

Original method of imprinting pores in scaffolds for tissue engineering

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Abstract

Results of the preparation of biodegradable porous scaffolds using an original modification of a wet phase inversion method were presented. Influence of gelatin nano-non-woven as a non-classic pore precursor and polyvinylpyrrolidone, Pluronic® as classic pore precursors on the structure of obtained scaffolds was analyzed. It was shown that the addition of gelatin nano-non-wovens enables the preparation of scaffolds, which allow for the growth of cells (size, distribution, and shape of pores). Mechanical properties of the obtained cell scaffolds were determined. The influence of pore precursors on mass absorption of scaffolds against isopropanol and plasma was investigated. Interaction of scaffolds with a T-lymphocyte line (Jurkat) and with fibroblasts (L929) was investigated. Obtained scaffolds are not cytotoxic and can be used as implants, e.g. the regeneration of cartilage tissue.

Introduction

In the human body, cartilage plays a very crucial role – it determines the growth of long bones and creates articular surfaces.[1] One of the most important and simultaneously, most frequently damaged human joints is the knee joint.[2] It is responsible for both horizontal (walking, running) and vertical (jumping) movements of the body. [3] The knee joint has a complex structure. Its primary function is to join the tibia with the femur and patella.[4] To alleviate the heavy burden, to which it is exposed, the knee joint is supported by additional elements, i.e. by ligaments and meniscus. The cartilaginous tissue plays one of the most critical functions in the knee joint – it forms the meniscus (fibrocartilage) and covers the surface of bones (hyaline cartilage).[4] The main task of this tissue is to intensify the movement, adjust articular surfaces, enable rotating movements in the bent knee (meniscus), and to cushion the movement (surfaces).[6] The cartilage is characterized by poor vascularisation – arteries, veins, and lymph vessels do not reach this type of tissue. Nutrients are transported by diffusion with synovial fluid and to a lesser extent, employing bones.[7] These limitations hinder self-regeneration of cartilages significantly and constitute a severe complaint affecting people of all ages. Therefore, regeneration of the cartilages constitutes a significant problem both to patients as well as to physicians [8]. Various factors may cause damages to joint cartilage.[9] They may be caused by direct injuries, repetitive micro-injuries, strains leading to gradual damages, abrasion of cartilage (degenerative lesions), and also by cartilage and bone necrosis.[10] Losses in the joint cartilage are manifested by pain, oedema, and inflammation, which are very troublesome to patients and limit his/her everyday functioning.[11] Previously used treatment methods aim mostly at minimization of symptoms and do not enable full remobilization of the patient [3, 9, 10].

Modern techniques of treatment the joint cartilage focused on biological methods that enable for self-regeneration potential of the body [3]. One such method is the technique of microfractures which is based on performing microdrillings in a subchondral layer of the joint where multipotent stem cells are released.[12] After some time, these cells differentiate into chondrocytes and then create the new surface of the joint. This method is often modified by using a collagen membrane (Autologous Matrix-Induced Chondrogenesis, AMIC) where cells released from the bone are settled. The microfracture technique has numerous advantages and is highly effective.[13] Unfortunately, they may not be used in cases of specific lesions located closely to each other. Moreover, one should keep in mind that newly formed cartilaginous tissue is only similar but not identical to the native tissue [14–16].

Another method is autogenic osteoarticular transplantation (Osteoarticular Transfer System, OATS).[15] In this method, damaged cartilage is removed along with the subchondral layer and bone lying underneath. Next, a cartilage part is taken (along with a subchondral layer and a bone fragment) out of a healthy surface of the bone, usually the femoral bone. The fragment of cartilage and bone is subsequently pushed in the place of the removed lesion. Healing of the lesion covered by the transplant occurs after 6–8 weeks.[17] This method is highly effective. However, it involves significant interference in the body of a patient. [18,19]

A technique using periosteal flaps and transplantation of bone marrow cells is implemented for more considerable cartilage damages.[20] After recessing damage and estimating its dimension, a fragment of the periosteum is taken from the femoral bone. It is then stitched to the site of the damage and sealed with the tissue glue forming a flap.[21] Furthermore, bone marrow drawn from the iliac bone or a suspension of cartilage cells cultivated in the laboratory is injected under the sewn and sealed flap. Like the previous techniques, this method is very effective but also involves significant interference, which diminishes the comfort of therapy for the patient. [22, 23]

A further method of cartilage regeneration is autologous chondrocyte implantation (ACI).[24] This technique is based on a biopsy of a small fragment of healthy cartilage. The chondrocytes are then multiplied for ca. 6–8 weeks.[25] After, the patient should undergo the next surgery. A fragment of the periosteum shinbone is collected and implanted in the place cleaned. Cultured cells of the cartilage tissue are then injected under the stitched periosteum which is multiplied once again and form a new fragment of the cartilage.[26] Unfortunately, this method requires two surgical procedures. [27–29]

Another way of cartilage regeneration involves modern biomaterials that are used for obtaining cellular scaffolds [30, 31]. They create a suitable spatial environment that enables the growth of chondrocytes retrieved from *in vitro* breeding or from stem cells of the bone marrow.[32] This scaffold should ensure the best distribution of cells, provide stability, and facilitate differentiation. The three-dimensional shape enables for forming of the structure that is very similar to natural cartilage.[33] Scaffolds are usually made of natural or synthetic polymers.[34–37] Synthetic and biodegradable polymers, i.e. polylactic acid (PLA), poly- ϵ -caprolactone (PCL), polyglycolide (PGA), and their copolymers constitute a popular and frequently used group of materials used for obtaining the scaffolds.[38–41] These compounds are characterized by biocompatibility, biodegradability, and degradation into products that are readily excreted from the body (CO_2 and H_2O). These polymers vary in degradation time which increases with increased length of the carbon chain of a polymer.[42] Among the mentioned polymers, the longest degradation time is attributed to PCL (over 2 years), followed by PLA (ca. 2 years) and PGA at the end (several months).[43] These polymers are characterized by simple processing (stability in variable temperature conditions) and excellent mechanical properties (especially in comparison to natural polymers – such as collagen or chitosan).[44,45] Moreover, by using biodegradable scaffolds, one improves the comfort of therapy because there is no necessity of removing an implant.[46] All the mentioned characteristics make biodegradable synthetic scaffolds very popular. [47–50]

The purpose of these studies was to obtain biodegradable scaffolds for the cultures of chondrocytes.[51] Scaffolds that were obtained should be characterized by a unique, three-dimensional spatial structure enabling proper differentiation of cells.[53] The strongly porous upper surface should allow for penetration of cells into scaffolds, and less porous bottom surface should prevent them from being "excluded" from the structure.[54]

The highly porous cross-section was supposed to contain large and interconnected pores with smaller pores (perforations), enabling intercellular communication and migration of nutrients and metabolites.[55]

Materials and methods

Materials

The following polymers were used for obtaining the scaffolds: poly-L-lactide (PLLA) having M_n 86 000 g/mol, Nature Works NW 2003D and poly- ϵ -caprolactone (PCL) having M_n 85 000 g/mol, poly(ϵ -caprolactone-*b*-lactide) (PCLA) having M_n 80 000 g/mol that was obtained in-house. Polymers like polyvinylpyrrolidone (PVP) of M_n 10 000 g/mol and Pluronic® F-127 from Sigma Aldrich were used as pore precursors. The following solvents were used: chloroform, methanol, ethanol 99%, (all from POCh SA). Ultrapure water of the resistance of 18.2 M Ω -cm was obtained using a MiliQ device.

For obtaining polymer nano-non-woven, the type A gelatin from the pork skin from Sigma Aldrich was used. Acetic acid and formic acid (p.a.) from Avantor PM Poland were used as solvents.

Jurkat cells were used for examining of cytotoxicity. The cytochemical reaction was performed with the use of propidium iodide from Sigma Aldrich.

Mouse fibroblasts from the L929 cell line were used to determine material toxicity and cell interaction with the scaffold surface. The Presto blue test determined cell viability.

Preparation of the polymer nano-non-wovens

The solution of the gelatin of the concentration of 15%_{mas} in the 9:1 (g/g) mixture of acetic acid and formic acid was prepared. The polymer was solved for 24 h at room temperature while stirring with a magnetic stirrer.

The electrospinning process was conducted in the horizontal mode. Two syringe pumps (New Era Pump Systems, NE-1000, and KD Scientific KDS-100-CE) were placed on the opposite sides of a grounded rotating drum collector. High voltage was provided by two generators that were connected with a positive terminal to stainless steel needles. Distance between needles and collectors was 15 cm with a flow rate of the solution on both sides counted to 2 mL/h and an inner diameter of the needle of 0.34 mm. The electrospinning process was run with 15–17 kV voltage, at humidity, ca. 50% and room temperature within 22–24 °C range.

Preparation of the scaffolds-forming solution

The polyesters had been dissolved in chloroform. The concentrations were adjusted (Table 1) so to obtain the viscosity of 350±50 cP. Polymers were dissolved in an organic solvent for 24 h at room temperature while stirring continuously with a magnetic stirrer. If pore precursors had been used, they were added to the polymer solution after the complete dissolution of the polyesters (minimum 24 h). PVP or PVP with the addition of Pluronic® were added in such amounts to keep an appropriate mass ratio to the polyester (Table 1). Next, the stirring was continued for the next 24 h.

Table 1. Scaffolds-forming solutions

no.	polymer	concentration, c_{mas} , %	pore precursor	mass ratio polymer/pore precursor
1	PLLA	6	-	-
2			PVP	1:1
3			PVP, Pluronic®	100:100:25
4	PCLA	8	-	-
5			PVP	1:1
6			PVP, Pluronic®	100:100:25
7	PCL	3.8	-	-
8			PVP	1:1
9			PVP, Pluronic®	100:100:25

Preparation of the scaffolds without polymer nano-non-wovens

Prepared solutions of polyesters in chloroform were poured on a glass base. The scaffold was gelled in methanol for 2 h at room temperature. After coagulation, the scaffold was dried out.

Preparation of the scaffolds using gelatin nano-non-woven

Solutions of polyesters in chloroform were poured on the glass base. After pouring the layer of the solution, a gelatin nano-non-woven was placed on it. The nano-non-woven was saturated with the solution of polyester. The residual air was removed by pressing it with a roller. Then, another layer of the solution was poured, and the polymer nano-non-woven was placed on it. Immediately after placing the second layer of the non-woven, it was pressed against the previous layer. The scaffold was gelled in methanol at $-18\text{ }^{\circ}\text{C}$ for 2 h. After the coagulation of the polymer, the scaffold was rinsed (MiliQ water, $40\text{ }^{\circ}\text{C}$, 2 h) to remove the gelatin nano-non-woven. After removing pore precursors, the scaffold was dried out at a temperature of $40\text{ }^{\circ}\text{C}$ under a vacuum of 1.5 hPa. Dry scaffolds were stored in 70% ethanol.

Scanning electron microscopy

Morphology of cross-sections of the scaffolds and both surfaces were examined using scanning electron microscopy (SEM) with the Hitachi TM1000 apparatus. Before the examination, samples of scaffolds were immersed in ethanol and then broken in liquid nitrogen. After drying, samples were covered with a 7–10 nm layer of gold using the K550X Sputter Coater. Samples covered with gold were examined at 300x and 1000x magnifications with the use of an acceleration voltage of 15 kV.

Mechanical properties

The mechanical strength of the scaffoldings was tested in a static tensile test using the Instron 5566 apparatus. Rectangular cut out of scaffoldings measuring 7x2 cm was tested. The samples were stretched at a speed of 5 mm/min, and then the mean values of Young's modulus (E), maximum stress (σ_{max}), maximum relative strain (ϵ_{max}) and their standard deviations were calculated. The analysis of each sample was done in 3 replicates.

Mass absorption

The dry sample was weighed on a Mettler Toledo XS 104 analytical balance and then placed in a 50 mL beaker filled with isopropanol. The beaker with the sample and isopropanol was placed in a desiccator, to which an oil pump was connected. The sample was degassed for 30 min. After this time, the sample in the isopropanol beaker was removed from the desiccator and beaker. Excess i PrOH was removed and weighed on an analytical balance. Each experiment was performed in 3 replications. Water absorption was calculated from the formula:

$$A_m = \frac{m_w - m_d}{m_d} \times 100\%$$

where:

m_w - mass of the wet air-weighed sample, g

m_d - dry mass of the air weighed sample, g

A_m - mass absorption, %

Elemental analysis

Elemental analysis of samples was performed with the use of the Elementar Vario EL III apparatus. Dry scaffolds without any previous preparation were used in the analysis.

IR Spectroscopy

IR spectra were obtained by the ATR technique using the Alpha Bruker apparatus. Dry samples without any previous preparation were used in the tests.

Cytotoxicity test

Scaffolds were rinsed in a 0.9% NaCl solution and then disks having a diameter of 7.0 mm were cut out of them and placed in 24-well plate. Jurkat cells (concentration 1×10^6 cells/mL) were placed on the prepared plates, along with the examined scaffolds. Cells were cultured for 8 days in the presence of examined samples at 37 °C, under an atmosphere of 5% CO₂. On the 1st, 4th, and 8th day of the culture, the cells were analyzed in a flow cytometer using a cytochemical reaction with propidium iodide.

Cell culture (fibroblasts L929)

Preparation of the culture medium: Culture medium consisting of 89% DMEM (with high glucose, L-glutamine, phenol red, without sodium pyruvate), 10% FBS, and 1% penicillin and streptomycin solution was prepared.

Cell cultures: Cells were grown in bottles with a capacity of 75 mL, filled with culture medium and placed in an incubator at 37 °C and 5% CO₂. Every 3 days, the medium was changed. To make suspensions with a specific cell density. The cells in the bottles were washed with PBS and then digested with 0.05% trypsin in PBS. After detaching the cells from the wall of the bottle, they were centrifuged. Next, the resulting pellet was resuspended in the medium and diluted to the density required for the test. To determine the appropriate cell density, they were counted on a TC2 Automated Cell Counter Bio-Rad flow cytometer.

Preparation of scaffolds samples for cell cultures: Samples with a diameter of 5 mm and 10 mm were cut from the scaffoldings, respectively, for 96 and 48-well plates. The cut samples were sterilized in 85% ethanol solution, irradiated with UV rays for 30 min on each side.

Non-contact test: Scaffold discs were placed in a 96-well plate. 200 mL of culture medium was added to each well. Samples were mixed, at 37 °C, for 24 h. Reference samples were wells containing only samples. At the same time, a cell suspension at a density of 1×10^4 cells / well was plated into a second 96-well plate and incubated for 24 h. After this time, the culture medium in the second plate was replaced with samples from the first plate and incubated for 24 h. After this time, the Presto Blue test was performed.

Contact test: Scaffold discs with a diameter of 10 mm were placed in 48-well plates. 0.6 mL of culture medium containing 1×10^4 cells was added to each. The control sample was well containing only culture medium. Plates were placed in an incubator at 37 °C, 5% CO₂. After 5 h and 1, 2, 3 days, the plates were removed from the incubator and analyzed.

Presto Blue test

The wells were washed with PBS and then 90 µl PBS and 10 µl Presto Blue reagent were added to each. The plate was again placed in an incubator for 40 min. Then 100 µl of suspension was withdrawn from each well and transferred to a new plate. Fluorescence was examined with a FLUOstar Omega apparatus at excitation and emission wavelengths of 530 and 620 nm, respectively.

Statistical analysis

The results of the measurements were expressed as means $\pm$ SD. Statistical significance of differences was analyzed using single-factor analysis of variance (ANOVA) for $p < 0.05$ (MS Excel 365).

Results and discussion

The major problem in obtaining polyester cellular scaffolds is their appropriate porosity and morphology of their pores. In the inversion phase method, it is not possible to obtain correct porous surfaces and large, interconnected pores inside the scaffold, without using pore precursors. Classic pore precursors, i.e. polymers (polyvinylpyrrolidone, polyethylene glycol) or crystals of salt that are being added into the polymer's solution and then washed away from coagulated scaffolds – do not give the desired effect.[56] The pores which

are formed this way are usually not large enough (over a dozen μm), unevenly distributed, and poorly interconnected.[57]

The scaffold for the culture of chondrocytes should have large (at least 30–40 μm) interconnected pores, distributed within the entire volume of the structure.[58] One of the scaffold surfaces must have high porosity, allowing for penetration of cultured cells inside the scaffold. On the other hand, the other surface should contain small, rare pores to prevent the cells from "falling out" from the polymeric scaffold.[59]

In the presented examinations, the scaffolds of poly-L-lactide were obtained without using any pore precursors and with the PVP or both PVP and Pluronic® as the classic pore precursors.

If no pore precursors have been used, then small and round pores having a diameter of 3–6 μm were observed (Fig. 1) on the bottom surface of the scaffold prepared from PLLA, from the side of glass base. The upper surface was more porous than the bottom one. Moreover, there were larger, round pores 5–10 μm in diameter, and the rest of the pores were covered with a thin covering layer preventing the cells from penetrating inside the scaffold. In the cross-section of the scaffold, there were some oval pores 5–15 μm in size and the wall thickness of 1–2 μm .

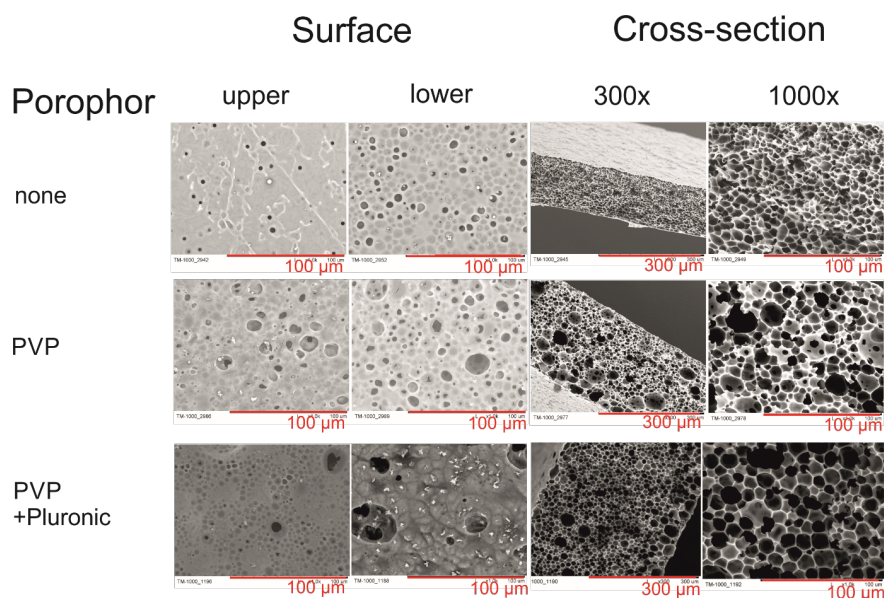


Fig. 1. SEM photomicrographs of PLLA, PCL, PCL scaffolds obtained with classic pore precursor.

Round pores were having a diameter of 5–15 μm . It was observed on the bottom surface of the scaffold prepared from PLLA with the addition of PVP as the pore precursor. The upper surface was more porous than the bottom one. Pores at this surface were round and of a larger diameter (2–20 μm) and other pores were covered with a thin covering layer. Numerous oval pores of a size of 5–15 μm were seen in the cross-section of the scaffold. Between them were also larger pores of the size of 20–30 μm .

Moreover, smaller pores of the size under 5 μm were observed in the walls of these pores (1–2 μm thick). This is a critical feature of the culture of the cells as perforations of walls of inner pores allow for the migration of nutrients and metabolites. On the bottom scaffold surface obtained from PLLA with the PVP or Pluronic® addition round pores of a diameter of 10–15 μm was observed. Microscopic pores of the size less than 1 μm were observed between the bigger ones. The majority of pores were covered with a thin covering layer. Rare, irregularly distributed pores having 10–40 μm in diameter were seen on the upper surface. In the cross-section of the scaffold, there were oval pores of the size of 10–15 μm . Larger pores, having 30–50 μm in

diameter were present between them. As in previous cases, the inner walls of pores had a thickness of 1–2 μm .

Considering that the addition of classic pore precursors didn't allow for obtaining the scaffolds of a proper morphology, because pores were still too small and weakly interconnected – an unconventional approach has been adopted. The previous studies have shown that the use of polymeric non-wovens as non-classic pore precursors leads to the forming of scaffolds of a desirable morphology [58–62]. Cellulose-based non-wovens, despite their high efficiency, were characterized by irregular structure. Moreover, their removal from the structure of the scaffold required several weeks.

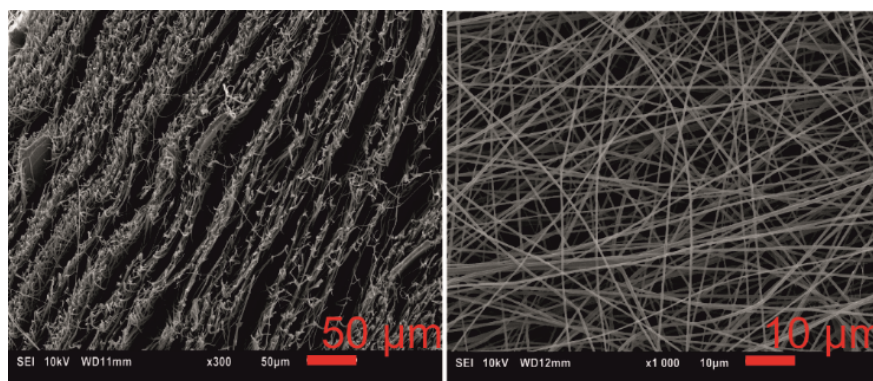


Fig. 2. SEM photomicrographs cross-section of a gelatin nano-non-woven. Magnification 300x (left) and 1000x (right)

On the other hand, polymeric nano-non-wovens obtained by electrospinning are characterized with strictly controlled morphology of pores, which depends on the material or the solvent used for the spinning and other parameters of the process (Fig. 2). From a large selection of available materials, one could choose materials that can be then easily removed from the obtained scaffolds. Such nano-non-wovens were regarded as non-classic pore precursors that enable for obtaining the structures of desired morphology (defined by nano-non-woven). Their additional advantage, in comparison to conventional precursors, is the formation of pores in the entire volume of the scaffold. It guarantees the formation of pores that are evenly distributed, not only inside the scaffold but also on one of the surfaces.

In the next stage of the study, poly-L-lactide scaffolds have been prepared using a gelatin nano-non-woven as a non-classic pore precursor. Also, a combination of a gelatin nano-non-woven and classic pore precursors was used (PVP or PVP and Pluronic®). Analogical tests were conducted for other polyesters (PCLA and PCL).

A small number of pores having a diameter of about 5 μm was observed on the bottom surface of the scaffold obtained from poly-L-lactide using a gelatin nano-non-woven (Fig. 3). On the other hand, numerous irregular pores of the size of 20–50 μm could be seen on the upper surface. Here, "imprints" of nanofiber were also observed, application, pressing and washing out of which have destroyed the thin covering layer. In the cross-section of the scaffold, there were irregular and mutually linked pores 20–80 μm in size. Their 2–5 μm thick walls contained oval pores of a size of 1–2 μm . Also, walls of the pores, similarly to those at the upper surface, contained "imprints" of nanofiber which proves the efficiency of using the non-wovens. In the scaffolds obtained with the nano-non-wovens, the "imprints" of gelatin nanofibers were observed.

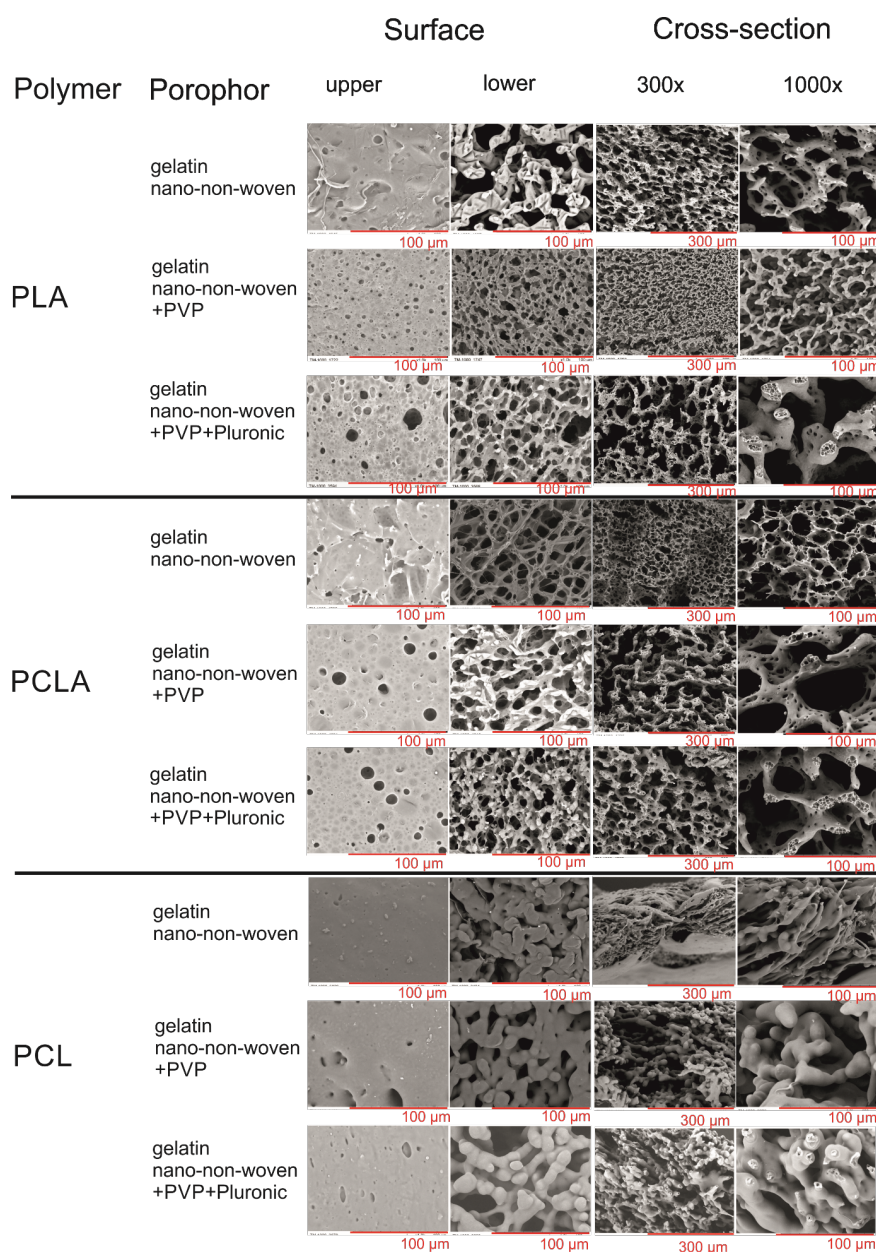


Fig. 3. SEM photomicrographs of PLLA, PCLA, PCL scaffolds prepared with the addition of a gelatin nano-non-woven.

On the bottom surface of the scaffold obtained from PLLA with the addition of PVP and gelatin nano-non-wovens, rare small pores of a diameter of 5–10 μm were observed. On the upper surface, numerous, irregular pores of the size of 20–40 μm were also present. Between them pores of 5–15 μm in size were present. In the cross-section of the scaffold, large irregular, nearly oval pores 20–70 μm , and up to about 100 μm in size could be seen. Compared to the scaffold obtained by using only a gelatin nano-non-woven, pores were more linked, and their walls were thinner (1–2 μm). As in the previous case, significantly smaller round pores (ca. 1 μm) could be seen in the walls of larger pores.

Rare pores were having 5–10 μm , sometimes 20 μm in diameter were observed on the bottom surface of the

scaffold prepared from PLLA with the addition of PVP, Pluronic®, and a gelatin nano-non-woven (Fig. 7). On the other hand, numerous irregular 20–40 µm pores could be seen on the upper surface. Irregular, nearly oval pores of the size of 20–80 µm are present in the cross-section of the scaffold. In contrast to the previous results, walls of these pores 5–10 µm thick, not only contained perforations but – first of all, their cross-section was distinctly divided into segments. It means that the inner walls of these pores did not have a solids structure.

Due to the promising results about the use of gelatin nano-non-woven in preparing scaffolds from poly-L-lactide, it was decided to test the effectiveness of the method for obtaining scaffolds from other biodegradable polymers: block copolymer poly(ε-caprolactone-*b* -lactide) and poly(ε-caprolactone).

Rare pores having less than 5 µm in diameter could be seen on the bottom surface of the scaffold prepared from block copolymer poly(ε-caprolactone-*b* -lactide) using gelatin nano-non-woven. Irregular, 20–50 µm pores are visible on the upper surface. Nearly oval pores of the size of 20–60 µm could be seen in the cross-section of the scaffold. Between them, there are smaller (10–15 µm) pores. These pores were also located in the inner 1–3 µm thick walls of larger pores.

Pores having 5–10 µm and 20 µm in diameter are present on the bottom surface of the scaffold prepared from PCLA with the addition of PVP and the gelatin nano-non-woven. Irregular 20–40 µm pores could be seen on the upper surface. In the cross-section of the scaffold oval, 50–80 µm pores are present in the cross-section of the scaffold. Additionally, the walls of these pores (3–5 µm thick) contained perforations.

Small pores (approx. 5–15 µm) could be seen on the bottom surface of the scaffold prepared from PCLA with the addition of PVP, Pluronic®, and the gelatin nano-non-woven. Irregular (20–40 µm) pores could be seen on the upper surface. Oval 40–80 µm pores could be seen in the cross-section of the scaffold. In the inner walls of these pores, smaller (1–2 µm) pores were present. Walls of these pores had 5–10 µm in thickness and were not stable – they consisted of some segments.

Another material tested was poly(ε-caprolactone). Unfortunately, scaffolds prepared from this polymer have shown severe contraction during a rinsing bath. Scaffolds were twisted, and their structure has wrinkles and discontinuities. The use of pressing roll to prevent contractions of the material failed to provide the desired effect. Although the scaffolds did not twist, numerous discontinuities of the material were seen in their structure. This effect could be caused by too low glass transition temperature of PCL, –60°C (for PLLA: +65°C), probably inadequate for the conditions used in this method (–18 and +40°C).

Small (less than 5 µm) and rarely distributed pores could be seen on the bottom surface of the scaffold prepared from PCL with the use of a gelatin nano-non-woven. On the upper surface, there were irregular 20–40 µm pores. Between them smaller (approx. 5 µm) pores were present. Oblong pores were having 40–80 µm in length were present in the cross-section. Walls of the pores were thick (5–15 µm) and twisted.

A number of pores of the size 5–10 µm could be seen on the bottom surface of the scaffold prepared from PCL with the addition of PVP and a gelatin nano-non-woven. Irregular pores of the size of 20–40 µm could be noted on the upper surface. Oblong (40–80 µm) pores could be seen in the cross-section of the scaffold. Walls of these pores were thick (5–15 µm) and twisted.

Few pores of the size of 5–15 µm could be seen on the bottom surface of the scaffold prepared from PCL with the addition of PVP, Pluronic®, and a gelatin nano-non-woven. Irregular pores of the size of 20–40 µm could be noted on the upper surface. Oblong pores of the size of 40–60 µm were present in the cross-section of the scaffold. As in the previous cases for this polymer, walls of pores were thick (5–15 µm) and twisted. Besides, their structure was not solid but divided into segments.

Table 2. Morphology of the membranes

	bottom surface	upper surface
PLLA	rare pores, 3–6 µm	oval pores, 5–10 µm

	bottom surface	upper surface
PLLA+PVP	rare, 5–15 μm	oval pores, 2–20 μm
PLLA+PVP+Pluronic®	rare pores, 10–15 μm and ~ 1 μm	oval pores, 10–40 μm
PLLA+gelatin nano-non-woven	rare pores, ~ 5 μm	multiple, irregular pores, 20–40 μm
PLLA+gelatin nano-non-woven+PVP	rare pores, 5–10 μm	multiple, irregular pores, 20–40 μm
PLLA+gelatin nano-non-woven+PVP+Pluronic®	rare pores, 5–10 and ~ 20 μm	multiple, irregular pores, 20–40 μm
PCLA+gelatin nano-non-woven	rare pores, <5 μm	multiple, irregular pores, 20–40 μm
PCLA+gelatin nano-non-woven+PVP	rare pores, 5–10 and ~ 20 μm	multiple, irregular pores, 20–40 μm
PCLA+gelatin nano-non-woven+PVP+Pluronic®	rare pores, 5–15 μm	multiple, irregular pores, 20–40 μm
PCL+gelatin nano-non-woven	rare pores, <5 μm	irregular pores, 20–40 and >40 μm
PCL+gelatin nano-non-woven+PVP	rare pores, 5–10 μm	irregular pores, 20–40 μm
PCL+gelatin nano-non-woven+PVP+Pluronic®	rare pores, 5–15 μm	irregular pores, 20–40 μm

Contents of scaffolds

IR spectra (Fig. 4–7) and elemental analysis (Table 3) of the scaffolds have been performed to check whether gelatin remained in their structure. Only scaffolds are having proper morphology and made of PLLA and PCLA before and after rinsing, and the bath was examined. Since PVP and Pluronic® are biocompatible, the scaffolds have not been tested for their presence. For comparison, analyses of raw materials (PLLA, PCLA, gelatin non-woven) and dried mixtures of raw materials, of which scaffolds were obtained, have been performed (before the gelling and rinsing baths).

IR spectra of mixtures of dried raw materials use to prepare scaffolds (before the gelling and rinsing baths), clearly show a significant presence of gelatin (Figs. 4, 6: bands within the range $3500\text{--}2800\text{ cm}^{-1}$). In the spectra, bands related to PLLA (1750 and 1180 cm^{-1}) (Fig. 4, 5) and PCLA (1420 and 1290 cm^{-1}) (Fig. 6, 7) respectively, could be observed. In the spectra of scaffolds after the gelling and rinsing baths (Fig. 5, 7) only the bands related to PLLA and PCLA are visible. There are no bands specific for gelatin.

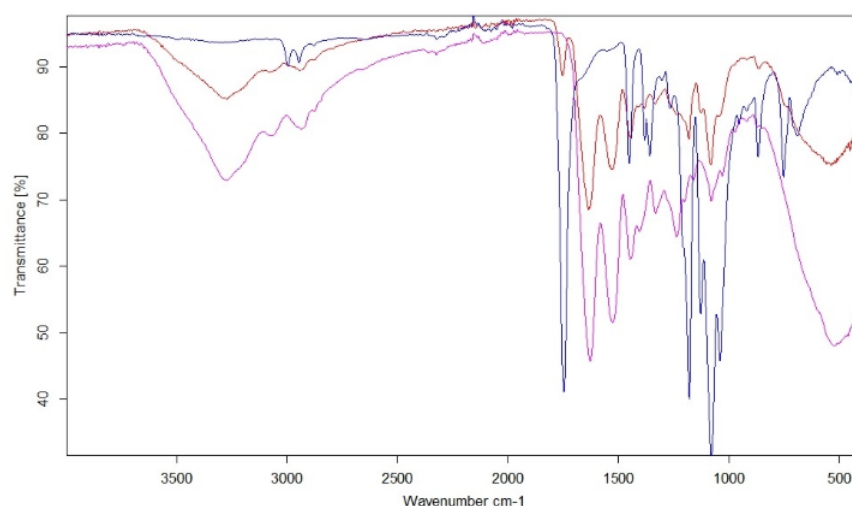


Fig. 4. ATR analysis. Blue line: PLLA, red line: PLLA scaffolds before gelling (with a gelatin nano-non-woven), violet line: gelatin

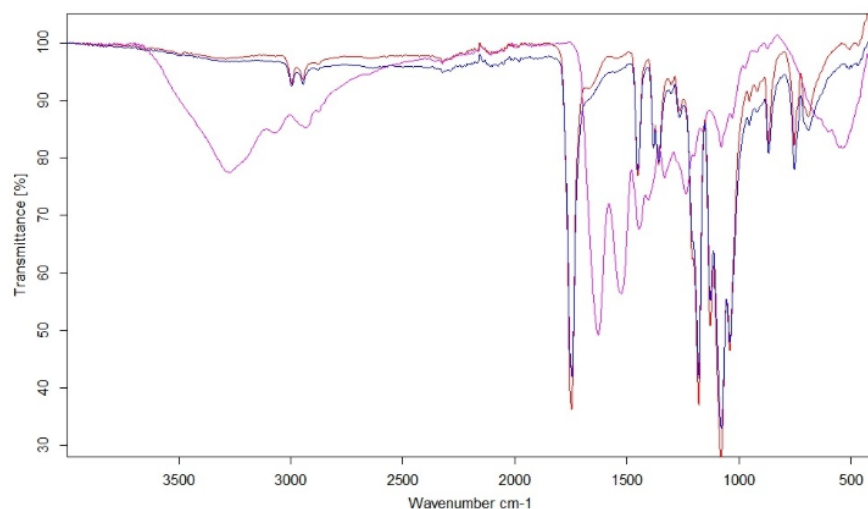


Fig. 5. ATR analysis. Blue line: PLLA, red line: PLLA scaffolds after gelling (without a gelatin nano-non-woven), violet line: gelatin

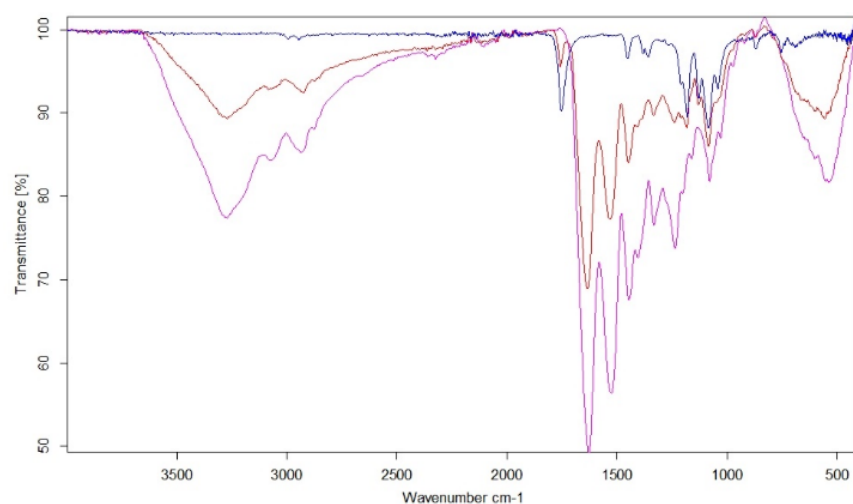


Fig. 6. ATR analysis. Blue line: PCLA, red line: PCLA scaffolds before coagulation (with gelatin nano-non-woven), violet line: gelatin

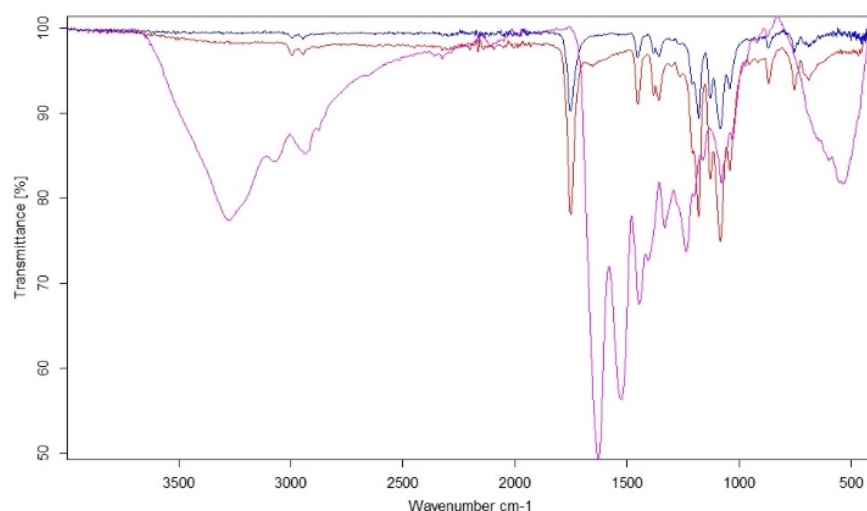


Fig. 7. ATR analysis. Blue line: PCL, red line: PCL scaffolds before coagulation (without a gelatin nano-non-woven), violet line: gelatin

Table 3. Results of elemental analysis (components and prepared scaffolds)

Components and obtained scaffolds	%C	%H	%N	%S
Gelatin nano-non woven	44.58	6.67	15.76	0.70
PLLA	49.87	5.62	0.23	0.23
PLLA + Gelatin nano-non woven before coagulation	45.58	5.74	7.22	0.38
PLLA + Gelatin nano-non woven after coagulation and washing	49.34	5.54	0.19	0.19
PCL	51.28	5.85	0.26	0.24
PCL + Gelatin nano-non woven before coagulation	45.92	5.97	9.48	0.39
PCL + Gelatin nano-non woven after coagulation and washing	51.57	5.36	0.34	0.25

The nitrogen content (PLLA 7.22%, PCL 9.48%) shows the presence of gelatin directly after the formation of the scaffold. After washing out the gelatin, the content of nitrogen decreased to the sensitivity limit of the analysis (0.3%). For 0.19% in the case of PLLA, and 0.34% in the case of PCL, respectively. Data from IR and elemental analyses prove that the gelatin non-woven was efficiently removed from the prepared scaffolds.

Mechanical properties

Mechanical properties were determined for suits obtained by wet phase inversion using a PVP blowing agent and those obtained using nanofibers. The obtained results were compared with the properties measured for a suit with PLA without the use of a blowing agent (Table 4). Stress curves of scaffoldings obtained without blowing agent both from PLLA and from PCL were characteristic for durable, hard materials, with yield stress. [60]

A higher Young's modulus was observed in scaffolds without porophores with PLLA (271.7 ± 17.1 MPa) than with PCL (48.2 ± 3.3 MPa) (Fig. 8). It was similar in the case of maximum stress where a higher value (9.46 ± 0.59 MPa) was obtained for PLLA than for PCL (5.68 ± 0.85 MPa). In contrast, scaffolds made of PCL ($647.6 \pm 91.6\%$) showed a higher maximum relative elongation than PLLA ($77.2 \pm 5.3\%$). All scaffolds tested exhibited excellent mechanical properties ($E > 10$ MPa), while scaffolds made of PLLA were more flexible than those made of PCL.[61] PLLA scaffolds with PVP (124.5 ± 20.8 MPa) and PCL scaffolds with

PVP (8.7 ± 1.3 MPa) were smaller Young's modulus.

All scaffolds obtained with the participation of nanofibers had a stress curve characteristic for elastic materials without a yield point.[60] In PLLA scaffolds obtained with the addition of non-woven fabric made of gelatin, Young's modulus was 231.6 ± 70.4 MPa, the maximum stress was 7.31 ± 0.07 MPa, and the maximum strain was $6.1 \pm 0.7\%$.

Table 4 Comparison of mechanical properties of selected suits that best meet the requirements for cartilage tissue regeneration.

Polymer	Porophore	E (MPa)	σ (MPa)	ϵ (%)
PLLA	-	271.1	94.6	77.2
PCL	-	48.2	56.8	467.9
PLLA	PVP	124.5	4.29	41.2
PCL	PVP	8.7	1.22	422.0
PLLA	Gelatin nano-non-woven	231.6	77.6	33.0
PCL	Gelatin nano-non-woven	45.4	49.3	411.0

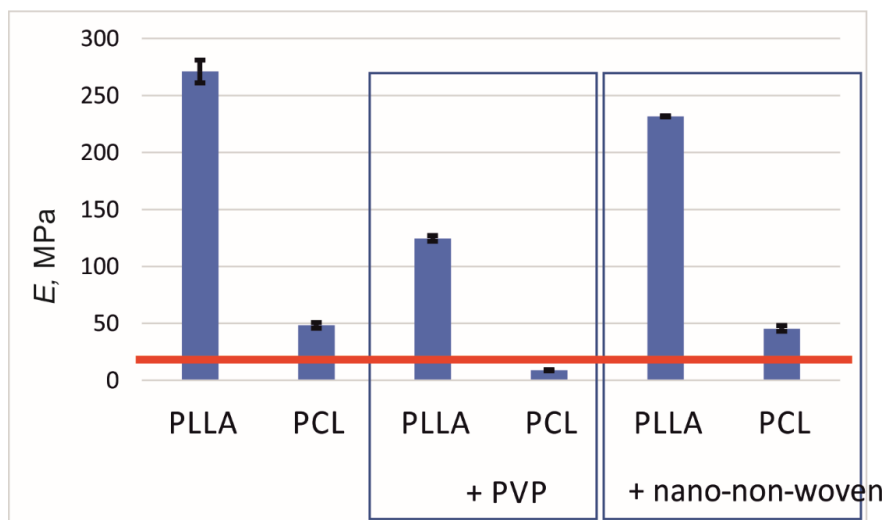


Fig. 8. The dependence of the scaffolds Young's moduli on the method of its production (red line – Young's moduli for cartilage)

Mass absorption

Mass absorption was determined for the best scaffolds obtained from PLLA. These scaffolds had the best internal morphology and mechanical properties suitable for cartilage. The results obtained were compared with suits obtained from PLLA obtained using PVP as a blowing agent. The highest absorbability (Fig. 9) was observed in scaffoldings obtained by the wet phase inversion method with a polymer nanofiber ($545.2 \pm 92.6\%$). Scaffolds obtained by wet ($315.7 \pm 7.2\%$) with PVP as a blowing agent showed less water absorption. The lowest through dry phase inversion ($124.3 \pm 12.1\%$).

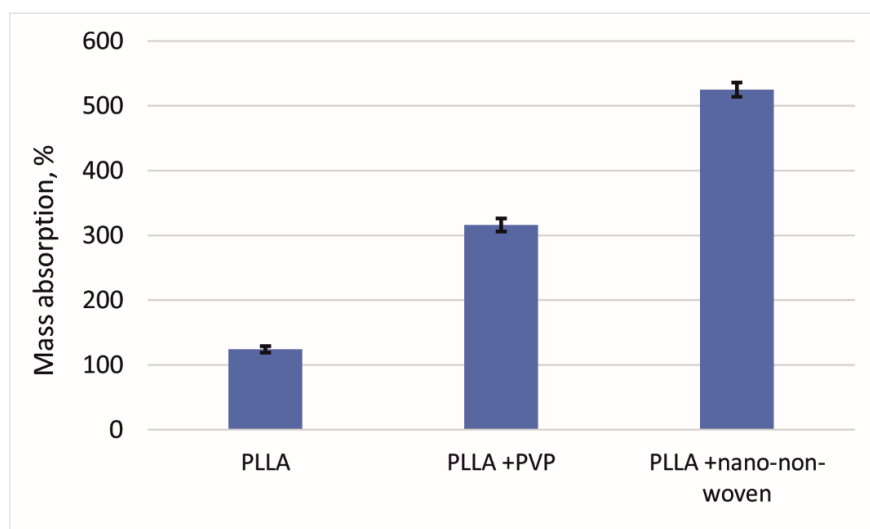


Fig 9. Dependence of scaffolds absorbability with isopropanol on the method of their preparation.

Cytotoxicity test

The cytotoxicity of scaffolds prepared from PLLA and PCLA using gelatin nano-non-wovens was examined against Jurkat cells. Due to poor morphology, no tests have been performed for PCL scaffolds. Since PVP and Pluronic® are biocompatible substances, their toxic impact was not examined. Material may be considered non-toxic if, during the entire period of the culture, the number of living cells that are in contact with such a material exceeds 50% of the initial state. The number of viable cells has remained over 95%. It means that these scaffolds are non-toxic to the examined cells (Fig. 10). Similar results for the scaffolds prepared from PCLA were slightly worse (but also non-toxic); a decrease in the viability of the cells (down to ca.70%) was observed on the 8th day.

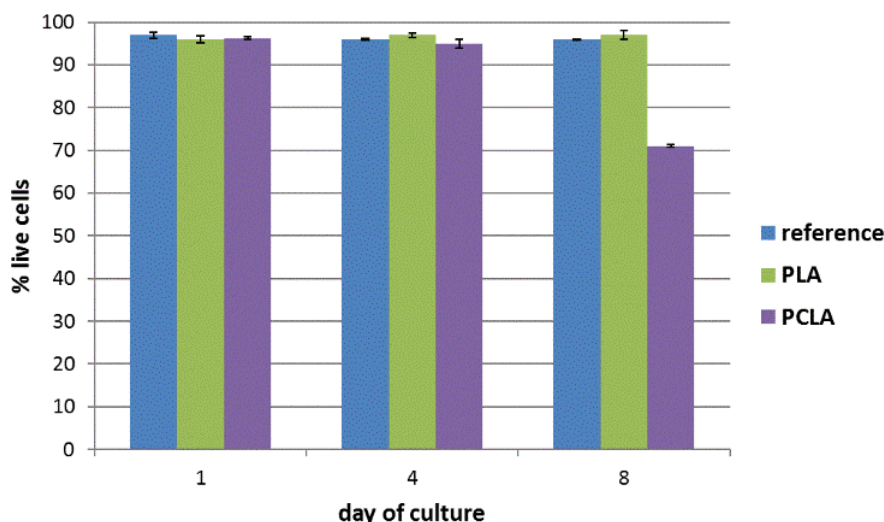


Fig. 10 . Results of the cytotoxicity test

Cell culture (fibroblasts L929)

Non-contact test

A much larger number of cells (Fig. 11) Were observed in scaffold samples obtained with wet phase inversion using gelatin nanofibers ($92 \pm 5\%$). A much smaller percentage of live cells contained scaffold extract obtained with dry phase inversion ($78 \pm 18\%$). All tested extracts had a cell viability of over 70%. Thus, scaffolding can be described as non-toxic.

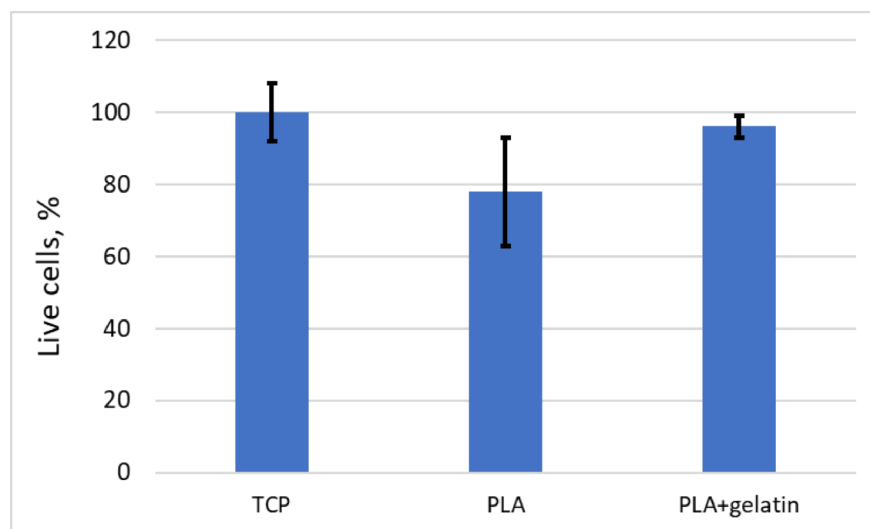


Fig 11. Percentage of viable fibroblasts after 24 h of culture on extracts from tested samples

Contact test

Both scaffolding surfaces were examined in a contact test. The obtained results were compared with TCP and PLA foil. The results are presented as the number of cells present in the sample after 1, 2, and 3 days of culture (Fig 12).

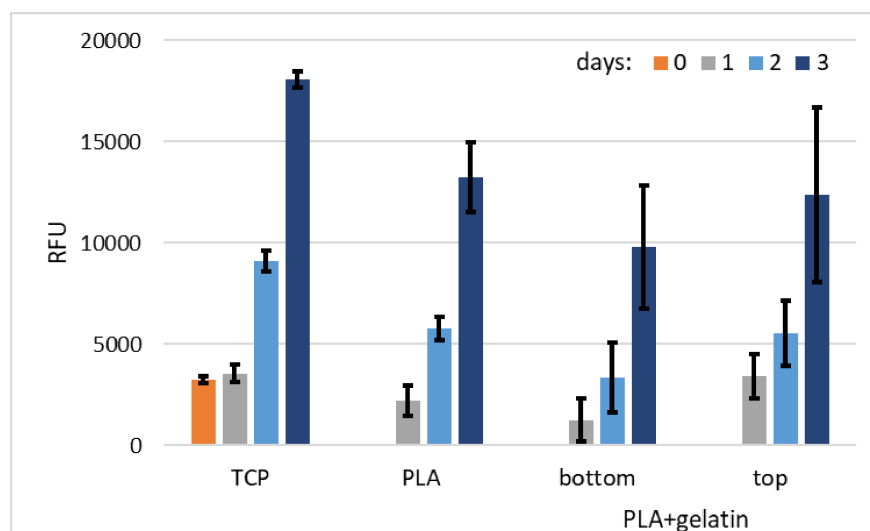


Fig 12. Cell growth after 3 days of fibroblast culture on TCP, PLA film, and a suit obtained with gelatin nano-non-woven.

After 24 h of culture, there were far fewer cells (2200 ± 800) on the surface of the PLA film than in the

control TCP (3500 ± 400) culture. It was similar in scaffolds obtained with wet phase inversion using gelatin nanofibers, were on the lower surface there were fewer cells (1300 ± 1500) than on the upper (3400 ± 1100), this result was burdened with a large error. After 48 h of culture, the cell number increased several times. In scaffoldings obtained with the addition of gelatin nanofibers, 3400 ± 1700 cells were observed on the lower surface and 5500 ± 1600 cells on the upper surface. After 72 h, the number of cells increased again. In scaffolds obtained using gelatin nanofibers, a larger number of cells were observed on the upper surface (9800 ± 3000) than on the lower surface (9800 ± 3000).

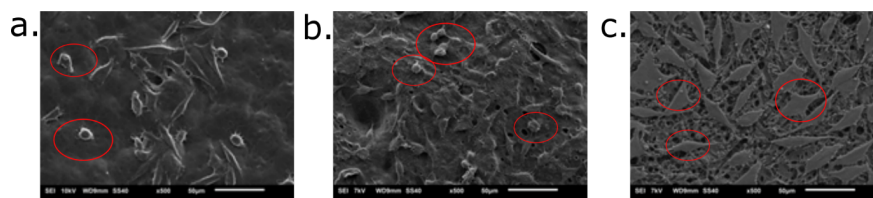


Fig 13. Evaluation of the fibroblast growth morphology on stable PLA (a), on the lower (b), and upper (a) scaffolds of PLA obtaining with gelatin nano-non-woven. Sample cells are marked with red circles. 50 μm

Cell growth morphology was assessed (Fig 13). Cells growing on stable PLA had the wrong morphology. They were rare, shrunken - rolled into balls (Fig 13.a). On both sides of the suit made using gelatin nanofibers, the cells had the correct morphology - they were uncracked and flattened. On the lower surface (less porous, Fig 13.b) the cells grew in the form of a solid layer tightly covering the surface of the suit. On the upper side (Fig. 13.c), they grew in rhomboid colonies and at specified intervals between them. The above observations show that fibroblasts prefer surfaces with smaller pores for growth.

Conclusions

An original method for preparing scaffolds for cell cultures was developed. It involves modification of the wet phase inversion method by the use of gelatin nano-non-wovens as a novel, non-classic pore precursor. The scaffolds obtained by this method have a unique characteristic. Their upper surface is highly porous, which allows for a "smooth penetration" of cells into the structure of a scaffold. The cross-section is characterized by the presence of numerous, large, interconnected pores. The bottom surface contains a few small pores which prevent the cells from "falling out" from the structure of the scaffolds. In comparison to pores of scaffolds obtained with conventional pore precursors (polymers added to the scaffold-forming solution), pores are significantly larger (usually 50–80 μm instead of 10–20 μm) and are evenly distributed.

The proper growth of chondrocytes requires three-dimensional space, which does not limit the shape they adopt during their growth. Moreover, the transfer of cultured cells into properly developed tissue requires interconnected pores since it allows for necessary intercellular communication. Due to their shape size and the level of interconnection of pores, scaffolds prepared from PLLA and PCLA, meet all these requirements. The most favourable morphology is attributed to scaffolds prepared from a gelatin nano-non-woven, PVP, and Pluronic®. In addition to the characters mentioned above, they contain perforations (addition of PVP) and inner walls of pores are divided into segments (due to addition of Pluronic®), which facilitates the migration of particles within the pores.

Cytotoxicity data obtained for the prepared scaffolds prove that none of the examined polymers is toxic concerning the Jurkat cells. Since the cells of that line are very susceptible to toxic substances, it has allowed us to conclude that these scaffolds may also be used for culturing other types of cells.

It was found that the original scaffolds developed did not show a cytotoxic effect on mouse fibroblasts since the number of viable cells after 24 hours of culture in a non-contact test is higher than 70% of their first number. Cells show an upward trend over time, and their morphology is normal after 5 days of culture. It is shown that the scaffolds tested can be used for other cell cultures.

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The authors declare that they have no conflict of interest.

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