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Polylactide/polycaprolactone asymmetric membranes for guided bone regeneration

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Abstract: The aim of this work was to develop bioresorbable, asymmetric membranes for guided bone regeneration (GBR). Two resorbable polymers – polylactide (PLA) and polycaprolactone (PCL) were used in fabrication process. Two different manufacturing methods were applied: electrospinning in the case of PLA and freeze-drying of PCL. Mechanical properties, stability in a water environment and biocompatibility of fabricated membranes were evaluated. Microstructure [scanning electron microscopy (SEM)] of the membranes was assessed in terms of level of porosity, as well as size and shape of the pores. Study showed that combination of electrospinning and freeze-drying methods allows biocompatible PLA/PCL bi-phasic materials of appropriate mechanical properties and diverse microstructure to be produced, that should on the one hand prevent soft tissue growth, and on the other hand be a suitable scaffold for the growth of bone cells.

Keywords: biocompatibility; electrospinning; freeze-drying; morphology; polymer membranes.

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1 Introduction

Traumas, infections and tumor resections may result in extensive bone tissue defects. Even though many elements of human body, including bone tissue, are capable of regeneration, this ability does not apply to so-called "critical size defects" (CSD) (1, 2). Thus, in the case of critical size defects it is necessary to support natural regenerative mechanisms of the human body. One of the possibilities is guided bone regeneration (GBR) when porous membranes are used as mechanical barriers separating the space around the defect. Thanks to such a separation, during the healing process the bone tissue defect is separated from the soft tissue which has much bigger regenerative potential (1, 3).

Nowadays the materials used for GBR membranes may be divided into two groups: stable [e.g. expanded – polytetrafloroethylene (ePTFE), titanium mesh] (4, 5) and resorbable ones (natural collagen and chitosan; synthetic PLLA, PGA and their co-polymers) (6–10). Biostable membranes are usually removed after they have performed their task, although they may also be left in the body when overgrown by the bone tissue. The intensive research is being conducted to find the best solution for membranes among resorbable materials. So far numerous studies (11–16) have specified the parameters that are key factors for guided bone regeneration materials. The most important requirement is the proper size of pores. The pores of the membrane serve two functions: they prevent the soft tissue invasion into the defect and they facilitate the formation of new blood vessels and regeneration of the bone tissue. The size of pores ranging from 50 to 100 μm allows adhesion and proliferation of osteoblasts (bone forming cells), while the size over 150 μm makes it possible to form osteons (fundamental functional units of cortical bones) (17). On the other hand, the pores have to be small enough to inhibit the migration and proliferation of fibroblasts. To fulfil both those requirements, the membrane needs to be asymmetric. The asymmetry may be achieved by multilayered and multiphased structure of the membrane (18). Moreover, the membrane as a barrier between the soft and bone tissue must fulfill mechanical tasks until the new tissue is formed. Thus, parameters of the membrane such as its strength, elongation and elasticity are crucial factors. The final requirement is that the membrane should undergo biodegradation and bioresorption at the rate matched with the bone regeneration process (6).

Taking into consideration both mechanical parameters and the processing possibilities, the most perspective materials seem to be polylactide (PLA) and polycaprolactone (PCL). They both belong to resorbable biomaterials group and are widely used for different medical applications (e.g. surgical sutures, drug delivery systems, tissue regeneration scaffolds) (19–22).

Recent years have brought much extremely intensive research on methods for fabrication of tissue engineering scaffolds, with electrospinning technique being the most versatile and the most promising one (23–26). It is possible to electrospin nanofibers from different materials, including biocompatible and biodegradable polymers, like PLA and PCL and their composites (9, 27–29). Electrospun scaffolds have high surface areas and interconnected pore networks, providing a facile transport of metabolic nutrients and waste through the nanometer-sized pores. Using electrospinning, it is possible to produce scaffolds which are similar to the natural extracellular matrix (ECM), which can thus enhance the adhesion, proliferation and growth of cells (30).

The aim of this work was to obtain polymer membranes for guided bone regeneration by combining two methods: electrospinning of polylactide and freeze drying of polycaprolactone. The resulting two-phased membranes combining PLA nanofibers and PCL porous matrix ensure differentiated porosity, asymmetric microstructure and proper mechanical parameters. *In vitro* biocompatibility studies proved proper cell viability and proliferation.

2 Experimental

2.1 Materials

Poly-L-lactide (PLA, Ingeo™ 3051D) was obtained from NatureWorks LLC, USA and polycaprolactone (PCL, M_n=80,000) was obtained from Sigma Aldrich, Poland. Chloroform, methanol and acetic acid (CH_3COOH 99.5%–99.9% CZDA) were purchased from Avantor Performance Materials Poland S.A. (previously POCH S.A.). Reagents were used as received, without purification.

2.2 Membrane fabrication

Polylactide nonwovens were obtained by electrospinning (TIC 1092012, Bielsko-Biala, Poland). To prepare the spinning solution, 2.5 g of polymer was dissolved in 30 ml of chloroform and 10 ml of methanol. The parameters of electrospinning were as follows: applied voltage 28.2 kV, collector revolutions 330 rpm, the distance between the tip and the collector 20 cm, needle diameter 0.7 mm. Polycaprolactone membranes were obtained by freeze-drying. In order to obtain the membranes two solutions of the polymer in the acetic acid were prepared: 1:20 g/ml and 1:40 g/ml, labeled, respectively as PCL20 and PCL40. Approximately 20 ml of each solution was spread evenly on a glass Petri dish measuring 10 cm in diameter. Next, the samples were kept for 30 min at -20°C to make the solvent change into solid state. The samples underwent lyophilization (Labconco Freezone, Kansas City, USA) for 24 h. In the combined method PLA nanofibers were placed on glass Petri dishes and covered by 15 ml of PCL40, then the total was frozen in -20°C for 30 min and lyophilized for next 24 h. Thus the two-phased PLA/PCL40 membranes were manufactured.

2.3 Characterization

A scanning electron microscope Nova Nano SEM 200 (FEI Europe Company) was used to assess the microstructure of the materials. The computer analysis of the images was performed with ImageJ software. Minimum of three representative images of each kind were used for pore size and diameter of pores and fibers measurements. Shape coefficient was calculated as the aspect ratio of largest diameter to smallest diameter orthogonal to it. Porosity was estimated by quantitative stereological methods (point fraction and line fraction). Statistical analysis was done using the Student's t-test where $n=20$ and $\alpha=0.05$. Mechanical parameters were measured in a static tensile test, using a universal testing machine Zwick 7000 type 1435. The tests (in quintuplicate) were performed in compliance with the PN-EN 10002-1:2004 norm. In order to describe changes in pH and ion conductivity of the material extracts, tests were performed during the 6-week incubation at 37°C in distilled water. The constant ratio of the sample weight and liquid amount was applied. Biological tests were conducted on human osteosarcoma cell line MG-63. The cells were grown on the tested materials for 3 and 7 days, and then the cytotoxicity levels were established (ToxiLight_BioAssay Kit; Lonza, USA). The assessment of the cell morphology was performed by optical fluorescence microscopy (Olympus, Japan).

3 Results and discussion

In this study, different kinds of membranes were produced and then three types were selected for further detailed testing: PLA nonwovens, PCL membranes and PLA/PCL systems.

3.1 Porous PCL membranes

The SEM images reveal that PCL membranes have a different microstructure, depending on the concentration of solutions. In the case of higher concentration (1:20 g/ml) there are significant non porous areas on the surface, while in lower concentration (1:40 g/ml) the pores are evenly distributed all over the surface (Figure 1). Total porosity of membranes reached approximately 80% for PCL40 and 20% for PCL20. The two materials also differed with respect to the size of pores, which is presented in a histogram in Figure 2.

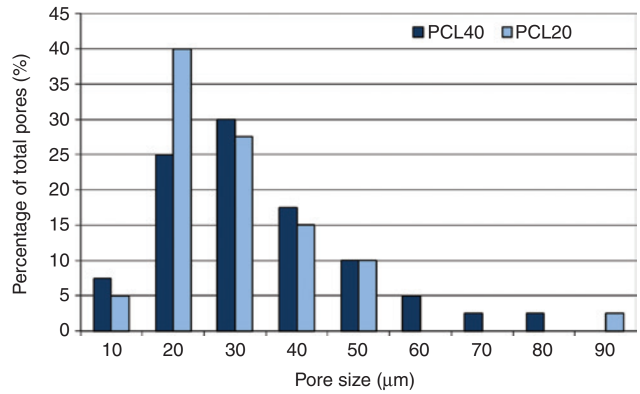

Figure 2: Average pore size distribution comparing PCL20 and PCL40.

The average size of pores in the PCL40 samples equals 80 μm, yet most of pores are in the 20–30 μm range. In the case of PCL20 the wider dispersion in results is observed, with the most dominant size of pores ranging from 10 to 20 μm. However, it is worth noting that the

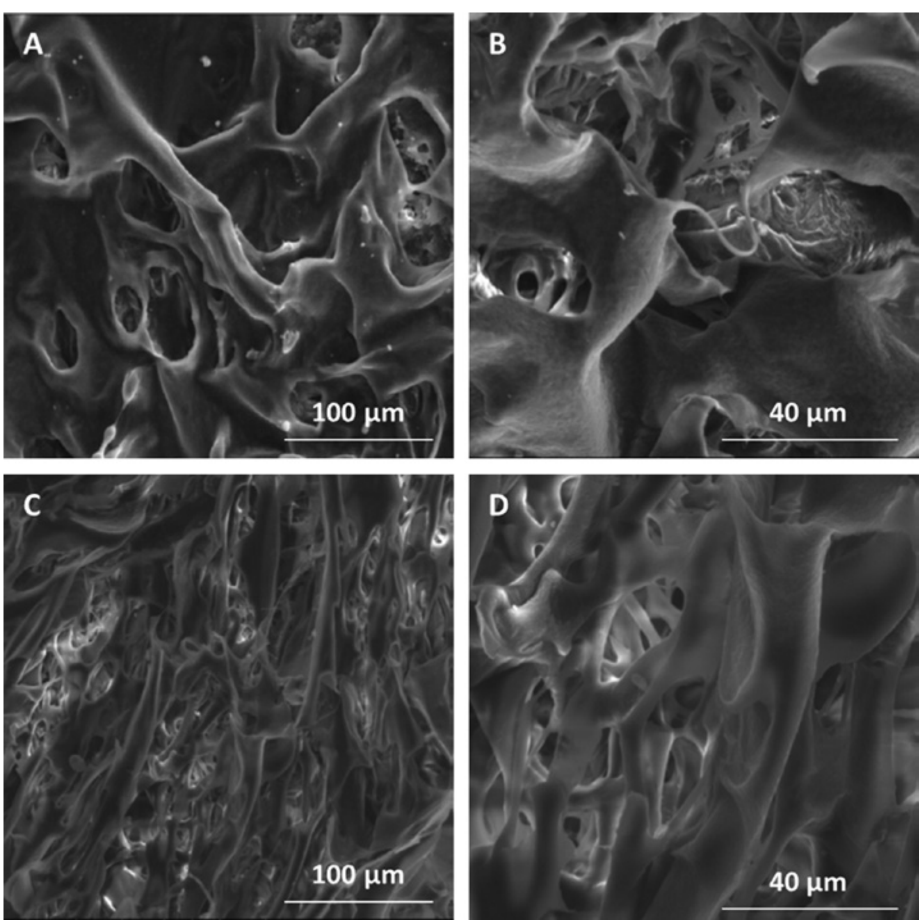

Figure 1: SEM images of PCL20 (A, B) and PCL40 (C, D) membranes.

shape of pores is irregular. The pores of the PCL40 membranes are elongated and the shape coefficient equals 3.6. The longer diameter of pores is up to 190 μm, although most of them are 40–60 μm long. Regardless of the size and number, in both cases the pores form a network of connected channels. Such open porosity is an essential factor in the transport of nutrients. The microstructure of membranes fabricated by freeze-drying is very different than that obtained by other methods, as for example, porogen leaching. Krok and Pamula (10) obtained a PLGA membrane for guide tissue regeneration with the use of polyethylene glycol as a pore former. The pores were spherical, of a diameter <20 μm. Gaona et al. obtained PLLA/PCL microporous membranes by the freeze extraction method with the pore diameter of about 20 μm (31). On the other hand, using freeze-drying Maji et al. prepared scaffolds for bone tissue engineering that exhibited >80% porosity with a mean pore size range between 100 and 300 microns (32).

3.2 Asymmetric PLA/PCL membranes

PLA nanofibers with a smaller size of pores were obtained by electrospinning (Figure 3). This method makes it possible to produce nanofibers with different morphology by controlling the solution parameters (viscosity, molecular weight, surface tension, concentration, etc.) and the electrospinning settings (the distance between dispenser and collector, applied voltage, speed of dispensing, rotational speed of the collector) as well as the external conditions (temperature, humidity, pressure) (9, 33–36). Having covered PLA fibers with PCL solution, an asymmetric membrane with differentiated porosity – depending on the side of the sample – was obtained (Figures 3 and 4). Such a conformation is advantageous, as it allows proliferation of bone cells on the PCL side which has bigger pores than the PLA fibers. The most favorable conditions to form the new bone tissue are provided by pores measuring 50–150 μm. The pores of this size guarantee the overgrowth

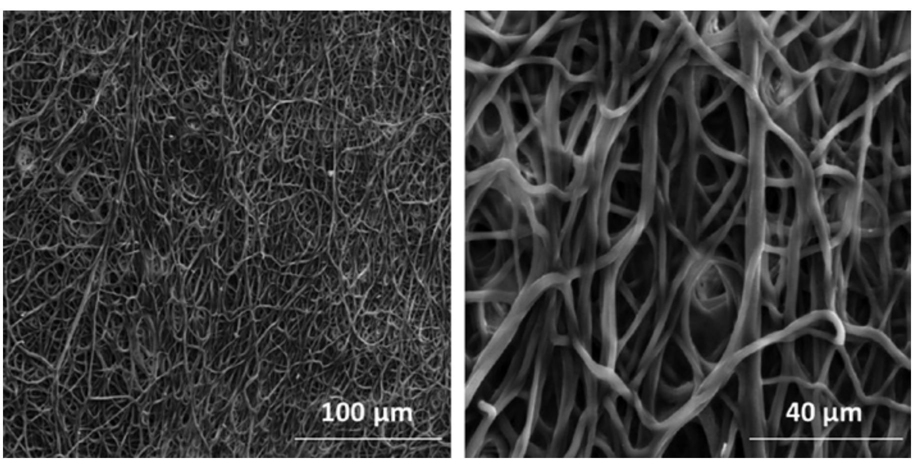

Figure 3: SEM images of PLA/PCL40 membrane, polylactide nanofibers side.

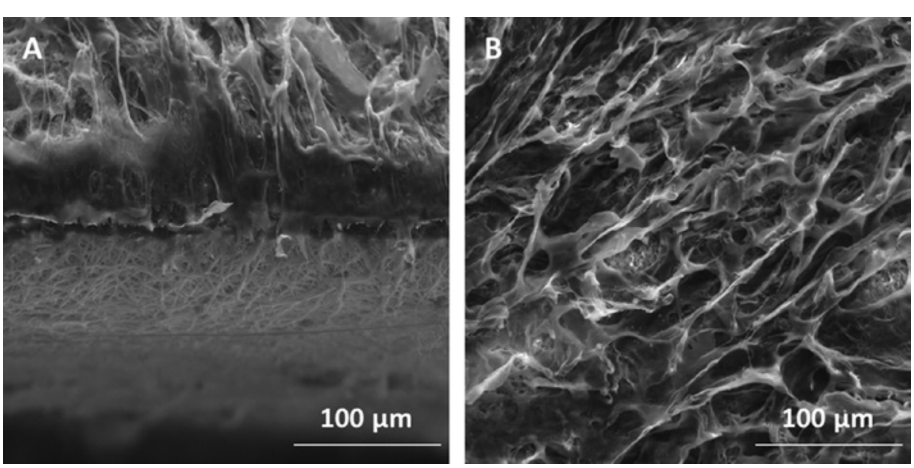

Figure 4: SEM images of PLA/PCL40 membrane: the cross-section (A) and PCL side (B).

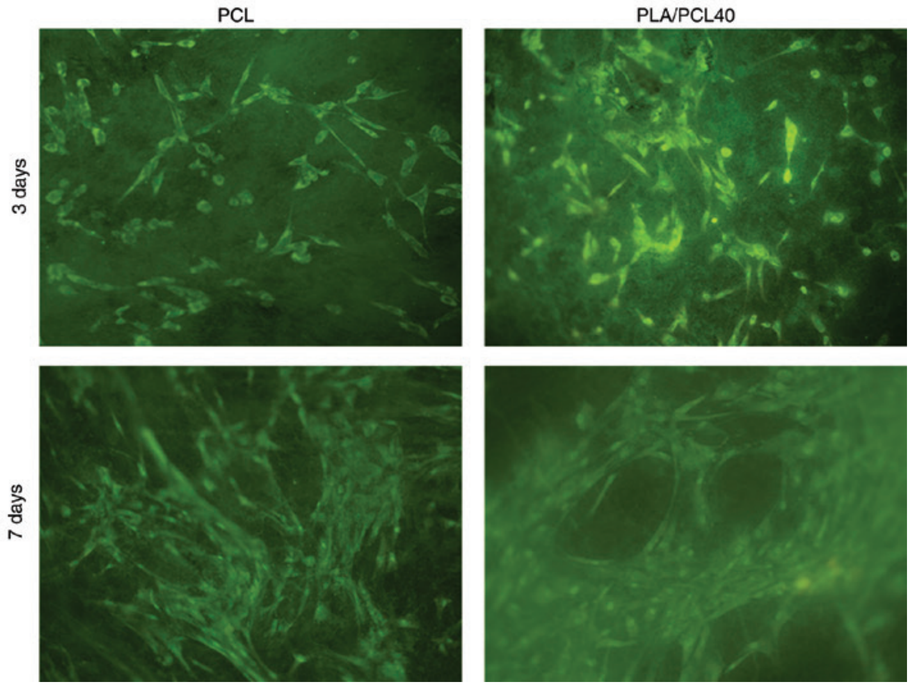

Figure 5: Morphology of MG-63 cells cultured on PCL and PLA/PCL40 membranes after 3 and 7 days.

of bone tissue and formation of new osteons (10). Due to the specificity of nanofibers, it is difficult to characterize the porosity of the PLA side, yet the size of these pores does not exceed 30 µm. The PLA microstructure with much smaller pores should prevent the overgrowth of soft tissue, at the same time allowing neovascularization and nutrition of the bone tissue forming in the defect area.

In vitro tests of the osteoblast-like MG-63 cell line performed on PCL40 and PLA/PCL40 membranes (on the PCL side) confirmed their biocompatibility (Figures 5 and 6). The research demonstrated that cells adhered well to the surface of both types of membranes. As compared to 3 days, after the 7-day cell culture, the proliferation and viability of cells on the tested substrates were higher. The morphology of the cells was correct. The obtained results were comparable to the control.

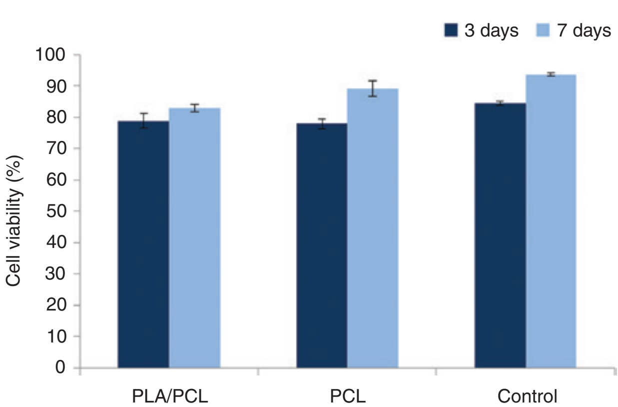

Figure 6: MG-63 cell viability after 3 and 7 days.

3.3 Mechanical properties and stability

The combination of electrospinning and freeze-drying made it possible to endow the two-phased material with higher strength, as compared to the parameters of its particular components (PLA and PCL) (Figure 7A). Along with the increase in strength, Young's modulus increased, whereas the deformability of membranes decreased (Figure 7B and C). The improvement of mechanical parameters probably results from the partial impregnation of PLA fibers with PCL, which leads to the formation of an intermediate composite layer. In this layer the PLA nanofibers constitute the reinforcing phase and the PCL matrix bonds them together, diminishing deformability of nanofibers. Mechanical properties strongly depend on the porosity of membranes and the size of pores, therefore the compromise is needed between high porosity and enough mechanical strength. Lou et al. observed an 80% increase of scaffolds compressive strength with an 11% decrease of their porosity (37). Poologasundarampillai et al. obtained, by electrospinning, hybrid, PLGA-based, fibermats with maximum value of tensile strength around 0.5 MPa (38). In our study, a combination of electrospinning with the

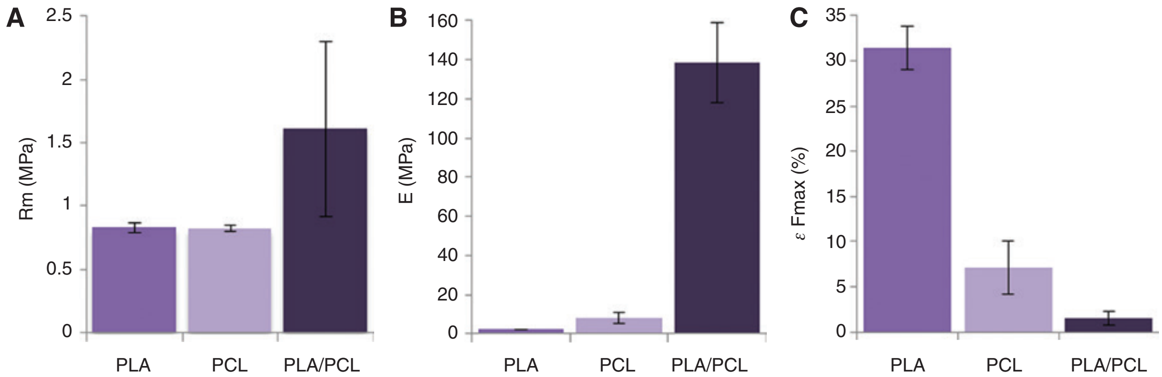

Figure 7: Tensile strength Rm (A), Young's modulus E (B) and maximum elongation εFmax (C) of studied membranes.

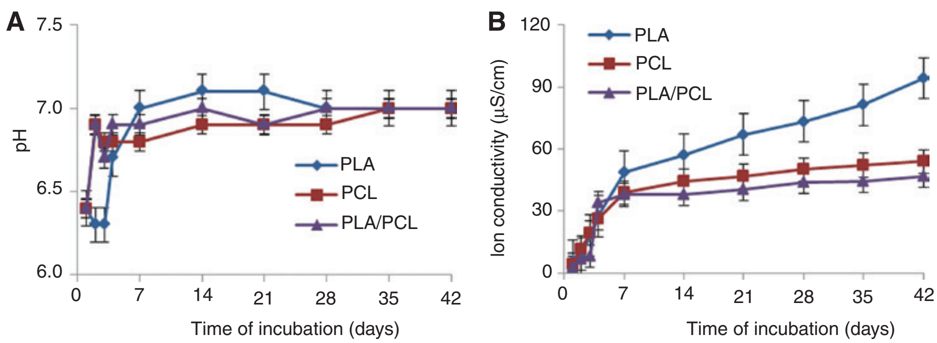

Figure 8: pH (A) and ion conductivity (B) changes during 6 weeks of incubation in distilled water.

freeze-drying method allowed fabrication of materials with higher tensile strength (about 1.6 MPa). Taking into consideration medical applications of the material, the obtained parameters seem to be beneficial. On the one hand, the membrane has to fit in the shape of the defect but, on the other hand, it has to provide the minimum of mechanical stability.

No significant pH or ion conductivity changes were observed during the 6-week incubation of the samples in distilled water (Figure 8A and B). Such results suggest that the membrane materials do not degrade rapidly, which could lead to premature failure of functionality (the proper growth of bone tissue should start up to 6 weeks after the injury) and induce inflammatory response of the organism.

4 Conclusions

The research conducted on the obtained materials serves as the preliminary study of their usability for guided bone regeneration. The results confirm that the freeze-drying method makes it possible to achieve open porosity of membranes. It is also possible to affect porosity and the size of pores by changing the concentration of polymer solution. The most favorable parameters were obtained for PCL in the ratio of 1:40 g/ml of acetic acid with the porosity above 80%. Fabrication of bi-phasic membranes by combining electrospinning with freeze drying significantly improved strength of the membrane, as compared to the parameters of its particular components (PLA and PCL). Obtained membranes remain stable during the 6-week incubation in distilled water, which corresponds to the period when the healing process of bone tissue regeneration should start. Resulting membranes are asymmetric and the size of pores of the PCL side is congruent with the demanding of bone cells, what was confirmed by *in vitro* biocompatibility assay. The smaller size of the pores on the PLA side ought to inhibit the overgrowth of soft tissue and facilitate neovascularization and nutrition of the bone tissue forming in place of the defect.

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