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# Bone scaffolds from electrospun fiber mats of poly(3-hydroxybutyrate), poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) and their blend

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**Abstract**

In the present contribution, electrospinning was used to fabricate ultrafine fiber mats from poly(3-hydroxybutyrate) (PHB), poly(3-hydroxybutyrate-*co*-2-hydroxyvalerate) (PHBV), and their 50/50 w/w blend for potential use as bone scaffolds. Cytotoxicity evaluation of these as-spun fiber mats with human osteoblasts (SaOS-2) and mouse fibroblasts (L929) indicated biocompatibility of these materials to both types of cells. The potential for use of these fiber mats as bone scaffolds was further assessed in vitro in terms of the attachment, the proliferation, and the alkaline phosphatase (ALP) activity of SaOS-2 that were seeded or cultured at different times. The cells appeared to adhere well on all types of the fibrous scaffolds after 16 h of cell seeding. During the early stage of the proliferation period (i.e., from ~24 to 72 h in culture), the viability of the cells increased considerably and appeared to be unchanged with further increase in the time in culture. In comparison with the corresponding solution-cast film scaffolds, all of the fibrous scaffolds exhibited much better support for cell attachment and proliferation. Lastly, among the various fibrous scaffolds investigated, the electrospun fiber mat of the 50/50 w/w PHB/PHBV blend showed the highest ALP activity. These results implied a high potential for use of these electrospun fiber mats as bone scaffolds.
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*Keywords:* Electrospun fibers; Poly(3-hydroxybutyrate); Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)

## 1. Introduction

Poly(hydroxyalkanoate)s (PHAs) are a class of biodegradable and biocompatible thermoplastic polyesters produced by various microorganisms, such as soil bacteria, blue-green algae, and some genetically-modified plants. In microorganisms, PHAs serve as an intracellular energy and carbon storage product in much the same way as glycogen in mammalian tissues. One of the most extensively-studied PHAs is poly(3-hydroxybutyrate) (PHB). Its copolymers in various ratios with hydroxyvalerate (HV) are the most widely-used members, due to the possibility for tailoring the physical characteristics and processability of the resulting copolymers. Incorporation of HV into the PHB main chains causes the chain flexibility to increase, hence the observed decrease in both the glass transition and the melting temperatures and an improvement in the processability. Due to their inherent biocompatible and biodegradable properties, these polymers are ideal for various biomedical applications [1,2].

So far, PHB, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), poly(4-hydroxybutyrate) (P4HB), poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx), and poly(3-hydroxyoctanoate) (PHO) are available in sufficient quantities for applicable researches. The in vitro biocompatibility studies of PHB and PHBV fabricated by both solvent-casting and solute-leaching techniques have been reported with

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various cell lines, including osteoblasts, epithelial cells, and ovine chondrocytes [3]. The adhesion of these various cells on the surfaces of PHB and PHBV films was good, suggesting a high possibility to further develop these materials as tissue scaffolds. The properties of PHB and PHBV films were proved to be fundamentally similar [4], but the potential utilization of PHB is somewhat restricted, due mainly to its fragility. Through copolymerization with a more flexible co-monomer, such an adverse property of PHB can be optimized, but the resulting copolymers (e.g., PHBV) are more expensive. Physical blending with PHBV was suggested to be an economical pathway [5].

Ultrafine polymeric fibers characterized by their small diameters in the range of several microns down to a few tens of nanometers are of considerable interest for various applications, due to their interesting characteristics such as high surface area to volume/mass ratio, vast possibilities for surface functionalization, and enhanced specific mechanical performance [6]. Some of the potential uses for these ultrafine polymeric fibers are reinforcing fillers [7–12], fibrous scaffolds for engineered tissues [13–15], carriers for delivery of drugs [16], and so on. A number of processing techniques such as template synthesis [17], phase separation [18], self-assembly [19], and electrospinning [20] have been used to prepare ultrafine polymeric fibers in recent years. Among these, electrospinning seems to be the simplest method that can be further developed for mass production of continuous ultrafine fibers from various polymers.

The electrospinning process involves the application of a strong electrical potential to a polymeric liquid contained in a reservoir (e.g., syringe) attached to a metal nozzle (e.g., needle) across a finite distance between the nozzle and a grounded collector (i.e., collection distance). Upon increasing the applied electrostatic field strength (i.e., electrical potential divided by collection distance) to a critical value, a pendant droplet of the polymeric liquid at the tip of the nozzle gradually changes its shape from partially-spherical into conical. Further increase in the electrostatic field strength causes an ejection of a stream of charged liquid (i.e., charged jet) from the apex of the liquid cone. The jet accelerates towards and finally rests on the grounded collector. Owing to the high enough viscosity of the polymeric liquid, the ejected, charged jet remains stable and does not break up into spherical droplets as commonly found in the electrospraying of low molecular weight liquids. This results in the deposition of ultrafine polymeric fibers on the collector as a non-woven mat [21].

In the present contribution, electrospinning was used to fabricate ultrafine fiber mats of poly(3-hydroxybutyrate) (PHB), poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), and their 50/50 w/w blend. The potential for use of these fiber mats as bone scaffolds was evaluated in vitro with human osteoblasts (SaOS-2), in which the attachment, proliferation, and alkaline phosphatase (ALP) activity of the cells were analyzed. Comparison was made against the tissue-culture polystyrene (TCPS) plates and the corresponding solution-cast films.

## 2. Experimental

### 2.1. Materials and preparation and characterization of spinning solutions

Poly(3-hydroxybutyrate) (PHB; $M_w = 300{,}000$ g mol$^{-1}$) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV; $M_w = 680{,}000$ g mol$^{-1}$) were purchased from Sigma–Aldrich, USA. The HV content in PHBV was 5 mol.%. Chloroform, used as the solvent, was purchased from Lab-scan (Asia), Thailand. PHB, PHBV, and 50/50 w/w PHB/PHBV solutions at a fixed concentration of 14% w/v were prepared in chloroform at 60 °C.

### 2.2. Electrospinning and characterization of electrospun fiber mats

In electrospinning, each of the as-prepared spinning solutions was contained in a 50-ml glass syringe, the open end of which was connected to a blunt-ended gauge-20 stainless steel needle (OD = 0.91 mm), used as the nozzle. The temperature of the solution was maintained at 50 °C via a home-made programmable heater band wrapped around the glass syringe. A Gamma High Voltage Research D-ES30PN/M692 power supply was used to generate a fixed DC electrical potential of 12 kV. The emitting electrode of positive polarity from the power supply was attached to the needle, while the grounded one was attached to a home-made rotating cylindrical collector (i.e., width and OD of the cylinder ≈ 15 cm and rotational speed = 1000 rpm), the surface of which was wrapped around by a piece of aluminum sheet. The distance between the tip of the needle and the surface of the rotating collector defined the collection distance, which was fixed at 20 cm. Each of the spinning solutions was electrospun continuously for about 8 h to obtain the as-spun fiber mats with thickness of about $85 \pm 5$ μm.

Films with thickness of about $65 \pm 5$ μm of the same materials were also fabricated by casting the solutions used to fabricate the electrospun fiber mats in a glass dish. The films were prepared to investigate how the surface topography affected the biological response of the cultured cells. Both the as-spun fiber mats and the as-cast films were dried in vacuo at 40 °C overnight to remove as much solvent as possible. The morphological appearance of the as-spun fiber mats was observed by a JEOL JSM-5200 scanning electron microscope (SEM). The specimens for SEM observation were prepared by cutting an Al sheet covered with the as-spun fiber mat and the cut sections were carefully affixed on copper stubs. Each specimen was gold-coated using a JEOL JFC-1100E sputtering device prior to SEM observation. Diameters of the as-spun fibers were measured directly from SEM images using a SemAphore 4.0 software, with the average value being calculated from at least 100 measurements.

Wettability of the as-spun fiber mat and the corresponding film surfaces was assessed by water contact angle measurements. The static water contact angles were measured by a sessile drop method using a Krüss contact angle measurement

system. A distilled water droplet of about 40 μl was gently plated on the surface of each specimen. At least 10 readings on different parts of the specimen were averaged to obtain a data point.

### 2.3. Cell culture and cell seeding

Human osteoblasts (SaOS-2) were cultured as monolayer in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, USA), supplemented by 10% fetal bovine serum (FBS; BIOCHROM AG, Germany), 1% L-glutamine (Invitrogen Corp., USA) and 1% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate, and amphopericin B (Invitrogen Corp., USA)]. The medium was replaced once in every 3 d and the cultures were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$. Each scaffold was cut into circular discs (about 15 mm in diameter) and the disc specimens were placed in wells of a 24-well tissue-culture polystyrene plate (TCPS; Biokom Systems, Poland), which were later sterilized in 70% ethanol for 30 min. The specimens were then washed with autoclaved de-ionized water and subsequently immersed in DMEM overnight. To ensure a complete contact between the specimens and the wells, the specimens were pressed with a metal ring (about 12 mm in diameter). SaOS-2 from the cultures were trypsinized [0.25% trypsin containing 1 mM EDTA (Invitrogen Corp., USA)], counted by a hemocytometer (Hausser Scientific, USA), and seeded at a density of about 40,000 cells/well on the scaffold specimens and empty wells of TCPS that were used as controls.

#### 2.3.1. Indirect cytotoxicity evaluation

The applicability of the as-spun fiber mats of PHB, PHBV, and their 50/50 w/w blend as bone scaffolds was first assessed by indirect cytotoxicity evaluation with SaOS-2 and mouse fibroblastic cells (L929) based on a procedure adapted from the ISO10993-5 standard test method. Extraction media were prepared by immersing samples cut from the as-spun fiber mats in wells of TCPS containing a measured quantity of serum-free medium (SFM; containing the same composition as DMEM, but without FBS) for 24 h. SaOS-2 and L929 were separately cultured in wells of TCPS in serum-containing DMEM for 16 h to allow cell attachment. The cells were then starved with SFM for 24 h. After that, the medium was replaced with an extraction medium and cells were re-incubated for 24 h. The viability of the cells cultured with fresh SFM was used as control. Finally, the viability of the cells cultured with fresh SFM (i.e., control) and the as-prepared extraction media was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma–Aldrich, USA) assay.

#### 2.3.2. Cell attachment and cell proliferation

For attachment study, SaOS-2 were allowed to attach to the scaffold specimens and TCPS for 4, 16, and 24 h, respectively. After each specified seeding period, the number of the attached cells was quantified by the MTT assay. Each sample was rinsed with phosphate buffer saline (PBS; Sigma–Aldrich, USA) to remove unattached cells prior to MTT assay. For proliferation study, the cells were first allowed to attach on the specimens for 24 h. The proliferation of cells on the specimens was determined after 1, 3, and 5 d, respectively. After attachment for 24 h, the cells were starved with serum-free medium twice (i.e., the medium was changed with SFM once after the 24 h-attachment period and again after 3 d). The number of viable cells was, again, quantified by the MTT assay. Morphological appearance of the cells during the attachment and the proliferation periods was observed by SEM.

#### 2.3.3. Quantification of viable cells (MTT assay)

The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells. First, each sample was incubated at 37 °C for 30 min with 250 μl/well of MTT solution at 0.5 mg/ml without phenol red. After incubation, MTT solution was removed. A buffer solution containing dimethylsulfoxide (DMSO; Carlo Erba, Italy) (900 μl/well) and glycine buffer (pH = 10) (125 μl/well) was added into the wells to dissolve the formazan crystals. After 10 min of rotary agitation, the solutions were then transferred into a cuvette and placed in a Thermospectronic Genesis10 UV–visible spectrophotometer, from which the absorbance at 540 nm representing the number of viable cells was measured.

#### 2.3.4. Morphological observation of cultured cells

After removal of the culture medium, the cell-cultured scaffold specimens were rinsed with PBS twice and the cells were then fixed with 3% glutaraldehyde solution [diluted from 50% glutaraldehyde solution (Electron Microscopy Science, USA) with PBS] at 500 μl/well. After 30 min, they were rinsed again with and kept in PBS at 4 °C. After cell fixation, the specimens were dehydrated in ethanol solutions of varying concentration (i.e., 30, 50, 70, 90, and 100%) for about 2 min at each concentration. The specimens were then dried in 100% hexamethyldisilazane (HMDS; Sigma, USA) for 5 min and later let dry in air after removal of HMDS. After being dried completely, the specimens were mounted on copper stubs, coated with gold, and observed by SEM.

#### 2.3.5. Production of characteristic protein of cultured cells

SaOS-2 were cultured on scaffold specimens for 5 or 10 d to observe the production of alkaline phosphatase (ALP). The specimens were rinsed with PBS after removal of culture medium. Alkaline lysis buffer (10 mM Tris–HCl, 2 mM $MgCl_2$, 0.1% Triton X-100, pH 10) (100 μl/well) was added and the samples were scrapped and then frozen at −20 °C for at least 30 min prior to the next step. An aqueous solution of 2 mg/ml *p*-nitrophenyl phosphate (PNPP; Zymed Laboratories, USA) mixed with 0.1 M amino propanol (10 μl/well) in 2 mM $MgCl_2$ (100 μl/well) having a pH of 10.5 was prepared and added into the specimens. The specimens were incubated at 37 °C for 2 min. The reaction was stopped by adding 0.9 ml/well of 50 mM NaOH and the extracted solution was

transferred to a cuvette and placed in the UV–visible spectrophotometer, from which the absorbance at 410 nm was measured. The amount of ALP was then calculated against a standard curve. In order to calculate the ALP activity, the amount of ALP had to be normalized by the amount of total protein synthesized. In the protein assay, the samples were treated in the same manner as the ALP assay up to the point where the specimens were frozen. After being frozen, bicinchoninic acid (BCA; Pierce Biotechnology, USA) solution was added into the specimens. The specimens were incubated at 37 °C for 2 min. The absorbance of the medium solution was then measured at 562 nm by the UV–visible spectrophotometer and the amount of the total protein was calculated against a standard curve.

### 2.4. Statistical analysis

Statistical comparisons were performed using one-way ANOVA with SPSS software (SPSS, Germany). $P$ values $<0.05$ were considered statistically significant ($n = 10$ for contact angle measurements, $n = 6$ for cell attachment and cell proliferation assays, and $n = 3$ for ALP assay).

## 3. Results and discussion

Successful fabrication of electrospun mats of smooth PHB/PHBV fibers without the presence of beads was reported and characterized in our previous related work [22]. With regards to the size of the fibers, it is interesting that the average diameter of the as-spun fibers from 50/50 w/w PHB/PHBV blend solution (i.e., 4.0 μm) was larger than those of the fibers from the pure polymer solutions (i.e., 3.7 μm for the as-spun PHB fibers and 2.3 μm for the as-spun PHBV fibers). This could be a result of the much greater electrical conductivity that the blend solution exhibited (i.e., 0.09 $\mu S\,cm^{-1}$) in comparison with those of the pure ones (i.e., about 0.07 $\mu S\,cm^{-1}$). The much greater electrical conductivity translated to the much greater electrostatic forces exerting on a jet segment. This led to decreased total path trajectory and/or increased material flow rate, with both being thought to be responsible for the observed larger diameters of the as-spun fibers from the blend solution.

Recently, Choi et al. [23] reported successful electrospinning (applied electrical potential = 15 kV and collection distance = 15 cm) of 20 wt.% PHBV ($M_w = 680{,}000\ g\,mol^{-1}$ and HV content = 6 mol.%) solution in chloroform that resulted in the fibers with diameters in the range of 1–4 μm. When small amounts of organo-soluble salts, i.e., benzyl trialkylammonium chlorides, were added to the PHBV solution, the average diameter of the as-spun fibers decreased to about 1 μm, due possibly to the marginal decrease in the surface tension and the monotonous increase in the conductivity of the resulting solutions. Additionally, Lee et al. [24] and Ito et al. [25] reported successful electrospinning (applied electrical potential = 15 kV and collection distance = 21 cm) of 2 wt.% PHBV ($M_w = 680{,}000\ g\,mol^{-1}$ and HV content = 5 mol.%) solution in 2,2,2-trifluoroethanol (TFE) that resulted in the fibers with an average diameter of 185 nm, due mainly to the greater dielectric constant of TFE in comparison with that of chloroform [24].

For further investigation, both the pure and the blend solutions of PHB and PHBV were electrospun continuously for about 8 h to obtain the as-spun fiber mats with thickness of about 85 ± 5 μm.

### 3.1. Wettability

The wettability of both the fiber mat and the corresponding film surfaces was assessed by evaluating the contact angle of a sessile droplet of distilled water on these surfaces. Evidently, all of the fiber mat samples exhibited the water contact angle in the range of about 116°–122°, while all of the film counterparts showed the value in the range of about 68°–75° (see Fig. 1). Ito et al. [25] also reported that the water contact angle of the nanofiber mat of PHBV from 2 wt.% PHBV solution in TFE was greater than that of the corresponding solution-cast film (i.e., ~110° versus 81°, respectively). The observed hydrophobicity of the fiber mats in comparison with that of the films was probably a result of the surface roughness that introduced multiple contacting points on the surface of the water droplet such that the interface between the water droplet and the fiber mat surface was not exactly solid/liquid.

### 3.2. Indirect cytotoxicity evaluation

Even though it is a known fact that PHB and PHBV are biocompatible with various cells [3], assessment with regards to the biocompatibility of the as-spun PHB, PHBV, and PHB/PHBV fibrous scaffolds is a prerequisite since chloroform, a known toxic organic substance, was used as the solvent to fabricate the scaffolds. Both human osteoblasts (SaOS-2) and mouse fibroblasts (L929) were used in the assessment. L929 were used to comply with the ISO10993-5 standard test method. For both types of cells, about 40,000 cells/well were seeded in empty wells of TCPS.

The viability of the cells after being cultured with an extraction medium, prepared by immersing the as-prepared

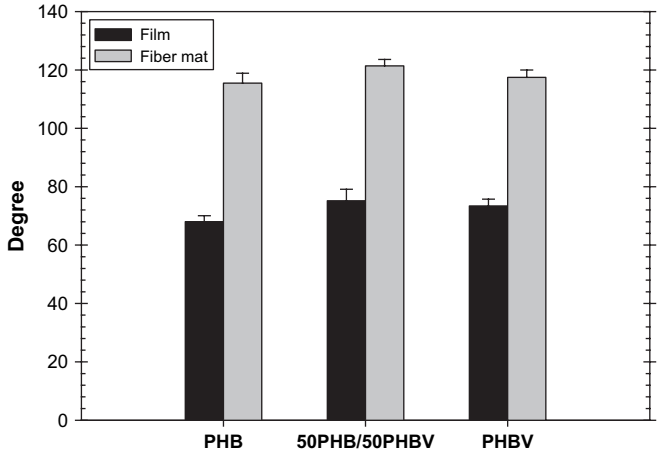



Fig. 1. Static contact angle of distilled water on surfaces of the as-spun PHB, PHBV, and PHB/PHBV fiber mats and the corresponding solution-cast films.

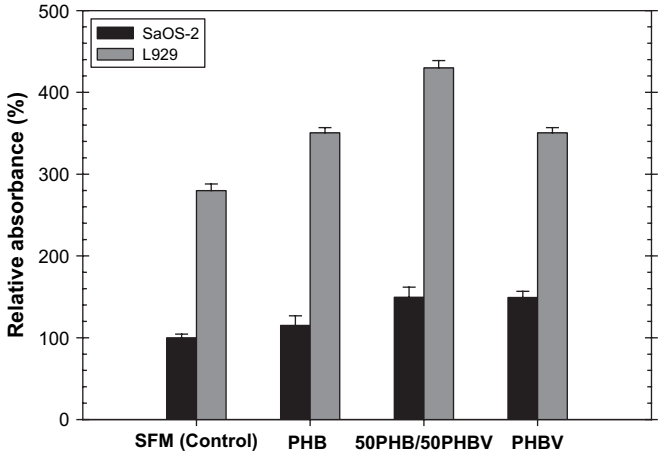



Fig. 2. Indirect cytotoxicity evaluation of the as-spun PHB, PHBV, and PHB/PHBV fiber mats based on the viability of human osteoblasts (SaOS-2) and mouse fibroblasts (L929).

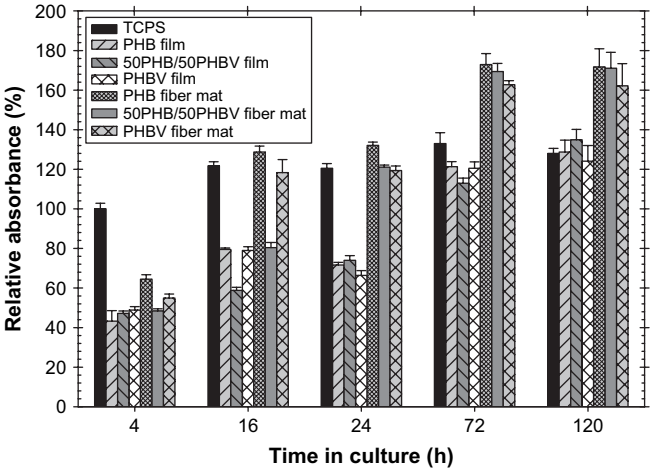



Fig. 3. Attachment and proliferation of SaOS-2 that were seeded or cultured on tissue-culture polystyrene plate (TCPS) and film and fibrous scaffolds of PHB, PHBV, and PHB/PHBV as a function of time in culture. During 4 and ~24 h of cell seeding signifies the attachment period, while during ~24 and 120 h of cell culture signifies the proliferation period.

fibrous scaffold sample in wells of TCPS containing a measured quantity of SFM for 24 h, is reported in Fig. 2 in terms of the relative absorbance with respect to the absorbance value of SaOS-2 that were cultured with fresh SFM for the same culture period. Evidently, for each type of the cells, all of the extraction media from all of the fibrous scaffolds investigated were non-toxic to the cells, since the average absorbance values were all greater than that of the control condition. Interestingly, all of the average absorbance values for L929 were much greater than those for SaOS-2, suggesting that L929 might be able to attach to TCPS much better than SaOS-2.

### 3.3. Cell attachment and cell proliferation

A tissue-engineered scaffolding material must support cellular attachment and growth. To evaluate cellular behavior on the as-spun fibrous scaffolds of PHB, PHBV, and 50/50 w/w PHB/PHBV as well as the corresponding film scaffolds, SaOS-2 were seeded and cultivated on these scaffolds in wells of TCPS. Fig. 3 shows both the attachment (during 4–24 h of cell seeding) and the proliferation (during 24 and 120 h in culture) in terms of the relative absorbance of SaOS-2 that were cultured on TCPS (i.e., control) and film and fibrous scaffolds of PHB, PHBV, and PHB/PHBV. The absorbance value signifying the viability of the cells that were cultured on TCPS was used as the reference to arrive at the various relative absorbance values shown in the figure.

At 4 h of cell seeding, the viability of the cultured cells on any type of both the film and the fibrous scaffolds was much less than that on TCPS, possibly due to the low hydrophilicity of the material. It was reported that an appropriate combination of hydrophilicity and hydrophobicity was an important factor determining the biocompatibility of poly(hydroxyalkanoate)s (PHAs), especially for the attachment of SaOS-2 on the surface of these materials [26]. Among the film and the fibrous scaffolds however, the attachment of SaOS-2 on the as-spun PHB and PHBV fibrous scaffolds was significantly better than that on the corresponding film counterparts. At 16 h of cell seeding, SaOS-2 appeared to attach on the as-spun PHB and PHBV fibrous scaffolds as good as they did on TCPS. Interestingly, at 24 h of cell seeding, the attachment of the cells on all of the fibrous scaffolds was comparable to that on TCPS. Nonetheless, at 16 and 24 h of cell seeding, all of the fibrous scaffolds showed much better attachment of SaOS-2 on their surface than the film counterparts (with an exception of the PHB/PHBV fibrous scaffold that was seeded with the cells for 16 h).

The proliferation of SaOS-2 on TCPS and both the film and the fibrous scaffolds could be assessed from the absorbance values after the cells were cultured for 24, 72, and 120 h. Again, at 24 h in culture, the viability of SaOS-2 on all of the fibrous scaffolds was comparable to that on TCPS and was significantly greater than that on all of the film scaffolds. At 72 h in culture, the viability of the cells on all of the film scaffolds increased considerably, but was still slightly lower than that on TCPS. Obviously, the viability of the cells that were cultured on all of the fibrous scaffolds was significantly greater than that on both TCPS and all of the film scaffolds. At 120 h in culture, the viability of the cells on all of the film counterparts was comparable to that on TCPS. Even though the viability of the cells on all of the fibrous scaffolds was practically unchanged from that at 72 h in culture, it was still significantly greater than that on both TCPS and all of the film counterparts.

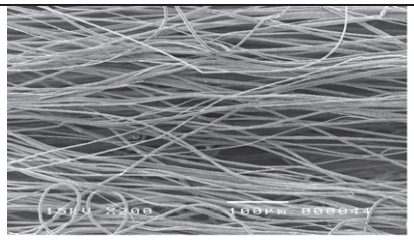
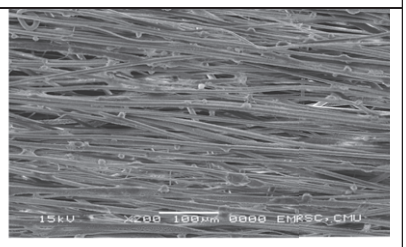
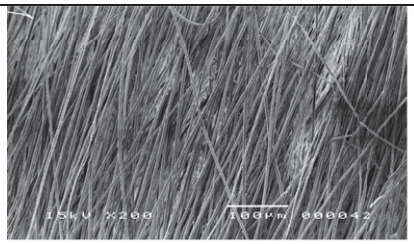
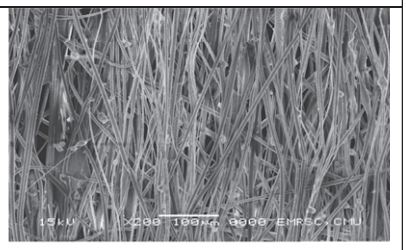
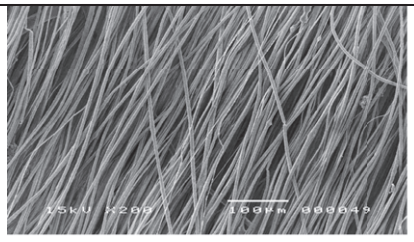
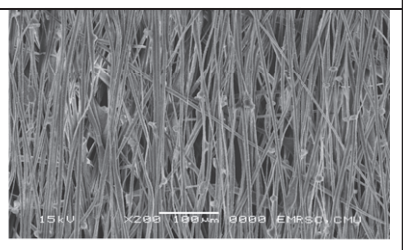
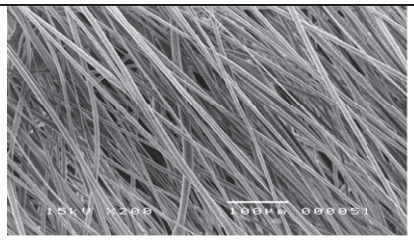
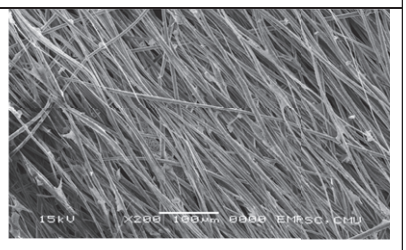
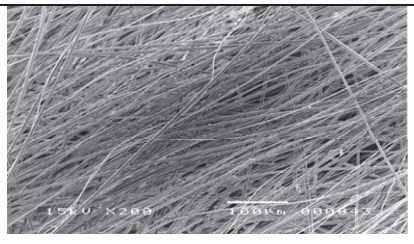
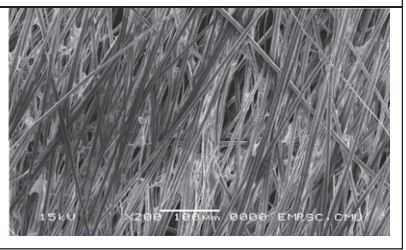
For both the attachment and the proliferation assays, about 40,000 cells/well were seeded and cultured; therefore, the results obtained from both assays can then be comparatively discussed. For TCPS, the viability of the cells increased significantly after 16 h of cell seeding and it was practically unchanged during the rest of the attachment period (~24 h of cell seeding). The viability of the cells increased slightly at the early stage of the proliferation period (from ~24 to 72 h in culture) and decreased slightly at longer culture time. The obtained results suggested that SaOS-2 appeared to attach well on TCPS after 16 h of cell seeding and that the cells only needed to proliferate slightly to form a monolayer

coverage on the surface of TCPS. For the film scaffolds, the cells appeared to attach well on the film surfaces after 16 h of cell seeding as well, but the ability of the films to support the attachment of SaOS-2 was apparently poorer than TCPS. During the proliferation period, the viability of the cells increased significantly to match that on TCPS (viz. the number of cells increased to realize the monolayer coverage on the flat surface of the films).

Like TCPS and the film scaffolds, SaOS-2 appeared to attach well on the fibrous scaffolds after 16 h of cell seeding as well (with an exception of the PHB/PHBV fibrous scaffold). Apparently, the ability of the fibrous scaffolds to support the attachment of the cells was as good as TCPS. During the proliferation period, the viability of the cells cultured on all of the fibrous scaffolds was far greater than that on both TCPS and all of the film scaffolds, as previously noted. The likely explanation for this should be due to the highly porous nature of the fibrous scaffolds that provided extra surface onto which the cells can propagate further.

Table 1 shows selected low-magnification SEM images (magnification = 200× and scale bar = 100 μm) of the PHB/PHBV fibrous scaffolds that were seeded with SaOS-2 at

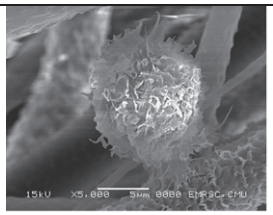
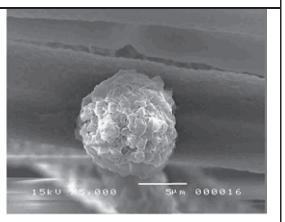
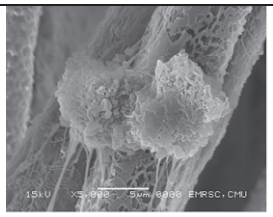
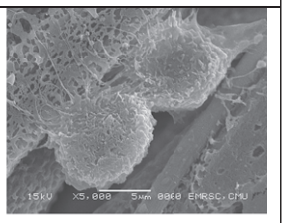
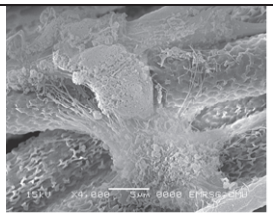
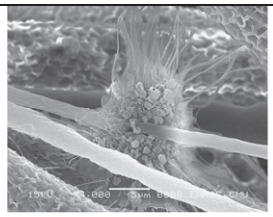
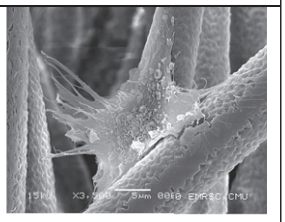
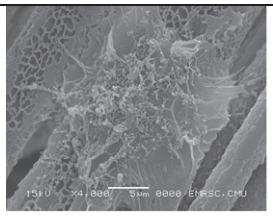
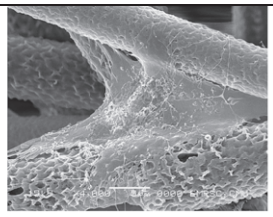
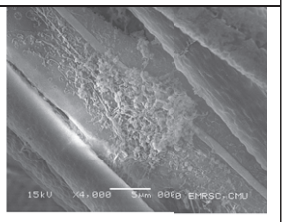
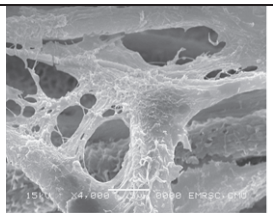
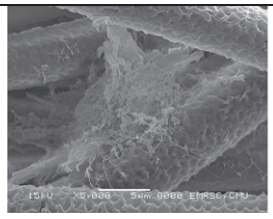
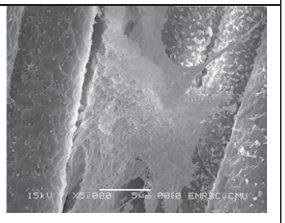
Table 1
Selected SEM images (magnification = 200×; scale bar = 100 μm) of neat and SaOS-2-cultured as-spun PHB/PHBV fibrous scaffolds at different times of cell seeding and in culture

| Time in culture (h) | As-spun PHB/PHBV fiber mat | SaOS-2-cultured as-spun PHB/PHBV fiber mat |
|---|---|---|
| 4 |  |  |
| 16 |  |  |
| 24 |  |  |
| 72 |  |  |
| 120 |  |  |

different times of cell seeding or in culture (i.e., 4, 16, 24, 72, and 120 h). Selected SEM images of the neat scaffolds (without cell seeding) after submersion in the culture medium for the same time intervals are also illustrated for comparison in Table 1. Clearly, the fibrous scaffolds retained their fibrous structure even after submersion in the culture medium for as long as 120 h. Though not shown, the neat and the cultured PHB and PHBV fibrous scaffolds were similar to those shown in Table 1. Despite the observed increase in the cell viability after the cells were seeded on these scaffolds for different times of cell seeding or in culture (see Fig. 3), such an increase could not be observed in these SEM images. The likely reason for this should be due to the relatively large pore sizes of these fibrous scaffolds that could allow the cells to penetrate into the inner side of the scaffolds.

Selected SEM images with a higher magnification (magnification = 5000× and scale bar = 5 μm) of PHB, PHBV, and PHB/PHBV fibrous scaffolds that were seeded with SaOS-2 at different times of cell seeding or in culture (i.e., 4, 16, 24, 72, and 120 h) are illustrated in Table 2. It is obvious now that the surface of the individual fibers of these fibrous scaffolds was rough. It was postulated that such a surface roughness was formed as a result of the rapid phase separation that occurred during rapid evaporation of chloroform (boiling point = 61.3 °C) [22]. Recently, it was demonstrated that both the relative humidity and solvent evaporation contributed to the formation of indentations on the surface of electrospun polystyrene fibers [27]. At 4 h of cell seeding, the morphology of the cells was largely in round shape with slight trace of filopodia. After 16 h of cell seeding, cell division started to be

Table 2
Selected SEM images (magnification = 5000×; scale bar = 5 μm) of as-spun PHB, PHBV, and PHB/PHBV fibrous scaffolds which were cultured with SaOS-2 at different times of cell seeding and in culture

| Time in culture (h) | Substrates | | |
|---|---|---|---|
| | PHB | 50/50 w/w PHB/PHBV | PHBV |
| 4 |  |  |  |
| 16 |  |  |  |
| 24 |  |  |  |
| 72 |  |  |  |
| 120 |  |  |  |

observed on all types of the fibrous scaffolds. During the proliferation period (from ~24 to 72 h in culture), the cells on all types of the fibrous scaffolds were well expanded, with a strong evidence of anchoring ligands reaching out to help support them on the fiber surfaces. Based on both the quantitative and the qualitative results, all types of the fibrous scaffolds appeared to support both the attachment and the proliferation of SaOS-2 particularly well, the results indicating the potential for use of these fibrous mats as bone scaffolds.

### 3.4. Alkaline phosphatase (ALP) activity

Alkaline phosphatase, an ectoenzyme produced by osteoblasts, is believed to involve in the degradation of inorganic pyrophosphate to provide a sufficient local concentration of phosphate or inorganic pyrophosphate for mineralization to proceed. Among the various biological functions of osteoblasts, secretion of alkaline phosphatase (ALP) is an important indicator determining the activity of the cells on a scaffold. The ALP activity of SaOS-2 on TCPS (i.e., control) and the fibrous scaffolds prepared from PHB, PHBV, and 50/50 w/w PHB/PHBV solutions was assessed on days 5 and 10 of cell culture (see Fig. 4). Evidently, for all of the fibrous scaffolds investigated, the ALP activity on day 10 was much greater than that on day 5 (especially for the PHB/PHBV fibrous scaffold), while for TCPS, the values were quite similar. On either day 5 or 10, the ALP activity on different fibrous substrates can be ranked as follows: PHB/PHBV > PHB > PHBV. The observed highest ALP activity of SaOS-2 on TCPS on day 5 compared with other substrates should be due to the fact that proliferation rate of the cells, after 24 h in culture, on TCPS was found to be lower than that of the fibrous scaffolds, while that on all of the fibrous scaffolds investigated still increased steadily. If we are to assume that the differentiation should begin as soon as the proliferation rate starts to decrease [28], the amount of ALP synthesized from the cells in culture on TCPS should be the highest, which is exactly what was observed here. According to Fig. 4, the cells that were cultured on the PHB/PHBV fibrous scaffold exhibited the highest ALP activity, which, in addition to the results on cell proliferation (see Fig. 3), suggests that the PHB/PHBV fibrous scaffold may be the best among the various fibrous scaffolds that could promote both proliferation and differentiation of SaOS-2 particularly well.

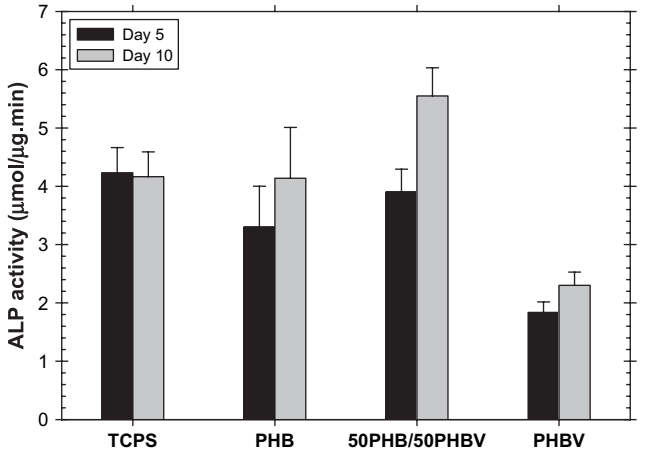



Fig. 4. Alkaline phosphatase (ALP) activity of SaOS-2 that were cultured on tissue-culture polystyrene plate (TCPS) and film and fibrous scaffolds of PHB, PHBV, and PHB/PHBV on days 5 and 10 of cell culture.

## 4. Conclusions

Ultrafine fiber mats from poly(3-hydroxybutyrate) (PHB), poly(3-hydroxybutyrate-*co*-2-hydroxyvalerate) (PHBV), and their 50/50 w/w blend were successfully prepared by electrospinning from pure and blend solutions of the polymers in chloroform at 50 °C. Apparently, the as-spun fiber mats appeared to be more hydrophobic than the corresponding solution-cast films. The potential for use of these fiber mats as bone scaffolds was first assessed by cytotoxicity evaluation with human osteoblasts (SaOS-2) and mouse fibroblasts (L929) and it was found that all of the fiber mats posed no threat to both types of cells. The potential for use of these fiber mats as bone scaffolds was further assessed by observing the cellular response of SaOS-2 that were seeded or cultured directly on these mats. The evaluation was carried out in terms of the attachment, the proliferation, and the alkaline phosphatase (ALP) activity of the cells. The cells appeared to adhere well on all types of fibrous scaffolds after 16 h of cell seeding. During the early stage of the proliferation period (i.e., from ~24 to 72 h in culture), the viability of the cells increased considerably and appeared to be unchanged with further increase in the time in culture. In comparison with the corresponding solution-cast film scaffolds, all of the fibrous scaffolds exhibited much better support for cell attachment and cell proliferation. Selected scanning electron microscopy (SEM) images showed that SaOS-2 maintained their phenotype during the cell culture. Lastly, among the various fibrous scaffolds investigated, the electrospun fiber mats from the 50/50 w/w PHB/PHBV blend solution showed the highest ALP activity. These results implied a high potential for use of these electrospun fiber mats as bone scaffolds.

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