Cell-compatible PHB/silk fibroin composite nanofiber mat for tissue engineering applications

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Abstract: Cell compatibility is one of the prominent requirements for the fabrication of tissue engineering materials. Silk fibroin (SF) is an excellent material for biomedical applications and shows desirable properties such as good compatibility, minimal tissue reaction, and tailorable degradability. Poly[(R)-3-hydroxybutyrate] (PHB) has a high percentage of crystallinity and a high melting temperature. In this study, PHB and SF were blended together to improve the mechanical properties of the SF fibrous structure and the crystallinity of PHB. Furthermore, the cell–biomaterial interaction on the PHB/SF composite scaffold was expected to be enhanced via the SF. For this purpose, PHB/SF composite scaffolds were prepared through the use of the electrospinning technique, which is a unique method for the production of biomaterials on the nanoscale intended for tissue engineering. PHB/SF scaffolds were prepared with different SF contents and a suitable electrospinning condition was chosen in terms of structure and fiber diameter. The average fiber diameter was 92 ± 2.6 nm at a flow rate of 0.4 mL/h, distance of 20 cm, polymer concentration of PHB/SF-0 of 7%:18% (w/w) or 1:1 (v/v), and voltage of 20 kV. Mechanical and crystalline properties of the PHB/SF scaffold were investigated. Adhesion and proliferation of L929 and HaCaT (mouse fibroblast and immortalized human keratinocyte cell lines) on PHB/SF-0 were examined.

Key words: Poly[(R)-3-hydroxybutyrate], silk fibroin, tissue engineering, electrospinning, biocompatibility

1. Introduction

Traditional synthetic biodegradable aliphatic polyesters such as poly(lactic-co-glycolic acid) (PLGA), polylactic acid (PLA), poly[(R)-3-hydroxybutyrate] (PHB), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), and polycaprolactone (PCL) have already been used in the electrospinning process due to their well-known good processability and mechanical properties. Here it is aimed to mimic the physical dimension and morphology of the major component, collagen, in the native extracellular matrix. However, there are some persistent problems related to the use of these synthetic polymers during applications. First, these synthetic polymers in the form of scaffolds do not have adequate cell recognition sites to improve the cell affinity (Hubbell, 1995; Cai et al., 2002; Rosso et al., 2005). Second, their hydrophobic nature affects cell seeding, cellular activities on the nanofibrous scaffolds (Chen et al., 2002; Cai et al., 2003), and the nanoscale effect (Feng et al., 2002; Neimark et al., 2003). These drawbacks create a need to develop a new bioactive and functional electrospun scaffold. Collagen is the most ideal candidate material in tissue engineering applications. Its inadequate mechanical properties coupled with the host immune response limit collagen’s use. In order to overcome these issues, surface chemical modification approaches or hybridization of synthetic polymers with bioactive natural polymers can be applied to improve the properties of synthetic nanofibers. The hybridization of polymers into nanofibers is a simple and cost-effective method. The composite nanofibrous scaffolds can be designed in randomly oriented or aligned forms through a versatile electrospinning method. These scaffolds provide better hydrophilicity and improved mechanical properties. Furthermore, the reinforcement of biological macromolecules such as growth factors or proteins into the nanofibrous scaffolds promotes cell–surface interaction (Drumheller and Hubbell, 2000).

Silk fibroin (SF) is a natural biopolymer used in the human body as a suture material. Silk proteins are obtained from various sources such as spiders, silkworms, scorpions, mites, and silkworms. Among them, silk fibers of silkworms have been commonly studied in biomedical device applications. Silk protein produced by Bombyx mori, a member of the family Bombycidae, is known as mulberry silk. Silk protein has several major advantages compared to other protein-based biomaterials. SF has been employed as a versatile material for tissue-engineered scaffolding.
due to its slow degradation profile, its biocompatibility, and the presence of easily accessible chemical groups for functional modifications (Altman et al., 2003; Wang et al., 2006). Furthermore, it can be processed easily into various forms such as nanofibrillar scaffolds, membranes, hydrogels, three-dimensional porous scaffolds, and nanoparticles (Kim et al., 2004, 2005; Kundu et al., 2010). SF has been extensively used for multiple types of scaffolds in tissue engineering of skin, bone, cartilage, tendons, and ligaments (Altman et al., 2003; Vepari and Kaplan, 2007). Silk-based biomaterials have showed excellent biocompatibility in different material forms for various tissue engineering applications. For example, Min et al. (2004) found that SF nanofiber nonwovens produced by the electrospinning process promoted normal human keratinocytes and fibroblast cell adhesion and spreading. Furthermore, in vitro cytocompatibility tests have indicated that electrospun SF fibrous scaffolds support cell viability, maintain the cell phenotype, and promote cell reorganization, and composite electrospun polymeric nanofibers could be promising as tissue engineering scaffolds by blending proteins and polysaccharides in the spinning solution (Jin et al., 2004; Marelli et al., 2010). Basal et al. (2016) produced olive leaf extract-loaded coaxial nanofibers made from a blend of SF and hyaluronic acid and evaluated the potential use of these nanofibers in wound dressing applications using the HS2 cell culture test. The prepared electrospun nanofibers had no obvious cytotoxic effect on HS2 human epidermal keratinocyte cells.

The polyhydroxyalkanoate (PHA) family is one of the attractive classes of microbial biopolymers due to its inherent biocompatible and biodegradable properties. These polyesters are produced by a wide variety of microorganisms under nitrogen-rich and glucose-limited conditions (Vepari and Kaplan, 2007; Silva et al., 2007). PHB has attracted the attention of tissue engineers and has become the most widely studied member of the PHA family. Moreover, PHB is known to be biocompatible to various cell lines and no report exists of its toxicity in medical applications (Suwantong et al., 2007; da Silva et al., 2016). These properties make it suitable for a variety of applications in the health industry, such as controlled release systems (Dararong et al., 2014), surgical sutures (Fedorov et al., 2007), wound dressings (Rennukka et al., 2014), and orthopedic uses (Alves et al., 2011). However, the intrinsic brittleness and high cost of PHB limits its use in tissue engineering applications. Therefore, recently, electrospun PHB scaffolds alone or in combination with other biodegradable polymers have become a topic of current interest. For instance, PHAs can be blended with SF to improve the morphology, structure, hydrophilicity, mechanical properties, and in vitro biocompatibility of tissue-engineered scaffolds. For example, Yang et al. (2011) reported SF-modified poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) scaffolds to improve the biocompatibility of PHBHHx. The study results, carried out using human fibroblasts, human smooth muscle cells, endothelial-like cell line ECV304, and umbilical vein endothelial cells, showed that SF-modified PHBHHx scaffolds are highly biocompatible and have the potential for extensive applications in cardiovascular regeneration. Similarly, Lei et al. (2015) prepared nanofibrous scaffolds by coelectrospinning from PHBV and fibroin regenerated from silk. The results showed that the surface hydrophilicity and water-uptake capability of the PHBV/SF nanofibrous scaffold were improved compared to the PHBV nanofibrous scaffold and the fibroblasts are more adhered to the PHBV/SF nanofibrous scaffold than the pure PHBV nanofibrous scaffold. As a result, the PHBV/SF nanofibrous scaffold may be a better candidate for wound dressing applications.

In the present work, PHB/SF hybrid nanofibrous scaffolds were fabricated through electrospinning to improve the surface hydrophilicity, brittleness, and biocompatibility of PHB. The cell viability and proliferation of L929 and HaCaT cells on contact with the new hybrid scaffolds were investigated as a wound dressing candidate. Furthermore, we focused on the effect of the blended form on the morphology, structure, hydrophilicity, and mechanical properties of the PHB/SF nanofibrous scaffolds for skin tissue engineering. The present contribution aims at highlighting the favorable properties of two biocompatible polymers through their use in the process of fabricating new blended nanofiber scaffolds, which have many advantages as well as disadvantages.

2. Materials and methods

2.1. Materials

PHB was obtained from Fluka (Buchs, Switzerland). All chemicals were purchased from Sigma-Aldrich, including disodium carbonate (Na₂CO₃), chloroform, trifluoroacetic acid (TFA), lithium bromide (LiBr), phosphate-buffered saline (1X PBS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Bombyx mori silkworm cocoons were obtained from Kozabirlik (Bursa, Turkey). The immortalized human keratinocyte cell line (HaCaT) was obtained from Lonza (Basel, Switzerland) while mouse fibroblast cells (L929) were purchased from the American Type Culture Collection (Rockville, MD, USA). The Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin used in the cultivation of mouse fibroblasts and the immortalized human keratinocyte cell line were purchased from Biological Industries (Beit Haemek, Israel).
2.2. Preparation of aqueous silk solutions
Cocoons of *B. mori* were provided by Kozabirlik. The SF aqueous solution was prepared as detailed in previously established protocols (Vepari and Kaplan, 2007). Briefly, *B. mori* silk cocoons were cut into small pieces and boiled in an aqueous solution of 0.05 M disodium carbonate at 80 °C for 30 min to remove the sericin shell from the surface of silk fibers and then rinsed with distilled water three times. The degummed SF fibers were dried for 24 h at 40 °C and then dissolved in 9.3 M LiBr solution at 60 °C. This solution was dialyzed through a cellulose membrane (12,000–14,000 MWCO) against distilled water for 5 days, which yielded an approximately 5 wt.% SF aqueous solution. The aqueous silk solution was centrifuged twice at 9000 rpm and the SF solution was freeze-dried. The SF solid after drying was dissolved in TFA overnight.

2.3. Electrospinning
Initially, PHB (7% wt/v) and SF (20% and 18% wt/v) were separately dissolved in TFA to prepare a stock solution. The solutions were stirred at room temperature overnight followed by sonication of 3 h at room temperature to achieve good dispersion, and the PHB solution was mixed well with the SF solution in ratios of 1:1, 3:1, and 1:3 (v/v). Four electrospinning conditions were varied in order to determine the optimum parameters for preparing fibrous PHB/SF scaffolds with the most suitable properties. The compositions of electrospinning groups are given in Table 1.

The electrospinning apparatus consisted of a high-voltage power supply (Spellman CZE1000R), a syringe pump (Goldman), and a collector made of stainless steel. A 5-mL plastic syringe containing PHB/SF solution in different ratios was connected to a stainless steel needle and the metal collector was wrapped with aluminum foil to collect nano- or macrofibers. The processing parameters were adjusted at a voltage of 20 kV at a feeding rate of 0.4 mL/h and the collector was fixed at 20 cm. All electrospinning procedures were carried out using the mixed solutions [PHB/SF: 1:1, 3:1, and 1:3 (v/v)]. The resultant composite nanofibrous scaffolds were collected on a stainless steel collector for further characterization. The composite nanofibrillar scaffolds were then placed in a vacuum dryer overnight to remove the solvents.

Table 1. The composition of electrospinning groups.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Composition</th>
</tr>
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<tbody>
<tr>
<td>PHB/SF-0</td>
<td>7:18% (w/v); 1:1, v/v</td>
</tr>
<tr>
<td>PHB/SF-1</td>
<td>7:20% (w/v); 1:1, v/v</td>
</tr>
<tr>
<td>PHB/SF-2</td>
<td>7:20% (w/v); 1:3, v/v</td>
</tr>
<tr>
<td>PHB/SF-3</td>
<td>7:20% (w/v); 3:1, v/v</td>
</tr>
</tbody>
</table>

2.4. Characterization
The structure and morphologies of the nanofibrous composite scaffolds were characterized by a scanning electron microscope (SEM), using a JEOL JSM700F at an operating voltage of 15 kV. The average fiber diameter was determined by measuring diameters of fibers at 100 points from approximately five images taken per area using image-processing software (Image, NIST). Furthermore, the composition of the PHB/SF nanofibrous scaffolds was characterized by energy dispersive X-ray spectroscopy (EDS/EDX). Fourier transform infrared spectroscopy (FT-IR) spectra of the electrospun blend scaffolds to analyze the chemical structure were obtained with an attenuated total reflection (ATR) technique at a resolution of 4 cm⁻¹ in the range of 500–4000 cm⁻¹. X-ray diffraction (XRD) analysis (Ultima IV, Rigaku, Japan) was carried out to characterize further crystallinity of the blend and PHB/SF fibrous structures alone. Scans were performed from 10° to 70° (2θ) with a speed of 2°/min.

Wettability of the PHB/SF nanofibrous scaffolds was assessed using static water contact angle measurements with the sessile drop method (DSA30, Krueess GmbH, Germany). The scaffolds were placed on a sample holder stage. One drop of distilled water was placed onto the sample surface and then the images were recorded using a video camera system. A tensile test to quantitatively evaluate the physical strength was performed on strips with dimensions of 1 cm × 1 cm using a universal testing machine (Zwick, 250 kN, USA). The testing was done at a constant speed rate of 10 mm min⁻¹ with load cell capacity of 100 N. The data were presented as an average of three tests. The tensile strength, elongation at break, and Young's modulus were determined from the stress–strain curves.

2.5. In vitro cell cultivation, cell viability, and adhesion
L929 and HaCaT cells were used to investigate cell behavior on the pure SF, PHB, and PHB/SF-0 nanofibrous scaffolds. L929 and HaCaT cells were cultured in DMEM containing 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin in a CO₂ incubator (37 °C, 5% CO₂). The cell medium was replaced every 2 days. When the cells reached 80% confluence, they were trypsinized and counted using a hemocytometer and seeded in a 96-well culture plate for indirect cytotoxicity tests.

In vitro cytotoxicity of the prepared nanofibrous scaffolds was observed using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) assay, which is based on the reduction of the tetrazolium salt to formazan crystals in metabolically active cells. Samples were incubated overnight in the culture medium (reacted medium) in order to investigate the biomaterial cytotoxicity of the nanofibrous blend scaffolds on HaCaT and L929 cell lines. The cells were seeded in a 96-well plate at a density...
of 7 × 10³ cells/well and incubated at 37 °C under 5% CO₂ in a humidified incubator overnight. After 1 day of cell culture, the culture medium was removed, and the reacted medium was pipetted in the 96-well plate and incubated at 37 °C in 5% CO₂ for another 1 day. Then 200 µL of MTT reagent was added to each well and wells were incubated in the dark for 4 h. The solution was removed at the end of 4 h and 200 µL of acidic isopropyl alcohol was added to the 96-well plate for the dissolving of formazan crystals. The optical density of the solution in the 96-well plate was measured using a microplate reader at 570 nm according to the manufacturer's instructions to determine the number of metabolically active cells. The tests were repeated 3 times and 3 parallel repeats were carried out for each sample. L929 cell proliferation on pure and blended nanofibrous scaffolds were measured by MTT solution as described above.

To examine the interaction of the pure and blended nanofibrous scaffolds with HaCaT and L929, the cells were seeded on the scaffolds and incubated in a humidified atmosphere containing 5% CO₂ at 37 °C for 3 days. The adhered cells were then evaluated using a SEM. Subsequently, the scaffolds were fixed in a 4% paraformaldehyde solution (pH 7.4) for 15 min at 4 °C and washed with PBS. Afterwards the samples were dehydrated in gradational alcohol solutions (50%, 60%, 70%, 80%, 90%, 95%, 100%) and dried in an oven for 24 h. Fixed samples were sputter-coated with a gold/palladium mixture and the surface was observed by SEM.

2.6. Live/dead cell analysis
A live/dead viability/cytotoxicity kit, consisting of calcein AM and propidium iodide (PI), was used to qualitatively assess cell morphology. L929 and HaCaT cells were grown in DMEM (high glucose) supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C and 5% CO₂. The cells (5 × 10³ cells per well) were seeded on pure and blended scaffolds for 3 days. The cells were then stained by a mixture of calcein-AM and PI solution for 10 min. Fluorescence images were taken using the Texas Red and FITC filters of a fluorescence inverted microscope (Leica, Germany). The calcein-AM in live cells is converted to calcein by intracellular esterase and emits a strong green fluorescence. PI can penetrate damaged membranes of dead cells and binds to nucleic acids, producing a red fluorescence signal.

2.7. Statistical analysis
The results of the present study are presented as mean ± standard deviation (SD) and were statistically analyzed using a two-tailed Student t-test. P < 0.05 and P < 0.005 were considered statistically significant.

3. Results and discussion
3.1. Morphology and surface properties
SEM micrographs were obtained in order to determine how to get the best electrospun fibers and select the best parameters. The optimized electrospinning conditions used in the present study were tip-to-collector distance of 20 cm, applied voltage of 20 kV, and flow rate of 0.4 mL/h. Figures 1A and 1B show the morphologies of pure PHB and SF electrospun fiber mats and the average fiber diameters were 365 ± 7.6 and 440 ± 12 nm, respectively.

The fibers of PHB/SF-2 were formed with beads when the SF concentration was increased, as shown in Figure 2A. On the contrary, the SEM image of PHB/SF-1 shows that the electrospun fiber diameters are in the range of micrometers with a mixing ratio of 1:1 (Figure 2B). Bead formation occurs if the electrospinning solution is not fully stretched. The increased charge, i.e. conductivity, increases

![Figure 1. SEM images of pure SF (A) and PHB (B) nanofiber scaffolds.](image-url)
the stretching of the spinning solution. Many studies reported that a polar polymer has a higher conductivity than a nonpolar polymer. SF is a polar biopolymer that includes many polar groups such as hydroxyl and carboxyl groups. When PHB was blended with SF and electrospun, the conductivity of the solution increased, thus leading to smooth fiber formation (Zhong et al., 2002; Lei et al., 2015).

In addition, SEMs of PHB/SF-3 revealed a matrix of fibers with a mean diameter of 252 ± 5.4 nm with a decrease in SF and PHB concentrations (Figure 2C; Table 2). Similarly, the electrospun PHB/SF-0 fibers showed a significantly smaller mean diameter of 92 ± 2.6 nm at a polymer concentration of PHB/SF of 7%:18%, w/w (Figure 2D; Table 2). Fiber diameters reduced with the decreasing of PHB content. It is well known that the fiber diameter increases with the increased viscosity at a rate dependent on the electric field present. Increased diameters of PHB/SF electrospun fibers as a result of increased viscosity of PHB concentration could be explained as reported previously (Sukigara et al., 2003; Ko et al., 2013). Furthermore, it was reported that smaller diameter fibers were obtained depending on the increased conductivity of the electrospinning solution when PHA was blended with SF and electrospun (Lei et al., 2015).

**Figure 2.** SEM images of PHB/SF-2 (A), PHB/SF-1 (B), PHB/SF-3 (C), and PHB/SF-0 (D). Insets show the histograms of fiber diameters.

**Table 2.** The average fiber diameter of the PHB/SF nanofibrous scaffolds.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Diameter (mean ± SD, nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure PHB</td>
<td>365 ± 7.6</td>
</tr>
<tr>
<td>Pure SF</td>
<td>440 ± 12</td>
</tr>
<tr>
<td>PHB/SF-0</td>
<td>92 ± 2.6</td>
</tr>
<tr>
<td>PHB/SF-1</td>
<td>1008 ± 21.4</td>
</tr>
<tr>
<td>PHB/SF-2</td>
<td>Bead</td>
</tr>
<tr>
<td>PHB/SF-3</td>
<td>252 ± 5.4</td>
</tr>
</tbody>
</table>
As can be seen from the SEM results, the smallest fiber diameter and best surface morphology were obtained for PHB/SF-0. Therefore, the PHB/SF-0 nanofibrous scaffold was considered to evaluate the crystallinity, wettability, and cell–biomaterial interactions in the following cell–culture studies.

Figure 2 reveals that blending PHB with SF changed the diameter distribution. While the bare SF scaffold exhibited a comparatively wide distribution of diameters, ranging from 250 to 950 nm, the electrospun PHB/SF fibers (PHB/SF-0 and PHB/SF-3) showed a narrow distribution range from 40 to 100 nm and 200 to 500 nm, respectively.

Blended solutions of PHB and SF were electrospun into nanofibrous scaffolds and the scaffolds were analyzed using FT-IR to confirm the presence of both PHB and SF in the fibers. This was performed in conjunction with an analysis of changes in chemical structure. The FT-IR spectrum given in Figure 3 shows peaks assigned to both the PHB and SF components. Figure 3A shows the prominent characteristic peaks of PHB at 1717, 1453, and 1370 cm⁻¹. The peak positions correspond to the stretching of the C-O bands and the asymmetrical deformation of the C-H bond in CH₂ groups and CH₃ groups (Karahaliloglu et al., 2013; Daranarong et al., 2014). In addition, the band observed at about 1273 cm⁻¹ can be attributed to the crystalline phase of PHB. In the case of SF, the absorption peaks observed at 1643 cm⁻¹, 1522 cm⁻¹, and 1186 cm⁻¹ can be assigned to amide I (C=O stretching), II (N-H bending), and III (C-N stretching) (Ha et al., 2005; Inpanya et al., 2012; Zhang et al., 2012). The peaks are characteristic for random coils and α-helix structures. After blending of the PHB with SF, the obtained FT-IR spectrum revealed the presence of three amides characteristic for the SF, suggesting that blending with PHB has no significant effect on the change of the secondary structure of SF. Furthermore, as can be seen in Figure 3B, the carbonyl band observed at about 1717 cm⁻¹ in pure PHB shifted to about 1726 cm⁻¹ in the case of electrospun PHB/SF and the vibration intensity apparently decreased with the addition of SF (Yang et al., 2011; Paşcu et al., 2013).

Table 3 shows the chemical composition of the electrospun scaffolds containing different concentrations of PHB and SF. N elemental percentages of these PHB/SF electrospun scaffolds were increased with increasing concentrations of SF. This phenomenon supports PHB with SF having good integration, as mentioned in previous reports (Bhattacharjee et al., 2015).

**Table 3. The chemical compositions of the electrospun scaffolds by EDX spectrometer.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Elemental (%)</th>
</tr>
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<tbody>
<tr>
<td>PHB/SF-0</td>
<td>C 59</td>
</tr>
<tr>
<td>PHB/SF-1</td>
<td>C 49</td>
</tr>
<tr>
<td>PHB/SF-2</td>
<td>C 49</td>
</tr>
<tr>
<td>PHB/SF-3</td>
<td>C 59</td>
</tr>
</tbody>
</table>

Figure 3. ATR-FT-IR spectrum of pure SF and PHB (A) and of electrospun PHB/SF composite scaffolds (B).
Figure 4 shows the XRD patterns of the pure SF, PHB, and PHB/SF nanofibrous scaffolds. The pure PHB nanofibrous scaffold showed diffraction peaks at 2θ = 13.3°, 16.7°, 21.8°, 25.4°, and 26.9° (Ikejima et al., 2000). On the other hand, the main peak at about 20.67° has to be associated to the SF component, corresponding to the β-sheet structure (silk II) (Kundu et al., 2010). However, in the case of PHB/SF-0 with 50/50, one broad peak, relatively intense in the region between 20° and 30°, can be seen. However, SF seems to dominate over PHB as shown in the diffraction peaks of PHB/SF and the individual peaks of PHB and SF were not clearly separated. Furthermore, the degree of crystallinity is calculated by dividing the total area of the crystalline peaks by the total area under diffraction peaks and the crystallinity percentage for PHB, SF, and PHB/SF nanofibrous scaffolds is 30.6%, 2.62%, and 0%, respectively. These results strongly suggest that the SF component influences the crystalline structure of the composite nanofibrous scaffolds through molecular interactions; in other words, after adding SF, the crystallinity of the PHB/SF composite decreased compared with pure PHB, with clearly reinforced mechanical properties of these blends.

The hydrophilicity of pure SF, PHB, and PHB/SF nanocomposite scaffolds can be observed in Figure 5. The pure PHB nanofibrous scaffold was apparently more hydrophobic, with a mean water contact angle of 112 ± 1.2° (Lei et al., 2015). In the case of pure SF, the water droplet spread out immediately and penetrated into the nanofibrous scaffolds, and the water contact angle showed a sharp decrease (4.6 ± 0.5°), indicating that SF has improved hydrophilicity compared to pure PHB. However, the water contact angle of the PHB/SF nanofibrous scaffold decreased with the addition of SF (55 ± 1.8°). This result demonstrates that the SF considerably affects the surface wettability of the nanofibrous scaffold, making the structure more hydrophilic.

The mechanical properties of the pure SF, PHB, and PHB/SF nanocomposite scaffolds were measured by tensile strength testing. Table 4 shows important mechanical parameters including tensile strength and elongation at break. Tensile strength measured for pure SF and PHB was 0.43 ± 0.06 and 6.23 ± 0.3 MPa, respectively. However, the combination of SF with PHB increased the tensile strength to 3.81 ± 0.1 when compared with pure SF. The yield strength measured at the yield point was 6.18, 0.3, and 1.49 for pure PHB, SF, and PHB/SF, respectively. Pure PHB had an elongation at break of 11.74% compared to 3.48% for pure SF. Further interaction of SF with PHB led to an even more significant increase in elongation at break for the PHB/SF composite of 17.10%. These results suggested that the addition of PHB to SF was beneficial for enhancing the mechanical properties of PHB/SF electrospun scaffolds. Recently, most composite SF fibrous scaffolds have been fabricated as a result of the incorporation of other polymer or mineral components such as bioactive glass and hydroxyapatite, which improve scaffold mechanical
properties. Hence, a fibrous structure is significantly weak against physical loadings compared to porous scaffolds (Yang et al., 2011; Zhang et al., 2013; Kim et al., 2014). For example, Cai et al. (2010) reported that the addition of SF enhanced the mechanical properties of CS/SF blends and the tensile strength of the cross-linked nanofibrous membranes increased from 1.3 to 10.3 MPa with an increase in the elongation at break. The data obtained here confirm the results of these mentioned reports. Similarly, Wang et al. (2009) fabricated a bilayer tube scaffold by electrosprining that included a PLA layer (outside layer) and a SF–gelatin layer (inner layer), and the results showed that the tensile properties of PLA/SF–gelatin composite tubular scaffolds, which had a higher breaking strength of 1.12 ± 0.11 MPa, were significantly different from the SF–gelatin (alone) scaffolds and PLA (alone) scaffolds.

### 3.2 In vitro cell cultivation, cell viability, and adhesion

Behaviors such as adhesion, proliferation, and differentiation are some of the primary markers for the biocompatibility of materials. Figure 6 shows the

**Table 4. Mechanical properties of pure PHB, SF, and PHB/SF composite nanofibrous scaffolds.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tensile stress (MPa)</th>
<th>Elongation at break (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure PHB</td>
<td>6.23 ± 0.3</td>
<td>11.74</td>
</tr>
<tr>
<td>Pure SF</td>
<td>0.43 ± 0.06</td>
<td>3.48</td>
</tr>
<tr>
<td>PHB/SF-0</td>
<td>3.81 ± 0.1</td>
<td>17.10</td>
</tr>
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</table>

**Figure 5.** The water contact angle of the pure PHB (A), SF (B), and PHB/SF-0 (C) nanofibrous scaffolds. Values are mean ± SEM; n = 8.

**Figure 6.** The viability percentage of L929 and HaCaT cell lines treated with pure and blend nanofibrous scaffolds (A) and L929 cell proliferation on pure PHB, SF, and PHB/SF-0 nanofibrous scaffolds (B). Values are mean ± SEM; n = 3; *P < 0.05, **P < 0.005 compared to the pure PHB nanofibrous scaffold.
compatibility of pure PHB, SF, and PHB/SF-0 composite nanofibrous scaffolds. Figure 6A shows the viability percentage of L929 and HaCaT on the pure and composite nanofibrous scaffolds. A significant difference in the viability percentage of cells was not observed between the PHB/SF-0 nanofibrous scaffold and the pure PHB nanofibrous scaffold. However, a cell proliferation study with L929 was performed at different time points over a period of days and, as can be seen from Figure 6B, mouse fibroblasts continued to proliferate with the increase of culture time on the PHB/SF-0 nanofibrous scaffolds. The OD values for PHB/SF-0 and pure SF nanofibrous scaffolds were significantly higher than those of the pure PHB nanofibrous scaffold at the end of 7 days (*P < 0.05, **P < 0.005).

The adhesion behavior of L929 and HaCaT cells on pure SF, PHB, and PHB/SF-0 nanofibers are shown in Figure 7. After 7 days of culture, fibroblasts and keratinocytes formed a continuous monolayer with elongated shape on the surface of nanofibrous scaffolds. Especially on PHB/SF-0 composite nanofibrous scaffolds, the cells spread with physical contacts and showed filopodial extensions with each other. The PHB/SF nanofibrous scaffold showed excellent attachment behavior to L929 and HaCaT cells, which could be attributed to the increased hydrophilicity. The combination of PHB and SF enhanced the adhesion behavior of L929 and HaCaT cells as expected.

3.3. Live/dead cell analysis
Figure 8 represents the results of the live/dead assay. Figures 8A and 8B show the HaCaT cells in contact with the pure PHB nanofibrous scaffold for 3 days. The cells show normal morphology; however, the cells on the pure SF (Figures 8C and 8D) and especially PHB/SF-0 (Figures 8E and 8F) nanofibrous scaffolds create a confluent layer. Furthermore, there are no dead cells on the nanofibrous scaffolds cultured with the HaCaT cell line. These results show that the combination of PHB and SF positively influences the cell proliferation and morphology in line with the previously presented MTT results.

3.4. Conclusions
Nanofibrous blend scaffolds made of PHB and SF were successfully prepared by electrospinning. As the SF blending in PHB decreased, the average fiber diameters in the electrospun scaffolds decreased significantly and SEM images showed that PHB/SF-0 blend nanofibrous scaffolds had well-interconnected porous structures. Similarly, the presence of SF affected the crystallization of PHB in the blend and mechanical and wettability properties were enhanced with SF loading. Furthermore, SF promoted the adhesion and growth of L929 and HaCaT cell lines compared to pure PHB and SF. Thus, a combination of PHB with SF supported the cell attachment and proliferation besides morphological and superficial changes. This study could be a guide for

Figure 7. The adhesion behavior for L929 and HaCaT cells after 90 min of culture on pure PHB (A, B), SF (C, D), and PHB/SF-0 (E, F) nanofibrous scaffolds, respectively.
further in vivo studies and the electrospun PHB/SF-0 blend scaffold can be used for wound dressing or tissue engineering scaffolds.

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References


