Engineering bone regeneration with novel cell-laden hydrogel microfiber-injectable calcium phosphate scaffold

Yang Song^{1,3*}, Chi Zhang^{2,3*}, Ping Wang³, Lin Wang^{3,4}, Chunyun Bao^{2,3}, Michael D. Weir³, Mark A. Reynolds³, Ren Ke⁵, Liang Zhao^{3,6}, Hockin H. K. Xu^{2,7,8}

¹ Department of Prosthodontics, Guanghua School of Stomatology, Guangdong Provincial Key Laboratory of Stomatology, Sun Yat-sen University, Guangzhou, Guangdong, China

² State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, Sichuan 610041, China

³ Department of Endodontics, Periodontics and Prosthodontics, University of Maryland School of Dentistry, Baltimore, MD 21201, USA

⁴ VIP Integrated Department, School and Hospital of Stomatology, Jilin University, Changchun, Jilin 130011, China

⁵ Department of Neural and Pain Sciences, School of Dentistry, & Program in Neuroscience, University of Maryland, Baltimore, MD 21201, USA

⁶ Department of Orthopaedic Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, China

⁷ Center for Stem Cell Biology and Regenerative Medicine, University of Maryland School of Medicine, Baltimore, MD 21201, USA

⁸ University of Maryland Marlene and Stewart Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD 21201, USA

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Correspondence: Dr. Ping Wang, University of Maryland School of Dentistry, Baltimore, MD 21201, email: dentistping@gmail.com; Dr. Liang Zhao, Department of Orthopedic Surgery, Southern Medical University, Guangzhou, Guangdong 510515, China, email: lzhaonf@126.com; Dr. Hockin H. K. Xu, Director of Biomaterials & Tissue Engineering Division, Department of Endodontics, Periodontics and Prosthodontics, University of Maryland Dental School, Baltimore, MD 21201, USA (Email: hxu@umaryland.edu).

* The first two authors contributed equally.

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Abstract

Cell-based tissue engineering is promising to create living functional tissues for bone regeneration. The implanted cells should be evenly distributed in the scaffold, be fast-released to the defect and maintain high viability in order to actively participate in the regenerative process. Herein, we report an injectable calcium phosphate cement (CPC) scaffold containing cell-encapsulating hydrogel microfibers with desirable degradability that could deliver cells in a timely manner and maintain cell viability. Microfibers were synthesized using partially-oxidized alginate with various concentrations (0-0.8%) of fibrinogen to optimize the degradation rate of the alginate-fibrin microfibers (Alg-Fb MF). A fibrin concentration of 0.4% in Alg-Fb MF resulted in the greatest enhancement of cell migration, release and proliferation. Interestingly, a significant amount of cell-cell contact along the long-axis of the microfibers was established in Alg-0.4%Fb MF as early as day 2. The injectable tissue engineered construct for bone reconstruct was fabricated by mixing the fast-degradable Alg-0.4%Fb MF with CPC paste at 1:1 volume ratio. *In vitro* study showed that cells re-collected from the construct maintained good viability and osteogenic potentials. *In vivo* study demonstrated that the hBMSC-encapsulated CPC-MF tissue engineered construct displayed a robust capacity for bone regeneration. At 12 weeks after implantation, osseous bridge in the rat mandibular defect was observed in CPC-MF-hBMSCs group with a new bone area fraction of $(42.1 \pm 7.8)\%$ in the defects, which was more than 3-fold that of the control group. The novel tissue-engineered construct presents an excellent prospect for a wide range of dental, craniofacial and orthopedic applications.

1. Introduction

With an aging population and numerous sports- and traffic-related injuries, there is an increasing need for bone repair and regeneration. In particular, bone defects with limited intrinsic regenerative potential represent a considerable surgical challenge. Cell-based tissue engineering approaches are highly promising to address this issue through the construct of a scaffold with living cells, with the aim to generate a cell-driven, functional tissue rather than to fill the defect with a nonliving scaffold [1-3]. An effective tissue-engineered construct should act both as a scaffold to bridge the defect and as a cell delivery vehicle to distribute cells throughout the scaffold to promote tissue regeneration [1, 4, 5].

Calcium phosphate cement (CPC), first developed in 1986 and approved in 1996 by the Food and Drug Administration (FDA) for craniofacial defect repairs, is one of the promising bone substitutes with excellent bioactivity, biocompatibility and osteo-conductivity [6, 7]. An aqueous paste can be injected into the osseous defects and set *in situ* during surgery, which can be resorbed and replaced by new bone *in vivo* [6-12]. A major challenge is how to efficiently deliver live cells in the CPC scaffold with the aim to boost bone regeneration. The cell delivery system critically affects cell viability, growth and differentiation, and thus plays a decisive role for the subsequent success of the tissue-engineered construct[13-15]. An ideal cell delivery vehicle should be able to protect cells from hazards during CPC setting and the injection operation, and is fast degradable to release the viable cells throughout the scaffold after CPC has set. To fabricate such a cell delivery system, natural hydrogel biopolymers, such as alginate, are of great interest, because they can be cross-linked under mild conditions to form highly hydrated gel with good biocompatibility and better

mechanical properties [16-18]. One major concern is that alginate degrades very slowly and uncontrollably in mammals[19]. The controlled degradability of a biomaterial is often a key factor in cell release and tissue replacement. The degradation rate of alginate can be controlled by manipulating polymer molecular weight by Gamma-irradiation[20], partial oxidation of alginate to make it susceptible to hydrolysis[21], and modification of the composition by adding fast-degradable component such as fibrin[22]. Fibrin is the primary protein component of a blood clot and an important regulator in the wound healing process. It provides binding sites for cell attachment, migration and proliferation[23], and can be degraded by plasmin-mediated fibrinolysis within a few days *in vivo*[24]. Such fast degradability is undesirable for fibrin to be solely used as a scaffold, but a valuable trait to be used as a component in alginate hydrogel to develop a fast-release cell delivery system.

In the present study, the alginate degradability was modified by partial oxidation and by incorporating fibrin into alginate. Human bone marrow mesenchymal stem cells (hBMSCs) were encapsulated in alginate-fibrin hydrogel microfibers (Alg-Fb MF). These microfibers were used as cell delivery vehicles to protect the encapsulated cells during CPC paste mixing, injection and setting reactions. The novel cell-laden microfiber-CPC construct could be injected or placed into bone defects and harden *in situ*. Once delivered, these microfibers could degrade inside the scaffold to release cells and subsequently create long macroporous channels in CPC for cell migration, re-organization and tissue ingrowth.

The objectives of this study were to: (1) synthesize a novel cell-encapsulating Alg-Fb MF that could quickly degrade and release the cells; (2) investigate the viability, proliferation and osteogenic differentiation of cells released from microfibers; and (3) develop an

injectable cell-encapsulating Alg-Fb MF-CPC tissue-engineered construct and investigate its bone regeneration in a rat mandibular bone defect model for the first time. The following hypotheses were tested: (1) Microencapsulation and injection in CPC paste would not harm the encapsulated cells; (2) the encapsulated cells could be quickly released from the microfibers after delivery; (3) hBMSC-encapsulated CPC-MF tissue-engineered construct would display a superior capacity for bone regeneration in vivo, compared to control.

2. Materials and methods

2.1 Cell culture

hBMSCs were obtained from Lonza (Allendale, NJ) and cultured in MSC medium, which consisted of low-glucose Dulbecco's modified eagle medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 1% penicillin/streptomycin, 0.25% gentamicin and 0.25% fungizone (Gibco). Routine characterization of hBMSCs was provided by the manufacturer (Lonza). It included characterization for surface markers and functional testing for tri-lineage differentiation. Passage 4 cells were used in the following experiments.

2.2 Synthesis of cell-encapsulating Alg-Fb MF

Alginate, a family of natural polysaccharides derived primarily from brown seaweed, could form hydrogel by fast cross-linking with multivalent cations under mild conditions[25]. In the present study, alginate (64% guluronic acid, MW = 75,000-220,000g/mol, ProNova, Oslo, Norway) was oxidized at 7.5% oxidation to increase its degradability, following

previous procedures[22]. The oxidized alginate was dissolved in saline at a concentration of 2%. Fibrinogen from bovine plasma (Sigma, St. Louis, MO) was added to the oxidized alginate solution to achieve a series of concentration of 0.1%, 0.4% and 0.8% (wt/volumn). hBMSCs were resuspended in the alginate-fibrinogen solution at a density of 1×10^6 cells/mL. Alg-Fb MF were synthesized by extruding the cell solution into a bath of calcium ions and thrombin. This system consisted of a syringe pump (NE-300, New Era Pump Systems, Farmingdale, NY), a 10 mL syringe (BD) loaded with cell solution, a 27 G stainless flat-end steel needle (210 μ m inner diameter), a laboratory stirrer (Daigger, Vernon Hills, IL) and a glass beaker containing 100 mL of bath fluid with 100 mmol/L calcium chloride (Sigma) and 1 NIH units per mL of thrombin (Sigma). The syringe pump was placed vertically and fed the cell solution into the needle tip at a rate of 6 mL/min. When the alginate-fibrinogen-based cell solution was sprayed into the bath fluid, calcium ions caused the alginate to cross-link, while the reaction between fibrinogen and thrombin produced fibrin. This produced hydrogel microfibers which were incubated in the bath for 20 min for complete gelation. The following fibrinogen concentrations were used in the alginate in order to determine the best cell delivery Alg-Fb MF:

- (1) Alginate microfibers without fibrinogen (control group, designated as “Alg-0%Fb MF”)
- (2) Alginate microfibers with 0.1% fibrinogen (designated as “Alg-0.1%Fb MF”)
- (3) Alginate microfibers with 0.4% fibrinogen (designated as “Alg-0.4%Fb MF”)
- (4) Alginate microfibers with 0.8% fibrinogen (designated as “Alg-0.8%Fb MF”)

The diameters of hydrogel fibers were determined by measuring 50 randomly-selected fibers (n = 50) using an optical microscope (Eclipse TE-2000S, Nikon, Melville, NY).

2.3 Degradation of Alg-Fb MF

The degradation of Alg-Fb MF was measured by calculating the percentage of dry weight loss of the microfibers with time, following previous studies[26-28]. Briefly, the hydrogel microfibers, fabricated from 1 mL of the cell solution, were collected in 40 μ m nylon cell strainers (BD Falcon, Franklin Lakes, NJ) which had been weighed and marked in advance. After washing with 5 mL of DMEM medium, the cell strainers with collected microfibers were inserted into 6-well plates, and 6 mL of MSC growth medium was added into each well to immerse the hydrogel microfibers. The 6-well plates with hydrogel microfibers were incubated in a humidified incubator (5% CO₂, 37 °C) for 8 days. The cell growth medium was changed every 2 days. Twenty-five wells were prepared for each group, with five wells for each time period (0, 2, 4, 6 and 8 days). On the day of harvest, the remaining hydrogel microfibers in the cell strainers were washed with distilled water gently and then moved to a ventilation hood for pre-dry for 6 hours, followed by lyophilization and weighing of the residual weight. To evaluate the degradation of the hydrogel microfiber with time, the percentage of dry weight loss (P_{DWL}) of the microfiber at each time period was calculated as $P_{DWL} = [(W_0 - W_T) / W_0]$, where W_0 is the dry weight of microfiber at 0 d, W_T is the dry weight of microfiber at one of the time periods tested[29].

2.4 Cell release and proliferation in Alg-Fb MF

hBMSCs were encapsulated in the aforementioned four groups of Alg-Fb MF. Cell-encapsulating Alg-Fb MF (200 μ L) was added to each well of 6-well plates with 2 mL

MSC growth medium and cultured up to 8 days. Cell medium was changed every 2 days.

A total of 24 wells per group were prepared, with six wells culturing for 2, 4, 6 and 8 days.

To examine cell release and viability, the samples were stained with a live-dead kit (Molecular Probes, Eugene, OR) for 30 min, which contained 2 μ M calcein-AM and 2 μ M ethidium homodimer-1. The samples were then examined via an epifluorescence microscopy (Eclipse TE-2000S, Nikon, Melville, NY). Live cells were stained green and dead cells were stained red. For quantification of cell viability and proliferation both inside and outside microfibers, a fluorescence microplate protocol was used. Cells were collected from five wells of each group at every time point for cell counting ($n = 5$). To collect both the cells retaining in the un-degraded Alg-Fb MF and those released to the tissue culture polystyrene, a dual-treatment was performed. Two mL of alginate depolymerization solution (110 mM sodium citrate, 40 mM EDTA and 150 mM sodium chloride, pH 6.8 in distilled water) was added to each well and incubated for 10 minutes[30]. Then, the dissolved Alg-Fb cell suspension was collected to a 50 mL centrifuge tube. The released cells that were adherent to the tissue culture polystyrene were collected via a regular cell detachment method using 0.25% trypsin-EDTA (Gibco) for 3-5 minutes. Cell suspension collected from microfibers and tissue culture polystyrene from one well was collected into the same centrifuge tube. The cells were centrifuged to pellet and re-suspended in 1 mL PBS. Then, 100 μ L of cell resuspension was added into each well of a 96-well plate and incubated with 100 μ L 2X concentrated Live-Dead staining solution (Molecular Probes, Eugene, OR) for 45 minutes. Three replicates from the same cell suspension were prepared for the measurement. The fluorescence emissions were measured via a microplate reader

(M5 SpectraMax, Molecular Devices, Sunnyvale, CA) at 530 nm for the live cells (calcein AM stain) and 645 nm for the dead cells (ethidium homodimer-1 stain, EthD-1). The live cell number at each time point was calculated via a standard curve made by a series of known live cell numbers. The cell proliferation index (CPI) was calculated by normalizing the cell counts for both the control and experimental groups to the initial numbers, following a previous study[31]. $CPI = N_T/N_0$, where N_T is the live cell number at the tested time, N_0 is the average live cell numbers at 0 day.

2.5 Fabrication of CPC-MF-hBMSC constructs

CPC powder was prepared following previous studies[32, 33]. Briefly, TTCP [$Ca_4(PO_4)_2O$] was synthesized using DCPA ($CaHPO_4$) and calcium carbonate (both from J.T. Baker, Philipsburg, NJ) which were mixed and heated at 1500 °C (Model 51333, Lindberg, Watertown, WI). The mixture was ground to obtain TTCP powder with a median particle size of 5 μm . DCPA was ground to obtain a median particle size of approximately 1 μm . Then the TTCP and DCPA powders at 1:3 molar ratio were mixed to form the CPC powder[4, 33]. CPC powder was mixed with distilled water at a powder to liquid mass ratio of 2 to 1. The CPC paste was mixed with cell-encapsulating Alg-Fb MF at a volume ratio of 1:1 to form the CPC-MF-hBMSC construct. Based on previous investigation of degradability and cell-releasing capability in Sections 2.3 and 2.4, Alg-0.4%Fb MF was selected for the following *in vitro* and *in vivo* studies.

2.6 Re-collected MF after extrusion

Immediately following the preparation of CPC-MF-hBMSC paste, the paste was loaded into a 5 mL syringe with a tip opening of 2.7 mm for injection. A syringe pump was used to control the extrusion speed at 4 mL/min. A 100 µm nylon cell strainer (BD Falcon, Franklin Lakes, NJ) was used to collect the extruded paste. The CPC paste was washed away by serum-free DMEM medium. The hydrogel microfibers left in the cell strainers after repetitive washing were referred to as the re-collected MFs.

2.6.1 Cell re-collection percentage

The re-collected MFs were dissolved in alginate depolymerization solution for 10 minutes. The resulting Alg-Fb cell suspension was centrifuged to obtain the re-collected hBMSCs. These cell samples were re-suspended in PBS for cell counting. The live-dead staining fluorescence microplate protocol was used to calculate the live cell percentage as stated in Section 2.4 ($n = 5$). The percentage of cell re-collection after extrusion was calculated as $P_{RE} = (N_{RE}/N_{TOTAL})$, where N_{RE} is the number of re-collected cell after extrusion, N_{TOTAL} is the number of cells in 1 mL cell solution used to produce the hydrogel microfiber.

2.6.2 Cytokinetic analysis by CCK-8

To investigate whether the paste mixing and extrusion would harm the encapsulated cells, a cell counting kit (CCK-8, Sigma) was used to evaluate cell viability after extrusion. The re-collected hBMSCs were resuspended at a density of 2×10^4 /mL. 100 µL of cell suspension was added into each well of a 96-well plate and cultured for 8 days. Cytokinetics of the re-collected cells was compared with untreated hBMSCs which were

seeded at the same density into the 96-well as control. At each time point, 10 μ L of CCK-8 solution was added to 100 μ L medium in each well and incubated at 37 °C for 2 hours. The optical density values (OD value) were measured on a microplate reader (M5 SpectraMax) at a wavelength of 450 nm and wavelength of 630 nm. The OD of eight consecutive days in each group was plotted to develop the growth curve of the seeded cells ($n = 5$)[34].

2.6.3. Osteogenic differentiation and mineralization

Cell-loaded Alg-0.4%Fb MF was collected after extrusion of CPC-MF-hBMSC construct from the syringe. 100 μ L recollected MFs, in which 1×10^5 cells were estimated to be encapsulated, was placed into a well of a 24-well plate and cultured for 3 weeks in osteogenic medium (OS), which consisted of MSC growth media plus 100 nM dexamethasone, 10 mM β -glycerophosphate, 0.05 mM ascorbic acid, and 10nM 1 α ,25-dihydroxyvitaminD3 (Sigma). Then the mineral deposited by hBMSCs in microfibers was stained with Alizarin Red S (ARS, Millipore, Billerica, MA) following the manufacturer's instruction[32].

2.7 *In vivo* mandibular bone defects in rats

The critical-sized circular defects were created in both sides of the mandibular rami of athymic nude rats (Hsd:RH-Fox1mu, male, 8 weeks old, 200-250 g, Harlan, Indianapolis, IN). The protocol was approved by University of Maryland Baltimore (IACUC # 0813018), following NIH guidelines. Under general anesthesia (75 mg/kg body weight of ketamine and 10 mg/kg body weight of xylazine), a mucoperiosteal flap was raised at the buccal side of the bone around the mandibular angle. A 5 mm diameter full-thickness circular defect was

created at the mandibular ramus under continuous saline irrigation. Cell-encapsulating CPC paste was prepared at the time of implantation. Alg-0.4%Fb MF was mixed with CPC paste at a volume ratio of 1:1. Six animals with bilateral defects, with a total of 12 defects, were tested. (1) For CPC-MF-hBMSC group ($n = 6$), the cell-encapsulating CPC-MF paste was implanted into one of the two defects in each rat. (2) For CPC-MF-Control ($n = 6$), CPC-MF paste without cells was implanted into another defect in each rat as a side-by-side control.

2.8 Histological and histomorphometric analyses

At 12 weeks after implantation, all animals were euthanized, and the implants embedded in the surrounding native bone were retrieved and fixed in 10% formalin for 48 hours. Specimens were decalcified and embedded in paraffin. The central part of the implant and defect was cut into 5 μm thick sections and stained with hematoxylin and eosin (HE). The slices were scanned using a Scanscope Digital Slide Scanner (Aperio, Vista, CA) and analyzed by Imagescope software (Aperio). New bone area, residual CPC area and total defect area were measured within the boundaries of defects in each section by Image Pro Plus Software (Media Cybernetics, Carlsbad, CA). New bone area fraction was calculated as the new bone area in each defect divided by the total defect area ($n = 6$). Residual CPC area fraction was calculated as the residual CPC area divided by total defect area ($n = 6$).

2.9 Statistical analyses

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS 17.0, Chicago, IL). All data were expressed as mean value $\pm$ standard deviation (SD). For

in vitro results, statistical significance was analyzed using one-way and two-way analyses of variance (ANOVA). A paired-sample t test was used to analyze the data of self-control comparisons for the *in vivo* results. A confidence level of 95% was considered significant.

3. Results

The alginate hydrogel microfibers were in a long and continuous form. An example of microfiber is shown in Fig. 1A. As the fibrinogen concentration increased from 0.1% to 0.8%, the mean diameters of the Alg-Fb MFs slightly increased from $251 \pm 25 \mu\text{m}$ to $276 \pm 32 \mu\text{m}$ (mean $\pm$ sd, $n = 50$) ($p > 0.05$). At 0 d (immediately after fabrication of cell-encapsulated Alg-Fb MFs), the encapsulated hBMSCs were spherical and distributed throughout the hydrogel microfibers (Fig. 1B). Live cells appeared as green dots, with a few dead cells (red dots) in the microfibers (Fig. 1C).

Controlled degradation is a key requirement for cell delivery vehicles. As illustrated in Fig. 2A, the percentage of dry weight loss (P_{DWL}) increased over time for all Alg-Fb MF groups. At 8 d, Alg-0%Fb MF control exhibited $\leq 20\%$ dry weight loss, indicating a slow degradation. In contrast, microfibers with fibrin demonstrated a much faster degradation rate. The Alg-0.4%Fb MF and Alg-0.8%Fb MF reached 90% dry weight loss at 6 d.

Cell viability was not adversely affected by encapsulation, as shown by live/dead staining (Fig. 1 & 2). At 8 d, Alg-0%Fb MF still maintained its integrity. Only a few cells demonstrated a spreading morphology, while most cells were in a spherical shape with no evidence of cell releasing from the Alg-0%Fb MF (Fig. 2B and 2C). In Alg-0.1%Fb MF group, cells started to migrate out of the microfibers at 4 d (Fig. 2D). The released cells

demonstrated a healthy polygonal morphology as indicated by black arrows in Fig. 2D. At 8 d, more cells were released and robustly proliferated (Fig. 2E). In the Alg-0.4%Fb MF group, cells were released from the microfibers as early as day 2 (arrowed in Fig. 1D). Interestingly, at day 2, cells still inside the Alg-0.4%Fb MF appeared to be elongated and spindle-like, extending their filopodia and establishing intercellular connections to create a complicated 3 dimensional cellular network (arrowed in Fig. 1E). Alg-0.4%Fb MF lost its integrity and released most of the encapsulated cells at 4 d (Fig. 1F). The released hBMSCs attached to tissue culture polystyrene and proliferated rapidly. Fluorescent images showed abundant live cells (green) with a few dead cells (red) at 8 d (Fig. 1H and 1I). The cells in Alg-0.8%Fb MF demonstrated similar behavior to that of the Alg-0.4%Fb MF group at day 2, in which cells started to stretch out and a few cells migrated out of the microfibers (Fig. 3A, 3B). However, at day 4, it appeared that cells in Alg-0.8%Fb MF would rather adhere to each other and assemble to the orientation along the long axis of the microfibers, instead of migrating outside (Fig. 3C, 3D). The contact-inhibition seemingly compromised the cell proliferation. Thus till 8 d, cell confluence for Alg-0.8%Fb MF was not as good as that for Alg-0.4%Fb MF (Fig. 3E). In addition, more dead cells were observed in Alg-0.8%Fb MF group than in the other groups (Fig. 3F).

The cell proliferation index (CPI) results are plotted in Fig. 4A. In Alg MF control, the number of hBMSCs appeared to be constant in the first 8 days. With the addition of fibrin, CPI increased over time. In Alg-0.1%Fb MF group, CPI underwent a slight decrease at 2 d, but then increased to 2.86 ± 0.16 at 8 d. Cells in Alg-0.4%Fb MF group demonstrated the earliest and fastest proliferation. CPI increased to 1.39 ± 0.22 , 2.33 ± 0.13 , 3.89 ± 0.15 ,

4.35 ± 0.14 at 2, 4, 6 and 8 days, respectively, significantly higher than other groups.

In Fig. 4 (B), $P_{\text{LIVE}} > 80\%$ in the freshly fabricated hydrogel microfibers was achieved in all groups ($p > 0.05$). In control, P_{LIVE} decreased to $76.2 \pm 4.4\%$ at 8 d. Alg-0.1%Fb MF remained a relatively stable level of P_{LIVE} over 80%. P_{LIVE} in Alg-0.4%Fb MF increased to $93.5 \pm 1.8\%$ at 8 d, which was significantly higher than other groups. In Alg-0.8%Fb MF group, P_{LIVE} declined to $64.3 \pm 4.1\%$ at 4 d.

Because of its fast-degradability and superior cell-releasing capability, Alg-0.4%Fb MF was selected for subsequent experiments. The freshly fabricated CPC-MF paste had good flowability and could be completely injected. The percentage of cells re-collected after extrusion (P_{RE}) was as high as $82.3 \pm 4.6\%$. P_{LIVE} of the re-collected cells was $84.9 \pm 2.1\%$, comparable to that of the freshly fabricated MF ($85.2 \pm 1.83\%$, in Fig. 4B, $p > 0.05$). CCK-8 assay showed that the growth of the re-collected hBMSCs was similar to control (Fig. 4C). Representative ARS staining image of mineral synthesis was showed in Fig. 4D. After 14 d osteogenic induction, the encapsulated hBMSCs formed abundant nodules of mineralization, which was stained red. These results indicate that the microfibers could protect hBMSCs during the CPC mixing and injection processes, and the delivered cells from CPC-MF construct maintained high viability and osteogenic differentiation potential.

Representative histological H&E images from the animal study are shown in Figs. 5 and 6. In CPC-MF control group (Fig. 5), only a limited amount of new bone was formed at the junction areas between the scaffold and native bone (Fig. 5A and 5B). Infiltration of connective tissue and muscular tissue into the defect was observed (Fig. 5C and 5D). In sharp contrast, samples in the hBMSC-encapsulated CPC-MF group showed nearly complete

osseous bridge of the defects and much greater amounts of new bone (Fig. 6A and 6B). Higher magnification examination revealed newly-formed trabecular and woven bone with a normal, organized and mature bone morphology in the defects (Fig. 6C and 6D).

As showed in Fig. 7A, the new bone area fraction of CPC-MF-hBMSC group was $(42.1 \pm 7.8)\%$, much higher than $(12.6 \pm 6.7)\%$ of CPC-MF-Control group ($p < 0.05$). In Fig. 7B, the residual CPC area fraction of CPC-MF-hBMSC was $(5.8 \pm 1.5)\%$, much less than $(17.3 \pm 2.7)\%$ of control ($p < 0.05$).

4. Discussion

In this study, an injectable Alg-Fb MF stem cell delivery system was developed, and a novel tissue engineered construct by dispersing the cell-encapsulating Alg-Fb MF in CPC for injectable bone tissue engineering was investigated. Alg-Fb MF protected the cells during CPC mixing and injection, and supported the viability, migration and differentiation of encapsulated hBMSCs. By implanting the CPC-MF construct in a mandibular bone defect model, more than 3-fold increase in new bone formation was achieved via the CPC-MF-hBMSCs group, compared to the control without cell delivery.

An efficient cell delivery system is of crucial importance for the success of tissue-engineered constructs[35]. The cell delivery vehicle needs to disperse cells evenly in a three-dimensional scaffold, to protect and maintain cell viability and functionality, and to deliver cells in a timely and controlled manner. Hydrogels, based on both natural and synthetic polymers, have been used for this purpose[17, 36]. Cells are typically encapsulated by gelling cell suspension in a hydrogel solution to form a solidified matrix. A

frequently-used material is alginate[37-39]. With advantages of mild gelation and easy manipulation, alginate can be produced in various forms, including microbeads[40, 41], microfibers[42,43], and hydrogel blocks[44]. Cell encapsulation in bulk alginate hydrogels offers the simplest encapsulation method. However, due to nutritional deficiency caused by the long diffusion path and accumulation of waste products inside the bulk, such an alginate hydrogel block often undesirably forms a central necrotic core[44]. The use of small microbeads can facilitate the gas, nutrition and waste diffusion, thus promote cell viability and regenerative potential of the constructs. Our previous studies successfully generated an oxidized alginate-fibrin microbead with several hundred microns in size[22]. These microbeads started to degrade at 4 d and released the encapsulated cells. In the present study, cells were encapsulated in alginate-fibrin hydrogel in the form of microfibers. Encapsulation of cells in microfibers possesses several advantages over microbeads. First, microfibers with tailored lengths and diameters can be fabricated via the simple method of needle extrusion/external gelation. In contrast, in order to break up the alginate droplets to form microbeads in size of several hundred microns, air injection and electronic injection are often required[45]. The air flow or applied electrostatic force during microbead formation may impose harsh shearing forces on the cells. Furthermore, air flow can form “tails” on microbeads which may cause an immune response[45]. Second, microfibers with diameters of several hundred microns and long lengths of millimeters are relatively easy to handle individually. Third, microfibers can provide more space for cellular self-assembly through which living cells may organize into functional units, allowing the cells to grow, migrate and differentiate in the extracellular matrix[46]. Fourth, when mixed together with another

scaffolding matrix such as CPC, microfibers can form long macroporous channels with excellent interconnectivity upon alginate degradation inside CPC, while microbeads can only form spherical pores with limited interconnectivity. These long macroporous channels could improve osteoconduction by facilitating ingrowth of cells and blood vessels into CPC scaffold.

Alg-0.4%Fb MF not only accelerated the degradation of alginate and thus promoted early cell release, but also markedly promoted cell migration inside microfibers to establish cell-cell interconnection as early as day 2. Initially, cells were suspended within the aqueous environment inside microfibers in all groups. For the Alg MF group, only a few cells appeared to spread inside the microfibers with no cell-cell interconnection at day 8. The majority of the encapsulated cells exhibited a spherical morphology which indicated an inactive state. The inactive cellular behavior is due to the fact that native alginate does not have receptors for mammalian cell attachment, which makes alginate gels relatively inert[47, 48]. In contrast, in Alg-0.4%Fb MF group, an extensive cellular network was formed and orientated along the long axis of the microfiber at day 2. This phenomenon indicates that the encapsulated cells were in an active state to self-assemble into functional units, which is important for robust cell proliferation and active involvement in regeneration *in vivo*. Moreover, such alignment of cells in a tubular network resembles the structures of blood vessels and nerve fibers and thus may be beneficial for vascularization and nerve regeneration. Thereafter, a significant amount of cell proliferation and cell release were observed. Fibrin is a natural component of provisional scaffolds in wound healing. It provides binding sites for cell attachment, migration, and proliferation[26]. Thus, cell

behavior can be steered by the addition of fibrin into alginate. In the present study, a series of fibrinogen concentrations from 0.1% to 0.8% with 1 U per mL thrombin was added to optimize the biodegradability and cell-releasing capability of Alg-Fb MFs. The 0.4% fibrinogen achieved the best results in cell spreading, migration, release and proliferation. These results are consistent with previous reports that cell behavior was strongly regulated by the fibrin concentration in hydrogels[49, 50]. A high concentration of fibrinogen such as 5% resulted in a stiffer, less malleable clot with a long degradation time, which hampered cell proliferation. On the other hand, low fibrinogen concentrations such as 0.5% significantly promoted cell proliferation[49]. In the present study, because of its fast degradability and favorable cell-releasing properties, the Alg-0.4%Fb MF group was selected to fabricate the novel cell-loading CPC-MF-hBMSC construct.

After injection, cells recollected from the tissue-engineered construct maintained high viability and osteogenic potential, which could play a key role in bone regeneration. The capability of CPC-MF-hBMSC construct for bone reconstruction *in vivo* was evaluated in rat mandibular critical-sized defects. Different from the cranial bone defect, the mandibular defect is surrounded by masticatory muscles and is subjected to the muscular forces during the animal's functional movements, such as breathing, chewing, and swallowing. Thus, it is challenging to repair mandibular defects. In the present study, the new bone area fraction of the CPC-MF-hBMSC group was nearly 4-fold that of the control without cell delivery. Compared to our previous *in vivo* studies of CPC bulk scaffold [32, 51] and injectable CPC-alginate microbeads paste[40], CPC-MF-hBMSC of the present study yielded a greater amount of new bone, along with less residual CPC. One reason may be that the

fast-degradable Alg-Fb MF delivered abundant vital pre-osteinduced hBMSCs throughout the implanted construct. The contribution of hBMSCs for new bone formation *in vivo* has been proved in many studies[52-54]. Another reason may be that with the fast-degradation of Alg-Fb MF, the long macroporous channels thus formed in CPC provided interconnected pathways in the CPC-MF-hBMSC group for cell migration, proliferation and organization, and facilitate diffusion of oxygen and nutrient. Further studies are needed to more systematically determine bone regeneration *in vivo* as a function of time such as 1, 3, and 6 months, and to test the novel injectable CPC-MF-hBMSC construct in larger animals.

5. Conclusion

A new injectable CPC-MF-hBMSC construct was demonstrated for the first time to regenerate more than 3-fold of new bone *in vivo* than control without cell encapsulation. Cell-encapsulating alginate-fibrin microfibers could quickly degrade and release the cells. A fibrin concentration of 0.4% in Alg-Fb MF resulted in the greatest enhancement of cell migration, release and proliferation. A large amount of cell-cell contacts were established in Alg-0.4%Fb MF as early as day 2. The injectable tissue-engineered construct for bone repair was fabricated by mixing the fast-degradable Alg-Fb MF with CPC paste. After mixing and injection, the cells re-collected from the construct maintained their viability and osteogenic potential *in vitro*. The hBMSC-encapsulated CPC-MF tissue-engineered construct *in vivo* displayed a robust capacity for bone regeneration, achieving complete osseous bridging in critical-sized mandibular defects in rats. The novel tissue-engineered injectable construct with stem cell encapsulation is promising to enhance bone regeneration

in a wide range of dental, craniofacial and orthopedic applications.

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Disclosure

There is no conflict of interest.

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Figure captions

- [1]. The cell encapsulation and release from alginate-0.4% fibrin microfiber (Alg-0.4%Fb MF) group: (A) 0 d; (B) 0 d, most of the encapsulated cells were spherical; (C) 0 d, live-dead staining. Arrows indicate that live cells appeared as small green dots in the hydrogel. Dead cells appeared as small red dots. (D) At 2 d, the boundary of microfiber started to become vague with a few cells migrating out (arrowed). (E) At 2 d, live-dead staining showed cell-cell contact inside microfibers (arrowed). (F) At 4 d, microfiber lost its structure. (G) At 8 d, cells grew robustly in and around the microfibers. Fluorescent images (H) and (I) showed abundant live cells (green in H) and few dead cells (red in I).
- [2]. Degradation of Alg-Fb MFs and cell encapsulation and release from Alg-0%Fb MF and Alg-0.1%Fb MF. (A) Percentage of dry weight loss (P_{DWL}): Each value is the mean of five measurements, with the error bar showing one standard deviation (mean $\pm$ sd; n = 5). (B) In control group, Alg-0%Fb MF remained its clear boundary at 8 d, with no evidence

of cell releasing; black arrows indicate that most of cells were spherical while a few cells spread inside the microfibers (red arrows). (C) Fluorescent live/dead image of Alg-0%Fb MF at 8 d. Arrows indicate cells stretching into a spindle shape. (D) In Alg-0.1%Fb MF, the majority of cells were in spindle morphology at 4 d, and a few cells were released (arrowed). (E) Live/dead staining of Alg-0.1%Fb MF at 8 d. The number of released cells greatly increased and attached on the tissue culture polystyrene.

[3]. The cell encapsulation and release from Alg-0.8%Fb MF group, (A) at 2 d, the boundary of microfiber started to become vague, with some cells migrating out (arrowed). (B) At 2 d, the encapsulated hBMSCs appeared elongated and spindle-like, with filopodia and intercellular connections to create a 3D cellular network. (C) At 4 d, the microfiber lost its integrity but most cells were still inside of the microfiber. (D) At 4 d, live-dead staining. (E) At 8 d, more cells were released. (F) At 8 d, live-dead staining showing dead cells in red.

[4]. Cell proliferation and mineralization. (A) Cell proliferation indexes, and (B) percentage of live cells at 2, 4, 6 and 8 d. (mean $\pm$ sd; n = 5). (C) Growth curves of the re-collected cells and the untreated control group. Cell growth was assessed using the CCK-8 cell proliferation assay (mean $\pm$ sd; n = 5). (D) Abundant small nodules of mineralization synthesized by the released BMSCs from the re-collected MF after 14 d osteogenic induction were stained red.

[5]. Representative HE images of CPC-MF control group at 12 weeks post-surgery. The decalcified specimens were sectioned along the coronal plane of the central defect area. No bone bridging was achieved in the defect. Only limited new bone was observed in an

adjacent area of host bon. (C) and (D) were high magnification images within the defects of the dotted-line rectangle areas in (B).

[6]. Representative HE images of the CPC-MF-hBMSC group at 12 weeks post-surgery.

Bone bridging was achieved in the critical-sized defects. The defect was closed with newly-formed woven bone and trabecular bone. (C) and (D) were high magnification images within the defects of the dotted-line rectangle areas in (B).

[7]. Quantification of new bone area fraction (A) and residual CPC area fraction (B). Bars with dissimilar letters indicate significantly different values ($p < 0.05$). Each value is mean $\pm$ SD ($n = 6$).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Step 1: Cell proliferation

Step 2: Microfiber fabrication

Cell encapsulated microfibers (MF)

Calcium phosphate cement (CPC)

Step 3: CPC-MF tissue engineered construct

Step 4: Bone defect repair