Mesoporous bioactive glass nanolayer-functionalized 3D-printed scaffolds for accelerating osteogenesis and angiogenesis

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The hierarchical microstructure, surface and interface of biomaterials are important factors influencing their bioactivity. Porous bioceramic scaffolds have been widely used for bone tissue engineering by optimizing their chemical composition and large-pore structure. However, the surface and interface of struts in bioceramic scaffolds are often ignored. The aim of this study is to incorporate hierarchical pores and bioactive components into the bioceramic scaffolds by constructing nanopores and bioactive elements on the struts of scaffolds and further improve their bone-forming activity. Mesoporous bioactive glass (MBG) modified β-tricalcium phosphate (MBG-β-TCP) scaffolds with a hierarchical pore structure and a functional strut surface (~100 nm of MBG nanolayer) were successfully prepared via 3D printing and spin coating. The compressive strength and apatite-mineralization ability of MBG-β-TCP scaffolds were significantly enhanced as compared to β-TCP scaffolds without the MBG nanolayer. The attachment, viability, alkaline phosphatase (ALP) activity, osteogenic gene expression (Runx2, BMP2, OPN and Col I) and protein expression (OPN, Col I, VEGF, HIF-1α) of rabbit bone marrow stromal cells (rBMSCs) as well as the attachment, viability and angiogenic gene expression (VEGF and HIF-1α) of human umbilical vein endothelial cells (HUVECs) in MBG-β-TCP scaffolds were significantly upregulated compared with conventional bioactive glass (BG)-modified β-TCP (BG-β-TCP) and pure β-TCP scaffolds. Furthermore, MBG-β-TCP scaffolds significantly enhanced the formation of new bone in vivo as compared to BG-β-TCP and β-TCP scaffolds. The results suggest that application of the MBG nanolayer to modify 3D-printed bioceramic scaffolds offers a new strategy to construct hierarchically porous scaffolds with significantly improved physicochemical and biological properties, such as mechanical properties, osteogenesis, angiogenesis and protein expression for bone tissue engineering applications, in which the incorporation of nano-structures and bioactive components into the scaffold struts synergistically play a key role in the improved bone formation.

Introduction

Bioactive materials have been proved to be one of the efficient strategies for repairing and regenerating large bone defects, which still remains a significant challenge in orthopedic clinics. To address the bone-related issue clinically, optimal bone biomaterials should be biocompatible, and possess osteogenic and angiogenic activities as well as satisfactory mechanical properties for enhancing tissue regeneration.1-3 Three dimensional (3D) porous scaffolds are most promising for the regeneration of bone defects. The scaffolds with 3D interconnected pores can enhance bone cell viability and induce early-stage osteogenesis by surrounding cells and tissues through cell attachment and migration, and hence promote tissue ingrowth and nutrient transport into interconnected macropores.4-6 It was found that the interconnected macropores with a size of 200–500 μm in scaffolds benefited bone ingrowth and capillary formation.7 Besides the large pore structure, more and more studies have shown that the hierarchically porous structure of scaffolds could distinctively enhance their osteogenic activity, in which the nanopores in...
the scaffolds can improve the bioactivity.\textsuperscript{8,9} Kim et al. have fabricated hierarchically micro and nanopatterned polyactic-co-glycolic acid (PLGA) patches by employing capillary force lithography in combination with a surface micro-wrinkling method.\textsuperscript{10} Oh et al. have developed a hierarchical structure in collagen scaffolds by using ice particulates or ice lines as a template.\textsuperscript{11} Previous studies demonstrated that the porous structures on the micrometer scale benefited the transport of nutrients and tissue ingrowth, and both the micro- and nanoscale structures in scaffolds could significantly stimulate cell proliferation, differentiation and further tissue regeneration.\textsuperscript{12–16} However, high-temperature sintering (1100–1300 °C) is generally necessary for bioceramic scaffolds with dense structures in struts and excellent mechanical properties. Nevertheless, nanoporous structures will be damaged after such high-temperature sintering. To our knowledge, the nanoporous structure in bioceramics is prepared at low temperatures (no higher than 700 °C) rather than at high temperatures. The conflict between obtaining nanoporous structures at low temperatures and achieving excellent mechanical properties at high temperatures makes the preparation of bioceramic scaffolds with a hierarchically porous structure challenging.

Besides the hierarchically porous structure of biomaterials, the surface of biomaterials is also of great importance to induce the osteogenic differentiation of stem cells. Nanoscale surfaces may lead to rapid protein adsorption in the early stages of implantation, which may further mediate cell attachment during the next stage of implant assimilation.\textsuperscript{17–19} Although surface modification of defined chemistry and topology has been widely applied in the metal implant surface, few studies have been conducted on the modification of the struts of bioceramic scaffolds in which most researchers focused on the optimization of their composition and large-pore structure, and ignored the surface/interface of struts in bioceramic scaffolds. Considering the importance of nanoporous structures and bioactive elements in the tissue engineering scaffolds, we supposed that if the nanoporous structure and bioactive elements can be prepared on the struts of bioceramic scaffolds, it may significantly improve their bioactivity for regeneration of large bone defects.

Mesoporous bioactive glass (MBG) possesses significantly improved specific surface area, pore volume, and apatite-mineralization activity due to its well-ordered mesoporous (nanoporous) structure as compared to conventional bioactive glasses (BG) without mesoporous structures. In 2010, MBG was successfully prepared as porous scaffolds by the polyurethane template method and presented excellent bioactivity.\textsuperscript{20} The major shortcoming for the prepared MBG scaffolds is their poor mechanical strength (<100 kPa), limiting their application. Previously, MBG has been incorporated into polymer scaffolds to improve their osteogenic activity.\textsuperscript{21–23} Considering the specific mesoporous structure and bioactivity of MBG, it is of great interest to apply MBG to modify porous bioceramic scaffolds, in which the nanopores (∼5 nm) and bioactive elements (Si, Ca and P) of MBG can be effectively combined with the inherent macropores (∼200–500 μm) and high strength of 3D-printed bioceramic scaffolds to achieve a hierarchically porous scaffold for bone tissue engineering. To the best of our knowledge, there has been no such design by incorporating nanopores and bioactive elements into the struts of porous bioceramic scaffolds to synergistically improve the osteogenesis and angiogenesis of tissue cells for bone regeneration. Therefore, the aim of this study is to prepare MBG-modified porous bioceramic scaffolds in order to achieve a hierarchically porous structure and functional surface on the struts, and further investigate the effect of the nano-structured surface on the osteogenesis and angiogenesis of tissue cells as well as the in vivo bone forming ability (as shown in the Graphical abstract). For this aim, β-tricalcium phosphate (β-TCP) bioceramics were applied for constructing porous scaffolds by the 3D-printing method as they are widely used as bone regeneration materials clinically.\textsuperscript{24–26} Then, the MBG nanolayer was uniformly deposited on the struts of β-TCP scaffolds by a spin coating strategy. The cell viability, in vitro osteogenic/angiogenic properties of rBMSCs and HUVECs as well as the in vivo bone-forming ability of the prepared scaffolds were systematically studied.

Materials and methods

Materials

Nonionic block copolymer EO\textsubscript{188}PO\textsubscript{24}EO\textsubscript{188} (P123, average $M_w = 5800$) was purchased from Sigma-Aldrich. Hydrochloric acid (HCl, 36.0–38.0%), tetraethyl orthosilicate (TEOS, 98%), calcium nitrate (Ca(NO\textsubscript{3})\textsubscript{2}·4H\textsubscript{2}O, 99%), triethyl phosphate (TEP, 99.8%), and polyvinyl alcohol (PVA, degree of polymerization: 1750 ± 50, 99%) were purchased from Sinopharm Chemical Reagent Co., Ltd. Bovine Serum Albumin (BSA, 98%) was purchased from Sangon Biotech. Beta-tricalcium phosphate (β-TCP) powders were purchased from Kunshan Chinese Technology New Materials Co., Ltd.

Fabrication of MBG–β-TCP scaffolds

The 3D plotting device applied in this study was designed by the Fraunhofer Institute for Materials Research and Beam Technology (Dresden, Germany). The printing β-TCP pastes were prepared by mixing β-TCP powders with polyvinyl alcohol (PVA) solution (6 w%) in a mass ratio of 65 : 35. After stirring homogeneously, the pastes were loaded into printing tubes and β-TCP scaffolds were generated by 3D printing via a dosing pressure of 3.0–5.5 bar and a printing speed of 6.5 mm s\textsuperscript{−1}. The printed scaffolds were then dried at room temperature for 24 h and then sintered at 1100 °C for 3 h to obtain pure β-TCP scaffolds.

To prepare MBG-modified β-TCP (MBG–β-TCP) scaffolds, the MBG nanolayer was uniformly coated on the struts of β-TCP scaffolds by the spin coating method. Firstly, the MBG precursor solution was prepared according to our previous study.\textsuperscript{27} 12 g of P123 was dissolved in 180 g of ethanol and stirred at room temperature for 1 h, then 20.1 g of TEOS, 4.2 g of Ca-
(NO$_3$)$_2$·4H$_2$O, 2.19 g of TEP and 3 g of 0.5 M HCl were added to the ethanol solution [Si/Ca/P = 80 : 15 : 5, molar ratio] and stirring was continued for 24 h. As control, the conventional bioactive glass (BG) precursor solution was synthesized similarly to the MBG precursor solution described above without P123. The obtained MBG precursor solutions were deposited on the surface of the struts of 3D-printed β-TCP scaffolds by spin-coating at 500 rpm for an initial 10 s and then at 2000 rpm for 20 s. Then, the coated scaffolds were kept at room temperature for 8 h to evaporate the volatile components, in which the MBG dry gel layers were formed via the evaporation-induced self-assembly (EISA) procedure. The coatings were repeated 9 times. Finally, the samples were heated at a rate of 1 °C min$^{-1}$ and annealed at 650 °C for 5 h to obtain MBG-β-TCP scaffolds. Bioactive glass (without mesopores)-modified β-TCP (BG-β-TCP) scaffolds were prepared using the BG precursor by the same methods. Pure β-TCP and BG-β-TCP scaffolds were used as the controls.

**Characterization of MBG-β-TCP scaffolds**

The phase compositions, surface and cross-section microstructures of MBG-β-TCP, BG-β-TCP and β-TCP scaffolds were analyzed by X-ray diffraction (XRD; D8 Advance, Bruker, Germany), scanning electron microscopy (SEM; Magellan-400, FEI, USA), and elemental analysis was carried out by using an energy dispersive spectrometer (EDS). The nanotexture in the MBG and BG nanolayers was observed by using a transmission electron microscope (TEM, 2100F, JEOL, Japan). The surface area and pore volume of the prepared scaffolds were analysed by N$_2$ adsorption–desorption analysis and calculated by Brunauer–Emmett–Teller (BET) and Barrett–Joyner–Halenda (BJH) models.

**Mechanical properties of MBG-β-TCP scaffolds**

The compressive strength of MBG-β-TCP, BG-β-TCP and β-TCP scaffolds (Ø 8 × 10 mm) was tested using a computer controlled universal testing machine (AG-I, Shimadzu, Japan) at a cross-head speed of 0.5 mm min$^{-1}$ and the porosity of scaffolds was measured according to Archimedes’ principle$^{10}$ ($n = 9$).

**The apatite mineralization and ion release of MBG-β-TCP scaffolds**

Simulated body fluids (SBF), which were prepared by the method described by Kokubo$^{11}$ were selected to investigate the apatite mineralization ability of MBG-β-TCP, BG-β-TCP and β-TCP scaffolds in vitro ($n = 3$). Three groups of scaffolds were soaked in SBF ($V_{\text{SBF}}/M_{\text{scaffolds}} = 100$ mL g$^{-1}$) for 1 and 3 d in a polyethylene bottle at 37 °C, respectively. The SBF solution was refreshed after soaking for 1 d. The scaffolds were collected from SBF solution after soaking, rinsed with distilled water 3 times and dried at 60 °C overnight. The apatite formation on the surfaces of scaffolds was observed by SEM and EDS (S-4800, Hitachi, Japan).

The ion release behavior of MBG-β-TCP scaffolds was carried out in Tris-HCl buffered solution ($V_{\text{Tris-HCl}}/M_{\text{scaffolds}} = 2$ mL g$^{-1}$) in a shaker at 37 °C for 1, 3, 5 and 7 d. Tris-HCl solution was collected and refreshed at each timepoint. The concentrations of Ca, P and Si ions in the collected solution were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Varian Co., USA).

**In vitro osteogenic ability of rBMSCs in MBG-β-TCP scaffolds**

**Cell attachment and viability.** In this experiment, rabbit bone marrow stromal cells (rBMSCs) came from the Department of Sports Medicine and Arthroscopy Surgery, Fudan University, China. They were seeded into MBG-β-TCP, BG-β-TCP and β-TCP scaffolds respectively in 24-well culture plates with a density of $1 \times 10^4$ cells per scaffold in DMEM (Dulbecco’s Modified Eagle’s Medium, HyClone, China) supplemented with 10% fetal calf serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). Then the cell-seeded scaffolds were incubated under a CO$_2$ (5%) atmosphere and at 37 °C and the culture medium was renewed every 2 d.

To evaluate the cell attachment, rBMSCs were cultured for 1 and 3 d. The samples ($n = 3$) were removed from the culture wells, rinsed with phosphate buffered saline (PBS) and fixed with 2.5% glutaraldehyde in PBS for 20 min, then the fixative was removed by washing in PBS 3 times. Subsequently, the samples were dehydrated sequentially in graded ethanol ($V_{\text{ethanol}}/V_{\text{distilled water}} = 50, 70, 90, 95$ and 100%) and hexamethyldisilazane ($V_{\text{hexamethyldisilazane}}/V_{\text{ethanol}} = 50$ and 100%) for 5 min, respectively. The morphology of the attached cells was observed using SEM (S-4800, Hitachi, Japan). To prepare samples for fluorescent photographs, scaffolds cultured for 3 d were rinsed in PBS and fixed by 4% paraformaldehyde followed by washing 3 times to remove excess paraformaldehyde. The fixed cells were incubated in freshly prepared rhodamine phalloidin (stock solution in methanol diluted in 1:100, Cytoskeleton Inc., USA) at room temperature for 20 min to stain the cytoskeleton and washed 3 times with PBS to reduce the nonspecific background. Finally, the nuclei of the cells were stained using DAPI solution (5 mg mL$^{-1}$). The confocal images were obtained on a confocal laser scanning microscope (Leica TCS SP8).

The viability of rBMSCs on scaffolds was determined by the MTT assay. After the rBMSCs were cultured on the scaffolds ($n = 4$) for 1, 3 and 7 d, DMEM was then removed from the culture plates and followed by the addition of 1 mL of MTT (Serva, Germany) solution (0.5 mg mL$^{-1}$). After incubation for 2 h, the MTT solution was removed and 150 μL of dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added. Finally, an aliquot of 100 μL solution was taken out from each well and transferred to a fresh 96-well plate. The absorbance value of these samples was measured at 590 nm with a microplate reader (Epoch Microplate Spectrophotometer, BioTek Instruments, USA). All the results were demonstrated as the optical density (OD) values minus the absorbance of blank wells.

**Alkaline phosphatase activity and osteogenic related gene expression.** The differentiation of rBMSCs was determined on the basis of alkaline phosphatase (ALP) activity, an early marker of osteoblastic cell differentiation and a measure of the
bone forming ability of osteoblast cells. In this experiment, ALP activity was assayed by the p-nitrophenylphosphate (PNPP) method. To investigate the early osteogenic differentiation of rBMSCs on MBG-β-TCP, BG-β-TCP and β-TCP scaffolds (n = 4), the rBMSCs were cultured following the protocol described in the “Cell attachment and viability” section, with the culture time prolonged to 7 and 14 d with the spent medium replaced every 3 d. On day 7 and 14, an aliquot of 400 μL of 0.05% Triton X-100 was added to the culture well and the mixture was incubated at 4 °C for 1 hour. Then, 50 μL of supernatant cell lysates were incubated with the reaction solution (containing 2-aminoo-2-methyl-1-propanol, MgCl2 and PNPP) for 45 min in a shaker at 37 °C and the absorbance at 405 nm was measured with the microplate reader. The ALP activity was normalized to the total intracellular protein content: relative activity of ALP = OD value/protein content.

The expression of osteogenic related genes (runx-related transcription factor 2 (RUNX2), bone morphogenetic protein-2 (BMP-2), collagen type I (COL-1) and osteopontin (OPN)) was measured using the real-time quantitative RT-PCR. Typically, rBMSCs with a density of 2 × 10^4 cells per well were cultured in MBG-β-TCP, BG-β-TCP and β-TCP scaffolds (n = 6) for 7 d and harvested using TRIzol reagent (Invitrogen Pty Ltd, Australia) to extract the RNA according to the manufacturer’s instructions. The obtained RNA was reverse-transcribed into complementary DNA (cDNA) using SuperScript III Reverse Transcriptase (Invitrogen) and the qRT-PCR analysis was performed on a StepOne Plus (Applied Biosystems) using SYBR Green detection reagent. The data were compared by the ΔΔCT method, and normalized to housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression of each condition and their sequences are summarized in Table 1. Then, the results were quantified relative to the corresponding gene expression from control sample (cells cultured with pure DMEM + 10% fetal calf serum), which were standardized to 1.

In vitro angiogenic ability of HUVECs in MBG-β-TCP scaffolds

Cell attachment and viability. Human umbilical vein endothelial cells (HUVECs) (ScienCell, USA) were cultured in the scaffolds to evaluate the in vitro angiogenesis. 1.5 × 10^4 HUVECs were seeded into scaffolds (MBG-β-TCP, BG-β-TCP and β-TCP) and placed in 24-well culture plates. The cells were cultured for 3 d in ECM (Endothelial Cell Medium, ScienCell, USA) under a CO2 (5%) atmosphere and at 37 °C and the culture medium was renewed every 2 d. Then, the cell attachment and cytoskeleton of HUVECs in the scaffolds were characterized by using SEM and a confocal laser scanning microscope as described in the “In vitro osteogenic ability of rBMSCs in MBG-β-TCP scaffolds” section.

To investigate the effect of MBG-β-TCP, BG-β-TCP and β-TCP scaffolds on the viability of HUVECs, the MTT assay was used in this study. Briefly, HUVECs were cultured on scaffolds for 1, 3, 7 d by the methods described above, with an initial density of 1 × 10^3 cells per scaffold in 24-well culture plates. The MTT assay was performed as described in the “In vitro osteogenic ability of rBMSCs in MBG-β-TCP scaffolds” section.

Western blot analysis for osteogenic and angiogenic protein expression of rBMSCs in MBG-β-TCP scaffolds

Western blot analysis was performed to detect the expression of the COL-1, OPN, VEGF, and HIF-1α proteins. Briefly, after being cultured with three kinds of scaffolds (n = 12) for 7 d, the whole-cell extracts were prepared in radioimmunoprecipitation assay buffer (20 mmol L^-1 Tris, 2.5 mmol L^-1 EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mmol L^-1 NaF, 10 mmol L^-1 Na3P2O7, and 1 mmol L^-1 phenylmethylsulfonyl fluoride) supplemented with proteinase inhibitor cocktail (Calbiochem). 40 μg of protein from each sample was separated on SDS-PAGE gels, and then transferred onto a nitrocellulose membrane. After being blocked for 1 h with Odyssey® blocking buffer (Millennium Science, Australia), the membranes were incubated with the primary antibodies against COL-1, OPN, VEGF, and HIF-1α and β-actin (antibodies against OPN, Col-1, VEGF and β-actin were obtained from Santa Cruz Biotechnology; HIF-1α was purchased from Cell Signaling Technology), overnight at 4 °C. The membranes were washed three times for 20 min in TBS containing 0.01% Tween-20, and then incubated with the corresponding fluorescent secondary antibodies (LI-COR Biosciences, USA) at 1 : 15 000 dilution for 1 h at room temperature. The protein bands were visualized using the Odyssey CLX (LI-COR Biosciences, USA).

Angiogenic related gene expression of HUVEC on the scaffolds. To investigate the effect of MBG-β-TCP, BG-β-TCP and β-TCP scaffolds on the angiogenic related gene expression (VEGF (vascular endothelial growth factor) and HIF-1α (hypoxia inducible factor-1α)), HUVECs with a density of 2 × 10^4 cells per ml were cultured on the scaffolds for 7 d and the total RNA was extracted from the HUVECs using TRIzol.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>Runx2</td>
<td>5'-TCAGGCAATGTCCTCCCTGATAT</td>
<td>5'-TGCGAGTAGTGGTGTGATGTTG</td>
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<tr>
<td>OPN</td>
<td>5'-CACCATGAAATCCGCGT</td>
<td>5'-CGTACTTGGGTTTTCACCC</td>
</tr>
<tr>
<td>COL-1</td>
<td>5'-CTTCTTGCCGGCTGTTGAAAGATG</td>
<td>5'-CCCAGTACAGTGGTTTCCAGTAG</td>
</tr>
<tr>
<td>BMP-2</td>
<td>5'-CGCCTCATAATTCCACGTCGTAAG</td>
<td>5'-GGGCAACATCCAGTCCGTTT</td>
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<tr>
<td>HIF-1α</td>
<td>5'-CCATGTGACCATGGAGAAAT</td>
<td>5'-GGCGATTCTGTTGTCGTTT</td>
</tr>
<tr>
<td>VEGF</td>
<td>5'-TGCCGATCAAACTCCAACCA</td>
<td>5'-CAGGATTCTGTTGTCGTTT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TCCACATCTTCCAGAGGCA</td>
<td>5'-CACATGCGGAATGGTGTG</td>
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</table>
protein adsorption analysis

The protein adsorption capacity of MBG, BG and β-TCP particles (0.1 g) (n = 5) was examined after immersion in BSA/PBS solution (0.2 mg ml⁻¹) at 37 °C for 6, 12, 24, 72, 120 hours. The particles were taken out at each timepoint by centrifugation and the concentration of BSA was tested using a microplate reader.

In vivo osteogenic evaluation of the MBG-β-TCP scaffolds

Eighteen male New Zealand White rabbits weighing 2–2.5 kg were used in the present study. All animal procedures were approved by the Animal Research Committee of the Ninth People’s Hospital affiliated to Shanghai Jiao Tong University School of Medicine (Shanghai, China). At the 4 or 8 week timepoint, nine rabbits were anaesthetized with ketamine (10 mg kg⁻¹) and xylazine (3 mg kg⁻¹), and then a 2.0 to 2.5 cm sagittal incision was made on the scalp, and the calvarium was exposed by blunt dissection. Two 8 mm full thickness parietal defects were created on the skull by a trephine bur and then randomly implanted with β-TCP, BG-β-TCP and MBG-β-TCP scaffolds (n = 6 for each group). Following the 0.9% saline rinse, the incision was closed in the layers using 2-0 Vicryl sutures as described in the previous study.

At 4 and 8 weeks after the operation, all the rabbits in each group were sacrificed by overdose of ketamine. The skulls were dehydrated in ascending concentrations of alcohols from 75% to 100%, and embedded in polymethylmethacrylate (PMMA). Three longitudinal sections for each sample were prepared and stained with Van Gieson reagent. The obtained RNA was reverse-transcribed, qRT-PCR analysed, normalized against GAPDH and calculated with the methods described in the “In vitro osteogenic ability of rBMSCs in MBG-β-TCP scaffolds” section.

Results

Characterization of MBG-β-TCP scaffolds

The XRD analysis shows that only the characteristic peaks of crystal β-TCP phase exist in the patterns of MBG-β-TCP, BG-β-TCP and β-TCP scaffolds (ESI Fig. 1†), indicating that the MBG and BG nanolayers have no effect on the crystal phase of β-TCP scaffolds.

The photographs and SEM micrographs of MBG-β-TCP, BG-β-TCP and β-TCP scaffolds are presented in Fig. 1. It can be seen that all the scaffolds have uniform macropores with a designed morphology, and the pore size is around 400 μm (Fig. 1a, b, d, e, g and h). High magnification of SEM images displays that the distinct grain boundary was clearly existed on the strut surface of β-TCP scaffolds (Fig. 1c), while no grain boundary on the strut surface of MBG-β-TCP scaffolds could be observed (Fig. 1l). A nanolayer of MBG was uniformly deposited on the strut surface of β-TCP scaffolds. The cross-section microstructure and EDS analysis of MBG-β-TCP scaffolds are shown in ESI Fig. 2†. The interface between MBG nanolayers and β-TCP scaffolds is not obvious (ESI Fig. 2a†) and the thickness of the MBG nanolayer is measured to be around 100 nm by the EDS analysis of two points in the cross section (ESI Fig. 2b and c†).

TEM analysis clearly shows the MBG nanolayers with well-ordered channels on the surface of MBG-β-TCP scaffolds (mesopore size: around 5 nm) (Fig. 2e). In contrast, the BG layer on β-TCP scaffolds has a disordered microstructure with an amorphous morphology (Fig. 2b). The electron diffraction (ED) pattern was further examined for the scaffolds, both the MBG and BG layers show distinct diffraction rings (Fig. 2a and d) while β-TCP presents specific diffraction spots (Fig. 2c and f). The results of N₂ adsorption–desorption, BET and BJH are shown in ESI Table 1†. The specific surface area (S₅₇₅) and pore volume (V₅₇₅) of MBG-β-TCP scaffolds increased as compared to those of the BG-β-TCP and β-TCP scaffolds.

Mechanical properties of MBG-β-TCP scaffolds

The compressive strengths of MBG-β-TCP, BG-β-TCP and β-TCP scaffolds are shown in Fig. 3. The compressive strengths of the three types of scaffolds were in the range of 8-11 MPa (Fig. 3c). It is clear that the MBG-β-TCP and BG-β-TCP scaffolds (~11 MPa) have a significantly higher compressive strength than pure β-TCP scaffolds (~8 MPa) while the porosity of the three scaffolds was similar (~56–58%).

Apatite formation ability and ion release of MBG-β-TCP scaffolds

SEM images and EDS analysis present the morphology of MBG-β-TCP, BG-β-TCP and β-TCP scaffolds after soaking in SBF for 1 d (ESI Fig. 3†). No obvious apatite formation was observed on the surfaces of β-TCP scaffolds (ESI Fig. 3a and b†). However, some lath-like apatite clusters were deposited on the surfaces of BG-β-TCP scaffolds (ESI Fig. 3c and d), while the surfaces of MBG-β-TCP scaffolds were fully covered with apatite crystals (ESI Fig. 3e and f). EDS analysis further con-
Fig. 1  Overview and SEM images of β-TCP (a–c), BG-β-TCP (d–f) and MBG-β-TCP (g–i) scaffolds. Low magnification SEM images (b, e and h) show the macropores with a size of 400 μm and high magnification SEM images (c, f and i) show that BG (f) and MBG (i) layers have been uniformly deposited on the struts of β-TCP scaffolds.

Fig. 2  TEM analysis for the coated BG (b) and MBG layer (e) on β-TCP scaffolds. The MBG layer presents a well-ordered mesoporous structure with a mesoporous pore size of 5 nm (e). Electron diffraction patterns reveal the amorphous states for the coated BG (a) and MBG (d) as well as the crystalline state of the substrate of β-TCP scaffolds (c, f).
firms the existence of Ca, P and Si elements in the formed apatite crystals on the surface of MBG-β-TCP and BG-β-TCP scaffolds (ESI Fig. 3g and h).

The release of Ca<sup>2+</sup>, PO<sub>4</sub><sup>3-</sup> and SiO<sub>4</sub><sup>4-</sup> ions in Tris-HCl buffered solution is shown in ESI Fig. 4.† It was obvious that the concentration of Ca<sup>2+</sup> ions in Tris-HCl increased on the first day and decreased after that along with the consumption of apatite mineralization (ESI Fig. 4a†). Meanwhile, a lower concentration of Ca<sup>2+</sup> ions was found for MBG-β-TCP scaffolds than that of BG-β-TCP scaffolds, which is consistent with the better mineralization ability in the early stages of MBG-β-TCP scaffolds. The PO<sub>4</sub><sup>3-</sup> ions maintained a continuous release behavior at first, while the apatite mineralization on the scaffolds led to the decrease of PO<sub>4</sub><sup>3-</sup> concentration in Tris-HCl solution at the later stage (ESI Fig. 4b†). The concentration of released SiO<sub>4</sub><sup>4-</sup> ions for MBG-β-TCP and BG-β-TCP scaffolds increased on the first day and remained constant after that (ESI Fig. 4c†).

Attachment, morphology, viability, ALP activity and osteogenic related gene expression of rBMSCs in MBG-β-TCP scaffolds

The attachment and morphology of rBMSCs on the strut surface of MBG-β-TCP, BG-β-TCP and β-TCP scaffolds were examined by SEM and confocal microscopy (Fig. 4). As shown in Fig. 4, all groups of scaffolds supported rBMSC attachment and the cells have close contact with the scaffolds by numerous filopodia after 3 d of culture. Furthermore, the stained cells in MBG-β-TCP scaffolds showed distinct and well-defined microfilaments as well as cytoskeleton (Fig. 4i) compared with BG-β-TCP (Fig. 4h) and β-TCP scaffolds (Fig. 4g) where the cells are observed.

The viability of rBMSCs cultured in MBG-β-TCP, BG-β-TCP and β-TCP scaffolds for 1, 3 and 7 d is shown in Fig. 5a. The cell viability of rBMSCs in all scaffolds increased significantly along with the culture time. Meanwhile, the viability of rBMSCs in MBG-β-TCP scaffolds was obviously higher than that in BG-β-TCP and β-TCP scaffolds throughout the whole culture time (1, 3 and 7 d) (P < 0.05). Meanwhile, the MBG-β-TCP scaffolds have significantly higher ALP activity of rBMSCs than the other two groups (Fig. 5b) (P < 0.05). The osteogenic differentiation of rBMSCs in MBG-β-TCP, BG-β-TCP and β-TCP scaffolds was further evaluated by RT-qPCR. The expression of osteogenic markers, RUNX2 (Fig. 5c), OPN (Fig. 5d), COL-1 (Fig. 5e) and BMP-2 (Fig. 5f) of rBMSCs in MBG-β-TCP scaffolds was obviously higher than that of BG-β-TCP and β-TCP scaffolds (P < 0.05).

The osteogenic and angiogenic protein expression of rBMSCs in MBG-β-TCP scaffolds

The expressions of COL-1, OPN, VEGF, and HIF-1α proteins were detected by western blot analysis. It is observed that the protein expressions of Col I, OPN, VEGF and HIF-1α for...
rBMSCs in MBG-β-TCP scaffolds have been significantly enhanced as compared to BG-β-TCP, β-TCP scaffolds and the blank control (Fig. 6), which is consistent with the results of the osteogenic and angiogenic related gene expression in the three scaffolds.

Attachment, morphology, viability and angiogenic related gene expression of HUVECs in MBG-β-TCP scaffolds

HUVEC attachment and morphology in MBG-β-TCP, BG-β-TCP and β-TCP scaffolds were examined by SEM and confocal microscopy (Fig. 7A). It is observed that after 3 d of culture, the three scaffolds support HUVEC attachment. The SEM images showed that the HUVECs spread well on the scaffolds and the confocal images revealed distinct and well-defined stress fibers, actin and cytoskeleton.

MTT analysis displays that the HUVEC number distinctly increased in MBG-β-TCP, BG-β-TCP and β-TCP scaffolds along with the culture time (Fig. 7B). After 1 d of culture, the viability of HUVECs on MBG-β-TCP scaffolds showed no difference compared with those of BG-β-TCP and β-TCP scaffolds, whereas the cell viability in MBG-β-TCP scaffolds was significantly higher than those in BG-β-TCP and β-TCP scaffolds at days 3 and 7 (P < 0.05). On day 7, angiogenic gene expressions of VEGF and HIF1-α for HUVECs in MBG-β-TCP scaffolds were significantly enhanced as compared to BG-β-TCP, β-TCP scaffolds and the blank control (P < 0.05) (Fig. 7B).

Protein adsorption of MBG, BG and β-TCP powders

The protein adsorption of MBG, BG and β-TCP particles is shown in ESI Fig. 5.† MBG groups show sustained adsorption of BSA (reached 80% at 72 h) while both BG and β-TCP groups show no obvious effect on the BSA concentration, indicating that the mesoporous structure endows MBG particles with significant capacity for protein adsorption.

The in vivo bone formation of MBG-β-TCP scaffolds in calvarial defects of rabbits

The 3D micro-CT images (Fig. 8A) showed that much more new bone formation was observed for MBG-β-TCP groups as compared with BG-β-TCP and β-TCP groups at 4 weeks or 8 weeks after operation. Moreover, the morphometrical analysis (Fig. 8B) showed that a significantly greater BV/TV value was detected for the MBG-β-TCP (18.02 ± 0.995% and 27.03 ± 2.18%) group as compared with BG-β-TCP (13.24 ± 1.58% and 22.12 ± 1.73%) and β-TCP (10.45 ± 1.58% and 18.69 ± 2.64%) groups at weeks 4 and 8, respectively. The value of Tb.Th in the MBG-β-TCP group (0.089 ± 0.014 mm and 0.17 ± 0.023 mm) was higher than that in BG-β-TCP (0.069 ± 0.013 mm and 0.13 ± 0.012 mm) and β-TCP (0.054 ± 0.0091 and 0.12 ± 0.010) groups at week 4 and 8, respectively (p < 0.05) (Fig. 8B).

The results of the undecalcified samples stained with Van Gieson’s picrofuchsin (Fig. 9A) show that the newly formed
bone in the \( \beta \)-TCP group was mainly detected in the periphery of the defect, while much more newly formed bone was detected in the periphery and center of the bone defect for BG-\( \beta \)-TCP and MBG-\( \beta \)-TCP groups, especially for the MBG-\( \beta \)-TCP group at week 4 after operation. When the implantation time was extended to 8 weeks, more newly formed bones were found in three groups as compared with that of the week 4 timepoints, while the most new bone formation was achieved in the MBG-\( \beta \)-TCP group. Under a histomorphometric assay, the higher percentage of new bone area was observed in the MBG-\( \beta \)-TCP group (14.99 ± 1.78% and 25.84 ± 2.02%) as compared with BG-\( \beta \)-TCP (11.83 ± 1.94% and 20.65 ± 2.98%) and \( \beta \)-TCP groups (9.34 ± 1.81% and 16.91 ± 2.54%) at weeks 4 and 8 (Fig. 9B), respectively.

Fig. 5 The viability (a), ALP activity (b) and osteogenic gene expression of Runx2 (c), OPN (d), COL-1 (e) and BMP-2 (f) of rBMSCs after being cultured with \( \beta \)-TCP, BG-\( \beta \)-TCP and MBG-\( \beta \)-TCP scaffolds for different time periods. rBMSCs cultured on MBG-\( \beta \)-TCP scaffolds showed the best viability, ALP activity, and osteogenic gene expression. (*\( P < 0.05 \), **\( P < 0.01 \)).

Fig. 6 Western blotting analysis of RUNX2, BMP-2, COL-1, OPN, VEGF, and HIF1-\( \alpha \) protein expression of rBMSCs cultured in MBG-\( \beta \)-TCP, BG-\( \beta \)-TCP and \( \beta \)-TCP scaffolds for 3 d. MBG-\( \beta \)-TCP scaffolds show the best protein expression.
Discussion

In this study, we set out to design and prepare a hierarchically porous bioceramic scaffold by incorporating a nanoporous structure onto the strut surface via combination of 3D printing with MBG modification. Our results suggest that the assembling strategy is very effective and the achieved scaffolds possess significantly improved osteogenic and angiogenic activity. It is known that 3D printing is a useful method to prepare scaffolds with controlled large-pore structures. However, 3D printing has a limited ability to prepare functional nanostructures in the scaffolds. Herein, we applied a combined strategy of 3D printing and spin coating to prepare a hierarchically porous structure composed of large pores (∼400 µm) and nanopores (∼5 nm) in β-TCP scaffolds. The strategy harnesses the advantages of the two techniques to
construct uniform macropores and well-ordered nanopores in one scaffold. To our knowledge, although there are different strategies for preparing hierarchically porous polymer scaffolds for tissue engineering, there are few methods for preparing macro and nanopores-combined bioceramic scaffolds. Our method by combining 3D printing and spin coating may be a universal strategy for constructing hierarchically porous scaffolds.

By using this strategy, the strut surface of β-TCP scaffolds was uniformly covered by a MBG nanolayer with a thickness of ~100 nm, which had no influence on the pore size of macropores, while significantly changing the microstructure of the struts in scaffolds. The grain boundary and micro defects in the strut surfaces of β-TCP scaffolds were fully filled by MBG nanolayers, which might be the main reason for reinforcement of the β-TCP scaffolds and the MBG-β-TCP scaffolds showed significantly enhanced mechanical properties. The compressive strength of the MBG-β-TCP scaffolds is around 11 MPa, which is significantly higher than the pure β-TCP scaffolds and is in the top range for cancellous bone (2–12 MPa). Therefore, the MBG-modified strut surfaces play a key role to improve the mechanical strength of β-TCP scaffolds.

For tissue engineering scaffolds and implants, the design of surface features, including surface chemistry and physics, is of great importance to influence the response of cells and tissues. In this study, the MBG nanolayer was successfully incorporated onto the strut surface of β-TCP scaffolds, which offers a functional interface with different surface chemistry (Si ions) and surface physics (nanopores). According to our obtained data, it was found that both BG-β-TCP and MBG-β-TCP scaffolds significantly enhanced ALP activity (a key marker for early mineralization), and the expression of impor-

Fig. 8  (A) 3D micro-CT images of the in vivo bone formation ability for β-TCP, BG-β-TCP and MBG-β-TCP scaffolds in rabbit calvarial defects at weeks 4 and 8 after operation; (B) morphometric analysis of the volume of the newly formed bone (BV/TV) and the trabecular thickness (Tb.Th) in the calvarial defect area at weeks 4 and 8. MBG-β-TCP scaffolds showed the best bone repair effect. Scale bar = 100 μm. (*P < 0.05).
 tant markers for osteogenic differentiation (RUNX2, OPN, COL-1 and BMP-2), angiogenic differentiation (VEGF and HIF-1α) and protein expression (OPN, Col I, VEGF, HIF-1α) as compared to pure β-TCP scaffolds. In the meanwhile, MBG-β-TCP showed higher osteogenic and angiogenic related gene expression than BG-β-TCP. It is reasonable to speculate that both the chemical (released Si ions) and physical features (nanopores’ structures) have positive effects on osteogenic differentiation of rBMSC and angiogenic differentiation of HUVECs, and more importantly, these effects could be doubled by combining both chemical and physical features. Firstly, previous studies have shown that Si and Ca ions could improve the osteogenic differentiation of rBMSCs.42–45 The orthosilicic acid (H₄SiO₄) could stimulate collagen I synthesis and osteoblastic differentiation in human osteoblasts,46,47 as well as the pure Si ions may improve the osteogenic differentiation by activating the Wnt-related signaling pathway of rBMSCs.48,49 Furthermore, the ionic products of calcium
silicate could stimulate HUVEC and HDF to over-express VEGF, and then induce HUVEC to form angiogenic network structures, which play an important role in bone regeneration because in vivo nearly all tissues are supplied with nutrients and oxygen by a highly branched system of larger blood vessels. The analysis of ionic concentration further indicated that Si also plays a pivotal role in stimulation of angiogenesis. On the basis of these studies, we speculate that the Si ions released from the MBG nanolayer of MBG-β-TCP scaffolds may be one of the key factors in enhancing the osteogenic differentiation and angiogenic differentiation of tissue cells. Secondly, the incorporation of nanoporous structure into the struts of scaffolds may be another important factor contributing to the improvement in both osteogenic and angiogenic differentiation of tissue cells. Previously, extensive investigations have confirmed that nanopores could lead to rapid protein adsorption in the early stages of implantation, which would affect the behavior of cells and then determine the bioactivity of the implant. Sang et al. found that the surface geometry and chemical properties of nanopores (pore size: 5.9 nm) dominates the conformation of a protein at the interface. Leong et al. found that the nanopores in the fibers of electrospun scaffolds enhanced the levels of adsorbed proteins from a dilute solution of fetal bovine serum and the amount of proteins adsorbed by nanoporous fiber scaffolds was 80% higher than the fiber scaffolds without nanopores.

In our study, MBG showed significantly improved protein adsorption (5–8 times, as shown in ESI Fig. 5f) as compared to BG and β-TCP, indicating that the incorporation of MBG into the struts of β-TCP scaffolds could improve protein absorption, which may further stimulate the in vitro osteogenic and angiogenic differentiation of tissue cells.

Finally, MBG-β-TCP scaffolds showed improved apatite mineralization as compared to BG-β-TCP and β-TCP scaffolds (as shown in ESI Fig. 3f), which may be one of the important factors contributing to the improved osteogenic ability of stem cells in MBG-β-TCP scaffolds. According to previous studies, the excellent apatite mineralization of biomaterials may contribute to the osteogenic activity of the materials. Wu et al. found that bioactive porous bredigite (Ca$_7$MgSi$_4$O$_{16}$) scaffolds with a biomimetic apatite layer have an active effect on cell viability and differentiation. Ohgushi and Labat et al. have found that the bone-like apatite layer possesses a good binding ability with serum proteins and growth factors, which could stimulate cell viability and activate cell differentiation. Chou et al. found that the morphology and structure of biomimetic apatite affected osteoblast viability, and gene expression. By comparison, we found that the apatite layer formed in MBG-β-TCP scaffolds is similar to the result in the study of Ohgushi et al. Likewise, it is reliable to believe that the quick apatite mineralization on the surface of MBG-β-TCP scaffolds benefits osteogenic differentiation of stem cells.

Moreover, the in vivo study demonstrated that MBG-β-TCP scaffolds significantly enhanced new bone formation as compared to BG-β-TCP and β-TCP scaffolds in the calvarial defects of rabbits, indicating that the hierarchically porous structure and functional surface of MBG-β-TCP scaffolds play important roles in enhancing the in vivo osteogenesis. The improvement of in vivo osteogenesis of MBG-β-TCP scaffolds is supposed to be closely related to the enhanced osteogenic differentiation of rBMSCs and angiogenic differentiation of HUVECs, which were clearly indicated by in vitro cell experiments. In consequence, our results suggest that the incorporation of hierarchically porous structures and functional strut surfaces into 3D-printed scaffolds is highly effective in stimulating the in vivo bone formation for bone tissue engineering application. Moreover, it is found that MBG-β-TCP scaffolds have significantly improved bone formation compared to BG-β-TCP scaffolds despite their having the same chemical composition, indicating that the nanopores on the scaffold strut are of great importance in improving their osteogenic activity. Besides the nanopores, it is found that both MBG-β-TCP and BG-β-TCP scaffolds have improved bone formation as compared to pure β-TCP scaffolds. The results suggest that the nanopores and bioactive components (Si, Ca and P) in MBG-β-TCP scaffolds synergistically stimulated bone regeneration.

**Conclusion**

In this study, MBG-β-TCP scaffolds with hierarchically porous structures and functional strut surfaces for bone tissue engineering were successfully fabricated via a combination of 3D printing and spin coating. The MBG and BG nanolayers on the strut surface of β-TCP scaffolds significantly enhanced their mechanical properties. The MBG-β-TCP scaffolds showed higher osteogenic and angiogenic related gene expression than BG-β-TCP due to the distinguished mesoporous structure. Meanwhile, through providing chemical (released Si ions) and physical (mesoporous structures) features on the strut surface, MBG-β-TCP scaffolds showed significantly improved apatite mineralization, osteogenic and angiogenic activities, and bone-forming efficiency as compared to β-TCP scaffolds. MBG-nanolayer modification of 3D-printed bioceramic scaffolds by incorporating nanopores and bioactive components into the scaffold struts offers a new strategy to construct hierarchically porous scaffolds with synergistically improved physicochemical and biological properties for bone tissue engineering applications.

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References


