Materials Science & Engineering C 110 (2020) 110668

Contents lists available at ScienceDirect

Materials Science & Engineering C

journal homepage: www.elsevier.com/locate/msec

Osteoblasts and fibroblasts attachment to poly(3-hydroxybutyric acid-*co*-3-hydrovaleric acid) (PHBV) film and electrospun scaffolds

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ARTICLE INFO

Keywords:
PHBV
Fiber
Thin film
Osteoblast
Fibroblast
Cell attachment

ABSTRACT

The cellular response is the most crucial *in vitro* research. Materials' biocompatibility is determined based on cell proliferation and growth. Moreover, the topography of the scaffold surface is the key to enhance cell attachment and anchoring that importantly control further tissue development. Individual cell types have specific preferences regarding the type of surface and its geometry. In our research, we used poly(3-hydroxybutyric acid-*co*-3-hydrovaleric acid) PHBV to produce two types of substrate: a 3D structure of electrospun fibers and 2D flat films. The PHBV products were morphologically characterized by scanning electron microscopy (SEM). The cytocompatibility was evaluated with cell viability and proliferation using two different types of cells: human osteoblast-like cells (MG-63) and NIH 3 T3 murine fibroblast cells. The behaviour of both cell types was compared on the similar PHBV fiber scaffolds and films using two types of polystyrene (PS) based substrate for the cell culture study: unmodified PS that is not favourable for the attachment of cells and on tissue culture polystyrene (TCPS) plates, which are chemically modify to enhance cells attachment. The results clearly showed high biocompatibility of PHBV as both types of cells showed similar proliferation. These results indicated that PHBV scaffolds are suitable for the development of multifunctional substrates facilitating the growth of different types of tissue regardless of the 3D and 2D designed structures for regeneration purposes.

1. Introduction

The effect of topography and substrate morphology are the main features of the biomaterials that affects the cellular response [1,2]. The scaffolds are the one of three basic elements (scaffolding, cells, growth factors) necessary for tissue engineering. To restore damaged tissue the crucial aspect of tissue regeneration is using the structurally similar scaffold to support cell attachment and growth [3]. Most of the biocompatibility studies of new materials are performed with osteoblasts [1,4,5] and fibroblast [6–8]. Osteoblasts are bone-forming (osteogenic) cells that occur in places where the bone tissue grows or rebuilds. Therefore, three-dimensional (3D) surfaces are preferred for this type of cells. This concept of bone repairing base on 3D scaffold structures, which promote the reconstruction of the natural structure of bone [4,9,10]. Fibroblasts are cells of connective tissue. They are settled cells, but they have the ability to move. Furthermore, they have a significant role in the wound healing process [2,7,11]. Fibroblasts generally spread well in hydrophilic surface [8]. Therefore, the morphological and chemical properties of the scaffolds have a significant impact on cell proliferation [2].

The electrospinning is commonly used method to produce micro- and nanofibers to fabricate scaffolds [12,13]. The obtained structure has a large surface area, high porosity and the diameter of the electrospun fibers can be controlled by process parameters e.g. humidity, voltage polarity or polymer flow rate [2,14]. Electrospun fibers has a structural similarity to ECM so they are often applied as scaffolds for bone or skin tissue regeneration. [2,8,9,15,16].

Poly(3-hydroxybutyric acid-*co*-3-hydrovaleric acid) (PHBV) is a natural, thermoplastic aliphatic polyester which is produced by bacteria. Due to its biocompatibility properties and good cellular response [7,17], PHBV is used in medicine and tissue engineering. PHBV degrades in vivo to hydroxybutyric acid, which is easily metabolized by the body [4,18]. This polymer has been known for many years [18–21], however, a significant increase in interest can be observed in the last 10–15 years [2,4,7,22,23]. Previous cell research conducted on cell lines was focused on cellular response of modified fibers by silk fibroin [24,25], chitosan [17] or collagen and graphene oxide [11]. The behaviour of osteoblasts [3,4,24] and fibroblasts [7,11,17,25] was studied, but the behaviour of both cell types has never been compared on the similar PHBV fiber scaffolds and films. Moreover, we used two types

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https://doi.org/10.1016/j.msec.2020.110668
Received 4 November 2019; Received in revised form 7 December 2019; Accepted 13 January 2020
Available online 16 January 2020

of polystyrene, based substrate for the cell culture study. Unmodified polystyrene (PS) is a material that is not favourable for the attachment of cells [26,27],therefore it needs modification to increase adhesion. Liquid surface deposition or energetic plasma activation have become significant in development and modification of cell culture plates [27]. Nowadays in vitro studies are usually done on tissueculture polystyrene (TCPS) plates [28]. TCPS plates are subjected to vacuum treatment with gas plasma to chemically modify the surface of the plate, which is well adapted to attach the cells [29].

In this study we performed the microscopy observations of cell attachment and proliferation assay on PHBV surface with two morphologies: fibers and films. We carefully analysed the differences in the behaviour of two types of cells: osteoblasts and fibroblasts and how surface topography affects their attachment, growth and proliferation, crucial in the assessment of the materials' biocompatibility. The comparison of both type of cells behaviour on PHBV scaffolds allow to develop the multifunctional scaffolds that are able to facilitate the growth of different types of tissue and are also suitable for co-culture studies.

2. Materials and methods

2.1. Materials, fibers and film production process

Poly(3-hydroxybuturic acid-*co*-3-hydrovaleric acid) (PHBV, PHV content 2 wt%, M_W = 450,000 $g{\cdot}mol^{-1}$) was purchased from Helian Polymers, The Netherlands. The PHBV fibers were prepared by electrospinning of PHBV solution. To obtain 8 wt% PHBV solution, polymer was dissolved in chloroform: DMF solution (*v*/v 9:1, Sigma Aldrich, UK) by stirring on the heated plate to 45 °C for 3 h at a speed of 1250 rpm. Prior solution preparation, PHBV powder was dried (Drying Over, POL-ECO Aparatura, Poland) at 40 °C for 4 h before using. Electrospinning of PHBV was carried out using the EC-DIG apparatus with the climate chamber system (IME Technologies, The Netherlands) at the temperature of 25 °C and the humidity of 40%. The voltage of 17 kV was applied to the stainless needle with the inner diameter of 0.8 mm, with solution flow rate of 0.1 $ml{\cdot}min^{-1}$, keeping 20 cm distance to the grounded collector. Thin PHBV films were prepared on glass circular slide using spin-coater (L2001A v.3, Ossila, UK; speed = 6000 rpm, time = 60 s).

2.2. Characterisation of PHBV fibers

2.2.1. Scanning electron microscopy (SEM)

The morphology of the PHBV fibers was observed by scanning electron microscopy (SEM, Merlin Gemini II, Zeiss, Germany), with the U = 3 kV, at the 5–8 mm of working distance. The samples were coated with 5 nm gold layer using the rotary pump sputter coater (Q150RS, Quorum Technologies, UK). Fiber diameter (D_f) of PHBV was measured from SEM images using ImageJ (v. 1.51j8, USA) to incorporate 100 measurements into histograms with the standard deviation calculations.

2.2.2. Contact angle

The wettability of the PHBV fibers was determined by static contact angle measurements using 3 μl droplet of deionized water (DI water, Spring 5UV purification system – Hydrolab, Straszyn, Poland) at *T* = 24 °C and H = 40%. The images of 10 droplets were taken within 3 s from the deposition using Canon EOS 700D camera with EF-S 60 mm f/2.8 Macro USM zoom lens (EOS 700D, Canon, Tokyo, Japan), and the contact angles were measured using ImageJ (v. 1.51j8, USA), with error based on the standard deviations.

2.3. In vitro studies

2.3.1. Cell culture studies

The biological tests have been carried out on the MG-63 human osteoblast - like cell line (Sigma Aldrich, UK) and NIH 3T3 murine fibroblast cell line (Sigma Aldrich, UK) in the amount 2×10^4 cells per well. The culture was conducted for 7 days using Minimum Essential Medium Eagle (Sigma Aldrich, UK) for osteoblast and Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, US) for fibroblasts both supplemented with 10% addition of bovine serum (FBS), 2% antibiotics (penicillin/streptomycin), 1% amino acids and 1% L-Glutamine (Sigma Aldrich, UK).

The both of the cell cultures were grown under standard conditions, i.e. at 37 °C, 90% of humidity and 5% CO_2 at the atmospheric pressure. As positive control, an empty TCPS well was used and an empty PS well as negative control. The cell culture medium was exchanged after 3 days and condition of the cells was checked after 1, 3 and 7 days.

2.3.2. Cell morphology

Cell morphology was evaluated using SEM. Culture medium was removed after each time point and samples were washed with PBS and fixed for SEM analysis with 2.5% glutaraldehyde (Sigma Aldrich, UK) for 2 h in 4 °C. Thereafter, samples were dehydrated in a series of ethanol solutions (50%, 70%, 96% and ~99,9%, Avantor, Poland), three times in each concentration for 3 min. Finally, dehydrated samples were immersed in hexamethyldisilazane (HMDS, Sigma Aldrich, UK) under a fume hood and left to dry overnight.

2.3.3. MTS cell viability assay

Cell proliferation was evaluated by colorimetric measurement of MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, the USA). Dehydrogenase enzymes in metabolically active cells convert the yellow tetrazolium to the purple soluble formazan. The quantity of formazan product was measured out at 490 nm using spectrophotometer (Microplate Reader LT-4000, Labtech International Ltd., UK).

2.4. Statistics

Analysis of variance (one-way ANOVA followed by Tukeys' post-hoc test) was used to determine the level of significance between the PHBV fibers and thin 2D films for cell proliferation, the statistical significance was evaluated at $p < 0.05$.

3. Results and discussion

3.1. Scaffolds morphology and surface properties

Using PHBV solution we produced two geometries in the form of flat film and porous electrospun scaffold as showed in Fig. 1A and B. The SEM image of PHBV film indicated an irregular surface giving slight roughness to this film.The average diameter of fibers was 2.79 ± 0.20 μm (Fig. 1C). The wetting properties of the fibers and films were verified using water contact angle as showed in Fig. 1D, demonstrating increased hydrophobicity for PHBV fibers, reaching contact angle of 114.6 ± 3.8°, whereas the PHBV film was hydrophilic with the contact angle of 83.4 ± 4.5°. The hydrophobic behaviour of electrospun surface was achieved with surface roughness as suggested in the previous studies [30,31]. As the roughness increases, the material becomes more hydrophobic reaching the Cassie-Baxter state [30,32]. In the case of fibers, the contact angle is greater due to the many contact points on the liquid-solid interface [33,34]. Interestingly, the water contact angle for flat TCPS and PS were 65.5 ± 1.4° and 68.6 ± 1.5°, respectively, marking no differences in terms of wettability of those surfaces [35,36].

3.2. Cell morphology

The detailed investigation using SEM allowed the evaluation of cellular attachment and anchoring to electrospun PHBV fibers as

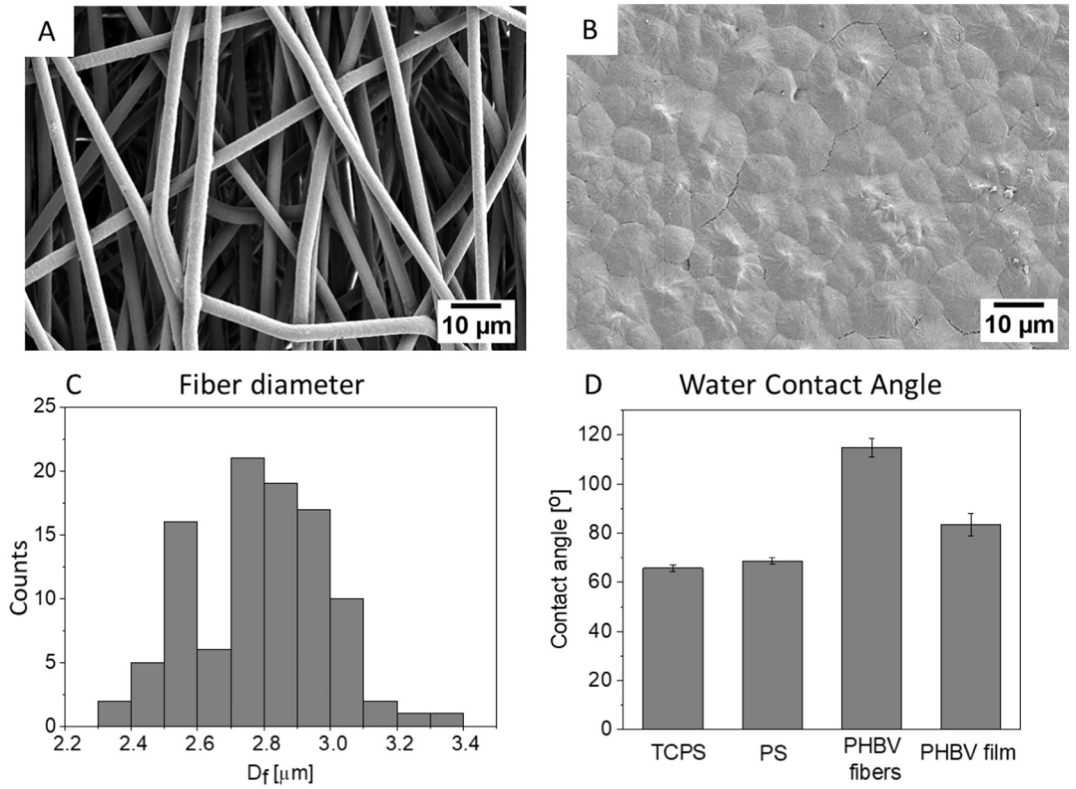

Fig. 1. SEM micrographs of (A) PHBV fibers and (B) film, histogram of (C)fiber diameter distribution (D_f)(D_f) in PHBV electrospun scaffold, and D) contact angle results for DI water, measured on TCPS, PS, PHBV fibers and films.

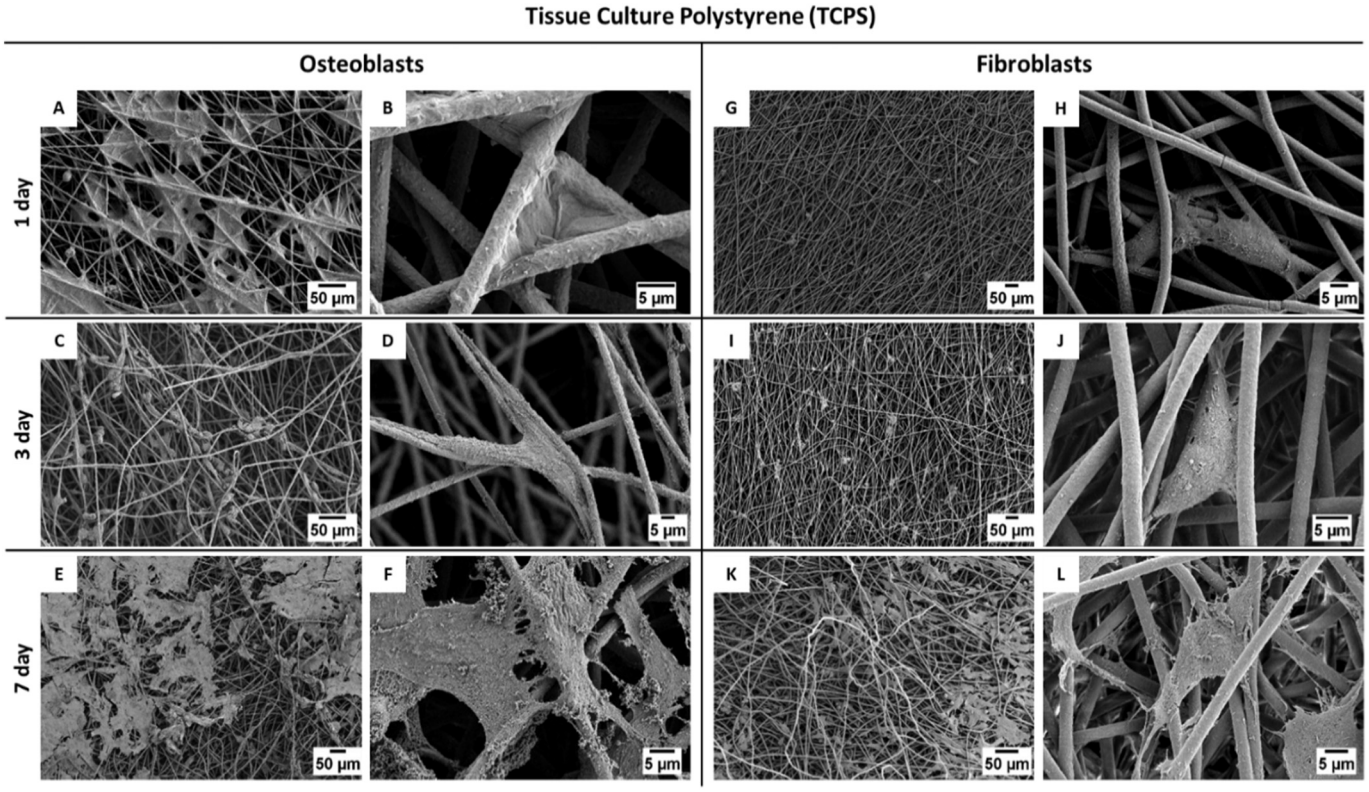

Fig. 2. SEM micrograph showing osteoblasts growth after 1 (A, B), 3 (C, D) and 7 (E, F) days and fibroblasts after 1 (G, H), 3 (I, J) and 7 (K, L) days on electrospun PHBV fibers using TCPS plates.

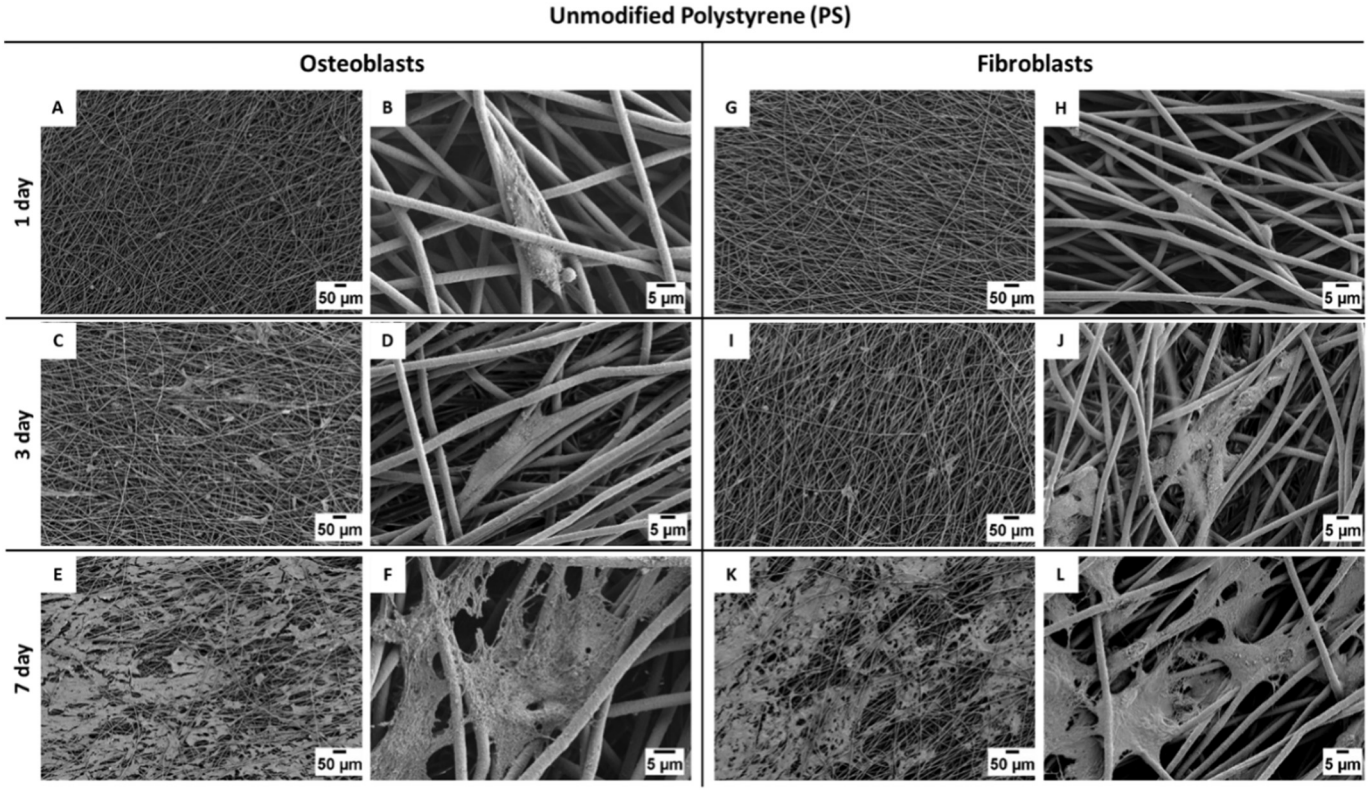

Fig. 3. SEM micrograph showing osteoblasts growth after 1 (A, B), 3 (C, D) and 7 (E, F) days and fibroblasts after 1 (G, H), 3 (I, J) and 7 (K, L) days on electrospun PHBV fibers using unmodified PS plates.

showed in Figs. 2 and 3 carried on TCPS and PS plates. The electrospun fibers created a 3D environment for osteoblasts and fibroblasts allowing to integrate them with scaffolds for further migration and proliferation and tissue formation.

From the perspective of using TCPS and PS as the substrate for cell culture on the electrospun scaffolds no significant differences were observed with SEM. Osteoblasts attached to the fibers from 1st day (Fig. 2 A). This cell type formed many filopodia to attach to surrounding fibers and protrude on and into the scaffolds starting from day 3 (Fig. 2C and D) and after 7th day osteoblasts completely covered fibrous scaffolds (Fig. 2E). For fibroblast after 1st day of the cell culture study, only a small number of the fibroblasts attached to the fibers (Fig. 2G). On the 3rd day significant increase of fibroblast was observed on the scaffolds (Fig. 2I), but on the other hand fibroblasts covered smaller area of the scaffold (Fig. 2K) in comparison to osteoblast (Fig. 2E).

Using PS plates, at the 1st day of cell culture, osteoblasts showed more spherical shape than fibroblast (Fig. 3 B, H). The increase in osteoblast and fibroblast proliferation on the fibers proceeded slowly during the first 3 days (Fig. 3), but significant increase was observed between 3rd and 7th day of incubation (Fig. 3), where both types of cells covered the entire surface of the fibers (Fig. 3 E, K).

The cell culture was also performed on PHBV film and TCPS considered as a 2D structure allowing easier comparison of changes of cell shape, because cells movement was limited to the surface. Generally, osteoblasts (MG-63) are not demanding cells and they are able to grow on many types of surface, also on 2D films [37]. After 1st day fibroblasts kept spherical shapes (Fig. 4H), whereas osteoblasts started to spread on the film surface (Fig. 4B). At 3rd and 7th day osteoblasts created filopodia allowing their migration (Fig. 4 D, F). Fibroblasts formed agglomeration and colonies of cells on the film surface (Fig. 4I).

While using PHBV films on PS plates for cell culture studies just a few cells, attached to the thin films on the 1st day, both osteoblast and fibroblast, see Fig. 5B and H. Osteoblasts connected to each other on the film surface, both after the 3rd (Fig. 5C) and the 7th days of culture (Fig. 5F). Especially fibroblasts agglomerated and formed aggregates (Fig. 5J, L) without spreading on the surface showing an effect of type of PS used in culture, as for TCPS large spreading was observed for both types of cells investigated in this study.

Many previous in vitro studies showed that osteoblasts prefer rough surfaces instead of flat, which one is preferred by fibroblasts. This behaviour of fibroblasts has been confirmed mostly on metal surfaces such as aluminium plates coated with Ti characterized with different roughness level [1]. Generally, fibroblasts spread well on the hydrophilic surface. However, the speed of the fibroblast movement and growth are affected by surface chemistry of produced scaffold [4,24,25]. Fibrous scaffolds have a larger surface area and generate more contact points for cells, which is reflected in a better spread of fibroblasts in the fibrous mat compared to film even though the roughness is higher in comparison to flat surfaces. These results are consistent with those published by Kuppan et al. [2], where the in vitro studies with PHBV fibers and 2D films were conducted to assess viability and proliferation of human skin fibroblasts.

3.3. MTS cell viability assay

To confirm the SEM observations showed in Figs. 2–5, we performed the proliferation tests, as presented in Fig. 6. The higher reagent reduction value (higher absorbance) indicates more living cells in the culture. TCPS showed the higher absorbance value than thin PHBV film or fibers (Fig. 6A, B). The absorbance values of the PS plates are almost the same for each sample, what testify that the PS plates are not appropriate for cell proliferation.

The proliferation of osteoblasts on PHBV fibers and film was at a similar level after the 1st and 3rd day of cell culture using TCPS and PS, see Fig. 6A, C. The absorbance value for osteoblasts on the fibers after the 7th day is close to the values for TCPS, which proves the very high biocompatibility of PHBV fibers (Fig. 6A). The electrospun PHBV fibers

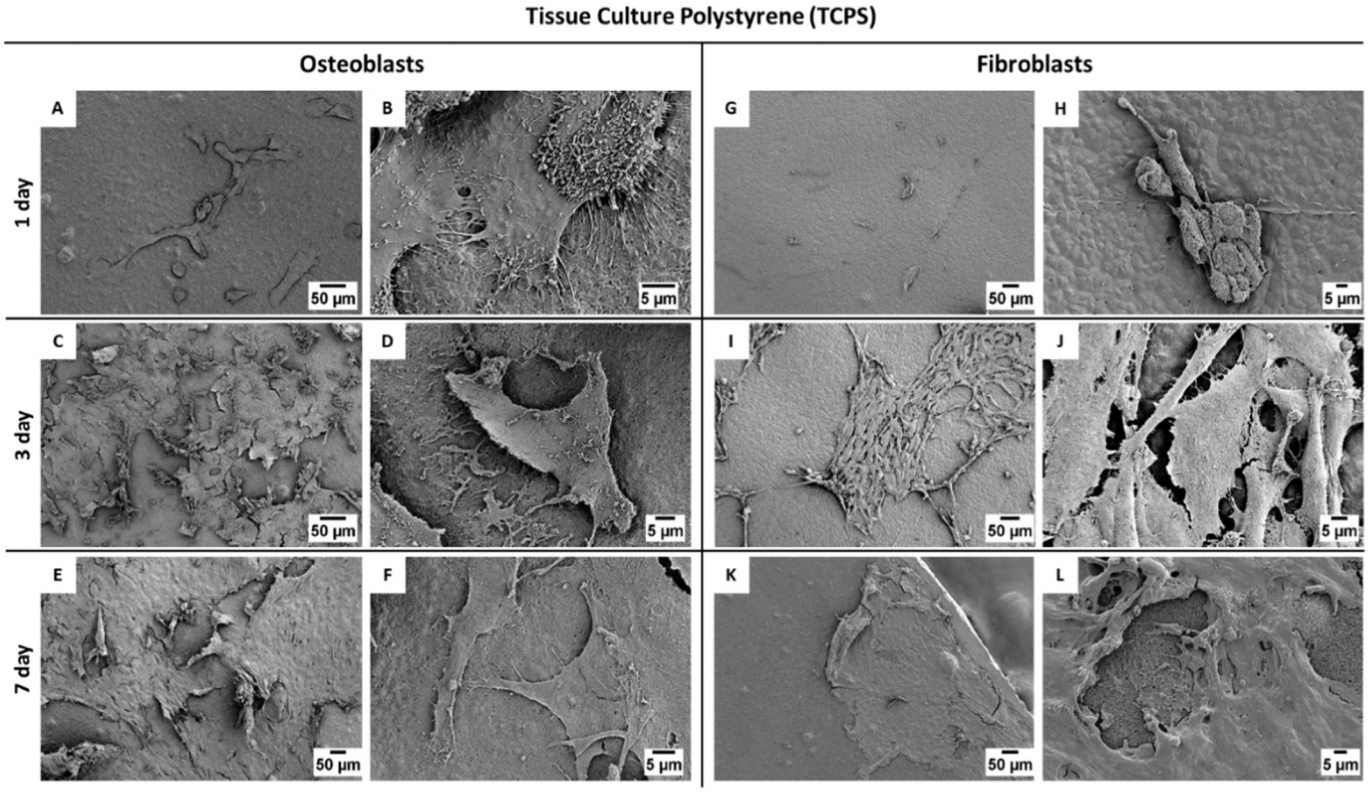

Fig. 4. SEM micrograph showing osteoblasts after 1 (A, B), 3 (C, D) and 7 (E, F) days and fibroblasts growth after 1 (G, H), 3 (I, J) and 7 (K, L) days on PHBV films using TCPS plates.

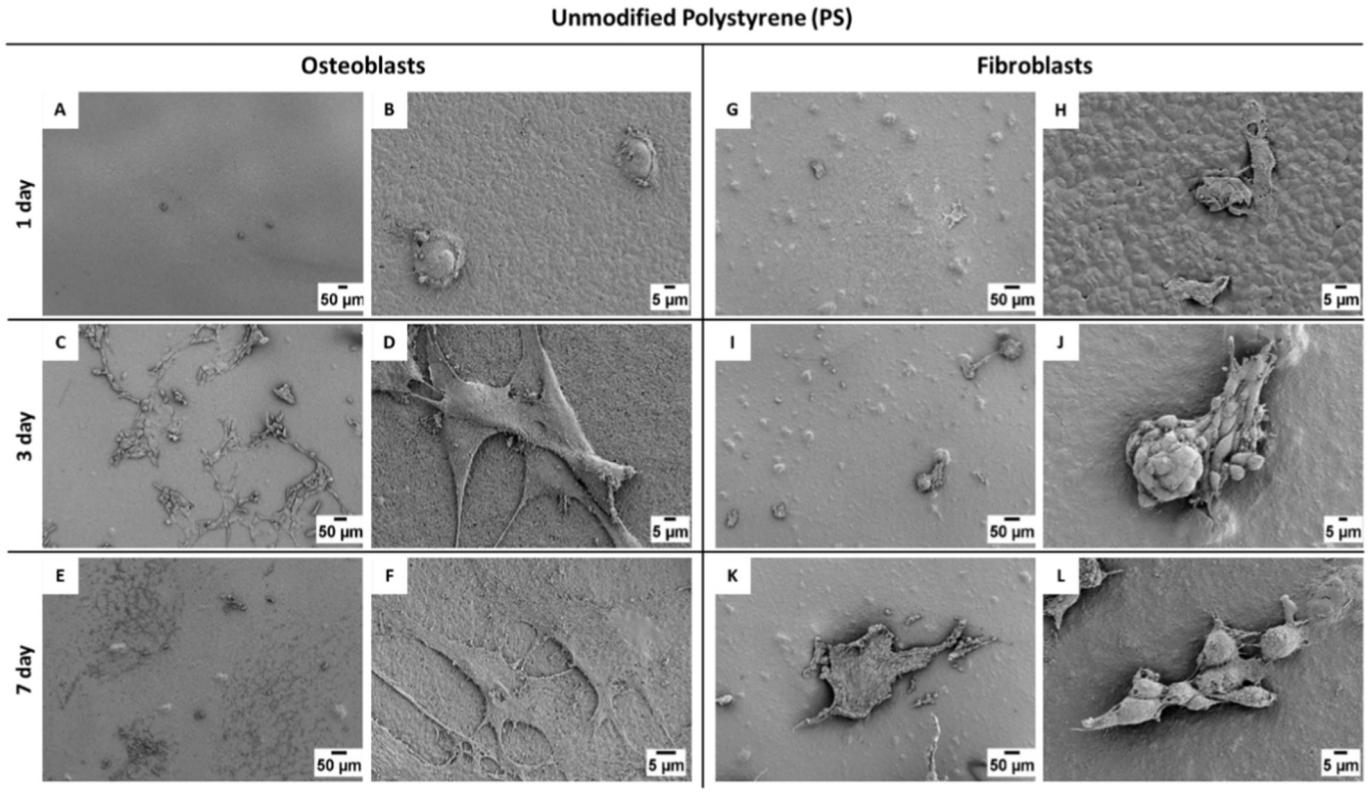

Fig. 5. SEM micrograph showing osteoblasts after 1 (A, B), 3 (C, D) and 7 (E, F) days and fibroblasts growth after 1 (G, H), 3 (I, J) and 7 (K, L) days on PHBV films using PS plates.

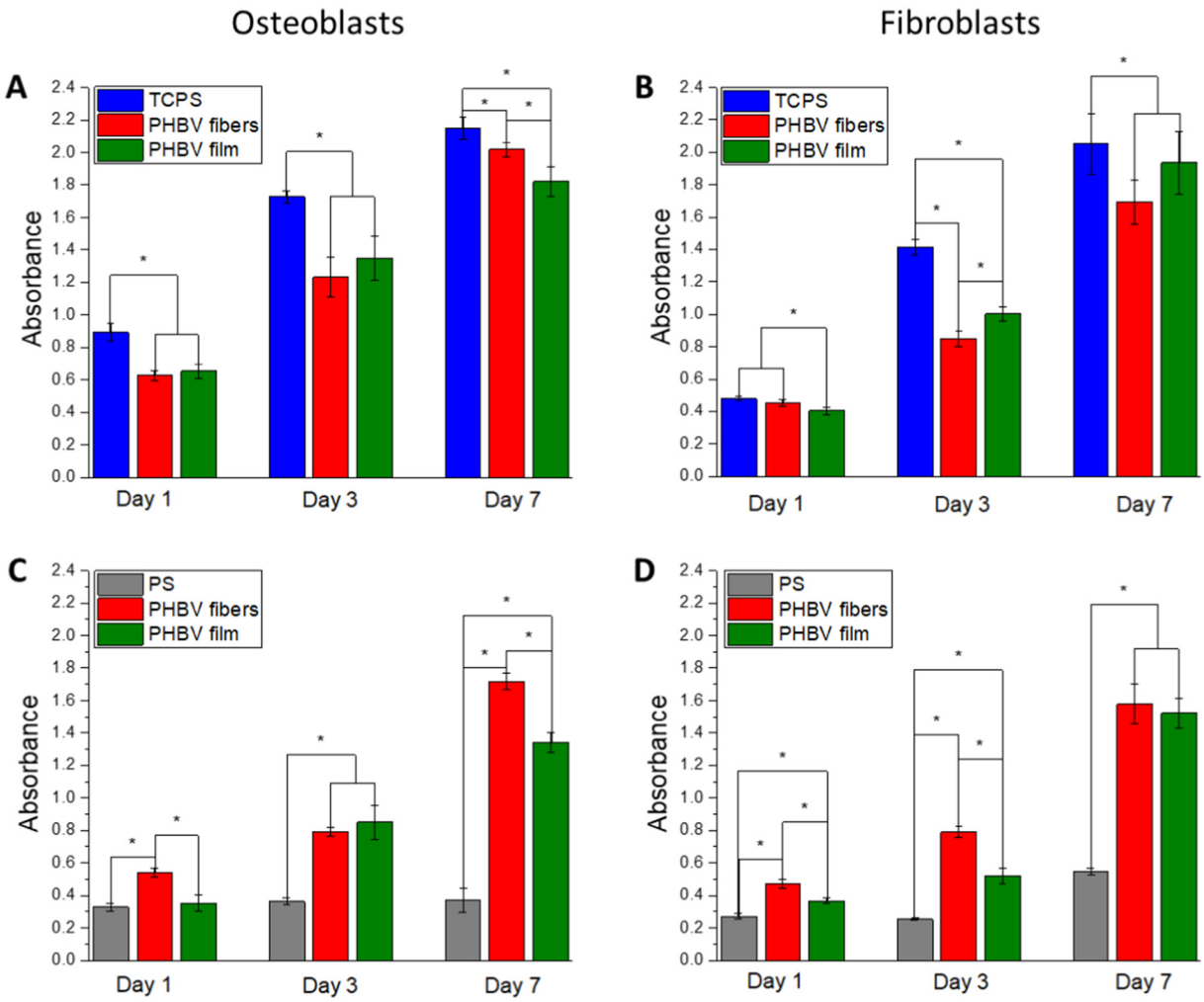

Fig. 6. Cell proliferation assessed by MTS assay performed on PHBV samples on TCPS (A, B) and PS (C, D) plates using MG-63 cell line (A, C) and NIH 3T3 cell line (B, D). *statistical significance calculated with ANOVA, followed by Tukey's post hoc test, $p < 0.05$.

showed higher absorbance values for osteoblasts than thin PHBV film after 7 day of culture (Fig. 6A, C), confirming that the osteoblasts preferred 3D structure of the scaffold, as previously suggested [4,9,10].

The absorbance values for fibroblast proliferation on the PHBV were comparable for each sample and plates at 1st day of the cell culture (Fig. 6B, D). Both after the 3rd and 7th day of fibroblast culture on a TCPS showed the higher proliferation on the films in comparison with the electrospun fibers (Fig. 6B), as fibroblasts prefer flat surface [8]. However, fibroblasts integrated inside PHBV fibrous scaffold (Figs. 2J and 3J) and upon the fiber surface (Figs. 2K and 3K), which can be observed on the 3rd day of culture on PS plates. At the 7th day of cell culture on PS, cell proliferation on PHBV fibers was slightly higher than on the film. As a result of agglomeration, fibroblasts showed high absorbance value, as previous SEM observation, see Fig. 5K, L.

Cells proliferated and grew on both types of surfaces PHBV fibers and films. However, osteoblast proliferation on PHBV fibers was more comparable to TCPS control, but for fibroblasts, the level of proliferation on the film surface was comparable to positive test control. To verify cells preferences often the studies are performed on flat and grooved films [38] to verify the formation of focal adhesions points [39], responsible for contact guidance and the formation of actin stress fibers. Therefore, surface properties in biomaterials are one of the most influencing parameters on cells and a lot of effort in tissue engineering is focused on surface modifications with roughness [40,41] to change the architecture of scaffolds [12,42] or designing antibacterial or antimicrobial properties [8]. Generally cell-material interactions are complex processes, controlling not only cells fate but also their adhesion and differentiation [43] by specific surface properties including topography [44], potential and surface charge [15,45]. Each type of cell has its own unique characteristics including how cells actually respond to scaffolds geometries and surface properties.

4. Conclusions

Both electrospun PHBV fibers and thin films showed a great potential to facilitate osteoblast and fibroblast in cell culture, what indicated high biocompatibility of the material regardless the 3D and 2D structure for these two most used cells types in tissue engineering. We performed a detail investigation of osteoblast and fibroblasts behaviour and their filopodia formation to attach to the 3D fibrous scaffolds and 2D flat film. The results clearly indicated that changes in the scaffold topography does not show much difference in cell attachment and proliferation. Generally fibroblasts are cells that prefer flat surfaces, whereas osteoblasts commonly show better growth and proliferation on more spatial structure. Nevertheless, conducting tests on TCPS and PS showed that there was a slightly difference in absorbance measurements for PHBV fibers and films contained in individual culture plates. Furthermore, the cell proliferation value on PHBV samples is similar for both TCPS and PS plates. We showed that surface topography does not significantly affect cell proliferation. Better cell growth and filopodia formation on fibrous 3D structures is a significant parameter in assessing cell behaviour. The study confirmed that electrospun PHBV

scaffolds are able to facilitate most often used cells types: osteoblast and fibroblast used in cell culture studies, indicating a universal application of fibrous scaffolds for different tissue regeneration. Further studies on PHBV scaffolds will include the surface properties effect on cells development and proliferation.

Author statement

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was conducted within “Nanofiber-based sponges for atopic skin treatment” project carried out within the First TEAM programme of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund, project no POIR.04.04.00-00- 4571/18-00.

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