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A comparative study of mesoporous-glass/silk and non-mesoporous-glass/silk scaffolds: physiochemistry and in vivo osteogenesis

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ABSTRACT

Mesoporous bioactive glass (MBG) is a new class of biomaterials with a well-ordered nano-channel structure, whose in vitro bioactivity is far superior to that of non-mesopore bioactive glass (BG); the material’s in vivo osteogenic properties is, however, yet to be assessed. Porous silk scaffolds have been used for bone tissue engineering, but this material’s osteoconductivity is far from optimal. The aims of this study were to incorporate MBG into silk scaffolds in order to improve their osteoconductivity, and then to compare the effect of MBG and BG on the in vivo osteogenesis of silk scaffolds. MBG/silk and BG/silk scaffolds with a highly porous structure were prepared by a freeze-drying method. The mechanical strength, in vitro apatite mineralization, silicon (Si) ion release and pH stability of the composite scaffolds were assessed. The scaffolds were implanted into calvarial defects in SCID mice and the degree of in vivo osteogenesis was evaluated by micro-computed tomography (μCT), hematoxylin and eosin (H&E) and immunohistochemistry (type I collagen) analyses. The results showed that MBG/silk scaffolds have better physiochemical properties (mechanical strength, in vitro apatite mineralization, silicon (Si) ion release and pH stability) compared to BG/silk scaffolds. MBG and BG both improved the in vivo osteogenesis of silk scaffolds. μCT and H&E analyses showed that MBG/silk scaffolds induced a slightly higher rate of new bone formation in the defects than did BG/silk scaffolds and immunohistochemical analysis showed greater synthesis of type I collagen in MBG/silk scaffolds compared to BG/silk scaffolds.
1. Introduction

A new class of bioactive glass, referred to as mesoporous bioactive glass (MBG), was first developed in 2004. This material has a highly ordered mesopore channel structure with a pore size ranging from 5–20 nm [1]. Compared to non-mesopore bioactive glass (BG), MBG possesses a more optimal surface area and pore volume, evident by greatly enhanced drug-delivery capability, *in vitro* apatite mineralization and degradation [1-6]. For this reason, MBG has received much attention for applications for bone tissue engineering [5,7-9]. Li et al. prepared hierarchically MBG scaffolds using polyurethane foam as template for the macroporous structure and showed that MBG scaffolds induced the formation of an apatite layer after soaking in SBF for 4 h [8]. We have recently shown that MBG scaffolds can support cell adhesion, proliferation and differentiation [7,9], and also that MBG powders, when incorporated into a poly(lactide-co-glycolide) (PLGA) film, significantly enhances the apatite-mineralization ability and cell response of PLGA films, compared to BG [5]. These studies suggest that MBG is a very promising bioactive material with respect to bone regeneration. Most studies of MBG have until now focused on *in vitro* studies, including the apatite mineralization in SBF and cell response [1,7,10] and there are, as far as we know, few, if any, studies that have investigated the *in vivo* osteogenic property of MBG and its composites.
Silk fibroin, as a new family of native biomaterials, has been widely studied for bone and cartilage repair applications in the form of pure silk or its composite scaffolds [11-16]. Compared to traditional synthetic polymer materials, such as PLGA and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), the chief advantage of silk fibroin is its water-soluble nature, which eliminates the need for organic solvents, that tend to be highly cytotoxic in the process of scaffold preparation [17]. Other advantages of silk scaffolds are their mechanical properties, controllable biodegradability and cytocompatibility [17-19]. However, for the purposes of bone tissue engineering, the osteoconductivity of pure silk scaffolds is suboptimal. We hypothesize that combining MBG with silk scaffolds would greatly improve silks osteogenic properties by virtue of the excellent in vitro bioactivity of MBG. The effect of MBG and BG on the in vivo osteogenesis of silk scaffolds has not been previously tested, therefore, the aim of this study is to incorporate MBG or BG powders into silk scaffolds and compare the physio-chemistry and in vivo osteogenesis of MBG/silk and BG/silk scaffolds.

2. Materials and methods
   2.1. Synthesis and characterization of MBG and BG powders

Mesoporous Bioglass (MBG) powders (molar ratio: Si/Ca/P = 80/15/5) were synthesized according to our previous publication [5]. In a typical synthesis, 4.0 g of Pluronic P123 (Mw=5800, Sigma), 6.7 g of tetraethyl orthosilicate (TEOS, 98%, Sigma), 1.4 g of Ca(NO₃)₂·4H₂O (Sigma), 0.73 g of triethyl phosphate (TEP, 99.8%, Sigma) and 1.0 g of 0.5M HCl were dissolved in 60 g of ethanol (Si/Ca/P = 80:15:5, molar ratio) and stirred at room temperature for 1 day. The resulting solution was
introduced into a petri dish for an evaporation-induced self-assembly process, and then the dry gel was calcined at 700ºC for 5 h to obtain MBG powders. Non-mesoporous bioglass (BG) powders were synthesized under the same preparation conditions, but without the addition of P123, and used as a control. The MBG and BG powders thus obtained were ground and sieved to 300 meshes. The inner microstructure of MBG powders was analyzed by transmission electron microscopy.

2.2. Preparation, characterization and mechanical strength of silk, MBG/silk and BG/silk porous scaffolds

Porous MBG/silk scaffolds with 10% MBG (w/w) were fabricated using a freeze-drying method: 0.1 g of MBG powders were added to 20 mL of 5% (w/v) silk water solutions under stirring for 2 h and ultrasonic dispersing for 10 min to form a uniform mixture, 0.4 mL of which was quickly transferred into the wells of a 96-well cell culture plate. The plate was then placed in a freezer at -20ºC overnight to solidify the mixture and induce solid–liquid phase separation. The solidified mixture was freeze-dried in a freeze-drying machine (Christ Alpha 1-2) for 48 h to obtain porous MBG/silk scaffolds. The scaffolds were soaked in ethanol (99%) for 10 min and then dried overnight at 40ºC. Pure silk and BG/silk scaffolds served as controls and were prepared the same way as the MBG/silk scaffolds. The pore morphology and surface microstructure of the scaffolds were characterized by scanning electron microscopy (SEM, Joel JSM 6510).

The porosity of the scaffolds was measured according to Archimedes’ principle. Samples with a size of Ø5×8 mm were used for the measurement and water was used as liquid medium. The porosity (P) was calculated according to the following formulation
P = ((W_2-W_1)/(W_2-W_3)) \times 100\%, where W_1 is the dry weight of the scaffolds, W_2 is the wet weight, and W_3 is the weight of scaffolds suspended in water.

For mechanical analysis, cylinder scaffolds with a size of Ø8×10 mm were prepared using the method described above and 1.2 ml of silk mixture was added to the wells of 48-well cell culture plate. The compressive strength and modulus of the scaffolds were measured using an Instron 5567 (Illinois Toolworks Inc, USA) computer-controlled universal testing machine at a crosshead speed of 1 mm/min by pressing the scaffolds until to a 60% strain.

2.3. The apatite-mineralization ability and ion release of the scaffolds in simulated body fluids

The apatite-mineralization ability and ion release of scaffolds were carried out using acellular simulated body fluids (SBF) [20,21]. The scaffolds were immersed in SBF with a ratio of 200 mL/g for SBF solution volume to scaffold mass, and kept at 37°C for 1, 3 and 7 days. SEM, energy dispersive spectrometer (EDS) and Fourier transform infrared spectra (FTIR) analysis was performed after sample scaffolds had soaked in SBF for 7 days. The concentration of Si ions released from the scaffolds was assayed by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Fresh SBF does not contain any Si ions, so these ions served as a measure of ion release from the scaffolds. The pH value of the SBF solution was also tested after soaking of the scaffolds for longer periods, up to 42 days.

2.4. Scaffolds transplantation into calvarial defects

The bone forming ability of the three scaffolds was assessed in a calvarial defect model
in adult severe combined immunodeficient (SCID) mice, following a previously
described method [16,22]. Surgery was carried out according to the guidelines of the
Animal Research and Care Committees of the Herston Medical Research Centre and the
Queensland University of Technology. The surgical procedures were performed in
aseptic conditions under general anesthesia. Briefly, a linear incision (1 cm long) was
made on the left side of the skull, exposing the bone surface. The periosteum was
dissected from the bone surface and a full-thickness calvarial bone defect, 3 mm in
diameter, was created with a trephine bur in the frontal and parietal bones using a
slow-speed dental drill. To avoid tissue damage due to overheating, 0.9% saline was
dripped onto the contact point between the bur and bone and great care was taken to
avoid injury to the dura mater. Scaffolds, with a size of 3×3×1 mm, were carefully
placed into the defects and soft tissue above the defect was covered by the skin which
was closed with skin staples.

2.5. Micro-computed tomography (μCT) and histology

The animals were euthanized 8 weeks after surgery and the defect areas collected. The
samples were fixed in 4% paraformaldehyde for 12 h at room temperature. All samples
were scanned for bone formation within the defect site using a Scanco μCT40 imaging
system (Scanco Medical, Bassersdorf, Switzerland) with the following scan parameters:
20 mm field of view, 55 kVp X-ray energy setting, 1024 reconstruction matrix, slice
thickness 0.02 mm, and a 250 ms integration time. Mineralized tissue was distinguished
from non-mineralized tissue using a global thresholding procedure with a value
approximating 1.20 g/cm³ (150 on micro-CT) (25% lower than 1.6 g/cm³) which is the
mineral density of normal human compact bone. Bone volume in each defect was recorded as the measure of defect bone regeneration.

After the µCT scanning, all tissue samples were decalcified in 10% EDTA, changed twice weekly, for 2 to 3 weeks, after which they were embedded in paraffin. Serial sections, 5 μm thick, were cut and mounted on polylysine-coated microscope slides. All sections were stained with hematoxylin and eosin (H&E), and a general assessment of the tissue and wound healing was performed using visual light microscopy.

2.6. Immunohistochemistry

Monoclonal antibody against human type I collagen (COL1) antibody was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Endogenous peroxidase activity was quenched by incubating the tissue sections with 3% H₂O₂ for 20 min before staining with immunoperoxidase. All sections were blocked by 0.1% bovine serum albumin (BSA) with 10% swine serum. Sections were then incubated with the optimal dilution of primary antibody for COL1 (1:100) overnight at 4°C. Sections were then incubated with a biotinylated swine anti-mouse, anti-goat antibody (Multilink; DakoCytomation, Carpinteria, CA) for 15 min, and then incubated with horseradish peroxidase conjugated avidin–biotin complex for 15 min. Antibody complexes were visualized with the addition of a buffered diaminobenzidine substrate for 4 min. The reaction was stopped by immersion and rinsing of sections in PBS. Sections were then lightly counterstained with Mayer’s hematoxylin and Scott’s blue for 40 s each, in between 3 min rinses with running water. Subsequently, sections were dehydrated with ascending concentrations of ethanol solutions, cleared with xylene, and mounted with a coverslip and DePeX
mounting medium (BDH Laboratory Supplies, Poole, UK).

Controls for the immunohistochemical staining procedures included conditions under which the primary antibody or the secondary (anti-mouse IgG) antibody was omitted; in addition, an irrelevant IgG, which should not have been present in the test sections, was used as a control. To ensure that the procedure itself was not resulting in nonspecific staining, various safeguards were used. These included elimination of the primary antibody incubation step, in the presence of all other steps; and normal primary antibody incubation followed by elimination of either the secondary antibody or one of the other subsequent detection steps.

2.7. Statistical analysis

The data was expressed as means ± standard deviation (SD) for all experiments and were analyzed using One-Way ANOVA with a Post Hoc test. A $p$-value<0.05 was considered statistically significant.

3. Results

3.1. Characterization and mechanical strength of the prepared porous scaffolds

Mesoporous MBG powders showed a well-ordered channel structure with a pore size of approximately 5 nm (Fig. 1). The silk, MBG/silk and BG/silk scaffolds were highly porous (Fig. 2), with near identical porosities, 78± 3%, 76±4% and 76±2%, respectively. The pure silk scaffolds had a flat pore morphology (Fig. 2a), whereas the MBG or BG composite scaffolds had a more open pore morphology (Fig. 2b and c) compared to the silk scaffolds. MBG or BG particles were clearly visible in the pore walls of composite
scaffolds (see arrows in Fig. 2b and c). It was noted that the MBG/silk scaffolds had more particles than BG/silk scaffolds due to the higher specific surface area for MBG powders (the specific area of MBG and BG is 400 and 57 m²/g, respectively [5]). The pore size of the pure silk scaffolds ranged from several tens to one hundred micrometers; The pore size of the composite scaffolds is larger than that of pure silk scaffolds (Fig. 2).

The compressive strength and modulus of MBG/silk scaffolds were 420kPa and 0.70MPa, respectively, figures that were comparable with those of pure silk scaffolds, and greater than those of BG/silk scaffolds (Fig. 3).

3.2. The apatite-mineralization ability and ion release of the scaffolds in SBF

The morphology of the three scaffold species, after soaking in SBF, is shown in Figure 4. There was no apatite particles deposit visible on the pore wall surfaces for pure silk and BG/silk scaffolds (Fig. 4a, b and c). However, a layer of apatite microparticles formed on the pore wall of MBG/silk scaffolds (Fig. 4d) and at higher magnification apatite was seen as nano-sized particles (Fig. 4e). EDS analysis revealed the ratio of Ca/P of the apatite to be 2.3. FTIR analysis has shown no obvious P-O peaks in the patterns after soaking silk and BG/silk in SBF (Fig. 4f); however, there are several weak P-O peaks in the pattern of MBG/silk after soaking in SBF (Fig. 4f).

Si ion release and pH values of SBF are shown in Figure 5. There was a sustained release of Si ions from both the MBG/silk and BG/silk scaffolds, even across an extended period of soaking and the MBG/silk scaffolds had a faster rate of Si ion release than BG/silk scaffolds (Fig. 5a). The pH value of SBF with MBG/silk scaffolds
stayed within a range of 7.25–7.5 throughout the 6 weeks of soaking. The pH values of the pure silk and BG/silk scaffolds resulted in a slight decreased in SBF, varying from 7.1 to 7.4 (Fig. 5b).

3.3. μCT analysis of the osteogenesis

Composite 3D μCT images of the bone defect repair with the three scaffold species are shown in Figure 6 and 7. Both BG/silk and MBG/silk scaffolds clearly showed better bone repair ability than pure silk scaffolds. The defects implanted with MBG/silk scaffolds had been completely filled with new bone mineral tissues (Fig. 6b). The BG/silk scaffolds also induced new bone formation in the defects (Fig. 6c). However, the skull defects implanted with pure silk scaffolds revealed little mineralized tissues around the border and no new bone formation at all in the middle of the defects (Fig. 6a). Quantitative analysis from μCT data showed that the mineralized tissue volume for MBG composite was a little higher than that of BG composite. The volume of mineralized tissue for silk, MBG/silk and BG/silk scaffolds was 2.5, 7.0 and 6.1 mm³, respectively (Fig. 7).

3.4. Histology analysis of the osteogenesis

Histological analysis by H&E demonstrated that more new bone formed in the defects filled with MBG/silk scaffolds, compared to BG/silk scaffolds (Fig. 8). New bone filled most of the MBG/silk scaffolds from the edge to the center and formed a continuous plate of bone area (Fig. 8a and b). Most of MBG/silk scaffolds had been degraded (Fig. 8a). In the BG/silk scaffolds the majority of the new bone was located in the periphery, with some bone islands forming centrally. There was only limited degradation of the
BG/silk scaffolds (Fig. 8c and d). In the skull defects implanted with pure silk scaffolds there was no evidence of bone formation (data not shown).

Immunohistochemical analysis revealed COL1 expression in the *de novo* bone in both MBG/silk and BG/silk scaffolds (Fig. 9); there was certainly slightly strong COL1 expression in the bone matrix of the MBG/silk scaffolds (Fig. 9a and b) and this expression was discernibly stronger compared to that in the BG/silk scaffolds (Fig. 9c and d).

4. Discussion

In this study, we have compared the physiochemical and *in vivo* osteogenic effects on silk scaffolds of mesoporous and non-mesoporous bioactive glass (MBG and BG respectively). We found that both materials significantly enhanced the *in vivo* osteogenesis of silk scaffolds. The significant finding was that in the case of MBG composites, they have the improved mechanical strength, dissolution, *in vitro* apatite mineralization and pH stability, compared to BG composite. This is a novel finding which confirms that MBG–a new class of bioactive inorganic materials–has improved *in vivo* bioactivity compared to BG when incorporated into polymer-based scaffolds. It is also proves, that preparing mesoporous biomaterials by sol-gel process and supramolecular chemistry is a conceptual advance for biomaterials science.

MBG/silk and BG/silk composite scaffolds with 10% of MBG or BG, have been successfully prepared using freeze-drying method. The composite scaffolds produced by this method are highly porous, which will benefit tissue ingrowth [23]. In this study we
incorporated various amounts of MBG into silk scaffolds, but we found that it was difficult to disperse the MBG uniformly in the silk solution if it exceeded 10% w/w due to the high surface area of MBG powders. Therefore, 10% w/w was the concentration used for both MBG and BG when preparing the composite scaffolds.

The mechanical tests revealed that MBG/silk scaffolds had greater mechanical strength than the BG/silk scaffolds. It is known that MBG powders have significantly greater specific surface area and pore volume compared to BG powders. The specific area of MBG and BG is 400 and 57 m²/g, and the pore volume of MBG and BG is 0.5 and 0.09 cm³/g according to our previous publication [5] and MBG powders have a highly ordered channel structure with a pore size of approximately 5 nm. In this study, the incorporation of BG particles into silk scaffolds may destroy the inner structure of silk and lead to the detrimental effect of the mechanical strength of silk scaffolds. Although MBG particles may also destroy the inner structure of silk, however, MBG has high surface area and pore volume, and parts of silk solution may enter into the nanopores of MBG during preparation, which leads to a strong bond between MBG particles and silk after freeze-drying. Thus, the incorporation of MBG into silk will not decrease the mechanical strength of silk scaffolds.

The MBG/silk scaffolds also have a faster rate of Si ion release than BG/silk scaffolds (Fig. 5a) and we estimated the Si content of MBG to be approximately 80%. It is reasonable to assume that MBG/silk scaffolds have greater rate of \textit{in vitro} dissolution than do the BG/scaffolds. The MBG/silk scaffolds maintained a stable pH value as the scaffolds degraded in SBF and this is beneficial for cell and tissue growth [24-26].
This, however, was not the case with BG/silk scaffolds in which the pH decreased. It is highly likely that the greater release of Si ions from the MBG/silk scaffolds buffered the SBF, thereby neutralizing the degradation products and maintaining a more stable pH value, compared to the BG/silk scaffolds.

Apatite mineralization of silicate materials, such as CaSiO₃ ceramics, 45S5 bioglass, etc., is thought to be an important phenomenon in the chemical interactions between the implant materials and the bone tissue, which ultimately affects the in vivo osteogenesis of the bone grafting materials [27-29]. In this study, MBG/silk scaffolds had an obvious apatite mineralization in SBF, whereas neither BG/silk nor pure silk scaffolds induced apatite mineralization. This suggests that MBG/silk scaffolds have an improved “in vitro bioactivity”, a term that has been used in previous studies [20,30,31]. Earlier studies have also demonstrated that MBG have a significantly improved apatite-mineralization ability in SBF than does BG [1,32] and our own work has shown that MBG improves attachment, proliferation and differentiation of human osteoblast on PLGA films [5]. Together these data leaves little doubt that MBG possesses excellent in vitro bioactivity. To the best of our knowledge, there are no reports describing the in vivo osteogenic properties of MBG. This study, therefore, is the first to compare the in vivo effect of MBG and BG on osteogenesis of silk scaffolds by implanting the scaffolds into calvarial defects of SCID mice. Micro-CT, H&E and immunohistochemical analyses all confirmed that MBG/silk scaffold were superior in all aspects compared to BG/silk scaffolds. Future work will be conducted in a bone defect model of immune-competent animals to investigate potential immunological reaction of the silk/BMG scaffolds, as
well as the bone forming capacity of this type of scaffold in immuno-competent models. There are three arguments that best explains why MBG/silk scaffolds have slightly improved new-bone formation, compared to BG scaffolds: (1) apatite mineralization plays an important role in bone repair and studies suggest that apatite mineralization of 45S5 bioglass [29], A–W bioactive glass ceramics [33] and CaSiO3 ceramics [27,34], is the direct factor influencing the in vivo osteogenesis potential of these materials. In the present study, we show that MBG/silk has a better apatite-mineralization ability than does BG/silk, leading us to draw the tentative conclusion that this may be one of the most important factors to improve new-bone formation. (2) The faster rate of dissolution and Si ion release of the MBG/silk scaffolds compared to BG/silk scaffolds may enhance new-bone formation; this is supported by a study that showed that CaSiO3 ceramics has significantly faster rate of degradation than does β-tricalcium phosphate ceramics and leads to an improved in vivo osseointegration [27]. It has been reported that Si ions may be associated with the initiation of pre-osseous tissue mineralization, both in periosteal or in endochondral ossification, in the early stages of calcification [35,36]. In vitro studies have confirmed that silicon released from the materials results in a significant up-regulation of osteoblast proliferation and gene expression [37-39]. The faster rate of degradation may in fact provide the space and environment for matrix deposition and tissue growth [40], and, at the same time, the quicker release Si ions from MBG/silk scaffolds may stimulate the viability of osteoblast around the defects, to the benefit of in vivo osteogenesis. (3) one cannot overlook the beneficial role that the stable pH environment of MBG/silk scaffolds has on in vivo osteogenesis [41,42].
5. Conclusions

We, for the first time, compared the physiochemistry and osteogenesis of MBG composite with BG composite. In the case of MBG composites, they have the improved physiochemical properties (mechanical strength, dissolution, in vitro apatite mineralization and pH stability), compared to BG composite. MBG/silk scaffolds induced a high rate of new-bone formation and type I collagen synthesis after implanted in calvarial defects. Our results confirm that MBG—a new class of bioactive inorganic materials—has significant capacity to improve the in vivo bioactivity of polymer-based scaffolds.

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References


**Figure captions**

**Figure 1.** TEM image of mesoporous bioactive glass powders show a well-ordered channel structure and the pore size of approximately 5 nm.

**Figure 2.** Surface morphology of porous silk (a), MBG/silk (b) and BG/silk (c) scaffolds. Arrows point to MBG or BG particles in silk scaffolds.

**Figure 3.** The mechanical strength of porous scaffolds. MBG/silk scaffolds have greater strength than BG/silk scaffolds.
Figure 4. Figure 4. SEM and EDS analysis silk (a), BG/silk (b, c) and MBG/silk (d, e) scaffolds after soaking in simulated body fluids for 7 days. Fig. (f) is FTIR analysis. Figure (c) is lower magnification of BG/silk scaffolds. Figure (e) is higher magnification of MBG/silk scaffolds. Apatite formed on the surface of MBG/silk scaffolds, but no apatite formed on the surface of pure silk and BG/silk scaffolds. The ratio of Ca/P for the apatite formed on MBG/silk scaffolds is 2.3.

Figure 5. Si ion release from the three scaffold species and their pH values in SBF solution. MBG/silk scaffolds have a faster rate of Si ion release and maintain a more stable pH value, than does BG/silk scaffolds.

Figure 6. The in vivo bone formation of (a) silk; (b) MBG/silk; and (c) BG/silk scaffolds after implantation in calvarial defects in SCID mice for 8 weeks as assessed by Micro-CT. New bone has completely filled in defects with MBG/silk scaffolds. MBG/silk scaffolds showed better bone-formation ability than did pure silk or BG/silk scaffolds. Size bar = 1 mm.

Figure 7. The new bone volume for silk; BG/silk; and MBG/silk scaffolds after implanted in calvarial defects of SCID mice for 8 weeks.

Figure 8. The in vivo bone formation was evaluated by hematoxylin and eosin staining. (a) and (b): MBG/silk; (c) and (d): BG/silk. (b) and (d) are higher magnification images. Arrows point to new formed bone. There is more new bone formed in the center of MBG/silk scaffolds than in the center of BG/silk scaffolds.

Figure 9. Immunohistochemical analysis by type I collagen staining on the new bone tissues. (a) and (b): MBG/silk; (c) and (d): BG/silk. (b) and (d) are higher magnification images. MBG/silk scaffolds show greater type I collagen expression than does BG/silk scaffolds.