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Poly(hydroxybutyrate-*co*-hydroxyvalerate) and bovine serum albumin blend prepared by electrospinning

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E. P., D. B., K. S., and D. K. developed the concept and designed the experiments. T. M., V. M., and G. B. obtained PHBV by bacterial synthesis and purified it. E. P. carried out the film preparation, their imaging by SEM, and the film dissolution experiments. D. K. carried out the elemental analysis. M. K. carried out the DSC measurements; A. B., D. S., and I. Z. carried out the experiments with the Vero cells. D. B. carried out the contact-angle measurements. Drafting of the manuscript was done by E. P., D. B., A. B., and K. S.

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ABSTRACT: Electrospinning is a method for the preparation of nanosized polymer fibers. Here, electrospinning is used to prepare a blend of a polyester, poly(hydroxybutyrate-*co*-hydroxyvalerate) (PHBV), and a globular protein, bovine serum albumin (BSA). The electrospun blend film is compared with a solution-cast blend film and with single-component electrospun films made of PHBV and BSA. In the electrospun blend films, BSA manifests itself as flat ribbons and a fine network formed from fibers less than 50 nm in diameter. The dissolution rate of BSA from the electrospun blended film is lower than from the solution-cast one. The films are characterized using scanning electron microscopy, differential scanning calorimetry, and contact-angle measurements. The obtained PHBV+BSA blend films have several emergent properties: a slow BSA dissolution rate, a fine BSA network, and unusual thermal behavior. Thus, the PHBV+BSA blend films introduce a new class of polymer–protein blends. © 2017 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2017**, *134*, 45090.

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INTRODUCTION

Polymers are poorly miscible; thus, polymer blends are susceptible to phase separation. This is usually due to the positive heat of mixing and small entropy of mixing.[1,2] There are only a few pairs of miscible polymers, such as poly(styrene) with poly(-methyl methacrylate) or poly(dimethylsiloxane). Despite their poor miscibility, polymer blends are often prepared as metastable materials with slow relaxation.

In this study, we used electrospinning to blend two immiscible polymers: the biodegradable and biocompatible copolymer poly(hydroxybutyrate-*co*-hydroxyvalerate) (PHBV) and the well-known protein bovine serum albumin (BSA). Electrospinning is a method to produce polymer fibers with diameters ranging from tens of nanometers to several micrometers via an electric field.[3,4] During the past decade, electrospinning has emerged as an irreplaceable polymer processing technique for both scientific laboratories and industry. Since an electrospun structure is fibrous and highly porous, it can be used to create filters, semipermeable membranes, wound dressing materials, tissue engineering scaffolds, and other polymer products. The advantages of electrospinning are its high flexibility (it can be used with virtually any polymer, it can process either solutions or melts, and so on) and the relative simplicity of the equipment (it is

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easily home-built). Electrospinning can produce nanosized fibers, which is almost impossible to achieve by three-dimensional printing or traditional extrusion. This is especially important when electrospinning is used to fabricate cell substrates, since the nanostructure promotes cellular adhesion.[3,4] However, for the current work, the most important advantage of electrospinning was the ability to process polymer mixtures.

The polymers used in this study were PHBV and BSA. PHBV is a hydrophobic polyester; the properties of PHBV blends and composites have been studied extensively.[5] It has relatively few industrial applications compared to other biodegradable polymers [such as collagen and poly(lactic-*co*-glycolic acid)]. One of the reasons for this is its high hydrophobicity. The addition of BSA could help to overcome this drawback. BSA (molecular weight ~66 kDa) is a globular protein that is the main component of bovine serum. Protein-based composites are usually made of structural fibrillar proteins, such as silk proteins, elastins, and collagen.[6] We used the albumin for two reasons. First, BSA is the most widely used model protein. Second, BSA is relatively cheap, which is crucial for the industrial application of novel materials.

PHBV and BSA were mixed in a common solvent: hexafluoroisopropanol (HFIP). We compared the properties of the electrospun films and the solution-cast ones. The blends, as well as the single-component films used for reference, were characterized using scanning electron microscopy (SEM), measurements of BSA dissolution kinetics, differential scanning calorimetry (DSC), contact-angle measurements, and *in vitro* cytocompatibility.

EXPERIMENTAL

Materials

PHBV, specifically poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate with an average molecular weight of 200 kDa, was synthesized as described below. BSA was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The 1,1,1,3,3,3-hexafluoroisopropanol was obtained from P&M-Invest (Moscow, Russia).

PHBV Synthesis and Characterization

The PHBV was synthesized from the *Azotobacter chroococcum* strain 7B by the precursor feeding approach as previously described.[7,8] Briefly, the culture was grown in shaker flasks at 30 °C in Burk's medium with 50 m*M* sucrose as primary carbon source supplemented with 20 m*M* sodium valerate (to produce the PHBV copolymer) and 50 m*M* sodium acetate (to regulate the PHBV molecular weight). The polymer was isolated and purified, the molecular weight was determined by viscosimetry, and the 3-hydroxyvalerate molar content in the PHBV copolymer was determined by ^{1}H-NMR.[7,8]

Preparation of Electrospun Films

The PHBV and BSA were separately dissolved in HFIP to prepare 6 wt % solutions. A blend solution of PHBV+BSA was made by mixing a 6 wt % solution of PHBV and a 6 wt % solution of BSA at a ratio of 70:30 with violent stirring. These solutions (PHBV and PHBV+BSA) were electrospun on the counterelectrode (collector). The solutions were delivered at 1 mL/h by a syringe pump. The accelerating voltage was 30 kV between the needle with an inner diameter of 0.7 mm and the counterelectrode. The distance between the needle and the collector was 15 cm. The film was detached from the collector and dried under vacuum for 20 h to remove the residual solvent.

Besides the blend films, control samples of electrospun PHBV and electrospun BSA were obtained under the same experimental conditions. The electrospun BSA film was relatively delicate, and it was difficult to detach it from the collector. However, we obtained enough electrospun BSA to examine it with SEM and DSC.

Preparation of Solution-Cast Films

Solution-cast films were prepared from the same solutions that were used for the electrospinning experiments. A 6% solution of PHBV or a PHBV and BSA 70:30 mixture was poured on a glass Petri dish and left covered overnight in a fume hood. Afterwards the films were dried under vacuum for 20 h to remove the residual solvent.

Film Characterization by SEM

The fiber films without cover were examined with a Zeiss Merlin microscope equipped with Gemini II Electron Optics (Zeiss, Oberkochen, Germany). The measurements were carried out at low accelerating voltage (1–3 kV) and low probe current (10–35 pA), so the conductive sample coating was not needed for the SEM measurements. The average fiber diameter was calculated using plugin DiameterJ[9] in the ImageJ software.[10]

Energy-dispersive X-ray spectroscopy (EDX) microanalysis was performed by SEM using the Silicon Drift Detector (SDD) X-Max^{N} 150 (Oxford Instruments, Abingdon, United Kingdom) and AZtecEnergy EDS Software (version 3.0).

Film Dissolution

The leakage of BSA from the films was examined by mass measurements. Pieces of the films were incubated in water for certain time periods, and afterwards their mass was measured. The relative mass loss was calculated for each piece of film. For each time point, the mass loss was averaged over three independent film pieces.

Differential Scanning Calorimetry

The thermal properties of the solution-cast and electrospun PHBV+BSA films were investigated by differential scanning calorimetry. Samples with a mass of 10–15 mg were placed in aluminum pans, and DSC measurements were performed at a constant rate of 10 °C/min in the inert atmosphere (argon) within the temperature range from 25 to 170 °C. Measurements were carried out on a Netzsch DSC 204 F1 Phoenix instrument (Selb, Germany). The measurements were repeated in triplicate and averaged for each film.

Contact-Angle Measurements

The hydrophilicity of the electrospun films was evaluated by measuring the contact angle between water drops and the film surface using a Drop Shape Analyzer DSA 25E (Kruss, Hamburg, Germany) at room temperature (22–25 °C). Water drops (1.5 µl) were placed on the film surface with a microsyringe. The contact-angle measurements were carried out each second over 3 min to capture the time-dependent behavior of the drop.

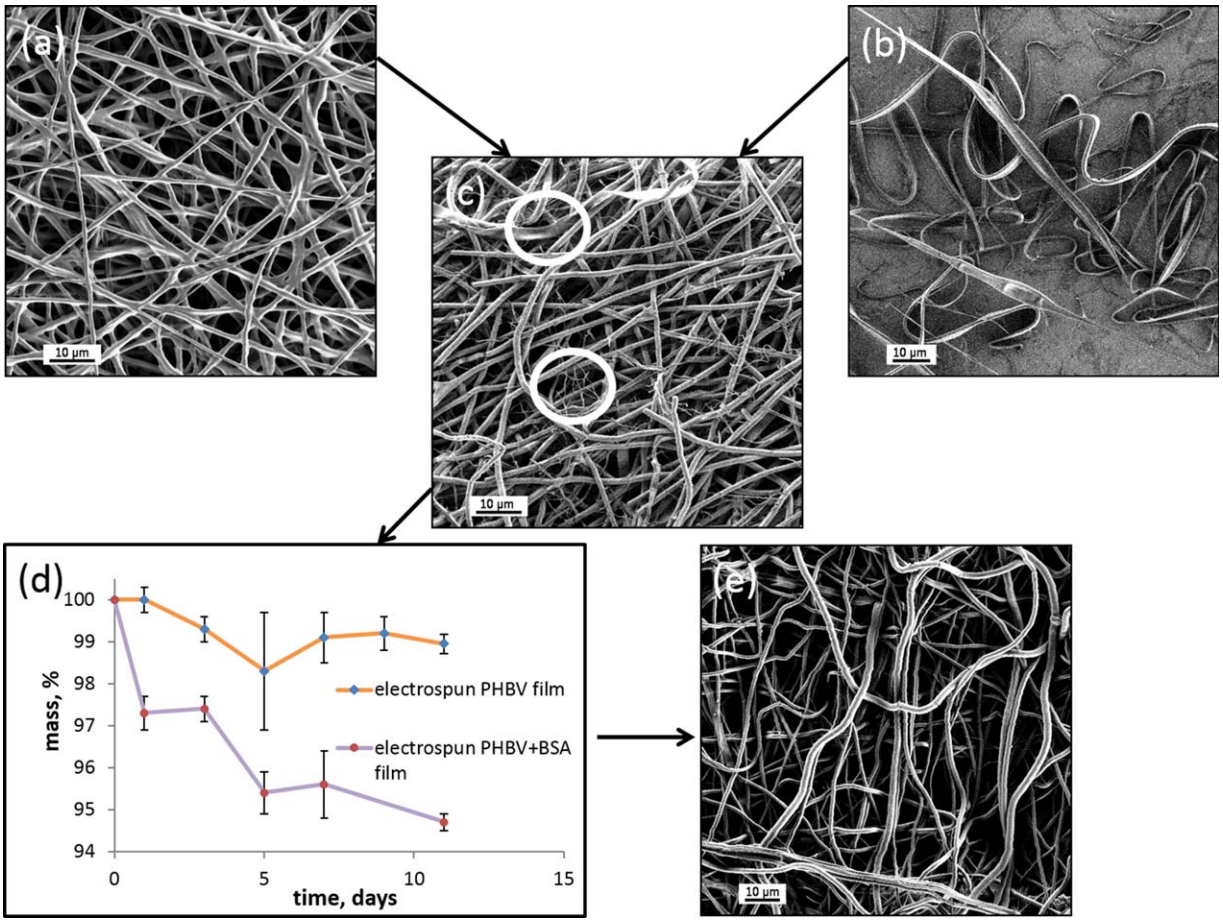

Figure 1. SEM images of the electrospun films: (a) PHBV, (b) BSA, (c) PHBV+BSA blend, (e) PHBV+BSA blend after 7 days of water treatment; (d) mass measurements of the electrospun PHBV and PHBV+BSA films upon water treatment. [Color figure can be viewed at wileyonlinelibrary.com]

Cytocompatibility

To estimate the cytocompatibility of the produced films, we used African green monkey kidney (Vero) cells. They were seeded onto 1 cm^2 films at 200,000 cells per sample concentration in 35 × 10 mm Petri dishes. The cells were cultured in Dulbecco's Modified Eagle medium supplemented with 10% fetal bovine serum and 50 U/mL penicillin and 50 μg/mL streptomycin at 37 °C and 5% CO_2.

A cell viability assay was performed on the films on the fifth day of culture by a Live/Dead Viability/Cytotoxicity Kit (Molecular Probes Thermo Fisher Scientific, Waltham, Massachusetts, USA). The kit consists of calcein AM (the stain for the live cells, used at 2 μ*M*) and ethidium homodimer (the stain for the dead cells, used at 4 μ*M*). After staining, the films were washed with excess PBS (Biolot, St. Petersburg, Russia) and transferred to the glass slides. We used a laser scanning confocal microscope Zeiss LSM 710 (Zeiss, Oberkochen, Germany). A 488 nm laser was used for fluorescence excitation, and the emission was recorded at 493–562 nm (green channel for calcein AM fluorescence) and 646–741 nm (red channel for ethidium homodimer). Images were acquired with an EC Plan-Neofluar 10×/0.3 objective. At least five images were captured for each sample. The films had uneven surfaces, so it was difficult to quantify the number of cells. Only some of the stained cells stayed in the focal plane, while the others produced a background glow. Thus, only a qualitative assessment of cell viability was possible.

RESULTS

Electrospun Film Morphology and BSA Dissolution

The film morphology was examined by SEM. Figure 1(a,b) shows images of the three electrospun films compared in this study: PHBV, BSA, and the PHBV+BSA blend. The PHBV film was composed of nanofibers with a roughly round cross section, which is common for electrospinning. The mean diameter was 500 nm. The fibrous BSA film contained many flat ribbons rather than round nanofibers. Similar results are reported in Nseir *et al.*[11] and Fleischer *et al.*[12] At a glance, the fibrous PHBV+BSA blend film looked similar to the fibrous film made of PHBV. However, it incorporated flat ribbons (as in electrospun BSA) and a fine network of nanofibers with diameters less than 50 nm. Some nanofibers looked broken, as if the polymer jet had stopped during their formation.

Many applications of electrospun films imply contact with water. PHBV is insoluble in water, while BSA is highly soluble in water, so the key characteristic of the electrospun PHBV+BSA films is the rate of BSA dissolution. Figure 1(d) shows the decrease in film mass over time. The electrospun PHBV films lost less than 3% of their mass during 11 days.

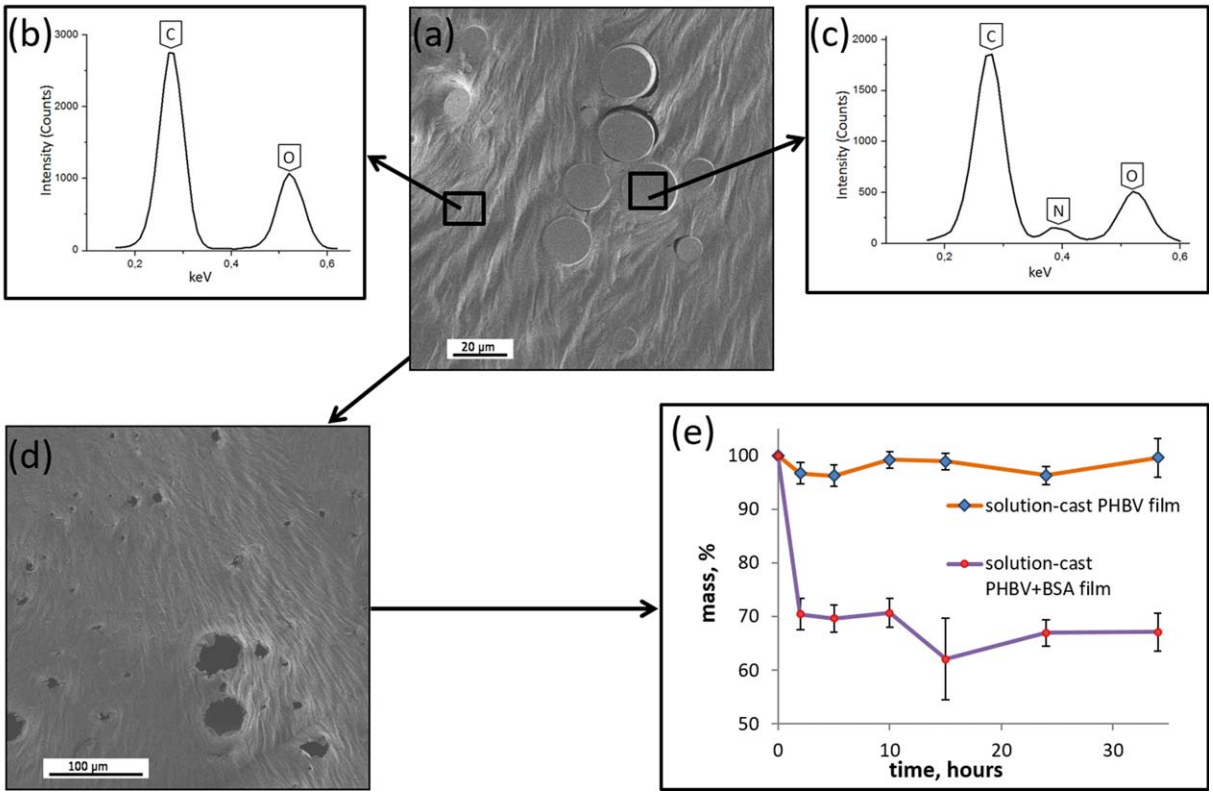

Figure 2. (a) SEM image of the solution-cast PHBV+BSA film; (b) and (c) EDX spectra obtained from the marked areas in (a); (d) SEM image of the solution-cast PHBV+BSA film after 2 h of water treatment; (e) mass measurements of the solution-cast PHBV and PHBV+BSA films upon water treatment. [Color figure can be viewed at wileyonlinelibrary.com]

PHBV is a slow-degrading water-insoluble polymer, so the observed mass loss could be attributed to the low-molecular-weight oligomers that are present in the PHBV sample.[13]

The PHBV+BSA blend film lost ~6% of its mass over 11 days, so the degradation occurs approximately two times faster than in the case of electrospun PHBV. Up to 3% of the lost mass could be accounted for by PHBV oligomers, so the mass of dissolved BSA was in the 3–6% range. The initial mass ratio of PHBV and BSA was 70:30. Thus, over the observation period the fraction of BSA dissolved from the electrospun PHBV+BSA films did not exceed 20% of the initial BSA mass in the film.

The PHBV+BSA film was examined with SEM after 7 days of water treatment [Figure 1(e)]. The flat ribbons and the fine network of nanofibers with diameter less than 50 nm disappeared from the film surface. These structures were more soluble than the round nanofibers, so we interpreted them as the BSA-enriched ones. The BSA dissolution caused a significant increase in pore area (Figure S1).

Solution-Cast Film Morphology and Dissolution

The solution-cast films had different surfaces: a smooth one, which formed in contact with the glass substrate, and a rough surface, which formed in contact with air. This feature is typical for films prepared by solution casting.[14] Only the bottom (smooth) surface was examined during our experiments.

The solution-cast PHBV+BSA film manifested a clear phase separation, as shown in Figure 2(a). The round regions with 5–35 µm diameter were interpreted as the BSA phase. We used elemental analysis to ensure that these regions were enriched with protein. The round regions contained nitrogen, which is found in BSA and is not found in PHBV [Figure 2(b,c)]. After ~2 h of incubation in water, these regions were transformed into holes because the BSA dissolved in the water. The dissolution was confirmed by SEM imaging [Figure 2(d)] and mass measurements [Figure 2(e)].

The elemental analysis measurements did not show any detectable amount of fluorine. The typical detection limit of EDX is 0.1 wt %. We used HFIP as the solvent, so the absence of fluorine proves that HFIP was reasonably removed from the films. We did not carry out EDX experiments on the electrospun films because their complex topography hindered data processing and interpretation.

Contact-Angle Measurements

PHBV is a hydrophobic polymer. At the moment of drop placement, the electrospun and solution-cast PHBV had high contact angles of about 120° and 75°, respectively (Figure 3). Similarly, PHB,[15] PHB+PHBV blend,[16] and poly(lactic acid) (PLA)[17] electrospun films had a greater contact angle than the solution-cast ones. The contact angle of PHBV films decreased gradually by 5–10° during the 3 min of observation. The electrospun

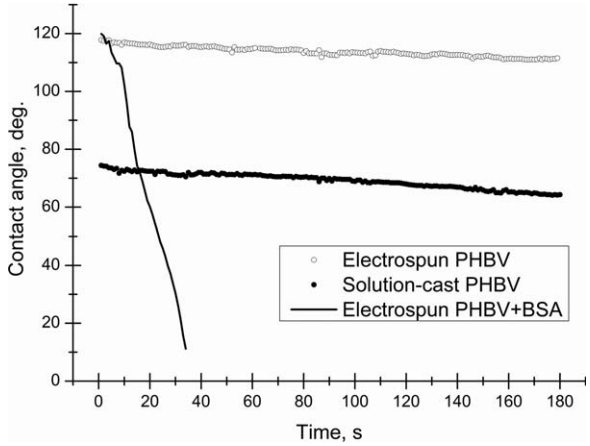

Figure 3. Contact-angle measurements of solution-cast PHBV film, electrospun PHBV film, and electrospun PHBV+BSA blend film.

PHBV+BSA blend films manifested a different behavior. They had a high contact angle (up to 130°) at the moment of drop placement, but it decreased rapidly as the drop spread on the surface and penetrated into the film over 25–120 s. Thus, the addition of BSA drastically changed the hydrophilicity of the electrospun films.

DSC Measurements

Differential scanning calorimetry measurements were performed for the solution-cast and electrospun PHBV+BSA films to investigate their thermal behavior, such as melting and crystallization (Figure 4). For each sample, three scans were recorded: a first heating, a cooling, and a second heating. The first heating was used to observe the melting behavior of the original crystalline entity of each sample; during cooling, the ability of the sample to crystallize at a constant cooling rate was studied; the second heating was performed to examine the melting behavior of the crystalline entity of the sample formed during the cooling scan. Experimental values for all of the DSC analyses are summarized in Table I.

The solution-cast PHBV+BSA film had a crystallization peak temperature (T_c) of ~96 °C, which shifted to a higher temperature (113 °C) for the electrospun film. The melting point corresponding to the melting of PHBV was calculated by the second heating data. It was also slightly higher for the electrospun film (147 °C) compared with the solution-cast one (133 °C). A

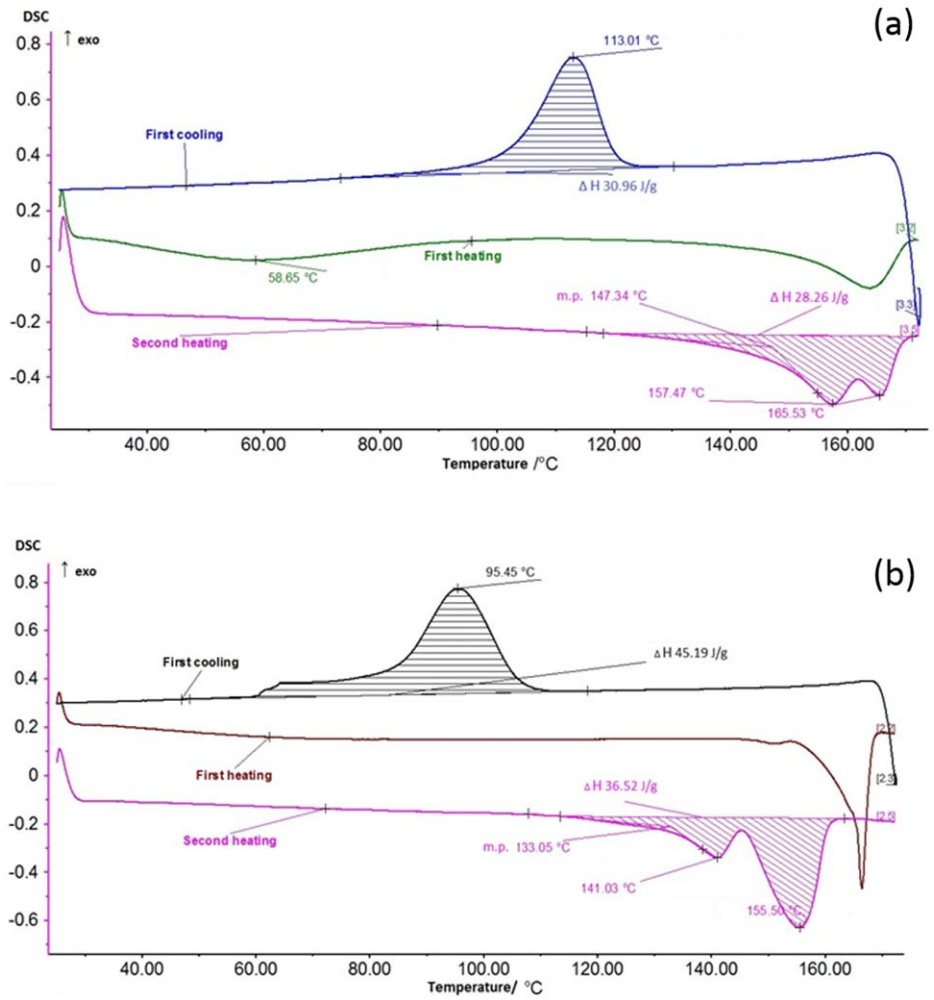

Figure 4. DSC measurements of the PHBV+BSA films: (a) electrospun film, (b) solution-cast film. [Color figure can be viewed at wileyonlinelibrary.com]

Table I. Thermal Characteristics of the PHBV+BSA Films

Film	T_m (°C)	ΔH_f (J/g)	T_c (°C)	ΔH_c (J/g)
Solution-cast PHBV+BSA film	133	37	96	45
Electrospun PHBV+BSA film	147	28	113	31

T_m, melting point (obtained from second heating run); ΔH_f, enthalpy of fusion (obtained from second heating run); T_c, crystallization peak temperature (obtained from first cooling); ΔH_c, enthalpy of crystallization (obtained from first cooling).

bimodal endothermic melting peak for PHBV was observed, as described previously for various polyesters.[18–20] There are several explanations for this phenomenon, the most recent one claiming that the bimodal peak appears to be due to the concurrent phenomena of melting and recrystallization, followed by final melting.[19] Both samples exhibited a low melting endotherm in the range of 141–158 °C.

An unusual endothermic peak (59 °C) was observed during the first heating of the electrospun PHBV+BSA film [Figure 4(a)]. This could be attributed to the relaxation of the stress that was present after electrospinning. This peak was not observed in the solution-cast PHBV+BSA film [Figure 4(b)]. Similarly, in one-component electrospun films made of either PHBV or BSA, this peak was not observed (Figure S2).

Cytocompatibility

The cytocompatibility of the electrospun PHBV+BSA blend film and the solution-cast and electrospun PHBV films was evaluated by growing Vero cells on them. The viability of the attached cells was evaluated after 5 days of culturing. Calcein labeled the viable cells green, and EthH labeled the dead cells red. Figure 5 shows the obtained images.

The cell viability on the electrospun films was higher than on the solution-cast one. This is evident from the increased number of dead cells on the solution-cast film. Furthermore, on the electrospun films (PHBV and PHBV+BSA), the cells had the characteristic, elongated fibroblast-like shape that indicated good cell viability. Significant differences in cell viability between PHBV and PHBA+BSA electrospun films were not detected. In the current case, the film morphology (bulk or fibrous) was more important than the film material.

DISCUSSION

There are only a few papers on electrospinning of globular proteins.[11,12,21,22] We think that the potential applications of electrospun protein films and protein-containing composites are underestimated. There are several examples of the use of electrospinning to produce polymer blends (often called composites) made of hydrophobic and hydrophilic components (Table II). These blends cover a wide range of applications, from biomaterials to selective filters. The novelty of the current study is that the electrospun PHBV+BSA films have been described for the first time. Some of the observed properties (simultaneous formation of flat ribbons and the nanonetwork, slow BSA dissolution) are absolutely new and, to our knowledge, have not been observed previously in electrospun polymer–polymer or polymer–protein blends.

The PHBV+BSA blend was made of two immiscible polymers. Phase separation was observed in both solution-cast and electrospun films, although it was less evident in the latter. We suppose that phase separation occurred before film formation, at the solution stage, when the PHBV and BSA solutions in HFIP were mixed. Although both solutions were transparent, their 70:30 mixture was turbid, indicating phase separation (Figure S3). The phases were dispersed by stirring and kinetically trapped due to the high viscosity.

If the film was produced by solution-casting, the polymers had enough time to form the macroscopic, BSA-enriched spherical regions with diameters of 5–35 μm [Figure 2(a)]. During electrospinning, the solvent was removed relatively quickly due to the high surface-to-volume ratio of the nanofibers. We proposed a qualitative description for the electrospinning process. When the PHBV+BSA emulsion was subjected to electrospinning, the randomly distributed PHBV-enriched and BSA-enriched volumes were randomly captured by the syringe and the jet. When the jet was converted to a plume, the descending jets were enriched either by PHBV or by BSA. They formed correspondingly the "normal" fibers and either the nanonetwork or the ribbons [Figure 1(c)]. When the PHBV+BSA solution was

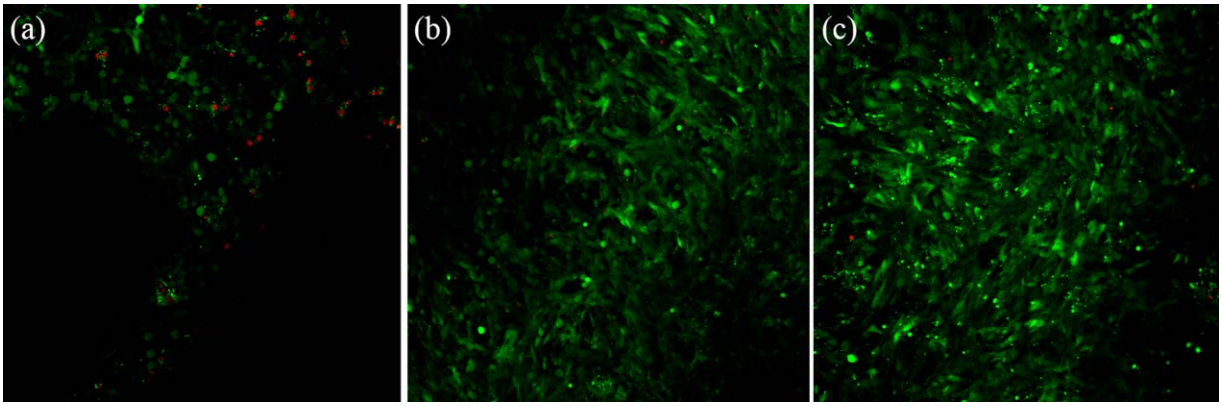

Figure 5. Images of Vero cells on the three films: (a) solution-cast PHBV film, (b) electrospun PHBV film, and (c) electrospun PHBV+BSA blend film. [Color figure can be viewed at wileyonlinelibrary.com]

Table II. Data on Electrospinning of Binary Polymer Mixtures of Hydrophobic and Hydrophilic Components

Hydrophobic component	Hydrophilic component	Solvent	References
Nylon 6	Methoxy poly(ethylene glycol) oligomer	Formic acid and acetic acid (4:1)	25
Nylon 6	Poly(acrylic acid)	Formic acid and acetic acid (4:1)	26
PLGA	Chitosan	Trifluoroacetic acid	34
PLGA	Dextran	Dimethylformamide and dimethyl sulfoxide (1:1)	38
Poly(ε-caprolactone)	Collagen (types I and III)	1,1,1,3,3,3-Hexafluoroisopropanol	39
Poly(ε-caprolactone)	Gelatin	2,2,2-Trifluoroethanol	40,41
Poly(ε-caprolactone)	Gelatin	1,1,1,3,3,3-Hexafluoroisopropanol	42,43
PLA	Gelatin	2,2,2-Trifluoroethanol	44
PLA	PEG	Dichloromethane	45
PHBV	Gelatin, collagen	2,2,2-Trifluoroethanol	46

stored over a month, the BSA-enriched phases grew larger in solution via the diffusion process. After electrospinning and imaging, we observed long flat ribbons accumulated in some areas of the film (Figure S4). Presumably, these areas were formed by the electrospinning of the BSA-enriched portions of the solution.

The measurements of dissolution kinetics showed that BSA solubility decreased when it was incorporated into a PHBV+BSA blend. The electrospinning process was insufficient to reduce BSA solubility. The single-component electrospun BSA films were soluble in water; the dissolution time was on the order of several seconds. They could be made insoluble if β-mercaptoethanol was added to the spinning solutions to disrupt the intramolecular S—S bonds.[12,22] In our case, the slow dissolution kinetics of the electrospun PHBV+BSA blend may be a consequence of BSA entrapment inside the film.

The presence of BSA in the electrospun PHBV+BSA blend gave rise to two different structures: the flat ribbons and the nanonetwork with fiber diameters less than 50 nm. We could not exclude that some of the ordinary fibrils were composed of BSA. The ribbons could be formed if the solvent was evaporated rapidly from the jet, resulting in a thin polymer skin.[23] After the skin formation, the atmospheric pressure collapsed the tube formed by the skin as the solvent evaporated. The fine nanonetwork, sometimes called a spiderweb, was observed in several electrospun compositions, including single-component electrospun films[24] and binary mixtures.[25,26] The mechanism of nanonetwork formation is currently unclear. We did not observe the nanonetwork in single-component electrospun BSA, so in our experiments the interaction between PHBV and BSA was necessary for its formation.

The water contact angle on the electrospun PHBV+BSA film was significantly dependent on time. At first, the film appeared hydrophobic, which can be explained by the BSA nanonetwork (the lower fiber diameter causes the higher contact angle[27] and the partial BSA denaturation (exposure of the hydrophobic amino acid residues to the fiber surface). There were at least two denaturation factors: HFIP as the solvent and the electrospinning process. HFIP disrupts the native protein structure and induces a transition to a highly helical state.[28,29] Electrospinning stretches the polymer chains by a strong electric field.[30–32] For collagen, the denaturating effect of HFIP is more important than the effect of electrospinning.[33] However, after a certain time period, the drop spread over the blend film surface, indicating a relatively high hydrophilicity. Usually the addition of a hydrophilic component to a hydrophobic water-insoluble polymer causes a decrease in the contact angle. This was observed, for example, for the electrospun composite made of nylon 6 and methoxy poly(ethylene glycol) oligomer,[25] the electrospun blend poly(lactic-co-glycolic acid), PLGA+chitosan,[34] and the solution-cast PHB films with the addition of poly(ethylene glycol) (PEG).[35]

DSC showed that PHBV+BSA blend films exhibited an endothermic peak at 59 °C that was not observed in the solution-cast PHBV+BSA film and single-component films made of PHBV or BSA (either solution-cast or electrospun). A similar peak was observed in a single-component electrospun PLA film.[36] This was attributed to the relaxation of the polymer chains in a mesophase consisting of oriented and extended polymer chains.

CONCLUSIONS

Electrospinning rapidly converts a polymer solution to nanofibers. We have prepared electrospun films made of PHBV and BSA and analyzed their properties: morphology, dissolution kinetics, hydrophilicity, thermal properties, and cytocompatibility. The electrospun PHBV+BSA blend films were compared with control ones: a PHBV+BSA film prepared by solution-casting and single-component electrospun PHBV and BSA films.

The electrospun PHBV+BSA films had several emergent properties that were not observed for either single-component films or the solution-cast film. These emergent properties were the nanonetwork (a feature of morphology), the capability of slow BSA dissolution, and the endothermic DSC peak.

When a protein is mixed with a polyester in solution, phase separation occurs. Upon electrospinning, it gives rise to a complex fibrous structure. On one hand, such a blend degrades faster than the bare polyester; on the other hand, it is capable of slow protein dissolution. These effects can be used for the optimization of film degradation kinetics (scaffold degradation kinetics is extremely important for medical applications, particularly for derivatives of poly(hydroxybutyrate)[13]) and for

prolonged drug release, if the protein is the therapeutic agent. Electrospun fibers can be loaded with drugs for their prolonged release.[37] Loading small molecules into the fibers is straightforward, but loading proteins can be relatively difficult. Loading of the protein into a polyester-based film, as shown for BSA in the current study, is a possible decision. The protein conformation and activity should be further examined to facilitate the usage of such blends in therapeutic agents.

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