

Cartilage tissue engineering using PHBV and PHBV/Bioglass scaffolds

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Abstract. Scaffolds have an important role in cartilage tissue engineering. Poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) has been demonstrated to have potential as a scaffold for the three dimensional construction of engineered cartilage tissue. However, the poor hydrophilicity and mechanical strength associated with PHBV affects its clinical applications as a scaffold in cartilage tissue engineering. The incorporation of Bioglass (BG) into PHBV has been shown to improve the hydrophilicity and mechanical strength of PHBV matrices. Therefore, this study aimed to compare the properties of PHBV scaffolds and PHBV scaffolds containing 10% BG (w/w) (PHBV/10% BG) and to investigate the effects of these scaffolds on the properties of engineered cartilage *in vivo*. Rabbit auricular chondrocytes were seeded onto PHBV and PHBV/10% BG scaffolds. Differences in cartilage regeneration were compared between the neocartilage grown on the PHBV and the PHBV/10% BG scaffolds after 10 weeks of *in vivo* transplantation. The incorporation of BG into PHBV was observed to improve the hydrophilicity and compressive strength of the scaffold. Furthermore, after 10 weeks incubation *in vivo*, the cartilage-like tissue formed using the PHBV/10% BG scaffolds was observed to be thicker, exhibit enhanced biomechanical properties and have a higher cartilage matrix content than that generated using the pure PHBV scaffolds. The results of this study demonstrate that the incorporation of BG into PHBV may generate composite scaffolds with improved properties for cartilage engineering.

Introduction

The emergence of engineered cartilage tissue has provided novel approaches for the repair of cartilage defects in plastic and reconstructive surgery. Seeding chondrocytes onto biodegradable scaffolds to construct three dimensional cartilage tissue for implantation may be a highly promising strategy for the repair of cartilage defects (1,2). Scaffolds represent one of the three essential factors necessary for tissue engineering and have a significant role in cartilage regeneration. Various biodegradable polymers have been explored for use as scaffolds for cartilage tissue engineering, including calcium alginate gel and polyglycolic acid (3-6). Among these, the novel polyhydroxyalkanoates (PHAs) have been shown to be biocompatible, biodegradable and thermoplastic polyesters, which, due to their enhanced biomechanical properties, may be ideal for use as biomedical materials. The PHA that has attracted the most interest is poly(3-hydroxybutyrate) (PHB). Numerous strains of bacteria have been identified to be capable of producing PHB in high yields. Poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV), a copolymer containing hydroxyvalerate and PHB in varying ratios, is a particularly useful material due to its less crystalline and more flexible structure, and greater ease of processability than PHB (7).

Previous studies have used PHBV as a biomaterial for cartilage tissue engineering. In one such study, Kose *et al* (8) investigated the use of macroporous PHBV scaffolds in the repair of full-thickness cartilage defects (side length, 4.5 mm; depth, 4 mm) in rabbits *in vivo*. At 8 and 20 weeks after seeding, minimal foreign body reaction was observed and the chondrocytes seeded onto the PHBV matrices showed early cartilage formation. Furthermore, the newly formed cartilage had the appearance of normal articular cartilage (8). Lu *et al* (9) confirmed the feasibility of engineering an entire meniscal structure in a total meniscectomy rabbit model using biodegradable PHBV scaffolds and cultured allogeneic meniscal cells. However, PHBV is a hydrophobic polyester. In a previous study, we revealed that the poor hydrophilicity and mechanical strength associated with PHBV resulted in a low percentage of cell adherence and the formation of thin cartilage layers with poor biomechanical properties (10). Therefore, the present study aimed to investigate the improvement of the

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hydrophilic characteristics and mechanical strength of PHBV scaffolds.

Bioglass® (BG) is a bioactive inorganic material composed of specific proportions of SiO₂, Na₂O, CaO and P₂O₅, and its incorporation into PHBV has previously been reported to be capable of improving the hydrophilicity and mechanical strength of the composites (7,11). However, few reports have shown whether the incorporation of BG into PHBV scaffolds has potential in cartilage tissue engineering. Therefore, in the present study it was hypothesized that the incorporation of BG into PHBV would generate composite scaffolds with enhanced properties for cartilage engineering compared with scaffolds solely composed of PHBV.

To investigate this hypothesis, PHBV scaffolds and PHBV scaffolds containing 10% BG (w/w) (PHBV/10% BG) were prepared. Chondrocytes were seeded onto the scaffolds and cultured *in vitro* for 24 h, prior to 10 weeks of *in vivo* implantation to observe the formation of engineered cartilage tissue on the different scaffolds. The extracellular matrix (ECM) production, size, structure and biomechanical properties of the neocartilage were analyzed to compare the structure and function of the engineered cartilage tissue produced by the different scaffolds.

Materials and methods

Ethics statement. All experimental procedures performed in this study were approved by the Ethics Committee of the Shanghai Jiao Tong University School of Medicine (Shanghai, China).

Preparation of PHBV and PHBV/10% BG scaffolds. PHBV, (molecular weight, 300,000 Da) containing 3 mol% 3-hydroxyvalerate was obtained from Tianan Biologic Material Co. Ltd. (Ningbo, China). A solvent casting/particulate leaching method was used in the preparation of the PHBV and composite PHBV/10% BG scaffolds as described in a previous study (12). PHBV powder (1 g) was dissolved in 10 ml chloroform to generate a solution with a concentration of 10% (w/v). To produce the PHBV/10% BG composite scaffolds, the solution was supplemented with 0.11 g BG powder and stirred continuously for 2 h to ensure uniform dispersal of the powder. NaCl particles were subsequently added to the solution as pore-generating additives. The solution was then cast in a 60-mm diameter, 3-mm long Teflon® mold and air-dried in a fume hood for 24 h to allow solvent evaporation. Any remaining solvent was eliminated through vacuum drying at 60°C for 48 h. The pore-generating additives were then leached from the dried samples by immersion in deionized water. Samples were subsequently subjected to further vacuum drying to produce porous scaffolds, which were referred to as PHBV and PHBV/10% BG scaffolds, respectively. Scaffolds were cut into identical rectangular prisms that measured 4 mm in length and were 3-mm thick. Additional PHBV and PHBV/10% BG films were prepared using an identical method; however, the porogen addition and particulate leaching processes were eliminated so that the water contact angles could be determined. AgNO₃ titration was performed to ensure complete NaCl leaching from the scaffolds (12).

Characterization of the PHBV and PHBV/10%BG scaffolds. Scaffold porosity was determined by measuring the mass and dimensions of the scaffolds as described previously (13). In order to test the compressive strength, PHBV and PHBV/10% BG scaffolds that measured 6 mm in diameter and were 3-mm thick were prepared. The compressive strength of the scaffolds was determined using an AG-1 Shimadzu mechanical tester (Shimadzu Co., Kyoto, Japan), with a cross-head speed of 0.5 mm/min.

The water absorptivity of the scaffolds was determined as described previously (14). Dry scaffolds were weighed to obtain the dry weight (W_{dry}), prior to being placed in deionized water at room temperature for 4 h. This ensured that water absorption was equilibrated. The hydrated scaffolds were then extracted from the water, filter paper was utilized to remove the free surface water and the scaffolds were weighed to obtain the wet weight (W_{wet}). The water absorption ratio was calculated as follows: Water absorption ratio (%) = (W_{wet} - W_{dry}) / W_{dry} × 100.

Determination of hydrophilicity. Scaffold hydrophilicity was analyzed by measuring the water contact angles of the nonporous composite cuboids. Contact angles were measured at 25°C using a contact angle goniometer (model SZ10-JC2000A; Maikailun Co., Xiamen, China). The sessile drop method was used to obtain the measurements, and five different locations were analyzed for each specimen. A total of 0.5 µl deionized water was deposited onto the surface of the specimen at each location. The degree of reproducibility for the different specimens was within ±4.0°. Three specimens were tested for each sample.

Cell isolation and construction of engineered cartilage. Auricular cartilage was harvested from the ears of New Zealand white rabbits (SLAC Lab. Animal Ltd., Shanghai, China), aged between three and five days, prior to being cut into small pieces as previously described (15). The cartilage slices were digested using 0.2% (w/v) collagenase II to release the chondrocytes, which were then cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The chondrocytes were incubated at 37°C in 5% CO₂ and the medium was changed every three days. Following two subcultures, 30 µl chondrocyte suspension (5 × 10⁷ cells/ml) at passage 2 was deposited onto each scaffold, prior to the addition of 5 ml DMEM after 4 h. After 24 h, the constructs were harvested and subcutaneously implanted into nude mice (SLAC Lab. Animal Ltd.). Ten weeks after implantation, the specimens were harvested.

Cell adhesion. The PHBV and composite PHBV/10% BG substrates were soaked in 75% ethanol for 48 h prior to overnight sterilization using ultraviolet radiation and washing with sterile phosphate-buffered saline (PBS, pH 7.4). Chondrocytes were then seeded onto the sterilized substrates in a 48-well plate (density, 70 cells/mm²) and maintained in a CO₂ incubator for 3 h, prior to the addition of 1 ml fresh medium to each well. Cell viability was determined using an MTT-based colorimetric assay and the percentage of adhered cells was calculated in accordance with a method described previously (16).

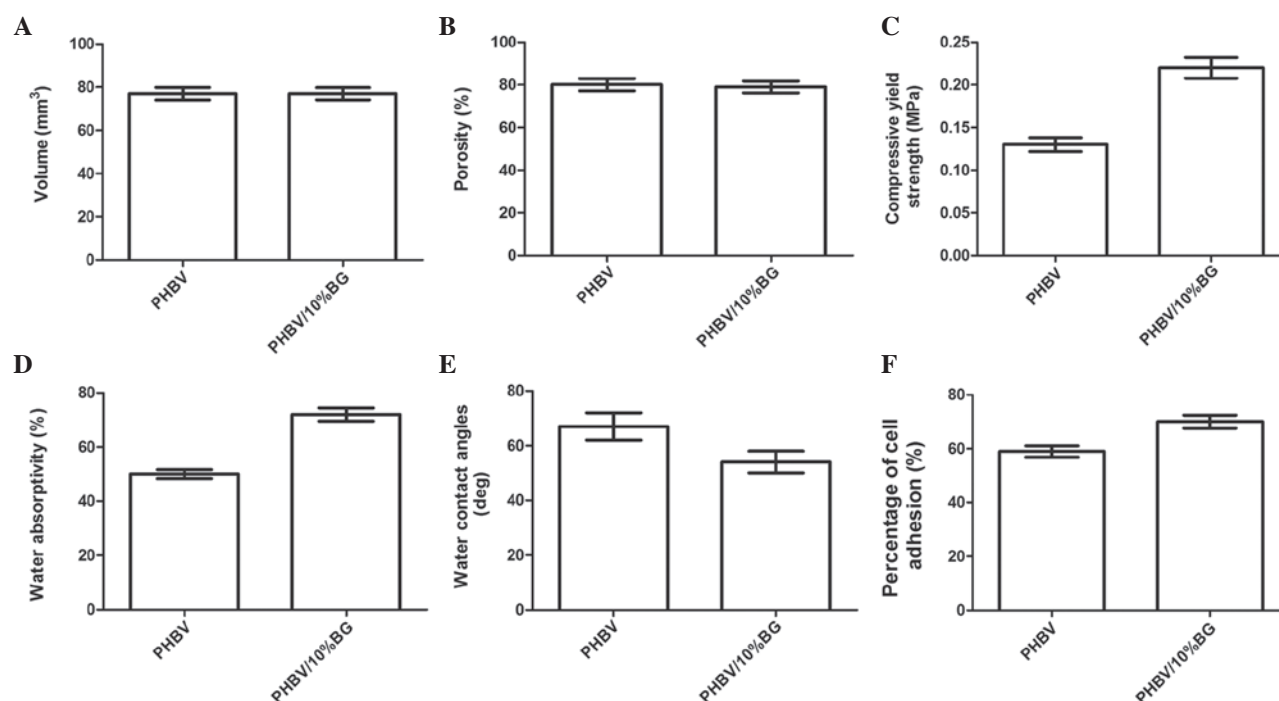


Figure 1. Scaffold characterization. No significant differences were observed in the (A) volume and (B) porosity between the PHBV and PHBV/10% BG scaffolds ($P>0.05$). However, the (C) compressive strength, (D) water absorptivity, (E) water contact angle and (F) cell adhesion were observed to be significantly higher in the PHBV/10% BG scaffolds than those in the PHBV scaffolds ($P<0.05$). PHBV, poly(hydroxybutyrate-co-hydroxyvalerate); BG, Bioglass.

Gross observation of *in vivo* engineered tissue. The constructs were harvested 10 weeks after implantation and images were captured so that the side length, volume and thickness of the constructs could be measured. At each time-point, the volume was determined using a volumenometer, and the side length and thickness were measured using a vernier caliper.

Quantitative analysis of *in vivo* cartilage formation. After 10 weeks *in vivo* culture, the Wwet, glycosaminoglycan (GAG) (17) and total collagen (18) content, and mechanical strength (19) were determined using previously described methods.

Histology evaluation. Ten weeks after implantation, representative cartilaginous tissue formed on the PHBV and PHBV/10% BG scaffolds was fixed in neutral buffered formalin, prior to being embedded in paraffin and sectioned into 5- μ m thick specimens. The cross-sections were stained using hematoxylin and eosin and Safranin-O. Immunostaining of the 10- μ m cryosections was performed with an anti-type II collagen antibody. Non-specific binding sites were blocked by immersing the samples overnight in PBS containing 1% goat serum at 4°C. The sections were then incubated for 4 h at 25°C in PBS containing 1% bovine serum albumin (BSA) and an anti-type II collagen antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a dilution of 1:100. Following three washes with PBS, samples were incubated in PBS containing 3% BSA and then in PBS containing 1% BSA and a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (Santa Cruz Biotechnology, Inc.) at a dilution of 1:150 at 25°C for 4 h. Color development was performed using diaminobenzidine tetrahydrochloride (Santa Cruz Biotechnology, Inc.) (2).

Statistical analysis. All data are presented as the mean \pm standard deviation for $n=6$. Differences between the PHBV and PHBV/10% BG scaffolds were analyzed using the Student's t-test. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Scaffold characterization. The PHBV and PHBV/10% BG scaffolds were cut into rectangular prisms with no significant differences in size or volume ($P>0.05$) (Fig. 1A). Furthermore, no significant difference was observed in the porosity of the scaffolds between the PHBV and PHBV/10% BG groups ($P>0.05$) (Fig. 1B). However, with the addition of 10% BG (w/w) to the PHBV scaffolds, compressive strength and water absorptivity were observed to increase from 0.13 ± 0.02 to 0.22 ± 0.05 MPa and from 50 ± 3 to $72\pm5\%$, respectively ($P<0.05$) (Fig. 1C and D).

Hydrophilicity and cell adhesion of different scaffolds. As shown in Fig. 1E, the water contact angles of the PHBV/10% BG composites ($54\pm1.5^\circ$) were observed to be significantly lower than those of the PHBV specimens ($66\pm2^\circ$) ($P<0.01$), indicating that the addition of BG to PHBV enhanced the surface hydrophilicity. Furthermore, the percentage of adhered cells increased from 59 ± 5 to $70\pm7\%$ with the addition of 10% BG (w/w) (Fig. 1F).

Gross evaluation of the *in vivo* engineered constructs. Variations in the gross shape and size of the *in vivo* engineered constructs were recorded to assess the impact of the two different scaffolds on three dimensional cartilage tissue formation. Following *in vivo* implantation, the cell-scaffold constructs were observed to maintain their original size in

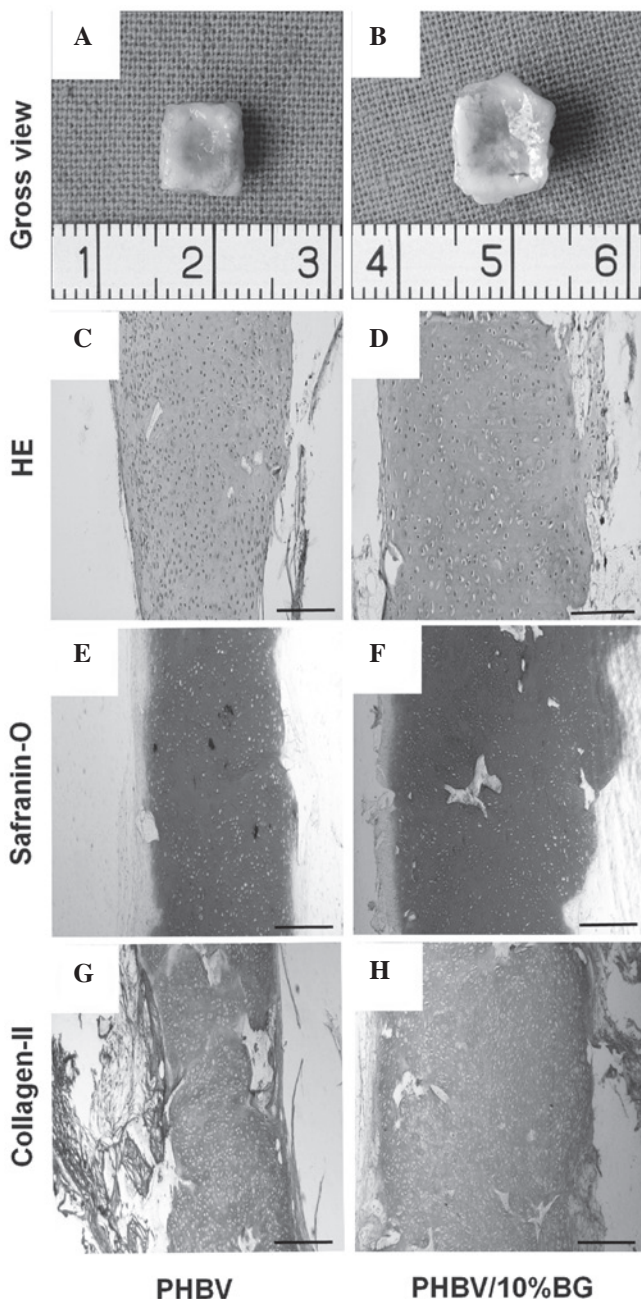


Figure 2. Gross view and full-thickness histological images of the *in vivo* engineered cartilage. After 10 weeks *in vivo* transplantation, the cell-scaffold constructs were capable of maintaining their original size and shape in the PHBV and PHBV/10% BG groups and formed cartilage-like tissues that were ivory-white. Constructs in the PHBV/10% BG group were thicker, with thicker cartilage-like tissue layers than those in the PHBV group. Scale bar=100 μ m. PHBV, poly(hydroxybutyrate-co-hydroxyvalerate); BG, Bioglass; HE, hematoxylin and eosin. Images were captured under 40X microscope and the scale bar is 100 μ m.

the two groups, and form an ivory-white cartilage-like tissue (Fig. 2A and B). Quantitative analysis revealed that in the PHBV/10% BG group, the thickness, side length, volume and Wwet were significantly higher than those in the PHBV group after 10 weeks *in vivo* transplantation ($P<0.05$) (Fig. 3).

Collagen and GAG content and compressive modulus. Quantitative analysis further revealed that there were significant differences in the ECM and mechanical properties

between the samples of regenerated cartilage in the PHBV and PHBV/10% BG groups ($P<0.05$) (Fig. 4A and B).

Histology and immunohistochemistry. The engineered tissue was subjected to histological and immunohistochemical analysis to assess the formation of neocartilage. Cartilage-like tissue was observed to have formed in the cell-scaffold constructs in the PHBV and PHBV/10% BG groups; this tissue exhibited a notable cartilage-like lacunar structure with strong expression of type II collagen (Fig. 4). However, the full-thickness histological and immunohistochemical staining revealed that the cartilage-like tissue layers generated by the cell-scaffold constructs in the PHBV/10% BG group were thicker than those generated in the pure PHBV group (Fig. 2).

Discussion

Engineered cartilage tissue is used to repair cartilage defects and has been suggested to be an ideal therapy for the clinic. The basic method underlying tissue engineering is to seed cells directly onto a biodegradable scaffold material and then implant the cell-scaffold complex subcutaneously to construct the required tissue (6,20). Therefore, scaffolds have a significant role in cartilage tissue engineering. The following properties are required for ideal scaffolds: (i) The mechanical strength necessary for the creation of a macroporous scaffold that retains its structure following implantation, particularly in the reconstruction of hard, load-bearing tissues; (ii) the ability to biodegrade at a controllable rate that approximates the rate of tissue regeneration under the culture conditions of interest; and (iii) histocompatibility that promotes cell-biomaterial interactions, cell adhesion and ECM deposition (21,22). On the scaffolds, chondrocytes should maintain their chondrogenic phenotype and produce ECM components to eventually replace the scaffolds (23,24). The chemistry and physicochemical properties of the scaffolds determine whether the seeded cells are able to grow and maintain their morphology and phenotype (25,26). Such properties include: (i) External geometry, including macro- and micro-structure and interconnectivity; (ii) surface properties, including surface energy, chemistry, charge and surface area; (iii) porosity and pore size; (iv) interface adherence and biocompatibility; (v) degradation characteristics, including biodegradation; and (vi) mechanical competence, including compressive and tensile strength (22,25-27). PHBV has demonstrated potential as a chondrocyte carrier for cartilage engineering (7). However, the cartilage tissue grown using PHBV matrices is not, at present, suitable for clinical application due to the poor hydrophilicity and mechanical strength associated with PHBV, which results in the engineered cartilage tissue exhibiting poor biomechanical properties. The hydrophilicity of a material has been reported to significantly influence cell adhesion, growth and proliferation. Improving the surface hydrophilicity of a material may enhance its ability to interact with cells and elicit controlled cellular adhesion, while maintaining a stable differentiated phenotype (28,29). Certain techniques have been reported to enhance the hydrophilicity of PHBV, including combining PHBV with the polymer poly[(R)-3-hydroxybutyrate]-alt-poly(ethylene oxide) (30),

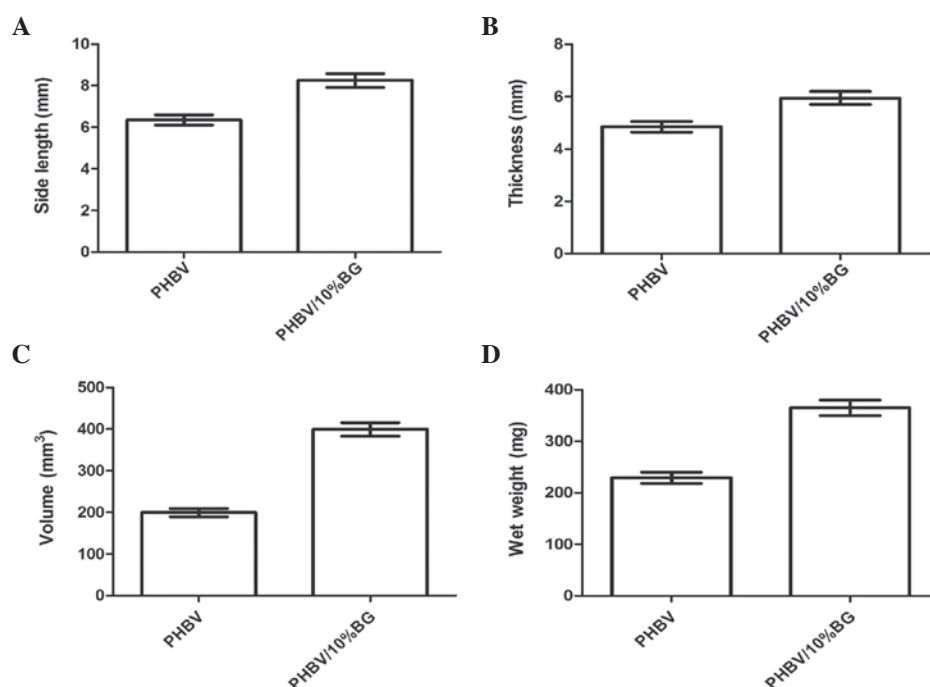


Figure 3. Gross evaluation of the *in vivo* engineered cartilage tissue constructs. The (A) side length, (B) thickness, (C) volume and (D) wet weight were observed to be significantly higher in the constructs generated using the PHBV/10% BG scaffolds compared with those generated using the PHBV scaffolds ($P < 0.05$). PHBV, poly(hydroxybutyrate-co-hydroxyvalerate); BG, Bioglass.

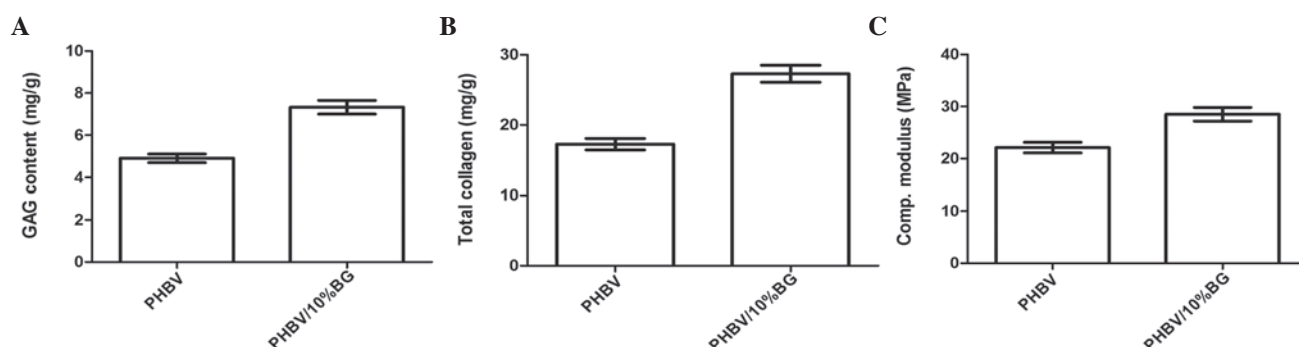


Figure 4. The (A) GAG content, (B) collagen content and (C) compressive modulus of the *in vivo* engineered cartilage tissue constructs. The collagen content, GAG content and compressive modulus were observed to be significantly higher in the constructs generated using the PHBV/10% BG scaffolds compared with those generated using the PHBV scaffolds after 10 weeks *in vivo* transplantation ($P < 0.05$). PHBV, poly(hydroxybutyrate-co-hydroxyvalerate); BG, Bioglass; GAG, glycosaminoglycan; comp. modulus, compressive modulus.

oxygen and nitrogen plasma treatment (31) and covalently immobilizing a water-soluble chitosan/chondroitin-6-sulfate polyelectrolyte complex onto the surface of PHBV membranes using ozone-induced oxidation and polyacrylic acid graft polymerization (32). Li *et al* (16) demonstrated that incorporating hydrophilic inorganic materials into hydrophobic polymers may be a feasible approach to improve the hydrophilicity of these composites.

Previous studies have indicated that the incorporation of bioactive glass into PHBV is capable of improving hydrophilicity and mechanical properties (11). BG is an inorganic material not normally present in bone or cartilage tissue; however, bioactive glass has been utilized in tissue engineering, and studies (33,34) have shown that this material is capable of promoting the growth and proliferation of osteoblasts. Bal *et al* (35) reported that bioactive glass

was superior to bone allografts with respect to integrating into the adjacent host bone, regenerating hyaline-like tissue at the graft surface and expressing type II collagen in the articular cartilage. Therefore, in the present study, 10% BG was incorporated into PHBV in order to generate porous composite scaffolds for *in vitro* and *in vivo* investigations. Hydrophilicity was observed to be significantly enhanced in the PHBV/10% BG scaffolds compared with that of the pure PHBV scaffolds, with the water contact angle decreasing from 66 to 54° with the addition of 10% BG (w/w). The enhanced hydrophilicity was associated with an increase in cell adhesion from 59 to 70%. Histological and immunohistochemical analyses of the *in vivo* engineered cartilage confirmed these findings. The cartilage-like tissue layers generated using the PHBV/10% BG scaffolds were thicker than those formed using the pure PHBV scaffolds, due to

the higher percentage of cell adhesion in the PHBV/10% BG group.

Ideal scaffolds require appropriate mechanical properties; therefore, various attempts have been made to improve the mechanical properties of PHBV scaffolds. Wang *et al* (36) reported that the addition of 5% (w/w) acetylated chitin nanocrystals to PHBV scaffolds improved the tensile strength and Young's modulus by 44 and 67%, respectively, compared with improvements of 24 and 43% in PHBV/chitin nanocrystal composites. Furthermore, it has been reported that combining PHBV with Ecoflex may improve the mechanical properties of PHBV and thereby promote its application in tissue engineering (37). The incorporation of bioactive inorganic materials into PHBV has also been reported to improve the mechanical strength of the scaffold (16). In the present study, the addition of BG into PHBV scaffolds was observed to generate scaffolds with enhanced mechanical properties and an increased capacity for cartilage formation. In addition, the composite scaffolds produced from the incorporation of 10% (w/w) BG into PHBV exhibited an increase in compressive yield strength, from 0.13 to 0.22 MPa. This suggests that BG may have a strengthening effect on PHBV scaffolds; however, the mechanism by which this is achieved is yet to be elucidated.

The compressive modulus of the cell-scaffold constructs in the PHBV/10% BG group in the present study was observed to be significantly higher than that in the pure PHBV group. This may be a consequence of the enhanced mechanical strength associated with the PHBV/10% BG scaffolds compared with that in the PHBV scaffolds, or the thicker cartilage-like tissue formed with the PHBV/10% BG scaffolds and the fact that the improved mechanical properties of the neocartilage tissue are determined by the content of the ECM. Studies have revealed that improvements in mechanical strength may be partially elicited by the homogeneous structure and the ECM content, specifically with regard to the GAG and total collagen content (38–40). This is consistent with the findings from the analysis of the GAG and total collagen content in the present study. It was observed in the present study that the GAG and total collagen content was significantly higher in the PHBV/10% BG group than that in the PHBV group, which contributed to the improved mechanical properties of the neocartilage tissue.

In conclusion, the present study has demonstrated that composite PHBV/10% BG scaffolds exhibit improved hydrophilicity and mechanical properties and also form neocartilage with enhanced biochemical and biomechanical properties. Although the specific mechanism by which this is achieved is yet to be elucidated, the incorporation of BG into PHBV may be beneficial for the generation of composite scaffolds with enhanced properties for cartilage engineering compared with pure PHBV scaffolds.

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