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Vascularization of plastic calcium phosphate cement in vivo induced by in-situ-generated hollow channels

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ABSTRACT

Despite calcium phosphate cement (CPC) is promising for bone repair therapy, slow biodegradation and insufficient vascularization in constructs negatively impacts its clinical application. A self-setting CPC composited with gelatin fiber is investigated to test the utility of this tissue engineering strategy to support rapid and extensive vascularization process. The interconnected hollow channels in CPC are formed after dissolution of gelatin fibers *in vivo*. The CPC-gelatin samples exhibit relatively decent/enhanced mechanical property, compared to the control. When implanted *in vivo*, the pre-established vascular networks in material anastomose with host vessels and accelerate vascular infiltration throughout the whole tissue construct. Different channel sizes induce different vascularization behaviors *in vivo*. Results indicate that the channel with the size of 250 μm increases the expression of the representative angiogenic factors HIF1α, PLGF and migration factor CXCR4, which benefit the formation of small vessels. On the other hand, the channel with the size of 500 μm enhances VEGF-A expression, which benefit the development of large vessels. Notably, the intersection area of channels has high invasive, sprouting and vasculogenesis potential under hypoxic condition, because more HIF1α-positive cells are observed there. Observation of the CD31-positive lumen in the border of scaffold indicates the ingrowth of blood vessels from its host into material through channel, benefited from gradually increased HIF1α expression. This kind of material was suggested to promote the effective application of bone regeneration through the combination of *in situ* self-setting, plasticity, angiogenesis, and osteoconductivity.

1. Introduction

The ideal bone substitutes have received much attention in the last four decades. Bone tissue engineering graft should have both excellent pro-osteogenesis and pro-angiogenesis to rapidly realize the bone regeneration *in vivo* [1]. It is important for bone regeneration to build up a functional vascular network within the defect site, which can provide sufficient oxygen and nutrients to facilitate growth, differentiation, and tissue functionality [2]. Recently, templates for the growth of tissue have led to clinical success for regeneration of skin [3] and cartilage [4], and made it promising for generalization to complex tissues or even large bone defects. Successful extension of template-based strategies to these contexts will require rapid and guided tissue ingrowth and vascularization for survival after grafting [5]. And some researchers have already presented the strategy to control the spatial patterns of cell and vascular ingrowth throughout the bioremodelable and resorbable matrix *via* well-defined micropores and networks of microchannels [5].

Since first being presented by Brown and Chow [6,7] in 1986, calcium phosphate cement (CPC), a mixture comprising several different kinds of calcium phosphates, has been developed as a successful material for bone defect repair. With its self-setting, easy plasticity and good biocompatibility, CPC had an extensive clinical application. However, most of the cement was degraded too slowly to match with the new bone ingrowth *in vivo*. CPC was degraded through a dissolution process of apatite associated with a cellular process *in vivo*. And it is difficult for tissue and blood vessels to grow into the compact structure of cement. With the research purpose of enhancing vascularization of CPC and subsequent replacement by bone tissue, macropores were introduced into CPCs [8–11]. Macropore allows for cell penetration as well as fluid flow throughout the material, thereby stimulating both active and passive degradation pathways. Hockin H. K. Xu et al. developed an injectable macroporous CPC using porogen and absorbable fibers. The macropore formed in the injectable CPC with a size ranged from 200 to 600 μm. With 5 wt.% absorbable fibers, the CPC composite paste

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was fully extruded under an injection force of 8.4 N. The flexural strength of the mocroporous CPC was 3.2 MPa, which approached the reported strength for sintered porous hydroxyapatite implants and cancellous bone [8]. Among the various strategies to introduce macropores into CPCs, the inclusion of poly (D, L-lactic-co-glycolic) acid (PLGA) microspheres as porogen is particularly promising due to the well-known biocompatibility and tailorable properties of PLGA [12–14]. But the degradation products of PLGA have a potential risk for vascularization. Marc Bohner et al. assessed the effects of macropore size on the *in vivo* behavior of ceramic scaffolds macroscopically, radiologically, histologically and histomorphometrically. Beta-tricalcium phosphate (β-TCP) blocks with different macropore diameters (150, 260, 510, and 1220 μm) were implanted in metaphysial or epiphysial defects in sheep. All β-TCP blocks were found to be biocompatible, osteoconductive, and to lead to a fast turnover from ceramic to bone [15].

The development of vascularization is critical for the regeneration of living bone, and the tissue will simply degenerate and die without it. The importance of blood vessels in the formation of the skeleton and bone repair was documented as early as the 1700s [16,17]. Various scaffold constructs have been employed in the development of tissue-engineered bone; however, an active blood vessel network is an essential pre-requisite for these constructs to survive and integrate with existing host tissue [18]. Various factors have been used to resolve the poor vascularization in large bone tissue engineering grafts, including use of angiogenic growth factors and cytokines [19], cells capable to express angiogenic factors [20,21], specific adhesion proteins and peptides favorable to angiogenesis [22,23]. The recent research into osteogenesis concentrated mainly on combination therapies of stem cells and growth factors released from scaffolds to promote angiogenesis and osteogenesis [24]. However, these bioactive strategies are not suitable to for CPC due to their special structure and unstable internal chemical environment as well as the uncontrollable released dosage of factors from CPC.

Generally, the newly generated blood will be supplied to the callus and cortical bone until the medullary blood supply is fully regenerated during bone repair. Thus it is necessary for the bone substitute materials to build up interconnective channels for tissue vascularization. Nonetheless, challenge still lies in developing techniques to form scaffolds with well-defined micro-scale connectivity, and understanding its mechanisms how such features influence cellular invasion and vascularization by micro-scale architecture.

In the present study, we report the design of CPC material with *in situ* generated hollow channel, which enhances vascularization when subcutaneously implanted in rat. The interconnective channels can facilitate the ingrowth of blood vessels (Fig. 1). The hollow channel arrays play a crucial role in enhancing cell infiltration, delivering oxygen and nutrient to the bulk of CPC construct, and promoting *in vivo* scaffold integration and vascularization. These findings are significant because they explicitly overcome the primary obstacle to build or regenerate large and complex tissues. Additionally, we conducted a research to figure out the impact of different channel diameters on the expression of the representative angiogenic factors.

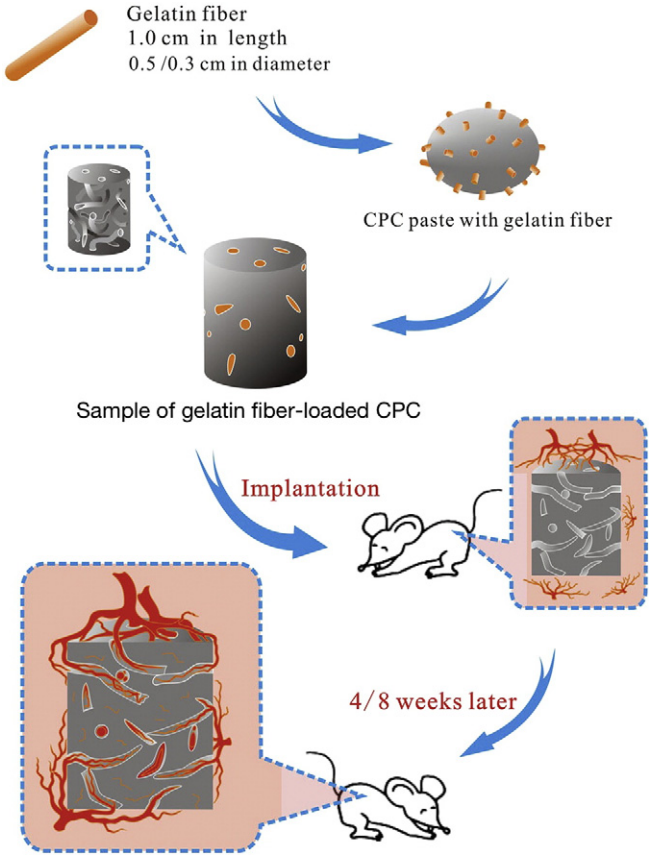

Fig. 1. Schematic for the experimental protocol of the vascularization strategy with *in situ* formed channel in CPC structure.

2. Materials and methods

2.1. Materials and preparation

The bone cement used in this study consisted of two components [25]: the partially crystallized calcium phosphate (PCCP, with the chemical formula of $Ca_3(PO_4)_2$) and dicalcium phosphate anhydrous ($CaHPO_4$, DCPA) with the weight ratio being 2:1; and deionized water was used as the liquid phase with a liquid/powder ratio being 0.5 ml g^{-1}. PCCP was synthesized from an aqueous solution of $Ca(NO_3)_2 \cdot 4H_2O$ (0.36 M) and $(NH_4)_2HPO_4 \cdot 12H_2O$ (0.15 M) by chemical precipitation method. The detailed synthetic method of PCCP has been described in our previous reports [25,26]. DCPA was purchased from Shanghai No.4 Reagent & H.V. Chemical Co. Ltd. (Shanghai, China). Alendronate was commercially obtained from Tianfeng Fine Chemicals Co. Ltd. (Xinxiang, China).

Synthesis of gelatin fiber: Gelatin was dissolved in deionized water at 60 °C, and then gelatin solution was put into the syringe; with iced water cooling needles, the gelatin fiber was extruded form syringe at a controlled extrusion speed. After dried, gelatin fiber rods were obtained (Fig. 2). Two kinds of gelatin fibers with diameters of 255 ± 8 μm and 507 ± 9 μm (named as F250 and F500, respectively) were obtained by adjusting the syringe needle. The synthesized gelatin fiber was cut

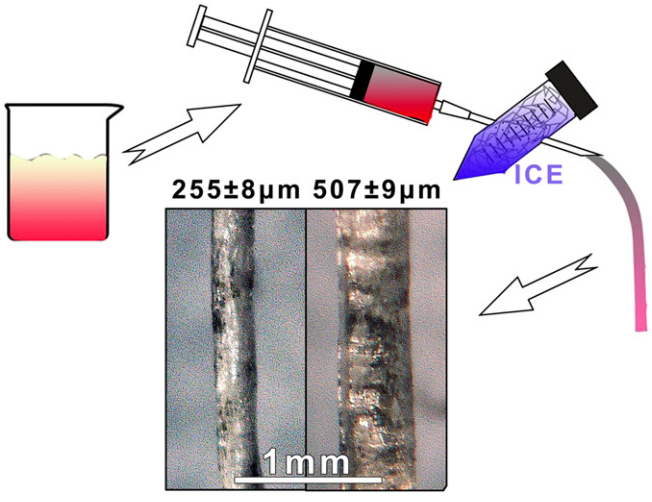

Fig. 2. Schematic for preparation of gelatin fiber.