induction medium and the scaffolds were separately placed on top of the cell layer. After 21 d induction, the scaffolds were removed and the cells were fixed with 2.5% GTA for 4 h. After thorough washing with PBS, 2% Alizarin Red S working solution (pH 4.1–4.3) was added for a 30 min staining at room temperature. With further washing, the stained cells were observed under an inverted microscope (Nikon Eclipse TE2000-U, Japan).

2.16. Statistical analysis
All statistical analyses were performed using the SPSS software (version 18). Numerical data are presented as the mean value ± standard deviation (SD). For statistical comparison, one-way analysis of variance (ANOVA) with the Student’s t test was applied. p < 0.05(∗) was considered to be statistically significant, while p < 0.005 (***) was considered to be highly significant.

3. Results

3.1. Emulsion formulation and scaffold formation
Through cryogenic 3D printing under the optimized condition, structurally stable scaffolds with a maximum 6-layer structure, in which struts with retentive shape were identical to that in the CAD model, could be formed. In the current investigation, a 4-layer structured CAD model was therefore used for producing scaffolds, enabling the desired solidification. It is known that the viscosity of printing inks can greatly affect the printability. It was shown in this investigation that PLLA scaffolds could be printed from emulsion inks with a solution concentration of 20% (w/v), which had a viscosity of 45 000 (mPa·s) (table 1). When the polymer solution concentration decreased to 5% (w/v) (PLLA−), the emulsion viscosity decreased to 4800 (mPa·s) and continuous patterns could not be drawn due to the high surface tension-induced breakup of emulsion trajectory. By contrast, when the water phase volume increased to 2.5 ml (PLLA+), the emulsion viscosity increased to 58 000 (mPa·s) and the emulsion pattern could be drawn continuously to
form 3D scaffolds. Figure 1(f) shows the morphology of the PLLA scaffolds at different magnifications (21 and 1200x). The pattern of the PLLA scaffolds was identical to the CAD model, suggesting that no obvious size shrinkage or expansion occurred before and after solvent evaporation. Moreover, the struts had a porous surface structure with a pore diameter of 7.5 ± 2.7 μm. By contrast, micropores with a much larger size (64 ± 32 μm) and an irregular shape were observed on the struts of the PLLA+ scaffolds (figure 1(g)).

BPLLA and CPLLA inks had a viscosity of 45 830 and 123 850 (mPa)-s, respectively. The 3D-printed BPLLA and CPLLA scaffolds also showed porous surface structure, in which the CPLLA scaffolds were more identical to the CAD model (figures 2(a) and (b)). The distribution of Ca-P nanoparticles in CPLLA scaffolds was investigated through energy dispersive x-ray (EDX) elemental mapping and peaks corresponding to the elements of calcium and phosphor were present in the EDX spectrum (figure 2(c)). Emulsion inks loaded with 30 wt% Ca-P nanoparticles were also employed to print CPLLA+ scaffolds. However, frequent nozzle clotting occurred during the printing process due to the much increased emulsion viscosity (382 560 (mPa)-s) and nanoparticle agglomeration, suggesting that the ink printability through a fine nozzle (<0.5 mm) was restricted when the viscosity or nanoparticle volume was too high.

3.2. Mechanical properties of cryogenic 3D-printed scaffolds

For bone tissue engineering, making scaffolds with sufficient mechanical strength is highly important. It can be seen from figures 3(a) and (b) that the compressive strengths and elastic moduli of scaffolds with various compositions were comparable to those of scaffolds fabricated through SLS and emulsion freezing/freeze-drying techniques [17, 18], as well as human cancellous bone [19]. Under the dry condition, the CPLLA scaffolds showed the highest compressive strength (1.12 ± 0.05 MPa). The compressive strength of the BCPLLA scaffolds (0.9 ± 0.04 MPa) was lower than that of the CPLLA scaffolds, but still significantly higher than that of the PLLA and BPLLA scaffolds (p < 0.005). A similar trend was observed for the elastic modulus, in which the PLLA, BPLLA, CPLLA, and BCPLLA scaffolds had an elastic modulus of 8.2 ± 0.2, 8.2 ± 0.25, 13.2 ± 0.3, and 9.8 ± 0.23 MPa, respectively. After 2 weeks of immersion treatment, the PLLA, BLLA, CPLLA, and BCPLLA scaffolds had a compressive strength of 0.77 ± 0.04, 0.76 ± 0.05, 1.06 ± 0.05, and 0.87 ± 0.04 MPa, respectively, which were about 2.5–4.6% lower than that of scaffolds tested under the dry condition. Similarly, the elastic modulus of all degraded scaffolds was about 3.0%–5.5% lower than that of scaffolds tested under the dry condition. Even with the slight decreases in the compressive strength and elastic modulus for scaffolds after 2 week immersion treatment, their mechanical properties were still comparable to those of human cancellous bone, making them suitable for bone tissue engineering.

3.3. Degradation behaviors of scaffolds and in vitro release profiles of Ca\(^{2+}\) and rhBMP-2 from scaffolds

The in vitro degradation behavior of scaffolds was investigated using different time points. Increased weight loss was observed with increasing degradation time for all scaffolds and CPLLA scaffolds had the highest weight loss after 8 week in vitro degradation (figure 4(a)) (p > 0.05, n = 5). However, after the 8 week in vitro degradation, no major morphological changes were found between the degraded samples and their respective undegraded samples (figure 4(b)). The in vitro releases of Ca\(^{2+}\) ions and rhBMP-2 could affect the functions and differentiation of hBMSCs. Ca\(^{2+}\) ions released from the CPLLA scaffolds showed an initial relatively rapid release of 5.0 ± 0.2% level in 24 h, followed by a slow but sustained release up to
22.5 ± 1.3% level at 30 d (figure 5(a)). No significant difference of Ca²⁺ ion release was found between the CPLLA and BCPLLA scaffolds. rhBMP-2 release from the BPLLA scaffolds consisted of a relatively rapid release of 20.5 ± 1.3% level in the initial 24 h, followed by a slower but steady release up to 67.5 ± 2.3% level at 30 d (figure 5(b)). In comparison, the BCPLLA scaffolds exhibited a similar but slightly
lowered rhBMP-2 release level \((p > 0.05)\). The possible reason for the lowered release level for the BCPLLA scaffolds was that a small quantity of released rhBMP-2 could be physically adsorbed to the surface of the Ca-P nanoparticles.

### 3.4. Viability, adhesion, and proliferation of hBMSCs on different types of scaffolds

The viability of hBMSCs on scaffolds was visualized using live and dead staining (figure 6(a)). After 4 d culture, hBMSCs were uniformly distributed on the scaffold struts and nearly all stained hBMSCs (>99%) on the PLLA, BPLLA, and CPLLA scaffolds were alive (green color), indicating an excellent scaffold biocompatibility. After 7 d culture, about 80% hBMSCs on the PLLA scaffolds were still alive, while more than 90% of the cells were found alive on the BPLLA and CPLLA scaffolds, in which the BPLLA group showed the highest cell viability \((p < 0.05, n = 5)\) (figure 6(b)). The initial cell attachment is also of great importance for subsequent cell expansion, proliferation, and differentiation. The focal adhesion plaques were visualized to investigate the initial cell adhesion to the scaffolds. After 1 d culture, distinct F-actin filaments were found in hBMSCs cultured on the PLLA, BPLLA, and CPLLA scaffolds (figure 6(c)). However, only a few vinculin adhesion plaques were found on the PLLA scaffolds, while many more vinculin dots were found on the BPLLA and CPLLA scaffolds \((p < 0.005, n = 5)\), suggesting that the single delivery of rhBMP-2 or Ca-P nanoparticles in scaffolds could enhance the formation of adhesion plaques (figure 6(d)). The morphology of hBMSCs on the PLLA, BPLLA, and CPLLA scaffolds was observed after 7 d culture (figure 6(e)). 70% cell confluence as well as individual hBMSCs were observed on the PLLA scaffolds, while more than 80% hBMSCs were observed on the BPLLA and CPLLA scaffolds \((p < 0.005, n = 5)\), suggesting that the single delivery of rhBMP-2 or Ca-P nanoparticles in scaffolds could enhance the formation of adhesion plaques (figure 6(d)). The morphology of hBMSCs on the PLLA, BPLLA, and CPLLA scaffolds was observed after 7 d culture (figure 6(e)). 70% cell confluence as well as individual hBMSCs were observed on the PLLA scaffolds, showing a normal phenotype and morphology with an expanded polygonal shape, indicating that the porous surface structure was a desirable microenvironment for cell expansion (figure 6(f)). In comparison, 95% and 84% cell confluence was observed on the BPLLA and CPLLA on the PLLA scaffolds, respectively.
indicating that the single delivery of rhBMP-2 or Ca-P could improve the cell expansion significantly \((p < 0.005, n = 5)\). The proliferation of hBMSCs on the scaffolds was quantified using MTT assay (figure 6(g)). The MTT absorbance increased with increasing culture time for all scaffolds. After 7 d culture, compared to the PLLA scaffolds, the CPLLA scaffolds slightly up-regulated cell proliferation, while the BPLLA and BCPLLA scaffolds significantly up-regulated cell proliferation \((p < 0.005, n = 5)\).

3.5. Effect of sustained release of Ca\(^{2+}\) or rhBMP-2 on the osteogenic induction of hBMSCs

As growth factors are very delicate biomolecules, maintaining the biological activity of growth factors at a high level during the scaffold fabrication process and also in the entire release periods is of great importance, but also highly challenging. To study the biological activity of rhBMP-2 released from scaffolds made by cryogenic 3D printing in the current investigation, ALP activities of hBMSCs regulated by the induction medium supplemented with released rhBMP-2 and virgin rhBMP-2 were compared. After 7 d osteogenic induction, the ALP activity of hBMSCs was significantly up-regulated by both the released rhBMP-2 and virgin rhBMP-2, compared to the group with no supplement \((p < 0.005, n = 5)\) (figure 7(a)). No significant difference was found in the two groups with rhBMP-2 administration, indicating that the biological activity of the released rhBMP-2 was maintained at a high level (about 90%).

The osteogenic differentiation of hBMSCs on the PLLA, CPLLA, BPLLA, and BCPLLA scaffolds after specific cell culture times was investigated by measuring the ALP expression of cells. For all scaffolds, the ALP activity first increased and then decreased with increasing induction time (up to 14 d) (figure 7(b)). After 14 d induction, the PLLA scaffolds induced limited ALP while the CPLLA scaffolds significantly up-regulated the ALP expression \((2.0\text{-fold compared to the PLLA scaffolds})\) due to the presence of Ca-P nanoparticles on the scaffold surface and the sustained Ca\(^{2+}\) ion release \((p < 0.005, n = 5)\). Moreover, the BPLLA and BCPLLA scaffolds further up-regulated the ALP activity by giving a 3.0- and 3.5-fold ALP expression, respectively \((p < 0.005, n = 5)\). The ALP staining was conducted after 7 d induction (figure 7(c)). Limited ALP area (stained in red) was observed on the PLLA scaffolds, whereas more ALP expression was detected on the BPLLA, CPLLA, and BCPLLA scaffolds, which was consistent with the results of the ALP activity assay. After 21 d induction,
the mineralization of hBMSCs treated with scaffolds was visualized using Alizarin Red S staining (figure 7(d)). No calcium nodule was observed in cells cultured with the PLLA scaffolds, while obvious calcium deposition was observed in cells cultured with the BPLLA, CPLLA, and BCPLLA scaffolds, respectively. These results demonstrated that compared to the PLLA scaffolds, the single delivery of Ca-P or rhBMP-2 from the scaffolds could significantly enhance the bone forming activity of these scaffolds, while dual delivery of rhBMP-2 and Ca-P could have a synergistic effect on the osteogenic differentiation of hBMSCs, although the amounts of rhBMP-2 or Ca-P nanoparticles loaded in the BCPLLA scaffolds was only 50% of those loaded in the BPLLA or CPLLA scaffolds.

4. Discussion

3D-printed hydrogels that have been increasingly investigated in tissue engineering as hydrogels of alginate, gelatin, polyethylene glycol diacrylate, or hyaluronic acid have advantages for making growth factors-loaded, cell-laden tissue constructs at room temperature. For instance, Luo et al fabricated nano-hydroxyapatite/alginate composite scaffolds through 3D plotting for bone tissue engineering [20]. They furthermore made concentrated gelatin/alginate bone tissue engineering scaffolds with the assistance of sequential crosslinking using Ca$^{2+}$ ion and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride [21]. Rajaram et al fabricated Schwan cell-loaded alginate/hyaluronic acid hydrogels through biplotting for potential peripheral nerve tissue engineering [22]. Zehnder et al made alginate-dialdehyde/gelatin hydrogel scaffolds using 3D plotting [23]. Adamkiewicz and Rubinsky also reported that 3D-printed hydrogels with a high resolution could be constructed in a cryogenic environment (e.g. liquid nitrogen) [24]. However, the cytotoxicity of crosslinking agents, which were commonly required for making these scaffolds, is always a problem. Apart from this, most hydrogels and natural polymer scaffolds have low mechanical strength and relatively high shrinkage percentage after fabrication [25]. By contrast, scaffolds made of biodegradable polyesters have improved structural stability and mechanical properties for implantation in the clinical situation. However, various challenges remain for the manufacture of these polyester-based scaffolds with well-defined structure and appropriate delivery of biomolecules and/or cells. Take scaffolds made through conventional FDM, as an example, which uses molten polymers as inks, the biggest shortcoming of this conventional 3D printing technology is that the melting of polymer wires to form fluidic inks mostly requires high temperature (220 °C for commercial PLA and acrylonitrile butadiene styrene) and hence this process definitely damages the incorporated biomolecules. In addition, patterned polyester scaffolds made through conventional FDM cannot form well-defined hierarchical porous structure, which resembles the anatomy of human cancellous bone. The primary objective of the current investigation was to investigate and develop a novel extrusion-based 3D printing technique for producing hierarchically structured polyester-based scaffolds with in situ Ca-P and/or rhBMP-2 delivery for bone tissue engineering. Compared to the molten polymer inks, pure PLLA solutions with an appropriate viscosity can be used as printable inks at room temperature to draw pre-designed patterns. However, the extrudate is quite difficult to solidify into struts with retentive shape even under a cryogenic environment, since most organic solvents have a very low freezing point (e.g. DCM at −95.1 °C). To overcome this limitation, water/oil emulsions, which had suitable viscosity and were readily able to be frozen in a short time, were prepared as inks in the current investigation, leading to the formation of structurally stable PLLA scaffolds through continuous 3D printing under a cryogenic environment. In the investigation, the scaffolds made by cryogenic 3D printing displayed hierarchical porous structure similar to human cancellous bone. The presence of micropores on the strut surface may be attributed to the formation of ice particles during the cryogenic 3D printing and the subsequent removal of solvents, while the larger pores on the strut surface of the PLLA+ scaffolds may be attributed to the higher water volume-induced water droplet fusion, which subsequently formed larger ice particles.

The compressive strength and modulus of scaffolds made in this investigation were comparable to those of human cancellous bone, and the CPLLA scaffolds exhibited the highest values. Wei et al reported that the addition of nanosized apatite to a polyamide 6 matrix could greatly improve the mechanical properties and integrity of the composite due to molecular interactions between the apatite nanoparticles and the polymer matrix [26]. Duan et al reported that Ca-P/PHBV and Ca-P/PLLA nanocomposite scaffolds produced by SLS had better mechanical properties than neat PHBV and PLLA scaffolds [17]. The results obtained in this investigation showed similar trends. It was also noted in the current investigation that the mechanical properties of 3D-printed scaffolds after 2 week immersion treatment were slightly lower compared to scaffolds tested under the dry condition. This result is similar to the findings on other scaffolds [17, 27].

The scaffold composition could affect scaffold biodegradation. Considering that the PLLA matrix had a low degradation rate, the highest weight loss of the CPLLA scaffolds could be partially attributed to the detachment of weakly bonded Ca-P nanoparticles from the strut surface. It was shown in this investigation that sustained releases of Ca$^{2+}$ ions and
rhBMP-2 were achieved. Compared to hydroxyapatite (HA), amorphous Ca-P nanoparticles had a much higher degradation rate. Therefore, the release of Ca\(^{2+}\) ions could be attributed to the decomposition of Ca-P nanoparticles located on the strut surface. Meanwhile, as PLLA slowly degraded \textit{in vitro}, a slow but sustained Ca\(^{2+}\) ion release was obtained. The initial relatively rapid release of rhBMP-2 may be attributed to the dissociation of rhBMP-2 from the micropores located on the strut surface, while the sustained release of rhBMP-2 could be attributed to the continuous dissociation and diffusion of rhBMP-2 from pores located at the inner parts of the struts.

As the cryogenic 3D printing established in the current investigation does not require the use of any crosslinking agent and all organic solvents could be completely removed through subsequent cryodrying, the 3D-printed scaffolds have shown high cytocompatibility, which would facilitate the growth of hBMSCs throughout the 21 d culture period. A number of factors including scaffold topography, surface chemistry, release of bioactive agents (including Ca\(^{2+}\) ions from Ca-P nanoparticles and rhBMP-2), etc., can influence the cytoskeleton organization and the formation of adhesion plaques of osteoblastic cells and hBMSCs. In this investigation, the CPLLA scaffolds up-regulated the expression of vinculin, suggesting that Ca-P nanoparticles on the strut surface provided the scaffolds with bioactivity and appropriate surface chemistry for cell adhesion. The BPLLA scaffolds also induced much higher vinculin expression than PLLA \((p < 0.005)\), indicating that the release of rhBMP-2 was favorable for the formation of adhesion plaques at the early stage. Kang \emph{et al} had similar observations that the rBMP-2 or Ca-P-incorporated ECM could enhance the F-actin and vinculin expression of MSCs \[13\]. Moreover, the presence of BMP-2 and Ca-P nanoparticles was also found to enhance the proliferation of MSCs. Compared to the PLLA scaffolds, the hBMSC proliferation was also significantly up-regulated by the BPLLA and BCPLLA scaffolds. As no harsh fabrication process was involved in the 3D printing process in the current investigation, the rhBMP-2 released from the scaffolds maintained its biological activity to a large extent, resulting in a very high level of biological activity. Compared to the PLLA scaffolds, the BPLLA, CPLLA, and BCPLLA scaffolds exhibited much improved bone forming activity by showing enhanced ALP activity, ALP staining area, and calcium deposition. These results suggested that the single delivery of rhBMP-2 or Ca-P nanoparticles could enhance the osteogenic differentiation of hBMSCs, while the dual delivery of rhBMP-2 and Ca-P nanoparticles could have a synergistic effect to further upregulate their osteogenic differentiation for accelerating bone tissue regeneration. It is known that bone regeneration is a coordinated cascade of events regulated by several cytokines and growth factors. Angiogenic factors such as vein endothelial growth factor and basic-fibroblast growth factor are predominantly expressed at the early phases for re-establishment of the vascularity, while osteogenic growth factors are continuously expressed during bone formation and remodeling. Therefore, in our future investigations, more attention will be paid to the multiple delivery of the angiogenic growth factors and osteogenic growth factor in a spatio-temporal manner to achieve enhanced bone tissue regeneration with required vascularization. This investigation has provided a new way to integrate polymers and biomolecules together to form scaffolds with a pre-designed architecture (both in macro- and micro-scales), controlled biomolecule release, sufficient mechanical strength, and enhanced bone forming ability for bone tissue engineering. In addition, \textit{in situ} cell delivery, which can endow scaffolds with better biological performance, may be expected by using this cryogenic 3D printing technique in the future.

5. Conclusions

New tissue engineering scaffolds were successfully produced by cryogenic 3D printing. They had a customized shape with hierarchical porous structure and tunable biological properties. The compressive strength and modulus of scaffolds were comparable to those of human cancellous bone. Ca-P nanoparticles and rhBMP-2 were incorporated \textit{in situ}. The sustained release of rhBMP-2 with well-preserved biological activity could be achieved. hBMSCs grown on the scaffolds made by cryogenic 3D printing exhibited good cell viability, attachment, and proliferation. Under the stimuli of both biomimetic structural cues of the scaffolds and suitable biochemical cues through the release of Ca\(^{2+}\) ions and rhBMP-2 from the scaffolds, the hBMSCs displayed enhanced osteogenic differentiation on the 3D-printed scaffolds, as the dual delivery of rhBMP-2 and Ca\(^{2+}\) ions provided a synergistic effect on hBMSC osteogenic differentiation and cell mineralization. Scaffolds made by the novel cryogenic 3D printing have shown great potential for bone tissue engineering.

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