Nor Faezah Ansari^{1,2}M. Suffian M. Annuar^{1,3}Belinda Pingguan Murphy⁴

Research Article

A porous medium-chain-length poly(3-hydroxyalkanoates)/hydroxyapatite composite as scaffold for bone tissue engineering

¹Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

²Department of Biotechnology, Kuliyyah of Sciences, International Islamic University of Malaysia, Kuantan, Pahang, Malaysia

³Centre for Research in Biotechnology for Agriculture (CEBAR), University of Malaya, Kuala Lumpur, Malaysia

⁴Department of Biomedical Engineering, Faculty of Engineering, University of Malaya, Kuala Lumpur, Malaysia

Polyhydroxyalkanoates (PHA) are hydrophobic biopolymers with huge potential for biomedical applications due to their biocompatibility, excellent mechanical properties and biodegradability. A porous composite scaffold made of medium-chain-length poly(3-hydroxyalkanoates) (mcl-PHA) and hydroxyapatite (HA) was fabricated using particulate leaching technique and NaCl as a porogen. Different percentages of HA loading was investigated that would support the growth of osteoblast cells. Ultrasonic irradiation was applied to facilitate the dispersion of HA particles into the mcl-PHA matrix. The different P(3HO-co-3HHX)/HA composites were investigated using field emission scanning electron microscopy (FESEM), X-ray diffraction (XRD) and energy dispersive X-ray analysis (EDXA). The scaffolds were found to be highly porous with interconnecting pore structures and the HA particles were homogeneously dispersed in the polymer matrix. The scaffolds biocompatibility and osteoconductivity were also assessed following the proliferation and differentiation of osteoblast cells on the scaffolds. From the results, it is clear that scaffolds made from P(3HO-co-3HHX)/HA composites are viable candidate materials for bone tissue engineering applications.

Keywords: Bone scaffold / Composite / Hydroxyapatite (30 %) / mcl-PHA / Porous biomaterial

Received: March 31, 2016; revised: July 1, 2016; accepted: September 19, 2016

DOI: 10.1002/elsc.201600084

1 Introduction

Polyhydroxyalkanoates (PHA) exhibit wide ranging physical and mechanical properties that arise from the diversity in their chemical structures. One of the unique properties of these PHA is the biodegradability with excellent biocompatibility, which makes them attractive as the potential biomaterial for various applications, particularly in biomedical field. PHA is classified based on the number of carbon atom present in the monomeric unit. Monomeric unit of short-chain-length PHA (scl-PHA) consisted of 3–5 carbon atoms while medium-chain-length PHA (mcl-PHA) consisted of 6–14 carbon atoms. Scl-PHA like poly

(3-hydroxybutyrate), P(3HB) is hard and brittle compared to mcl-PHA and their copolymers like poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate), P(3HHx-co-3HO), which are soft and elastomeric. Mcl-PHA and its copolymers exhibit low crystallinity, low glass transition temperature, low tensile strength and high elongation-to-break ratio compared to scl-PHA, which is brittle and stiff [1,2]. Mcl-PHA biosynthesis is a general property of the fluorescent pseudomonads belonging to the rRNA homology group I. Most of these bacteria are able to grow on various carbon sources that can be incorporated into mcl-PHA. Depending on the nature of the carbon substrate available, the hydroxyacyl monomers are derived from the intermediates of fatty acid β -oxidation or *de novo* fatty acid biosynthesis pathways [3,4]. In addition, the removal of possible presence of cellular endotoxin from Gram-negative bacteria is needed for further application especially in biomedical field. Solvent extraction has clear advantages over the other extraction methods of PHA in terms of efficiency, and this method is also able to remove bacterial endotoxin and causes negligible degradation to the polymers [5–8].

Bacteria-originated mcl-PHA are more structurally diverse than scl-PHA such as PHB, this provides a wider and crucial

Correspondence: Prof. Mohamad Suffian Mohamad Annuar (suffian_annuar@um.edu.my), Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

Abbreviations: ALP, alkaline phosphatase; EDXA, energy dispersive X-ray; FESEM, field emission scanning electron microscopy; FTIR, Fourier transform infrared spectra; HA, hydroxyapatite; mcl-PHA, medium-chain-length poly(3-hydroxyalkanoates); P(3HO-co-3HHX), poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate); XRD, X-ray diffraction

flexibility in determining the physical and mechanical properties of mcl-PHA in order to meet the requirements of the engineered tissue [1, 4, 9]. Concomitantly, mcl-PHA such as poly(3-hydroxyoctanoate), poly(3-hydroxyhexanoate), copolymers like poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) are being increasingly studied to develop osteosynthetic materials, surgical sutures, stents, scaffolds for tissue engineering and matrices for drug delivery [5]. Nevertheless, extensive studies on mcl-PHA in general remain limited because of inavailability of these polymers in testing quantities [2].

Synthetic and natural hydroxyapatites (HA) ($\text{HCa}_5\text{O}_{13}\text{P}_3$) have similar chemical composition and crystallographic properties to a human bone [6]. Their biocompatibility and osteoconductive behavior are suitable for making bone implants. Studies have shown that incorporation of HA into biomaterials could help to enhance mechanical performance and osteoblast responses [7, 8]. Currently, composites of polymers and ceramics are being developed with the aim to increase the mechanical scaffold stability and to improve tissue interactions. In addition, efforts have also been invested in developing scaffolds with drug-delivery capacity. These scaffolds allow for local release of growth factors or antibiotics and enhance bone in-growth to treat bone defects and even support wound healing [9].

Polymer-based composite scaffold showed great potential in bone tissue engineering. Efforts have thus been made to form porous PHB/HA and PHBV/HA composites for bone tissue repair by utilizing the osteoconductivity property of HA [10–13]. For instance, particulate hydroxyapatite (HA) incorporated into poly(3-hydroxybutyrate) (PHB) formed a bioactive and biodegradable composite for applications in hard tissue replacement and regeneration [11]. Jack et al. [14] fabricated PHBV/HA composite scaffolds with high porosity and controlled pore architectures. They found that incorporation of HA nanoparticles increased the stiffness and strength, thus improved the *in vitro* bioactivity of the scaffolds. Baek et al. [10] incorporated collagen into PHBV/HA scaffold fabricated by using a hot-press machine and salt leaching method. Their results showed that the PHBV/HA/Col composite scaffolds allowed for better cell adhesion and significantly higher proliferation and differentiation than the PHBV/HA composite scaffolds and the PHBV scaffolds. Furthermore, various PHA blends have been developed to improve the performance of scaffold for bone defect repairs or bone tissue engineering. Meischel et al. [15] reported that PHB composite with Herafill® showed higher accumulation and in-growth of bone tissue. Their results showed that the PHB composite with ZrO_2 and 30% of Herafills® as well as the Mg-alloy WZ21 yielded the highest values of bone mass accumulation around the implant compared to PHB without Herafills®. They reported that the moduli of elasticity, tensile strength and strain properties of PHB composites were similar to natural bone components. Scaffolds should exhibit high porosity, high interconnectivity and proper pore sizes in order to facilitate cell adhesion, tissue in-growth and mass transfer. The appropriate pore characteristics of scaffolds are vital in tissue engineering particularly during the late stage of implantation when cells need to migrate deep into the scaffold [13]. The scaffold should positively interact with cells, enhance cell adhesion, growth, migration and differentiated function. The basic

challenges to the material selection and scaffold design are to achieve the initial strength and stiffness. For instance, the material for the scaffold must have the sufficient inter-atomic and inter-molecular bonding or physical and chemical structures that allow for hydrolytic attachment and breakdown. In addition, porosity and proper pore size are important design parameters for the scaffold design, and high surface area necessary for mechanical stability [16].

Pores are necessary for bone tissue formation because they allow migration and proliferation of osteoblasts and mesenchymal cells as well as vascularization. In addition, a porous surface improves mechanical interlocking between the implant biomaterial and the surrounding natural bone thus providing for greater mechanical stability at this critical interface [17]. The most common techniques applied to create porosity in a biomaterial are salt leaching, gas foaming, phase separation, freeze-drying and sintering depending on the material used to fabricate the scaffold [18].

Several studies on bone tissue engineering have been conducted using PHA/HA such as poly(3-hydroxybutyrate), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) [6, 11, 12]. To date, there are still limited studies on mcl-PHA as a composite scaffold for bone tissue engineering. In this paper, a mcl-PHA *viz.* poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) P(3HO-co-3HHX) was investigated as a potential material for bone cells regeneration scaffold both in its pure form and as P(3HO-co-3HHX)/HA composite. The physical, thermal and mechanical properties of the composite P(3HO-co-3HHX)/HA scaffold were investigated. The biocompatibility and osteoconductivity of the porous composite P(3HO-co-3HHX)/HA scaffold was also studied.

2 Materials and methods

Hydroxyapatite (HA) powder ($\text{HCa}_5\text{O}_{13}\text{P}_3$, MW 502.31, CAS 12167-74-7) was purchased from Sigma Aldrich ($\sim 15\text{--}20\ \mu\text{m}$). Octanoic acid (CAS 124-07-2) and acetone (CAS 67-64-1) were purchased from Merck Millipore, Darmstadt, Germany. Analytical grade chemicals were used throughout the study. Human osteoblast cells (hFOB 1.19) were purchased from American Type Culture Collection (ATCC® CRL-11372™), and were used as a model cell line to evaluate the growth of cell on the biopolymer composite scaffold.

2.1 Biosynthesis of mcl-PHA in fed-batch cultivation

Medium-chain-length poly(3-hydroxyalkanoates) (mcl-PHA) was produced in a fed-batch fermentation process by *Pseudomonas putida* BET 001. This microorganism is known to be a fast growing bacterium that accumulates mcl-PHA during its growth phase [19]. Fed-batch fermentation was employed in order to extend the cell growth phase and concomitant mcl-PHA accumulation, with the culture broth gradually supplied with octanoic acid at 12- and 24-h. Nitrogen source was provided to the culture broth, along with the carbon source feeding, at constant C/N mole ratio of 10.

2.1.1 Cell harvesting and PHA extraction

The cells with accumulated PHA were harvested by centrifugation at 3,578 *g* for 10 min at 4°C. Pellets were washed twice with saline solution (90 %) and *n*-hexane to remove excess fatty acids. The pellets were dried in dry-air oven (50°C) until constant weight. Cell concentration was expressed as total cell dry weight (CDW, g/L) concentration. Extraction was carried out by re-suspending the dried cells in acetone and then refluxed for 4 h at 70 ± 5°C. Each suspended solution was filtered and the acetone was evaporated using rotary evaporator at 60°C and 70 rpm until about one-sixth of the initial volume. A beaker containing excess cold methanol was used to precipitate the PHA, in the volume ratio of 1:4. Purification steps were repeated three times by dissolving the PHA in acetone followed by re-precipitation in cold methanol. The PHA composition and its cellular content were analyzed using gas chromatography (GC) [19, 20]. The extracted mcl-PHA composed of three different monomers *viz.* 3-hydroxyoctanoate, 3-hydroxyhexanoate and 3-hydroxydecanoate at 90 mole %, 8 mole % and 2 mole %, respectively.

2.2 P(3HO-co-3HHX)/HA scaffold preparation

The P(3HO-co-3HHX)/HA composite scaffold was fabricated using a solvent casting-particulate leaching technique. Agglomeration of the particles was prevented by placing the solution in a Multifrequency Ultrasonic Bath SB-300DTY (Ningbo Scientz Biotechnology Co., Zhejiang, China). A combination of ultrasonication and a solution casting method was applied to achieve a well-dispersed P(3HO-co-3HHX)/HA matrix. The scaffold was fabricated as follows: 0.6 g of the polymer was dissolved with 6 mL of acetone followed by the addition of HA powder. The amount of HA particles was 10 wt% relative to the polymer. After 20 minutes ultrasonication (25 kHz, 340 W), NaCl was added to the mixture and stirred for 15 min. The size range of salt particles was 100 to 200 μm and its weight fraction was 90 %, based on the total mass of polymer and salt. Subsequently, the mixture was instantly poured into a glass Petri dish (6-cm diameter) as a casting mold. After 48 h of drying at room temperature (25 ± 1°C) samples were dried in an oven (40°C) under vacuum for 24 h. After the drying process, dried and solidified sample was removed from the Petri dish. For salt leaching, the samples were soaked in deionized water (minimum 300 ml) with light stirring using magnetic stirrer. The soaking was carried out for five consecutive days with daily changes of fresh deionized water. Then, the samples were dried out at 25 ± 1°C under vacuum for 48 h. The pure P(3HO-co-3HHX) scaffold for control experiment was fabricated using the same method without the addition of HA particles.

2.3 Characterization of polymer composite

2.3.1 FTIR-ATR spectroscopy

A nondestructive attenuated total reflectance Fourier transform infrared spectra of the control references and the composite

polymer were recorded on Perkin-Elmer Spectrum 400 FT-IR and FT-NIR Spectrometer (Perkin-Elmer Inc., Wellesley, MA, USA) equipped with PIKE GladiATR hovering monolithic diamond ATR accessory (Pike Technologies Inc., Fitchburg, USA). Control samples and their composites were placed on the monolithic diamond ATR probe and clamped against the diamond crystal plate using the force adapter. Thereafter, the samples were scanned over a range of 4000–400 cm⁻¹ at 25°C [21].

2.3.2 X-ray diffraction (XRD) analysis

Crystallinity of mcl-PHA was investigated using a PANalytical EMPYREAN (PANalytical, Almelo, Netherlands). Five milligrams of PHA was dissolved in 2 mL dichloromethane. Then, it was casted on a glass slide, and was allowed to dry overnight. A 20-min scan was run on each sample with the X-ray settings held at 40 kV and 40 mA. The scan range was from 10° to 70° 2θ.

2.3.3 Differential scanning calorimetry (DSC)

The analysis was done using a Mettler-Toledo differential scanning calorimeter (DSC 822e; Mettler-Toledo, Columbus, OH, USA) running on STARe DSC ver 8.10 software, equipped with a HAAKE EK90/MT digital immersion cooler (Thermo Fischer Scientific, USA). About 5 mg sample was encapsulated in an aluminium pan. Analysis was performed at a programmed temperature range of –50 to 200°C with a heating rate of 10°C min⁻¹ under a nitrogen flow rate 50 mL/min at a head pressure of 1.5 bars. The melting temperature (*T_m*) was taken at the endothermic peak of the DSC thermogram. The degree of crystallinity in polymer was calculated based on the endothermic melting enthalpy (ΔH_m) value obtained from DSC endotherm with respect to ΔH_m of PHB with 100 % crystallinity (140.1 J/g) [22].

2.3.4 Surface analysis

The morphological characteristics of both control and composited polymers were viewed in a high-resolution field emission scanning electron microscope (FESEM) (Quanta FEG 450) (FEI, Oregon, USA). The microscope was operated at high vacuum mode with an electron acceleration voltage of 5 kV and a working distance of about 10 mm. Thin films of neat polymer and polymer composites were mounted on brass stubs using double-sided cellophane tape and introduced into the viewing chamber of the instrument [21]. Energy dispersive X-ray spectrometry (INCAEnergy200, Oxford Inst., UK) was performed in order to determine the presence and distribution of HA particles in the composite scaffolds [13].

2.3.5 Porosity of the scaffold

To evaluate the porosity of the composite scaffolds, they were weighed and immersed in 95 % ethanol for one hour. Then, the samples were soaked in deionized water overnight and wet weight of samples were recorded. The percentage of porosity of the composite scaffolds was calculated by using the following equation [11, 23]:

$$\text{Porosity (\%)} = \frac{W_w - W_d}{V_a}$$

where W_w is the wet scaffold weight (g), W_d is the dry scaffold weight (g) and V_a is the apparent scaffold volume (mL).

2.4 Biocompatibility study

2.4.1 In vitro cell culture

Prior to culture initiation, 5-mm diameter disks were cut from both pure P(3HO-co-3HHX) and P(3HO-co-3HHX)/HA composite scaffolds, and were sterilized through immersion in 70 % ethanol for 15 min. Then, the scaffolds were rinsed with sterilized phosphate-buffered saline and were retained at room temperature for 2 hours under aseptic condition prior to cell culture. Then, osteoblast cells were suspended in 500 μ L Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS), 2 mM *L*-glutamine, penicillin and streptomycin (all from Gibco-Invitrogen, USA), and were placed on the surfaces of the disks located in wells of 24-well culture plates. Osteoblast cells were inoculated in a plating density of 1×10^5 per scaffold. Cell cultures were incubated at 37°C, 5 % CO₂ and saturated humidity to allow cell adhesion to the surface of the materials and infiltration into the porous structure. Three samples of each material were used in replicated experiments.

2.4.2 Alamar blue assay

Alamar Blue (Biosource Int, Camarillo, USA), which detects the number of viable cells, was used to measure the growth of cells on polymer scaffolds. At the indicated time endpoints, the wells received 100 μ L Alamar Blue per well. After incubation at 37°C for 3 h, the reduced resazurin dye was quantitated by FLUOstar® Omega (BMG Optima, Ortenberg, Germany) fluorescence cell reader at 570 to 595 nm wavelengths. The percentage of resazurin dye reduction was calculated in order to determine the growth of osteoblast cells.

2.4.3 Alkaline phosphatase (ALP) activity

The differentiation of osteoblast cells was evaluated by the expression of alkaline phosphatase activity [10]. For ALP activity measurement, total protein of cells on scaffolds was extracted using 100 μ L M-PER mammalian protein extraction reagent to lyse the cells. The lysate was then centrifuged at 14 000 g at 4°C for 15 min to separate cell debris. Supernatant was collected and ALP activity was measured using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate. Through the reaction, *p*-nitrophenylphosphate was converted to *p*-nitrophenol and the absorbance at 405 nm was measured with a microplate reader.

3 Results and discussion

3.1 Fed-batch culture of *P. putida* BET001

Culture condition is one of the main factors that affects the production of PHA. Various types of mcl-PHA monomeric composition can be synthesized by providing different carbon sources in the cultivation medium [20]. In this study, mcl-PHA was

Figure 1. Biosynthesis of mcl-PHA by *P. putida* BET 001.

produced through fed-batch fermentation process by *P. putida* BET 001. This microorganism is known to be a fast growing bacterium that accumulates mcl-PHA during its growth phase [19]. Fed-batch fermentation was employed in order to extend the cell growth phase and concomitant mcl-PHA accumulation, with the culture broth gradually supplied with octanoic acid. From Fig. 1, a feeding strategy at C/N 10 mole ratio was employed to increase the cell density. Octanoic acid and $\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$ in the mole ratio of C/N 10 were fed at 12 h and 24 h, respectively. 3 g/L of octanoic acid were supplied to the culture every 4 hours in order to maintain the concentration of carbon substrate during fermentation process. Chardon et al. [3] reported that fatty acids are toxic to cells of *P. putida* even at low concentration and for octanoic acid the inhibition was observed when its concentration exceeds 4 g/L. Therefore, efficient production of mcl-PHA on octanoic acid requires control of the acid concentration. Consequently, 8.6 g/L of cell dry weight with the 63% mcl-PHA content was obtained after 48 h fermentation. The cell mass and mcl-PHA content increased with the fermentation time (Fig. 1). It has been reported that *P. putida* BET 001 is a growth-associated mcl-PHA producer whereby the PHA fraction from the total biomass increases with the specific growth rate [19]. Analyses showed that the mcl-PHA produced was composed of three different monomers viz. 3-hydroxyoctanoate, 3-hydroxyhexanoate and 3-hydroxydecanoate at 90 mole %, 8 mole % and 2 mole %, respectively, with weight average molecular weight of ~130 kDa.

3.2 Characterization of polymer composite

The chemical bonding structure of P(3HO-co-3HHX), P(3HO-co-3HHX)/10% HA and P(3HO-co-3HHX)/30% HA and HA were examined by FTIR spectroscopy, as shown in Fig. 2. Absorptions at 2926 and 2861 cm^{-1} were attributed to both asymmetric CH₃ and symmetric CH₂ vibrations in the samples, respectively. The presence of carbonyl ester bond in pure mcl-PHA sample was assigned to the absorption at 1725 cm^{-1} (Fig. 2A). In the composite of P(3HO-co-3HHX)/10% HA and P(3HO-co-3HHX)/30% HA, carbonyl band absorption were shifted to 1726 cm^{-1} and 1727 cm^{-1} , respectively. It was reported that spectral changes (intensities and position) of carbonyl band at 1740–1720 cm^{-1} was observed during PHA crystallization

Figure 2. FTIR spectra of (A) P(3HO-co-3HHX); (B) P(3HO-co-3HHX)/10% HA; (C) P(3HO-co-3HHX)/30% HA; and (D) HA powder.

[24, 25]. The bands at 563, 601, 604, 1030, 1032 and 1092 cm^{-1} corresponded to the phosphate group of HA [7, 26, 27]. The spectra of P(3HO-co-3HHX)/HA composite (Fig. 2B and C) showed the vibrational bands at 1727 cm^{-1} based on $\text{C}=\text{O}$ of P(3HO-co-3HHX) and 1043 cm^{-1} based on PO_4^{3-} of the hydroxyapatite, indicating the presence of P(3HO-co-3HHX) and HA. Figure 2D showed the bands at 563, 600 and 1032 cm^{-1} corresponding to the phosphate group of HA. At 1042 cm^{-1} , different intensities of phosphate group for HA of P(3HO-co-3HHX)/10% HA and P(3HO-co-3HHX)/30% HA were observed. Peak shifts from 1043 cm^{-1} (C–O) to 1021 cm^{-1} (P–O) were found for both on P(3HO-co-3HHX)/10% HA and P(3HO-co-3HHX)/30% HA after blending, which indicated that HA had been well blended into P(3HO-co-3HHX).

Figure 3. XRD spectra of (A) P(3HO-co-3HHX); (B) P(3HO-co-3HHX)/10% HA; (C) P(3HO-co-3HHX)/30% HA and (d) HA.

The X-ray diffraction patterns of the P(3HO-co-3HHX), P(3HO-co-3HHX)/10% HA and P(3HO-co-3HHX)/30% HA composites were shown in Fig. 3A–C. The crystalline nature of P(3HO-co-3HHX)/HA composite scaffold was further confirmed following XRD analysis. Taking into account the broadening of each peak in XRD, mean crystallite size was calculated using Scherrer's equation, that is, $D = 0.9 \lambda / \beta \cos \theta$, where D is the average crystallite size in $\text{\AA}$, β is the peak broadening of the diffraction line measured at half of its maximum intensity in "radian," λ is the wavelength of X-rays, and θ is the Bragg's diffraction angle [27]. The major HA reflection peaks, such as (002), (211), (300), (004) at 25.8°, 31.7°, 32.1°, 32.9° 2θ , were shown in Fig. 3D. The standard P(3HO-co-3HHX) was observed to display the typical polymeric crystallite reflection

Table 1. Physical and mechanical properties of P(3HO-co-3HHX) and P(3HO-co-3HHX)/HA composites

Scaffolds	^{a)} T_g (°C)	^{a)} T_m (°C)	^{a)} ΔH_m (J g ⁻¹)	^{a)} X_c (%)	^{b)} D_{040} (nm)	Porosity %
P(3HO-co-3HHX)	-33.7	54.9	11.2	7.9	17.9	80.2 ± 1.2
P(3HO-co-3HHX)/10% HA	-32.9	55.2	10.4	7.4	14.9	75.4 ± 0.8
P(3HO-co-3HHX)/30% HA	-33.1	55.7	9.7	6.9	10.1	78.1 ± 1.6

^{a)} Calculated from DSC analysis. T_g : Glass transition temperature; T_m : Melting temperature; ΔH_m : Enthalpy of melting; X_c : polymer crystallinity.

^{b)} Calculated from XRD analysis. D_{040} : Crystallite size at 040 plane.

Figure 4. EDX spectrum obtained at 10 keV on the (A) P(3HO-co-3HHX); (B) P(3HO-co-3HHX)/10% HA and (C) P(3HO-co-3HHX)/30% HA.

at (020), (110), (111) and (040) planes at 17.9°, 19.5°, 21.9°, 26.5° 2θ . It has been reported that the 040 reflection peak in the neat PHA was observed to be broader due to the presence of less perfect crystal structure in the PHA [21]. In composite scaffold (P(3HO-co-3HHX)/HA), the peaks HA (211) and (300) were observed. This suggested the presence of interface binding between HA particles and polymer matrix. In addition, P(3HO-co-3HHX)/30% HA showed higher intensity peak of HA compared to the P(3HO-co-3HHX)/10% HA, which indicated that the HA was well blended with the polymer matrix. It was

Table 2. Elemental analysis of HA using EDX analysis of P(3HO-co-3HHX), P(3HO-co-3HHX)/10% HA and P(3HO-co-3HHX)/30% HA scaffolds

Element	Percentage of elements	
	Weight %	Atomic %
P(3HO-co-3HHX)		
C	74.4 ± 0.5	79.4 ± 0.4
O	25.6 ± 0.5	20.5 ± 0.4
P(3HO-co-3HHX)/10% HA		
C	71.9 ± 0.8	78.0 ± 0.7
O	26.2 ± 0.7	21.3 ± 0.6
P	0.4 ± 0.0	0.2 ± 0.0
Ca	1.5 ± 0.1	0.5 ± 0.0
P(3HO-co-3HHX)/30% HA		
C	64.4 ± 1.7	74.1 ± 1.1
O	25.7 ± 0.3	22.2 ± 0.5
P	2.9 ± 0.5	1.3 ± 0.3
Ca	7.0 ± 0.9	2.4 ± 0.3

found that the crystallite size of the pure P(3HO-co-3HHX) was decreased from 17.9 to 14.9 nm and 10.1 nm after incorporation of 10 % and 30 % of HA particles into polymer matrix (Table 1). The crystallinity of the P(3HO-co-3HHX) scaffold was decreased after composite formation indicating that crystal structures of both P(3HO-co-3HHX) and HA have changed after composite formation. Similarly, it was reported that addition of HA particles has led to lower degree of crystallinity of the PHBV matrix in composite scaffold [12]. The XRD peaks, (211) and (300) were shifted to higher 2θ values in case of P(3HO-co-3HHX)/HA composite as compared to pure HA (i.e. from $2\theta = 31.9$ to 32.1 and 32.9 to 33.3 , respectively). This is possibly due to compression from the contracting polymeric matrix through interfacial bonding [27]. In addition, the crystalline peak of pure PHO at $2\theta = 26.7$ has shifted to 25.8 . The shift and decrease in crystallinity of each peak of the polymer as well as HA after composite formation clearly indicated the presence of interface binding between HA particles and porous wall of polymer matrix.

The thermal properties, molecular weight and porosity data of the scaffold were shown in Table 1. The T_m values of the polymers were increased from 54.9°C to 55.2°C and 55.7°C after blending P(3HO-co-3HHX) with 10% HA and 30% HA, respectively. The crystallinity of the polymers was decreased from 7.9 to 6.9% after blending with HA. The observed decrease in polymer crystallinity as a result of HA composition was corroborated by the data obtained from XRD analysis. It has been documented that polymer with the lower degree of crystallinity is degraded faster [28]. Thus, it can be expected that P(3HO-co-3HHX)/HA composite scaffold would exhibit higher rate of degradation in

Figure 5. FESEM image of the scaffolds (A) P(3HO-co-3HHX) (B) cells on scaffold surface P(3HO-co-3HHX) (C) composite P(3HO-co-3HHX)/10% HA (D) cells on scaffold surface P(3HO-co-3HHX)/10% HA (E) composite P(3HO-co-3HHX)/30% HA (F) cells on scaffold surface P(3HO-co-3HHX)/30% HA (magnification 5000 ×).

vitro and *in vivo* than pure P(3HO-co-3HHX) scaffolds. In contrast, the porosity of the polymer did not show significant differences before and after incorporation of HA particles.

Energy Dispersive X-ray Analysis (EDX, map of Ca) was performed in order to investigate the distribution of hydroxyapatite particles in the P(3HO-co-3HHX)/HA composite scaffold. Figure 4A showed the EDX spectra of neat P(3HO-co-3HHX) with the presence of carbon and oxygen elements. In contrast, P(3HO-co-3HHX) composite with HA showed the presence of carbon, oxygen, calcium and phosphorus elements (Fig. 4B and C), which clearly showed the presence of HA in the composite matrix. EDX analysis showed a relatively higher level of calcium and phosphorus on the surfaces of P(3HO-co-3HHX)/30% HA composite scaffolds compared to P(3HO-co-3HHX)/10% HA. Ca/P molar ratios on the surfaces of P(3HO-co-3HHX)/10% HA and P(3HO-co-3HHX)/30% HA were 2.48 and 1.85, respectively. In the EDX spectra, the carbon and oxygen peaks

were derived from the polymer (Table 2). The presence of HA was revealed by the Ca and P peaks [29]. In addition, EDX was applied to the composites to monitor HA exposure at the composite surface of porous PHO/HA scaffold. The weight and atomic percentage of Ca and P element in P(3HO-co-3HHX)/30% HA were higher (2.9, 7.0, 1.3, 2.4) as compared to the P(3HO-co-3HHX)/10% HA (0.4, 1.5, 0.2, 0.5), respectively. These results showed that the HA particles were successfully incorporated in the P(3HO-co-3HHX) matrix. Moreover, it is suggested that high energy ultrasound irradiation (25 kHz) helped to enhance the dispersion of HA particle in the polymer matrix. According to the previous study, ultrasonication has been shown to be an effective means to overcome the agglomeration of particles in the biopolymer [8]. Furthermore, the good dispersion of inorganic filler in the composite helped to improve the mechanical properties of the composite material [30].

The morphologies of the scaffolds were shown in Fig. 5. Both the pure P(3HO-co-3HHX) and composite scaffolds showed spongy appearance, high porosity and extensive inter-pore connectivity (Fig. 5A, C and E). The porosity of the scaffolds was observed to be in the range of 75 to 80% (Table 1). The P(3HO-co-3HHX)/HA scaffolds (Fig. 5C and E) retained porous morphology as that of pure P(3HO-co-3HHX) scaffolds (Fig. 5A). Pores are necessary for bone tissue formation because they allow migration and proliferation of osteoblasts and mesenchymal cells as well as vascularization. In addition, a porous surface improves mechanical interlocking between the implant biomaterial and the surrounding natural bone, thus providing the greater mechanical stability at this critical interface [18, 27]. The average pore size of the scaffolds viewed using FESEM was approximately 100–180 μm . Previous research suggested that human osteoblasts cells were penetrate faster within the scaffolds containing large pores ($>100 \mu\text{m}$), meanwhile the extent of mineralization was not affected by the pore size [31, 32].

It was found the osteoblast cells were favorably attached to the P(3HO-co-3HHX)/30% HA scaffold (Fig. 5F) as compared to P(3HO-co-3HHX)/10% HA scaffold (Fig. 5D), and significantly less osteoblast cells were found to be able to attach themselves to pure P(3HO-co-3HHX) scaffolds (Fig. 5B). This was attributed to the increased concentration of dispersed HA that helped to promote the proliferation of osteoblast cells throughout the polymer matrixes. As far as the composite scaffolds prepared in this study were concerned, their porosities were sufficient for good interconnection and transportation of nutrition as evidenced by good cell growth. The pore size of the scaffolds also supported good cell growth. Jack et al. [14] fabricated the PHBV/HA composite scaffolds with high porosity and controlled pore architectures. Their results showed that the incorporation of HA microparticles increased the stiffness and strength and improved the in vitro bioactivity of the scaffolds. For bone tissue engineering, biodegradable composite scaffolds containing HA hold great promises. The current investigation has also demonstrated that HA particles could be homogeneously incorporated into the composite scaffolds.

3.3 Biological response of osteoblast cells to P(3HO-co-3HHX)/HA composite scaffolds

Further investigation was carried out with osteoblasts cells seeded on pure P(3HO-co-3HHX) and P(3HO-co-3HHX) matrices containing 10% and 30% HA. The attachment and growth of osteoblast cells were assessed using the Alamar Blue Assay (Fig. 6A). The metabolic activities of the osteoblast cells were analyzed at day 1, 7 and 14. It was found that the percentage of resazurin reduction increased with culture time. From calibration data (fluorescence of resorufin at 570/595 nm against cell density [$\times 10^5 \text{ cfu ml}^{-1}$]), it was found that the percentage of resazurin reduction was positively correlated with the cell density. All matrices were able to support the growth of osteoblast cells during 14 days of culture. The percentage of resazurin reduction in pure PHO scaffolds was lower compared to P(3HO-co-3HHX)/10% HA and P(3HO-co-3HHX)/30% HA scaffolds, which indicated that the HA composite scaffold provided more

Figure 6. (A) Growth of human osteoblast cells (Alamar Blue Assay) (B) ALP activity of human osteoblast cells on P(3HO-co-3HHX) PHO, P(3HO-co-3HHX)/10% HA and P(3HO-co-3HHX)/30% HA scaffolds. ($n = 6$).

favorable condition for cell attachment and growth. It has been reported that when the HA content were increased, more particles will be exposed on the surface of the porous scaffold, hence favored the increase in the proliferation of the cells [7, 33]. No significant differences were found between P(3HO-co-3HHX)/10% HA and P(3HO-co-3HHX)/30% HA scaffold after 7 and 14 days of culture. Both P(3HO-co-3HHX)/10% HA and PHO/30% HA showed highest percentage of resazurin reduction on day 14 indicating that the recorded cells activities was primarily attributed to their response from HA exposure. In addition, incorporation of hydroxyapatite particles into the composite scaffold greatly increased the protein adsorption [17]. The microporous structure of composite scaffold provided for greater surface to volume ratio, which could have helped to further increase the proliferation of the cell.

The differentiation process of osteoblast cells on pure P(3HO-co-3HHX) and P(3HO-co-3HHX)/HA composites have been analyzed by the alkaline phosphatase (ALP) activity (Fig. 6B). Generally, the ALP activities of cells grown on all scaffolds increased continuously until day 14. In contrast, there was no significant difference on osteoblast cells proliferation between P(3HO-co-3HHX)/10% HA and P(3HO-co-3HHX)/30% HA scaffold after 7 and 14 days of culture. Both P(3HO-co-3HHX)/30% HA and P(3HO-co-3HHX)/10% HA scaffolds showed the highest ALP activities on day 14. The ALP activities of osteoblast cells grown on the pure P(3HO-co-3HHX) scaffolds for various culture times were significantly lower than that of the composite HA scaffolds. The results suggested that the presence of dispersed HA within the polymer matrix helped to improve the differentiation of osteoblast cells. It is consistent with the previous reports that the polymer–HA scaffolds are superior to the pure polymer scaffolds for tissue engineering because the presence of HA hydroxyl groups promote calcium and phosphate precipitations hence improve interactions with osteoblast cells [6].

4 Concluding remarks

The successful fabrication of composite scaffolds of P(3HO-co-3HHX)/HA using facile solvent particulate leaching technique followed by its promising performance in terms of cell attachment, proliferation and differentiation widens the range of options for bone tissue engineering applications. It is further envisaged that advanced exploration with regards to other composite materials and compositing methods for fabrication of biologically active biomaterials using medium-chain-length PHA with diverse composition would undoubtedly discover more potentialities, hence future applications.

Practical application

Medium-chain-length poly(3-hydroxyalkanoates) (mcl-PHA) are structurally diverse polyester and readily tailored for various biomedical applications. They are biodegradable, biocompatible and thermoprocessable, hence suitable platform materials for applications in both conventional medical devices and tissue engineering (e.g. sutures, cardiovascular application, bone marrow scaffolds, matrices for controlled drug delivery etc.) Composite of mcl-PHA with hydroxyapatite is a highly promising candidate material for bone tissue engineering applications. A porous composite scaffold made of mcl-PHA and hydroxyapatite (HA) was successfully fabricated using facile particulate leaching technique. The scaffolds were found to be highly porous with interconnecting pore structures and the HA particles were homogeneously dispersed in the polymer matrix. The scaffolds showed excellent biocompatibility and osteoconductivity following strong proliferation and differentiation of osteoblast cells on them. Thus, scaffolds made from P(3HO-co-3HHX)/HA composites are viable candidate materials for bone tissue engineering applications.

The authors acknowledged University of Malaya for research grants PG043-2014A and RP031C-15AET.

The authors declare no conflict of interest, financially or otherwise.

5 References

- [1] Muhr, A., Rechberger, E. M., Salerno, A., Reiterer, A. et al., Biodegradable latexes from animal-derived waste: Biosynthesis and characterization of mcl-PHA accumulated by *Ps. citreololus*. *React. Funct. Polym.* 2013, 73, 1391–1398.
- [2] Rai, R., Keshavarz, T., Roether, J., Boccaccini, A. R. et al., Medium chain length polyhydroxyalkanoates, promising new biomedical materials for the future. *Mat. Sci. Eng. R* 2011, 72, 29–47.
- [3] Chardron, S., Bruzaud, S., Lignot, B., Elain, A. et al., Characterization of bionanocomposites based on medium chain length polyhydroxyalkanoates synthesized by *Pseudomonas oleovorans*. *Polym. Test* 2010, 29, 966–971.
- [4] Zinn, M., Witholt, B., Egli, T., Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. *Adv. Drug. Deliver. Rev.* 2001, 53, 5–21.
- [5] Chen, G. Q., Wu, Q., The application of polyhydroxyalkanoates as tissue engineering materials. *Biomaterials* 2005, 26, 6565–78.
- [6] Xi, J., Zhang, L., Zheng, Z. A., Chen, G. et al., Preparation and evaluation of porous poly (3-hydroxybutyrate-co-3-hydroxyhexanoate)—Hydroxyapatite composite scaffolds. *J. Biomater. Appl.* 2008, 22, 293–307.
- [7] Wang, Y. W., Wu, Q., Chen, J., Chen, G. Q., Evaluation of three-dimensional scaffolds made of blends of hydroxyapatite and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) for bone reconstruction. *Biomaterials* 2005, 26, 899–904.
- [8] Baei, M. S., Rezvani, A., Nanocomposite (PHBV/HA) Fabrication from Biodegradable Polymer. *Middle. East. J.* 2011, 7, 46–50.
- [9] Rezwani, K., Chen, Q. Z., Blaker, J. J., Boccaccini, A. R., Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. *Biomaterials* 2006, 27, 3413–31.
- [10] Baek, J.-Y., Xing, Z.-C., Kwak, G., Yoon, K.-B. et al., Fabrication and characterization of collagen-immobilized porous PHBV/HA nanocomposite scaffolds for bone tissue engineering. *J. Nanomater.* 2012, 2012, 171804.
- [11] Saadat, A., Behnamghader, A., Karbasi, S., Abedi, D. et al., Comparison of acellular and cellular bioactivity of poly 3-hydroxybutyrate/hydroxyapatite nanocomposite and poly 3-hydroxybutyrate scaffolds. *Biotechnol. Bioproc. E* 2013, 18, 587–593.
- [12] Sultana, N., Khan, T. H., In vitro degradation of PHBV scaffolds and nHA/PHBV composite scaffolds containing hydroxyapatite nanoparticles for bone tissue engineering. *J. Nanomater.* 2012, 2012, 190950.
- [13] Sultana, N., Wang, M., PHBV/PLLA-based composite scaffolds containing nano-sized hydroxyapatite particles for bone tissue engineering. *J. Exp. Nanosci.* 2008, 3, 121–132.

- [14] Jack, K. S., Velayudhan, S., Luckman, P., Trau, M. et al., The fabrication and characterization of biodegradable HA/PHBV nanoparticle–polymer composite scaffolds. *Acta Biomater.* 2009, 5, 2657–2667.
- [15] Meischel, M., Eichler, J., Martinelli, E., Karr, U. et al., Adhesive strength of bone-implant interfaces and in-vivo degradation of PHB composites for load-bearing applications. *J. Mech. Behav. Biomed.* 2016, 53, 104–118.
- [16] Sabir, M. I., Xu, X., Li, L., A review on biodegradable polymeric materials for bone tissue engineering applications. *J. Mater. Sci.* 2009, 44, 5713–5724.
- [17] Wei, G., Ma, P. X., Structure and properties of nano-hydroxyapatite/polymer composite scaffolds for bone tissue engineering. *Biomaterials* 2004, 25, 4749–4757.
- [18] Karageorgiou, V., Kaplan, D., Porosity of 3D biomaterial scaffolds and osteogenesis. *Biomaterials* 2005, 26, 5474–5491.
- [19] Gumel, A. M., Annuar, M. S., Heidelberg, T., Biosynthesis and characterization of polyhydroxyalkanoates copolymers produced by *Pseudomonas putida* Bet001 isolated from palm oil mill effluent. *PLoS One* 2012, 7, e45214.
- [20] Razaif-Mazinah, M., Rafais, M., Annuar, M., Suffian, M. et al., Effects of even and odd number fatty acids cofeeding on PHA production and composition in *Pseudomonas putida* Bet001 isolated from palm oil mill effluent. *Biotechnol. Appl. Biochem.* 2015, 63, 92–100.
- [21] Gumel, A., Annuar, M., Ishak, K., Ahmad, N., Carbon nanofibers-poly-3-hydroxyalkanoates nanocomposite: Ultrasound-assisted dispersion and thermostructural properties. *J. Nanomater.* 2014, 2014, 264206.
- [22] Doi, Y., *Microbial Polyesters*. VCH Publishers, Inc., New York 1990.
- [23] Kuo, Y. C., Leou, S. N., Effects of composition, solvent, and salt particles on the physicochemical properties of polyglycolide/poly (lactide-co-glycolide) scaffolds. *Biotechnol. Progr.* 2006, 22, 1664–1670.
- [24] Kansiz, M., Domínguez-Vidal, A., McNaughton, D., Lendl, B., Fourier-transform infrared (FTIR) spectroscopy for monitoring and determining the degree of crystallisation of polyhydroxyalkanoates (PHAs). *Anal. Bioanal. Chem.* 2007, 388, 1207–1213.
- [25] Xu, J., Guo, B.-H., Yang, R., Wu, Q. et al., In situ FTIR study on melting and crystallization of polyhydroxyalkanoates. *Polymer* 2002, 43, 6893–6899.
- [26] Moradi, A., Dalilottojari, A., Pingguan-Murphy, B., Djordjevic, I., Fabrication and characterization of elastomeric scaffolds comprised of a citric acid-based polyester/hydroxyapatite microcomposite. *Mater. Design* 2013, 50, 446–450.
- [27] Pramanik, N., Mishra, D., Banerjee, I., Maiti, T. K. et al., Chemical synthesis, characterization, and biocompatibility study of hydroxyapatite/chitosan phosphate nanocomposite for bone tissue engineering applications. *Int. J. Biomater.* 2009, 2009, 512417.
- [28] El-Hadi, A., Schnabel, R., Straube, E., Müller, G. et al., Correlation between degree of crystallinity, morphology, glass temperature, mechanical properties and biodegradation of poly (3-hydroxyalkanoate) PHAs and their blends. *Polym. Test* 2002, 21, 665–674.
- [29] Rizzi, S. C., Heath, D., Coombes, A., Bock, N. et al., Biodegradable polymer/hydroxyapatite composites: surface analysis and initial attachment of human osteoblasts. *J. Biomed. Mater. Res.* 2001, 55, 475–486.
- [30] Chen, W., Tao, X., Xue, P., Cheng, X., Enhanced mechanical properties and morphological characterizations of poly (vinyl alcohol)–carbon nanotube composite films. *Appl. Surf. Sci.* 2005, 252, 1404–1409.
- [31] Nguyen, L. H., Annabi, N., Nikkhah, M., Bae, H. et al., Vascularized bone tissue engineering: approaches for potential improvement. *Tissue Eng Part B Rev.* 2012, 18, 363–382.
- [32] Akay, G., Birch, M., Bokhari, M., Microcellular polyHIPE polymer supports osteoblast growth and bone formation in vitro. *Biomaterials* 2004, 25, 3991–4000.
- [33] Huang, J., Lin, Y. W., Fu, X. W., Best, S. M. et al., Development of nano-sized hydroxyapatite reinforced composites for tissue engineering scaffolds. *J. Mater. Sci. Mater. Med.* 2007, 18, 2151–7.