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Incorporation of BMP-2 loaded collagen conjugated BCP granules in calcium phosphate cement based injectable bone substitutes for improved bone regeneration

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ABSTRACT

The objective of the present study was to incorporate surface modified porous multichannel BCP granule into CPC to enhance its *in vivo* biodegradation and bone tissue growth. The multichannel BCP granule (15 wt%) was first coated with collagen subsequent to BMP-2 loading (ccMCG-B). It was then embedded into CPC to form CPC-ccMCG-B system. The newly developed CPC-ccMCG-B system was then examined for SEM, EDX, XRD, setting time, compressive strength, injectability, pH change, BMP-2 release, *in vitro* as well as *in vivo* studies and further compared with CPC. Optimized CPC (0.45 mL/g) was found based on setting time and compressive strength studies. *In vivo* studies exhibited improved new bone formation and better degradation of CPC after 2 and 4 weeks of implantation as compared to CPC as resulted from effective BMP-2 signaling. Our results suggest that CPC-ccMCG-B system might be used as a promising injectable bone substitutes in clinical applications.

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1. Introduction

The demand for injectable bone substitutes (IBSs) such as cements, pastes, putties, and gels is growing for minimally invasive surgery in the field of bone regeneration. IBS presents advantages over preset/solid scaffolds as it can be easily molded into any shape of bone cavity. In addition, it can harden *in situ* at defect site. Moreover, it can be injected by using a syringe only, thus eradicating the need of complex surgery which in turn can reduce patient discomfort and shorten the healing time. Apart from the above mentioned advantages, application of *in situ* self-setting IBS has numerous benefits in clinical situations such as augmentation of osteoporotic fractures, certain spine indications, maxilla facial defects, and deformities treatment *etc.* [1–9].

Calcium phosphate cement (CPC) is used as the most promising injectable filler material because its composition is identical to mineral part of bone. In addition, it is easy to use it at bone defect site. Moreover, CPC has superior biocompatibility and osteoconductivity [10–17]. CPC primarily consists of tetracalcium phosphate (TTCP) and dicalcium phosphate dihydrate (DCPD) or dicalcium phosphate anhydrous (DCPA) as reported by Brown and Chow in 1986 [18]. CPC offers excellent properties such as good self-setting ability, injectability, mouldability, reactivity, and great feasibility in controlled drug delivery [19]. Various modified CPC based IBS systems have been studied in the literature to enhance its performance in clinical applications *via* improving properties such as compressive strength, setting time, and biocompatibility [18–24]. Apatite based CPC made by TTCP and DCPD can slowly be resorbed under physiological conditions during the curing process. Brushite based CPC made by MCPM and TCP degrades faster than apatite based CPC but they have comparatively less strength and biocompatibility than apatite based CPC. Also, lack of interconnected porosity and inadequate pore size distribution in CPC bone substitute systems can adversely affect the bone tissue growth potential. Pore sizes of around 100–500 μm are desired for fast osteoconduction and remineralization [24–26]. Hence, CPC based bone substitutes mainly rely on either cellular interaction or surface degradation for rapid bone tissue growth. There is a clinical need for simultaneous degradation along with the removal of degraded products from the site of interest prior to bone tissue growth. The degradation of CPC is essential for complete ossification of implant site with respect to bone healing.

The incorporation of secondary degradable phase with desired size distribution and interconnected pores has been used to improve the biocompatibility of CPC [27]. In this regard, a cylindrical shaped multichannel BCPgranule (MCG) can be a good choice as the secondary phase with respect to porosity incorporation and cell migration. BCP granule composing of HAp (hydroxyapatite) and β-TCP (beta-tricalcium phosphate) has been widely used as an ideal biomaterial for bone replacement applications due to its optimum porosity, good

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mechanical strength, and biodegradability [28–30]. Porous BCP granule with suitable pore size and interconnected pores can provide easy and uniform cell bone tissue growth passage. HAp may also promote interfacial bonding between the CPC matrix and the granule surface [28,31].

Surface morphological feature plays a vital role in cellular adhesion and proliferation [32]. Collagen, a primary organic phase of bone, significantly promotes cell adhesion and differentiation through specific interactions with ligands and adhering cells [33]. As a major component of *in vivo* bone extracellular matrix (ECM), collagen is a good candidate for bone tissue engineering [34]. It has been reported that collagen can be adsorbed onto HAp surface to form a nanopatterned layer [35–37]. The incorporation of growth factors is a good alternative to further accelerate tissue regeneration. Bone morphogenetic protein-2 (BMP-2), a potent osteoinductive growth factor, has been well accepted as the most notable cytokine that can promote bone formation with clinical success [38,39]. At the same time, desired BMP-2 release in appropriate physical environment is pivotal for optimum bone remodeling [40]. Stabilized crosslinked collagen can act as a temporary reservoir matrix for BMP-2. In addition, it can stimulate cell adhesion and proliferation [41,42].

Thus, the aim of this study was to develop a sound CPC bone substitute system by incorporating biodegradable porous material into CPC to enhance bone tissue growth. The idea is that incorporating multifunctional granule into injectable calcium phosphate based bone grafting might yield favorable outcomes such as inducing proper signaling as per physiological demand, higher healing rate, sequential drug delivery, and tailored degradation. In this work, multichanneled granules were first modified by collagen coating subsequent to BMP-2 loading. They were then incorporated into compositional modified CPC to enhance bone regeneration. The developed CPC system was investigated for its microstructural, phase, setting time, mechanical, injectability, pH change, BMP-2 release, *in vitro* biocompatabilty, and *in vivo* studies with respect to CPC.

2. Materials and methods

2.1. Surface modification of multichannel granules with collagen and BMP-2 loading

Commercially available multichannel BCP granules (MCG; Innobone Pvt. Ltd.) were used as precursors. All reagents used in this study were procured from Sigma-Aldrich (USA). Multichannel granules were initially treated with bovine serum albumin (1% BSA) solution at 80 °C for 8 h. For crosslinking, these granules were immersed in 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (20 mM EDC)-*N*-hydroxysuccinimide (50 mM NHS) solution (pH 8.5) at 4 °C for 1 h with continuous shaking. MCGs were washed with phosphate buffered saline (PBS) twice after each step. These MCGs were then soaked in collagen solution (5 mg/mL) and kept at 4 °C for 8 h under mechanical shaking. The crosslinked and collagen coated MCGs (ccMCG) were finally dried at −20 °C for 8 h followed by freeze drying overnight. In order to incorporate bone morphogenetic protein 2 (BMP-2) onto the coated MCGs, BMP-2 solution at a concentration of 10/240 μg/μL in 0.1% w/v BSA solution was prepared. Collagen coated MCGs (0.24 g) were then soaked in BMP-2 solution and vortexed for 5 min. MCGs were then dried again at −20 °C and subsequently freeze dried for 12 h. These samples indexed as ccMCG-B.

2.2. Synthesis of tetracalcium phosphate (TTCP)

Dicalcium phosphate anhydrous (DCPA, $CaHO_4P$, Sigma, USA) and calcium carbonate ($CaCO_3$, DC chemical, South Korea) with a purity of 99.9% were used as the staring materials. DCPA and $CaCO_3$ powders were mixed in a molar ratio of 2:1 using high energy planetary ball mill (Wisemix Ball mill, South Korea) at 250 rpm with a ball to powder ratio of 1:1 for 8 h. For mechanical mixing, alumina balls and jars were used as media. Milling was carried intermittently for 2 h with a break of half an hour to avoid over heating of the media. Heat treatment of the mixture was carried out at 1450 °C for 6 h followed by air cooling [43]. The sintered powder was used for later preparation of calcium phosphate cement.

2.3. Preparation of CPC and ccMCG-B conjugated CPCs

Monocalcium phosphate monohydrate (MCPM, $H_4CaO_8P_2 \cdot H_2O$, Sigma, USA), DCPA, and the synthesized TTCP powders were used as raw materials for the preparation of base calcium phosphate cement (CPC). MCPM, TTCP, and DCPA were first mixed at weight ratio of 1:3:1. Sodium phosphate dibasic (0.25 M, Na_2HPO_4, Sigma, USA) solution was then slowly added to the mixture to initiate cementing reaction. Different CPC pastes with various liquid to powder (L/P) ratios (0.4, 0.45, 0.5 mL/g) were prepared. All cement mixtures were kneaded thoroughly for 1 min at room temperature to achieve a homogenous cement paste. Surface modified multichannel granules (ccMCG, ccMCG-B) (15 wt%) were carefully embedded into CPC at each ratio to form separate granule-loaded CPC systems. Different CPC samples with and without ccMCG-B were developed and poured into cylindrical molds (8 mm in diameter, 15 mm in height). These samples were stored at room temperature. In brief, we investigated three CPC systems with different compositions (varied L/P ratio) and assessed their performance after loading with multichannel granules.

2.4. Characterizations

2.4.1. Setting time

Gilmore needle method was employed to measure the setting time of different CPC and multichannel granule loaded CPC (CPC-ccMCG) samples with various L/P ratios according to international standard ISO 9917. The setting time was distinguished, in other words, cement was considered as set when a needle (1 mm tip diameter) loaded with a 400 g mass failed to create an indentation on the surface of the cement sample. The entire procedure was conducted at 37 °C with 100% humidity. Mean value of five samples from each set was computed to represent the setting time.

2.4.2. Compressive strength

The compressive strength for each set of CPC and CPC-ccMCG with various L/P ratios was evaluated using a computer-controlled Universal Testing Machine (R&B Unitech, Korea). A standard ring with a span of 20 mm was used in the ring test to load samples. It was tested at a crosshead speed of 1 mm/min using Helio-X software. Prior to the test, samples were first incubated under dry (air) or wet (saline solution) conditions in a closed vessel at 37 °C and 100% relative humidity for one day. An average of five samples was taken from each set to evaluate the compressive strength.

2.4.3. Morphology

Morphological studies of 'as received' MCGs, ccMCG-B, and optimized CPC-ccMCG-B were carried out using scanning electron microscopy (SEM; JSM-635F, JEOL, Tokyo, Japan) equipped with energy dispersive X-ray spectroscopy (EDX). Before SEM imaging, samples were sputter coated with platinum (Cressington 108 Auto, JEOL, Tokyo, Japan) for conduction. An accelerating voltage of 10 kV was used to acquire SEM images.

2.4.4. Phase analysis

The phase structure of precursor powders such as MCPM, TTCP, DCPA along with MCG, prepared CPC as well as CPC-ccMCG were determined using X-ray diffraction (XRD, D/MAX-250, Rigaku, Japan) with CuKα radiation. XRD was operated at an accelerating voltage of 30 kV and current of 15 mA. The diffracted beam intensity against 2θ values

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