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Properties and Biocompatibility of Poly-3-Hydroxybutyrate-co-3-Hydroxyvalerate/ Poly-ε-Caprolactone Blends

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Blends consisting of the poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) copolymer and poly-ε-caprolactone (PCL) were used to prepare polymer films by casting solution technique. The structure and physical-mechanical, and biological properties of the films were examined as influenced by the ratios of the blend components. The microstructure of the films was determined by the dominant component: the films had porous surface if the percentage of PHBV was higher than that of PCL and were relatively smooth if the dominant component was PCL. The highest elongation at break (190-368 %) was exhibited by the films prepared from the blends containing 5-25 % PHBV; at 25 % PHBV, local maxima of tensile strength (16 MPa) and Young's modulus (529 MPa) were observed. Biocompatibility of the films prepared from the blends was studied in the culture of NIH 3T3 mouse fibroblast cells. Results of SEM and DAPI staining, determination of the number of attached cells, and MTT assay showed the absence of cytotoxic effects and high cell adhesion and proliferation, which in some cases were higher than on films prepared from pure PHBV and PCL. Thus, PHBV/PCL blends are suitable materials for constructing cell scaffolds for tissue engineering and other reconstructive technologies.

Keywords: poly-3-hydroxybutyrate-co-3-hydroxyvalerate copolymer, poly-ε-caprolactone, polymer blends, biopolymers, biocompatibility.

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Свойства и биосовместимость смесей поли-3-гидроксибутирата-3-гидроксивалерата с поли-ε-капролактоном

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Смеси, состоящие из сополимера поли-3-гидроксибутирата-3-гидроксивалерата (ПГБВ) и поли-ε-капролактона (ПКЛ), были использованы для получения полимерных пленок методом испарения из раствора. Исследованы структура, физико-механические и биологические свойства пленок в зависимости от соотношения компонентов смеси. Микроструктура пленок определялась преобладающим компонентом: пленки имели пористую поверхность, если содержание ПГБВ было выше, чем ПКЛ, и относительно гладкими при преобладании ПКЛ. Наибольшее значение удлинения на разрыв (190-368 %) отмечалось для пленок, полученных из смесей с содержанием 5-25 % ПГБВ; при 25 % ПГБВ наблюдались локальные максимумы предела прочности на разрыв (16 МПа) и модуля Юнга (529 МПа). Биосовместимость полученных из смесей пленок была изучена в культуре фибробластов мыши линии NIH 3T3. Результаты растровой электронной микроскопии, окрашивания DAPI, определения численности прикрепленных клеток и MTT-тест показали отсутствие цитотоксических эффектов и высокий уровень адгезии и пролиферации клеток, в ряде случаев превосходившие соответствующие значения для пленок, приготовленных из чистых ПГБВ и ПКЛ. Таким образом, смеси ПГБВ/ПКЛ являются материалами, подходящими для конструирования клеточных матриксов для тканевой инженерии и других реконструктивных технологий.

Ключевые слова: сополимер поли-3-гидроксибутират-3-гидроксивалерат, поли-ε-капролактон, смеси полимеров, биополимеры, биосовместимость.

Introduction

Biocompatible and biodegradable polymers are in high demand in various branches of modern reconstructive medicine. In this group of polymers, a special place is occupied by polyhydroxyalkanoates (PHAs) – microbial polymers of hydroxy derivatives of fatty acids, which are highly biocompatible and biodegradable in biological media (Volova, 2004). PHAs comprise polymers consisting of various monomers and, thus, having widely varying physicochemical properties: from high-crystallinity thermoplastics to rubber-like elastomers (Sudesh et al., 2000; Steinbüchel, 2003). The best-known representative of PHAs is a homopolymer – poly-3-hydroxybutyrate (PHB), which has high crystallinity (over 70 %); the products based on this material are rigid and prone to "physical ageing", which narrows down the range of uses for them.

PHA copolymers are more readily processible, and the 3-hydroxybutyrate/3-hydroxyvalerate copolymer (PHBV) is the most commonly used PHA copolymer now. Its crystallinity varies between 50 and 70 %, depending upon the ratio of its monomer molar fractions. Synthesis of other types of PHA copolymers requires rather complicated approaches including the search for new PHA producers, testing of nutrient media and culture conditions, or construction of genetically modified strains (Madison, Huisman, 1999; Volova, 2004).

One of the approaches to improving mechanical properties of pure polymers and eliminating their drawbacks, making them stronger and more ductile, is preparation of polymer-based blends. The common condition for all medical uses is biocompatibility of both components of the blend. Blending may result in three different types of systems (Avella et al., 2000). One type is a homogenous blend with a uniform structure and averaged properties of the initial pure components. If components are immiscible for thermodynamic reasons, the resulting system may be a two-phase blend whose material structure changes qualitatively under varied concentrations of components. If components effectively adhere to each other, this blend may have good mechanical properties. One more type of the composite system may consist of the matrix with reinforcing elements. In these systems, the effectiveness of reinforcement is also controlled by the degree of adhesion between components.

Polymer blends and composites can be prepared by using the following main methods: 1) extrusion of the mechanical mixture of polymers; 2) preparation of a mixed solution of the components in a common solvent and production of films by the solvent evaporation technique; 3) preparation of suspension of the insoluble component (which then becomes the reinforcing element) in the solution of the polymer (the future matrix) and preparation of the products by the solvent evaporation technique. Although extrusion methods are technically simple, they are not quite suitable for PHAs, which show poor thermal stability at temperatures close to their melting points (Hablot et al., 2008), and, thus, solvent techniques are more appropriate.

Poly-ε-caprolactone (PCL) is a translucent polyester that has a strength of about 0.4 GPa (Eshraghi, Das, 2010), which is regarded as an outstanding synthetic biodegradable polymer. The structure of PCL enables the manufacture of elastic and mechanically strong products. PCL is also well known for its biocompatibility. However, being very hydrophobic, PCL does not favor cell adhesion and proliferation on the surface of tissue-engineered constructs produced from pure PCL (Abedalwafa et al., 2013).

There are a number of studies addressing the miscibility behavior of different PHAs with PCL, which describe thermodynamic properties of the blends produced. In the majority of these studies, the blends contained poly-3-hydroxybutyrate (PHB). Lovera et al. (2007) investigated miscibility of PCL and PHB in mixtures produced by evaporation of the chloroform solutions with different ratios of components and found that blends with high molecular weight PCL (M_w 120 kDa) remained immiscible in the entire composition range. The immiscibility of PHB (M_w 349 kDa) with PCL (M_w 120 kDa) was also shown in the compositions prepared by melt extrusion (Hinuber et al., 2011); the authors noted a decrease in the PHB and PCL crystallization temperatures in the blend. In the blends with the composition of PHB/PCL 70/30 and 30/70, fractionated crystallization was observed, while in the intermediate, 60/40 and 50/50, blends, the phenomenon of fractionated crystallization was absent; a possible reason

for this might be the bicontinuous structure of both polymers. However, in a study by Gassner and Owen (1994), although the authors also found PHB and PCL immiscible, they noted a decrease in the melting temperature of PHB, which suggested certain mutual solubility of the components. The study performed by Chun and Kim (2000) with films prepared by evaporation of the chloroform solution proved that the PHBV (7 % 3-hydroxyvalerate) copolymer was also immiscible with PCL. The degree of crystallinity of the copolymer remained almost unchanged (decreasing from 51 to 46 %) when PCL concentration was increased from 0 to 50 %.

Thus, abundant data have been accumulated on the miscibility behavior of polymers and the properties of the blends, mainly their thermal properties. The studies were usually conducted with PHB. There are scant data in the literature on biocompatibility of blends of PHAs with PCL or other polymers.

The purpose of this study was to prepare blends of the PHBV copolymer with PCL, and to investigate the effects of variations in the PHBV/PCL composition on the structure, physicochemical, and biological properties of the films produced from these blends.

Materials and Methods

Material

Poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV; weight average molecular weight M_w=701 kDa, polydispersity Đ=3.27) was produced by microbial biosynthesis by using bacterial strain *Cupriavidus eutrophus* B-10646 (registered in the Russian Collection of Industrial Microorganisms). Cells were batch-cultured under strictly aseptic conditions, in a 7.5-L BioFlo 115 fermenter (“New Brunswic”, U.S.) on the mineral medium with glucose (Volova et al., 2014).

Poly-ε-caprolactone (PCL; M_w=169 kDa, Đ=1.86) was manufactured by Sigma-Aldrich (U.S.).

Preparation of films

PHBV and PCL were separately dissolved in chloroform to prepare 2 % (w/v) solutions. Then, the solutions were mixed to produce the following compositions (w/w): PHBV: PCL = 19:1 (5 % of the second polymer), 9:1, 3:1 (25 %), 1:1 (50 %), 1:3 (75 %), 1:9 (90 %), and 1:19 (95 %). The mixtures were kept for three hours with periodic stirring.

The mixtures of the two polymers and solutions of pure polymers (PHBV and PCL) were used to prepare films by the solvent evaporation technique, with 20 ml of each solution preheated to 35°C and placed onto a degreased Petri dish. The films were dried at ambient temperature for 3-5 days in a laminar flow cabinet.

Analysis of the properties of the blends

Crystallinity of films prepared from PHBV/PCL blends was determined by X-Ray structure analysis, with a D8 ADVANCE X-Ray powder diffractometer equipped with a VANTEC fast linear detector, using CuKa radiation (‘‘Bruker, AXS’’, Germany). The error in determination was 2 % or less (Volova et al., 2014).

The microstructure of the surface of the films was analyzed in a TM 3000 scanning electron microscope (Hitachi, Japan). The specimens were preliminarily sputter-coated with gold (10 mA, 40 s), employing an Emitech K575X sputter coater.

Surface properties of the specimens were examined using a DSA-25E drop shape analyzer (Krüss, Germany) and software DSA-4 for Windows. Drops of water and diiodomethane, 1.5 µl each, were alternately placed on the specimen surface, and contact angles of these liquids were measured in a semiautomatic mode, by the

"Circle" method. The results of measurements were used to calculate surface free energy (SFE) and its dispersive and polar components by the Owens, Wendt, Rabel and Kaelble method (Owens and Wendt, 1969; Kaelble, 1970).

Physical and mechanical properties of films (in a form of dumbbell-shaped samples 50 mm long and 6.1 mm wide) were examined at room temperature using an Instron 5565 electromechanical tensile testing machine (U.K.), with the clamping length of the samples 30 mm and the speed of the crosshead 3 mm/min. The thickness of films was previously measured by a "LEGIONER EDM-25-0.001" electronic digital micrometer. Samples were maintained under normal conditions for at least two weeks to reach equilibrium crystallization. A minimum of five samples were tested for each type of films. Young's modulus (E, MPa), tensile strength (s, MPa) and elongation at break (e, %) were calculated using the Bluehill 2 software (Instron, France). Young's modulus was calculated by the slope of each stress-strain curve in its elastic deformation region. Measurement error did not exceed 10 %.

Evaluation of biocompatibility of the blends

Adhesive properties of the surface of the films were examined as follows. Disks of 15 mm diameter were cut out with a mold cutter. The disks were placed into 24-well plates (TPP, Switzerland) and sterilized in a Sterrad NX plasma system (Johnson & Johnson, U.S.). Then, polymer disks were seeded by NIH 3T3 mouse fibroblast cells (10×10^3 cells/well/ml of the medium). Cells were cultivated in DMEM medium supplemented with 10 % fetal bovine serum and antibiotics (streptomycin 100 μg/ml, penicillin 100 IU/ml) (Gibco, Invitrogen) in a CO_2 incubator with 5 % CO_2 concentration, at a temperature of 37°C. The medium was replaced every three days.

Comparison of adhesive properties of films prepared from polymer blends was based on the attachment, number and morphology of cells, from scanning electron microscope images and images obtained by using the DAPI fluorescent stain (a nuclear DNA marker). A 300 nM solution of DAPI was used. The cells were fixed, rinsed three times with DPBS, incubated in the DAPI solution for 5 min, and thoroughly rinsed with DPBS.

Cell viability was assessed in a MTT assay, which is based on the ability of mitochondrial dehydrogenases of live cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to crystals of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-formazan. Optical density was measured after MTT-formazan was dissolved in DMSO, with an iMark microplate absorbance reader (BioRad, U.S.). The number of viable cells was determined using the calibration curve. Cells were fixed with a 4 % formaldehyde solution; then, for electron microscopy, films with cells attached to them were rinsed in ethanol solutions of escalating concentrations and, finally, in anhydrous ethanol.

Statistical analysis

Statistical analysis of the surface properties of the films was done by using built-in methods of DSA-4 software. Statistical analysis of the other results was performed using the standard software package of Microsoft Excel 2003. Arithmetic means and standard deviations were found. Significant differences between mean values were tested using Student's t test (significance level: $p=0.05$) by standard methods.

Results

Description of the films prepared from PHBV/PCL blends are given in Table 1. Films produced from the blends with different ratios

Table 1. Visual characteristics of the films prepared from blends of poly-3-hydroxybutyrate-co-3-hydroxyvalerate and poly-ε-caprolactone (PCL).

Percentage of PCL in the blend, w%	Description
0	Homogeneous, translucent
5	Homogeneous, translucent
10	Heterogeneous, translucent with opaque inclusions
25	Homogeneous, opaque white
50	Heterogeneous, transparent-opaque, fine-grained
75	Homogeneous, translucent
90	Homogeneous, translucent, flexible
95	Homogeneous, translucent, flexible
100	Translucent

of components differed in their transparency, flexibility, and degree of homogeneity. Films prepared from the blends with equal fractions of the components had a distinct heterogeneous structure, while most of the films in which one or the other component prevailed were visually homogeneous or almost homogeneous (films prepared from the blend with 10 % PCL had small opaque inclusions on their surface).

Electron microscopy revealed differences in the surface microstructure of PHBV/PCL films (Fig. 1). Films in which PHBV prevailed were porous and structurally similar to the films prepared from pure PHBV. The largest pores, whose size varied widely between 1 and 7 μm, were observed on films prepared from the 50/50 PHBV/PCL blend. Films with the higher percentage of PCL were smoother, and their surface was similar to that of the films prepared from pure PCL.

The surface properties of the PHBV/PCL films were evaluated by measuring water contact angles and calculating the polar and dispersive components of interfacial tension (IFT) (Fig. 2a), which are indirect indicators of the hydrophilic/hydrophobic behavior of the surface and its biological compatibility. The low percentage of PCL (5 %) in the PHBV/PCL blend caused a decrease water contact angle from 78.5° to 75.6° and an increase in the polar component of IFT (pIFT) from 7 to 8.3 mN/m. As the PCL fraction was increased to 10-25 %, water contact angles increased to 88.4°-90.6° and pIFT decreased to 3.3-3.6 mN/m. However, a further PCL increase, to 50-75 %, led to a pIFT increase to levels comparable with the pIFT values for pure PHBV which correlated with decreasing water contact angles. The dispersive component of IFT changed insignificantly at PCL levels of 50 % or lower.

The study of physical and mechanical properties of the blends containing different ratios of the components (Fig. 2b) showed that they differed considerably and changed nonlinearly. Pure PHBV films showed the greatest tensile strength (48.20 MPa). As the percentage of PCL was increased to 25 %, this parameter gradually decreased to 7.42 MPa. A further increase in the PCL fraction to 75 %, however, caused an increase in tensile strength, which reached 16.24 MPa. Then, with a PCL increase above 75 %, this parameter decreased, declining to 9.42 MPa for the pure PCL. Young's modulus gradually decreased from 3818.70 MPa for pure PHBV to 238.35 MPa for pure PCL. However, for blends containing 75 % PCL, Young's modulus locally increased to 529

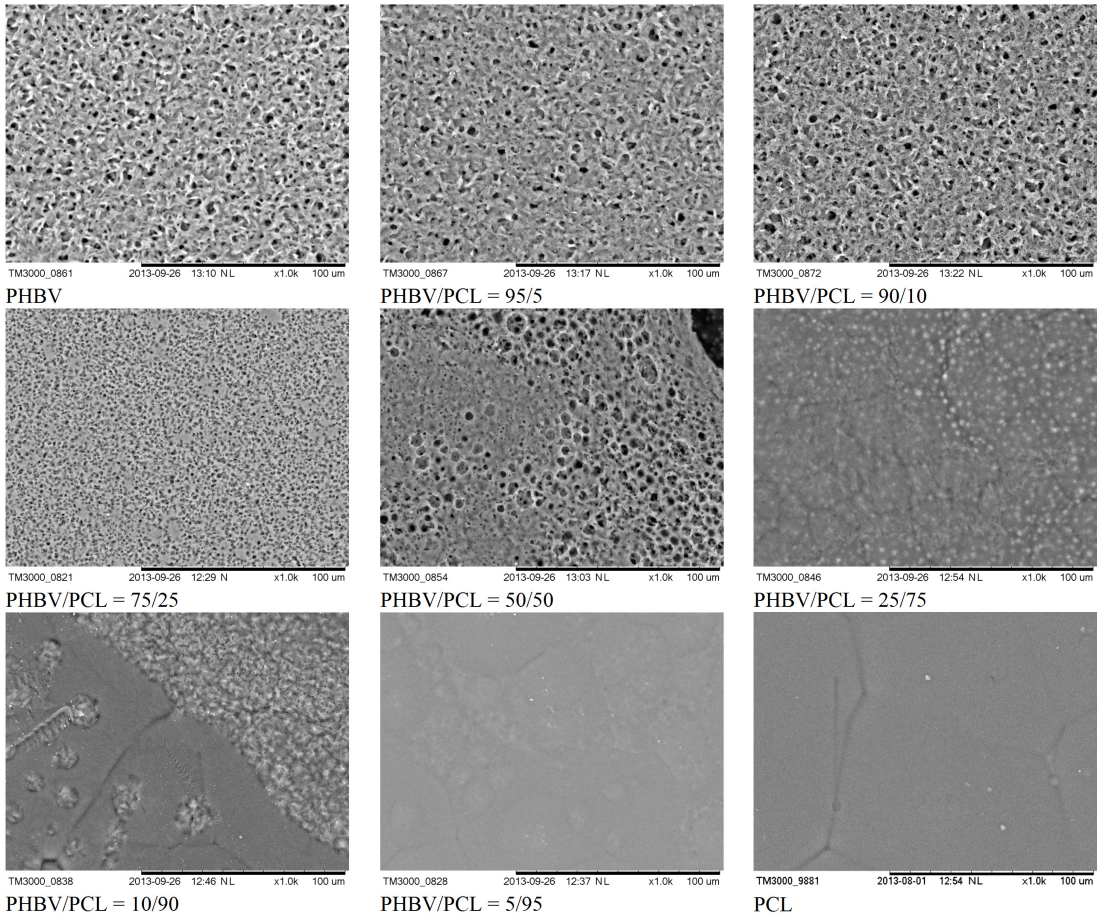

Fig. 1. Surface microstructure of the films prepared from PHBV/PCL blends. Scanning electron microscopy

MPa. Elongation at break, as an indicator of elasticity, was about 2 % for PHBV but much greater, almost 130 %, for PCL; as the ratio of components in the blends was varied, no clear trend in the change of this parameter was observed. When the percentage of PCL was increased from 0 to 10 %, elongation at break did not change noticeably. Elongation at break of the films prepared from the blend containing 25 % PCL was 106 %; in the films with equal percentages of PHBV and PCL (50/50), it dropped to 45 %, but then increased again, reaching the highest values, over 360 %, in the films with 75 % and 95 % PCL. The degree of crystallinity (Fig. 2c) was the highest for PHBV, 52 %, and it gradually decreased to 37 % for PCL.

Results of assessing adhesive properties and biological compatibility of films prepared from PHBV/PCL blends in a culture of mouse fibroblast NIH 3T3 cells are shown in Figures 3 – 5. None of the polymer films tested in the experiments showed any cytotoxic effects on the fibroblast cells cultured on them; all films favored cell attachment and facilitated cell proliferation. However, the number of cells in the culture varied, but that was not directly related to the surface properties of the films. On PHBV, PCL, and 10/90 and 5/95 PHBV/PCL films, the number of cells was lower than on other films used in the study.

Scanning electron microscopy (Fig. 4) showed that the best cell attachment was achieved

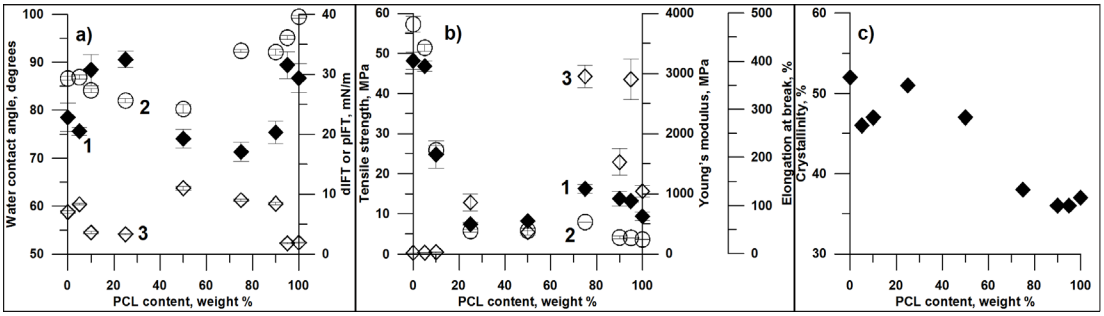

Fig. 2. Characterization of the films prepared from PHBV/PCL blends as related to PCL percentage: a) Surface properties: 1– Water contact angle, degrees; 2 – Interfacial tension, dispersive component (dIFT), mN/m; 3 – Interfacial tension, polar component (pIFT), mN/m; b) Mechanical properties: 1 – Tensile strength, MPa; 2 – Young's modulus, MPa; 3 – Elongation at break, %; c) Crystallinity, %

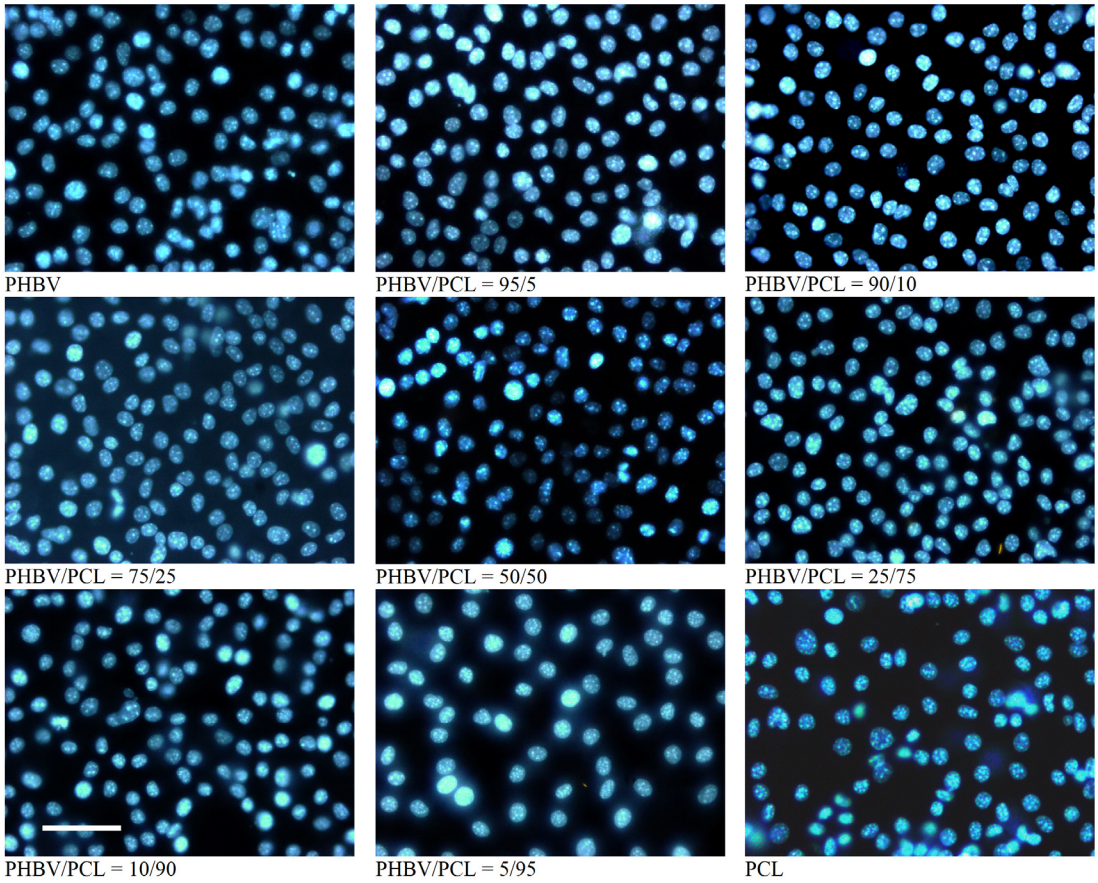

Fig. 3. Results of cultivating NIH 3T3 mouse fibroblast cells on the films prepared from PHBV/PCL blends, Day 7. DAPI staining, bar = 50 μm

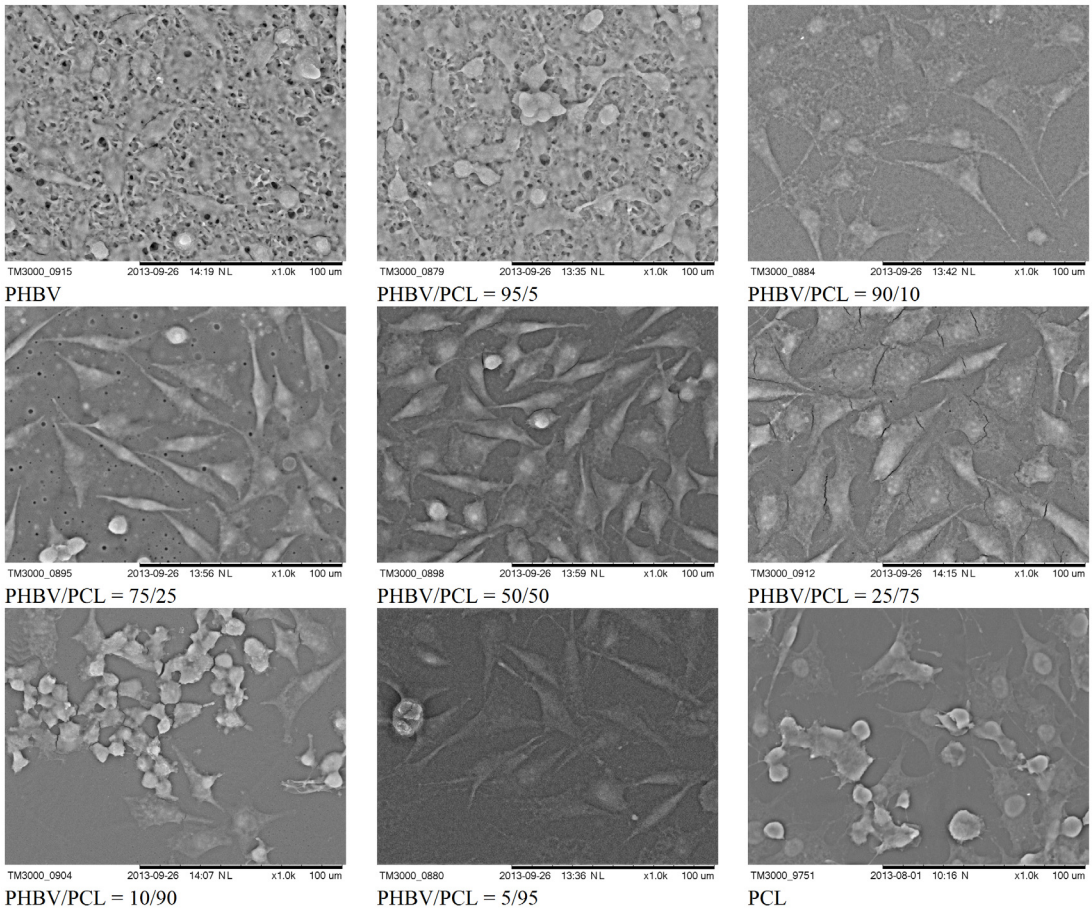

Fig. 4. Scanning electron microscopy of the surface of films prepared from PHBV/PCL blends populated by NIH 3T3 mouse fibroblast cells, Day 7

on films prepared from 90/10, 75/25, 50/50, and 25/75 PHBV/PCL blends. On these films, the cells were well spread, in contrast to cells on other films, where a large number of loosely attached spherical cells were observed.

Results of MTT assay (Fig. 5) were consistent with the results obtained by using the DAPI stain and scanning electron microscopy. At Day 7, the largest number of viable cells was observed on the films prepared from 95/5 and 90/10 PHBV/PCL: 4.71×10^5 and 5.46×10^5 cells/cm^2, respectively. The number of cells attached to the 75/25 PHBV/PCL films was similar to the number of cells on the 50/50 PHBV/PCL films: $4.12\text{-}4.14\times10^5$ cells/cm^2. On the 25/75 PHBV/PCL films, the number of cells was somewhat lower: 3.74×10^5 cells/cm^2. The lowest number of cells was observed on PHBV, PCL, 10/90 PHBV/PCL, and 5/95 PHBV/PCL films ($2.55\text{-}2.99\times10^5$ cells/cm^2) and the highest – on the films containing 5-10 % PCL.

Discussion

Preparation of polymer blends is one of the approaches to overcoming the limitations of pure polymers. The limitation of PHBV is its relatively high brittleness (although it is lower than the brittleness of the homopolymer, poly-3-hydroxybutyrate); one of the parameters showing this is the low elongation at break of this polymer.

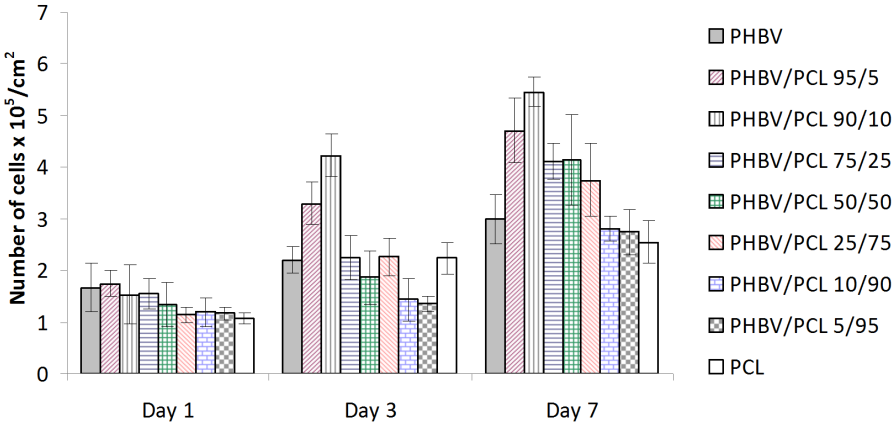

Fig. 5. Results of MTT assay of NIH 3T3 mouse fibroblast cell growth on the films prepared from PHBV/PCL blends

By contrast, PCL exhibits considerable elasticity but lower mechanical strength than PHBV.

Results of our study suggest that these parameters may be varied by preparing blends of polymers with different ratios of their components. The addition of PCL to PHBV generally led to a gradual shift in the values of physical and mechanical parameters of the blends towards the values characteristic of PCL, although there were departures from this trend in the behavior of blends with certain ratios of components. For instance, films prepared from the blend containing 75 % PCL had higher tensile strength, Young's modulus, and elongation at break than the films with the compositions nearest to them (50/50 and 10/90 PHBV/PCL). Elongation at break of the films prepared from the blends containing 75-95 % PCL was considerably higher than elongation at break of both PHBV and PCL. For the films with 25 % PCL, we observed a local increase in elongation at break.

The surface properties of products (topography, hydrophilic/hydrophobic properties, phase composition) are important indicators of the biocompatibility of materials and products, as they influence their interactions with tissues and cultured cells.

Although biocompatibility of the blends and composites, including those containing a PHA and PCL, is a critical issue for their use for biomedical purposes (such as construction of tissue engineered scaffolds), very few studies addressing this aspect have been reported so far. Possible reasons for this may be the great variety of combinations of components in the blends and numerous methods of biocompatibility assessment, on the one hand, and expected biocompatibility of the blends that contain polymers known to be biocompatible, on the other hand.

A rather recent study (Lim et al., 2013) proved that the blending of poly-3-hydroxybutyrate-3-hydroxyhexanoate with poly-ε-caprolactone did not affect the biocompatibility of the copolymer. In the present study, experiments with mouse fibroblasts showed high biocompatibility of all PHBV/PCL blends used as cell scaffolds. No adverse effects on the cultured fibroblasts were observed, as confirmed by morphological examination and MTT assay. Film scaffolds prepared from PHBV/PCL blends that contained 5 and 10 % PCL by mass were found to be the most favorable for cultivation of cells, but as the percentage of poly-ε-caprolactone in the blend

was increased further, cell adhesion strength became somewhat weaker, and the number of viable cells tended to decrease. At the same time, physical and mechanical properties of the films prepared from these blends were rather poor, and this may be their limitation for the construction of tissue engineered scaffolds. Films prepared from the blend containing 25 % PHBV and 75 % PCL had the best mechanical properties, and their biocompatibility was sufficiently high to make this blend a promising material for constructing cell scaffolds.

Conclusion

The present study showed that PHBV/PCL blends could be used to prepare polymer products whose physical and mechanical properties would be different from those of the pure polymers. Despite the differences in the surface structure, all films favored cell attachment and facilitated cell proliferation in experiments with NIH 3T3 mouse fibroblast cells, and, thus, they may be suitable for cell technologies. Based on their mechanical properties, films prepared from the 25/75 PHBV/PCL blend were evaluated as the most promising ones.

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