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Research Article

Factorial Study of Compressive Mechanical Properties and Primary *In Vitro* Osteoblast Response of PHBV/PLLA Scaffolds

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For bone tissue regeneration, composite scaffolds containing biodegradable polymers and nanosized osteoconductive bioceramics have been regarded as promising biomimetic systems. Polymer blends of poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) and poly(L-lactic acid) (PLLA) can be used as the polymer matrix to control the degradation rate. In order to render the scaffolds osteoconductive, nano-sized hydroxyapatite (nHA) particles can be incorporated into the polymer matrix. In the first part of this study, a factorial design approach to investigate the influence of materials on the initial compressive mechanical properties of the scaffolds was studied. In the second part, the protein adsorption behavior and the attachment and morphology of osteoblast-like cells (Saos-2) of the scaffolds *in vitro* were also studied. It was observed that nHA incorporated PHBV/PLLA composite scaffolds adsorbed more bovine serum albumin (BSA) protein than PHBV or PHBV/PLLA scaffolds. *In vitro* studies also revealed that the attachment of human osteoblastic cells (SaOS-2) was significantly higher in nHA incorporated PHBV/PLLA composite scaffolds. From the SEM micrographs of nHA incorporated PHBV/PLLA composite scaffolds seeded with SaOS-2 cells after a 7-day cell culture period, it was observed that the cells were well expanded and spread in all directions on the scaffolds.

1. Introduction

In scaffold-based tissue engineering (TE), scaffolds play several important roles. Scaffold material and scaffold fabrication techniques should be selected carefully as the scaffold properties are crucial to determine the success of a TE approach [1, 2]. One of the basic requirements for polymer-based scaffolds is that the scaffolds should have controllable porous architecture that can allow cell migration, attachment, and growth, leading to tissue regeneration. The degradation product(s) of scaffolds should be nontoxic and easily taken up or excreted via metabolic pathways. Scaffolds should have sufficient mechanical strength to maintain structural integrity during culture. Some of the commonly used biodegradable polymers that are used as scaffold materials include poly(glycolic acid) (PGA), poly(l-lactic acid) (PLLA), and their copolymer poly(lactic acid-co-glycolic acid) (PLGA) [2]. Poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV), which is a natural, biodegradable polymer, possesses good biocompatibility, also being used to made into TE scaffolds. The *in vivo* degradation product of PHBV is a normal constituent of human blood [2]. PHBV has been investigated for tissue engineering application [3–5]. Due to the slow degradation rate, PHBV can be blended with PLLA in order to control degradation rate and time [6, 7]. Hydroxyapatite (HA) being the mineral component of living bones has gained much recognition as a scaffolding material [8, 9]. Nanosized HA (nHA) and biodegradable polymer blends containing biocomposite scaffolds can offer a promising strategy for bone tissue regeneration.

The freeze-drying technique has been used for constructing TE scaffolds due to its usefulness to create highly porous

scaffolds and controlled pore sizes [10, 11] although there are many techniques available to fabricate tissue engineering scaffolds. If freeze-drying technique is used, a number of factors are concerned for the ultimate morphology and properties of the scaffolds. It is possible to achieve highly organized three-dimensional structures if the parameters are selected and employed properly. In the first part of the study, the factorial design was used to investigate the material parameters to fabricate TE scaffolds based on PHBV and PLLA polymers and nHA.

Protein adsorption on the substrate is of importance in evaluating a TE scaffold as cell adhesion and survival could be modulated by adsorption capacity on the substrate [12]. Within seconds of implantation, protein adsorption onto the foreign surface occurs when biomaterials are implanted into animals or humans. By rapid protein adsorption, it is meant that the arriving cells at the biomaterial surface interact with the adsorbed protein layer instead of the material itself. The response between implanted biomaterial and the body depends on the initial protein adsorption onto a biomaterial surface. Thus adsorption of protein plays a key role. The three primary plasma proteins are albumin, immunoglobulin, and fibrinogen. Among these three proteins, albumin is the predominant plasma protein which makes up 60–70% of plasma. It was reported that albumin could "passivate" the surface of biomaterial and reduced the acute inflammatory response to the biomaterial. It was reported that osteoblast adhesion was significantly greater on nanophase alumina, titania and HA which was due to enhanced adsorption of protein vibronectin by the bioceramics [12]. Protein adsorption of cerium oxide nanoparticles as a function of zeta potential was studied and it was reported that electrostatic interactions played an important role in protein adsorption of nanoparticles [13]. The adhesion of protein on low density polyethylene (LDPE) was studied and it was described that the surface wettability and contact time had significant effects on protein adhesion to the surface of biomaterials. In the second part of this study, the protein adsorption behavior by the scaffolds and the response of osteoblast attachment and morphology was also investigated for the composite scaffolds.

In the present study, a two-level fractional factorial design was formulated to determine the compressive properties of the scaffolds *in vitro* and then the scaffolds were used for protein (bovine serum albumin, BSA) adsorption study. The attachment and morphology of osteoblast-like cells (Saos-2) when cultured on the scaffolds were also studied.

2. Materials and Methods

2.1. Materials. PHBV (6% of 3-hydroxyvalerate) was purchased from Sigma-Aldrich and PLLA with an inherent viscosity 1.6 dL g^{-1} and 1.9 dL g^{-1} (Medisorb 100L 1A) was purchased from Lakeshore Biomaterials (Birmingham, AL). The nHA nanoparticles used for composite scaffolds were produced in-house through a nanoemulsion process [14]. All chemicals used in this investigation were analytical grade.

2.2. Methods

2.2.1. Factorial Design. To overcome the limitations of one-factor-at-a-time method, a factorial design study was performed to investigate the effects of material parameters to produce tissue engineering scaffolds. Five processing parameters as variables were tested in a two-level fractional factorial design [15] and compressive properties were determined from compressive stress-strain curves. The variables in the experimental design were (1) the polymer concentration, (2) water phase fraction, (3) blend composition of PLLA in PHBV, (4) the amount of HA as filler, and (5) molecular weight of PLLA.

2.2.2. Scaffold Fabrication. For scaffold fabrication, polymer was weighed accurately and poured into a centrifuge tube. Then an accurately measured amount of chloroform was added to the tube to make a solution with a desired polymer concentration. After obtaining the homogeneous polymer solution, the water phase (aqueous acetic acid solution or ultra pure water) was added to make an emulsion. The emulsion was homogenized by using a homogenizer (Ultra-Turrax, T-25; IKA-WERKE). Polymer or HA/polymer composite scaffolds were made via freeze-drying technique [5, 16].

In order to solidify the emulsion, the beaker containing the emulsion was rapidly transferred into a deep freezer at a preset temperature for overnight. The frozen emulsion was then placed into a freeze-drying vessel (LABCONCO-Freeze dry system, USA). To remove the solvent and the water phase completely, the samples were freeze-dried for at least 46 hrs. The polymer and composite scaffolds were subsequently obtained. The scaffolds were stored in a vacuum dessiccator at room temperature for storage.

2.2.3. Characterization and In Vitro Mechanical Properties of the Scaffolds. The porous structures of PHBV scaffolds were studied through scanning electron microscopy (SEM; Stereoscan 440, Cambridge, UK). The pore diameters were calculated using SEM micrographs. Using an Instron mechanical tester (Instron 5848, USA) with a 100 N load cell and at a crosshead speed of 0.5 mm/min, the compressive mechanical properties of PHBV and PHBV-based composite scaffolds were obtained for dry cylindrical samples with height of 5 mm and diameter of 10 mm at room temperature The compressive yield strength and compressive modulus was calculated from the initial linear region of stress-strain curves.

2.2.4. Protein Adsorption Study. Experiments of protein adsorption on scaffolds (100/0 PHBV/PLLA, 50/50 PHBV/-PLLA, and 10% HA in 50/50 PHBV/PLLA) were performed by incubating scaffold samples at 37°C in PBS containing bovine serum albumin (BSA) for predetermined periods of time. The samples were pretreated by ethanol and washed by PBS before incubation. A commercial protein assay kit, Micro BCA (Thermo Scientific, Pierce, Rockford, IL, USA) using BSA standards, was used to quantify the

concentration of protein in BSA/PBS solutions containing scaffold samples and the control (without scaffold samples), respectively, under the same incubation conditions. The amount of adsorbed proteins was determined by subtracting the amount of protein which was left in the BSA/PBS solution from the amount of protein in the control.

2.2.5. Cell Culture and Cell Seeding. Human osteoblast cells (Saos-2) were cultured with Dulbecco's modified Eagle medium (DMEM, Gibco, UK). The medium was supplemented with L-glutamine, 1% streptomycin, 0.04% fungi zone, and 10% fetal calf serum (FCS) at 37°C in a 5% (v/v) CO_2 incubator. The cells were detached using 0.25% (w/v) trypsin-ethylenediamietetraacetic acid (EDTA) (Invitrogen, USA) when the cells reached about 80% confluence and subcultured for subsequent use. Scaffold samples were cut to the correct height (1.5 mm) and diameter (10 mm) and were prewetted in ethanol for 24 h and then washed with PBS. Then they were sterilized by ^{60}Co γ-irradiation. 2.2×10^5 cells/well in the wells of a 24-well plate were then seeded separately onto scaffold samples as well as onto a tissue culture polystyrene (TCPS) as control. Then the culture wells were filled with 1 mL of culture medium. The cell-scaffold constructs were maintained until 1, 7, or 14 days postseeding.

2.2.6. Cell Attachment and Morphology. The cells were cultured for 3 h and 24 h in order to examine the cell attachment to the scaffolds and TCPS; Triplicate samples were used for each time. The culture medium was removed after each time point and then the wells were gently washed with PBS to remove unattached cells. The adherent cells were enzymatically released from the specimens with the aid of 0.25% trypsin-EDTA. In order to count the cells, a haemocytometer (Tiefe Depth Profounder, Marienfeld, Germany) was used.

The viability and the morphology of cells around the scaffold specimen on the bottom of the cell culture plate was observed through an optical microscope (Leica MZFLIII, Germany) equipped with a digital camera. For scanning electron microscopy (SEM), cells were grown and prepared for SEM by fixing with 2.5% glutaraldehyde in cacodylate buffer and dehydrated with ethanol. Then the samples were dried in a critical point dryer using liquid carbon dioxide as the transition fluid. The samples were sliced, sputter coated, and analyzed with a SEM (Hitachi S3400N).

2.2.7. Statistical Analysis. All quantitative data of this study were obtained from triplicate samples and were presented as mean ± standard deviation (SD). To test the significance, an unpaired Student's t test (two tail) was applied. A value of $P < 0.05$ was considered to be statistically significant.

3. Results and Discussion

3.1. Factorial Design Study. Tables 1 and 2 show the low and high levels of the variables which are represented by "+" and "−" and the combinations of the variables in total of eight runs. The results of factorial design on compressive properties are given in Table 3. For the composite scaffolds, the effects of the five variables in the fractional factorial design on the compressive strength and modulus with their standard errors are given in Table 4. Figure 1 is the typical compressive stress-strain curves of a scaffold specimens cut from one scaffolds. The compressive stress-strain curve had three distinct regions: linear elasticity, long plateau, and densification region. The compressive modulus and compressive yield strengths were calculated from initial linear region. It can be demonstrated that the positive number indicates that the particular variable has an effect to increase the modulus as it goes from its low level to high level and a negative number indicates that the variables causes the modulus to decrease as the variable goes from its low level to its high level. As it is important to have some methods for interpreting the effects whether they are certainly real and whether they might be explained readily by chance variation, a rough rule is that effects greater than 2 or 3 times their standard error cannot be explained by chance alone [15].

Table 1: Low levels (−) and high levels (+) of variables in the fractional factorial design.

Factors	Levels	
	−	+
(A) Polymer concentration (%)	7.5	10
(B) Water phase fraction	0.5	0.66
(C) Composition of PLLA in PHBV	50/50	75/25
(D) Amount of HA (%)	5	10
(E) Molecular weight of PLLA (dL g^{-1})	1.6	1.9

In Table 4, the main effects which are not due to noise are given in italic form. It can be seen from Table 4 that the polymer concentration, amount of HA, and the molecular weight of PLLA had the effects to increase the compressive modulus and yield strength as they go from low levels to high levels. On the other hand, the ratio of water phase and blend composition have the effects to decrease the compressive modulus and yield strength when they go from low to high levels.

Yaszemski et al. investigated the effects of several composite material formulations and conducted a factorial study to calculate initial mechanical properties *in vitro* and studied the association with histological characteristics of the resulting material *in vivo* [17]. It was also reported that the synthesis of polymer allowed alterations of the composition as well as of the physical properties to effect resulting composites. It was also studied and reported that the polymer molecular weight, presence of a leachable salt, and amount of cross linking monomer had strong effects on the final strength and modulus of the composite, which were on the order of 5 MPA, an appropriate magnitude for a temporary trabecular bone substitute [18]. In this study, the effects of five processing parameters were studied for the determination of the effects of these processing parameters on the compressive properties. It was possible

Table 2: Eight-run experimental design for studying the effects of the five factors.

Run number	Polymer concentration (A)	Water phase composition (B)	Blend composition PLLA/PHBV (C)	Amount of HA (D)	PLLA molecular weight (E)
1	+	−	−	−	−
2	+	−	+	+	−
3	+	−	−	+	+
4	−	+	+	+	−
5	−	+	−	−	−
6	+	+	+	−	+
7	−	+	−	+	+
8	−	−	+	−	+

Table 3: Compressive yield strength and compressive modulus of scaffolds.

Run number	Compressive yield strength* (MPa)	Modulus* (MPa)
1	0.25 ± 0.07	4.0 ± 1.2
2	0.50 ± 0.04	11.0 ± 3.4
3	0.80 ± 0.06	12.0 ± 4.1
4	0.20 ± 0.01	1.0 ± 0.3
5	0.11 ± 0.01	1.2 ± 0.4
6	0.32 ± 0.08	0.8 ± 0.2
7	0.23 ± 0.04	10.0 ± 3.1
8	0.34 ± 0.05	2.0 ± 0.8

*mean ± standard deviation.

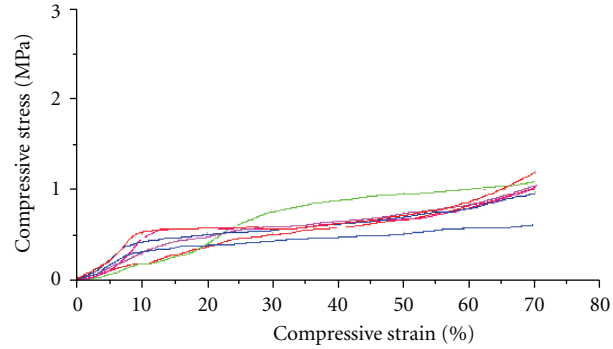


Figure 1: Typical compressive curves of the scaffold specimens cut from one scaffolds fabricated from combination of run 3 of Table 3.

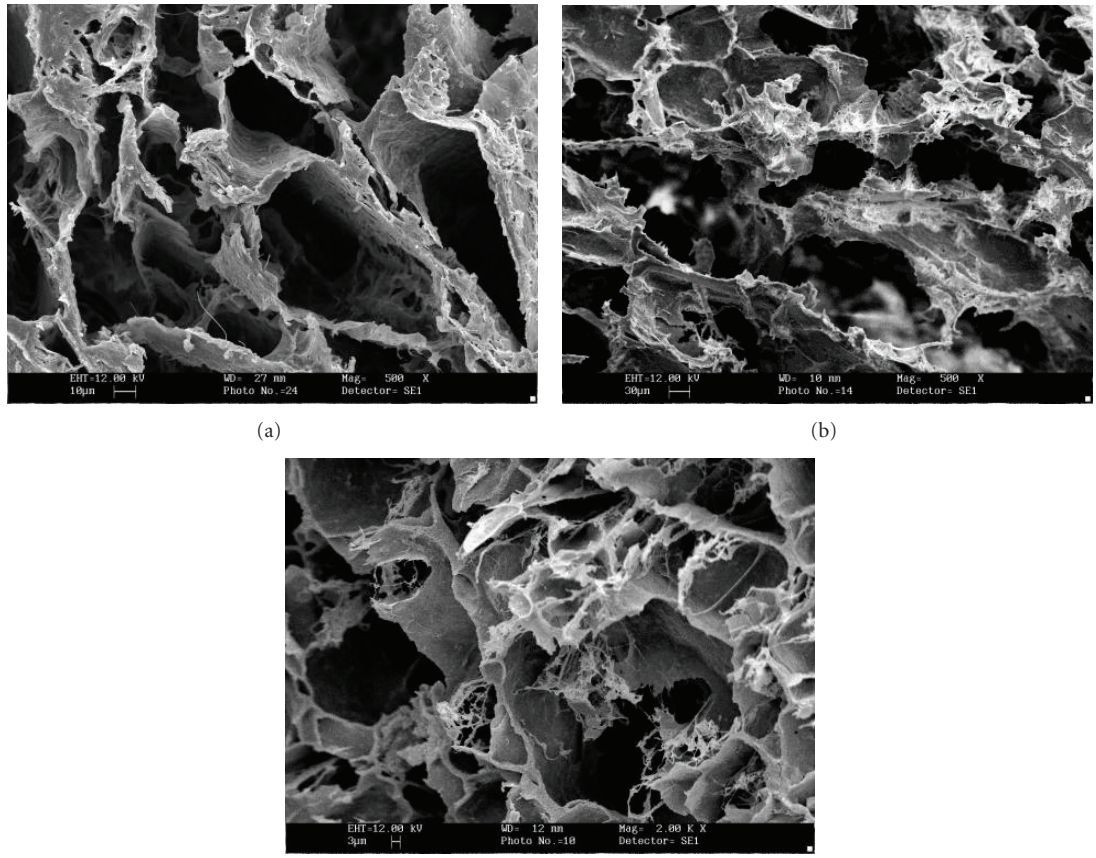
to produce scaffolds by altering the combinations of these parameters. Polymer concentration and amount of HA had the maximum effects on the scaffold compressive properties. It was observed that the scaffolds produced from these processing parameters which had highest effects on the compressive properties would facilitate bone formation *in vitro* [5].

3.2. Scaffold Properties. For the second part of the study, based on the parameters and main effects as given in Table 1 and Table 4, three types of scaffolds fabricated from 10% (w/v) polymer concentration, 0.5 water phase fraction, 100/0 PHBV/PLLA, 50/50 PLLA/PHBV and 10% HA incorporated 50/50 PHBV/PLLA scaffolds were chosen for protein adsorption and in vitro cell culture study. SEM micrographs of these three types of scaffolds are given in Figures 2(a), 2(b), and 2(c). It was observed that the scaffolds exhibited a large pore size distribution (from several μm to 300 μm) and had interconnected pores. It was also observed that the HA nano particles were homogeneously distributed in the 10% HA incorporated 50/50 PHBV/PLLA composite scaffolds described elsewhere [5].

3.3. Protein Adsorption. Figure 3(a) shows the physical appearance of the scaffolds. All the scaffolds were three-dimensional in structure and physically manageable. UV-visible spectroscopy measurements were carried out for known concentrations of BSA at the absorbance of 562 nm. Noncoated polymer and composite scaffolds of different composition were incubated for 3 hrs, 22 hrs and 42 hrs in BSA/PBS solution in order to assess protein adsorption behavior. After 22 hrs, protein adsorption of all the scaffolds reached equilibrium as there was no significant change observed from 22 hrs to 42 hrs (Figure 3(b)). Figure 4 shows the effect of polymer blend composition and incorporated nHA on the protein adsorption capacity of composite scaffolds. The amount of adsorbed protein was calculated from the standard curve. After 22 hr, of immersion in BSA/PBS solution, 50/50 PHBV/PLLA scaffold adsorbed more BSA proteins than 100/0 PHBV/PLLA scaffold and 10% nHA incorporated 50/50 PHBV/PLLA composite scaffolds adsorbed more BSA proteins than plain PHBV scaffold.

Protein adsorption is of importance to evaluate a scaffold for the tissue engineering application. It was observed from this part of the study that composite scaffolds containing nanosized HA could adsorb more protein than pure PHBV or PHBV/PLLA blend scaffolds. Incorporation of HA can alter the pore surface morphology of the scaffolds and may made them more suitable for increased protein adsorption. As HA has better affinity towards protein adsorption, particles exposed on the pore walls in the composite scaffolds had increased capacity of protein adsorption.

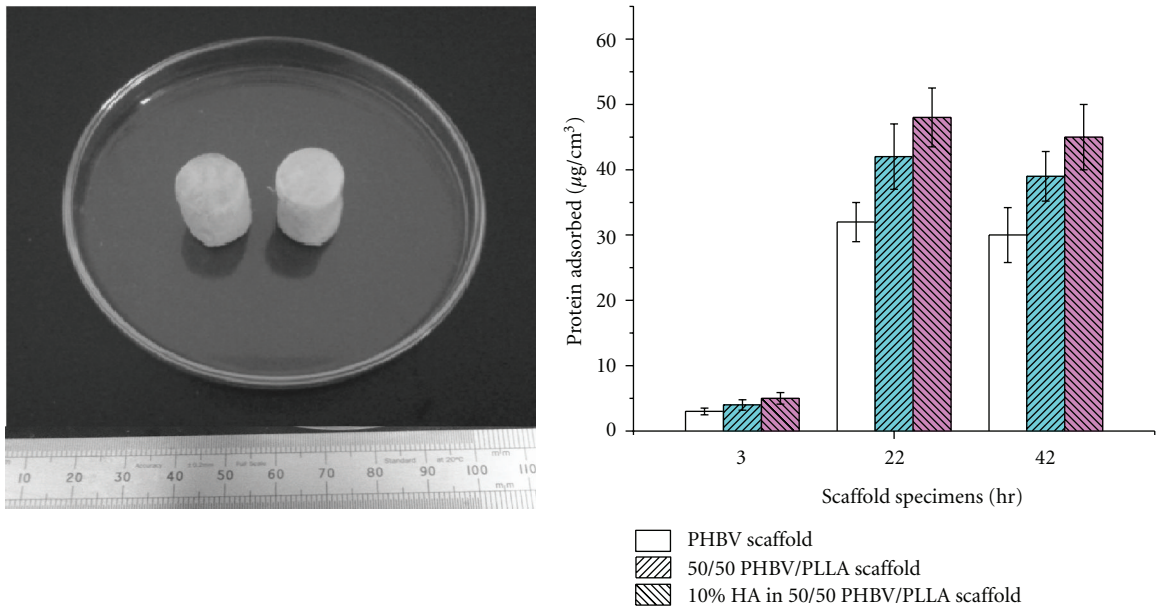
Andrade et al. and Sun et al. studied the principles of protein adsorption extensively and summarized that the



(a) (b)

(c)

Figure 2: Microstructures examined by SEM of three types of scaffolds: (a) 100/0 PHBV scaffold, (b) 50/50 PHBV/PLLA scaffold, and (c) 10% HA in 50/50 PHBV/PLLA scaffold.



(a) (b)

Figure 3: (a) General appearance of the scaffolds and (b) protein adsorption of different scaffolds at 3 hrs, 22, hrs and 42 hrs (data are expressed as mean ± SD; $n = 3$).

Table 4: Calculated main effects of the variables on compressive strength and modulus.

Main effects	Effect on compressive yield strength (MPa)*	Effect on modulus (MPa)*
(A) Polymer concentration	$+0.25 \pm 0.03$	$+3.4 \pm 1.12$
(B) Water phase composition	-0.26 ± 0.03	-4.0 ± 1.12
(C) Ratio of PHBV/PLLA	-0.01 ± 0.03	-3.1 ± 1.12
(D) The amount of HA	$+0.18 \pm 0.03$	$+6.5 \pm 1.12$
(E) Molecular weight of PLLA	$+0.16 \pm 0.03$	$+1.9 \pm 1.12$

*mean ± standard error.

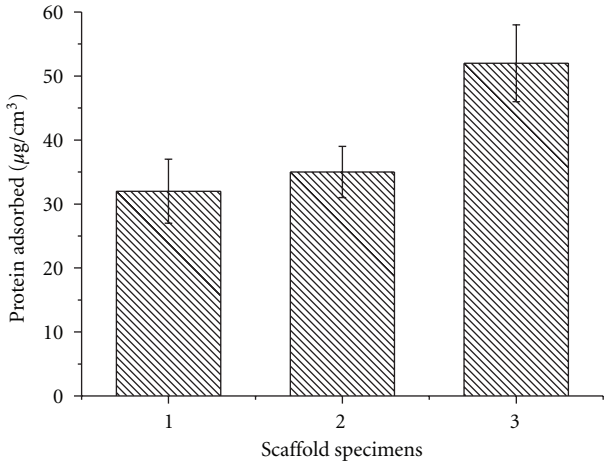


Figure 4: Protein adsorption of different scaffolds: (1) PHBV scaffolds, (2) 50/50 blend scaffolds, and (3) 10% HA in 50/50 blend scaffolds; (Data are expressed as mean ± SD; $n = 3$).

arrival of protein at the interface is driven solely by diffusion process which is dependent on bulk concentration and diffusion coefficient [18, 19]. It was also demonstrated that the particular surface chemistry of the protein also controls the adsorption. When protein adsorption takes place on neutral hydrophilic surfaces, it will be relatively weak; on the other hand, adsorption of proteins on hydrophobic surfaces tends to be very strong and irreversible. When adsorption takes place on charged surfaces, it tends to be a strong function of the charge character of the protein, the pH of the medium, and ionic strength. In this study, protein adsorption onto the scaffolds was reported. In order to study the mechanisms whether the protein is adsorbed on the scaffolds strongly or moderately, protein release study is needed.

3.4. Cell Morphology and Attachment. Both optical microscopy (Figure 5) and SEM (Figure 6) were used to examine the state and morphology of Saos-2 cells. Figure 5 shows the state and morphology of Saos-2 cells after biocompatibility test for 3 days. It was observed that the cell population and cell morphology of all specimens of PHBV scaffolds, 50/50 PHBV/PLLA scaffolds, and 10% HA in 50/50 PHBV/PLLA scaffolds were comparable to the control. This can indicate that the cell viability remained high after being in contact with all three types of scaffolds.

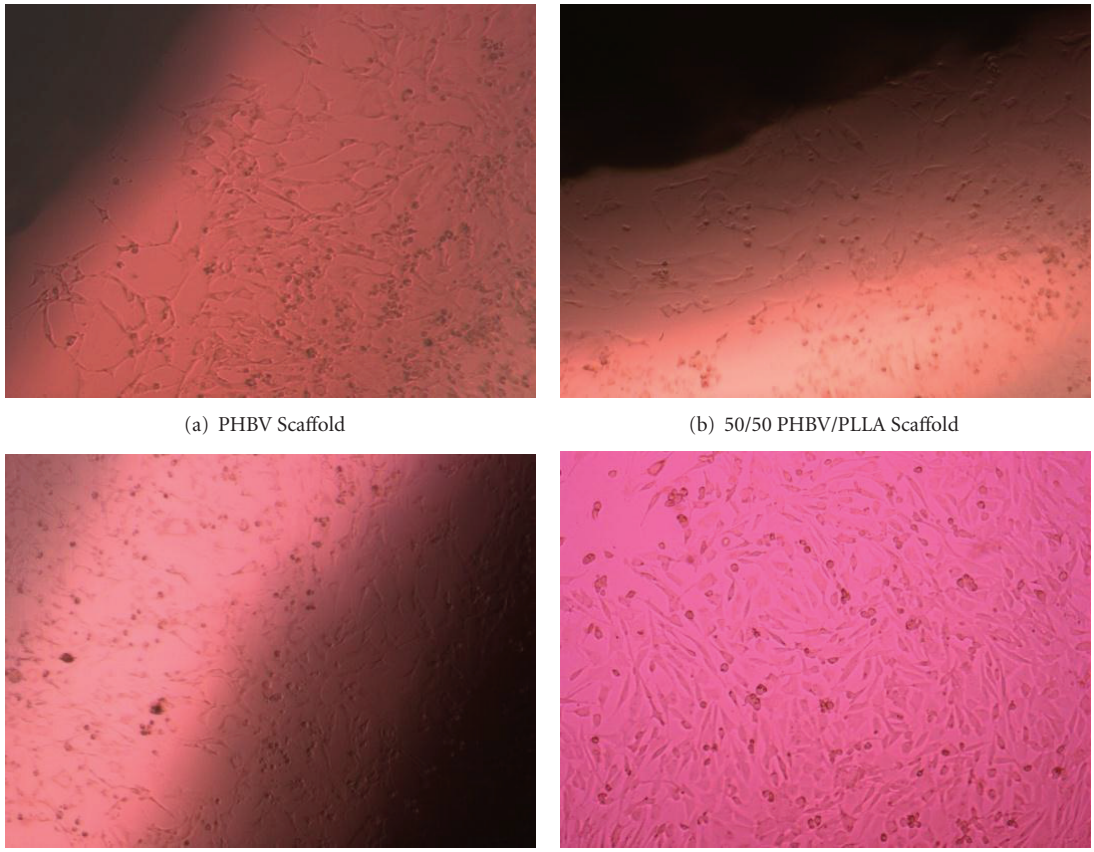
Using SEM analysis, the morphology of Saos-2 cells on the surface of the scaffolds specimens was also studied. Cells were appeared with clear substrate attachments and cellular processes. After culturing for 1 day, flat cells were observed by SEM to be well attached on all three types of scaffolds. At day 7 of Saos-2 cultures on all types of scaffolds, SEM analysis verified that the Saos-2 cells were well proliferated. The cells were more elongated as well as very well attached and spread on the surface of the scaffolds. The difference in morphology observed among the cells grown on three types of scaffolds was not obvious. The cells were found anchored to the surface by discrete filopodia on the composite scaffolds (Figure 6).

In this study, all three types of scaffolds were evaluated for cell attachment (Figure 7). It was observed that after 24 hours, the number of cells attached to HA incorporated composite scaffolds was significantly different ($P < 0.05$) than that of 100/0 PHBV/PLLA scaffolds or 50/50 PHBV/PLLA blend scaffolds. On the other hand, the number of Saos-2 cells attached on the PHBV/PLLA scaffold was apparently higher than PHBV scaffolds. The difference was not statistically significant.

In our previous studies [5–7], we reported the fabrication, *in vitro* degradation and biological evaluation of PHBV-based scaffolds using emulsion freezing/freeze-drying process. In the present paper, a factorial design approach was used to systematically investigate the influence of different material parameters on the compressive mechanical properties of the PHBV-based scaffolds. Besides the other requirements, the scaffolds should possess appropriate mechanical properties. In order to control the compressive stress and compressive modulus of the polymer and composite scaffolds, careful analysis was possible using factorial design approach. The protein adsorption and osteoblast (Saos-2) cell response were also studied in order to evaluate the scaffolds for bone tissue engineering application. Successful control of compressive properties is important for constructing composite scaffolds for bone tissue engineering applications.

4. Conclusions

A factorial design study was performed to assess the main effects of different material parameters on the compressive properties of the scaffolds. Polymer concentration and the amount of HA had the strongest effect to increase the compressive modulus and yield strength. 100/0



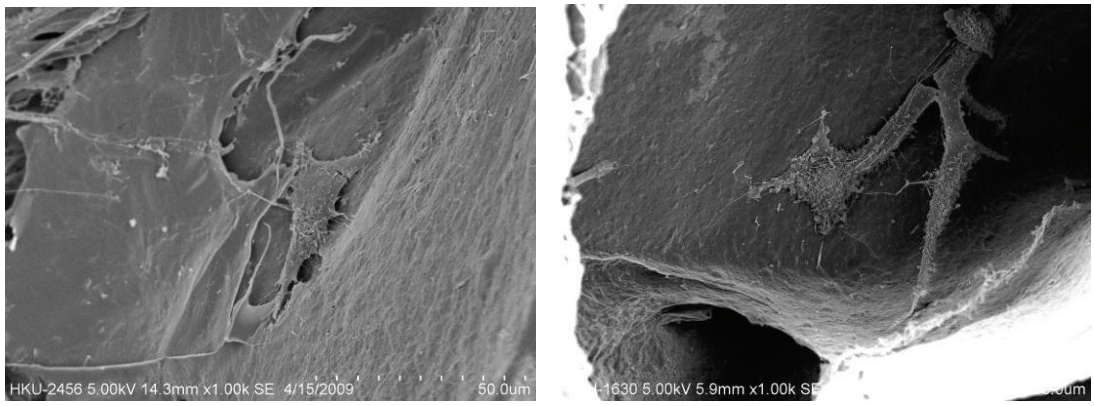
(a) PHBV Scaffold

(b) 50/50 PHBV/PLLA Scaffold

(c) 10% HA in PHBV/PLLA Scaffold

(d) Control

Figure 5: Optical microscopic image of state and morphology of Saos-2 cells in contact with scaffold specimens, (the dark area in (a), (b), (c) is the test specimen (d) control). (Magnification ×10).



(a)

(b)

Figure 6: SEM micrographs of 10% HA in 50/50 PHBV/PLLA composite scaffold after 7-day culture with Saos-2 cells (a, b).

PHBV/PLLA scaffolds, 50/50 PHBV/PLLA scaffolds, and 10% HA incorporated 50/50 composite scaffolds showed satisfactory adsorption of BSA protein. Adsorption of protein of all the scaffolds reached equilibrium after 22 hrs. The HA incorporated composite scaffolds exhibited more affinity towards protein adsorption than pure other two types of scaffolds. The attachment of Saos-2 cells was also significantly higher in 10% HA incorporated 50/50 composite scaffolds.

Conflict of Interests

The authors declare that they do not have any conflict of interests.

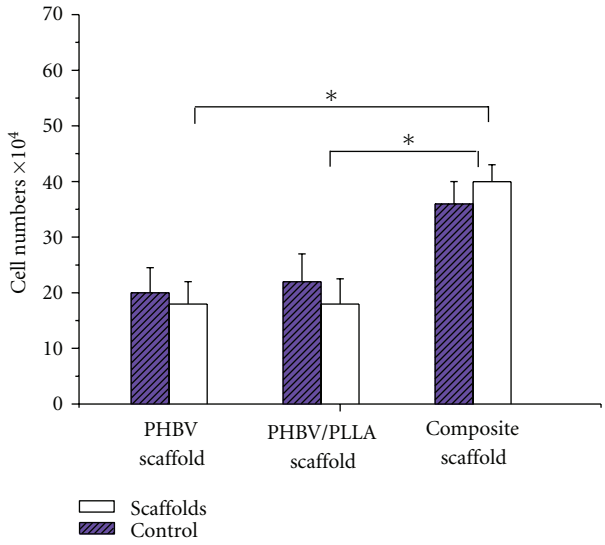


Figure 7: The attachment of Saos-2 cells on PHBV and PHBV-based composite scaffolds (data are expressed as mean ± SD; 2 = 3. $^{*}P < 0.05$).

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