Sequential and sustained release of SDF-1 and BMP-2 from silk fibroin-nanohydroxyapatite scaffold for the enhancement of bone regeneration

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A R T I C L E   I N F O

Article history:
Received 5 June 2016
Received in revised form 14 August 2016
Accepted 15 August 2016
Available online 17 August 2016

Keywords:
Bone regeneration
Controlled release
Stromal cell derived factor-1
Bone morphogenetic protein-2
Silk fibroin
Nano-hydroxyapatite

A B S T R A C T

In this study, a cell-free bone tissue engineering system based on a silk fibroin (SF)/nano-hydroxyapatite (nHAp) scaffold was developed, in which two bioactive molecules, stromal cell derived factor-1 (SDF-1) and bone morphogenetic protein-2 (BMP-2), were embedded and released in a sequential and controlled manner to facilitate cell recruitment and bone formation, respectively. BMP-2 was initially loaded into SF microspheres, and these BMP-2 containing microspheres were subsequently encapsulated into the SF/nHAp scaffolds, which were successively functionalized with SDF-1 via physical adsorption. The results indicated rapid initial release of SDF-1 during the first few days, followed by slow and sustained release of BMP-2 for as long as three weeks. The composite scaffold significantly promoted the recruitment of bone marrow mesenchymal stem cells (BMSCs) and osteogenic differentiation of them in vitro. Further, the in vivo studies using D-Luciferin-labeled BMSCs indicated that implantation of this composite scaffold markedly promoted the recruitment of BMSCs to the implanted sites. Enhanced bone regeneration was identified at 12 weeks' post-implantation. Taken together, our findings suggested that the sequential and sustained release of SDF-1 and BMP-2 from the SF/nHAp scaffolds resulted in a synergistic effect on bone regeneration. Such a composite system, therefore, shows promising potential for cell-free bone tissue engineering applications.

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1. Introduction

Large bone defects associated with trauma, tumor, and infection frequently require surgical intervention [1,2]. Currently, the transplantation of allogeneic and autologous bones remains the mainstream of treatment for bone defects. However, the risks of infection, rejection, and donor site morbidity and the limited supply prevent its clinical application and remain serious problems that must be resolved [1,3,4]. Bone tissue engineering, which is promising alternative strategy for inducing tissue regeneration, has thus attracted considerable attention [4,5]. Conventional tissue engineering involves the use of a combination of biomaterials, appropriate bioactive molecules, and cells, which may suffer from the problems related to long-term in vitro culture for the formation of new bones, such as the high cost of cell isolation, proliferation and maturation, as well as the risk of pathogens [6]. Alternatively, in situ bone tissue engineering provides a better solution by taking advantage of the in vivo microenvironment, in which the cell-free, bone-specific biomaterials alone or in combination with bioactive molecules are directly implanted in the defect sites to recruit host circulating stem cells in situ, which thus aims to guide the osteogenic differentiation of mesenchymal stem cells (MSCs) for bone regeneration [7,8].

Biomaterial comprises a key element in bone tissue engineering and provides a three-dimensional (3D) scaffold to support cell growth and the formation of the extracellular matrix (ECM) [9]. Silk fibroin (SF), a biologically derived protein harvested from...
domesticated silk worm (*Bombyx mori*) cocoons, has been recognized as a favorable scaffold material for bone tissue engineering because of its unique mechanical properties, tunable biodegradation rate, good preservation of bioactive molecule activity and the ability to support the osteogenic differentiation of MSCs [10,11]. Moreover, hydroxyapatite (HAp) is chemically similar to the natural bone and exhibits excellent osteoconductivity [12,13]. It has been demonstrated that the incorporation of nanoparticle HAp (nHAp) in SF scaffolds may promote bone regeneration through the osteoinductive effects of nHAp on bone marrow mesenchymal stem cells (BMSCs) [12,13].

In addition to biomaterials, bioactive molecule comprises another key element in bone tissue engineering to endow the functionality of the biomaterial to stimulate the recruitment and osteogenic differentiation of BMSCs for bone regeneration [9]. Stromal cell derived factor-1 (SDF-1 or CXCL12) is a member of the CXC chemokine family, the recognition of SDF-1 by its main receptor, CXCR4, triggers the migration of CXCR4-positive stem cells and progenitor cells to injury sites during the acute phase of bone repair and participates in regeneration [14,15]. More importantly, it has been reported that SDF-1 signaling contributed to the osteogenic differentiation of mesenchymal C2C12 cells induced by bone morphogenetic protein-2 (BMP-2) [14]. BMP-2 comprised an important osteoinductive growth factor, which is approved by the US Food and Drug Administration (FDA) for clinical use to induce bone formation via the enhancement of osteoblast progenitor cell recruitment, angiogenesis, and the stimulation of the osteogenic differentiation of MSC [16–18]. Taking advantage of the unique properties of SDF-1 and BMP-2, several groups have attempted to utilize their combination for bone regeneration [19,20]. However, the concomitant use of BMP-2 and SDF-1 mixture had no additive effects on osteoblastic differentiation, cell migration or angiogenesis [20]. In Hwang’s work, the sequential treatment of collagen scaffolds with BMP-2 following SDF-1 demonstrated the strongest degree of new bone regeneration compared with the simultaneous treatment with BMP-2 and SDF-1 [21]. This phenomenon may be explained by the enhancement of stem cell migration in the early stage by SDF-1 and the activation of osteoblastic differentiation in the later stages by BMP-2, considering that SDF-1 is not required following the activation of osteogenic induction [19,21]. It should be noted that in Hwang’s work, BMP-2 was applied to the implanted scaffolds via periodic percutaneous injections, which is not appropriate for some fracture sites in deep locations of the body (e.g., lumbar vertebra). In addition, because of the uncontrolled and offsite release of BMP-2, high doses of BMP-2 must be used to compensate for the short half-life of BMP-2 in vivo to achieve effective clinical bone regeneration [18], which may cause side effects, such as heterotopic bone formation, edema or inflammatory reactions [22–24]. It is suggested that this problem may be solved by the encapsulation of BMP-2 into microspheres prior to incorporation into a polymeric scaffold [25]. For example, SF microspheres have been demonstrated to be efficient in delivering BMP-2 with the controlled release kinetics for MSC chondrogenesis [25–27].

In this work, we developed a novel scaffold for effective bone regeneration with the capability to sequentially release SDF-1, followed by the release of BMP-2 in a controlled manner. A composite of SF and nHAp porous scaffold was used to incorporate SF microspheres encapsulated with BMP-2, and SDF-1 was then physically adsorbed onto the scaffold to construct the cell-free scaffold. The SF microspheres were prepared via lumen jet break-up technology to endow the high encapsulation efficiency of BMP-2 [28]. With the combination of physically adsorbed SDF-1 and microspheres encapsulated with BMP-2 in the SF/nHAp scaffold, we expected enhanced synergistic effects for bone regeneration, in which the initial release of SDF-1 facilitates the recruitment of MSCs, and the controlled release of BMP-2 subsequently induces the osteogenic differentiation of MSCs. The effects of the sequential and sustained release of SDF-1 and BMP-2 on the migratory and osteogenic capacity of MSCs and the bone regeneration were assessed by different and complementary assays.

2. Materials and methods

2.1. Preparation of scaffolds that contain both SDF-1 and BMP-2

2.1.1. Preparation of SF solutions

Aqueous SF solutions from cocoons of *B. mori* (RudongXinsilu Co., Ltd., Jiangsu, China) were prepared as previously described [13]. Briefly, the cocoons were boiled for 1 h in an aqueous solution of Na2CO3 (0.5% w/w) and subsequently rinsed with water to remove the sericin. The extracted SF was solubilized in 9.3 M LiBr (Strem Chemicals Inc., MA, USA) solution at 60 °C for 4 h to produce a 20% (w/v) solution. This solution was dialyzed against water for 4 d with 8 times water changes in between. Finally, the purified SF solution was dialyzed against polyethylene glycol 20,000 (Biosharp, Shanghai, China) powder to obtain a concentrated SF of 15.0%. The solution was filtered through a syringe filter and stored at 4 °C prior to use.

2.1.2. Preparation of SF microspheres

The SF microspheres were fabricated using the laminar jet break-up method as previously described with a slight modification [28]. A syringe connected to a nozzle with a diameter of 200 μm was fixed on the micro-injection pump (LSP02-1B, Baoding Longer Precision Pump Co., Ltd., China). The SF solution with concentrations at 2%, 4% and 6% (w/v) in the injector underwent jet break-up into droplets under a high voltage of 10 kV. The produced pellets were collected in a liquid nitrogen bath. The frozen spheres were freeze dried (Alpha 1–2 LD plus, Christ, Germany) for 2 d. The freeze-dried microspheres were subsequently treated with methanol at room temperature for 30 min to induce SF structural transformation. Finally, the insoluble SF microsphere in water was obtained after being lyophilized again. The SF concentration at 6% was used in the following experiment. To produce BMP-2 (or rhBMP-2, Shanghai Rebone Biomaterials Co. Ltd., China) loaded SF spheres, the growth factor was added to the aqueous SF solution with a mass ratio of BMP-2 to SF at 1:50 (w/w).

2.1.3. Fabrication of SF based composite scaffold

SF microspheres loaded with BMP-2 were incorporated into a mixture of nHAp (50–100 nm; Aladdin, Shanghai, China) and SF suspension to prepare the composite scaffolds via freeze drying; SDF-1 (or rhSDF-1z, Peprotech, USA) was then physically adsorbed onto the surface of the scaffold. First, nHAp was dispersed in phosphate buffer solution (PBS, 0.001 M, pH 6.8) by sonication for 1 min to achieve a suspension concentration at 20% (w/v). Then HAp and SF solution with a mass ratio of nHAp to SF at 1:20 (S1, supporting information, Fig. S1) was subsequently prepared via the addition of the desired amount of nHAp/PBS into SF solution (6%, w/v) under sonication for 1 min and magnetic stirring for 30 min. SF microspheres loaded with BMP-2 were suspended into the mixture of SF/nHAp with the mass ratio of microspheres to nHAp at 2:3. The prepared composite suspension (50 μL) was then transferred to a mold plate (5 mm in diameter and 3 mm in height) and frozen in liquid nitrogen for freeze drying. The freeze-dried scaffold was treated with methanol for 30 min to induce SF structural transformation to develop a cylindrical insoluble SF scaffold that contained SF microspheres loaded with BMP-2 (BMP-2 (E)). The SF/nHAp scaffold was prepared and used as a control. The
scaffold was subsequently stored at scaffold, and SDF-1

2.3. Determination of SDF-1 and BMP-2 release from scaffold to prepare the SDF-1 scaffold, SDF-1 dropped onto the control scaffold, BMP-2 (P) scaffold and BMP-2 (E) scaffold, and SDF-1+BMP-2 (E) (S + B (E)) scaffold, respectively. The scaffold was subsequently stored at –20 °C and sterilized with ultraviolet light (λ = 254 nm) for 4 h prior to use.

The abbreviations and the preparation of the scaffolds used in this work were summarized in Table 1.

2.2. Characterization of microspheres and scaffolds

2.2.1. Scanning electron microscopy
To observe the morphology of the SF microspheres and scaffolds, the samples were examined using a scanning electron microscopy (SEM, S-4800; Hitachi, Kotoyo, Japan) at an accelerating voltage of 10 kV. Prior to characterization, the samples were added directly on top of conductive tapes mounted on the SEM sample stubs and sputter coated with gold for 60 s using gold sputter coating equipment (SC7620, Quorum Technologies, UK).

2.2.2. Fourier transform infrared spectroscopy
The structure of the scaffold was analyzed via Fourier transform infrared spectroscopy (FTIR, Nicolet 6700; Thermo scientific, USA). For each measurement, 128 scans were obtained with a resolution of 4 cm⁻¹, with wavelengths that ranged from 650 to 4000 cm⁻¹.

2.3. Determination of SDF-1 and BMP-2 release from scaffold

The release of SDF-1 and BMP-2 from the scaffold in vitro was quantified using ELISA. First, the scaffolds were incubated in 2 mL PBS with vigorous shaking at 37 °C. At the desired time points (3 h, 12 h, 24 h, 4 d, 7 d, 10 d, 13 d, 19 d, 25 d, 31 d and 34 d), PBS was collected and stored at –20 °C prior to use, and the scaffold was incubated in 2 mL fresh PBS again. Finally, the SDF-1 and BMP-2 concentrations in the collected PBS were quantified with a SDF-1 and BMP-2 ELISA kit (R&D, USA) according to the manufacturer’s instructions. The cumulative release ratio was calculated as the ratio of the cumulative mass of SDF-1 or BMP-2 released at each time interval to their initial input amount in the scaffold, which is 2.0 μg and 400 ng for BMP-2 and SDF-1, respectively.

2.4. Migratory response of BMSCs to SDF-1 modified scaffold

To evaluate the recruitment capacity of SDF-1 on BMSCs, the Transwell chemotactic migration model was used. Briefly, 1 × 10⁴ cells were seeded in the upper chamber of a 24-well Transwell plate (pore size: 8 μm, Corning, USA), and scaffolds were placed in the lower chamber. After 24 h, the upper surface of the Transwell membrane was initially scraped with a cotton swab to remove the adherent cells and subsequently detached from the inserts. The cells that migrated to the lower side of the membrane were fixed with 4% paraformaldehyde (Biosharp, Shanghai, China) for 30 min and stained with 0.1% crystal violet (Solarbio, Beijing, China) for 10 min. The number of cells on the lower surface of the membrane was counted in five random high power (200×) microscopic fields in each well.

2.5. Cell adhesion and proliferation on scaffold

Cylinder scaffolds were used for the cell adhesion studies. Prior to the cell culture work, the scaffolds were sterilized under UV, followed by immersion in Oricell™ SD Rat MSC Growth Medium (Cyagen, Guangzhou, China) overnight. BMSCs were seeded into scaffolds in 96-well culture plate at a density of 2.0 × 10⁵ cells/well. The samples were placed in an incubator at 37 °C, 95% relative humidity and 5% CO₂ partial pressure. The culture medium was replaced every second day.

The morphology of the cells seeded on the cultured scaffold surface was examined via SEM. The cells were fixed in 4% paraformaldehyde at 4 °C for 1 h, followed by rinsing with deionized water three times. The samples were subsequently dehydrated through a gradient series of ethanol/water solution (10%, 20%, 35%, 50%, 70%, 85%, and 100%). Finally, the sample was dried using the CO₂ critical-point drying method and sputter coated with gold. The electron microscope was operated at 5 kV to image the samples.

Cell proliferation was measured using cell counting kit-8 reagent (CCK-8, Dojindo, Kumamoto, Japan). At the desired time points (1 d, 3 d, 5 d, 7 d and 9 d), the BMSC-seeded scaffolds were incubated in CCK-8 solution at 37 °C in an incubator with 5% CO₂ for 2 h. The solution obtained was measured at 450 nm using a spectrophotometer.

2.6. In vitro osteogenesis study

The alkaline phosphatase (ALP) activity, calcium content and expression of osteocalcin (OCN) proteins and genes related to osteogenesis were measured to assess the osteogenic differentiation of BMSCs. Prior to the analyses, all scaffolds were sterilized with UV. Osteogenic induction medium (OM, Cyagen, Guangzhou, China) was used to evaluate the ALP activity, calcium content and gene expressions. The scaffolds were placed in the upper chamber of a 6-well Transwell plate (pore size: 8 μm, Corning, USA), and 3 × 10⁵ cells were cultured in the lower chamber.

2.6.1. Alkaline phosphatase activity
The activity of ALP, a widely exploited early biochemical marker for osteogenic activity, was measured. After 7 d of culture, the cells were fixed in 4% paraformaldehyde, followed by a reaction with ALP staining solution (Yeasen, Shanghai, China) for 20 min. The ALP-positive cells were stained blue and visualized with microscopic fields.

2.6.2. Calcium content
Mineralization was evaluated by quantifying the formation of calcium phosphate by cells using Alizarin Red S (ARS) staining

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**Table 1**

Sample abbreviations used in this work.

<table>
<thead>
<tr>
<th>Name of sample</th>
<th>Description of sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Original scaffold of SF/nHAP without any biomolecules</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Scaffold with physically adsorbed SDF-1</td>
</tr>
<tr>
<td>BMP-2 (P)</td>
<td>Scaffold with physically adsorbed BMP-2</td>
</tr>
<tr>
<td>BMP-2 (E)</td>
<td>Scaffold with BMP-2 encapsulated into SF microspheres</td>
</tr>
<tr>
<td>S + B (P)</td>
<td>Scaffold with physically adsorbed SDF-1 and BMP-2</td>
</tr>
<tr>
<td>S + B (E)</td>
<td>Scaffold with physically adsorbed SDF-1 and BMP-2 encapsulated into SF microspheres</td>
</tr>
</tbody>
</table>

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2.6.4. Expression of osteogenic genes

Nuclei were stained with DAPI. OCN proteins, which are commonly used as late markers of osteogenic differentiation, were examined using immunofluorescence staining. After 21 d, the cells were fixed with 4% paraformaldehyde, treated with 0.5% Triton X-100 (Sigma-aldrich, USA) and blocked with 2% BSA (Solarbio, Beijing, China). After washing with PBS, the cells were stained against OCN primary antibody (Abcam, USA) at 10 μg/ml at 4 °C overnight. The cells were subsequently incubated in the secondary antibody of goat anti-mouse IgG H&L (1:500, Cy3®; Abcam, USA) for 1 h at 37 °C, and the nuclei were stained with DAPI.

2.6.3. OCN immunofluorescence staining

OCN proteins, which are commonly used as late markers of osteogenic differentiation, were examined using immunofluorescence staining. After 21 d, the cells were fixed with 4% paraformaldehyde, treated with 0.5% Triton X-100 (Sigma-aldrich, USA) and blocked with 2% BSA (Solarbio, Beijing, China). After washing with PBS, the cells were stained against OCN primary antibody (Abcam, USA) at 10 μg/ml at 4 °C overnight. The cells were subsequently incubated in the secondary antibody of goat anti-mouse IgG H&L (1:500, Cy3®; Abcam, USA) for 1 h at 37 °C, and the nuclei were stained with DAPI.

2.6.4. Expression of osteogenic genes

The expression of genes related to osteogenesis, including Alp, Runt-related factor-2 (Runx2), Osteopontin (Opn), and Ocn was analyzed by quantitative reverse transcription polymerase chain reaction (RT-qPCR). The RT-qPCR assay was performed as previously described to evaluate the expression levels of the genes of BMSCs cultured with the scaffold at 3, 7 and 14 d [29,30]. The primer sequences used for PCR amplification are listed in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an internal control, and all primers were synthesized by Invitrogen.

2.7. In vivo bone formation study

Sprague-Dawley (SD) male rats were purchased from the Experimental Animal Center of Soochow University (Suzhou, China). The animal handling and surgical procedures were conducted in accordance with protocols approved by the Ethics Committee at the First Affiliated Hospital of Soochow University.

2.7.1. Rat calvarial critical size defect model and scaffold implantation

The SD rats were randomly assigned to groups, including control, scaffold of SDF-1, BMP-2 (P), BMP-2 (E), S + B (P) and S + B (E), to evaluate the osteogenic potential in a cranial defect on the implantation of scaffold with or without BMP-2 and/or SDF-1, as well as the difference between the sequential release and the concomitant release of BMP-2 and SDF-1 from the scaffold. The scaffolds were implanted in defects (5 mm in diameter, one defect at the center of parietal bone for bioluminescence imaging (BLI) and two bilateral defects for micro computed tomography (μCT) imaging and histological analysis) in the calvarium of the SD, which were anesthetized using 2% isoflurane inhalation 3 days after the surgery. At 8 and 12 weeks post-surgery, a total of 72 SD rats (average weight of 300 g) were sacrificed using CO2 suffocation, and the calvarial specimen was harvested and fixed in 10% formalin for μCT imaging and histological analysis. The three dimensional (3D) structures of the regenerated bone tissue within the cranial defect area were evaluated with μCT (SkyScan 1176, SkyScan, Aartselaar, Belgium) with the following settings: 65 kV, 385 μA, and 1 mm Al filters. 3D reconstructions were performed with software software. A cylinder ROI of 4.8 mm in diameter was used for the bone volume fraction (bone volume (BV)/tissue volume (TV), means ± standard deviations of 6 rats).

2.7.2. BMSC recruitment in vivo

In vivo BLI was performed using the IVIS lumina series III imaging system (Caliper Life Science, Hopkinton, MA). A total of 18 SD rats (average weight of 120 g) were divided into six groups and were anesthetized using 2% isoflurane inhalation 3 days after the creation of a calvarial defect model. Each rat was injected with 1.0 × 10^6 BMSCs labeled with the firefly luciferase (Fluc) reporter gene (Synchem, Shanghai, China) through the tail vein. After injection at 30 min, 1 d, 3 d, 7 d and 14 d whole-body images of each rat were obtained. Imaging was conducted 20 min after the intra-peritoneal injection of the reporter probe of D-luciferin (Synchem, Shanghai, China). The BLI signals at the standardized region of interest (ROI) were investigated.

2.7.3. Micro-computed tomography analysis

After 8 and 12 weeks post-surgery, a total of 72 SD rats (average weight of 300 g) were sacrificed using CO2 suffocation, and the calvarial specimen was harvested and fixed in 10% formalin for μCT imaging and histological analysis. The three dimensional (3D) structures of the regenerated bone tissue within the cranial defect area were evaluated with μCT (SkyScan 1176, SkyScan, Aartselaar, Belgium) with the following settings: 65 kV, 385 μA, and 1 mm Al filters. 3D reconstructions were performed with software software. A cylinder ROI of 4.8 mm in diameter was used for the bone volume fraction (bone volume (BV)/tissue volume (TV), means ± standard deviations of 6 rats).

2.8. Statistical analysis

Experiments were performed in triplicate unless otherwise indicated. The data were expressed as the means ± standard deviations. Statistical analysis (GraphPad Software, Inc.; USA) was evaluated using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test to evaluate the differences between the groups. Differences at p < 0.01 and p < 0.05 were considered statistically significant.

3. Results

3.1. Characterization of composite scaffolds

The SF/nHAp based scaffold impregnated with BMP-2 and/or SDF-1 was prepared as illustrated in Fig. 1A. First, SF microspheres loaded with BMP-2 were fabricated using laminar jet break-up technology. The diameter of the spheres was controlled by adjusting the SF concentration. The microspheres were subsequently mixed with a suspension of SF and nHAp to prepare the SF/ nHAp scaffolds loaded with BMP-2 (BMP-2 (E)); SF/nHAp scaffolds were prepared as the control. Finally, the SDF-1 was physically adsorbed onto the control, BMP-2 (E) by dropping SDF-1 solution onto the scaffold to obtain scaffolds of SDF-1 and S + B (E), respectively. The difference between the sequential release and concomitant release of SDF-1 and BMP-2 on the effect of bone regeneration was also investigated by preparing the scaffold of
S + B (P), in which BMP-2 was directly adsorbed onto the control scaffold instead of encapsulated into microspheres.

The influences of the SF concentration and methanol treatment on the size and morphology of the microspheres were investigated. The diameter increased with increasing concentration. The spheres had average diameters of 189 ± 32, 201 ± 34, and 227 ± 40 μm for the SF concentrations at 2%, 4% and 6%, respectively. The SF concentration at 6% was used in the following experiment. Methanol treatment has been widely used to make SF microspheres insoluble via conformational transitions of SF from random coil to β-sheet [31,32]. As shown in Fig. 1B and C, the methanol treatment resulted in a decrease in the diameter of SF microspheres from 227 ± 40 to 112 ± 28 μm, which is consistent with the results reported by other groups [28]. Moreover, after treatment, the microspheres exhibited a porous structure and clearly rougher surface compared with the non-treated microspheres. A cross-section of these spheres indicated a porous structure, as shown in Fig. 1F. There are no obvious changes in the morphology or size of the microspheres with the loading of BMP-2 (SI, Fig. S2). Microspheres loaded with BMP-2 were incorporated into the suspension of SF and nHAp during the scaffold fabrication. The FTIR spectra of pure SF, SF/nHAp and SF/nHAp scaffold loaded with microspheres that contained BMP-2 are shown in Fig. 1D. The peaks at 1650 cm⁻¹ (amide I) and 1516 cm⁻¹ (amide II) correspond to the SF. The existence of nHAp in the SF scaffold is confirmed by the appearance of a peak at 1025 cm⁻¹, which is ascribed to the PO₄³⁻ characteristic peaks, and its intensity decreased with the incorporation of the microsphere loaded with BMP-2. The SEM characterization of the scaffold (Fig. 1E and F) indicated that the microspheres are integrated and homogeneously distributed within the scaffold (Fig. 1F). The average pore size of the SF scaffolds is 124 ± 42 μm, which decreased to 85 ± 25 μm following microsphere incorporation.

3.2. In vitro release of SDF-1 and BMP-2

The release profiles of SDF-1 and BMP-2 from the SF/nHAp scaffolds loaded with SDF-1 and/or BMP-2 in vitro were investigated using the corresponding ELISA kits, and the results are presented in Fig. 2. There was a burst release with 47% of the total SDF-1 released from the scaffolds in the first day; the release was subsequently slowed down with approximately 85% of the total SDF-1 released after 7 d. In contrast, the overall release of BMP-2 from the scaffold was comparatively slow and sustained compared with SDF-1; a less initial burst release with 21% of the total BMP-2 released was identified for the first day, and the BMP-2 was released for as long as three weeks. At 10 d, nearly 90% of the SDF-1 was released from the scaffold, whereas the remaining percent of BMP-2 was more than 50%, indicating the sequential release behavior of the two bioactive molecules from S + B (E) scaffold (Fig. 2A). For the scaffolds of SDF-1 and BMP-2 (E), irrespective of the combination, SDF-1 or BMP-2 exhibited nearly the same release behavior as that for the scaffold of S + B (E) (SI, Fig. S3). While for the scaffold of S + B (P) in which BMP-2 was directly adsorbed onto scaffold without using microspheres, the release profile of BMP-2 was found to be similar to that of SDF-1. A burst release with 54% of BMP-2 released from the S + B (P) scaffold was found in the first day; at 10 d, 85% BMP-2 was already released from the S + B (P) scaffold (Fig. 2B), indicating the concomitant release characteristic
from $S + B$ (P) scaffold in contrast to the sequential release characteristic from $S + B$ (E) scaffold.

3.3. In vitro cell migration

The properties of BMSCs were confirmed by expression of BMSC associated surface markers by flow cytometry and immunofluorescence staining (SI, Figs. S4 and S5). The ability of SDF-1 from the scaffold to mobilize BMSCs in vitro was evaluated using a Transwell system [15,33]. As shown in Fig. 3, cell migration across the Transwell membrane was identified as a result of the released SDF-1 from the scaffold. The numbers of the recruited BMSCs in the groups that contained SDF-1 were significantly increased compared with the control or scaffold loaded with BMP-2 alone, and the recruited BMSCs of the scaffold loaded with SDF-1 + BMP-2 were slightly higher than the scaffold loaded with SDF-1 alone.

3.4. Cell adhesion and proliferation on the scaffold

The biocompatibility of the SF/nHAP scaffold loaded with SDF-1 or/and BMP-2 was evaluated via a cell adhesion and growth assay. BMSCs were seeded and cultured on the scaffolds, and the morphology of the adhered cells was assessed via SEM after culture for 1, 5 and 9 d. The SEM results in Fig. 4A demonstrated that BMSCs adhered to the surfaces of all scaffolds. As the culture time increased, there was an increase in the number of cells on the scaffolds, and the cell morphology changed from round to spindle-shaped, which indicates that the SF/nHAP based scaffolds are biocompatible.

The proliferation of BMSCs on the scaffold loaded with BMP-2 and/or SDF-1 was assessed using a CCK-8 assay from 1 to 9 d. As shown in Fig. 4B, the cells exhibited a good proliferating ability on the scaffolds loaded with BMP-2. After 3 d of culture, the CCK-8 reading was slightly increased in the scaffolds with SDF-1 + BMP-2 compared with BMP-2 alone both in sequential and concomitant release systems ($S + B$ (E) vs BMP-2 (E) and $S + B$ (P) vs BMP-2 (P)). The results suggested that the composite scaffold with both bioactive molecules has a good ability to support cell proliferation, and $S + B$ (E) scaffold can support cell proliferation in a relatively long time. The cells exhibited the most apparent viability in the scaffold with SDF-1 + BMP-2 compared with the other scaffolds (SI, Fig. S6), and the result is consistent with that of the CCK-8 assay.

3.5. In vitro osteogenesis study

The ability of scaffolds to promote osteogenesis was assessed. The ALP activity, calcium content and osteogenesis marker of OCN of the BMSC culture with scaffolds incorporated with SDF-1 and/or BMP-2 were investigated, and the results are presented in Fig. 5. All the scaffolds contained BMP-2 exhibited higher ALP activity (no remarkably difference among these scaffolds) compared with the scaffolds without BMP-2. The scaffolds of $S + B$ (E) and BMP-2 (E) exhibited an obvious increase in the calcium content and OCN content measurements in 21 d, when compared to the $S + B$ (P) and BMP-2 (P); the scaffold of $S + B$ (E) exhibited the highest level among all groups.

To further investigate the effects of SDF-1 and BMP-2 on osteogenic differentiation, the expression of osteogenic genes,
including Alp, Runx2, Ocn and Opm, was quantified by RT-qPCR at 3, 7 and 14 d. Fig. 6A and B shows the expression levels of the Alp and Runx2 genes for the BMSCs cultured with the scaffolds incorporated with SDF-1 and/or BMP-2. The expression patterns were similar for these two genes. The scaffolds contained BMP-2 exhibited a significantly up-regulated expression of both genes compared with control scaffold, and the scaffold of S + B (E) exhibited the highest level at 7 d and 14 d. Fig. 6C and D shows the Opm and Ocn gene...
expression results, and these two gene expression patterns were also similar. There was no obvious difference among the six scaffolds at 3 d. However, at a prolonged period (7 d and 14 d), there were significantly increased levels of \textit{Opn} and \textit{Ocn} expression in the cells cultured in the scaffold of \( S + B \) (E) compared with the other scaffolds. The results demonstrated the promotion effect of the scaffold \( S + B \) (E) incorporated with SDF-1 and BMP-2 in a sequential release manner on the stimulation of osteogenic genes in BMSCs.

3.6. In vivo studies

The migration of BMSCs injected through the rat tail vein to the defects was assessed \textit{in vivo} via BLI (Fig. 7). Cell recruitment to the tissue around the site of the scaffolds incorporated with SDF-1 and/or BMP-2 was identified. At 30 min and 1 day after the injection, the D-Luciferin-labeled BMSCs were predominately retained in the lungs for all groups, with the exception of a slightly enhanced migration to the defect site for the SDF-1+BMP-2 group at 1 d. At 3 d, the luminescence intensity increased for all groups, particularly for the groups contained SDF-1, and the strongest signal was identified in the scaffold loaded with SDF-1+BMP-2. As the time increased to 7 d and 14 d, a significant decrease in the intensity of the bioluminescence signals was identified for all groups; however, the SDF-1 and SDF-1+BMP-2 groups continued to exhibit a higher intensity than the other three groups without SDF-1.

To evaluate the ability of the scaffolds loaded with SDF-1+BMP-2 as grafts to facilitate bone formation within a full-thickness bone defect \textit{in vivo}, we surgically created bilateral cranial bone defects in the rats. The implant area and bone formation in the defects were imaged with \( \mu \)CT (Fig. 8A and B). The images indicated that the bone content in the scaffolds of BMP-2 (E) and \( S + B \) (E) was increased compared with the other four scaffolds at both 8 and 12 weeks. New bone was formed from the edge toward the center of the defects. The bone volume faction was the highest in the scaffold of \( S + B \) (E) where SDF-1 and BMP-2 were released in a sequential manner. The bone content at 12 weeks was increased compared with 8 weeks, which indicates that the bone content in this group increased over time. At 12 weeks, the bone completely bridged the injury site for the scaffold of \( S + B \) (E). Histological results in Fig. 8C indicated the formation of new bone with the typical structure of the graft group at 12 weeks post-implantation. There was a thicker bone matrix in the scaffolds of \( S + B \) (E) compared with the other groups.

4. Discussion

\textit{In situ} tissue engineering for bone regeneration comprises a powerful strategy to treat bone defects, which are regulated by the interaction between bone progenitor cells and their environment, including biomaterials and bioactive molecules (e.g., chemokines and growth factors). The objective of this study was to develop a composite scaffold to initially enhance MSC homing through the delivery of the chemokine SDF-1 and then stimulate the osteogenic differentiation of these MSCs via the subsequent sustained release of BMP-2 from the scaffold. To accomplish this design, SF/nHAp scaffolds were used to incorporate SF microspheres encapsulated with BMP-2, and the prepared scaffolds physically absorbed SDF-1 to achieve cell-free scaffolds \( S + B \) (E) for the enhanced bone regeneration used in \textit{in situ} tissue engineering. Here, BMP-2 was loaded into SF microspheres to fulfill the subsequent controlled release purpose. For comparison, the scaffold of \( S + B \) (P) in which SDF-1 and BMP-2 were released in a concomitant way was prepared. In this \( S + B \) (P) scaffold, BMP-2 was directly dropped onto scaffold instead of encapsulated into SF microspheres. nHAp was assembled into the SF scaffold to promote bone regeneration through its osteoinductive effects [13].

Many techniques have been used to manufacture SF microspheres, including ball milling, spray-drying, double emulsion, self-
assembly, microfluidics and electro-spraying [34]. Of these methods, the laminar jet break-up technology has been demonstrated to be an efficient approach to produce SF microspheres with a high encapsulation efficiency of biomolecules. This technology is also scalable, simple, and may be operated using only aqueous SF solutions. Wenk et al. reported that SF microspheres prepared via this approach not only preserved the bioactivity of insulin-like growth factor I (IGF-I) but also enabled a striking sustenance of its release profile [28]. SF microspheres were prepared from a series of SF solutions with different concentrations. The SF microparticle size increased with an increasing SF concentration from 2% to 6%, which was attributed to the process in which the higher SF concentration induced a higher SF viscosity, thereby reducing the likelihood of the break-up of the solution into small droplets. However, further increase in the SF concentration to 9% is difficult to fabricate SF microspheres because of the nozzle blockage. Previous studies have confirmed that the SF concentration comprises a key factor in drug release properties, and the initial burst release and the release rates of the drugs encapsulated within the SF microspheres were reduced with the SF concentration increase [28]. These findings are likely a result of the bigger spheres prepared at a high SF concentration, which reduces the solvent penetration and limits its disruption of the interaction between BMP-2 and SF that control release [35]. In our study, the SF concentration at 6% was used to encapsulate BMP-2 for the sustained release purpose.

Fig. 7. BMSC migration to the defect cranial site. (A) Rats are shown at 30 min, 1, 3, 7 and 14 d post-injection; data indicate the systemic cell distribution of reporter cells. Red circles indicate the ROI. (B) Quantitative analysis of the luminescent signal on the ROI in (A). *p < 0.05 and **p < 0.01 compared with control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
the scaffold). However, it is suggested that the bioactivity of encapsulated BMP-2 could be well maintained even with methanol treatment [26,38]. BMP-2 loaded SF microspheres were subsequently incorporated into an SF/nHAp suspension to prepare the composite scaffold, followed by physically adsorbed SDF-1 on the surfaces to achieve an SDF-1+BMP-2 loaded scaffold of S + B (E). The release behaviors of SDF-1 and BMP-2 in S + B (E) scaffold are different; the SDF-1 exhibited a burst release in the first day, followed by a slower release for a period of 7 d, whereas the release of BMP-2 exhibited a substantially lower extent of burst release in the first day and a substantially longer release for up to 3 weeks. While for the S + B (P) scaffold, both SDF-1 and BMP-2 showed a burst release profile. This difference may be explained by the different incorporation methods [34]. Two mechanisms of both the diffusion of BMP-2 and degradation of SF may participate in the BMP-2 release process in S + B (E) scaffold. The former causes a burst release in the early stage, whereas the latter leads to the prolonged release of BMP-2 from microspheres. SDF-1 or BMP-2 from S + B (P) scaffold predominately attached at the scaffold surface via physical adsorption, whereas its burst release was mainly attributed to the diffusion mechanism [39].

We hypothesized that compared to the concomitant release from S + B (P), the sequential release of these two bioactive molecules from S + B (E) are beneficial for the promotion of bone regeneration because the initial burst release of BMP-1 promotes MSC homing to the graft areas in the early time, whereas the sustained release of BMP-2 maintains an effective concentration of BMP-2 to promote the transformation of MSCs into osteoblasts. To test this hypothesis and its potential application in bone defects, the in vitro cell migration, proliferation, and osteogenic differentiation of BMSCs, as well as the in vivo bone formation were assessed.

It has been reported that SDF-1 enhances the migration of MSCs; however, controversial results exist regarding the threshold of SDF-1 used to induce MSC mobilization [15,33]. Ji et al. indicated that the membranes loaded with SDF-1 in the range of 50–400 ng significantly induce MSC chemotaxis without dose-dependent effects [15]. In our experiment, 400 ng SDF-1 was used. Both the concomitant and sequential release of SDF-1 and BMP-2 has a synergistic effect on the chemotactic capability of BMSCs, which is consistent with previous reports [14,15,40]. This synergistic effect may be explained by the fact that in addition to the SDF-1/CXCR4 pathway involved in the process of BMSC homing induced by SDF-1 [14,15], BMP-2 may also recruit the cells [40], and the placental growth factor (PIGF) pathway plays an important role in the recruitment of BMSCs triggered by BMP-2 [16,40].

To determine the effects of SDF-1 and BMP-2 on the proliferation and osteogenic differentiation of BMSCs in vitro, a CCK8 assay, the ALP activity, the calcium content and the osteogenesis marker of OCN were measured. The scaffolds that contained BMP-2 induced significantly increased cell proliferation and osteoblastic activity compared with the scaffolds without BMP-2. The treatment with SDF-1 and BMP-2 together resulted in an even higher proliferation and osteoblastic activity and a greater potential to accelerate new bone formation compared with the scaffold loaded with BMP-2 alone, in both concomitant and sequential release systems. However, the controlled release of BMP-2 from the sequential release system induced higher osteoblastic activity compared with the concomitant release system at a prolonged period. Considering the addition of SDF-1 alone only slightly influences BMSC proliferation and ALP activity, it is suggested that SDF-1 alone cannot effectively enhance osteogenic differentiation in vitro [21]. The ability of osteogenic differentiation of BMSCs cultured in the scaffolds incorporated with SDF-1 and/or BMP-2 was also investigated by evaluating the expressions of a series of osteogenic genes, including Alp, Runx2, Ocn and Opn. Alp and Runx2 are typically used to confirm the early differentiation of osteoblastic cells, whereas Ocn and Opn are representative indexes for further bone maturation [18,40,41]. It is thus reasonable that the up-regulation of Alp and Runx2 is initiated as early as 3 d, whereas Ocn and Opn begin at 7 d. The scaffold of S + B (E) exhibited the highest gene expression for all markers after culture for 7 d, which supports the previously described results regarding the calcium content and ALP activity measurement.

The migration of BMSCs is of great importance for the bone regeneration [42,43], however, so far there are fewer reports on the in vivo investigation of migration of BMSCs compared with corresponding in vitro investigation. Tracking the injected cells via

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**Fig. 8.** (A) μCT results of the specimen of calvarial critical-sized defects and (B) the bone volume faction. The circle plates in (A) represent original calvarial defects. The white area above the circle plate and light area in the plate represent newly formed bone. (C) Sectional structure of calvarias repaired by scaffolds at 12 weeks post-implantation (25×). Scale bar – 1 mm. Bone-like structures were detected in all graft groups after H&E staining. The second row represents higher magnification images (200×) of the corresponding square boxes in the first row in (C). NB, HB and RSF represent new bone, host bone and remnant silk fibroin, respectively. Red arrow shows remnant of microspheres. Blue arrows indicate newly formed blood vessels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
imaging offers the feasibility to determine their survival, migration, immunogenicity and their function in bone regeneration in living animals. Among the optical imaging techniques, BLI is a promising tool for semi-quantitative measurements of biological processes because of a strong relationship between live cell number and the bioluminescence signal detected both in vitro and in vivo [44,45]. In this work, D-Luciferin-labeled BMSCs were injected through the rat tail vein and were found to be mainly trapped in the lungs within 1 d after injection (Fig. 7), in consistent with previous report [46,47]. At 3 d, BMSCs were gradually recruited to the defect site for all groups. Particularly, the groups containing SDF-1 showed stronger signal compared with the other groups, suggesting that SDF-1 is critical to induce the migration and homing of stem cells to a targeted site within the body [48]. The scaffolds of S + B (P) and S + B (E) exhibited slightly higher signal than the scaffold loaded with SDF-1 alone, indicating that the released BMP-2 did not interfere the function of SDF-1 but might provide a synergistic effect to improve the recruitment of BMSCs to the defect sites, which is in consistent with our experimental data of in vitro migration assay. Bioluminescence decreased over time, suggesting that most of the injected cells were dead after 14 d following the transplantation. The lower survival rate for the injected cells was also found in former group [47]; this phenomenon may likely be attributed to the effect of immune rejection [44].

Besides recruiting the BMSCs to the implanted area, the SF/nHAp scaffolds loaded with SDF-1+BMP-2 may substantially enhance bone regeneration in rat cranial defects; After 12 weeks of implantation of S + B (E), the bone completely bridged the injury site as supported by the BLI, μCT and histological results. The extent of bone regeneration was formed in the order of S + B (E) > BMP-2 (E) > S + B (P) > BMP-2 (P) > SDF-1 > control. There was almost no bone regeneration at the sham-surgery control sites (SL Fig. S7). In comparison with the in vitro results, the scaffolds loaded with SDF-1 alone may also promote bone regeneration. These phenomena may be explained by the fact that there are many other types of cells, such as osteoblast progenitor cells (OPCs) and endothelial progenitor cells (EPCs), in the circulating blood [49,50]. OPCs and EPCs have the potential to facilitate osteogenesis [51] and angiogenesis [52], respectively. SDF-1 has an inherent ability to enhance the recruitment of these cells [53,54], and BMP-2 has a potential to promote the osteogenic differentiation of bone marrow-derived stem/progenitor cells and recruit the cells [40,55]; thus, the release of these two factors from the scaffold may promote the recruitment of hematopoietic and bone marrow-derived stem/progenitor cells. The former cells will generate new blood vessels, whereas the latter cells exhibit the ability to be osteoblasts by BMP-2 stimulation and result in enhanced bone regeneration [40]. In addition, SDF-1 provides synergistic effects that support BMP-2-induced, osteogenic differentiation of mesenchymal C2C12 cells [56] or BMSC-mediated bone formation [19]. Hosogane et al. demonstrated a regulatory role of SDF-1 in BMP-2-induced osteogenic differentiation of MSCs, in which perturbation of the SDF-1 signaling affected the differentiation of MSCs towards osteoblastic cells in response to BMP-2 stimulation [14]. It was further demonstrated that the effect of SDF-1 on BMP-2-induced osteogenesis was mediated via intracellular Smad and Erk activation [14,21] or Smad and MAPK activation [56].

Angiogenesis and the vascularization capability are critical for the survival of engineered grafts following implantation [57]. Histological results (Fig. 8C) show the presence of blood vessel in newly formed bones. Thus, further experiments are being conducted in our group, such as testing angiogenesis after the implantation of S + B (E). Moreover, histological results (Fig. 8C) indicate that most SF scaffold was degraded following an implantation period of 12 weeks; thus, the influence of the scaffold degradation behavior on bone formation will also be investigated.

5. Conclusions

In the present study, we developed a SF/nHAp based scaffold incorporated with SDF-1 and BMP-2 loaded SF microspheres to induce the sequential and controlled release of SDF-1 and BMP-2. The rapid initial release of SDF-1 promoted BMSC recruitment, and the relatively slow and sustained release of BMP-2 facilitated osteogenic differentiation both in vitro and in vivo. Compared with the concomitant release, the sequential release of SDF-1 and BMP-2 from scaffolds increased bone regeneration in rat cranial defects, and the bone completely bridged the injury site after 12 weeks of implantation. The application of these scaffolds may comprise a powerful platform in bone tissue engineering to treat bone defects.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81371930, 21604059). Key Talented Man Project of Jiangsu Province (RC2011102) and Standardized Diagnosis and Treatment Project of Key Diseases in Jiangsu Province (BE2015641). Y. Zhang also acknowledges the Natural Science Foundation of Jiangsu Province (BK20160321) and the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (16KJB430029).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2016.08.023.
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