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# Engineering of bone using rhBMP-2-loaded mesoporous silica bioglass and bone marrow stromal cells for oromaxillofacial bone regeneration

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ABSTRACT

In present study, $CaO–P_2O_5–SiO_2$-system mesoporous silica (MS) scaffolds were synthesized and loaded with recombinant human bone morphogenetic protein-2 (rhBMP-2), while their protein release properties and other characteristics were investigated. Furthermore, rabbit bone marrow stromal cells (bMSCs) were cultured and seeded on rhBMP-2-loaded MS (rhBMP-2/MS) scaffolds. Cell adhesion and proliferation were evaluated by scanning electron microscopy (SEM) and MTT assays, while osteogenic differentiation was measured by ALP activity and real-time PCR analysis on the osteogenic markers of runt-related transcription factor 2 (Runx2), collagen type 1 (COL1), osteocalcin (OCN), and osteopontin (OPN). Finally, twenty-four rabbits received unilateral maxillary sinus floor elevation surgery at each time point (2 and 8 weeks), and randomly filled with one of the following four materials: MS alone; autologous bMSCs/MS complexes; rhBMP-2/MS complexes; or autologous bMSCs/rhBMP-2/MS complexes. New bone formation and mineralization were detected by histological/histomorphometric analysis, and fluorochrome labeling. The results showed that MS scaffolds presented excellent hierarchically large pore and well-ordered mesopore properties; moreover, rhBMP-2/MS scaffolds efficiently released rhBMP-2 in a sustained manner. Furthermore, rhBMP-2/MS scaffolds significantly enhanced the proliferation and osteogenic differentiation of bMSCs. In the maxillary sinus floor elevation experiments, rhBMP-2/MS scaffolds promoted new bone formation and augmented the height of the sinus floor, while the addition of bMSCs further enhanced new bone formation and mineralization. The present study revealed that $CaO–P_2O_5–SiO_2$-system MS scaffolds could act as drug delivery carriers for rhBMP-2 and could be used to construct tissue-engineered bone with bMSCs for oromaxillofacial bone regeneration.



## 1. Introduction

Tissue regeneration for jaw bone defects caused by tumor resection, infection, trauma, congenital malformation, or bone deficiencies with aging and anatomic etiologies, remains a challenging problem in oromaxillofacial medicine [1–3]. Autogenous bone grafting is as the "gold standard" for bone regeneration, but has major disadvantages including infection, pain, and loss of function, limiting its clinical application. Moreover, the use of allograft or xenograft tissue as a substitute for autogenous bone, may be limited by the complications such as disease transmission, immunogenic response and limited supply [4–6]. Fortunately, with the development of stem cells and biomaterial scaffold technology, tissue-engineered bone could be developed as an alternative to achieve better outcomes in oromaxillofacial bone regeneration. Bone tissue engineering is defined as construction of new bone tissue in vitro or in vivo using biomaterials, cells, and growth factors, alone or in combination, based on the basic principles of biology and engineering [7,8].

Biomaterial scaffolds for bone regeneration should possess a series of biological properties, such as good biocompatibility, biodegradability and osteoinductivity. As a synthetic bone graft substitutes, bioactive glass has been studied extensively and used clinically for bone regeneration to induce bone formation and to strongly bond to surrounding bone tissue in vivo [9,10]. However, the new bone growth stimulated by bioactive glass is often limited by several drawbacks. For example, their osteoconduction may not closely approximate that of the host bone, and they may not

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achieve osteoinduction in nature [9]. To solve these problems, the strategies involving the loading of osteoinductive growth factors on bioactive glass scaffolds have been attempted to enhance the ability to stimulate cell differentiation and tissue growth. However, traditionally bioactive glass scaffolds lack efficient delivery capabilities [11].

Recently, mesoporous bioglass (MBG), which has a highly ordered mesopore channel structure with a pore size ranging from 5 to 50 nm, has attracted significant attention in the field of bone regeneration [12,13]. Moreover, MBG scaffolds exhibit greatly enhanced in vitro apatite mineralization, degradation, and drug delivery capability compared with non-mesopore bioglass, due to their more optimal surface area and pore volume [11,12]. It has been reported that MBG scaffolds could support cell adhesion, proliferation and differentiation in vitro. More importantly, MBG might be an excellent drug delivery carrier for osteogenic growth factors or drugs [14–16]. Recently, it was reported that recombinant human bone morphogenetic protein-2 (rhBMP-2) was successfully loaded on calcium/magnesium-doped mesoporous silica (MS) scaffolds and preliminarily applied for bone regeneration [17]. However, no systematic analysis of the rhBMP-2 delivery properties of the MS scaffolds, and their effects on cell proliferation, differentiation without magnesium interference, in vivo bone regeneration or material degradation, has been conducted. More importantly, it is largely unknown whether this MS delivery system could be combined with bone marrow stromal cells (bMSCs) to achieve better bone regeneration.

In present study, we hypothesized rhBMP-2-loaded MBG scaffolds could release protein in a sustained manner and that tissue-engineered bone combined with rhBMP-2-loaded MBG and bMSCs may be a better alternative technique for oromaxillofacial bone regeneration. To confirm our hypothesis, $CaO–P_2O_5–SiO_2$-system MS scaffolds were synthesized, characterized, and then loaded with rhBMP-2. Further, the rhBMP-2 delivery properties of the scaffolds and their effects on proliferation and differentiation of rabbit bMSCs were evaluated systematically in vitro. Finally, a tissue-engineered bone was constructed using the rhBMP-2-loaded MS scaffolds and bMSCs, and used for maxillary sinus floor elevation in a rabbit model, which served as a standardized model of oromaxillofacial bone regeneration [2,18–22].

## 2. Materials and methods

### 2.1. Preparation and characterization of rhBMP-2-loaded MS scaffolds

The MS scaffolds were fabricated using the replication technique (also called polymeric sponge method), as previously described [17]. Briefly, the MS bioglass species were synthesized by a modified template-induced and self-assembly method, while nonionic block copolymer $EO_{20}PO_{70}EO_{20}$ (Pluronic P123, BASF) and tetraethylorthosilicate (TEOS, Sigma, USA) acted as a structure directing agent and silica source, respectively. In a typical reaction, 1 g HCl and 4.0 g P123 were added to 60 mL ethanol with stirring for 3 h; then 0.74 g of $Ca(NO_3)_2{\cdot}4H_2O$, 0.3833 g of triethyl ephosphate (TEP) and 6.7 g of TEOS were added to the above solution, and stirred at room temperature for 24 h. Subsequently, the polyurethane foam was immersed into the silica sol and compressed to ensure complete penetration of the sol throughout the entire foam structure, while the excess sol was squeezed out to provide a reasonably homogeneous coating on the struts so that the pores remained open. The same procedure was repeated after the foams were dried under a fume hood with air at room temperature for 24 h. Finally, an optimized heat treatment program was applied to eliminate the surfactant and polyurethane template and to increase the density of the struts. The calcination was carried out in air at 600 °C for 10 h at a ramping rate of 5 °C/min. The polyurethane foams were cut to the desired size ($5 \times 5 \times 5$ mm$^3$) and immersed in 0.1 M NaOH solution, rinsed with deionized water, and then dried and stored in a vacuum desiccator before use. To prepare the rhBMP-2-loaded MS scaffolds, 20 μL of 1 mg/mL rhBMP-2 solution (Rebone, China) was added to the MS scaffolds (20 μg rhBMP-2 for each sample) using a pipette and freeze dried for 24 h.

The ordered mesoporous structure of MS scaffolds was evaluated by small-angle X-ray diffraction (SAXRD, Rigaku, Japan) and high-resolution transmission electron microscopy (HRTEM, JEOL, Japan). The surface area and the pore size distribution were evaluated by Brunauer-Emmett-Teller (BET) and Barrett-Joyner-Halenda (BJH) analysis (Micrometrics, USA). The microstructure of MS scaffolds was determined by scanning electron microscopy (SEM) observation.

### 2.2. Measurement of rhBMP-2 release from rhBMP-2-loaded MS scaffolds

To measure rhBMP-2 release from rhBMP-2-loaded MS (rhBMP-2/MS) scaffolds, 3 mL simulated body fluid (including NaCl, $NaHCO_3$, KCl, $K_2HPO_4{\cdot}3H_2O$, $MgCl_2{\cdot}6H_2O$, $CaCl_2$ and $Na_2SO_4$) solution was added to rhBMP-2/MS scaffolds, and the scaffolds were then placed into the thermostat oscillator (37 °C, 50 r/min). The rhBMP-2 protein release from the samples at 6, 12, 24, 72, 144, 216, 288, 360, 432, and 504 h was determined using an rhBMP-2 enzyme-linked immunosorbent assay (ELISA) Kit (R&D Systems, USA) according to the manufacturer's instruction. The results were reported as the ratio of the rhBMP-2 released at various time points to the total rhBMP-2.

### 2.3. Isolation and culture of rabbit bMSCs

Forty-eight male New Zealand White rabbits weighing 2–2.5 kg were used in this experiment. All animal procedures in present study were approved by the Animal Research Committee of the Ninth People's Hospital affiliated to Shanghai Jiao Tong University, School of Medicine.

A 3 mL bone marrow sample was aspirated from a rabbit fibula, and then cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) in a humidified 37 °C and 5% $CO_2$ incubator. On day 5, the medium containing non-adherent cells was removed and replaced with fresh medium; while the remaining adherent cells were mainly mesenchymal stromal cells. The cells were passaged once for expansion, at a confluence of approximately 80%. After the first passage, the growth medium was replaced with osteogenic medium (DMEM, 10% FBS, 50 μg/mL L-ascorbic acid, 10 mM glycerophosphate and 100 nM dexamethasone). bMSCs after passage 2 cultured in osteogenic medium with a seeding density of $2 \times 10^4$ cells/mL were used for each in vitro assay, as described previously [2,19].

### 2.4. Cell adhesion and growth assay

The adhesion and growth of rabbit bMSCs seeded on MS and rhBMP-2/MS scaffolds were determined by SEM observation. At days 1 and 4 after cell seeding, the cell/scaffold complexes were fixed in 2% glutaraldehyde for 2 h, and then dehydrated in increasing concentrations of ethanol. Finally, the samples were dried by hexamethyldisilazane, sputter-coated with gold and observed by SEM (JEOL, Japan).