PHBV microspheres – PLGA matrix composite scaffold for bone tissue engineering

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ABSTRACT

Polymer scaffolds, particularly in the form of microspheres, have been employed to support cells growth and deliver drugs or growth factors in tissue engineering. In this study, we have established a scaffold by embedding poly (β-hydroxybutyrate-co-β-hydroxyvalerate) (PHBV) microspheres into poly (L-lactic-co-glycolic acid) (PLGA) matrix, according to their different solubility in acetone, with the aim of repairing bone defects. PLGA/PHBV scaffolds had good pore parameters, for example, the porosity of PLGA/30% PHBV scaffold can reach to $81.273 \pm 2.192\%$. Besides, the pore size distribution of the model was evaluated and the results revealed that the pore size mainly distributed between 50 μm and 200 μm. With increasing the amount of PHBV microspheres, the compressive strength of the PLGA/PHBV scaffold enhanced. The morphology of the hybrid scaffold was rougher than that of pure PLGA scaffold, which had no significant effect on the cell behavior. The in vitro evaluation suggested that the model is suitable as a scaffold for engineering bone tissue, and has the potential for further applications in drug delivery system.

1. Introduction

Several polymers have been used to fabricate porous scaffolds for three-dimensional cell or tissue culture to repair bone defect, including poly (L-lactic-co-glycolic acid) (PLGA). It is one of the most widely used biodegradable polymers because of its good biocompatibility and regulable degradation rate which can be readily modulated by varying the copolymer ratio of lactic to glycolic acid [1]. Several methods have been adopted to fabricate porous biodegradable PLGA scaffold, including solvent casting/particulate leaching [2,3], phase separation [4,5], emulsion freeze-drying [6], gas foaming [7,8], electrospun [9], 3-D printing [10], fiber bonding [11] and microspheres sintering [12–15]. An ideal scaffold is supposed to bear the characteristics of excellent biocompatibility, cytocompatibility, controllable biodegradability, suitable microstructure (pore size and porosity) and mechanical properties [16,17].

Poly (β-hydroxybutyrate-co-β-hydroxyvalerate) (PHBV) copolymers, especially PHBV with 8% HV component have drawn many attention, owing to its low cytotoxicity and cell compatibility [18].

Chen et al. have studied the interactions between PHBV and human keratinocytes [19], allogeneic chondrocytes [20], glial cells [21], fibroblast and osteoblast [22], and these studies proved that PHBV can support the above cells to adhere and proliferate. In addition, PHBV matrices have been proved to support fibroblast proliferation while maintaining the structural integrity [23]. When PHBV was used as sutures, it did not bring about acute inflammation, necrosis or malignant tumor within 1 year [24,25].

There are several approaches to promote tissue formation on scaffolds so that it can receive and respond to specific biological signals, which can direct and facilitate cell attachment, proliferation, differentiation and tissue regeneration [26]. Most of them took the idea of improving bioactivity by coating some proteins. For instance, Shen et al. immobilized rhBMP-2 on PLGA films via plasma treatment and found that the immobilized rhBMP-2 can stimulate the differentiation of OCT-1 cell and accelerate its mineralization [27]. Wang et al. immobilized collagen on PHBV surface by chemical treatment to improve chondrocytes compatibility [28]. However, numerous researchers found that micrometric topographies such as grooves and ridges can affect the cell responses because cells can orient themselves along the groove and ridge-so called "contact guidance" [29,30]. Surface topography on the macroscale can also affect tissues such as bone or cartilage significantly [31].

In this work, PHBV microspheres were embedded in a PLGA matrix in order to change the topological structure of the PLGA matrix and enhance the compressive strength of PLGA scaffold. The

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novel PLGA/PHBV scaffold was prepared using particle-leaching method. The diameters of PHBV microspheres were comparable with the thickness of PLGA matrix walls, which can change the topology of PLGA surface. The morphology, mechanical strength, pore parameters, and human mesenchymal stem cells (hMSCs) compatibility of the new scaffold were evaluated, as well as its ability to support hMSCs. Furthermore, the potential applications of new scaffold model were also discussed.

2. Materials and methods

2.1. Materials

PHBV with 8% hydroxyvalerate (HV) content was purchased from Goodfellow Cambridge Limited, UK. PLGA (lactic/glycolic 1:1; Mw 31,000 Da; inherent viscosity 0.30 dL/g in chloroform at 30 °C) was purchased from Daigang Biomaterials Inc. (Jinan, China). Methyl cellulose M20 (MC) was obtained from Guoyao chemical reagents Limited, Shanghai, China. Most of cell-culture related reagents were purchased from Gibco (Invitrogen, USA) except specialized.

2.2. PHBV microspheres

One gram PHBV was dissolved in 6 ml methylene chloride. The solution was poured into 15 ml 0.4% MC aqueous solution and homogenized at a speed of 10000 rpm for 1 min. After that, the mixture was poured into 500 ml 0.4% MC aqueous solution and stirred at a speed of 400 rpm for 12 h. The resultant PHBV microspheres were centrifuged and washed with deionized water then vacuum dried.

2.3. PLGA/PHBV scaffold

Two grams of PLGA was dissolved in acetone at room temperature for 12 h at a concentration of 200 mg/mL. Then 0.6 g or 1 g PHBV microspheres together with 10 g sodium chloride (200–300 μm) were suspended in the PLGA solution to fabricate PLGA/30% PHBV and PLGA/50% PHBV scaffolds, respectively. The mixture was airproofed immediately and transferred to disperse using ultrasonic dispersion. The following steps [32] can be briefly described below: fetched out the above mixture into a cylindrical mold and then exposed the mold in the air for 48 h to evaporate the solvent. In the end, took out the mixture and dropped it into the deionized water to remove the NaCl particles, resulting in porous PLGA/PHBV scaffold. PLGA scaffolds prepared by the same method were set as a control.

2.4. Microsphere size analysis

A light-scattering particle size analyzer (Matersizer 2000, Malvern Instrument Ltd., British) was used to determine the size distribution of the PHBV microspheres. The desiccated microspheres were suspended in a large amount of distilled water (about 800 ml) and analyzed after continuous stirring.

2.5. Morphological characterization

Morphological characterization was conducted using scanning electron microscopy (SEM, FEI Quanta 200, Netherlands). PHBV microspheres and the cross-section of PLGA/PHBV scaffolds were fixed on a cupreous stub and sparked with gold. PHBV microspheres were sprinkled over conductive adhesive and their morphology was characterized by SEM using an accelerating voltage of 15 kV. PLGA/PHBV scaffolds were coated two times before observation.

2.6. Pore parameters of PLGA/PHBV scaffold

The porosity and other pore parameters of the scaffold were determined according to the method described in the reference [33]. Firstly, certain amount of ethanol was added into the weighing bottle and then a scaffold (dry weight, W_s) was put into it until utterly wetting. Subsequently, weighed the bottle together with the scaffold and marked it as W_a. Record the mass of the remaining liquid together with bottle after getting out the scaffold as W_b. The weight of a pycnometer which was full of ethanol was recorded as W_1. The wet scaffold was transferred to the pycnometer quickly, then marked their total weight as W_2. The pore parameters of the scaffolds were calculated by the equations listed below:

$$\varepsilon = (W_a - W_b - W_s)/[(W_a - W_b) - (W_2 - W_1)] \tag{1}$$

$$\rho_s = \rho W_S/[(W_a - W_b) - (W_2 - W_1)] \tag{2}$$

$$\pi = (W_a - W_b - W_s)/\rho W_s \tag{3}$$

In which ρ, ε, ρ_s and π represent the density of ethanol, porosity, scaffold density and pore volume per gram respectively.

2.7. Pore size distribution of PLGA/PHBV scaffold

The pore size distribution of the scaffold was determined by a Quantachrome (USA)
PoreMaster 33 mercury intrusion porosimetry (MIP). The pore diameter (D) was calculated according to the Wasburn equation [34]:

$$D = -\frac{4\gamma \cos \theta}{p} \tag{4}$$

in which p, θ and γ are the adopted pressure, contact angle and hydrargyric surface tension of mercury on solid surface, respectively. The tests were conducted under low pressure condition with the minimum starting pressure.

2.8. Compressive test of the scaffolds

The compressive strength of cylindrical scaffolds (diameter = 6 mm, height = 6 mm) was measured using a universal material testing machine (Instron 5567, Instron Corp., USA) at a crosshead speed of 1 mm min^{-1}.

2.9. Cell culture

Human mesenchymal stem cells (hMSCs) were a kind gift donated by the Second Affiliated Hospital of Sun Yat-Sen University and were propagated in Dulbecco's Modified Eagle's Medium (DMEM) with supplements of 1.5 mg·ml-1 sodium bicarbonate, 4.5 mg ml-1 glucose, 10% (v/v) fetal bovine serum (FBS), 3 mg ml-1 4-(2-hydroxyerhyl)piperazine-1-erhanesulfonic acid (HEPES). The cells were kept in a humidified incubator at 37 °C and 5% CO$_2$, and the medium was changed every 3 days.

2.10. Cell seeding on the scaffolds

PLGA/PHBV scaffolds were cut into disks of 6 mm in diameter and 2 mm in height and sterilized by exposing to gamma radiation (15 kGy). The pure PLGA scaffolds of the same size as that of PLGA/PHBV were immersed in 75% (v/v) ethanol aqueous
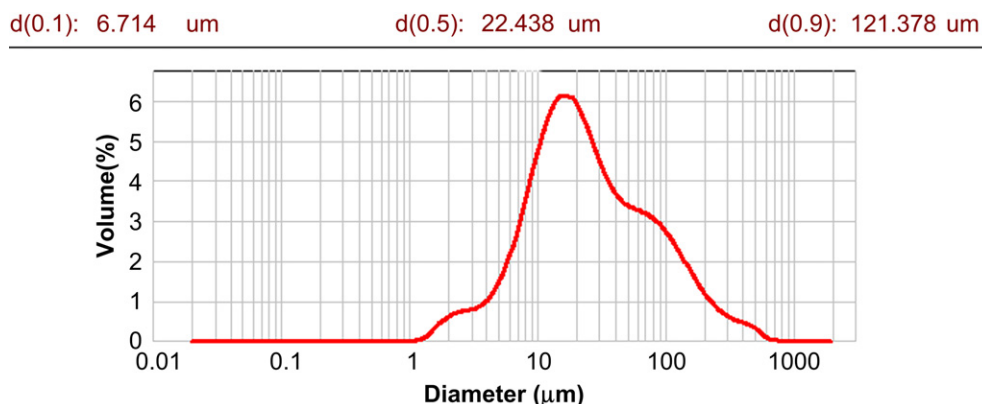
d(0.1): 6.714 um   d(0.5): 22.438 um   d(0.9): 121.378 um

Fig. 1. Size distribution of PHBV microspheres.
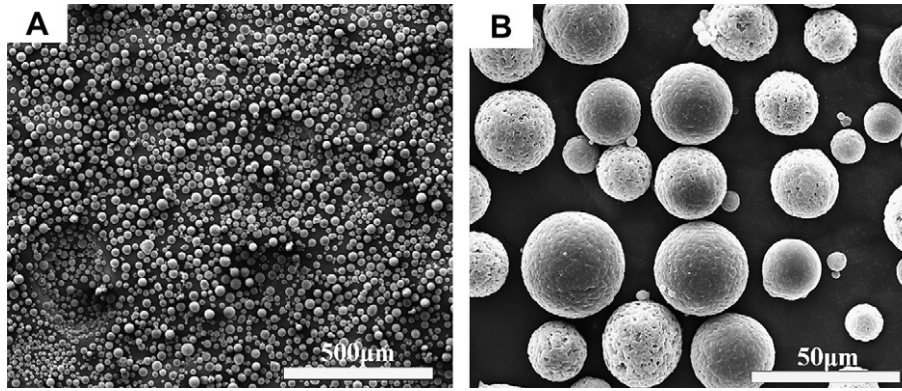
Fig. 2. SEM micrographs of PHBV microspheres: (A) magnification of 200×, (B) magnification of 2000×.

solution for 2 h and followed by ultraviolet radiation for 30 min to sterilize. All the scaffolds were prewet in the DMEM solution for 24 h. Fifteen microlitres of passage 6 cells in suspension (7.5×10^4 cells/well) were seeded on each scaffold. 2 h later, 700 μl of culture medium was added into each well. The cells/scaffold constructs were incubated at 37 °C in a humidified incubator of 5% CO_2 for pre-set days.

2.11. Cell adhesion evaluation of the scaffolds

After 12 h of incubation, the constructs were washed with 1×PBS three times then immobilized with 2.5% (v/v) glutaraldehyde at 4 °C for 2 h. The resultant constructs were subjected to sequential dehydration for 10 min each with ethanol series (30, 50, 70, 90 and 100%). The morphology of cells on the constructs was observed under SEM.

2.12. Cytotoxicity and cell viability

Cytotoxicity of the scaffolds was measured using a widely used method as follows: The cell-scaffolds constructs after 1, 3 and 7 days of culture were washed three times with PBS and then incubated at 37 °C for 4 h in 700 μl of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT, Sigma) PBS solution. The following assay was conducted following the standard protocol. Cells seeded on the pure PLGA scaffolds were also evaluated as a control.

Cell viability was evaluated using a Live/Dead assay kit (Biotium, USA) following the standard protocol given by the manufacturer. Briefly, the cells/scaffold constructs were first washed with PBS then incubated in standard working solution at room temperature for 45 min. Washed the constructs with PBS two times before observation under the fluorescence microscopy (Zeiss Axioskop 40, Germany)

2.13. Statistical analysis

Experiments were repeated with $n = 4$ biological replicates. The results were expressed as means ± standard deviations. MTT evaluation and Compressive strength results were assessed by one way analysis of variance (ANOVA). The comparison between two means was analyzed using Tukey's test which $p < 0.05$ was considered statistical significance.

3. Results

3.1. PHBV microspheres size distribution

The size distribution of the pre-prepared PHBV microspheres was shown in Fig. 1. The result demonstrated that the PHBV microspheres made in the method of the single emulsion technique
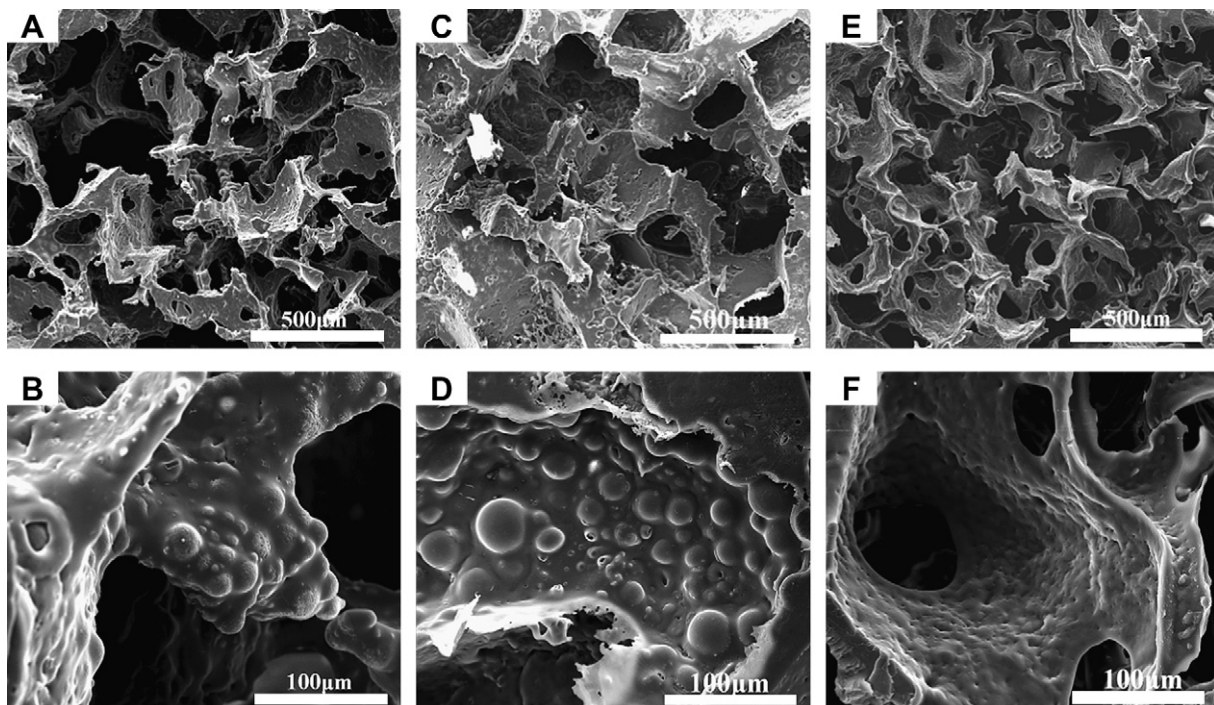

Fig. 3. SEM micrographs of PLGA/30% PHBV (A and B), PLGA/50% PHBV (C and D) and pure PLGA (E and F) scaffods (A, C and E: 200×; B, D and F: 1000×).
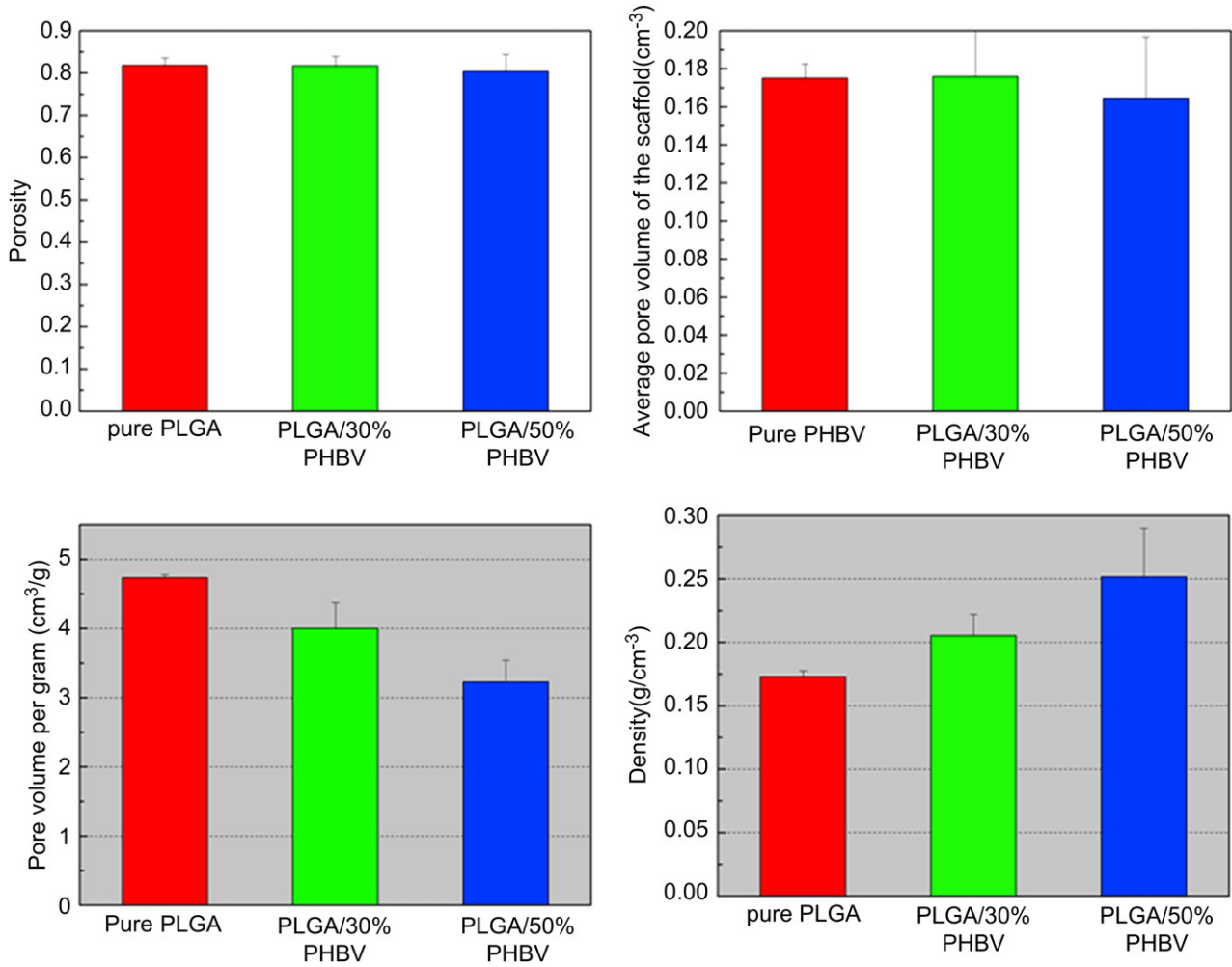
Fig. 4. Pore parameters of pure PLGA, PLGA/30% PHBV and PLGA/50% PHBV scaffolds.

had a small size and narrow particle size distribution. The median particle diameter reached to 22.438 μm.

3.2. SEM micrographs of PHBV microspheres

The morphology of the PHBV microspheres was analyzed with SEM (Fig. 2). Fig. 2 showed that PHBV microspheres possessed spherical shape and relative rough surface. The diameters of most of the microspheres were below 40 μm which might be fit for further usage in PLGA/PHBV scaffold. The result accorded well with the result of the size distribution test.

3.3. SEM micrographs of PLGA/PHBV scaffold

The morphology of the pure PLGA scaffold and PLGA/PHBV scaffolds containing different amount of PHBV microspheres were shown in Fig. 3. The SEM graphs (A, B, C and D) showed the bonding between PHBV microspheres and PLGA matrix. The PHBV microspheres were distributed all across the PLGA matrix and most of them were buried in the PLGA matrix which might make them hard to separate from the matrix. Graphs A, C and E demonstrated that all of the three kinds of scaffold possessed interconnected porous structure. It can be observed from graphs A and C that the addition of PHBV microspheres made the morphology of the PLGA/PHBV scaffold much rougher than that of

pure PLGA scaffold. However, with the increase of PHBV microspheres, the pore size of PLGA/PHBV scaffolds changed little. The PLGA matrix walls were decorated with PHBV microspheres which can be seen from graphs B and D and they were seldom
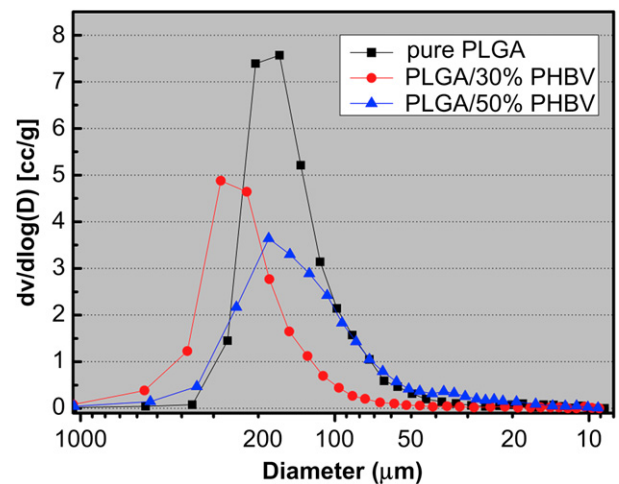

Fig. 5. Pore size distribution of pure PLGA, PLGA/30% PHBV and PLGA/50% PHBV scaffolds.
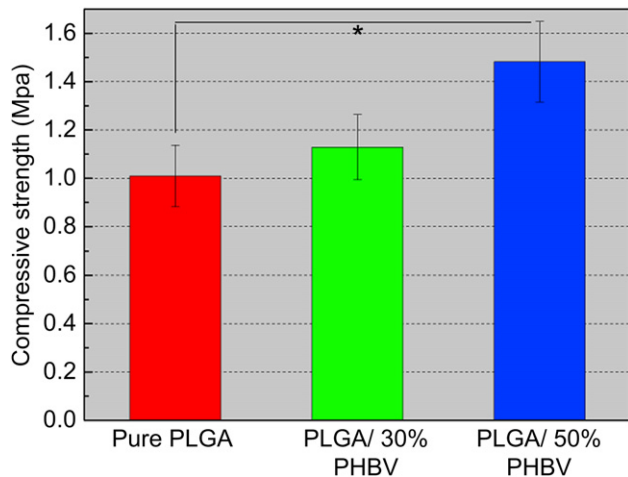
Fig. 6. Compressive strength of pure PLGA, PLGA/30% PHBV and PLGA/50% PHBV scaffolds (*: $p < 0.05$).

influenced by PHBV microspheres, which can be convinced by the well-connected walls.

3.4. Porosity of PLGA/PHBV scaffold

Some parameters related to the scaffolds such as porosity, average volume, pore volume per gram and density were exhibited in Fig. 4. The porosities of three kinds of scaffold were slightly below the pre-set value. The porosity of pure PLGA scaffolds reached to $81.830 \pm 1.723\%$, which was close to that of PLGA/30% PHBV scaffolds ($81.273 \pm 2.192\%$). When the PHBV microspheres content came to 50%, the porosity of the scaffolds still showed no statistical significant when compared with that of pure PLGA scaffolds. The result demonstrated that the PHBV microspheres had little influence on the porosity of PLGA scaffold. With the increase

of PHBV microspheres, the density of scaffolds increased accordingly (from 0.17290 ± 0.00461 of pure PLGA to 0.25160 ± 0.03828 of PLGA/50% PHBV). At the same time, the pore volume per gram decreased with the increase of PHBV microspheres. The above two graphs coincided well with each other. Because of the augment of density, the volume of every gram of the scaffolds should have reduced.

3.5. Pore size distribution of PLGA/PHBV scaffold

Pore size distribution of the scaffolds acquired by MIP test was shown in Fig. 5. The results seemed a little out of expectation. After adding the PHBV microspheres, the pore size of the scaffold should be influenced in some extent, but the PLGA/30% PHBV scaffold showed bigger pore size than that of pure PLGA. However, the PLGA/50% PHBV exhibited reasonable pore size distribution compared with PLGA/30% PHBV scaffold. Besides, most of pore sizes of the scaffolds were located between 50 μm and 300 μm, which were suitable for cell and tissue penetration.

3.6. Compressive strength of PLGA/PHBV scaffold

The compressive strength of the scaffolds was tested on a universal material testing machine and the results were shown in Fig. 6. The value of PLGA/50% PHBV scaffolds was the highest which came to (1.48232 ± 0.16643) Mpa. It was obviously higher than that of pure PLGA and PLGA/30% PHBV scaffolds. However, the strength of PLGA/30% PHBV scaffold only possessed 1.12844 ± 0.13511 Mpa, which was of no significant differentiation to that of pure PLGA scaffold (1.00937 ± 0.12762 Mpa).

3.7. Cell morphology adhered on PLGA/PHBV scaffold

As shown in Fig. 7, the cell morphology of hMSCs grown on three kinds of scaffolds showed some differences at 12 h. The cells had grown into the surface by forming filopodia between some
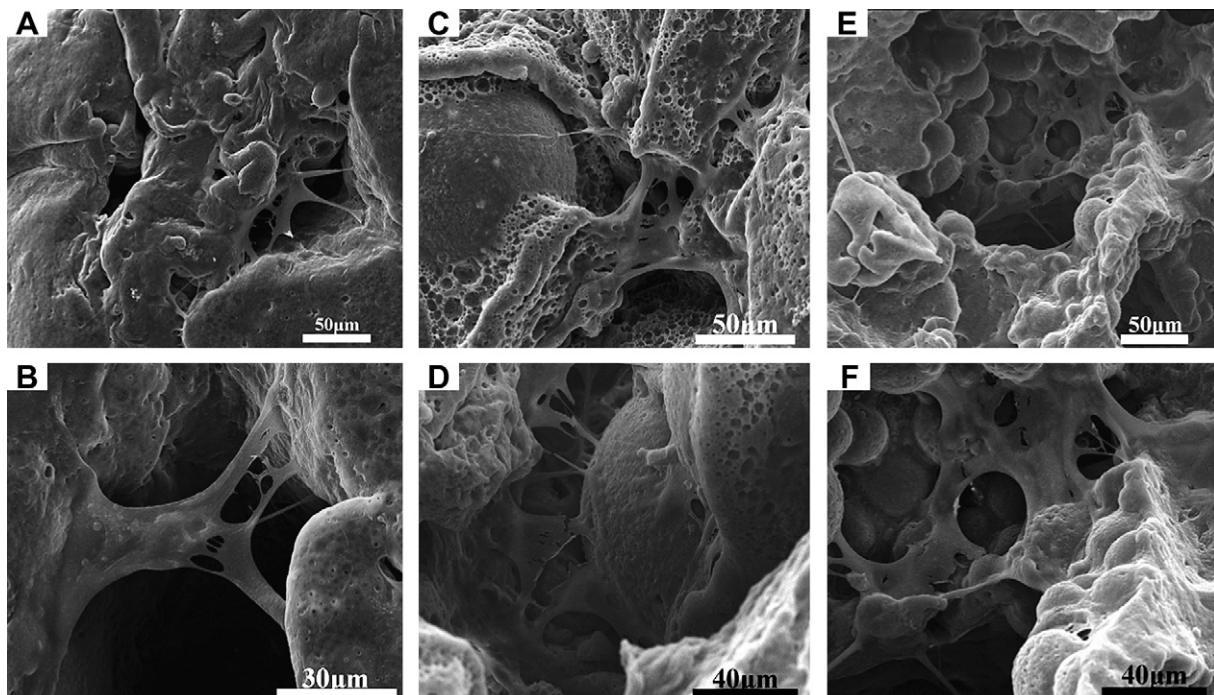

Fig. 7. Cell morphology adhered on pure PLGA, PLGA/30% PHBV and PLGA/50% PHBV scaffolds (Pure PLGA: A and B; PLGA/30% PHBV: C and D; PLGA/50% PHBV: E and F).

apertures. Most of the cells seemed to prefer to adhere onto the surface of PHBV microspheres than onto the morphology of PLGA matrix made by NaCl particles. This point can be approved by graphs C, D, E, and F in Fig. 7. Most of the visible cells were lain on or between PHBV microspheres.

3.8. Viability of cells cultured on PLGA/PHBV scaffolds

Viability of cells cultured on PLGA/PHBV scaffolds was evaluated by means of kinetic examinations and observation of hMSCs, which was quantified by MTT and Live/Dead assay respectively. As shown in Fig. 8, cell viability of the cells on each group of scaffolds increased gradually when the culture days went longer. However, there was no significant differentiation between the cell viability of all groups on day1, day 3 and day 7. The result indicated that the adding of PHBV microspheres had no significant influence on the cell viability. The viability of hMSCs on the scaffolds was generally visualized using fluorescent Live/Dead assay. Fig. 9 indicated the cells status at day 7 and 14 after being seeded on three kinds of scaffold. On day 7, few dead cells (red spots) can be observed, which indicated that at the first few days there were several dead cells with damaged membranes [35]. With the increase of incubation time, more and more green spots can be observed which indicated that the cell proliferated well on the scaffolds. Besides, some graphs showed that the green light formed several circles which indicated that the cells were attached on the microspheres, which was coincident with the results of SEM micrographs.

4. Discussion

Particle-leaching technique has been broadly employed to fabricate porous materials, which mainly forms 3D scaffolds instead of 2D membranes. Some important parameters of porous scaffold, such as porosity, pore size distribution and inter-connectivity can be exactly regulated by tailoring the fabrication parameters. For example, the pore size can be controlled by varying the size of sodium chloride particles. While increasing the amount of porogen, the interconnectivity of scaffold can be improved. At the same time, the compressive strength of the scaffold might decrease significantly. In general, interconnectivity and pore size distribution of scaffold should be balanced according to specific application requirement. As to this study, we employed macropores (50 μm–300 μm) to investigate the influence of spherical

topography generated by PHBV microspheres on cells adhesion and proliferation.

After Campbell and Von Recum found that micropore with 1–2 μm was most favorable for cell adhesion [36], many researchers began to fabricate various specific surfaces to study the relationship between surface topography and cell attachment, migration
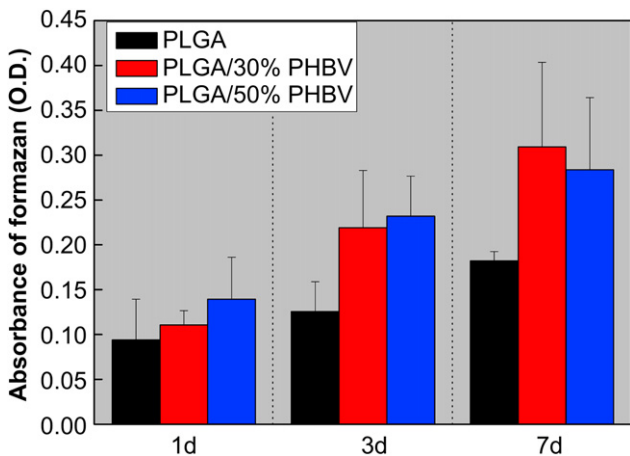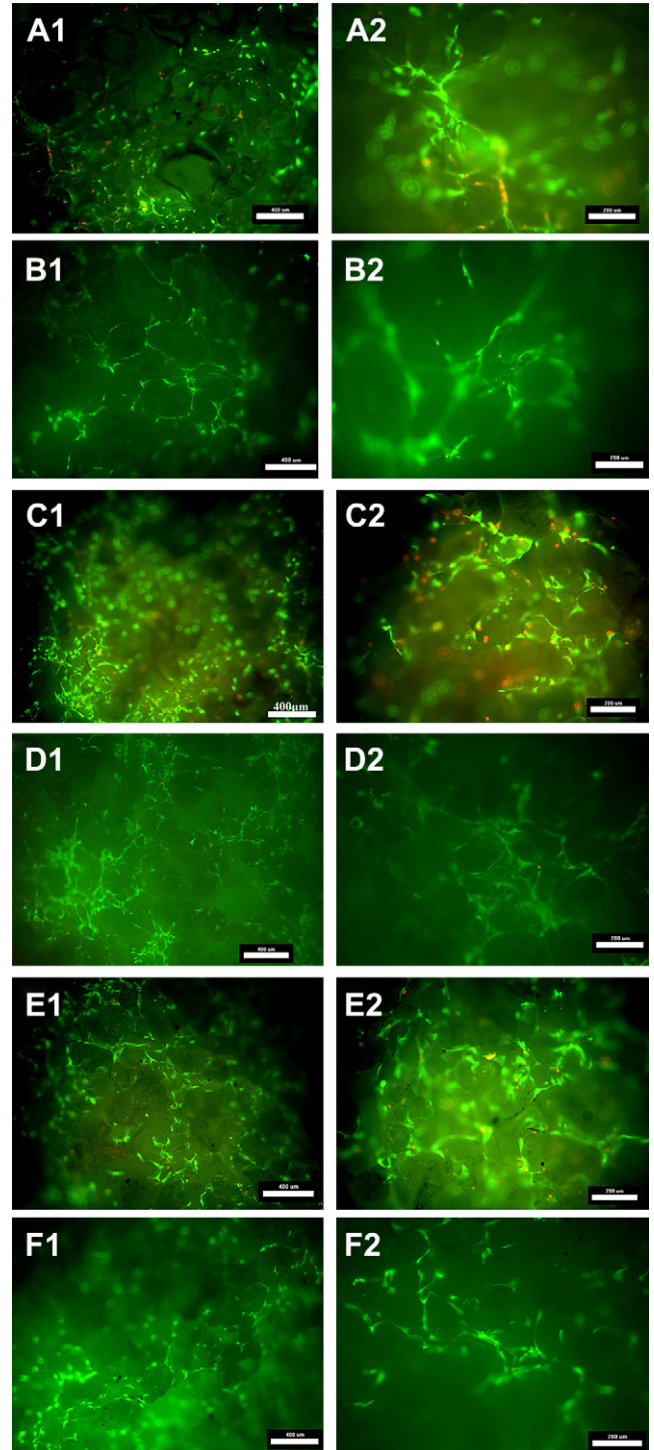
Fig. 9. Fluorescence photographs of hMSCs on pure PLGA, PLGA/30% PHBV and PLGA/ 50% PHBV scaffolds determined by Live/Dead assay (pure PHBV: A1, A2 (7d); B1, B2 (14d). PLGA/30% PHBV: C1, C2 (7d); D1, D2 (14d). PLGA/50% PHBV: E1, E2 (7d); F1, F2 (14d). A1, B1, C1, D1, E1, F1 scale bars: 400 μm; A2, B2, C2, D2, E2, F2 scale bars: 200 μm).

Fig. 8. hMSCs proliferation after 1, 3 and 7 days of culture on pure PLGA, PLGA/30% PHBV and PLGA/50% PHBV scaffolds evaluated by MTT.

[37–39]. Most of them found that micro- and nano-scale topography had certain effect on different types of cell. In the present study, we prepared a new model in which we decorated the PLGA matrix walls with PHBV microspheres. From Fig. 3, we can find that with the increase of PHBV microspheres content, the morphology of the scaffold changed little, which suggest that the addition of PHBV microspheres did not damage the structure of the scaffold. Appropriate aperture is a necessity for an ideal tissue engineering material. The porosity can be modulated by adding different amount of sodium chloride particles in this study. Theoretically, the porosity should be influenced by the amount of PHBV microspheres added. However, we did not see any significant difference in porosity after adding PHBV microspheres. It seemed that the diameters of the PHBV microspheres were small enough for this model.

Because of the decoration of PHBV microspheres, the surface of PLGA matrix turned to be rougher than before. The exposed microspheres can be regarded as some "islands", which had been proved to be active in facilitating the adhesion and proliferation of some kinds of cells [39,40]. It showed that some pores on the scaffolds were disappeared because of the shrink after dehydration, which made it hard for us to observe some details, especially the cell morphology in the pores. There were some micropores which were generated by γ-ray appeared in (Fig. 7) graphs C and D. The gamma radiation sterilization can destroy PLGA matrix by reducing its molecular weight. After incubation in the DMEM, some parts underwent degradation, which resulted in the micropores appeared in graphs C and D. Besides, the viability results we got turned out to be not so optimistic (Figs. 8 and 9) because the cell viability showed no significant difference between PLGA/PHBV scaffold and pure PLGA scaffold (Fig. 8). This might imply that the topography change on large scale cannot significantly influence the cell activity.

In spite of these, we suppose this new model can be used as a drug or bioactive factor carrier as well as load-bearing scaffold and this model deserves further research for bone repair applications.

5. Conclusion

In this study, we set up a new model which was composed of PLGA and PHBV for bone repair. After incorporating PHBV microspheres into PLGA scaffold, morphology, pore size distribution, porosity, compressive strength and in vitro experiments were employed to evaluate it. The PLGA/PHBV scaffold has good pore interconnectivity and macropores which is suitable for cells to grow in. Besides, by mixing the PHBV microspheres into PLGA matrix, the compressive strength of the scaffold was improved obviously while the morphology was not destroyed. The in vitro evaluation showed that the new model can facilitate the cells to proliferate well. In a word, the PLGA/PHBV model is worth further studying for a bone repair application.

Acknowledgements

Appendix

Figures with essential colour discrimination. Figs. 1, 4–6, 8, 9 in this article may be difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.01.059.

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