

In vitro evaluation of PLLA/PBS sponges as a promising biodegradable scaffold for neural tissue engineering

İrem Ayşe KANNECİ ALTINIŞIK^{1,2}, Fatma Neşe KÖK^{1,2,3}, Deniz YÜCEL^{2,4}, Gamze TORUN KÖSE^{2,5,*}

¹Molecular Biology-Genetics and Biotechnology Program (MOBGAM), İstanbul Technical University, Maslak, İstanbul, Turkey

²Center of Excellence in Biomaterials and Tissue Engineering (BIOMATEN), Middle East Technical University, Ankara, Turkey

³Department of Molecular Biology and Genetics, İstanbul Technical University, Maslak, İstanbul, Turkey

⁴Department of Histology and Embryology, Faculty of Medicine, Acıbadem University, İstanbul, Turkey

⁵Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University, İstanbul, Turkey

Received: 04.01.2017 • Accepted/Published Online: 13.04.2017 • Final Version: 10.11.2017

Abstract: In tissue engineering, the use of poly-L-lactic acid (PLLA)/polybutylene succinate (PBS) blend for the construction of scaffold is very limited. Moreover, polymeric sponges fabricated from PLLA/PBS have not been studied for neural tissue engineering. In the present study, the potential of the utility of PLLA/PBS polymeric sponges seeded with Schwann cells was investigated. PLLA and PBS were blended in order to increase the processability and tune the crystallinity, porosity, and degradation rate of the resulted polymeric sponges. These sponges were then seeded with Schwann cells. Porosity analysis showed that there were no significant differences between different compositions of PLLA/PBS blends; however, the porosity was slightly higher in PLLA/PBS (3%, w/v, 2:1) scaffold. Degradation profiles were also investigated for 120 days and almost 25% weight of PLLA/PBS (6%, 4%, 2%, w/v, 1:1) scaffolds and 18% weight of PLLA/PBS (3%, w/v, 2:1) scaffolds were lost at the end of 120 days. In vitro cell culture studies were also performed and the results proved that all PLLA/PBS blended scaffolds were biocompatible. The highest cell proliferation was observed for PLLA/PBS (3%, w/v, 2:1) scaffolds and this construct can be considered a promising biodegradable scaffold for neural tissue engineering.

Key words: Biomaterials, neural tissue engineering, polybutylene succinate, poly-L-lactic acid, Schwann cells

1. Introduction

Tissue engineering aims to repair, rebuild, and regenerate damaged tissues or organs by using and combining cells, scaffolds, and biologically active molecules such as growth factors and cytokines (Bonassar and Vacanti, 1998; Neel et al., 2014). There are various approaches for tissue engineering strategies. However, from the point of view of the biomaterials field, one of the most preferred approaches is the utilization of biomaterials to construct implants in order to replace damaged tissues and support the cells to build up new ones. These temporary scaffolds serve as a template for the renewing tissues, and by addition of bioactive substances to these scaffolds, differentiation and growth of cells are provided and enhanced.

Different sources of biomaterials can be used to construct the scaffolds; however, the following criteria should be considered for the choice of a suitable platform. Biodegradation rate is an important parameter that affects cell proliferation and growth, implant integrity, and host response. Another factor is the porosity of three-dimensional scaffolds. The interconnected porous

structure of the scaffold enhances cellular adherence and tissue regeneration (Martina and Huttmacher, 2007). Biocompatibility, suitable surface chemistry, and mechanical properties are also crucial parameters that influence the success rate of the implant.

Biodegradable aliphatic polyesters have great potential to supply the above criteria, and among these polymers, poly-L-lactic acid (PLLA) is one of the most seasoned thermoplastics that is utilized extensively to build tissue engineering scaffolds. PLLA has many advantages, such as renewability, biocompatibility, processability, and energy efficiency (Rasal et al., 2010). However, the brittle and hydrophobic nature of PLLA complicates its usage for tissue engineering applications. Therefore, further modifications are applied to PLLA. Blending PLLA with another polyester that has a more flexible nature and rapid crystallization rate is a strategy to improve the thermal and mechanical characteristics of the polymer. This blend will exhibit new features that can be optimized to be used in scaffold construction. Polybutylene succinate (PBS) is a suitable candidate for this application. It has a low glass

* Correspondence: gamzekose@yeditepe.edu.tr

transition temperature and higher crystallization rate and processability. In addition, the degradation product of PBS is succinic acid, which is converted to CO₂ and H₂O in the tricarboxylic acid cycle. Blending PLLA and PBS results in a lower crystallization temperature (Shibata et al., 2006). Besides, by determining a convenient composition, the blend can have high flexibility. This parameter is very important for neural tissue engineering as tubular forms are more attractive for scaffold design. In addition to flexibility, the porous structure of the scaffold is also a desired property. This porous structure enables the release and permeation of bioactive substances incorporated into the scaffold or released by the cells cultured on the scaffold, which supports regeneration of the surrounding tissue and cells.

In this study, the potential usage of PLLA/PBS blends for neural tissue engineering was investigated for the first time. First, different compositions of PLLA/PBS blends were prepared in a sponge structure and the degradation rates of these blends in the physiological environment were determined. In addition to scaffold characterization, biocompatibility of PLLA/PBS blended scaffolds was examined by culturing Schwann cells on these scaffolds for 21 days.

2. Materials and methods

2.1. Materials

PLLA was purchased from AppliChem (A5489, 0100, Germany) and PBS was purchased from Sigma-Aldrich (448028, USA). Dichloromethane (Riedel-34856, Germany) was used as a solvent. For in vitro studies, Schwann cells were obtained as an immortalized cell line from the ATCC (SW10, CRL-2766). The CellTiter 96 aqueous nonradioactive cell proliferation assay (Promega, G5421, USA), Dulbecco's modified Eagle's medium (DMEM) (GIBCO, 41966-029, USA), Dulbecco's phosphate-buffered saline (DPBS) (PAN Biotech, P04-37500, Germany), TrypLE Express Enzyme (GIBCO, 12605-010, USA), fetal bovine serum (FBS) (GIBCO, 10108-165, USA), dimethyl sulfoxide (Applchem, A3006, 0100, Germany), penicillin-streptomycin solution (GIBCO-15140-122, USA), glutaraldehyde solution (Sigma-Aldrich, G5882, USA), cacodylic acid sodium salt trihydrate (AppliChem, A2140, 0100, Germany), Alexa Fluor 546 phalloidin (Thermo Fisher, A22283, USA), DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) (Thermo Fisher, D1306, USA), formaldehyde solution (Sigma, F8775, USA), and Tween 20 (Sigma, P9416, USA) were used for degradation and in vitro cell culture studies.

2.2. Scaffold fabrication

Sponges from biodegradable PLLA and PBS polymers at different blend ratios were prepared using solvent casting and the particulate leaching method. Polymer blends (1:1

and 2:1 w/v) were weighed respectively and dissolved in dichloromethane at different total polymer concentrations (6%, 4%, 3%, and 2% w/v). Sieved sodium chloride particles (NaCl; size: between 300 µm and 500 µm) were added to the solution in order to create pores. The solution was then cast onto glass templates and allowed to evaporate overnight at room temperature. NaCl particles were removed from the sponges in dH₂O. After removal of NaCl, the scaffolds were freeze-dried and cut into equal sizes (square shaped, 1 cm²) for scaffold characterization and in vitro studies (Figure S1).

2.2.1. Scanning electron microscopy (SEM) analysis

Fabricated scaffolds were coated with gold before SEM observations. SEM was carried out with a Carl Zeiss instrument (EVO, Germany). In order to analyze and calculate the percent porosity of scaffolds and pore size distribution, ImageJ porosity analysis software was utilized.

2.2.2. Degradation study

Degradation of the polymeric sponges was performed in DPBS 1X solution (pH 7.4) at 37 °C in a shaking water bath for 120 days. The dry samples were first weighed and then sterilized in 70% EtOH. After sterilization, the samples were placed in 10 mL of DPBS solution separately. The pH of the solutions was measured at days 7, 15, 30, 45, 60, 75, 90, and 120. Designated samples for the determined time intervals were removed from the solutions and freeze-dried for weight measurement. All experiments were performed in triplicate.

2.2.3. In vitro cell culture studies

2.2.3.1. Cell seeding on polymeric sponges

Schwann cells were used for in vitro cell culture studies and grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution in a humidified 5% CO₂ atmosphere at 37 °C. Schwann cells were harvested from a monolayer Schwann cell culture by TrypLE Express Enzyme. The PLLA/PBS sponges were sterilized in 70% EtOH for 3 hours at 4 °C. After removal of EtOH, the sponges were incubated in DMEM overnight and then the cells were seeded on sponges at a density of 50,000 cells/sample. Growth medium was renewed every 3 days and the cells were incubated in a CO₂ incubator at 37 °C and 5% CO₂.

2.2.3.2. Cell proliferation assay

The proliferation of the Schwann cells seeded on the polymeric sponges was measured at days 1, 7, 14, and 21. The CellTiter 96 nonradioactive MTS cell proliferation assay was used to determine the number of cells on the polymeric sponges. MTS is a tetrazolium compound that is reduced and converted to formazan in viable cells by NAD(P)H-dependent dehydrogenase enzymes. Formazan

is a colored compound and the metabolic activity of viable cells is measured by the absorbance of the generated formazan dye at 490–500 nm. MTS reagent was added to each well of the 24-well plates and incubated for 2 hours at 37 °C in a CO₂ incubator. Absorbance was determined at 490 nm using an ELx808 Absorbance Microplate Reader (BioTek, USA) and converted to cell number using a calibration curve. All experiments were performed in triplicate.

2.2.3.3. SEM analysis

In order to examine cell adhesion and proliferation, cells (20,000 cells/sample) were seeded on polymeric sponges and the samples were analyzed by SEM on days 7, 14, and 21. In order to prepare the scaffolds for SEM analyses, the cells on the scaffolds were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature. After the fixation, the samples were incubated in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C until observation. The samples were air-dried and coated with gold before SEM observation. SEM analysis was carried out with a Carl Zeiss instrument (EVO, Germany).

2.2.3.4. Immunocytochemistry analysis by confocal microscopy

To investigate the cell adherence and cell morphology on porous sponges, cells (20,000 cells/sample) were seeded on polymeric sponges and the samples were stained with Alexa Fluor 546 for F-actin and DAPI for nuclear DNA. The samples were analyzed with a confocal microscope on days 1, 7, 14, and 21. The samples were fixed with 3.7% formaldehyde including 0.1% Tween 20 for 30 min. After fixation, the cells were rinsed with DPBS 1X and incubated with 3% FBS in DPBS 1X for 30 min at room temperature. Then the cells were incubated in 1:100 dilute Alexa Fluor 546 solution (1% FBS in DPBS 1X) for 45 min. After labeling the cells, samples were rinsed again and cell nuclei were stained with DAPI solution at a 1:1000 dilution (1% FBS in DPBS 1X). The samples were then rinsed, mounted, and incubated in DPBS 1X (pH 7.4) at 4 °C until observation. The analysis was performed with a Carl Zeiss LSM 700 confocal microscope.

2.2.4. Statistical analysis

Statistical analysis was performed by two-tailed t-test using Excel. Differences were evaluated as statistically significant at $P \leq 0.05$.

3. Results

3.1. Scaffold characterization by SEM

The blends of PLLA/PBS polymeric sponges were visualized by SEM (Figure 1) and the porosity of the samples was analyzed. Porosity percentages of different polymer blends were calculated and are listed in the Table. As seen in Figure 1, no significant difference was observed

with respect to pore size (around 280 μm) among the samples. Still, porosity analysis showed that the porosity of PLLA/PBS (3%, w/v, 2:1) samples was slightly higher compared to other samples (Table), and this was correlated with the higher PLLA ratio in the blend. As the PBS ratio increases in the blend, PBS is aggregating in PLLA as islands and the nucleation sites are increasing in the structure. In addition, the viscosity of the blend decreases and the pores collapse (Ma et al., 2012), which leads to a lower number of pores.

3.2. In vitro degradation study: pH changes and weight loss

Throughout 120 days of incubation, there was a significant decrease ($P \leq 0.05$) in the pH of the sample solutions (Figure 2). A slower decrease in pH was observed in the PLLA/PBS (3%, w/v, 2:1) sponges compared to PLLA/PBS (6%, 4%, 2%, w/v, 1:1) sponges. This was correlated with a higher concentration of PLLA in the blend. At the end of 120 days of incubation, the pH differences between PLLA/PBS 6% and 4%, w/v, 1:1, were not significantly different ($P \leq 0.05$), and it was around 6.9. There was not a significant difference ($P \leq 0.05$) between PLLA/PBS 2%, w/v, 1:1, and PLLA/PBS, 3%, w/v, 2:1, which was around 7.1. These two groups had a significant ($P \leq 0.05$) difference between them, but the pH decrease throughout 120 days was not sharp in all sponges. The minor pH reduction was caused by acidic groups that formed due to the biodegradation of PLLA.

In order to determine the percentage of weight loss, all samples were incubated in a DPBS solution (1X) for 120 days at 37 °C in a shaking water bath. It was observed that almost 25% of the weight of PLLA/PBS (6%, 4%, 2%, w/v, 1:1) and 18% of the weight of PLLA/PBS (3%, w/v, 2:1) were lost at the end of 120 days of incubation (Figure 3). The weight loss percentages among the blends of PLLA/PBS 6%, w/v, 1:1, PLLA/PBS 4%, w/v, 1:1, and PLLA/PBS 2%, w/v, 1:1 were not significantly different ($P \leq 0.05$), but the weight loss of the PLLA blends 1:1 group was significantly higher ($P \leq 0.05$) than PLLA/PBS 3%, w/v, 2:1 at the end of 120 days of incubation. The PLLA ratio and percentage distinctly affected the degradation trend of the scaffold. The structural integrity of sponges was lost after day 30 for PLLA/PBS (6%, w/v, 1:1), while other samples retained their structural integrity until day 45. At the end of 120 days of incubation, PLLA/PBS (3%, w/v, 2:1) and PLLA/PBS (2%, w/v, 1:1) retained most of their physical state. However, PLLA/PBS (2%, w/v, 1:1) lost its structure and the width of the scaffold decreased extensively. This may cause physical collapse of the scaffold implanted in vivo. Therefore, at the end of characterization studies, the most promising blend was determined to be PLLA/PBS (3%, w/v, 2:1).

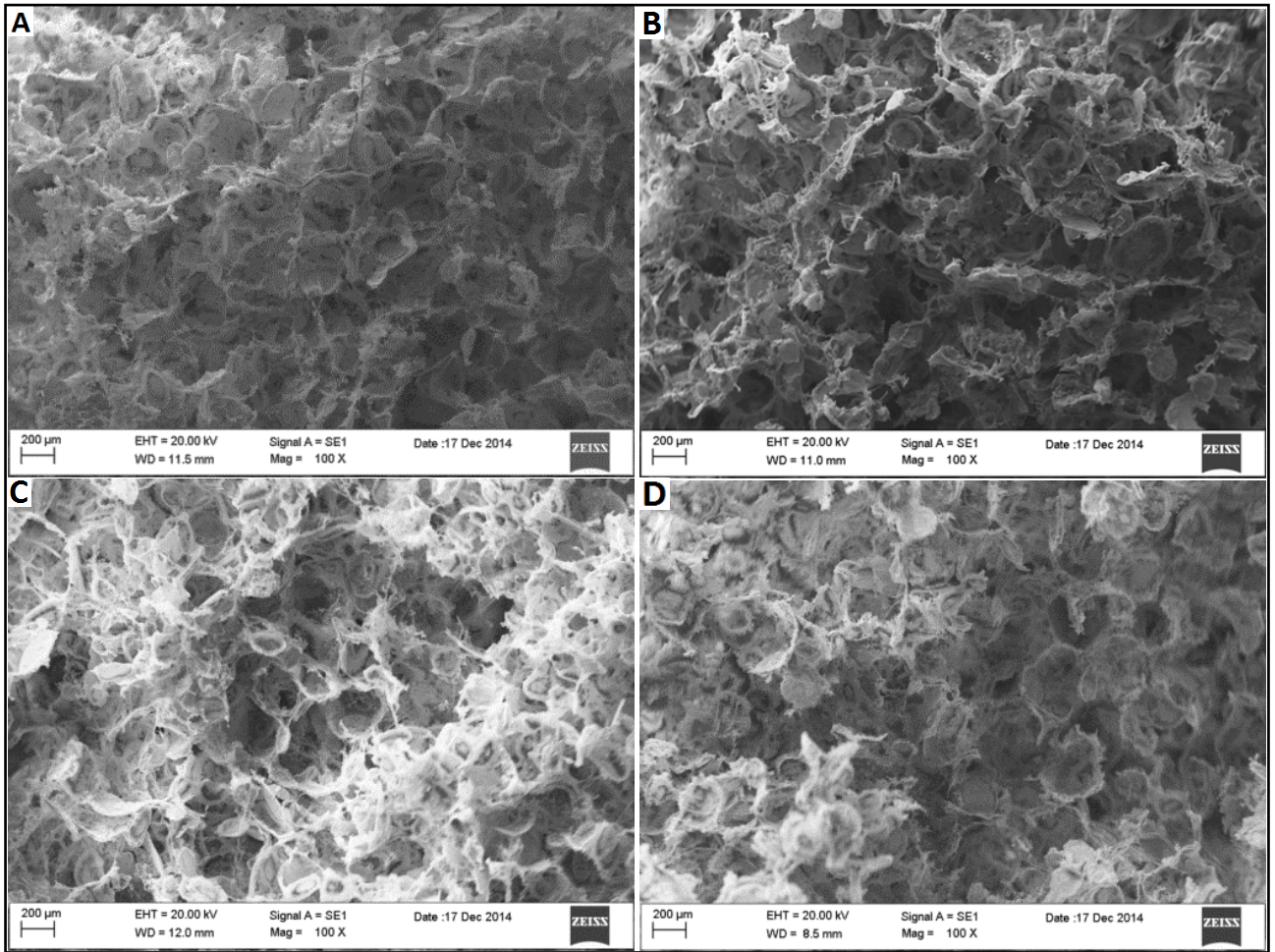


Figure 1. SEM of PLLA/PBS sponge scaffolds: (A) PLLA/PBS (2%, w/v, 1:1); (B) PLLA/PBS (3%, w/v, 2:1); (C) PLLA/PBS (4%, w/v, 1:1); (D) PLLA/PBS (6%, w/v, 1:1); scale bar – 200 µM, magnification – 100×.

Table. Porosity percentages of PLLA/PBS sponges.

Samples	Porosity %
PLLA/PBS (2%, w/v, 1:1)	32.49 ± 1.2
PLLA/PBS (3%, w/v, 2:1)	36.60 ± 0.67
PLLA/PBS (4%, w/v, 1:1)	32.12 ± 0.31
PLLA/PBS (6%, w/v, 1:1)	30.46 ± 1.1

3.3. Schwann cell proliferation on PLLA/PBS sponges by MTS assay

The CellTiter 96 nonradioactive cell proliferation MTS assay was performed to examine the effect of PLLA/PBS sponges on Schwann cell proliferation for 1, 7, 14, and 21 days of incubation (Figure 4).

The results show that all the blends were biocompatible and not cytotoxic. On day 7, when cell proliferation rates

of the groups were compared to each other, the highest cell number was observed in PLLA/PBS (3%, w/v, 2:1), and it was significantly ($P \leq 0.05$) higher than in other blends. However, due to focal contact inhibition, the cell number decreased on day 14 and day 21 for PLLA/PBS (3%, w/v, 2:1), and this blend presented a slightly lower cell number than PLLA/PBS 6%, w/v, 1:1, PLLA/PBS 4%, w/v, 1:1, and the control groups at the end of 21 days. At the end of the

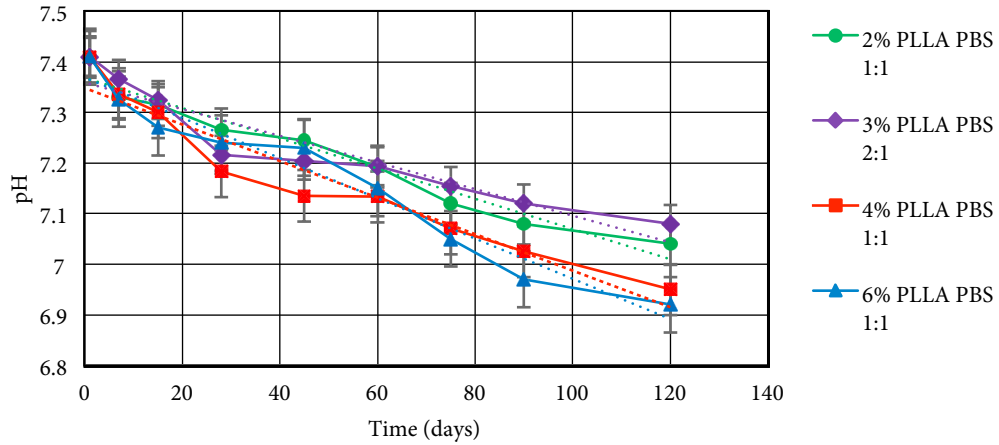


Figure 2. pH change of PLLA/PBS sponges during 120 days of degradation study.

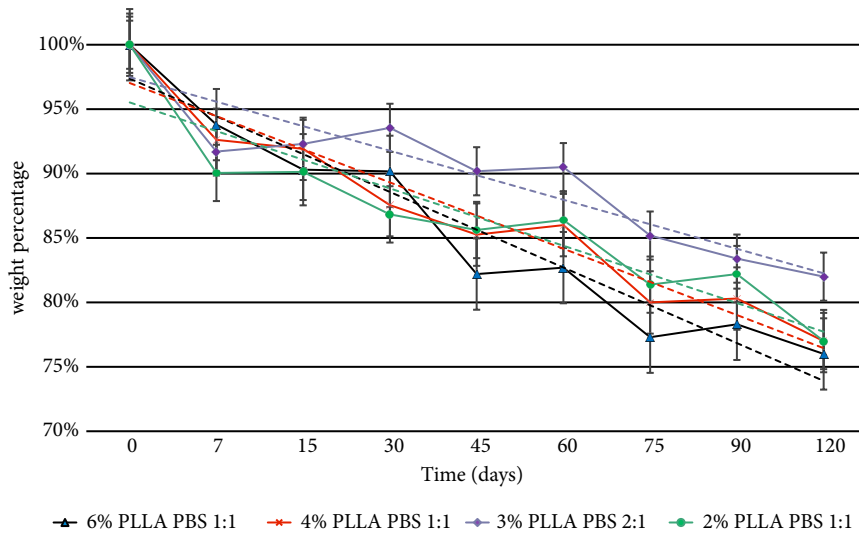


Figure 3. Percentage weight loss of PLLA/PBS sponges during 120 days of degradation study.

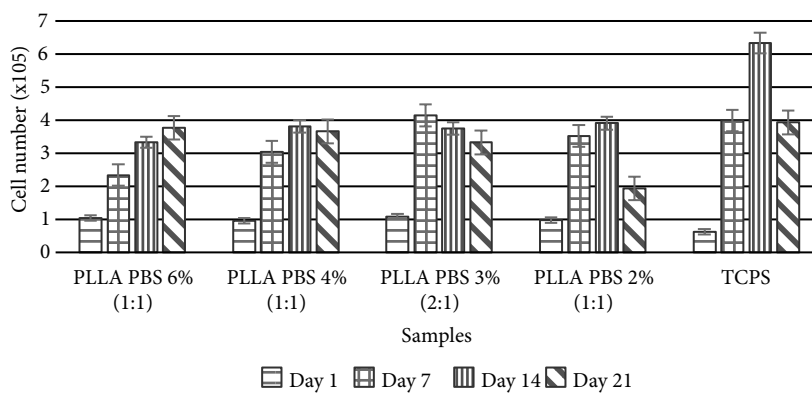


Figure 4. Proliferation of Schwann cells on PLLA/PBS sponges throughout 21 days of incubation. Initial cell seeding number was 50,000 cells/sample.

study, the cell number differences between the PLLA/PBS 6%, w/v, 1:1 blend, PLLA/PBS 4%, w/v, 1:1 blend, and the control group were not significantly different ($P \leq 0.05$). However, the cell number for PLLA/PBS 2%, w/v, 1:1 was distinctly lower than in all other groups, and the difference was statistically significant ($P \leq 0.05$). On day 21 it was also seen by light microscopy that the cells tended to detach from the scaffold surfaces as cellular sheets (data not shown), and cell numbers decreased for all blends except PLLA/PBS (6%, w/v, 1:1).

3.4. Scanning electron microscopy

Schwann cells (20,000 cells/sample) were seeded on PLLA/PBS sponges and incubated for 21 days. Cell adherence and proliferation were visualized by SEM (Figures 5A–5L). Localization of cells around the interconnected pores was investigated. The results showed that the cells adhered in the pores of the sponges and cellular interactions were provided. The results also demonstrated that the 3-D porous structure of polymeric sponges was maintained for 21 days, and the cells elongated in these pores and underwent cellular interactions. For PLLA/PBS (2%, w/v, 1:1) sponge, the cells adhered to the material surface on day 7, and cellular sheets occurred on day 14 (Figures 5A and 5B). However, the cell number decreased on day 21 due to the degradation of scaffolds and focal contact inhibition (Figure 5C). A similar situation was observed for sample PLLA/PBS (3%, w/v, 2:1) (Figures 5J–5L); however, the porosity was lower compared to PLLA/PBS (2%, w/v, 1:1). It was also seen that the cells adhered in the pores of PLLA/PBS (4%, w/v, 1:1) (Figures 5D–5F) and PLLA/PBS (6%, w/v, 1:1) (Figures 5G–5I) sponges, and the cellular proliferation rate was lower in these samples compared to PLLA/PBS (2%, w/v, 1:1) and PLLA/PBS (3%, w/v, 2:1), as supporting results were also obtained from the MTS assay.

3.5. Confocal microscopy

The morphology and proliferation of Schwann cells on PLLA/PBS sponges were visualized by confocal microscopy throughout 21 days of incubation (Figures 6A–6P). It was observed that there were a few cells on the scaffolds and the cells were rounder on day 1 (Figures 6A, 6E, 6I, and 6M). However, on days 7 and 14 the cells showed ordinary Schwann cell morphology with neural cell extensions (Figures 6B, 6C, 6F, 6G, 6J, 6K, 6N, and 6O). It was observed that the polymer sponges were biocompatible and the cells were able to survive on these sponges. Cellular attachment was detected around and in the inner part of the pores. The presence of cell sheets was observed in PLLA/PBS (2%, w/v, 1:1) and PLLA/PBS (3%, w/v, 2:1) samples especially (Figures 6J, 6K, and 6O). The cells were localized in the pores and tended to form cell sheets on sponge structures. However, due to the degradation of the scaffold and contact inhibition, the cell number decreased on day 21 (Figures 6D, 6H, 6L, and 6P), as was also confirmed by MTS and SEM results.

4. Discussion

The goal of this study was to evaluate the potential of PLLA/PBS sponges as a scaffold for neural tissue engineering. PLLA is one of the most preferred polyesters in tissue engineering that has been used in many forms such as sponges, fibers, and films (Roether et al., 2002; Yang et al., 2004a, 2004b; Wojasinski et al., 2013; Caldara et al., 2014). PBS has also been used in bone and cartilage tissue engineering with several applications including coating, micropatterning, and electrospinning (Ishioka et al., 2002; Coutinho et al., 2012; Wang et al., 2012; Wei et al., 2012; Arphavasin et al., 2013). Oudega et al. (2001) investigated the effects of PLLA tubular scaffold implantation on spinal cord injury. Schwann cells were seeded on scaffolds and implanted in a transected adult rat thoracic spinal cord. It was observed that axonal growth was provided with extensive vascularization after 4 months. However, it was also found that in some animal studies the tubular implants broke into large polymer pieces because of the crystalline structure and brittle nature, and this breakage diminished the regeneration (Oudega et al., 2001). PBS is known to have better processability and mechanical properties than PLLA (Ishioka et al., 2002; Li et al., 2005; Xu et al., 2010; Arphavasin et al., 2013). Besides, PBS has a very high tensile strength that makes the polymer suitable for tissue engineering applications (Tserki et al., 2006). Therefore, we have used blends of PLLA with PBS in order to increase the processability of the polymer. There are some studies investigating the biocompatibility of PBS/PLA blends (Hua et al., 2010, 2012). However, the potential of Schwann cell-seeded PLLA/PBS polymeric sponges has not been studied in the literature yet. In our study it is shown that PLLA/PBS sponges can be promising scaffolds for neural tissue engineering. According to Shibata et al. (2006), PLA/PBS blends have a higher tensile strength compared to pure PLA or PBS. It was demonstrated by Hua et al. (2012) that PLA/PBS blends have good biocompatibility and result in a mild and acceptable inflammatory response when implanted in rats subcutaneously.

In the present study, MTS analysis showed that PLLA/PBS scaffolds were not cytotoxic, and Schwann cells survived on these scaffolds for up to 21 days. On day 21, the cell numbers were very similar and close to the tissue culture polystyrene (TCP) control for PLLA/PBS blends, except for PLLA/PBS (2%, w/v, 1:1). In this blend, the cell number decreased significantly on day 21. This might be related to the degradation of the scaffold and focal inhibition, as it was observed that cellular sheets were detaching from the sponge surfaces. Supporting our findings, it was stated in one of the studies (Casella et al., 2000) that there was a significant decrease in the rate of Schwann cell growth when the cell densities were higher, and the Schwann cell response to mitogen was

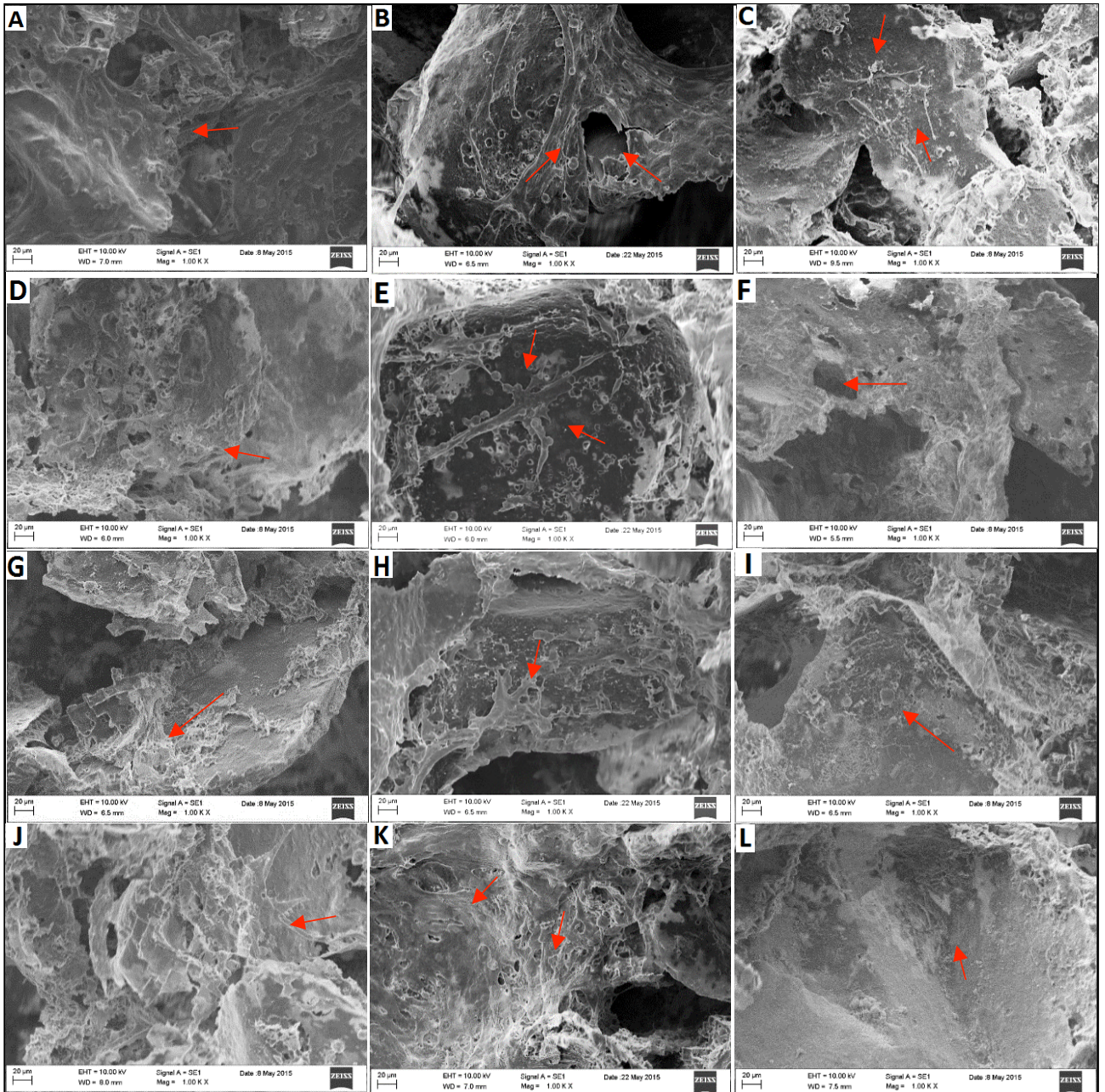


Figure 5. SEM images of Schwann cells seeded on (A, B, C) PLLA/PBS (2%, w/v, 1:1), (D, E, F) PLLA/PBS (4%, w/v, 1:1), (G, H, I) PLLA/PBS (6%, w/v, 1:1), and (J, K, L) PLLA/PBS (3%, w/v, 2:1) throughout (A, D, G, J) 7 days, (B, E, H, K) 14 days, and (C, F, I, L) 21 days of incubation. Scale bar – 20 µm, magnification – 1000×. Arrows show Schwann cells.

regulated by a density-dependent mechanism that might be autocrine inhibitor production or contact inhibition. In other studies (Augenlicht et al., 1974; Bard et al., 1986), it was also mentioned that the proliferation rate decreased significantly in confluent cell cultures compared to sparsely seeded cells. It was also stated by Park et al. (2013) that when there was less available space for the cells to proliferate, there was a smaller number of adhered cells

and less proliferation. These results also supported our findings both in the TCPS (control) group and in PLLA/PBS 2%, w/v, 1:1 and 3%, w/v, 2:1 blends for day 21. In addition, Schwann cells tend to form cellular sheets, and this is a desired feature for neural tissue engineering. Cell sheet engineering is another promising approach for nerve tissue engineering, and it was demonstrated that Schwann cell sheets can support neural regeneration by

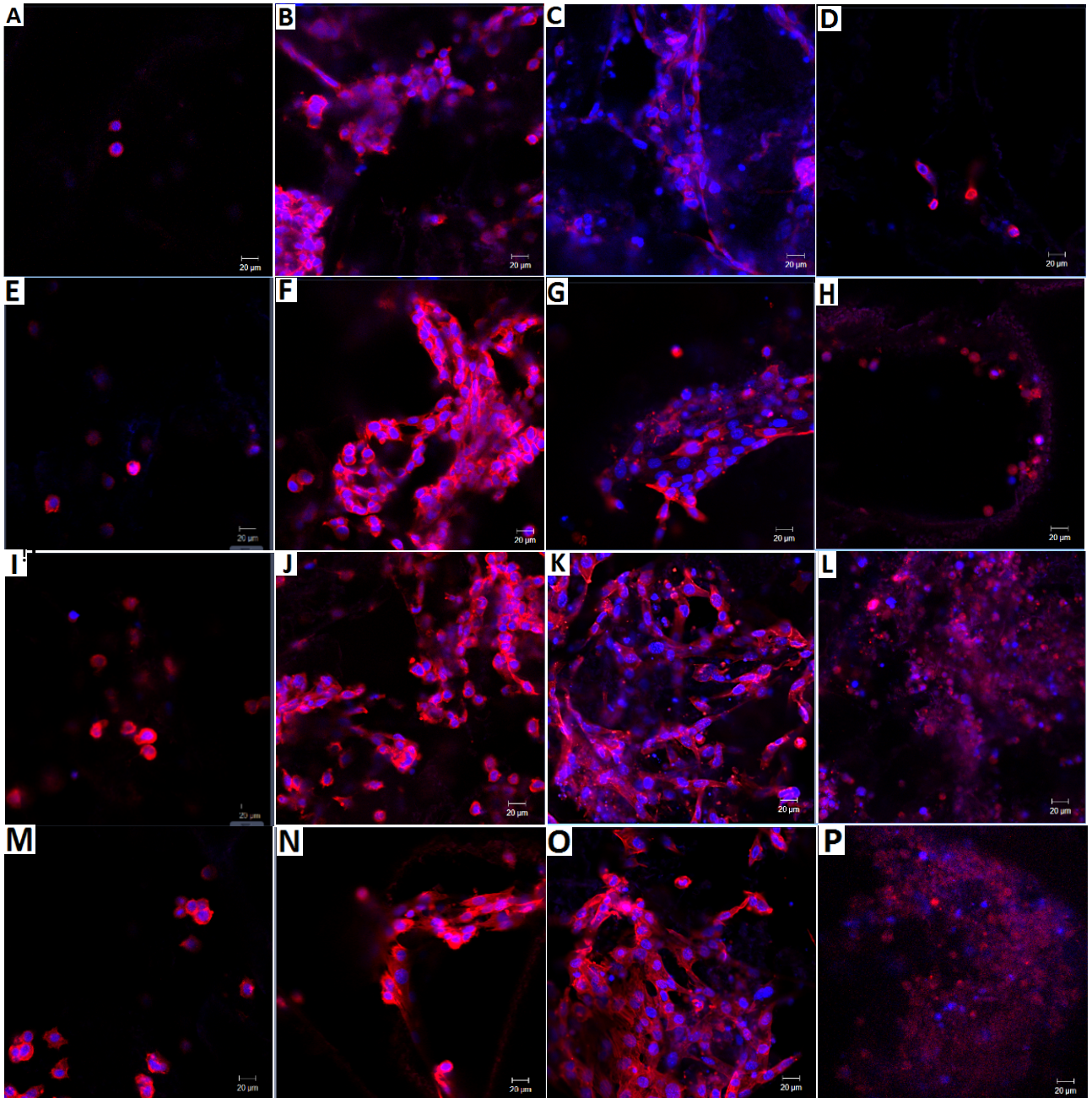


Figure 6. Confocal microscopy images of Schwann cells seeded on (A, B, C, D) PLLA/PBS (6%, w/v, 1:1), (E, F, G, H) PLLA/PBS (4%, w/v, 1:1), (I, J, K, L) PLLA/PBS (3%, w/v, 2:1), and (M, N, O, P) PLLA/PBS (2%, w/v, 1:1) throughout (A, E, I, M) 1 day, (B, F, J, N) 7 days, (C, G, K, O) 14 days, and (D, H, L, P) 21 days of incubation. Scale bar – 20 µm, magnification – 20×. Red: F-actin stained with Alexa Fluor 546; blue: nuclear DNA stained with DAPI.

secreting a high amount of neurotrophins (Pesirikan et al., 2013). The scaffolds that we constructed also enabled the Schwann cells to form cellular sheets that can enhance the regeneration process. These data were also supported by SEM and confocal microscopy results. Cellular sheets were visualized around and inside the pores of the scaffolds by confocal microscopy and all of these results supported the

potential of the polymeric sponge to be used in neural tissue engineering. Supporting our results, Vilarinho-Feltre et al. (2016) asserted that Schwann cell sheets on hyaluronic acid conduits can be used to restore damaged neural tracts. It was demonstrated that the seeded Schwann cells covered the inner surface of the constructed conduit after 10 days of culture and formed an inner continuous

cell tube from the cellular sheet. It was concluded in the study that these biohybrid tubular structures may have the potential for reconnecting the neural cells both for central and peripheral nerve tissues (Vilariño-Feltrer et al., 2016). In our study, it was observed that PLLA/PBS (2%, w/v, 1:1) and PLLA/PBS (3%, w/v, 2:1) blends triggered cellular sheet formation after 7 days of incubation, but after day 14, due to contact inhibition, Schwann cell sheets detached from the sponge surfaces.

One of the main reason for choosing a sponge structure was to construct a suitable environment for Schwann cells, which naturally secrete cellular extracts and neurotrophic factors such as ciliary neurotrophic factor and neurotrophins. The diffusion of these agents to the environment provides nutrition, axonal growth, differentiation, and survival of neural cells, which contributes to the regeneration process (Schmidt et al., 2003; Angelov et al., 2007). Schwann cells are known as support cells of the peripheral nervous system, and they are utilized to promote axonal regrowth both in peripheral and in central nervous system defects for neural tissue engineering (Blakemore et al., 1977; Frostick et al., 1998; Nomura et al., 2006). There are also studies showing that the usage of Schwann cells in central nervous system injuries enhanced the regeneration process, and this effect was suspected to be provided by the secreted neurotrophins from the Schwann cells. According to Wang and Xu (2014), injection of Schwann cells directly into the lesion site at the midthoracic level in adult rats promoted myelination of regenerating axons. The transplanted cells survived until week 24 and migrated along the central canal. However, they concluded that the Schwann cell transplantation strategy should be combined with other approaches for significant functional recovery. The idea was also supported by Yuan et al. (2009), who showed that transplantation of Schwann cells alone had drawbacks such as short migration distance and that 3-D scaffolds gain importance for providing growth of nerve fibers and regeneration. Kamada et al. (2005) seeded bone-marrow stromal cell-derived Schwann cells on a nondegradable tubular scaffold made of polyether sulfone ultrafiltration membrane and implanted the scaffold in a transected rat spinal cord. It was observed that central axon regrowth was promoted and functional recovery was enhanced as a result of implantation.

In terms of biodegradability of the polymer sponge, a 120-day degradation study was performed and different blend ratios were analyzed. In one of the earlier studies it was stated that in vitro degradation behavior of polymers can be utilized to predict the in vivo degradation profile. To mimic in vivo conditions, polymers are generally incubated at 37 °C in PBS and either static or continuous agitation is performed (Costa-Pinto et al., 2014). We used a similar approach for the degradation study and

we monitored both the pH difference and the weight loss of the sponges. The structural integrity of the sponges was also examined. For the performance of cell-seeded scaffolds, the degradation behavior is important for cell proliferation, regeneration, and immune response. The rate of degradation should be compatible with the healing rate of the targeted tissue (Babensee et al., 1998). There are limited studies considering hydrolytic PBS biodegradation for medical applications. However, PBS is frequently used for nonmedical applications and there are different studies investigating PBS degradation in soil-burial biodegradation tests (Liu et al., 2009; Zhang et al., 2013). Liu et al. (2009) performed a biodegradation study of PBS film and PBS/jute fibers; the degradation was carried out by burying the sample in compost soil. It was observed that 30% of the pure PBS film was degraded by the end of 180 days. Using the same strategy that we used in our study, Li et al. (2005) performed a hydrolytic degradation of poly(butylene succinate) films, and at the end of 9 weeks, $90 \pm 5.2\%$ of the initial weight remained. However, after 15 weeks, this value dropped to $35 \pm 4.0\%$ (Li et al., 2005). Regarding PLLA degradation behavior that depends on the crystallinity of the polymeric structure (Miller et al., 1977), Wu and Ding (2004) determined that PDLLA porous scaffold weight did not change significantly at the end of a 26-week degradation study. Supporting this study, Lu et al. (2000) studied the degradation profile of a PLLA foam structure with different porosity levels (0.67, 0.79, and 0.91 porosities for the constructs treated with 70%, 80%, and 90% initial salt weight fraction, respectively) and different thicknesses for 46 weeks. It was determined that the initial weights and thicknesses did not change in the constructs treated with salt particles of 106–150 µm during the degradation study. It was also stated in this study that thicker pore walls resulted in faster foam degradation due to an autocatalytic effect, and as the degradation products and carboxylic groups were accumulating inside the pores, faster degradation was triggered (Lu et al., 2000). In our study, similar to this result, we have also observed faster degradation of the sample PLLA/PBS (6%, w/v, 1:1) polymer blend. Since the PBS concentration in 1:1 blends was higher than in the 2:1 blend, PBS degraded faster than PLLA in the sponge, which led to the loss of structural integrity in these blends. Similar results were obtained in different studies (Can et al., 2011). There is a limited number of studies in the literature considering PLLA/PBS blend degradation. Therefore, each polymer was investigated for its own characteristics. Recently, it was reported by Kimble et al. (2014) that after a 24-week degradation study, the molecular weight of 75:25 wt.% PLLA/PBS decreased to 2.8×10^3 g/mol from an initial molecular weight of 49×10^3 g/mol. Zhang et al. blended PLLA with PBS in a melt state and carried out a biodegradation study

for 60 days by burying the samples in soil (Zhang et al., 2013). Different blend ratios were prepared and 8.5% and 9.5% of initial weights were lost for the samples of PLLA/PBS 60:40 and 40:60, respectively, at the end of 60 days. Although a different methodology was tested to determine the biodegradation rate of PLLA/PBS blend in this study, Zhang et al. (2013) determined that higher PLLA amount in a PLLA/PBS blend resulted in a slower degradation rate compared to the PLLA/PBS blend with a higher PBS ratio in a soil-burial test, and these data also support our results. In our study, after 120 days of incubation, almost 25% of total weight was lost in PLLA/PBS sponges (6%, 4%, 2%, w/v, 1:1), while this value was around 18% for PLLA/PBS (3%, w/v, 2:1). It can be asserted from these results that the degradation rate is relevant to the PBS ratio in the polymer sponges. Regarding the pH differences of the samples during 120 days of incubation, there was a pH decrease of the incubation solution from 7.4 to around 7.1–6.9, respectively, for PLLA/PBS 3%, w/v, 2:1 and PLLA/PBS 6%, w/v, 1:1. We did not observe a sudden decline in pH that could harm the implanted tissue. There was no burst of material during the degradation study that could cause a significant problem in vivo. Increased acidity can cause the decrease of cell viability (Wuertz et al., 2009). During the degradation, pH decrease showed a smooth pattern, which also proved that the constructed sponges were convenient for tissue engineering applications.

In this study, the potential in vitro usage of PLLA/PBS polymeric sponges for neural tissue engineering was investigated. Different concentrations and ratios of polymeric blends were used to prepare tissue engineering scaffolds and their in vitro characterization was carried out in terms of porosity, biodegradation behavior, and biocompatibility using Schwann cells. At the beginning of our experiments, PLLA/PBS (8%, w/v, 6:2), PLLA/PBS (6%, 4%, 3%, w/v, 2:1), and PLLA/PBS (8%, 6%, 4%, 2%, w/v, 1:1) blend ratios were prepared and characterized (data not shown). It was determined that the morphological

structure of the blends PLLA/PBS 8%, w/v, 6:2, 6%, 4%, w/v, 2:1, and 8%, w/v, 1:1 were not suitable for further analysis. Phase separation and nonhomogeneous pores were observed (Figure S2). Therefore, PLLA/PBS (6%, 4%, 2%, w/v, 1:1) and PLLA/PBS (3%, w/v, 2:1) were chosen for further characterization studies.

According to the results, the PLLA/PBS (3%, w/v, 2:1) blend is more convenient for supporting Schwann cell attachment and proliferation compared to other blends. The PLLA/PBS (3%, w/v, 2:1) blend retained its structural integrity until day 120 of the biodegradation study. Furthermore, the acidity of the incubation solution did not increase significantly. In terms of biocompatibility studies, cell number was almost the same as in the control group at the end of 21 days of culture. Confocal images and SEM images also showed that the cellular attachment was higher on these scaffolds and the cells penetrated inside the pores. There are very limited studies regarding PBS in tissue engineering due to its lower biocompatibility compared to other polyesters. However, the flexible nature of the polymer makes it convenient for the construction of tissue conjugates with different 3-D structures, especially when blended with different materials. Our study supported the finding that PBS, when blended with PLLA, was a suitable alternative for neural tissue engineering studies. Schwann cells are known as supportive cells of the peripheral nervous system. The positive effects of Schwann cell transplantation not only for peripheral nervous system injuries but also for injuries of the central nervous system were proven in many studies in the literature. Therefore, in this study, PLLA/PBS sponges were combined with Schwann cells and the construct's performance was investigated in vitro to show the efficiency for neural tissue engineering. Based on these results, this tissue-engineered construct will be further investigated in in vivo experiments.

Acknowledgement

This work was funded by L'Oréal Türkiye.

References

- Angelov DN, Ceynowa M, Guntinas-Lichius O, Streppel M, Grosheva M, Kiryakova SI, Skouras E, Maegele M, Irintchev A, Neiss WF et al. (2007). Mechanical stimulation of paralyzed vibrissal muscles following facial nerve injury in adult rat promotes full recovery of whisking. *Neurobiol Dis* 26: 229-242.
- Arphavasin S, Singhatanadgit W, Ngamviriyavong P, Janvikul W, Meesap P, Patntirapong S (2013). Enhanced osteogenic activity of a poly(butylene succinate)/calcium phosphate composite by simple alkaline hydrolysis. *Biomed Mater* 8: 055008.
- Augenlicht LH, Baserga R (1974). Changes in the G0 state of WI-38 fibroblasts at different times after confluence. *Exp Cell Res* 89: 255-262.
- Babensee JE, Anderson JM, McIntire LV, Mikos AG (1998). Host response to tissue engineered devices. *Advanced Drug Delivery Reviews* 33: 111-139.
- Bard J, Elsdale T (1986). Growth regulation in multilayered cultures of human diploid fibroblasts: the role of contact, movement and matrix production. *Cell Tissue Kinet* 19: 141-154.
- Blakemore WF (1977). Remyelination of CNS axons by Schwann cells transplanted from the sciatic nerve. *Nature* 266: 68-69.
- Bonassar LJ, Vacanti CA (1998). Tissue engineering: the first decade and beyond. *J Cell Biochem* 30: 297-303.

- Caldara G, Rigogliuso S, Pavia FC, Brucato V, Ghersi G (2014). Biocompatibility evaluation of PLLA scaffolds for vascular tissue engineering. *Italian Journal of Anatomy and Embryology* 119: 31.
- Can E, Udenir G, Kanneci AI, Kose G, Bucak S (2011). Investigation of PLLA/PCL blends and paclitaxel release profiles. *AAPS PharmSciTech* 12: 1442-1453.
- Casella GT, Wieser R, Bunge RP, Margitich IS, Katz J, Olson L, Wood PM (2000). Density dependent regulation of human Schwann cell proliferation. *Glia* 30: 165-177.
- Costa-Pinto AR, Martins AM, Castelhana-Carlos MJ, Correló VM, Sol PC, Longatto-Filho A, Battacharya M, Reis RL, Neves NM (2014). In vitro degradation and in vivo biocompatibility of chitosan-poly(butylene succinate) fiber mesh scaffolds. *J Bioact Compat Pol* 29: 137-151.
- Coutinho DF, Gomes ME, Neves NM, Reis RL (2012). Development of micropatterned surfaces of poly(butylene succinate) by micromolding for guided tissue engineering. *Acta Biomater* 8: 1490-1497.
- Frostick SP, Yin Q, Kemp GJ (1998). Schwann cells, neurotrophic factors, and peripheral nerve regeneration. *Microsurgery* 18: 397-405.
- Hua K, Wang XW, Zhang W, Zhou J, Yang XB, Ji JH (2010). Cytocompatibility of PBS/PLA blend as the sternal fixation material. *Journal of Southern Medical University* 30: 1501-1508.
- Hua K, Zhang W, Liu X, Yang XK (2012). Biocompatibility of a novel poly(butyl succinate) and polylactic acid blend. *ASAIO J* 58: 262-267.
- Ishioka R, Kitakuni E, Ichikawa Y (2002). Aliphatic polyesters: Bionolle. In: Steinbüchel A, editor. *Biopolymers Online*. New York, NY, USA: Wiley, pp. 275-297.
- Kamada T, Koda M, Dezawa M, Yoshinaga K, Hashimoto M, Koshizuka S, Nishio Y, Moriya H, Yamazaki M (2005). Transplantation of bone marrow stromal cell-derived Schwann cells promotes axonal regeneration and functional recovery after complete transection of adult rat spinal cord. *J Neuropath Exp Neur* 64: 37-45.
- Kimble LD, Bhattacharyya D (2014). In vitro degradation effects on strength, stiffness, and creep of PLLA/PBS: a potential stent material. *Int J Polym Mater* 64: 299-310.
- Li H, Chang J, Cao A, Wang J (2005). In vitro evaluation of biodegradable poly(butylene succinate) as a novel biomaterial. *Macromol Biosci* 23: 433-440.
- Liu L, Yu J, Cheng L, Yang X (2009). Biodegradability of poly(butylene succinate) (PBS) composite reinforced with jute fibre. *Polym Degrad Stabil* 94: 90-94.
- Lu L, Peter SJ, Lyman MD, Lai HL, Leite SM, Tamada JA, Vacanti JP, Langer R, Mikos AG (2000). In vitro degradation of porous poly(L-lactic acid) foams. *Biomaterials* 21: 1595-1605.
- Ma P, Wang X, Liu B, Li Y, Chen S, Zhang Y, Xu G (2012). Preparation and foaming extrusion behavior of polylactide acid/polybutylene succinate/montmorillonoid nanocomposite. *J Cell Plast* 48: 191-205.
- Martina M, Hutmacher DW (2007). Biodegradable polymers applied in tissue engineering research: a review. *Polym Int* 56: 145-157.
- Miller RA, Brady JM, Cutright DE (1977). Degradation rates of oral resorbable implants (polylactates and polyglycolates): Rate modification with changes in PLA/PGA copolymer ratios. *J Biomed Mater Res* 11: 711-719.
- Neel EAA, Chrzanowski W, Salih VM, Kim HW, Knowles JC (2014). Tissue engineering in dentistry. *J Dent* 42: 915-928.
- Nomura H, Tator CH, Shoichet MS (2006). Bioengineered strategies for spinal cord repair. *J Neurotrauma* 23: 496-507.
- Oudega M, Gautier SE, Chapon P, Frago M, Bates ML, Parel JM, Bunge MB (2001). Axonal regeneration into Schwann cell grafts within resorbable poly(alpha-hydroxyacid) guidance channels in the adult rat spinal cord. *Biomaterials* 22: 1125-1136.
- Park S, Ahn SH, Lee HJ, Chung US, Kim JH, Koh WG (2013). Mesoporous TiO₂ as a nanostructured substrate for cell culture and cell patterning. *RSC Advances* 3: 23673-23680.
- Pesirikan N, Chang W, Zhang X, Xu J, Yu X (2013). Characterization of Schwann cells in self-assembled sheets from thermoresponsive substrates. *Tissue Eng Pt A* 19: 1601-1609.
- Rasal RM, Hirt DE (2010). Poly(lactic acid) toughening with a better balance of properties. *Macromol Mater Eng* 295: 204-209.
- Roether JA, Boccaccini AR, Hench LL, Gautier VMS, Jérôme R (2002). Development and in vitro characterisation of novel bioresorbable and bioactive composite materials based on polylactide foams and Bioglass® for tissue engineering applications. *Biomaterials* 23: 3871-3878.
- Schmidt CE, Leach JB (2003). Neural tissue engineering: strategies for repair and regeneration. *Annu Rev Biomed Eng* 5: 293-347.
- Shibata M, Inoue Y, Miyoshi M (2006). Mechanical properties, morphology, and crystallization behavior of blends of poly(L-lactide) with poly(butylene succinate-co-L-lactate) and poly(butylene succinate). *Polymer* 47: 3557-3564.
- Tserki V, Matzinos P, Pavlidou E, Vachliotis D, Panayiotou C (2006). Biodegradable aliphatic polyesters. Part I. Properties and biodegradation of poly(butylene succinate-cobutylene adipate). *Polym Degrad Stabil* 91: 367-376.
- Vilarinho-Feltrer G, Martínez-Ramos C, Monleón-de-la-Fuente A, Vallés-Lluch A, Moratal D, Albacar JAB, Pradas MM (2016). Schwann-cell cylinders grown inside hyaluronic-acid tubular scaffolds with gradient porosity. *Acta Biomater* 30: 199-211.
- Wang H, Xu M, Wu Z, Zhang W, Ji J, Chu PK (2012). Biodegradable poly(butylene succinate) modified by gas plasmas and their in vitro functions as bone implants. *ACS Applied Materials and Interfaces* 4: 4380-4386.
- Wang X, Xu XM (2014). Long-term survival, axonal growth-promotion, and myelination of Schwann cells grafted into contused spinal cord in adult rats. *Exp Neurol* 26: 308-319.

- Wei JD, Tseng H, Chen ET, Hung CH, Liang YC, Sheu MT, Chen CH (2012). Characterizations of chondrocyte attachment and proliferation on electrospun biodegradable scaffolds of PLLA and PBSA for use in cartilage tissue engineering. *J Biomater Appl* 26: 963-985.
- Wojasinski M, Faliszewski K, Ciach T (2013). Electrospinning production of PLLA fibrous scaffolds for tissue engineering. *Challenges of Modern Technology* 4: 9-15.
- Wu L, Ding J (2004). In vitro degradation of three-dimensional porous poly (D,L-lactide-co-glycolide) scaffolds for tissue engineering. *Biomaterials* 25: 5821-5830.
- Wuertz K, Godburn K, Iatridis JC (2009). MSC response to pH levels found in degenerating intervertebral discs. *Biochem Biophys Res Commun* 379: 824-829.
- Xu J, Guo BH (2010). Poly(butylene succinate) and its copolymers: research, development and industrialization. *Biotechnology Journal* 5: 1149-1163.
- Yang F, Murugan R, Ramakrishna S, Wang X, Ma YX, Wang S (2004a). Fabrication of nano-structured porous PLLA scaffold intended for nerve tissue engineering. *Biomaterials* 25: 1891-1900.
- Yang F, Xu CY, Kotaki M, Wang S, Ramakrishna S (2004b). Characterization of neural stem cells on electrospun poly(L-lactic acid) nanofibrous scaffold. *J Biomat Sci-Polym E* 12: 1483-1497.
- Yuan JD, Nie WB, Fu Q, Lian XF, Hou TS, Tan ZQ (2009). Novel three-dimensional nerve tissue engineering scaffolds and its biocompatibility with Schwann cells. *Chinese Journal of Traumatology* 12: 133-137.
- Zhang SJ, Tang YW, Cheng LH (2013). Biodegradation behavior of PLA/PBS blends. *Adv Mat Res* 821: 937-940.

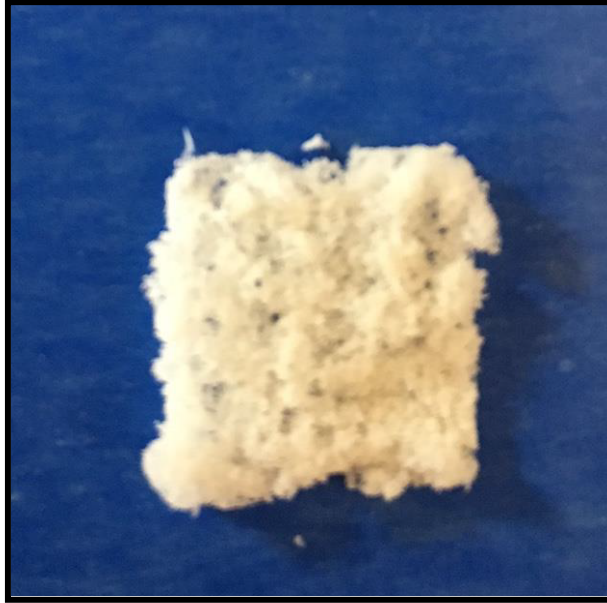


Figure S1. Square sponge scaffolds prepared for characterization and in vitro studies. Total area: 1 cm².

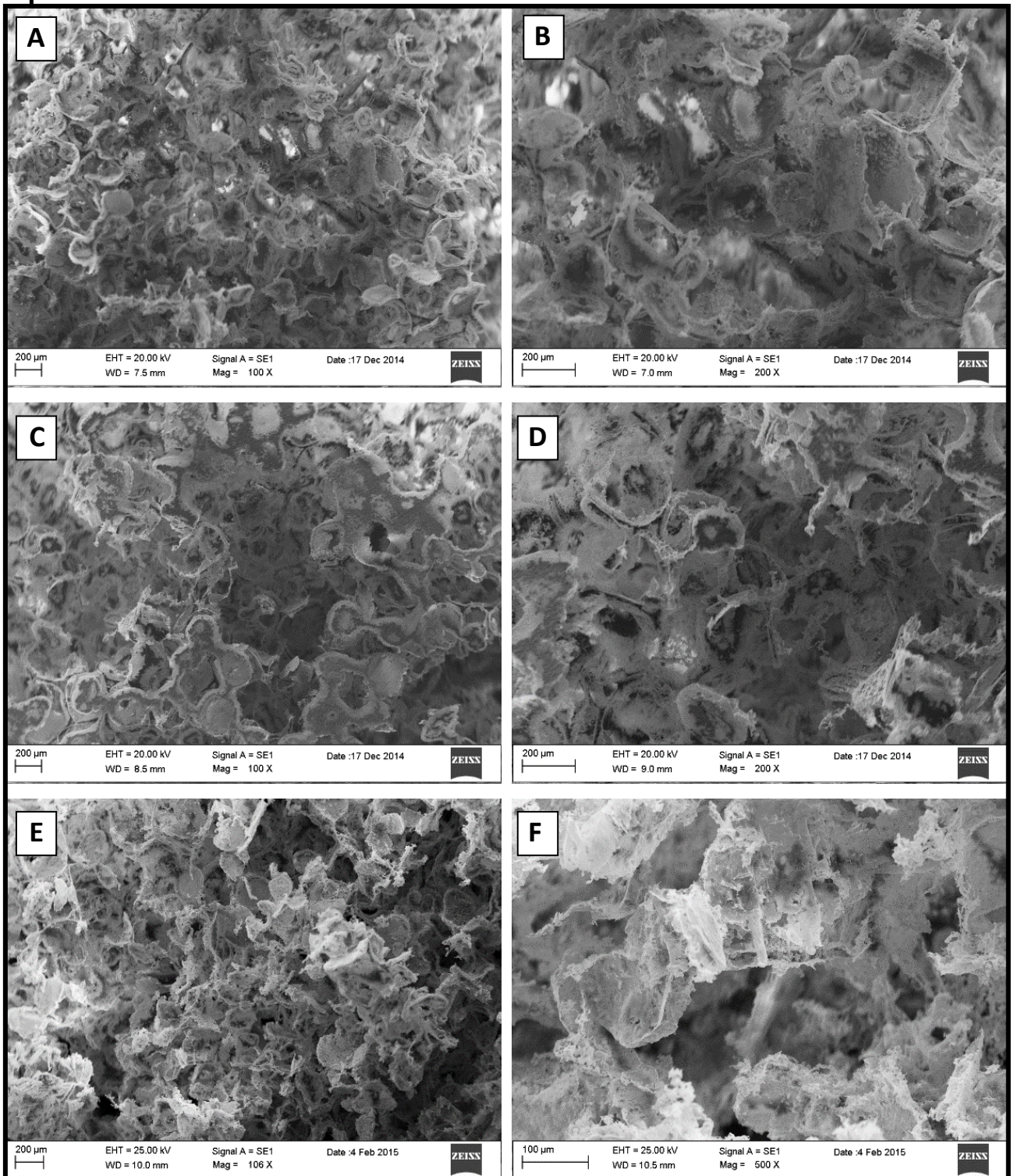


Figure S2. SEM of PLLA/PBS sponge scaffolds: (A, B) PLLA/PBS (8%, w/v, 6:2), (C, D) PLLA/PBS (6%, w/v, 2:1), (E, F) PLLA/PBS (8%, w/v, 1:1).