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# Osteogenic evaluation of calcium/magnesium-doped mesoporous silica scaffold with incorporation of rhBMP-2 by synchrotron radiation-based μCT

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ABSTRACT

The regenerative treatment of large osseous defects remains a formidable challenge in orthopedic surgery today. In the present study, we have synthesized biodegradable calcium/magnesium-doped silica-based scaffolds with hierarchically macro/mesoporous structure (CMMS), and incorporated recombinant human bone morphogenetic protein-2 (rhBMP-2) into the scaffolds to obtain a hybrid system for osteogenic factor delivery in the functional repair of bone defects. The developed CMMS/rhBMP-2 scaffolds presented interconnected porous network, macropores (200–500 μm) and mesopores (5.7 nm), as well as good bioactivity and biocompatibility and proper degradation rate. Combined with the capacity to deliver ions and growth factors, the CMMS/rhBMP-2 scaffolds significantly promoted the in vitro osteogenic differentiation of bone marrow stromal cells (bMSCs), as evidenced by the enhanced expression of Runx-2, osteopontin, osteocalcin and bone sialoprotein, and induced the ectopic bone formation in the thigh muscle pouches of mice. We further assessed the in vivo effects of CMMS/rhBMP-2 scaffolds in a rabbit femur cavity defect model by using synchrotron radiation-based μCT (SRμCT) imaging and histological analysis, indicating that the CMMS/rhBMP-2 scaffolds resulted in more bone regeneration compared to that observed with the CMMS scaffolds without rhBMP-2. Moreover, scaffolds with or without rhBMP-2 underwent gradual resorption and replacement with bone and almost disappeared at 12 weeks, while the dense CMMS/rhBMP-2 material showed slower degradation rate and promoted the least extensive neo-bone formation. This study suggested that the hybrid CMMS/rhBMP-2 scaffolds system demonstrates promise for bone regeneration in clinical case of large bone defects.


## 1. Introduction

Biomaterials suitable for bone tissue regeneration should be endowed with biologically instructive properties such as bioactivity, biocompatibility, mechanical strength and controllable biodegradability [1]. A variety of synthetic bone graft substitutes, such as calcium phosphate ceramics, calcium phosphate cement, bioglass, polymers and composites [2], have been well studied for clinical application over the past decades. In particular, synthetic three-dimensional scaffolds with an interconnective porous structure have been developed for osteogenesis and received a significant amount of attention in recent years. They were designed to mimic the morphology, structure and function of host bone in order to optimize integration into surrounding tissues, and showed higher resorbability than that of a dense product of the same chemical composition [3]. Moreover, though scaffolds serve primarily as osteoconductive moieties, they can be used as delivery vehicles for osteoinductive growth factors as well, thus providing osteoinduction. For example, recombinant human bone morphogenetic protein-2 (rhBMP-2) products have been approved by the FDA by delivery in a purified collagen matrix (Infuse® Bone Graft, Medtronic) that provides release and improved local retention, and demonstrated some clinical success for bone repair [4]. However, the drug delivery properties of this collagen sponge system are less than optimal owning to its bolus release of rhBMP-2 at early period [5], as a number of studies have implied that the sustained release of rhBMP-2 results in stronger osteogenic outcome than a burst release in the healing of critical-size bone defects [6]. Therefore, it is imperative to develop a more effective delivery system that provides enhanced bone ingrowth into the supportive matrix and

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thereby presents a bona fide alternative to autograft and BMP therapy with equal performance in bone healing.

Over the past several years, silica-based ordered mesoporous matrices have been largely employed as drug delivery systems, since they have distinct advantages like, as the name implies, highly ordered mesoporous structure, large surface area and porosity that provide excellent drug release profiles [7]. Thereafter, the mechanically stable 3D scaffolds of mesoporous silica with architecture similar to that of cancellous bone have been synthesized and recently introduced as potential bioactive materials for skeletal tissue applications. These amorphous silica-based materials produced by the sol–gel process tend to be more bioactive and bioresorbable compared with bioceramics or traditional melt derived bioglasses of similar composition, and can also contain fewer components while maintaining bioactivity [8]. Therefore, the evidence of the beneficial effects of mesoporous scaffolds as well as the osteogenic factors mentioned above motivated us to incorporate these two factors together to achieve effective local delivery of rhBMP-2 into the injured bone site. In addition, some in vitro studies revealed that elements leaching from osteogenic materials into dissolution medium have effects on osteoblast activity. For example, Ca, P and Si ions could affect the behavior of osteoblasts or primary osteoblasts related to proliferation and differentiation [9–11]. Mg ions were reported to associate with the mineralization of calcified tissues and could influence mineral metabolism through the activation of alkaline phosphatase [12,13]. An earlier study by our group has shown the effects of Mg ions in magnesium–calcium phosphate scaffolds in bone restoration, suggesting that Mg ions can significantly increase the alkaline phosphatase activity of MG63 cells (human osteosarcoma cell line), accelerate the dissolution of materials in vitro and hence improve the bone ingrowth into the scaffolds [14]. However, to the best of our knowledge, there are no previous reports describing the incorporation of rhBMP-2 into calcium/magnesium-doped mesoporous silicas (CMMS), and their efficacy in guiding bone regeneration in vivo also remains to be investigated. The objectives of this work, therefore, were to examine the possibility of developing a hybrid growth factor delivery system utilized in bone-grafting substitution and to provide enhanced de novo bone formation in a osteoinductive way.

In order to investigate the osteogenic effects on quantitative understanding of bone microstructure as well as to quantify the scaffold degradation within biopsy specimens sampled three months after implantation, histomorphometric quantification using synchrotron radiation-based micro-computed tomography (SRμCT) was performed in the present study. SRμCT is a nondestructive X-ray based volume imaging methods in medical imaging applications, by which we can acquire quantitative 3D image analysis subsequent to data reconstruction [15]. Compared with the conventional bone histomorphometry, 3D SRμCT yields higher spatial resolution and contrast, and facilitates a more accurate distinction between newly formed bone and residual bone substitute materials [16]. The data presented here were obtained at beamline BL13W of the Shanghai Synchrotron Radiation Facility (SSRF) in China.

In the present study, we first synthesized and characterized calcium/magnesium-doped silica-based hierarchically mesoporous scaffolds impregnated with rhBMP-2 and assessed the bioactivity and biocompatibility of the scaffolds in vitro. Furthermore, we investigated the ectopic bone formation in mouse hindlimb muscle pocket and the bone regeneration in a rabbit femur cavity defect model by using the SRμCT imaging and histological analysis. The combination of mesoporous scaffolds and osteoinductive growth factors here is hypothesized to achieve more effective bone regeneration in vivo in comparison with biomaterial matrices alone.

## 2. Materials and methods

### 2.1. Preparation of CMMS/rhBMP-2 scaffolds

The silica-based scaffolds with hierarchically macro/mesoporous structure have been fabricated using the replication technique (also called polymeric sponge method). Firstly, the calcium/magnesium-doped mesoporous silica (CMMS) species were synthesized by a modified template-induced and self-assembling method using nonionic block copolymer $EO_{20}PO_{70}EO_{20}$ (Pluronic P123, BASF) as structure-directing agent and tetraethylorthosilicate (TEOS, Sigma–Aldrich) as silica source. In a typical reaction, 4.0 g of P123 ($M_w = 5800$) was dissolved in 60 ml ethanol under stirring for 1h, after which 6.7 g of TEOS and 2 g of 1 M HCl were added to the P123-ethanol solution. Then 2.4 g of $MgCl_2 \cdot 6H_2O$, 1.4 g of $Ca(NO_3)_2 \cdot 4H_2O$ and 0.73 g of triethylephosphate with a molar ratio of Mg:Ca:P ≈ 2:1:1.5 were added as magnesium, calcium and phosphorus oxide precursors, respectively, and stirred magnetically at room temperature for 24 h. Afterward, the polyurethane foam was immersed into the silica sol and compressed to ensure complete penetration of the sol throughout the entire foam structure. The excess sol was then squeezed out to give a reasonably homogeneous coating on the struts so that the pores remained open. After the foams were dried under a fume hood in air at room temperature for 24 h, the same procedure was repeated. An optimized heat treatment program was applied to eliminate the surfactant and sacrificial polyurethane template and to densify the struts. The calcination was carried out in air at 600 °C for 10 h at a ramping rate of 5 °C $min^{-1}$. Previously, the polyurethane foams were cut to the desired size ($5 \times 5 \times 5$ $mm^3$) and immersed in 0.1 M NaOH solution before rinsed clean with deionized water, then dried and stored in a vacuum desiccator until use. Two kinds of foams with different porosities were used to prepare scaffolds with small and large pore sizes, labeled as CMMS-S and CMMS-L, respectively. For the purpose of comparison, samples without macroporous structure were prepared when some scaffolds were ground into powders and conformed as 0.05 g nonporous cylinders (Φ 6 × 5 mm) by uniaxial (1 MPa) and isostatic pressure (1 MPa). Finally, 40 μl of rhBMP-2 solution at a concentration of 0.5 mg $ml^{-1}$ was added dropwise to the scaffolds or cylinders (20 μg rhBMP-2 for each sample) using a pipette and freeze dried for 24 h.

### 2.2. Characterization

The ordered mesoporous structure of CMMS was confirmed by small-angle X-ray diffraction (SAXRD, Rigaku D/max 2550VB/PC, Japan) and high resolution transmission electron microscopy (HRTEM, JEM-2010, JEOL, Japan). Brunauer–Emmett–Teller (BET) and Barret–Joyner–Halenda (BJH) analyses were used to determine the surface area and the pore size distribution with a Micromeritics porosimeter (TRISTAR 3000, Micrometrics Instrument Corp., Norcross, GA, USA).

The bioactivity of the scaffolds in vitro was tested by immersing the samples in 25 ml of simulated body fluid (SBF) at 37 °C for 7 d and 14 d in polyethylene bottles according to ISO standard (10993-14:2002) in order to monitor the hydroxyapatite (HA) formation on sample surface with time. The scaffolds before and after immersing in SBF were evaluated by scanning electron microscopy (SEM, JSM-6360LV, JEOL, Japan) and energy dispersive spectroscopy (EDX, Falcon, USA) for morphological and compositional analysis, respectively. The ionic products of scaffold dissolution was investigated using inductively coupled plasma-atomic emission spectroscopy (ICP-AES, IRIS 1000, Thermo Elemental, USA) after soaking the samples in 0.05 M Tris[hydroxymethyl] aminomethane–HCl (Tris–HCl, Sinopharm, China) buffer solution (pH 7.40) at 37 °C for different time periods.

### 2.3. Cell culture

Rat bMSCs used for study in vitro were enzymatically lifted from culture dishes with trypsin/EDTA (0.25% and 0.53 mM, respectively), and centrifuged for 5 min at 1000 rpm. CMMS discs were obtained by pressing CMMS powders uniaxially into discs typically 15 mm in diameter and 1.5 mm in thickness. The cells were resuspended in fresh culture medium, and then seeded on CMMS and rhBMP-2 loaded CMMS discs, respectively. A seeding density of $5 \times 10^3$ cells/disc was applied for studies on cell proliferation, while a higher density of $2 \times 10^4$ cells/disc (near confluence) was used for osteogenic differentiation assays.

#### 2.3.1. Cell proliferation assay

MTT assay was used for the cell proliferation assay at day 1, 4 and 7 after rat bMSCs seeded on CMMS and rhBMP-2 loaded CMMS discs in 24-well plates. Five pieces of co-cultured discs for each group were washed twice with phosphate buffered saline. 400 μl DMEM with supplement 40 μl 5 mg/ml MTT (Amresco, Solon, OH, USA) solution was added and incubated at 37 °C for 4 h to form MTT formazan. Then the medium was replaced with 400 μl dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) and vibrated for 15 min in order to dissolve the formanzan. Finally, the absorbance was measured at 490 nm by ELX Ultra Microplate Reader (Bio-tek, Winooski, VT, USA).