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Fabrication of HA/PHBV composite scaffolds through the emulsion freezing/freeze-drying process and characterisation of the scaffolds

Naznin Sultana · Min Wang

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Abstract Biodegradable polymer-based scaffolds containing osteoconductive hydroxyapatite (HA) particles can be very useful for bone tissue engineering. In this investigation, HA nanoparticles were incorporated in poly(hydroxybutyrate-*co*-valerate) (PHBV) polymer to fabricate osteoconductive composite scaffolds. PHBV and HA/PHBV scaffolds were made using an emulsion freezing/freeze-drying technique. The scaffolds produced were subsequently characterized using several techniques. It was found that the scaffolds were highly porous and had interconnected porous structures. The pore size ranged from several microns to around 300 μm. The spherical HA nanoparticles which were produced in-house through a nanoemulsion process could be incorporated into composite scaffolds although some of these nanoparticles existed on the surface of pore walls when a relatively large amount of HA was used for composite scaffolds. The incorporation of HA nanoparticles also enhanced compressive mechanical properties of the scaffolds.

Introduction

The aim of tissue engineering is to develop cell, construct, and living system technologies to restore the structures and functions of damaged or degenerated tissues [1, 2]. For the therapeutic strategies of the treatment of injured tissue or organ disease, tissue engineering relies extensively on the use of porous scaffolds to provide an appropriate environment for tissue formation. Tissue engineering scaffolds are characterized by three dimensional, open porous structures made of biocompatible and biodegradable polymers [3]. The polymer scaffolds must be designed in such a way that they maximize diffusion of species, permit vascular ingrowths into the implanted structure and are resorbed when the support degrades, leaving only the newly formed tissue. The products of biodegradation of scaffolds should be non-toxic and be able to exit the body without interfering with tissues and organs in the body. The scaffolds should have mechanical properties consistent with the anatomical site into which they are to be implanted. They should have a high surface area to volume ratio in order to allow sufficient mass transport for cells within the scaffold and surrounding tissue and to provide space for the ingrowth of tissue and vascularization [4]. The most frequently used biodegradable polymers which have been fabricated into tissue engineering scaffolds are poly(lactic acid) (PLA) and poly(lactic acid-*co*-glycolic acid) (PLGA) copolymers.

Biocompatible and biodegradable polymers such as polyhydroxybutyrate (PHB) and its copolymer poly(hydroxybutyrate-*co*-valerate) (PHBV) are linear aliphatic polyesters which can be produced by microorganisms via fermentation [5]. Together with high biocompatibility, PHB and PHBV polymers have longer degradation time than PLA and PLGA polymers, which is useful for tissue engineering scaffolds as it allows scaffolds to maintain mechanical integrity until there is sufficient tissue formation in the constructs. Compared to PHB, PHBV copolymers are less brittle and are easier to be thermo-mechanically processed [6]. PHBV copolymers have been found to cause minimal inflammatory reactions in long-term studies of implants in mice and rats [7] because the

N. Sultana · M. Wang (✉)
Department of Mechanical Engineering, The University of Hong Kong, Pokfulam Road, Hong Kong, China
e-mail: memwang@hku.hk

ultimate degradation product of these polymers is (*R*)-3-hydroxybutyric acid which is a normal constituent of human blood. Over the past three decades, hydroxyapatite (HA), which is similar to the mineral component of natural bone, has been extensively studied and now used for bone tissue repair [8]. HA is considered to be osteoconductive, i.e. capable of promoting bone formation, due to its capability to bind and concentrate bone morphogenetic proteins (BMPs) in vivo. Efforts have thus been made to form non-porous HA/PHB and HA/PHBV composites for bone tissue repair utilizing the osteoconductive property of HA [9–12]. For bone tissue engineering, biodegradable composite scaffolds containing HA appear to hold great promises.

Depending on the polymer and the intended applications of scaffolds, a number of techniques such as immersion precipitation, freeze-drying, and thermally induced phase separation (TIPS) have been developed to fabricate polymer-based tissue engineering scaffolds [2]. In this investigation, highly interconnected PHBV and composite scaffolds were made using an emulsion freezing/freeze-drying technique. The effects of polymer solution concentration, solvent and water phase, as well as the feasibility of incorporating nano-sized HA particles, were studied. The scaffolds were characterized using several techniques.

Materials and methods

PHBV in the powder form was commercially available (Tianan Biologic Material Ltd., China). The PHBV with 2.9% of 3-hydroxyvalerate (HV) had the molecular weight of 310,000 and its purity was 98.8%. It was used without further purification. All chemicals such as chloroform and acetic acid were analytical grade and were used in their as-received state. The spherical HA nanoparticles used for composite scaffolds were produced in-house through a nanoemulsion process [13].

PHBV and HA/PHBV scaffolds were fabricated using an emulsion freezing/freeze-drying technique which was originally developed for making pure polymer scaffolds [14]. Typically, the polymer was weighed accurately and put into a centrifuge tube. An accurately measured amount of chloroform was then added to the tube to make a polymer solution with a desired solution concentration which was to be used to fabricate scaffolds. The mixture of polymer powder and chloroform was kept at 50 °C in a water bath for a few minutes for several times and mixed thoroughly. After obtaining a homogeneous polymer solution, the water phase (acetic acid) was added to produce an emulsion. At this stage, for manufacturing composite scaffolds, an amount of HA nanoparticles was dispersed in the emulsion. The emulsion was homogenized using a homogenizer (Ultra-Turrax, IKA-WERKE, Germany) at a high speed**.** The scaffolds were fabricated in the following steps: 10 mL of PHBV or HA/PHBV emulsion was put into a beaker (30 mL, pre-warmed to 50 °C). The beaker containing the emulsion was then rapidly transferred into a freezer at a preset temperature to solidify the emulsion. The solidified emulsion was maintained at that temperature overnight. The frozen emulsion was subsequently transferred into a freeze-drying vessel (LABCONCO-Freeze dry system, USA) which had been set at the temperature of –35 °C. The samples were freeze dried for at least 72 h to remove the solvent and the water phase completely. The scaffolds produced were stored in a vacuum desiccator at room temperature for storage and further removal of any residual solvent until being used for characterization.

Thermal properties of the PHBV matrix of composite scaffolds were measured using a differential scanning calorimeter (Pyris 6, Perkin-Elmer, USA). The degree of crystallinity, X_c, of a specimen was calculated using the following equation [15]:

$$X_c\,(\%) = \frac{\Delta H_m/\phi_{PHBV}}{(\Delta H_{m100\%})} \times 100\% \quad (1)$$

where ΔH_m and $\Delta H_{m100\%}$ are the measured enthalpy of PHBV matrix of composite scaffolds and the enthalpy of melting for 100% crystalline PHBV polymer (114 J/g [6]), respectively, and ϕ_{PHBV} is the weight fraction of PHBV matrix in the composite scaffold specimen.

In order to estimate the porosity of scaffolds, the volume of the scaffolds was calculated using the measured diameter and height of cylindrical scaffold samples. The mass of the scaffold was measured using an analytical balance. The measured density of scaffolds was calculated from this volume and mass. The porosity, ε, of a scaffold was calculated from the measured density D_m and the skeletal density D_s of the scaffold. The skeletal density of the composite scaffold was calculated using the densities of amorphous and crystalline PHBV polymer and the density of HA. The porosity of a scaffold is given by

$$\varepsilon = \frac{D_s - D_m}{D_s} \quad (2)$$

D_s can be calculated using the following formula:

$$D_s = \frac{1}{(1 - X_h)/D_p + X_h/D_h} \quad (3)$$

where X_h is the weight percentage of HA in the composite and D_h is the density of HA (3.16 g/cm^3 [8]). And the density of PHBV polymer D_p can be calculated as

$$D_p = \frac{1}{\frac{1-X_c}{D_a} + \frac{X_c}{D_c}} \quad (4)$$

where X_c is the degree of crystallinity of the PHBV matrix. For PHB-based polymers, the amorphous density (D_a) is 1.177 g/cm^3 and the crystalline density (D_c) is 1.260 g/cm^3 [6].

The porous structures of PHBV and composite scaffolds were studied using a scanning electron microscope (SEM, Stereoscan 440, UK) at 12 kV. Cubic SEM specimens were cut from cylindrical scaffold samples using a sharp razor blade after scaffold samples were frozen at –35 °C for 1 day. SEM specimens were sputter-coated with a thin layer of gold before examination. Energy dispersive X-ray spectrometry (EDX, INCA 300, UK) was performed in order to determine the presence and distribution of HA nanoparticles in composite scaffolds. Compressive mechanical properties of scaffolds were determined using a mechanical tester (Instron 5848, USA) at a crosshead speed of 0.5 mm/min. From each scaffold sample, cubic specimens of 5 mm × 5 mm × 5 mm were prepared for compression tests. The compressive modulus of a scaffold specimen was determined from the initial linear region of the stress–strain curve. At least three scaffold specimens were tested for each scaffold condition. Statistical analysis was performed to determine the statistical significance ($p < 0.05$) of differences in mechanical properties of scaffolds of different conditions.

Results

Porosity

For the pure PHBV scaffolds, it was found that the polymer solution concentration and the volume fraction (φ) of the water phase had significant influences on the density and porosity of scaffolds (Table 1). At the same volume fraction of the water phase ($\varphi = 0.5$), the porosity of scaffolds dropped from 88% to 80% when the polymer solution concentration increased from 2.5% (w/v) to 12.5% (w/v). When the volume fraction of the water phase was increased from 0.5 to 0.66, the porosity was found to be increased.

A series of composite scaffolds containing 5–20% of HA nanoparticles were fabricated using PHBV emulsions having the polymer solution concentration of 5% (w/v), 7.5% (w/v) and 10% (w/v), respectively. With an increase in the amount of HA nanoparticles in the composite scaffolds, the density of scaffolds increased while the porosity decreased to some extent (Table 2). All composite scaffolds exhibited high porosity levels of at least 79%.

Porous structure

Using optimized polymer solution concentration, PHBV scaffolds exhibiting interconnected but anisotropic porous networks with pore sizes ranging from several microns to around 300 μm (Fig. 1) were made. Good distribution and good adhesion of HA nanoparticles in the porous PHBV matrix were found according to SEM and EDX analyses of scaffolds. Using the same processing parameters, the HA/PHBV composite scaffolds fabricated possessed nearly the same porous morphology (Fig. 2) as that of pure PHBV scaffolds. At low contents (5–10%) of HA, HA/PHBV scaffolds maintained internal ladder-like pore structure which was similar to that of PHBV scaffolds. It was found that HA nanoparticles were mostly distributed within the pore walls of scaffolds. At high contents (>10%) of HA, some aggregates of HA particles appeared on pore surfaces. Compared to PHBV scaffolds, with the incorporation of HA nanoparticles, the pore size of composite scaffolds decreased slightly and the scaffolds exhibited both open and closed pore morphologies. The freeze-dried HA powders used in this investigation consisted of tiny agglomerates of HA nanocrystallites, as shown in Fig. 2a. From SEM micrographs in Fig. 2, it can be seen that a small percentage of HA nanoparticles existed on the surface of pore walls. Fibrous and loose network of semicrystalline PHBV polymer could be seen at high magnifications and HA nanoparticles were observed to adhere to polymer fibrils (Fig. 2d). EDX analyses at different locations of composite scaffolds confirmed the presence of HA particles inside pore walls (Fig. 3).

Table 1 Porosity and compressive modulus of PHBV scaffolds made from different polymer solution concentrations

Polymer solution concentration (%) for making PHBV scaffolds	Volume fraction (φ) of the water phase	Porosity (%) of PHBV scaffolds	Compressive modulus (MPa) of PHBV scaffolds
5.0	0.50	88	0.3
7.5	0.50	86	1.1
10.0	0.50	85	3.0
10.0	0.66	94	0.2
12.5	0.50	80	5.0

Table 2 Density and porosity of PHBV and HA/PHBV scaffolds

Scaffold[a]	Pore type	Quenching temperature (°C)	Measured density (g/cm^3)	Porosity (%)
PHBV	Open	–35	0.1961	85
5% HA/PHBV	Open	–35	0.2110	84
10% HA/PHBV	Open + closed	–35	0.2601	81
20% HA/PHBV	Open + closed	–35	0.3102	79

[a] The polymer solution concentration was 10% w/v for all scaffolds

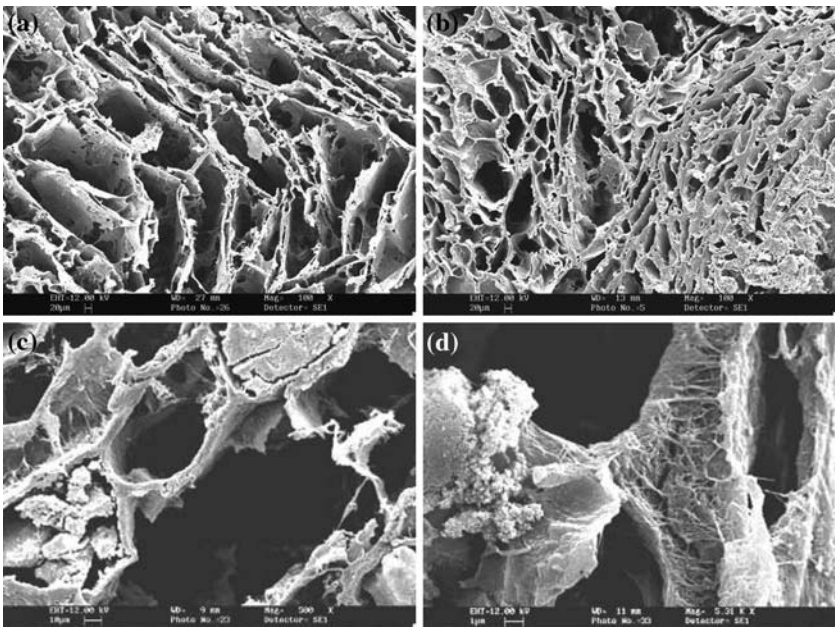

Fig. 1 SEM micrographs of the porous structure of scaffolds (All scaffolds were made using the 10% (w/v) PHBV polymer solution): (**a**) pure PHBV scaffold; (**b**) 10% HA/PHBV scaffold; (**c**) 20% HA/PHBV; (**d**) 20% HA/PHBV

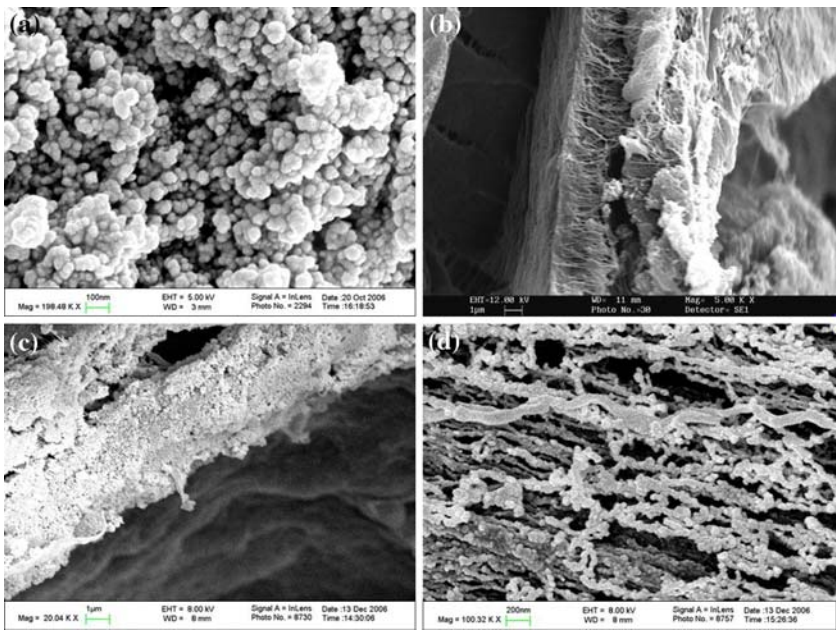

Fig. 2 SEM micrographs of HA nanoparticles and HA/PHBV scaffolds: (**a**) freeze-dried HA powders synthesized in-house; (**b**) HA nanoparticles adhering to the surface of pore walls; (**c**) HA nanoparticles adhering to the surface of pore walls (another location); (**d**) a high magnification view showing the fibrous morphology of composite scaffolds

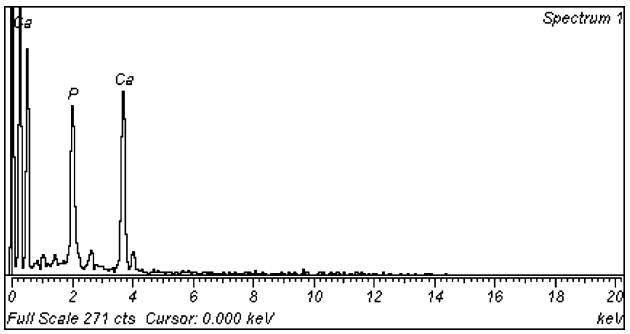

Fig. 3 An EDX spectrum confirming the presence of HA nanoparticles in the HA/PHBV composite scaffold

Compressive mechanical properties

For PHBV scaffolds, it was found that the compressive modulus increased with an increasing polymer solution concentration that was used for making the scaffolds (Table 1). Under the same fabrication conditions, the compressive modulus of PHBV scaffolds varied from 0.31 to 5.0 MPa for scaffolds of polymer concentrations of 5% (w/v) to 12.5% (w/v). Statistical analysis of modulus data showed that the compressive modulus of PHBV scaffolds from high polymer concentrations (e.g. 10% (w/v)) was significantly different ($p < 0.05$) from those of PHBV scaffolds from low polymer concentrations (e.g., 5% (w/v)). A high polymer solution concentration for making scaffolds certainly had a positive effect on the compressive mechanical properties of resultant scaffolds. It can be seen from Fig. 4b that there was a ten-fold increase in compressive modulus when the polymer solution concentration was changed from 5% (w/v) to 10% (w/v). Figure 4a shows the effect of incorporation of HA nanoparticles on the compressive mechanical properties of HA/PHBV scaffolds. The compressive curves of PHBV and HA/PHBV scaffolds displayed in Fig. 4a indicated that the scaffolds underwent three distinctive stages of deformation, with HA/PHBV scaffolds exhibiting a higher level of resistance to compressive load than pure PHBV scaffolds. This is a clear demonstration of the benefit of adding HA nanoparticles to reinforce polymeric scaffolds. There was a threefold increase in compressive modulus when 20% of HA nanoparticles was incorporated in the scaffolds.

Discussion

This investigation has demonstrated that the emulsion freezing/freeze drying technique can be used to produce highly porous and three dimensionally interconnected PHBV scaffolds. It was reported previously that the porous structure of other polymeric scaffolds could be controlled by varying the processing or formulation parameters such as polymer, polymer solution concentration, solvent and water phase concentration, quenching temperature, etc. [16, 17]. The careful selection of various processing parameters is crucial in creating an emulsion from two immiscible phases. The volume fraction of the dispersed water phase φ has great influence on the emulsion stability. (φ is defined as the volume of water phase divided by the total volume of the solvent and water phase.) It was found in the current study that when $\varphi = 0.5$ was used instead of $\varphi = 0.66$, better mechanical properties of the scaffold could be obtained even though the porosity was slightly sacrificed (Table 1). With the optimal processing parameters (among them, $\varphi = 0.5$), polymer scaffolds exhibited greater than 80% porosity levels and also a better handling property. This investigation has further demonstrated that polymeric scaffolds containing bioceramic nanoparticles could be made using the emulsion freezing/freeze drying technique. At $\varphi = 0.5$, both polymer and composite emulsions did not show significant artefacts such as creaming, flocculation and coalescence that can cause poorly distributed pores in the resultant scaffolds. In the scaffolds manufactured, the pore size ranged from several microns to a few hundred microns. Detailed SEM analysis of these scaffolds revealed that the scaffolds possessed oriented tubular macro-pores (Fig. 1). The thickness of the pore walls changed with the polymer solution concentration, which was similarly observed in another investigation that used a different fabrication technique and a different polymer [18]. The formation of such tubular porous structure is due to the scaffold fabrication technique employed. In the current investigation, the crystallization of the solvent and water phase took place when the emulsion temperature was lowered to be below crystallization temperatures of the solvent and water phases, and the PHBV polymer phase was excluded from the crystallization front. As a result, a continuous polymer-rich phase was formed which was in fact the aggregation of excluded polymer from every single liquid crystal. After the sublimation of the solvent and water phases, scaffolds with pores of the similar geometry of the solvent and water phase crystals were formed. A similar process took place in forming HA/PHBV composite scaffolds, notwithstanding that the PHBV polymer solutions now contained HA nanoparticles. At low contents of HA, due to their nanometer size nature, the resultant HA/PHBV composite scaffolds exhibited an almost uniform distribution of HA particles and the scaffolds maintained similar microstructures to that of pure PHBV scaffolds. In tissue engineering, scaffolds with highly interconnected macro porous structures having pores above 100 μm in size are required for cell-penetration in vitro. This investigation shows that the PHBV and HA/PHBV scaffolds fabricated using the

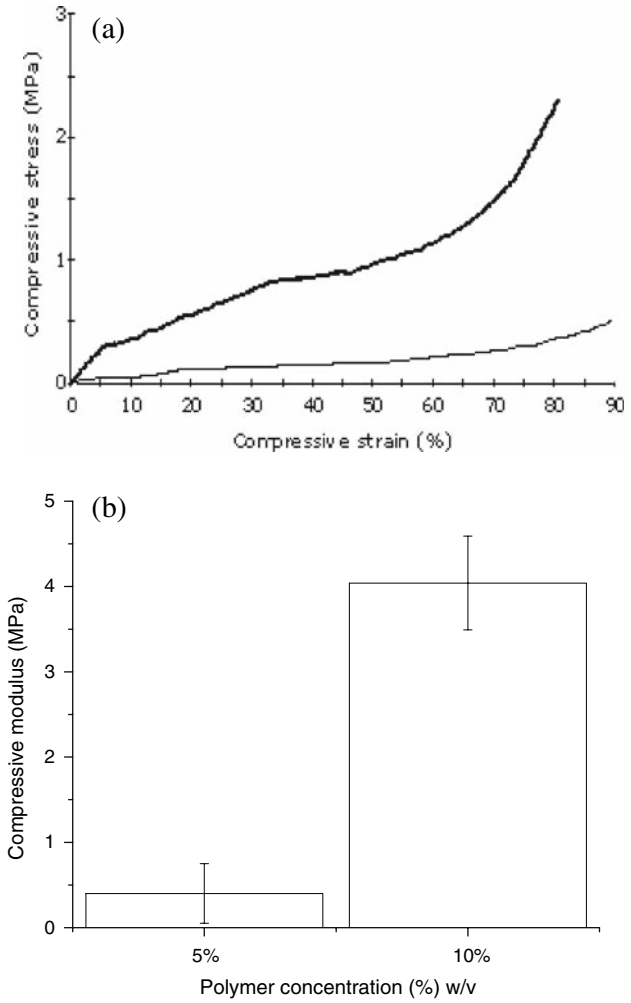

Fig. 4 Compressive behaviour and properties of PHBV and HA/PHBV scaffolds: (**a**) typical compressive stress–strain curves of PHBV (lower curve) and 20% HA/PHBV (upper curve) scaffolds; (**b**) effect of polymer solution concentration on the compressive modulus of scaffolds, (A) 5% (w/v) PHBV emulsion, (B) 10% (w/v) PHBV emulsion

emulsion freezing/freeze drying technique can have pores of these characteristics.

In the current investigation, HA nanoparticles were used to form HA/PHBV composite scaffolds. It is believed that the use of the high degree of flowability of freeze-dried HA powder consisting of nanoparticles [13], which was produced in-house, is beneficial for the production of composite scaffolds as these nano-sized spherical particles were easier to be dispersed in polymer solutions than powders of low flowability provided the amount of the nanoparticles was not large. (In the current investigation, the maximum amount of HA incorporated was 20%.) The good dispersion of HA nanoparticles in polymer solutions and hence good distribution of these particles in the resultant composite scaffolds can contribute to both good mechanical properties and uniform bioactivity of scaffolds. It is also believed that the incorporation of nano-sized HA is more advantageous than micro-sized HA because the later can make the pore structure of scaffolds more irregular.

The current study shows that HA nanoparticles could be incorporated successfully in composite scaffolds, albeit some HA particles were distributed on pore walls (Fig. 2). There is certainly a limit for the amount of HA particles that can be totally incorporated in the scaffolds [19]. Nevertheless, HA particles adhering to pore walls also contribute to the osteoconductivity of HA/PHBV composite scaffolds. The composite scaffolds exhibited a fibrous morphology (Fig. 2) which had a much greater surface-to-volume ratio than the scaffolds with solid pore walls. This type of porous structure can be beneficial for protein adsorption and cell attachment and proliferation, thus enhancing the biological performance of scaffolds. The incorporation of HA particles in composite scaffolds renders scaffolds osteoconductive. Besides this, the current study also shows that the addition of HA particles has led to lower degrees of crystallinity of the PHBV matrix in composite scaffolds. Polymers of a lower crystallinity degrade faster [2]. It can be expected that HA/PHBV scaffolds have higher degradation rates in vitro and in vivo than pure PHBV scaffolds.

Compressive stress-strain curves of PHBV and HA/PHBV scaffolds exhibit three regions, as shown in Fig. 4a, which are commonly observed for porous structures (or termed "cellular structures" in solid mechanics) [20]. Under compression, the scaffolds exhibited linear elasticity at low stresses followed by a long plateau of cell wall collapse and then a regime of densification in which the stress rose steeply. The linear elasticity is controlled by cell wall bending, the plateau is associated with collapse of the cells (of the "cellular structure") and when the cells have almost completely collapsed, opposing cell walls touch, with further strain compressing the solid itself, giving the final region of rapidly increasing stress [20]. Compressive modulus is obtained from the initial slope of the stress–strain curve. As the relative density increases, the cell walls thicken and the pore space shrinks. Increasing the relative density of the scaffold increases the compressive modulus, raises the plateau stress and reduces the strain at which densification starts. Due to these reasons, the compressive modulus of PHBV scaffolds increased with increasing polymer solution concentration and decreasing porosity (Table 1, Fig. 4b). According to composite theories [21], the addition of HA nanoparticles had certainly reinforced the scaffolds as the incorporated HA is a stronger and stiffer material than PHBV (Young's modulus of 80 GPa for HA [8] versus Young's modulus of 2.9 GPa for PHBV [6]), resulting in higher compressive modulus and strength

of composite scaffolds (Fig. 4a). Additionally, as revealed by SEM examination, HA/PHBV scaffolds had a higher level of closed pores than pure PHBV scaffolds (Table 2), which is another source for higher strength and modulus of composite scaffolds, even though the closed pore morphology should be avoided for tissue engineering scaffolds. (In the composite scaffolds produced, there appeared to be only a small percentage of closed pores.) Overall, the HA/PHBV composite scaffolds possess high porosities, satisfactory pore characteristics (Table 2, Fig. 2) and enhanced mechanical properties.

Conclusions

(1) PHBV and HA/PHBV tissue engineering scaffolds can be fabricated using the emulsion freezing/freeze-drying technique. The fabrication parameters should be carefully controlled in order to produce scaffolds of appropriate pore characteristics.
(2) The scaffolds produced are highly porous and exhibit interconnected porous structures with pore sizes ranging from several microns to a few hundred microns. Below a certain limit for the amount of HA, HA nanoparticles can be incorporated in scaffolds which will render the scaffolds osteoconductive and hence suitable for bone tissue engineering.
(3) The incorporation of HA nanoparticles lowers the crystallinity of PHBV matrix of composite scaffolds, which will lead to higher in vitro and in vivo degradation rates of the scaffolds. The incorporation of HA nanoparticles also enhances compressive mechanical properties of scaffolds, which is beneficial for bone tissue engineering.

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