



In Vitro Characterization of Poly(Lactic Acid)/ Poly(Hydroxybutyrate)/ Thermoplastic Starch Blends for Tissue Engineering Application

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Abstract

Complex in vitro characterization of a blended material based on Poly(Lactic Acid), Poly(Hydroxybutyrate), and Thermoplastic Starch (PLA/PHB/TPS) was performed in order to evaluate its potential for application in the field of tissue engineering. We focused on the biological behavior of the material as well as its mechanical and morphological properties. We also focused on the potential of the blend to be processed by the 3D printer which would allow the fabrication of the custom-made scaffold. Several blends recipes were prepared and characterized. This material was then studied in the context of scaffold fabrication. Scaffold porosity, wettability, and cell-scaffold interaction were evaluated as well. MTT test and the direct contact cytotoxicity test were applied in order to evaluate the toxic potential of the blended material. Biocompatibility studies were performed on the human chondrocytes. According to our results, we assume that material had no toxic effect on the cell culture and therefore could be considered as biocompatible. Moreover, PLA/PHB/TPS blend is applicable for 3D printing. Printed scaffolds had highly porous morphology and were able to absorb water as well. In addition, cells could adhere and proliferate on the scaffold surface. We conclude that this blend has potential for scaffold engineering.

Keywords

PLA/PHB/TPS blend, 3D printing, viscosimetry, porosity, biocompatibility, tissue engineering

Introduction

Tissue engineering (TE) is an interdisciplinary field that aims to repair various damaged tissues. Scaffolds belong to its major building blocks. They provide mechanical support for the proliferating cells and enable them to create newly formed 3D tissue by directing cell growth. For in vivo application, the material used for the scaffolding has to meet several requirements, most importantly biocompatibility and biodegradability^{1–3}. The importance of biodegradation is underlined with the fact that this process enables tissue remodeling. Therefore, current studies are mainly oriented on the materials, which can provide this requirement. In addition to this, proper architecture together with the physical properties facilitate cell adherence, distribution as well as differentiation into desired cell lines^{4,5}. When suitable polymers are applied, their degradation rate respects the regeneration process of the damaged organ so the formation

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of the new tissue is a regulated and balanced process⁶. Despite the fact that various materials (both natural and synthetic origin) had been already studied in the context of TE and regenerative medicine, reconstruction of the large tissue defects still remains challenging as the sufficient vascularization for the newly engineered tissue seems to be the burning issue. Sufficient neovascularization requires a highly porous structure of the scaffold⁷. It has been previously described that micropores facilitated ingrowth of the blood vessels and macropores influenced cell distribution and cell to cell interactions⁸. In the presented study, we focused on the blended scaffold consisting of Poly(Lactic Acid), Poly(Hydroxybutyrate), and Thermoplastic Starch (PLA/PHB/TPS). Each polymer had been already investigated alone or as part of various blends in various medical fields (e.g., orthopedics, cardiology, neurology, urology)^{9–11}. However, according to the best of our knowledge, no research in the field of tissue engineering and regenerative medicine has studied a blend composed of this particular formula so far.

Poly (lactic acid) (PLA) belongs to a poly-lactone family and is currently considered to be the most promising component for scaffolding because of its excellent biological and mechanical properties and processability. PLA is synthesized from lactic acid, which exists in two optically active stereoisomers, L and D. Ratio of L and D form of lactic acid in PLA macromolecules has a significant effect on its resulting properties (crystalline phase volume, mechanical properties, thermal properties, degradation rate)¹². The first step of PLA degradation is run by hydrolysis in the amorphous phase. Optically pure PLA (semicrystalline polymer) degrades slower than racemic (amorphous polymer). Choosing appropriate PLA enables to set biodegradation rate according to the needs of the tissue. However, characteristics such as degradability during processing, brittleness present PLA disadvantages. Moreover, some studies also pointed out that degradation metabolites could cause an inflammatory reaction *in vivo*. On the other hand, these disadvantages can be reduced or completely suppressed by combination with other polymers or by the addition of specific additives¹³.

Poly (3-hydroxybutyrate) (PHB) is a polyhydroxyalkanoate which can be produced by prokaryotic cells. Within the field of material science, its attractiveness is related to the good biocompatibility, biodegradability, and mechanical properties of this polymer¹⁴. PHB is a highly crystalline polymer with a crystalline phase content from 50% to 80%¹⁵. The high content of the crystalline phase makes this polymer brittle. Another disadvantage is susceptibility to thermal degradation, which complicates its thermoplastic processing. PHB degradation *in vivo* is a similar process as PLA degradation but lasts longer due to the high content of the crystalline phase¹⁶. PHB hydrolysis metabolites are non-toxic and physiologically occur in the human body¹⁷. When combined with PLA, flexible material from these brittle polymers can be engineered¹⁸.

Starch is a polymeric carbohydrate consisting of two different polysaccharides which are amylose and amylopectin. It had been described as a promising material for scaffold engineering thanks to its properties such as natural occurrence, biodegradability, and cost-effectiveness. Hydroxyl groups from D-glucopyranose units make starch highly hydrophilic and therefore proper biodegradation rate can be obtained. When combined with other polymers, biocompatible matrices with highly porous structure and good mechanical properties can be fabricated¹⁹. For the purpose of this study, thermoplastic starch was prepared from maize starch with glycerin and water.

This study is focused on *in vitro* complex characterization of five blends (labelled as 1–5) based on the combination of PLA, PHB, and TPS. Presented manuscript is divided into two main parts: material studies and biological studies. Within the evaluation of the material characteristics, the attention was also drawn on the possibility of tested blends to be used for 3D printing. The aim was to engineer tubular scaffolds, as repairing of the tubular tissues such as urethra or blood vessels is still challenging for the scientists. Moreover, computer aided technology is currently one of the most applied technologies within the fields of TE and regenerative medicine. The influence of the viscosity of each component on the scaffold morphology was studied as well. Other material properties included the analysis of the morphology, porosity, and ability of the material to absorb the water. The main aim of the biological studies was to evaluate biocompatibility of the tested blends as well as the cell-scaffold interactions. For the biocompatibility studies, blends were molded into thin 2D films. Cell adherence on the scaffold surface was performed on 3D planar scaffolds.

Materials and Methods

Preparation of the PLA/PHB/TPS Blend

To prepare the blends, we used PLA from Nature Works LLC (Minnetonka, MN, USA) marked Ingeo Biopolymer 4060D, PHB Biomer[®] batch T22 (TianAn Biopolymer, China) and TPS obtained from the company Panara (Panara Ltd., Nitra, Slovakia). We worked with three different types of TPS with different viscosity. Five different blends with different concentrations or types of TPS were tested. Blends recipes are described in Table 1. PLA/PHB ratio was 60:40. For the better mechanical properties, citrate was used as a plasticizer. Detailed recipes of the blends are under patent protection.

Blends were prepared by twin screw extruder from Lab-Tech (LabTech, Bergamo, Italy) with co-rotating and fully intermeshing screws. Diameter of screws was 16 mm, L/D ratio 40.

3D Printing of the Scaffolds

For the engineering of the scaffolds, we used the filament which was prepared on a single screw extruder Plasticorder

Brabender (Brabedner, Duisburg, Germany) with the following parameters: $L/D = 25$, diameter 19 mm, and screw compression ratio 1:3. The melt was extruded through a perpendicular nozzle with a diameter of 2.5 mm. In the next step, the melt was cooled by the water. The filament was processed by 3D printer Prusa I3 (Prusa Research, Prague, Czech Republic). Two types of scaffolds were prepared in the form of tubular or planar structures (Fig. 1). The initial input data for the printing of the planar scaffolds were $3 \times 3 \times 0.2$ cm. For the tubular scaffolds, following parameters were set: diameter 0.8 cm, wall thickness 0.2 cm and the height 2 cm.

Viscosimetry

The viscosity of the blend units was evaluated by the capillary rheometer GOTTFERT RG 20 (Göttfert, Buchen, Germany). The length of the capillary was 20 mm and it had a diameter of 2 mm. Tests were run at a temperature of 185°C. Shear rate range was from 36 to 2160 s^{-1} . The flow curves were corrected for non - Newtonian fluid by Rabinovich's correction. The rheological data and coefficients "n" were adjusted for the Ostwald-de Waele model.

$$\tau = K \cdot \dot{\gamma}^n$$

$$\eta = d\tau/d\dot{\gamma}$$

Table 1. Composition of Studied Blends.

Sample No	TPS viscosity* (Pa.S)	TPS contains (wt%)	TPS contains (vol%)
1	0.26	30	26.2
2	0.26	20	17.6
3	0.26	10	8.4
4	0.66	20	17.6
5	1.67	20	17.6

*Viscosity determined by capillary rheometer Goettfert RG 20 at shear rate 2000 1/s according to procedure in 2.1.3.

$$\eta = n \cdot K \cdot \dot{\gamma}^{n-1}$$

Mechanical Properties of the Filaments for 3D Printing

Tensile tests were performed on a Zwick-Roell testing machine (Zwick Roell Group, Ulm, Germany) at a crosshead speed of 50 mm/min in accordance with ISO 527. The tensile strength at break (σ_b) and the relative elongation at break (ϵ_b) were evaluated from the tensile curve.

Morphological Analysis of the Scaffold

The microarchitecture of the tubular scaffolds was analyzed by scanning electron microscope JEOL 7500F (Jeol Ltd., Tokyo, Japan). All samples had been pretreated in the boiling water for 3 hours in order to leach out the starch which served as a porogen. Samples were longitudinally broken in liquid nitrogen and brittle fractions were fixed on the mounting discs by 2-component epoxy paste. Specimens were sputter-coated with gold and platinum in an argon atmosphere for 60 s. ImageJ software was used to measure pore size. Fifty pores from each sample were randomly chosen at different sites and measured.

Porosity

The porosity of the tubular scaffolds was determined using Archimedes' principle with the ethanol used as a liquid medium. Porosity was calculated via the following equation:

$$\text{Porosity (\%)} = (w_2 - w_3 - w_s) / \rho_E / (w_1 - w_3) / \rho_E,$$

where w_1 is the weight of the cylinder tube filled with ethanol, w_2 is the weight of the cylinder tube containing ethanol and scaffold, w_3 is the weight of cylinder tube taken out of ethanol—saturated scaffold, w_s is the weight of the scaffold, and ρ_E is the density of the ethanol. The experiment was run three times in order to obtain statistically relevant results.

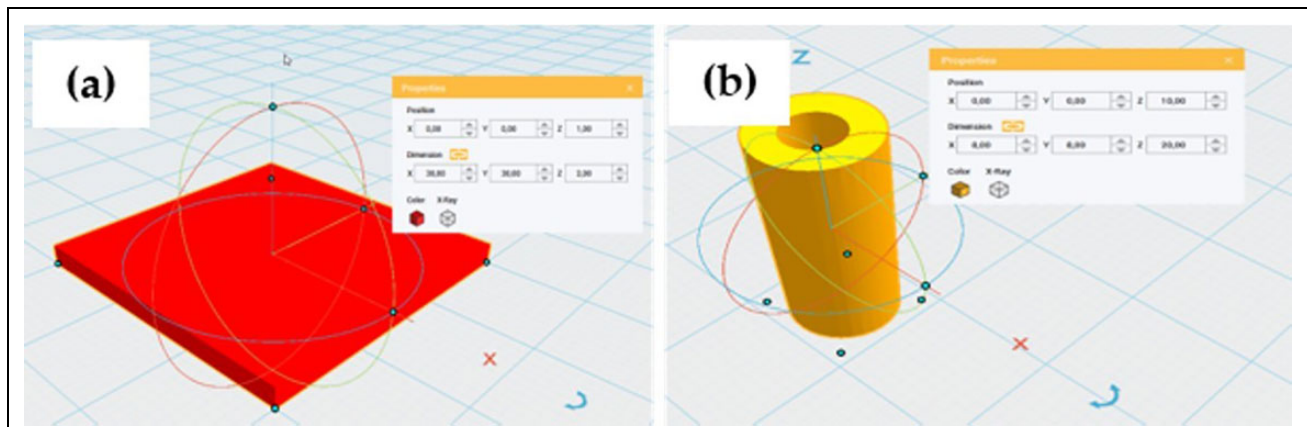


Figure 1. The scheme of the scaffolds for intended for 3D printing. Schemes were processed by software XYZmaker 1.2 (XYZprinting, Inc., Taiwan).

Water Uptake Test

The swelling ratio of the tubular scaffolds was calculated via the following equation:

$$\text{Swelling ratio (\%)} = (\text{Ww} - \text{Wd})/\text{Wd} \times 100$$

Firstly, all samples needed to be pretreated in the boiling water as mentioned above. Scaffolds were placed into a dry incubator for 48 hours and their dry weight (Wd) was measured on analytical weights (Kern, Frankfurt am Main, Germany). Subsequently, scaffolds were immersed in PBS and their wet weight (Ww) was measured at the predetermined time (24 hours, 2 weeks). Finally, the swelling ratio was calculated and collected data were transferred into a graph. All samples were tested in triplicates.

Cell Culture

Because of the large supply of the chondral tissue, chondrocytes were chosen to be used for the further experiments. Samples were harvested in accordance with The Helsinki Declaration and isolated cells were stored in liquid nitrogen at the Institute of the Medical Biology, Genetics, and Clinical Genetics of the Comenius University (Bratislava, Slovak Republic). After the refreezing, chondrocytes were cultured in Gibco DMEM/Ham's F12 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum (FBS, Biosera, Nuaille, France), 1 mmol/L of glutamine (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL of Penicillin and 100 µg/mL of Streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were plated into Petri dishes and cultured in an incubator at 37°C in a humidified atmosphere containing 5% CO₂. After 24 hours, medium together with non-adherent cells were removed. Subsequently, the medium was changed every 3 days. After reaching 80% confluence, cells were passaged using 0.05% trypsin (Sigma-Aldrich, St. Louis, MO, USA).

Testing of the Biocompatibility

Tested samples differed in the content of the TPS. Therefore, sample 1 was used because it contained the highest ratio of TPS, for the biocompatibility studies, as we had not had information about its biological behavior, which needed to be established. Cell proliferation served as an indicator of the material biocompatibility which was detected by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) test and direct contact cytotoxicity test²⁰. Firstly, the MTT test was performed on chondrocytes in the co-culture with the tested material. Cells at the density of 0.4×10^5 cells per well were seeded in 24-well plates and left to adhere for 24 hours. Sample 1, in the form of 2D molded films, was cut into squares with a size of 0.5×0.5 cm. Samples were sterilized by UV light (30 minutes per each side) and immersed in a chondrocyte culture medium for 24 hours. On the next day, films were added to the cell cultures

and left to affect the culture system for 24, 48, and 78 hours. After these time intervals, films together with the culture medium were removed. Subsequently, 300 µL of the fresh medium together with 30 µL of the MTT reagent (Cell Titer 96[®] Aqueous One Solution Reagent, Promega, Madison, WI, USA) were added into each well. Cells were cultured at 37°C with 5% humidified CO₂ for 3 hours. ELISA micro-reader BioTek EL800 (BioTek Instruments Inc., Winooski, VT, USA) was used to measure the absorbance at the wavelength of 490 nm. Chondrocyte cell cultures without PLA/PHB/Starch blend were used as positive controls. All MTT tests were performed in triplicates. Obtained data were analyzed and processed into graphs.

For the direct contact cytotoxicity test, 0.6×10^5 cells per well were seeded in 24 well-plates and cultured in DMEM/Ham's F-12 medium supplemented with 1mmol/l of glutamine, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 10% FBS at 37°C with 5% humidified CO₂ atmosphere. Sterilized 2D films, from the sample 1, were added into seeded wells and left to affect cell culture for 7 days. Cell proliferation and morphological changes were studied using an inverted light microscope (Zeiss Axiovert 100, Carl Zeiss, Jena, Germany).

Cell Seeding of the Scaffolds

Scanning electron microscopy (SEM) was used to evaluate the cell attachment and proliferation on the scaffold surface. According to the previous tests, sample 4 was chosen for the cell seeding experiments because of its high porosity. In brief, sterile planar 3D scaffold was placed at the bottom of the 24-well plate and seeded via droplet technique with chondrocytes. 8×10^4 cells were resuspended in 30 µL of culture medium and placed as a droplet on scaffold. After the 3 hours of the initial cell adhesion, 0.5 mL of culture medium was added into each well. Samples were cultured for 2 weeks and the medium was changed every 3 days. For the SEM analyses, sample was prepared as follow. At first, specimen was washed twice with PBS and fixed in 2.5% glutaraldehyde. Increasing concentrations of ethanol were used for dehydration and sample was subsequently left to air-dry overnight. On the next day, sample was mounted using carbon adhesive tape, sputter-coated with gold and palladium, and observed by SEM JEOL 7500F. Experiment was run in triplicate.

Statistical Analysis

All experiments were run in triplicates. Values were expressed as mean \pm standard deviation (SD). One-way ANOVA followed by a Bonferroni and Holm post hoc tests for multiple comparisons were used when appropriate. $P < 0.01$ and $P < 0.05$ were considered as statistically significant. Presented study was not blinded.

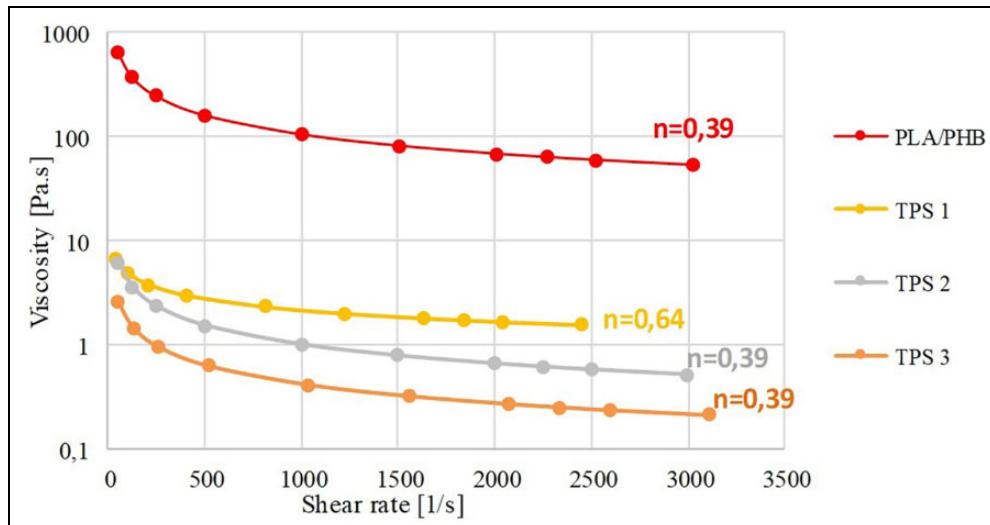


Figure 2. The flow curves of TPSs and PLA/PHB matrix at temperature 185°C. Correlation coefficients n from the Ostwald-de Waele's model.

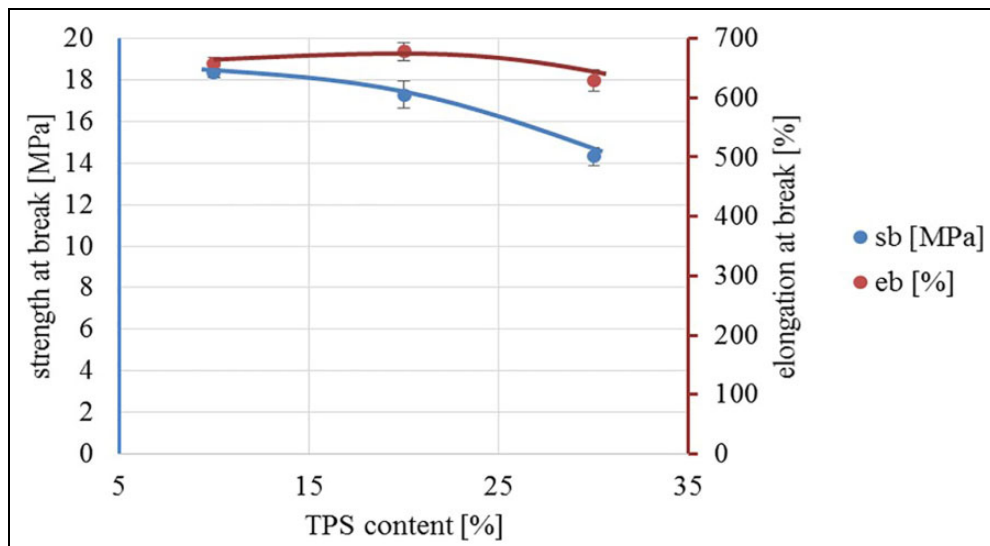


Figure 3. The dependence of strength at break (σ_b) and the relative elongation at break (ϵ_b) on TPS concentration.

Results

Viscosimetry

In the case of polymer blending, the blend configuration preferably depends on the viscosity ratio of the blended polymers. For this reason, the rheological properties of the blends were monitored, based on these data, it is possible to predict the distribution/dispersation of TPS domains in the PLA/PHB matrix.

All polymers had a pseudoplastic flow character. The individual types of TPS have sufficiently different viscosity, especially at higher shear rates (Fig. 2). The PLA/PHB matrix has a significantly higher viscosity than TPSs. Therefore, it is an assumption that TPS would form a continuous phase even at lower concentrations than 50% in the PLA/

PHB matrix due to the lower viscosity of TPS and at the same time, the PLA/PHB domains would be relatively large due to the big difference in the viscosities of the components.

Mechanical Properties of 3D Filaments

For the processability in a 3D printer, it is necessary that 3D filament has adequate mechanical properties that ensure its printability. The filament must be strong and flexible enough to ensure correct feeding and to prevent its destroying before entering to the nozzle. The continuous supply of printed material to the 3D printer's nozzle has to be ensured as well. The dependence of strength at break (σ_b) and the relative elongation at break (ϵ_b) on TPS concentration was evaluated (Fig. 3). As the TPS content increases, the tensile strength of

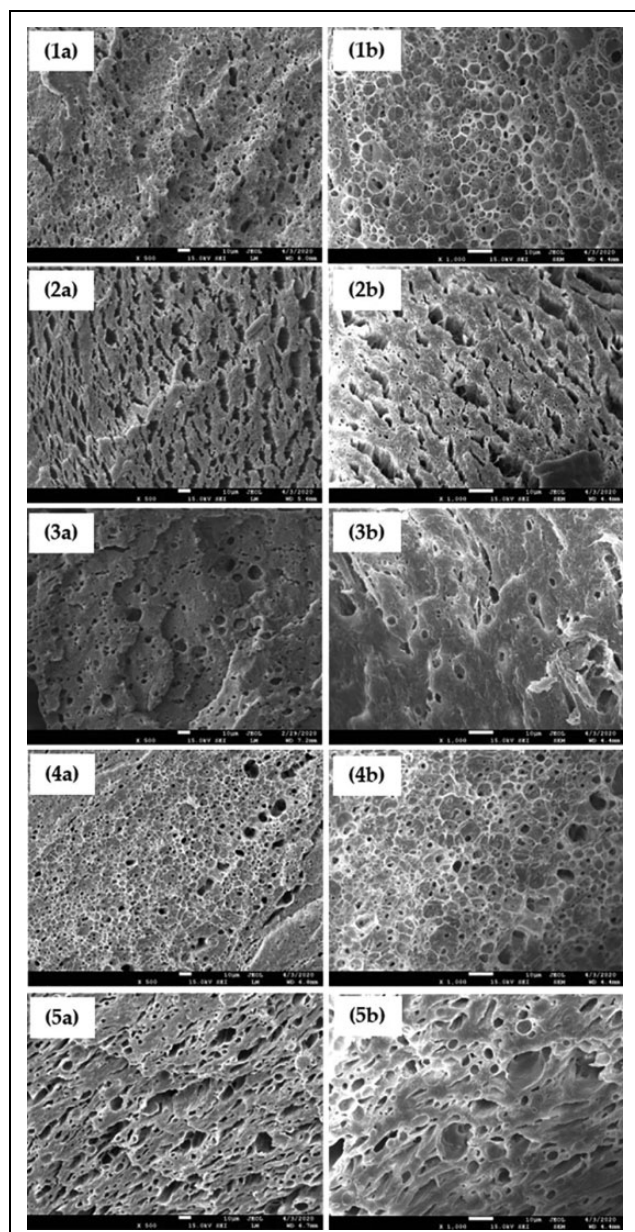


Figure 4. Microstructure of the 3D tubular scaffolds. Morphological analysis performed on SEM (JEOL 7500F, Japan) revealed that different porous structures had been engineered (1-5). Interconnected micropores with the round shape were observed in 3 samples (1, 3, 4). Elongated pores with the different orientation were detected as well (2, 5). Mean largest pore size obtained by starch leaking was 7.55 μm (3a, 3b). For the image acquisition, accelerating voltage was set to 15 kV and all samples were magnified 500 \times and 1000 \times .

the filament decreases. At the lowest TPS content, the tensile strength was approximately 18 MPa and at the highest content approximately 14 MPa. The TPS content, in the range from 10 wt.% (8.4 vol.%) to 30 wt.% (26.2 vol.%), does not have a significant effect on the elongation at break of the filament. Blends have relatively high elongation - approximately 600% in the whole range of TPS content. When these value are compared with mechanical properties of

Table 2. Pore Measurement of the 3D Printed Scaffolds. According to Our Results, Micropores, with the Size Varying form Approximately 1.6–19.4 μm , Were Formed After the Starch Leaked Out from its Domain. All Samples Were Pre-Treated in the Boiling Water.

Sample No	Mean pore size (μm)	SD	min. (μm)	max. (μm)
1	6.53	2.363	2.522	15.561
2	6.078	4.385	1.258	22.359
3	7.55	3.49	3.273	21.664
4	5.818	2.282	1.838	10.48
5	7.032	3.769	1.602	19.419

Table 3. Summary of the Obtained data Applied in the Calculation of the Porosity. Each Sample Was Measured Three Times. We Assigned the Result of the Low Porosity, Calculated in Sample 5, to the Insufficient Leakage of the Starch.

Sample No	\varnothing w1 (g)	\varnothing w2 (g)	\varnothing w3 (g)	\varnothing ws (g)	ρ ethanol (g/cm^3)	\varnothing porosity (%)	\varnothing STDV
1	10.875	11.862	10.612	1.033	0.789	82.6	0.062
2	10.875	11.793	10.631	0.994	0.789	81.5	0.050
3	10.875	11.727	10.681	0.891	0.789	80.0	0.086
4	10.875	11.833	10.661	0.986	0.789	86.7	0.096
5	10.875	11.266	10.666	0.537	0.789	30.2	0.090

polycaprolactone (PCL) it can be see they reached a similar value of elongation at break but PCL had higher value of tensile strength (26,1 MPa). It should be noted that while we observed the mechanical properties of the filament (round cross section), in the previously published article authors observed the properties of the thin sheet (rectangular cross section)²¹.

These parameters guarantee sufficient mechanical properties of the studied 3D filament for its application in a 3D printer.

Scaffold Morphology and Porosity

In accordance with the previous results, we managed to engineer both planar and tubular scaffolds using computer-aided technology (3D printing). When analyzed under SEM, starch proved to be a suitable porogen as we harvested highly porous structures. According to the composition of the blends, we obtained five types of scaffolds with different pore sizes, pore distribution, and orientation (Fig. 4). Pores observed in samples 1, 3, and 4 had circular shape. When compared with samples 2 and 5, harvested images depicted more elongated pores. Pore size measurement revealed that the mean size of the pores was smaller than 10 μm (Table 2). The calculated porosity of most of the structures was $\geq 80\%$ except for one sample, in which calculations revealed a small porosity index. We assigned this phenomenon to the insufficient leak of the starch (Table 3).

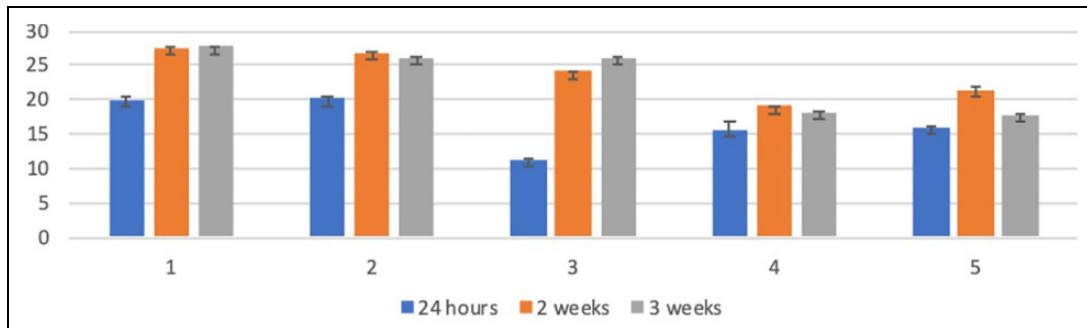


Figure 5. Water uptake test. During the first 24 hours, the highest rate of water uptake was approximately $20\% \pm 0.35$ SD. When immersed in PBS for 2 weeks, an increased swelling ratio was measured in several samples ($27\% \pm 0.07$ SD). Weight loss detected after the 3-week analysis might have indicated the ongoing process of biodegradation.

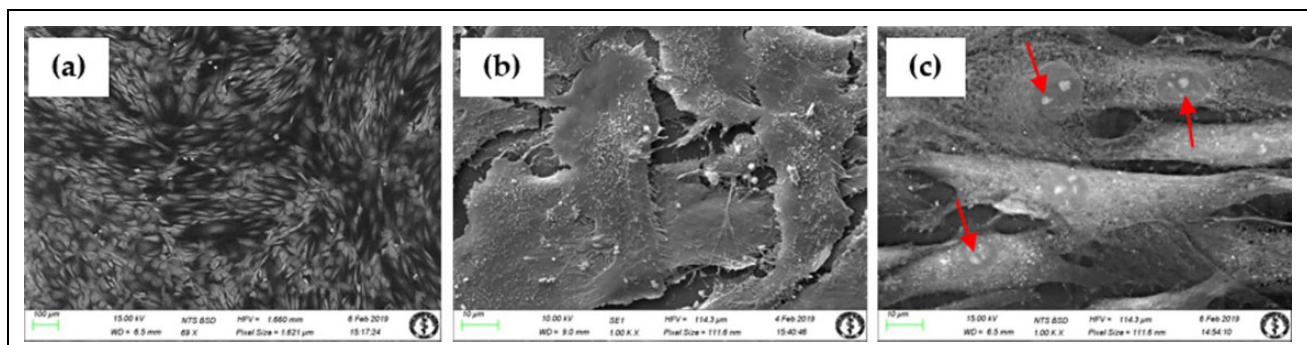


Figure 6. Morphological analysis of the cell culture. After the 7 days of the initial seeding, cells formed a confluent colony (a). Detailed shape analysis showed that chondrocytes had flattened shape (b). Application of the backscattered electron detector revealed multiple nucleoli present in the cells' nuclei, which indicated protein translation activity (c, red arrows). Images were acquired at accelerating voltage of 10 kV (b) or 15 kV (a, c). Images were magnified $60\times$ (a) and $1000\times$ (b, c).

Swelling Behaviour of the Scaffolds

According to our results, all samples were able to absorb water while being immersed in PBS for 24 hours. When hydrated for a longer time period, only discrete water uptake was detected in 2 samples (1 and 3). What is more, weight loss was measured as well which reflected the process of biodegradation (Fig. 5).

Morphology of the Cell Culture

Morphological analysis of the cell cultures intended for the biocompatibility studies was performed using SEM (Fig. 6). Within a week after the initial seeding, chondrocytes formed a confluent colony and maintained their typical fibroblast-like shape (Fig. 6a). During the passaging, cell proliferation capacity did not decrease and no morphological changes were detected. This suggested that this cell line was stable and therefore suitable for our further experiments. Flattened cell morphology was observed when cell culture was analyzed by SEM (Fig. 6b). We could also observe nucleoli after backscattered electron detector had been used. Nucleoli are parts of the cell nucleus and their multiple occurrences

indicated high proliferation activity of the chondrocytes (Fig. 6c). Passage number 10 was used in all cell tests.

Biocompatibility Studies

The results of the MTT test (Fig. 7) evaluated after 24, 48, and 72 hours showed that material composition of the sample 1 did not have any significant inhibitory effect on cell proliferation. A slight decrease in the proliferation level was measured during 48 hours of the culture when compared to control group at 24 hours. However, we contributed this phenomenon to the cell adaptation process. Importantly, the proliferation capacity of the chondrocytes was up to 99% after 92 hours of the co-culture with the PLA/PHB/TPS blend. This indicated that the tested material, especially TPS, did not have a toxic effect on the cell culture.

Figure 8 presents the result of the direct contact cytotoxicity test performed with the sample 1. After 7 days of co-culture, PLA/PHB/TPS blend (did not influence the morphology and proliferation capacity of the cells. When compared to the control group (Fig. 8a), cells maintain fibroblast-like shape and could form a compact monolayer in the co-culture with the tested material. This finding also supported the fact that no toxic metabolites had been leaked out.

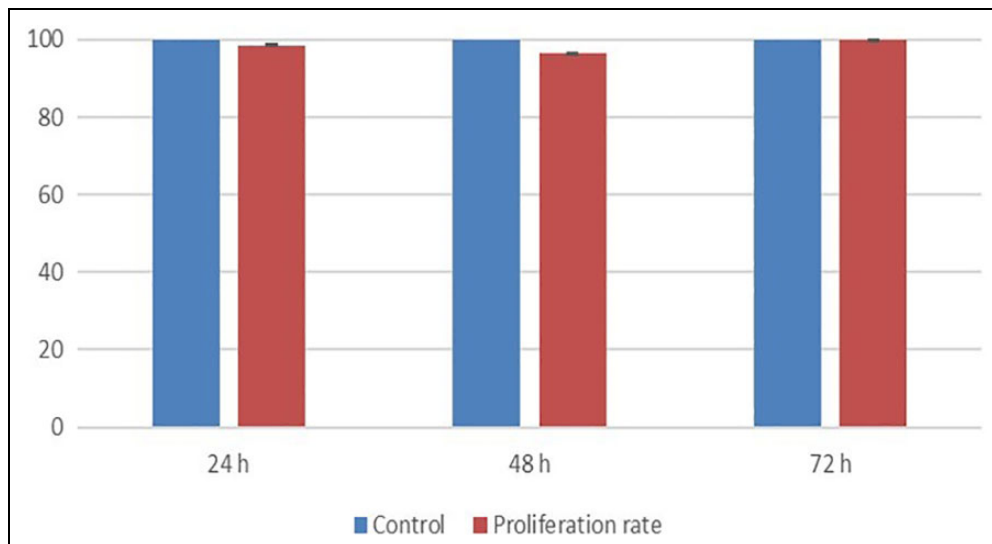


Figure 7. The MTT test results. Data are presented as the mean \pm SD. No significant decrease of the cell proliferation rate was detected when co-cultured with the PLA/PHB/TPS blend.

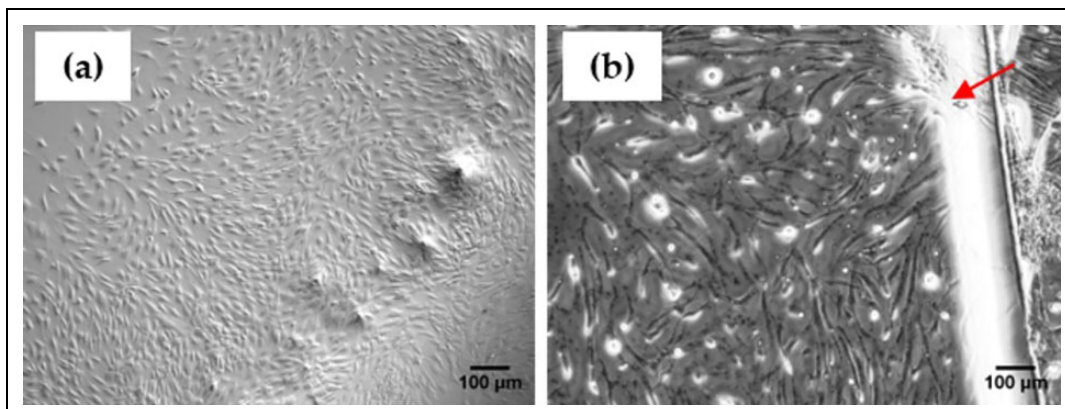


Figure 8. The evaluation of the cytotoxic potential of the sample I according to direct contact cytotoxicity test. The control group was presented as a standard culture of chondrocytes with fibroblast-like morphology (a). PLA/PHB/TPS scaffold, which influenced cell culture for a week, did not alter cell morphology or viability, as they could form overgrown structure in the scaffolds vicinity (b, arrow depicting the edge of the 2D film). The Images were taken by inverted light microscope (Zeiss Axiovert 100, Carl Zeiss, Germany).

Evaluation of the Cell Adherence on the Scaffold

SEM analysis of the interference between cells and 3D planar scaffold showed that cells could attach, proliferate, and form multilayered structure on the scaffold surface, covering its entire area (Fig. 9). The surface of the unseeded scaffold seemed to be slightly rough, which could have been caused by the presence of the TPS in the blend (Fig. 9a). Multiple filopodia, which are cytoplasmic projections of the migrating cells, underlined the successful attachment (Fig. 9b). The highest cell concentration was observed in the center of the cell-seeded construct. Cells had typical fibroblast-like morphology. Regarding the mentioned results, we assumed that the tested PLA/PHB/TPS blend is biocompatible and therefore has the potential for the tissue engineering application.

Discussion

The presented study was focused on the characterization of the blended material based on PLA/PHB/TPS in the context of scaffold fabrication. The potential of the single polymers had been already studied and the outcomes had confirmed that these biocompatible and biodegradable polymers were suitable for application in TE^{22–24}. Moreover, their significance is in fact that they belong to materials approved by the U.S. Food and Drug Administration (FDA) for clinical applications, such as surgical suture material and implants.²⁵ However, we had not worked with TPS in the past so we needed to prove its biocompatibility along with applied plasticizers. Because of this, the sample with the highest ratio of TPS was chosen for evaluation of biocompatibility.

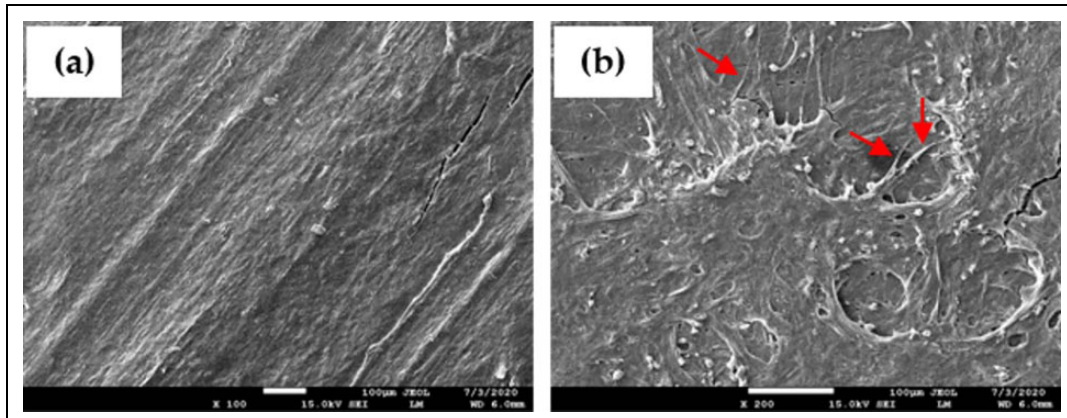


Figure 9. Cell-scaffold interaction. Unseeded PLA/PHB/TPS scaffold presented a control group (a) for cell-seeding experiments. After 2 weeks of culture, cells could form a multilayered structure on the scaffold surface (b). The presence of the multiple filopodia indicated effective attachment to the surface (b, red arrows). Images were acquired at the accelerating voltage of 15 kV and were magnified 100 \times (a) or 200 \times (b).

At first, properties of the blend were assessed. Viscosity was evaluated using a capillary rheometer that simulates the flow of material through a 3D printer nozzle. It was determined that the viscosity of the individual components had a significant effect on the morphological properties of the scaffolds. It will be possible to regulate the structure of scaffolds based on PLA, PHB, and TPS according to the tissue needs by influencing the viscosity (the composition of the blend, technological parameters). TPS has a lower viscosity than PLA/PHB matrix and therefore, it is an assumption that TPS would form a continuous phase even at lower concentrations than 50%. If the concentration of TPS in the PLA/PHB matrix would be low enough to form a continuous phase it can be assumed that the TPS domains in the highly viscous matrix at sufficiently high shear rates will be small. It is possible to expect that not only the concentration of TPS but also shear rates could cause a significant change in the morphology of the scaffold. Also, slow cooling during scaffold construction can lead to the formation of clumps and larger domains. This could influence the morphology by creating larger pores. It could be possible to regulate the morphology of the scaffolds by choosing the proper processing parameters.

Another goal of the material study was to evaluate the printability of the material. The prepared filaments fulfilled both physical and mechanical requirements for the 3D printing applications. The flow in the printer nozzle was continuous, there was no tearing of the filament.

MTT assay and direct contact cytotoxicity test were applied in order to evaluate the biological behavior of composite material. Chondrocytes were chosen as an affected cell type for both biocompatibility tests. MTT assay was applied in order to study the instant impact of the polymers on the cell culture during short-time cultivation. We considered results obtained from the 3-day culture as the most relevant because, during the first 48 hours, the process of the cell adaptation might have influenced their vitality. Most

importantly, the proliferation rate after 72 hours of co-culture with the tested material was nearly 100%. This phenomenon result indicated that scaffold did not negatively affect the biological behavior of the cell culture and created a suitable environment for cells to proliferate. A recent study carried out by Ehlert et al. also described MTT assay as the test of choice for biocompatibility studies of specific alloy material²².

The potential toxic effect of the scaffold during long-term cultivation was analyzed by direct contact cytotoxicity test. Adverse influence might have been caused by the degradation products of the polymers. Although metabolites derived from PLA, TPS, and PHB do not present any biological risk but their altered properties might be caused by additives needed for polymer processing. These can negatively affect the pH of the culture environment and thus decrease cell viability and proliferation. According to our results, PLA/PHB/TPS scaffold did not negatively affect surrounding cells. Moreover, cell migration from the scaffold back to the culture well was observed as well. This fact indicated that cells could attach and fully cover the surface of the matrix so extensive proliferation was present. This phenomenon was also confirmed by SEM.

Morphological analysis of the scaffold revealed a highly porous structures which is the crucial property as the inner porous architecture enables effective and throughout cell incorporation. Moreover, pores also play role in neovascularization and mass transfer. The importance of the porosity was highlighted in a study carried out by Liu et al.²³. In this research, a cell-seeded silk fibroin/3D bladder acellular matrix was used as a composite scaffold in order to repair the complex defects of the urethral wall (animal model). High density of the macropores was achieved by electrospinning and was crucial for successful and efficient cell seeding. In our study, we attained porous architecture by starch leaching. Although we were satisfied with the overall highly porous structure, a bigger pore size would be needed. 3D printed

tubular scaffolds had the biggest pore size of approximately 22 μm . However, it has been pointed out that macro pores ($>100 \mu\text{m}$) are essential for cell distribution, nutrition flow, and vascular ingrowth. On the other hand, micro pores play role in ECM formation^{26,27}. Ideally, scaffold should contain a mix of the micro- and a macro-pores in order to provide the best conditions for cell seeding and nutrition supply. Our pores were created only by starch leaching during pre-treatment in the boiling water. The solution to this problem might be the pore size modulation through the starch domains. Then, the blend would contain both bigger and smaller starch grains which could create the desired porous structure. Another possibility presents the control of the porosity by more sophisticated 3D printing. In this case, the exact pore size and pore distribution could be obtained. Moreover, time-consuming scaffold pre-treatment could be omitted and a precise custom-made scaffold could be fabricated. The importance of 3D printing for the clinical application of tissue-engineered constructs was described in a study by Xu et al.²⁸.

Results of the porosity calculation correlated with findings gathered from the electron microscopy in 4 samples. The porosity of sample 5 measured according to the Archimedes principle was only 30%. However, SEM analysis of the longitudinal sections revealed its highly porous structure. We assign this outcome to thick material layering during the 3D printing, which did not allow the effective starch leak during the pre-treating process. In this case, the modification of 3D process could solve this problem.

The swelling behavior of polymers accounts for the scaffold capability to provide the required inner environment for the mass transfer, vascular supply, and cell to cell communication. Water uptake also enlarges pore size and thereby maximizes the internal surface for cell proliferation and migration. The swelling ratio of our scaffold varied from 11% to 20% during the first 24 hours. Samples with a higher concentration of TPS absorbed more water during the first 24 hours. This might have been caused by the strong hydrophobic character of TPS. When comparing the samples which contained different types of TPS, we have observed that the increasement of the viscosity was related to the lower water absorption during the first 24 hours of incubation in the water environment. This phenomenon might occur due to the used modifiers which influence the hydrophilicity of TPS. The advantage of TPS implementation into the blend is that we could modulate water absorption of the scaffold when the proper type of TPS is chosen. For further experiments, it would be useful to examine hydrophilic character of each type of TPS. A slight increasement in water adsorption was detected during scaffold incubation in the water for 2 weeks. Further analyses showed that 3 samples continued to absorb water after this time period. However, the increase was still low. After the long-time scaffold hydration, we detected that their weight started to decrease. The ratio behind this finding is probably assigned to TPS dissolution. We have also observed that the sample with the lowest

content of TPS increased its weight after 3 weeks of incubation in the water – the amount of the dissolved TPS was probably lower than the amount of the absorbed water. Several studies described that water absorption should have been higher than 120%²⁹. On the other hand, excessive swelling could alter mechanical properties and degrade supporting function of the scaffold. The stiffness of the material as well as the thickness of the samples could affect our results of the water uptake. Stiffness of the scaffold needs to be altered mainly because of the potential further applications in animal models or clinical medicine because good surgical manipulation with the cell-seeded construct is essential.

Scaffold was also seeded with cells in order to evaluate cell attachment and proliferation on the surface. Regarding the results from the SEM analysis, cells could interact and survive on the scaffold. Several scaffold seeding techniques had been described³⁰. We applied the droplet seeding technique. Despite we achieved successful colonization, cells were not homogenously spread and were mainly oriented in the center of the sample. Homogenous colonization can be obtained by the dynamic culture conditions. Bioreactors play a crucial role in this process and also promote tissue maturation. Moreover, this system enables the application of the physical stimuli which is crucial for the TE of the elastic and complex tissues such as blood vessels or the urethra^{2,31}. The disadvantage of the bioreactor system is its cost extensiveness. Characteristic of the surface roughness plays also an important role in the cell-seeding process. Compared with the smooth surface, it had been described that scaffolds with the rough surface area were better colonized, as the overall area was bigger. In addition, spatial restriction did not allow flattening of the cells, so they maintained their shape as in a confluent population²³. The surface of our scaffold was smooth but starch grains could be used to modulate the roughness and create matrices with ragged topography.

Another crucial step for the accurate evaluation of cell-seeded construct's topography under the electron microscope is precise specimen preparation. Standard protocol involves the application of various chemicals. If handled improperly, natural shape of the cells is altered. We observed shrinkage as well as tearing of the cells in several samples after this process. Cryo-electron microscopy could offer a more sensitive approach. This method applies rapid freezing of the sample which results in the maintenance of the natural cell morphology. Moreover, cross-sections can be also performed in the vacuum atmosphere.

Conclusions

We have successfully managed to develop, engineer, and characterize the PLA/PHB/TPS blend in the context of tissue engineering and regenerative medicine. Thanks to its biocompatibility and printability, we assume it is possible to apply this blend in a form of 3D scaffold *in vivo*. However, further studies need to be carried out in order to alter scaffold pore size and stiffness.

Ethical Approval

Ethical Approval is not applicable for this article.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.


Declaration of Conflicting Interests

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