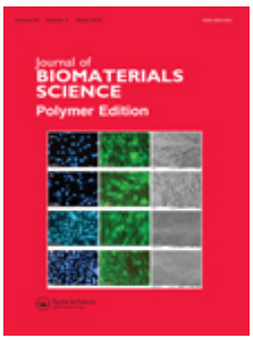

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Electrospinning of polyhydroxyalkanoate fibrous scaffolds: effects on electrospinning parameters on structure and properties

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In this study, electrospinning was used to prepare ultrafine fibers from PHAs with different chemical compositions: P(3HB) and copolymers: P(3HB-*co*-4HB), P(3HB-*co*-3HV), and P(3HB-*co*-3HHx). The main process parameters that influence ultrafine fiber diameter and properties (polymer concentration, solution feeding rate, working distance, and applied voltage) have been investigated and their effects evaluated. The study revealed electrospinning parameters for the production of high-quality ultrafine fibers and determined which parameters should be varied to tailor the properties of the products. This study is the first to compare biological and physical-mechanical parameters of PHAs with different chemical compositions as dependent upon the fractions of monomers constituting the polymers and ultrafine fiber orientation. Mechanical strength of aligned ultrafine fibers prepared from different PHAs is higher than that of randomly oriented ones; no significant effect of ultrafine fiber orientation on surface properties has been found. None of the fibrous scaffolds produced by electrospinning from PHAs had any adverse effects on attachment, growth, and viability of NIH 3T3 mouse fibroblast cells, and all of them were found to be suitable for tissue engineering applications.

Keywords: electrospinning; polyhydroxyalkanoates; ultrafine fibers; physical-mechanical properties; fibroblast cells

1. Introduction

Electrospinning (electrostatic spinning) is a promising technique that can be used for fabricating micro and ultrafine fibers and fibrous scaffolds (mats) and membranes. This technique was introduced in the 20th century to fabricate synthetic fibers. Several variants of production of fibers and devices using electrostatic force were patented in the 1930–1940s.[1,2] 'Electrospinning' has become a widely used term quite recently (1994), but theoretical underpinning of electrospinning was produced by Taylor in the 1960s.[3] In the electrospinning process, ultrafine fibers are formed between two oppositely charged electrodes: one is placed in a polymer solution or melt and the counter electrode is a collecting metal screen. Electrospinning is used to produce ultra and nanofine fibers and porous structures based on them from solutions and melts of

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polymers with different structures. Electrospun products have been used in various applications such as filtration and protective apparel, tissue scaffolding and drug delivery, nanocomposites, and sensor applications. Fiber constructions prepared from various materials by electrospinning are promising candidates to be used as scaffolds for *in vitro* cell cultures and as medical devices for surgical reconstruction (wound dressings, barrier membranes for guided tissue regeneration in maxillofacial surgery, etc.).[4,5]

The process of electrospinning has been found to have great potential in cell and tissue engineering.[6] Several types of electrospinning process have been described, which are used to produce fibers and membranes from both synthetic and natural polymers such as gelatin, carboxymethyl cellulose, polyethylene oxide, polylactide, polyurethanes, polyvinyl alcohol, polytrimethyl terephthalate, dimethylformamide, etc.[7–13] A number of processing parameters (applied voltage, polymer solution feeding rate, solution density, working distance, capillary diameter, etc.) can greatly affect fiber formation and structure. In order to be able to tailor the properties of the resulting products, one needs to determine the processing parameters for each particular type of polymer material.

In recent years, electrospinning techniques have been increasingly used to process biodegradable polymers intended for biomedical applications. Polyhydroxyalkanoates (PHAs) are a class of polymers of microbial origin with different chemical structure and diverse physicochemical properties. Similarly to polylactides and polyglycolides, PHAs are biocompatible materials capable of biodegradation. The most extensively studied PHAs are a high-crystallinity polymer of 3-hydroxybutyric acid [P(3HB)] and more readily processable polyesters: copolymers of 3-hydroxybutyrate with other monomers such as 4-hydroxybutyrate [P(3HB-*co*-4HB)], 3-hydroxyvalerate [P(3HB-*co*-3HV)], and 3-hydroxyhexanoate [P(3HB-*co*-3HHx)].[14,15] Numerous studies have shown that PHAs are suitable materials for fabricating medical devices and can be effectively used in reconstructive medicine. FDA has authorized the clinical use of a number of devices produced by Tepha (USA).[16] Our research team has carried out many studies of PHAs and PHA-based biomedical devices.[17]

Electrospinning studies using PHAs have not been conducted until quite recently. The first papers reporting the employment of this technique to produce ultrafine fibers from poly-3-hydroxybutyrate and copolymers of 3-hydroxybutyrate with 3-hydroxyvalerate were published in 2006.[18,19] So far, PHA electrospinning studies have been mainly conducted using P(3HB-*co*-3HV) copolymers. In a number of papers, Tong et al. [20–23] and Sombatmankhong et al. [19,24] reported detailed studies of the effects of the electrospinning parameters on the properties of fibers produced from P(3HB-*co*-3HV) (5 mol.% 3HV) and evaluated the potential of ultrafine fibers and mats as cell scaffolds; they also described the effects of the process parameters on the morphology and mechanical properties of fibers and fibrous scaffolds. More recent publications report results of processing and characterization of electrospun poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) nanofibrous membranes and describe structure and mechanical properties of the electrospun fibrous blends of PHBHHx/PDLLA, showing the influence of the molar fraction of 3HHx on mechanical strength and elasticity of the fibers.[25–28] Ying et al. [29] reported results of a comparison study of three types of electrospun PHA scaffolds – P(3HB), P(3HB-*co*-3HHx), and P(3HB-*co*-4HB) – and described morphology and diameter of the fibers and some of their mechanical properties (such as Young's modulus) as dependent on the type and molecular weight of the polymer used.

Much research has been done to evaluate the biocompatibility of electrospun fibrous scaffolds in various cell cultures (fibroblasts, osteoblasts, bone marrow mesenchymal stem cells etc.) and the results show that they are good candidates for cell technologies.[19,21,22,24,27,28,30]

Although the number of studies describing results of using the electrospinning method to prepare PHA-based products is increasing, the available data are not complete and cannot provide an estimate of the effects of the PHA chemical composition and electrospinning parameters on the physical-mechanical and biological properties of ultrafine fibers and fibrous scaffolds prepared from this type of polymers.

The purpose of this study was to produce electrospun ultrafine fibers differing in their physicochemical properties using PHAs with dissimilar chemical structures and to investigate the influence of electrospinning parameters and PHA chemical composition on the morphology of ultrafine fibers and physical-mechanical and biological properties of fibrous scaffolds.

2. Materials and methods

2.1. Materials

2.1.1. Bacterial strain synthesizing PHAs

In our work, we used polymers synthesized in the Institute of Biophysics SB RAS [15]. *Ralstonia eutropha* B5786 and B8562 and *Cupriavidus eutrophus* B10646, hydrogen-oxidizing bacterial strains, were used as PHA producers. The strains were grown under different conditions of carbon nutrition, which had been developed previously, and this enabled production of PHAs with different chemical compositions.[31,32]

2.1.2. Media

Cells were grown on Schlegel's mineral medium [33]: $Na_2HPO_4{\cdot}H_2O$ – 9.1; KH_2PO_4 – 1.5; $MgSO_4{\cdot}H_2O$ – 0.2; $Fe_3C_6H_5O_7{\cdot}7H_2O$ – 0.025; NH_4Cl – 1.0 (g/L); the medium was supplemented with glucose in amounts corresponding to cell concentration in the medium. Due to the use of urea, no pH adjustment was needed. A solution of iron citrate (5 g/L), which was used as a source of iron, was added to reach a concentration of 5 ml/L. Hoagland's trace element solution was used: 3 ml of standard solution per 1 L of the medium. The standard solution contains H_3BO_3 – 0.288; $CoCl_2{\cdot}6H_2O$ – 0.030; $CuSO_4{\cdot}5H_2O$ – 0.08; $MnCl_2{\cdot}4H_2O$ – 0.008; $ZnSO_4{\cdot}7H_2O$ – 0.176; $NaMoO_4{\cdot}2H_2O$ – 0.050; $NiCl_2$ – 0.008 (g/L).

To achieve synthesis of PHA copolymers, the culture medium was supplemented with additional carbon substrates (γ-butyrolactone; propionic, valeric, and caproic acids in the form of potassium salts) – precursors of the synthesis of different monomers.

2.1.3. Growth conditions

Cells were grown in batch culture, as developed previously for PHA synthesis.[15] Inoculum was produced using an Innova® 44 constant temperature incubator shaker ('New Brunswick Scientific', US). Inoculum was prepared by resuspending the museum culture maintained on agar medium. Museum culture was grown in 1- to 2-L glass flasks half-filled with saline liquid medium, with the initial concentration of glucose 10 g/L. Depending on the length of this phase (10–20 h), cell concentration of the inoculum varied from 1 to 2 g/L and intracellular polymer content was no higher

than 10%. In the phase of bacterial culture in the fermenter, the lowest initial cell concentration was 1.0–2.0 g per L.

In order to synthesize necessary amounts of PHAs, the process was scaled up in a BioFlo-155 automated fermentation system of total volume 12 L and working volumes 3–6 L, under strictly aseptic conditions. The air was pumped through the culture medium using a system of microbiological filters with an air pump. When cell concentration reached 2.0–2.5 g/L, nitrogen (60 mg/g biomass) and glucose solution were fed to the culture medium in two separate flows. Substrate feed rates were regulated with a peristaltic pump. Nitrogen concentration in the culture medium was maintained at trace levels, and glucose concentration did not exceed 10 g/L. Nitrogen supply was stopped after 20–25 h and the second phase was started; it lasted for 20–55 h. Glucose concentration was determined using a 'Glucose – FKD' kit, which contained chromogenic enzyme substrate and a calibrator (a glucose solution of a known concentration). Optical density of the study sample and calibration sample was compared photometrically with the optical density of the blank, with optical path length 10 mm at wavelength 490 nm. Glucose concentration in the samples was calculated using the following formula: $C = (E_0/E_K) \cdot 9$.

Synthesis of PHA copolymers was achieved as follows: after 4–6 h of cultivation, nitrogen supply was discontinued and the culture medium was supplemented with precursor substrates (γ-butyrolactone; propionic, valeric, and caproic acids in the form of potassium salts). Concentrations of these substrates in the culture medium were controlled using chromatographic analysis of the culture medium samples, which was done after preliminary extraction with chloroform from acidified samples.

2.1.4. Characterization of PHA samples

PHA samples were extracted from bacterial biomass with chloroform and precipitated in hexane. The optimized extraction procedure enabled the production of medically pure specimens that contained no organic impurities (proteins, carbohydrates or lipids, including fatty acids) and were suitable for use in contact with blood.[34]

Ultrafine fibers were prepared by electrospinning from high-purity PHA specimens with different chemical structure containing different monomer fractions. The first type was a homopolymer of 3-hydroxybutyric acid, an isotactic polyester with regular identically oriented (head-to-tail) sequential units: [–O–CH(CH_3)–CH_2–CO–]x. The second type was copolymers of 3-hydroxybutyric and 3-hydroxyvaleric [–CH(C_2H_5)–CH_2–CO–]y acids. The third type was copolymers of 3-hydroxybutyric and 3-hydrohexanoic [–O–CH(C_3H_7)–CH2–CO–]z acids. The fourth type was copolymers of 3-hydroxybutyric and 4-hydroxybutyric [–O–CH_2–CH_2–CH_2–CO–]v acids. PHA specimens differed significantly in a number of important parameters: molecular weight (Mw and Mn) and polydispersity (Đ); thermal properties (T_m and T_d); and degrees of crystallinity (C_x) (Table 1).

Molecular weight and molecular weight distribution of PHAs were examined using a gel permeation chromatograph ('Agilent Technologies' 1260 Infinity, USA) with a refractive index detector, using an Agilent PLgel Mixed-C column. Chloroform was the eluent at a flow rate of 1.0 ml/min at 40 °C. Typical sample volumes were 50 µl at a polymer concentration of 2 mg/ml. Narrow polydispersity polystyrene standards (Agilent, USA) were used to generate a universal calibration curve, from which molecular weights (weight average, M_w, and number average, M_n) and polydispersity (Đ) were determined.

Table 1. PHA chemical composition and properties.

No.	PHA composition	M_w (kDa)	Đ	T_m (°C)	T_d (°C)	C_x (%)
1.	P(3HB) (100 mol.%)	922	2.51	179.0 ± 1.6	294.8 ± 2.6	75
2.	P(3HB-*co*-4.5 mol.%-3HV)	1184	2.42	146.2 ± 1.3	281.1 ± 2.5	67
3.	P(3HB-*co*-10.5 mol.%-3HV)	695	3.15	173.1 ± 1.5	283.5 ± 2.5	59
4.	P(3HB-*co*-32.8 mol.%-3HV)	1336	2.67	179.6 ± 1.6	284.3 ± 2.5	51
5.	P(3HB-*co*-4.9 mol.%-3HHx)	527	2.64	169.4 ± 1.5	278.1 ± 2.4	64
6.	P(3HB-*co*-10 mol.%-3HHx)	647	3.30	173.8 ± 1.5	246.1 ± 2.2	62
7.	P(3HB-*co*-13.6 mol.%-3HHx)	924	4.10	174.7 ± 1.5	283.8 ± 2.5	35
8.	P(3HB-*co*-6.1 mol.%-4HB)	547	3.28	169.0 ± 1.4	284.3 ± 2.5	44
9.	P(3HB-*co*-10 mol.%-4HB)	473	3.2	153.8 ± 1.4	286.3 ± 2.6	50
10.	P(3HB-*co*-13.6 mol.%-4HB)	401	5.01	171.0 ± 1.5	265.8 ± 2.4	40
11.	P(3HB-*co*-20.3 mol.%-4HB)	605	4.19	167.8 ± 1.5	288.0 ± 2.5	38
12.	P(3HB-*co*-38 mol.%-4HB)	418	3.71	169.64 ± 1.5	287.5 ± 2.6	35
13.	P(3HB-*co*-48.3 mol.%-4HB)	507	3.79	167.76 ± 1.5	289.1 ± 2.6	51

The PHA samples were considerably different. PHA copolymers [P(3HB-*co*-4HB), P(3HB-*co*-HV), and P(3HB-*co*-3HHx)] had lower melting points and thermal decomposition temperatures than P(3HB) (179 ± 5 °C and 294 ± 5 °C, respectively). Melting points and thermal decomposition temperatures of PHAs with molecular weight greater than 1000 kDa tended to increase. The degree of crystallinity of PHA copolymers was considerably lower (35–67%) than the degree of crystallinity of the poly(3-hydroxybutyrate) homopolymer (75 ± 5%).

2.2. *Analytical procedures*

At least three samples of each type were used to study their properties; parameters were determined by measuring each sample five times. Samples were prepared using different techniques, depending on the method of analysis.

2.2.1. *Analysis of PHA structure*

Intracellular PHA content was determined by analyzing samples of dry cell biomass. Intracellular PHA content and composition of polymer samples extracted with chloroform were analyzed by a GC-MS (6890/5975C, 'Agilent Technologies', USA). Both lyophilized cells and extracted PHAs (4 mg) were subjected to methanolysis in the presence of sulfuric acid, and PHA was extracted and methyl esterified at 100 °C for 4 h. Benzoic acid was used as an internal standard to determine the total intracellular PHA.[32,33] Monomer units were identified in the extracted and purified PHA samples based on their retention times and mass spectra. Sensitivity of the method was 10^{-11} g.

2.2.2. *Physicochemical investigations*

Molecular weight and molecular weight distribution of PHAs were examined using a gel permeation chromatograph ('Agilent Technologies' 1260 Infinity, USA) with a refractive index detector, using an Agilent PLgel Mixed-C column. A 4-g polymer sample was dissolved in chloroform. Chloroform was the eluent at a flow rate of 1.0 ml/min at 40 °C. Typical sample volumes were 50 µl at a polymer concentration of 2 mg/ml. Narrow polydispersity polystyrene standards (Agilent, USA) were used to generate a universal calibration curve, from which molecular weights (weight average,

M_w, and number average, M_n) and polydispersity (Đ) were determined. The measurement accuracy was 2%.

Thermal analysis of PHA specimens was performed using a DSC-1 differential scanning calorimeter (METTLER TOLEDO, Switzerland). Powdered samples (4.0 ± 0.2 mg each) were placed into the aluminum crucible and compressed prior to measurement. Every sample was measured at least thrice. Samples were preheated to 60 °C and cooled to 25 °C. The specimens were heated to temperatures from 25 °C to 300 °C at 5 °C × min^{-1} (measurement precision 1.5 °C); melting point (T_m) and thermal decomposition temperature (T_d) were determined from exothermal peaks in thermograms. The thermograms were analyzed using the STARe v11.0 software.

In order to determine the crystallinity of the PHAs, three film samples 2 cm in diameter and 0.15 mm thick were prepared from a 2% polymer solution in chloroform. The samples had a circular shape because during measurement the sample spins in a direction perpendicular to the surface. X-ray structure analysis and determination of crystallinity of PHAs were performed employing a D8ADVANCE X-ray powder diffractometer equipped with a VANTEC fast linear detector, using CuKa radiation ('Bruker, AXS', Germany). The scan step was 0.016°, measurement time in each step 114 s, and scanning range from 5° to 60° (from 48° to 60° there only was a uniformly decreasing background); the registered parameter was intensity of X-rays scattered by the sample; 55°/0.016° = 3438 times. The degree of crystallinity was calculated as a ratio of the total area of crystalline peaks to the total area of the radiograph (the crystalline + amorphous components). Measurement accuracy: point measurement accuracy ± 0.4 PPS, with the lowest intensity 1.5 PPS and the highest intensity 32 PPS; the error in determination of the degree of crystallinity, which was calculated based on multiple measurements, was 2% or less.

2.3. Fabrication of PHA ultrafine fibers

Ultrafine fibers were electrospun from PHA solutions using a Nanon 01A automatic setup (MECC Inc., Japan). Chloroform solutions with polymer concentration varied from 1 to 10 wt.% were prepared from all types of PHAs. The polymer solution was poured into a plastic syringe (13 mm inside diameter). The syringe was fixed horizontally in the setup, the solution feeding rate varied from 4 to 8 ml/h, the applied voltage from 15 to 30 kV, and the working distance from 11 to 15 cm. Randomly oriented or aligned ultrafine fibers were collected on a flat steel plate or a rotating drum (at 1000 rpm), respectively; both collectors were covered with aluminum foil to collect ultrafine fibers more effectively.

2.4. Structure and properties of ultrafine fibers and fibrous scaffolds

2.4.1. Electron microscopy of surface morphology

The microstructures of the surface of electrospun ultrafine fibers and fibrous scaffolds were analyzed using scanning electron microscopy (TM-3000, Hitachi HT Corporation) at 5 kV. Samples of size 5 × 5 mm were attached to the microscope stage. Prior to microscopy, the samples were sputter-coated with gold (10 mA, 40 s) using an Emitech K575X sputter coater.

2.4.2. Determination of average ultrafine fiber diameters and spaces between fibers

Fiber diameters and spaces between fibers were measured by analyzing SEM images with image analysis program Image Processing and Data Analysis in Java (ImageJ). The diameters of 50 individual ultrafine fibers and spaces between them were measured in each SEM micrograph. Diameters and spaces between ultrafine fibers were analyzed in 10 fields of SEM images in triplicate.

2.4.3. Physical-mechanical properties

Physical-mechanical properties of fibrous scaffolds prepared from PHAs with different compositions were investigated using an Instron 5565 electromechanical tensile testing machine (UK). Dumbbell-shaped samples, 50 mm long, 6.1 mm wide, and 25–30 μm thick, were prepared for studying physical-mechanical properties of the scaffolds. The thickness of fibrous scaffolds was measured prior to testing, using a 'LEGIONER EDM-25-0.001' electronic digital micrometer.

Samples were maintained under normal conditions for at least two weeks to reach equilibrium crystallization. At least five samples were tested for each type of electrospun fibrous scaffold. Measurements were conducted at room temperature; the clamping length of the samples was 30 mm. The speed of the crosshead was 3 mm/min at room temperature. Young's modulus (E, MPa), tensile strength (σ, MPa), and elongation at break (ε, %) were automatically calculated by the Instron software (Bluehill 2, Elancourt, France). To obtain Young's modulus, the software calculated the slope of each stress–strain curve in its elastic deformation region. Measurement error did not exceed 10%.

2.5. Assays of cell attachment and viability

Fibrous scaffolds were cut into disks of 10 mm diameter, using a mold cutter. The samples were packed using an NS 1000 shrink-wrapping machine (Hawo Gmbh, Germany) and sterilized with H_2O_2 plasma in the Sterrad NX system (Johnson & Johnson, USA) for 45 min.

2.5.1. Assays of cell attachment

The ability of PHA ultrafine fibers to facilitate cell attachment was studied using NIH 3T3 mouse fibroblast cells. Cells were seeded into 24-well cell culture plates (Greiner-Bio-One, USA) (1×10^3 cells/ml per well). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and a solution of antibiotics (streptomycin 100 μg/ml, penicillin 100 IU/ml) (Sigma–Aldrich) in a CO_2 incubator with CO_2 level maintained at 5% at a temperature of 37 °C. The medium was replaced every three days.

Cell morphology and distribution on the scaffolds were investigated using SEM. The cells attached to scaffolds were fixed with a 2.5% glutaraldehyde solution (Sigma–Aldrich) for 60 min at room temperature, rinsed thrice in absolute ethanol, and freeze dried. Cells were examined using a scanning electron microscope (Philips SEM 525 M).

Cell cytoplasm and nuclear DNA molecules were stained with phalloidin conjugated with fluorescein (FITC) and DAPI, respectively (Sigma–Aldrich), to observe cell morphology and to count the cells attached to scaffolds.

The scaffolds with cells were fixed with a 3.7% formaldehyde solution (Chimreactivsnab, Russia) for 60 min and then rinsed in phosphate buffered saline to remove the fixative. After that, scaffolds with attached cells were incubated in a Triton X-100 solution (0.1%) (GERBU Biotechnik GmbH, Germany) for 5 min and in a 1% bovine serum albumin solution (Ampresco, USA) for 30 min at room temperature. Cell cytoplasm was stained with a FITC solution (a 1/100 dilution) for 60 min at room temperature; nuclei were contrasted with DAPI (a 300 nM solution, for 5 min). The cells were analyzed using an Axiovert 40 fluorescence microscope (Carl Zeiss).

The areas of cells were measured by analyzing images of FITC and DAPI stained cells with an image analysis program Image Processing and Data Analysis in Java (ImageJ). At least 50 cells were analyzed to calculate the areas of cells.

2.5.2. Assays of cell viability

Cell viability was evaluated using MTT assay at Day 7 after cell seeding onto scaffolds. Reagents were purchased from Sigma–Aldrich. A 5% MTT solution (50 μl) and complete nutrient medium (950 μl) were added to each well of the culture plate. After 3.5 h of incubation, the medium and MTT were replaced by DMSO to dissolve MTT-formazan crystals. After 30 min, the supernatant was transferred to the 96-well plate, and optical density of the samples was measured at wavelength 540 nm, using a Bio-Rad 680 microplate reader (Bio-Rad LABORATORIES Inc., USA). Measurements were performed in triplicate. The number of viable cells was determined from the calibration graph.

2.6. Statistics

Statistical analysis of the results was performed by conventional methods, using the standard software package of Microsoft Excel. Arithmetic means and standard deviations were found. The statistical significance of results was determined using Student's test (significance level: $p \leq 0.05$).

3. Results

To determine optimal conditions for the production of ultrafine fibers and fibrous scaffolds, we studied the effects of electrospinning parameters on morphology and physical-mechanical and biological properties of electrospun products. The main parameters of electrospinning in a Nanon 01A setup investigated in this study were concentration of the polymer solution, PHA chemical composition, polymer solution feeding rate, applied voltage, and the type of the collector.

3.1. The effect of concentration of the polymer solution on production and morphology of ultrafine fibers

The effect of the density of polymer solutions on fiber properties was studied using the homopolymer of 3-hydroxybutyric acid, in order to avoid the influence of chemical composition of the PHA on the electrospinning process and properties of the products. P(3HB) chloroform solutions with polymer concentration varied from 1 to 10 wt.% were used. The process parameters were as follows: needle diameter – 1 mm, applied voltage – 30 kV, solution feeding rate – 5 ml/h, and working distance – 15 cm, a flat steel collection plate.

Polymer concentration directly affects the quality of electrospun fibers. Polymer solutions of the density under 2 wt.% could not yield high-quality fibers, due to their low viscosity (below 100 cP). These solutions yielded a few very thin ultrafine fibers and fine spray, which consisted of microdrops rather uniformly distributed on the collector. As P(3HB) concentrations increased, the solution viscosities increased too (from 60 to 800 cP) because the entanglement of polymer molecular chains prevented the breakup of the electrically driven jet and allowed the electrostatic stresses to further elongate the jet.[25]

Stable electrospinning of ultrafine fibers in the Nanon 01A setup was attained from P(3HB) solutions with polymer concentrations from 2 to 8 wt.% (solution viscosity 200–800 cP). Polymer concentration significantly influenced the diameter of the ultrafine fibers, which varied from 0.45 to 3.14 μm. Within the study range of polymer concentration, the diameter of the ultrafine fibers is linearly related to the solution density. The viscosity of the solutions with polymer concentrations above 8 wt.% was too high (about 1000 cP) to allow successful formation of ultrafine fibers.

Figure 1 shows SEM images of electrospun P(3HB) ultrafine fibers prepared from the polymer/chloroform solutions with different polymer concentrations. Most of the fibers were cylindrical and smooth; their surface was virtually defect-free; there were spaces between the fibers. As polymer concentration of the solution was increased, the range of fiber diameters became wider (1–3 μm). As the fiber diameter increased,

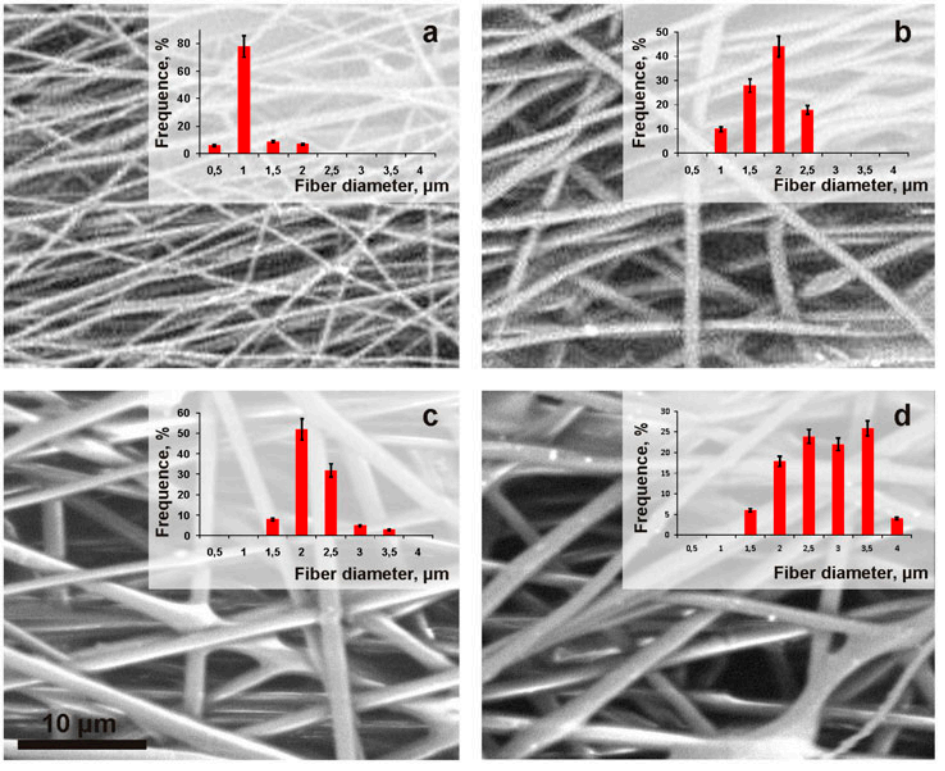

Figure 1. The effect of the density of P(3HB) chloroform solution on morphology of ultrafine fibers.

spaces between fibers in the fibrous mat widened from about 2 to about 10 μm and the thickness of the mats increased too (from 10 to 75 μm). P(3HB) concentrations of the solution ranging from 2 to 8 wt.% yielded good-quality fibers. However, some of the fibers produced by electrospinning from solutions with P(3HB) concentration of 8 wt.% fused together at several points (Figure 1(d)), probably because of the presence of residual solvent in large-diameter fibers.

The diameter of P(3HB) ultrafine fibers significantly influenced the physical-mechanical properties of fibrous mats comprised of them (Figure 2). As fiber diameter increased from 0.45 to 3.14 μm, the mats became more elastic, which was expressed as a more than twofold increase in elongation at break (from 4.5 to 10.6%), but much less mechanically strong, with tensile strength decreasing from 23.16 to 6.65 MPa and Young's modulus from 1.16 to 0.3 GPa. Thus, by varying the density of polymer solution only, one can produce fibers with significantly different mechanical properties.

3.2. The effect of electrospinning parameters on morphology of ultrafine fibers

The effects of the major electrospinning parameters (polymer solution feeding rate and applied voltage) on the process and quality of the electrospun fibers were also studied using P(3HB), and the fibers were collected on a flat steel plate. The effect of the voltage range from 15 to 30 kV was studied at constant values of other electrospinning parameters: needle diameter 1 mm, concentration of polymer solution 5 wt.%, solution feeding rate 5 ml/h, and working distance 15 cm.

Good fiber morphology was achieved at the voltage ranging from 15 to 30 kV. Within this voltage range, the average fiber diameter changed from 1.25 to 2.5 μm. At 15 kV, polymer junction formation was observed; beaded fibers were formed when the applied voltage was very low (at 15 kV). At voltages below 10–12 kV, no fiber

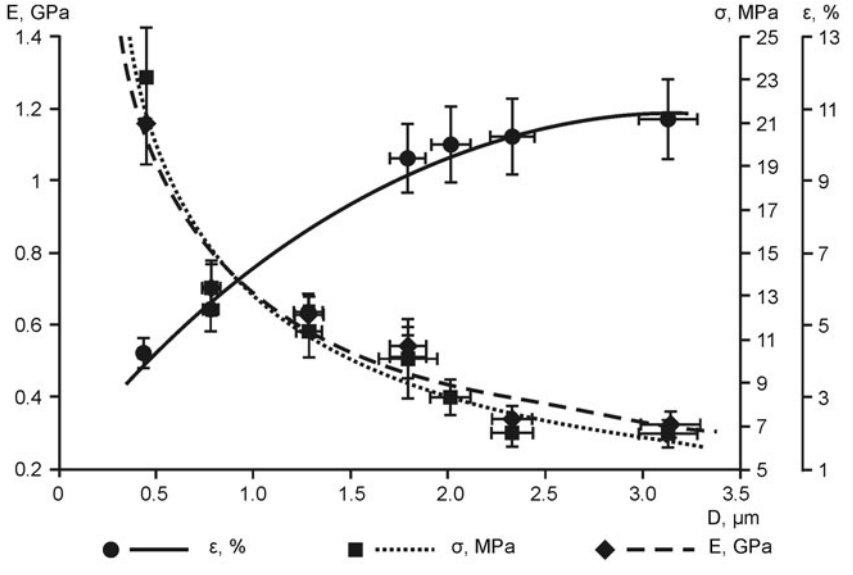

Figure 2. The effect of the density of P(3HB) chloroform solution on physical-mechanical properties of ultrafine fibers.

formation occurred whatever the polymer concentration used. Performance characteristics of the setup used did not allow us to investigate the effect of the voltage above 30 kV.

The effect of the polymer solution feeding rate on the properties of the fibers was studied by varying this parameter from 2 to 8 ml/h. Other electrospinning parameters were maintained constant: needle diameter 1 mm, solution concentration 5 wt.%, working distance 15 cm, and applied voltage 30 kV. At the solution feeding rate below 2 ml/h, the jet was unstable; when the solution feeding rate reached 8 ml/h, polymer junctions were formed. The fiber morphologies were similar. The relationship between solution feeding rate and the fiber diameter shows that diameter varied from 1.2 to 2.6 μm.

Thus, the influence of these electrospinning parameters on P(3HB) fiber morphology was much weaker than the influence of polymer solution concentration. This study shows that high-quality ultrafine fibers can be produced under the following electrospinning conditions: applied voltage 25–30 kV, solution feeding rate 4–6 ml/h, and polymer concentration 2–8 wt.%.

3.3. The effect of PHA chemical composition on morphology and properties of ultrafine fibers

We prepared ultrafine fibers using PHAs with different chemical structures (Table 2) and studied the effect of polymer composition on the surface structure and physical-mechanical properties of the fibers.

Chloroform solutions were prepared from all types of PHAs (5% concentration); the following electrospinning parameters were used: solution feeding rate 5 ml/h, working distance 15 cm, applied voltage 30 kV, and flat steel collection plate.

Figure 3 shows SEM images of the fibers prepared from chloroform solutions of different PHAs that contained similar molar fractions of their second monomers (3HV, 3HHx, and 4HB) (samples 1, 3, 6, and 9 in Table 2). The fibers were collected on a flat steel plate, i.e. randomly oriented fibers were produced. As Figure 3(A) shows, the fibers were good quality but different in diameter. The average diameter of the fibers prepared from P(3HB) was 2.9 μm, and the thickness of the fibrous scaffolds was 25 μm. Measurements of diameters of fibers prepared from copolymers with similar

Table 2. Values of tensile strength and elongation to break for fibrous mats comprised of aligned ultrafine fibers.

No.	Polymer composition	E (MPa)	σ (MPa)	ε (%)
1.	P(3HB) (100 mol.%)	543.03 ± 72.45	11.17 ± 2.33	9.90 ± 3.21
2.	P(3HB-*co*-4.5 mol.%-3HV)	824.04 ± 78.23	14.18 ± 2.17	10.11 ± 1.24
3.	P(3HB-*co*-10.5 mol.%-HV)	463.52 ± 49.08	12,26 ± 2.65	11.56 ± 2.10
4.	P(3HB-*co*-32.8 mol.%-HV)	321.90 ± 27.33	14.13 ± 2.05	91.16 ± 7.32
5.	P(3HB-*co*-4.9 mol.%-3HHx)	563.61 ± 61.56	16.27 ± 1.91	14.83 ± 2.13
6.	P(3HB-*co*-10 mol.%-3HHx)	431.04 ± 38.42	10.26 ± 2.23	20.69 ± 3.61
7.	P(3HB-*co*-13.6 mol.%-3HHx)	479.95 ± 52.62	12.71 ± 2.32	50.00 ± 4.66
8.	P(3HB-*co*-6.1 mol.%-4HB)	817.50 ± 76.81	15.33 ± 3.71	13.10 ± 2.00
9.	P(3HB-*co*-10 mol.%-4HB)	193.70 ± 22.30	5.70 ± 0.82	52.46 ± 6.32
10.	P(3HB-*co*-13.6 mol.%-4HB)	113.54 ± 9.85	9.30 ± 1.24	180.33 ± 20.71
11.	P(3HB-*co*-20.3 mol.%-4HB)	77.94 ± 8.41	15.74 ± 2.12	262.29 ± 32.41
12.	P(3HB-*co*-38 mol.%-4HB)	5.50 ± 4.22	11.91 ± 1.56	245.90 ± 22.54
13	P(3HB-*co*-48.3 mol.%-4HB)	4.12 ± 1.68	4.92 ± 0.62	311.47 ± 45.37

molar fractions of their second monomers showed that they had different average diameters and ranges of diameters, but the fibrous mats had similar thickness – about 20 μm. The average diameter of P(3HB-*co*-10 mol.%-3HV) fibers was 2.3 μm, while the average diameter of P(3HB-*co*-4HB) fibers was significantly smaller (1.7 μm). By contrast, the diameter of the fibers prepared from the solution of P(3HB-*co*-10 mol.%-3HHx) of the same concentration (5%) was twice larger (4.2 μm).

Results of comparing physical-mechanical properties of fibrous scaffolds prepared from PHAs with different chemical compositions are given in Table 2. It is apparent from this table that all copolymer fibrous scaffolds, whatever their composition, had higher elongation at break, i.e. greater elasticity, than P(3HB) fibrous mats, but considerably reduced mechanical strength, as indicated by Young's modulus and tensile strength.

Comparison of samples with equal molar fractions of the second monomers (samples 3, 6, and 9 in Table 2) showed that the second monomers influenced these parameters to different degrees. All fibrous scaffolds prepared from PHA copolymers showed lower mechanical strength than those prepared from P(3HB), but the presence of 4HB (sample 9) decreased mechanical strength of fibrous scaffolds more significantly than 3HV or 3HHx did: Young's modulus dropped from 356.23 ± 40.62

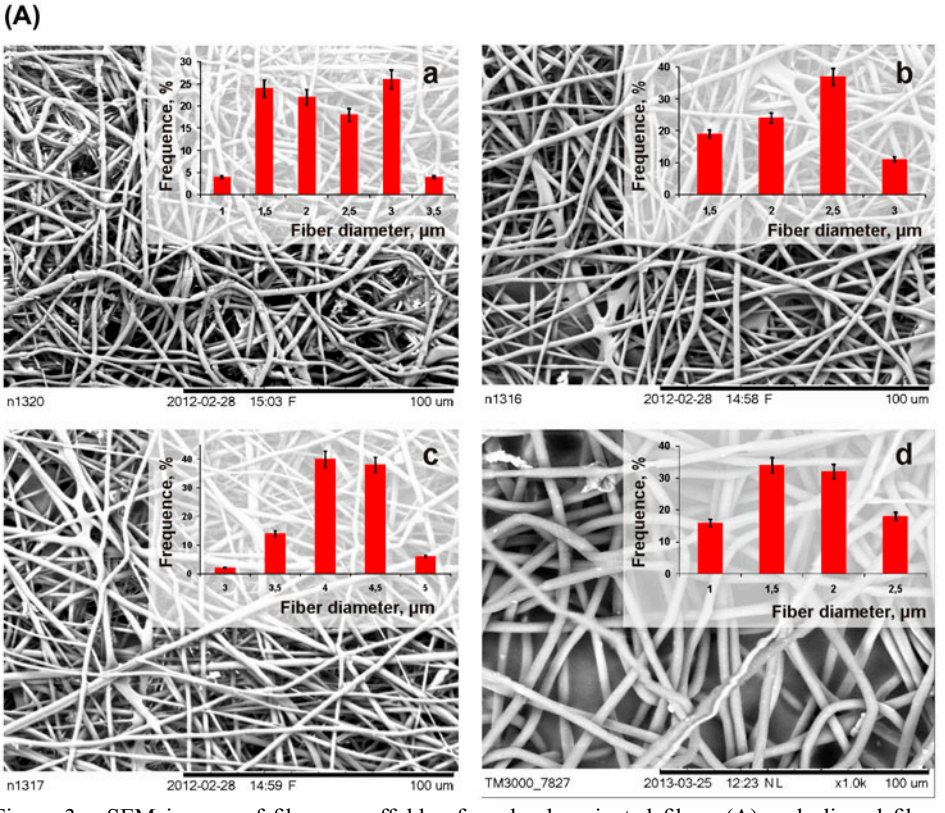

Figure 3. SEM images of fibrous scaffolds of randomly oriented fibers (A) and aligned fibers (B) produced by electrospinning from solutions of PHAs with different chemical compositions: a – P(3HB); b – P(3HB-*co*-10.5 mol.%-3HV); c – P(3HB-*co*-10.0 mol.%-4HB); d – (3HB-*co*-10.0 mol.%-3HHx).

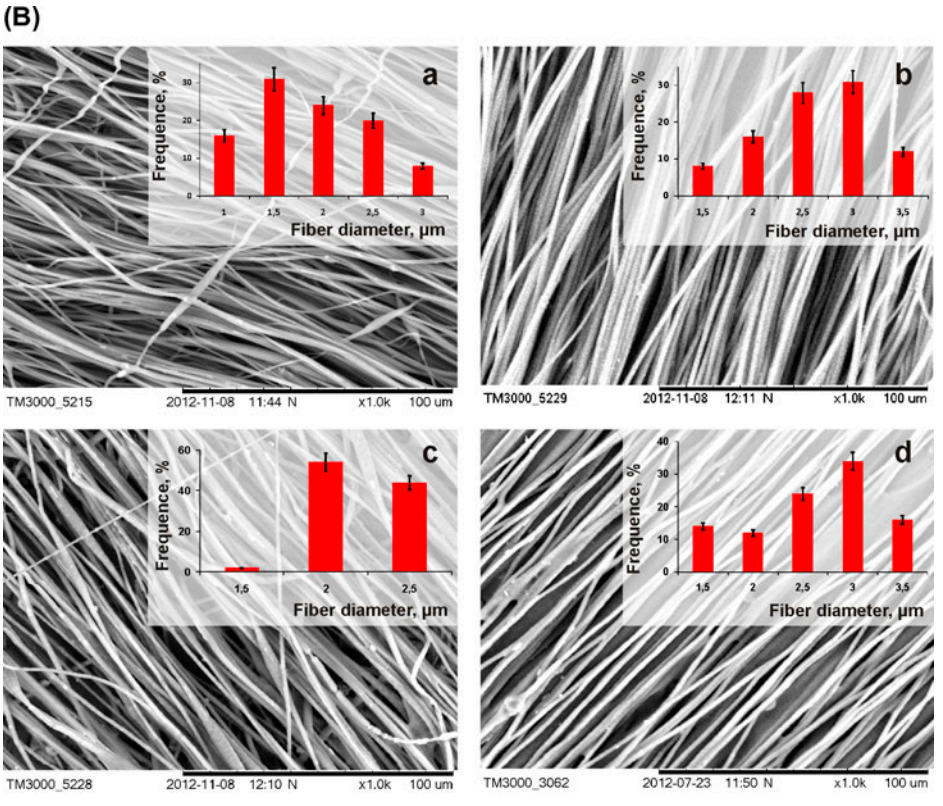

Figure 3. (*Continued*).

to 1.59 ± 0.30 MPa and tensile strength from 9.32 ± 2.54 to 0.42 ± 0.08 MPa. The presence of 3HV (sample 5) decreased Young's modulus and tensile strength of fibrous scaffolds to 5.81 ± 1.06 and 0.67 ± 0.06 MPa, respectively, and Young's modulus and tensile strength of copolymers containing 3HHx were 18.68 ± 2.85 and 0.76 ± 0.10 MPa, respectively. The presence of these monomer units had an opposite effect on elongation at break of the fibrous scaffolds. Elongation at break of P(3HB)-based products was 13.3 ± 3.11%, while copolymer fibers showed considerably higher values: 29.45 ± 4.61% in P(3HB-*co*-10 mol.%-3HHx) fibers and 40.72 ± 6.20% in P (3HB-*co*-10.5 mol.%-3HV) ones, and the most significant increase, to 72.98 ± 10.33%, was revealed in P(3HB-*co*-10 mol.%-4HB) fibers.

For randomly oriented fibers, no obvious relationship between their physical-mechanical properties and the molar fraction of the second monomer was observed. A more than sevenfold increase in the molar fraction of 3HV in P(3HB-*co*-3HV) (from 4.5 to 32.8%) caused a 1.6-fold increase in Young's modulus, but did not induce any change in tensile strength or elongation at break. However, a smaller (threefold) increase in the molar fraction of 3HHx (from 4.9 to 13.6%) did not have any significant effect on Young's modulus but reduced the tensile strength by half and caused a threefold decrease in elongation at break. A 7.5-fold increase in the molar fraction of 4HB in P(3HB-*co*-4HB) did not affect mechanical strength of the samples but increased their elasticity considerably.

The PHA samples with different chemical compositions had dissimilar degrees of crystallinity (Table 1), which varied from 35 to 75%. These dissimilarities may be accounted for by differences in the micromolecular structure of polymer chains and crystallization processes of PHAs with different chemical compositions. Further work is required to establish this.

3.4. *The effect of orientation of ultrafine fibers on morphology and properties of fibrous scaffolds of different chemical compositions*

Aligned ultrafine fibers were prepared from PHAs of the same composition as those used in the previous study and under the same electrospinning conditions, but with a rotating drum (at 1000 rpm) used as a collector (Figure 3(B), Table 3).

The average diameter of aligned fibers prepared from P(3HB) was 2.1 μm. The diameter of copolymer aligned fibers, in contrast to randomly oriented ones, was similar to the diameter of P(3HB) fibers. The diameter of aligned P(3HB-*co*-3HV) fibers was similar to that of the corresponding randomly oriented fibers (about 2.5 μm); the average diameter of P(3HB-*co*-3HHx) fibers was 2.2 μm and that of P(3HB-*co*-4HB) fibers 2.7 μm. The diameter range of the aligned fibers was about 2 μm and that was close to the diameter range of randomly oriented fibers. The thickness of fibrous scaffolds comprised of aligned fibers was 25–30 μm, i.e. comparable to the thickness of randomly oriented scaffolds.

The physical-mechanical properties of the fibrous mats comprised of aligned and randomly oriented ultrafine fibers differed significantly (Table 3).

First, electrospun aligned fibrous scaffolds differed from randomly oriented ones in that they had much higher mechanical strength. Second, the effects of the second monomers of the copolymers used to prepare the fibers on the properties of the aligned fibrous mats were different from their effects on the properties of the randomly oriented fibrous mats. The aligned fibrous mats prepared from copolymers containing 3HV and 3HHx (samples 3 and 6 in Table 3) had similar values of tensile strength and Young's modulus, and they were not significantly lower than those of P(3HB) fibers, but their elasticity values differed by a factor of two. In P(3HB-*co*-10 mol.%-4HB) fibers, both parameters characterizing mechanical strength were lower than in P(3HB) ones, but this

Table 3. Values of tensile strength and elongation to break for fibrous mats comprised of randomly oriented ultrafine fibers.

No.	Polymer composition	E (MPa)	σ (MPa)	ε (%)
1.	P(3HB) (100 mol.%)	356.23 ± 40.62	9.32 ± 2.54	13.3 ± 3.11
2.	P(3HB-*co*-4.5 mol.%-3HV)	9.61 ± 1.85	0.75 ± 0.08	47.36 ± 7.21
3.	P(3HB-*co*-10.5 mol.%-HV)	5.81 ± 1.06	0.67 ± 0.06	40.72 ± 6.20
4.	P(3HB-*co*-32.8 mol.%-HV)	13.91 ± 2.07	0.80 ± 0.09	38.33 ± 5.34
5.	P(3HB-*co*-4.9 mol.%-3HHx)	22.58 ± 3.24	0.94 ± 0.12	63.25 ± 9.85
6.	P(3HB-*co*-10 mol.%-3HHx)	18.68 ± 2.85	0.76 ± 0.10	29.45 ± 4.61
7.	P(3HB-*co*-13.6 mol.%-3HHx)	21.28 ± 3.64	0.58 ± 0.11	21.59 ± 3.17
8.	P(3HB-*co*-6.1 mol.%-4HB)	3.61 ± 0.85	0.82 ± 0.09	58.95 ± 8.51
9.	P(3HB-*co*-10 mol.%-4HB)	1.59 ± 0.30	0.42 ± 0.08	72.98 ± 10.33
10.	P(3HB-*co*-13.6 mol.%-4HB)	2.91 ± 0.75	1.26 ± 0.23	102.37 ± 12.55
11.	P(3HB-*co*-20.3 mol.%-4HB)	2.32 ± 0.62	1.01 ± 0.15	117.33 ± 14.30
12.	P(3HB-*co*-38 mol.%-4HB)	1.22 ± 0.36	1.46 ± 0.21	177.74 ± 20.67
13.	P(3HB-*co*-48.3 mol.%-4HB)	1.60 ± 0.54	0.81 ± 0.09	118.93 ± 15.76

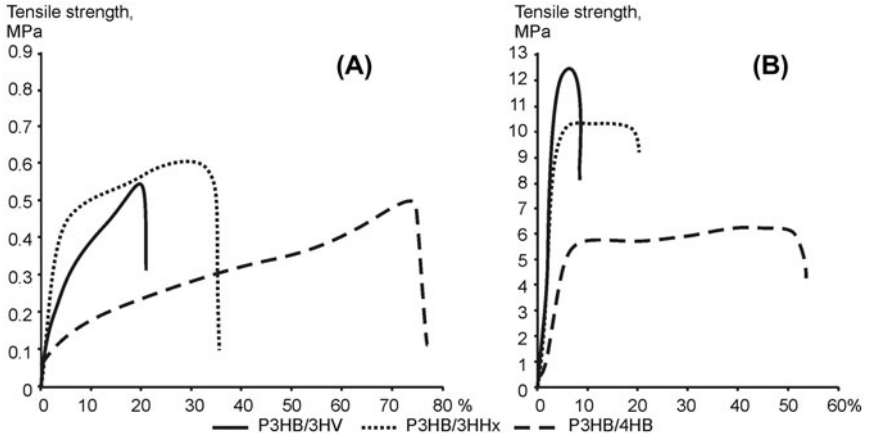

Figure 4. Tensile strength: A – randomly oriented ultrafine fibers, B – aligned oriented ultrafine fibers produced by electrospinning from solutions of PHAs with different chemical compositions: a – P(3HB); b – P(3HB-*co*-10.5 mol.%-3HV); c – P(3HB-*co*-10.0 mol.%-4HB); d – (3HB-*co*-10.0 mol.%-3HHx).

difference was not as significant as in randomly oriented fibers, while elasticity was more than four times higher.

The most important difference between randomly oriented and aligned copolymer fibrous scaffolds was that in the latter, increased molar fractions of the second monomers had pronounced effect on the properties of the scaffolds (Table 3).

As the molar fraction of 3HV in P(3HB-*co*-3HV) was increased from 4.5% to 32.8%, Young's modulus consistently decreased from 824.04 ± 78.23 to 321.90 ± 27.33 MPa, while tensile strength remained practically unchanged, amounting to 14 MPa, and elongation at break increased from 10.11 ± 1.24% to 91.16 ± 7.32%. For P(3HB-*co*-4HB), the increase in the molar fraction of 4HB from 6.1% to 48.3% also caused a gradual and more pronounced decrease in Young's modulus, from 817.50 ± 76.81 to 4.12 ± 1.68 MPa, and an insignificant decrease in tensile strength, with elongation at break rising from 13.10 ± 2.00 to 311.47 ± 45.37%. In the fibers prepared from P(3HB-*co*-33HHx), the increase in the molar fraction of 3HHx from 4.9% to 13.6% did not cause any significant change in Young's modulus, but elongation at break gradually increased threefold.

Figure 4 clearly shows the effects of orientation and PHA composition on mechanical properties of electrospun fibrous scaffolds, demonstrating changes in tensile strength and elongation at break of randomly oriented (Figure 4(A)) and aligned (Figure 4(B)) fibrous scaffolds prepared from PHA copolymers that had different structures but contained the same molar fractions of their second monomers.

Scaffolds comprised of aligned ultrafine fibers prepared from P(3HB-*co*-10.5 mol.%-3HV) had the highest values of elongation at break, while scaffolds comprised of randomly oriented ultrafine fibers prepared from P(3HB-*co*-10.0 mol.%-4HB) showed the highest elasticity. The effects of the parameters investigated in this study on physical-mechanical properties of electrospun fibers can be ranked from strongest to weakest as follows: fiber orientation – PHA chemical composition – polymer solution density. The factor that has the strongest effect on fiber diameter and morphology is polymer solution density, while the effect of PHA chemical composition is less significant. Thus, by using

different types of PHAs and different collectors, one can prepare electrospun products with tailored strength and elasticity.

3.5. Study of cell attachment and viability

Biological properties of fibrous scaffolds were studied in the culture of NIH 3T3 fibroblast cells. Investigation of cell attachment and proliferation on scaffolds prepared from solutions of the P(3HB) homopolymer of different densities (2, 4, 6, and 8 wt.%) did not reveal any significant differences at $p \leq 0.05$, showing that all fibrous mats were suitable for use as cell culture scaffolds. At 24 h after seeding, the number of cells on scaffolds was 1.52–1.95 × 10^3 cells/cm^2 irrespective of fiber diameter; at Day 3, the number of cells reached 6.55–8.02 × 10^3 cells/cm^2. No statistically significant differences were found in the counts of proliferating and viable cells between scaffolds of different types. Hence, the diameter of electrospun P(3HB) fibers did not influence the attachment and growth of NIH 3T3 mouse fibroblast cells.

In contrast, considerable differences in cell culture parameters were revealed in experiments with fibrous scaffolds prepared from PHAs with different chemical compositions. The number of cells on the reference scaffold (polystyrene) was lower than the number of cells on PHA scaffolds (half or even less than half of that number). At Day 7 after seeding, counts of viable cells using MTT assay (Figure 5) showed that on randomly oriented fibrous scaffolds prepared from copolymers containing 3HV or 4HB as their second monomer, the increase in the number of cells was about 1.4 times higher than on P(3HB) scaffolds or on the scaffolds prepared from the copolymer containing 3HHx. The number of cells on randomly oriented P(3HB), P(3HB-*co*-3HV), P(3HB-*co*-4HB), and P(3HB-*co*-3HHx) scaffolds reached 1.81 ± 0.15 × 10^5, 2.33 ± 0.17 × 10^5, 2.41 ± 0.20 × 10^5, and 1.803 ± 0.21 × 10^5 cells/cm^2, respectively. No statistically significant ($p \leq 0.05$) differences were found between P(3HB-*co*-3HV) and P(3HB-*co*-4HB) scaffolds (Figure 5(A)). Also, fiber orientation had a significant effect on the growth and viability of fibroblast cells (Figure 5). It is apparent from Figure 5(B) that the number of viable fibroblasts on all aligned scaffolds was significantly lower (1.14 ± 0.19 × 10^5, 1.56 ± 0.25 × 10^5, 1.83 ± 0.31 × 10^5, and

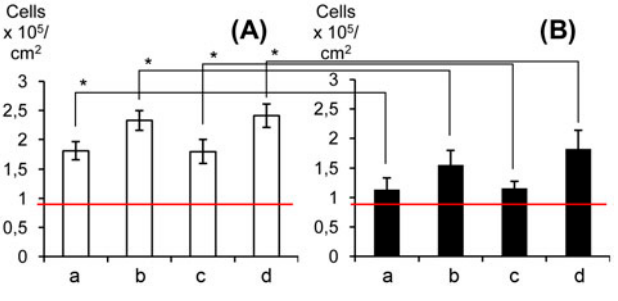

Figure 5. The results of MTT assay in the culture of NIH 3T3 fibroblast cells on fiber scaffolds at Day 7: A – randomly oriented scaffolds; B – aligned scaffolds, prepared from P(3HB) and copolymers with different compositions and similar molar fractions of their second monomers: a – P(3HB); b – P(3HB-*co*-10.5 mol.%-3HV); c – P(3HB-*co*-10.0 mol.%-4HB); d – (3HB-*co*-10.0 mol.%-3HHx) (the line shows the number of cells on polystyrene). $^*p \leq 0.05$ for randomly oriented fibers relative to the aligned ones.

$1.16 \pm 0.12 \times 10^5$ cells/cm^2 at Day 7) than on randomly oriented fibrous scaffolds (Figure 5(A)). The effect of the PHA chemical composition on the number of cells on aligned scaffolds was similar to that on randomly oriented ones; scaffolds prepared from P(3HB-*co*-4HB) copolymers provided the highest increase in cell numbers. On the reference scaffolds (polystyrene plates), the number of proliferating cells was lower too.

Thus, MTT assay showed that all PHA fibrous scaffolds facilitated fibroblast cell growth better than the reference scaffolds. Thus, results of MTT assay suggest that all types of fibrous scaffolds facilitate proliferation of fibroblast cells more effectively than

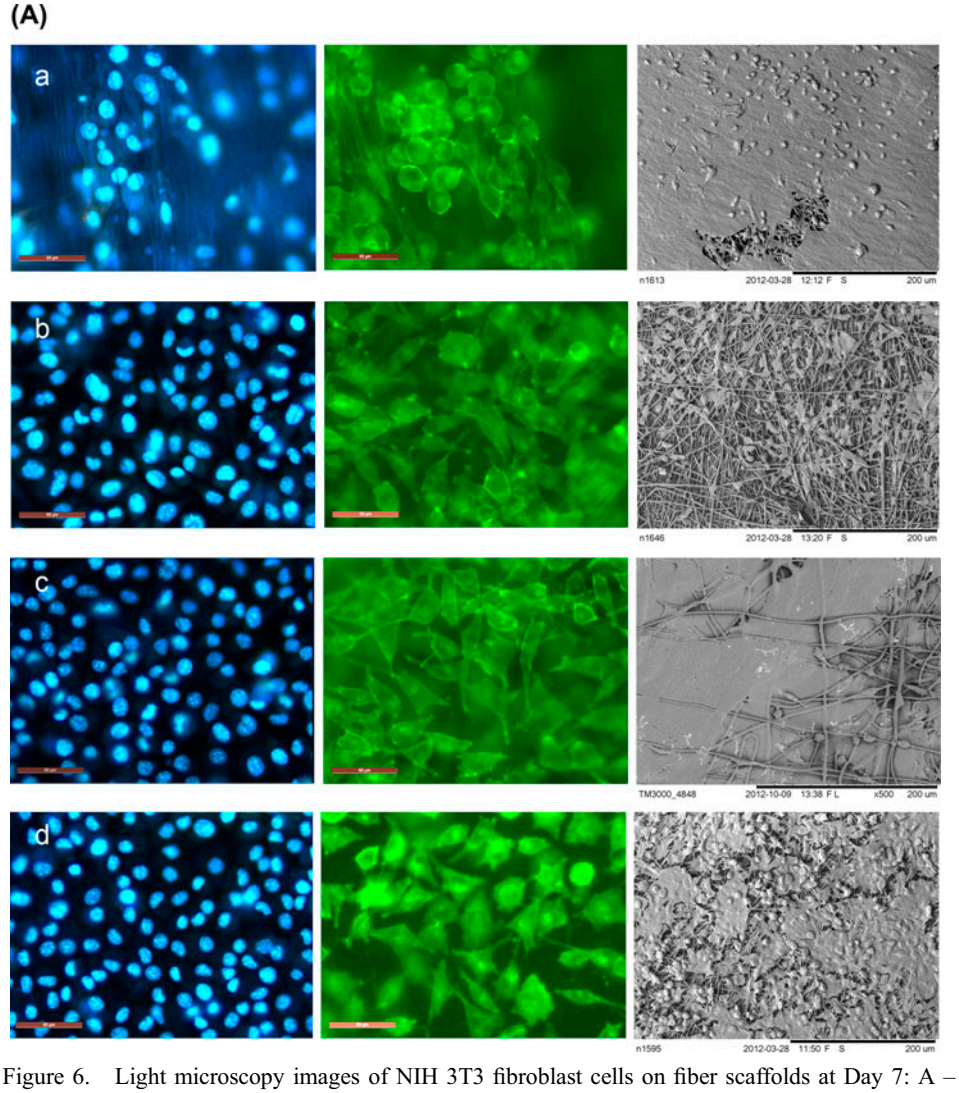

Figure 6. Light microscopy images of NIH 3T3 fibroblast cells on fiber scaffolds at Day 7: A – randomly oriented scaffolds; B – aligned scaffolds, prepared from P(3HB) and copolymers with different compositions and similar molar fractions of their second monomers: a – P(3HB); b – P(3HB-*co*-10.5 mol.%-3HV); c – P(3HB-*co*-10.0 mol.%-4HB); d – (3HB-*co*-10.0 mol.%-3HHx): 1– DAPI staining, bar = 50 μm, 2 – FITC staining, bar = 50 μm, 3 – SEM, bar = 200 μm.

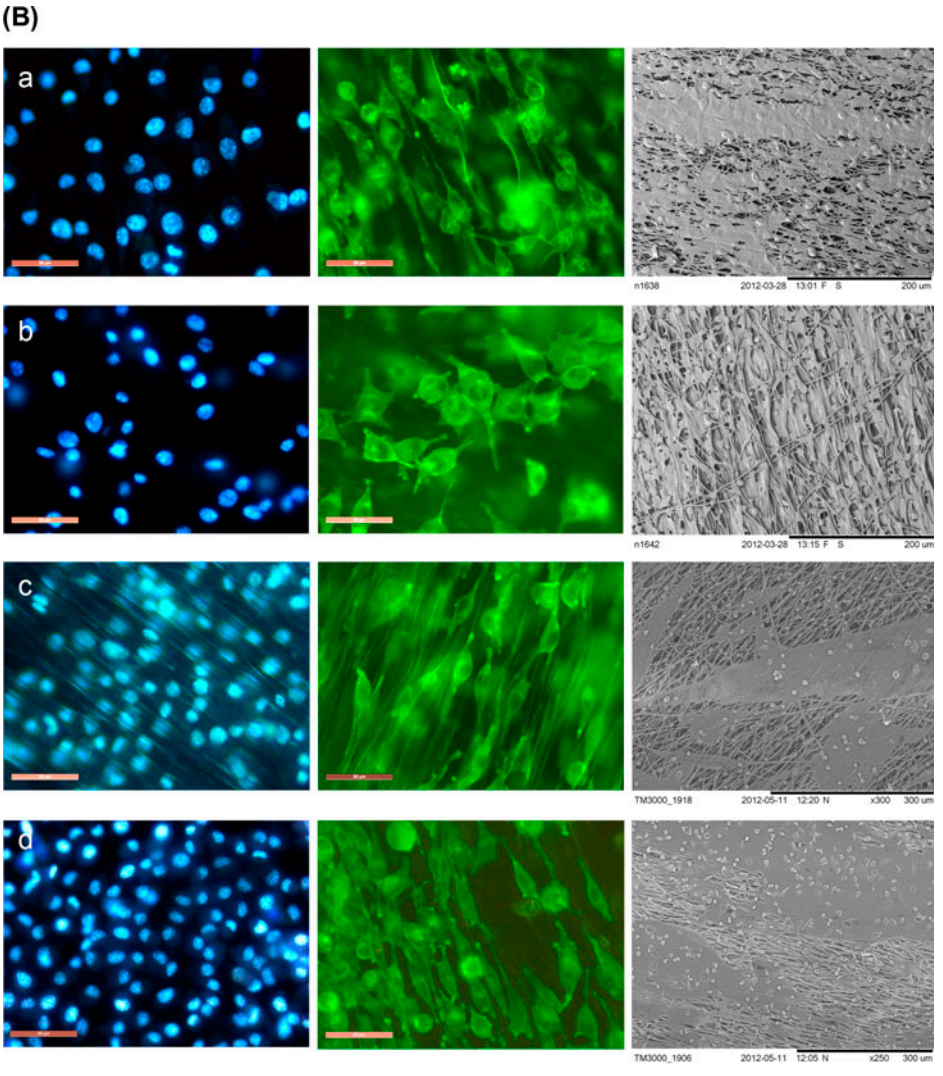

Figure 6. (*Continued*).

the reference scaffolds and that randomly oriented scaffolds are more advantageous for growth and development of this kind of cells than aligned ones.

Figure 6(A) and (B) shows results of investigating fibroblast cell morphology in experiments with cells stained with phalloidin conjugated to FITC and DAPI and SEM images showing the development of extracellular matrix. Results of quantification of cells stained with fluorescent dyes are comparable with results of MTT assay.

On aligned scaffolds (Figure 6(B)), cells, mostly spindle-shaped ones, were arranged along the fibers. On randomly oriented scaffolds, cells were also arranged along the fibers; most cells were triangular or rectangular, with 3–4 appendages, used by cells to attach to the fibers. Larger cells were observed on the randomly oriented fibrous scaffolds. On the aligned P(3HB) scaffolds, the average cell size was $338.06 \pm 45.19\ \mu m^2$, while on randomly oriented scaffolds it was $177.05 \pm 45.20\ \mu m^2$. On randomly oriented scaffolds prepared from PHA copolymers (Figure 6(A)), the

average cell sizes were also greater than on copolymer aligned scaffolds: 381.73 ± 74.97 μm^2 on P(3HB-*co*-3HV), 398.77 ± 92.59 μm^2 on P(3HB-*co*-3HHx), and 429.93 ± 158 μm^2 on P(3HB-*co*-4HB) vs. 387.38 ± 99.31 μm^2 on P(3HB-*co*-3HV), 370.82 ± 91.87 μm^2 on P(3HB-*co*-3HHx), and 274.79 ± 28.59 μm^2 on P(3HB-*co*-4HB).

However, significant differences ($p \leq 0.05$) were only obtained by comparing the results on aligned fibrous scaffolds prepared from P(3HB) and from copolymer PHAs. The differences between randomly oriented scaffolds were insignificant.

SEM images of cells proliferating on aligned and randomly oriented electrospun fibers are shown in Figure 6. Spherical cells prevailed on all types of scaffolds; cell infiltration between fibers was observed. Greater deposits of extracellular matrix protein were recorded on randomly oriented scaffolds (Figure 6(A)).

Thus, all PHA scaffolds tested in this study were biocompatible and facilitated attachment and proliferation of fibroblast cells.

4. Discussion

Electrospinning is a promising and actively developed technique that can be used to produce materials and devices for a wide range of applications, including biomedical ones. By using different materials and varying electrospinning conditions, one can prepare products with tailored properties, which may be used in tissue engineering, including reconstruction of skin, tendons, nervous and bone tissues, etc. For cell and tissue engineering applications, electrospinning can produce structures that have an extensive surface area and closely mimic natural extracellular matrix.[4–7]

In recent years, there has been an increasing interest in the use of biodegradable polymers of biological origin – PHAs – as materials for electrospinning. Owing to high biocompatibility and superior mechanical properties of PHA-based products and the diversity of PHAs, which have different chemical structure and very dissimilar physicochemical properties, these polymers may be promising materials for biomedical uses, including cellular and tissue engineering processes. PHA scaffolds have been successfully used to culture cells of different origins: fibroblasts, osteoblasts, and bone marrow mesenchymal stem cells.[15,19–24,26–28,30,35]

Of all PHA fibers prepared by electrospinning, only the fibers prepared from P(3HB-*co*-3HV) with a low molar fraction of 3HV (2.9–5%) have been investigated in detail.[20–23] The authors found the process that enabled the production of high-quality fibers.[23] Another type of PHA, P(3HB-*co*-3HHx) copolymers containing low molar fractions of 3-hydroxyhexanoate (4 and 8%), was processed by electrospinning and investigated by Wang et al. [27]. Blending of P(3HB-*co*-3HHx) with PLA increased elongation at break of electrospun fibers, probably due to low crystallinity of polylactide, which contains a larger fraction of amorphous regions.[26]

In this study, we used electrospinning technique to produce fibers from PHAs with different chemical compositions and investigated the effects of process parameters and polymer properties on the quality of the products. The use of a high-density P(3HB) solution had a considerable effect on the diameter of electrospun fibers, which increased from 0.45 to 3.14 μm, and this finding was in good agreement with the data reported by other authors.[21] Elongation at break of fibrous scaffolds increased two times, while the strength parameters (tensile strength and Young's modulus) dropped dramatically. A similar effect of fiber diameter on tensile strength and tensile modulus was reported for fibers prepared by electrospinning from different materials, such as

caprolactone fibers.[36,37] Results showing the favorable effect of higher applied voltage on fiber diameter are consistent with the data reported by Tong et al. [21] for fibers produced by electrospinning from P(3HB-*co*-3HV). Cheng et al. [25], however, reported different results: the diameter of the fibers prepared by electrospinning from P (3HB-*co*-3HHx) did not change as the applied voltage was increased. The data reported on other polymers are contradictory: some authors reported an increase in fiber diameter with increasing voltage for polystyrene [38] and polylactide [39] fibers, while others observed opposite results.[40] We also studied the effect of polymer solution feeding rate on fiber properties. As the solution feeding rate was increased, fiber diameter increased. A similar though less pronounced effect was reported by Tong et al. [21] for electrospun P(3HB-*co*-3HV) fibers. In other studies [41,42], no effect of this parameter on fiber diameter was observed. Variations in the working distance from 15 to 30 cm in the Nanon setup did not influence the quality and properties of electrospun P(3HB) fibers. Tong et al. [21], however, reported a significant decrease in fiber diameter (from 5 to 2 μm) with an increase in the working distance from 12 to 22 cm; this result is consistent with the observation made in [43], for electrospinning from polyacrylonitrile solutions.

Differences in the literature data on the effect of electrospinning parameters on fiber properties suggest that special studies need to be conducted for each polymer type in order to determine optimal conditions for the production of high-quality fibers that would differ in their diameter and other properties.

In this study, electrospinning technique was first used to process PHAs with different chemical compositions. These were a homopolymer of 3-hydroxybutyric acid [P(3HB)] and three types of 3HB copolymers with molar fractions of the second monomer units varied from a few percent to tens of percent: P(3HB-*co*-4HB) containing from 6.1 to 48 mol.% 4HB; P(3HB-*co*-3HV) with 4.5 to 19.4 mol.% 3HV; and P (3HB-*co*-3HHx) with 4.9, 10.0 or 13.6 mol.% 3HHx. Such widely varying PHAs were taken for electrospinning because, firstly, PHA properties are determined not only by the second monomer contained in them but also by its molar fraction [44] and, secondly, there are only scant data on the effects of PHAs with different compositions on the properties of electrospun ultrafine fibers.

Fibrous scaffolds produced by electrospinning from different PHAs that contained similar molar fractions (about 10%) of their second monomers (3HV, 3HHx, and 4HB) had similar thicknesses (20 μm) but different average fiber diameters, which varied from 1.7 to 4.2 μm. All copolymer fibers had higher values of elongation at break but lower strength parameters than P(3HB) fibers. Second monomers influenced these parameters to different degrees: all fibrous scaffolds prepared from PHA copolymers showed lower mechanical strength than those prepared from P(3HB), but the presence of 4HB decreased the mechanical strength of fibrous scaffolds more significantly than 3HV or 3HHx did.

The presence of these monomer units had an opposite effect on elongation at break of the fibrous scaffolds. Elongation at break of P(3HB)-based products was the lowest, 13.3 ± 3.11%, while copolymer fibers showed considerably higher values, which increased from 3HHx to 3HV to 4HB. Suwantong et al. [45] compared mechanical strength of the P(3HB) and P(3HB-*co*-3HV) fibers and obtained similar results. Cheng et al. [25] showed that an increase in the HHx fraction from 4 to 8 mol.% caused an increase in Young's modulus but a decrease in elongation at break; the authors concluded that increased 3HHx content did not seem to affect mechanical properties because the effect of the nonuniform fiber diameters became greater than the effect of

3HHx content. Ying et al. and Ishii et al. [29,46] reported that Young's modulus of the as-spun fibrous scaffolds increased in the order of P(3HB-*co*-97 mol.%-4HB) < P (3HB-*co*-7 mol.%-4HB) < P(3HB) < P(3HB-*co*-5 mol.%-3HHx); the authors ascribed the increase in Young's modulus to a decrease in the degree of crystallinity of the scaffolds, caused by an increase in the amorphous phase. It appears to be impossible to compare the literature data and our results in greater detail as published studies describe electrospinning of just a few PHAs, none of which containing high molar fractions of the second monomer (3HV or 3HHx); moreover, there are no available data on electrospinning of PHAs with varied monomer fractions.

The differences revealed in properties of electrospun scaffolds were, in our opinion, caused by differences in PHA properties. The polymers used in this study differed significantly, especially in the degree of crystallinity, which varied from 35 to 76%, depending of the chemical composition of the PHA employed. Also, it is well known that the degree of crystallinity of P(3HB-*co*-3HV) is lower than that of P(3HB) and can vary rather widely: from 39 to 69%.[47] As the molar fraction of 3HV is increased, the polymer becomes less crystalline and more elastic. The presence of 3HV in the polymer has a considerable effect on the crystallization rate and the size of the resultant spherulites. In contrast to P(3HB), P(3HB-*co*-4HB) copolymers are highly elastic polymers, with elongation at break reaching 1000%, or two orders of magnitude greater than elongation at break of P(3HB); the copolymer containing 20–40 mol.% 4HB is a rubber-like material, whose degree of crystallinity is reduced from 60 to 14%.[48] Copolymers of 3HB and 3HHx have rather low melting points; they are rubber-like materials with a low degree of crystallinity (about 40–18%).

The data on the effect of the molar fraction of the second monomer on the properties of the PHA products obtained in this study deserve special attention. Pronounced effect of the molar fraction of the second monomer on the physical-mechanical properties of fibrous scaffolds, Young's modulus, and elongation at break, in particular, was revealed for aligned scaffolds. As the molar fractions of 3HV in P (3HB-*co*-3HV) and 4HB in P(3HB-*co*-4HB) were increased, Young's modulus of the polymers consistently decreased; a particularly significant (40-fold) decrease in Young's modulus was recorded in the polymer containing 48.3 mol.% 4HB. All aligned copolymer scaffolds became more elastic as the molar fractions of the second monomers (3HB, 3HHx, and 4HB) of the PHAs were increased; the increase in molar fractions of 3HV and 4HB resulted in the most remarkable increase in elasticity – by almost 9 and 20 times, respectively. The study revealed electrospinning parameters for the production of high-quality PHA fibers and determined which parameters should be varied to tailor the properties of the products.

Electrospun fibrous scaffolds differing in fiber diameter, PHA composition, and fiber orientation were studied in cell culture. It is well known that surface morphology and fiber orientation of electrospun scaffolds influence cell attachment and growth.

The diameter of electrospun P(3HB) fibers in the range 0.45–2.7 μm seeded with NIH 3T3 mouse fibroblast cells did not influence cell attachment, development, and viability. In contrast, fiber orientation had a significant effect on the growth and viability of fibroblast cells. Results of this study confirm the data reported by other authors,[49,50] suggesting that fiber orientation influences cell morphology irrespective of PHA composition. Which orientation – aligned or random – is more favorable depends on the type of the cells. Aligned fibers are more advantageous for growth and development of osteoblasts, vascular endothelial cells, and neurons than randomly

oriented ones.[6,21,27] Fibroblast cells, however, are rather affected by the porosity of the scaffold than by its being aligned or randomly oriented. [49,51]

Results of MTT assay showed that the number of viable cells on randomly oriented scaffolds made of P(3HB), P(3HB-*co*-4HB), P(3HB-*co*-3HV), and P(3HB-*co*-3HHx) was significantly greater than on aligned scaffolds. Analysis of the effect of the PHA chemical composition on attachment and growth of fibroblast cells showed the best results for P(3HB-*co*-4HB) copolymers. These results are in good agreement with the data reported in other studies, which characterize P(3HB-*co*-4HB), along with P(3HB-*co*-3HHx), as the most suitable type of PHA for cell cultivation.[52] None of the fibrous scaffolds from PHAs, though, had any adverse effects on fibroblast cells, and all of them facilitated cell attachment and growth.

5. Conclusion

This study investigated the main parameters of electrospinning of fibers from solutions of PHAs with different compositions that influenced fiber diameter and properties. The study revealed electrospinning parameters for the production of high-quality fibers from different types of PHA and determined which parameters should be varied to tailor the properties of the products (fiber diameter, surface morphology, and physical-mechanical properties). This study was the first to compare biological and physical-mechanical parameters of PHAs with different chemical compositions as dependent upon the fractions of monomers constituting the polymers and fiber orientation. None of the fibrous scaffolds produced from PHAs by electrospinning had any adverse effects on attachment, growth, and viability of NIH 3T3 mouse fibroblast cells, and all of them were found to be suitable for tissue engineering applications.

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