MANUFACTURING OF BIODEGRADABLE SCAFFOLDS TO ENGINEER ARTIFICIAL BLOOD VESSEL

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IZRADA BIORAZGRADIVIH MATRICA ZA VEŠTAČKI KRVNI SUD

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ABSTRACT

Blood vessels diseases such as cardiac infarction with coronary artery occlusion, peripheral arterial disorders, or stroke of carotid or cerebral arteries, are the leading causes of death in the world. One of medical procedures for clinical treatment of vascular diseases is the blood vessels grafting. As the autologous blood vessels, which are the "golden standard" for coronary grafting, are not always suitable for blood vessels grafting, there is a need to develop artificial blood vessels as a vascular prostheses, either from natural and synthetic materials, permanent synthetic or biodegradable scaffolds which would be suitable for vascular grafts. Considering this to be our study goal we made bilayered biodegradable polycaprolactone scaffolds with different properties and evaluated their morphological and biomechanical characteristics.

Keywords: *blood vessel, vascular grafts, biodegradable scaffolds, electrospinning*

SAŽETAK

Oboljenja krvnih sudova, kao što su infarkt miokarda sa okluzijom koronarne arterije, poremećaji perifernih arterija, ili infarkt karotidnih ili cerebralnih arterija su vodeći uzrok smrti u svetu. Jedna od medicinskih procedura za klinički tretman vaskularnih oboljenja je primena graftova krvnih sudova. Kako autologi krvni sudovi, koji predstavljaju „zlatni standard" u primeni koronarnih graftova, nisu uvek pogodni, postoji potreba za razvojem veštačkih krvnih sudova kao vaskularnih proteza, od prirodnih ili sintetičkih materijala, permanentnih ili biorazgradivih skafolda koji bi bili pogodni da se primene kao graftovi. Imajući ovo u vidu, cilj naše studije je bio da se naprave dvoslojni biorazgradivi skafoldi od polikaprolaktona sa različitim svojstvima i da se zatim procene njihove morfološke i biomehaničke karakteristike.

Ključne reči: *krvni sud, vaskularni graft, biorazgradiva matrica, elektrospining*

ABBREVIATIONS

DMF - N,N – dimethylformamide
ECs – endothelial cells
ECM – extracellular matrix
PCL – polycaprolactone
PEG – polyethyleneglycol
SEM – scanning electron microscopy
SMCs – smooth muscle cells

INTRODUCTION

Blood vessels diseases such as cardiac infarction with coronary artery occlusion, peripheral arterial disorders, or stroke of carotid or cerebral arteries are the leading causes of death worldwide (1). Clinical treatments of vascular diseases include various drugs and medical procedures with different success of treating. One of medical procedures for clinical treatment of vascular diseases is the blood vessels grafting. As the autologous blood vessels, which are the "golden standard" for coronary grafting, are not always suitable for blood vessels grafting, there is a need to develop artificial blood vessels as a vascular prostheses. In recent years, a significant progress has been made in the development of prosthetic grafts from natural (2, 3) and synthetic materials (4, 5). Tissue engineering offers an attractive option to vascular grafting, particularly for creating small diameter vessels using biodegradable scaffolds technologies for vascular grafts in which layers of endothelial cells (ECs), smooth muscle cells (SMCs), or fibroblasts are grown to resemble the blood vessel structure. These scaffolds are designed to degrade over time as vas-

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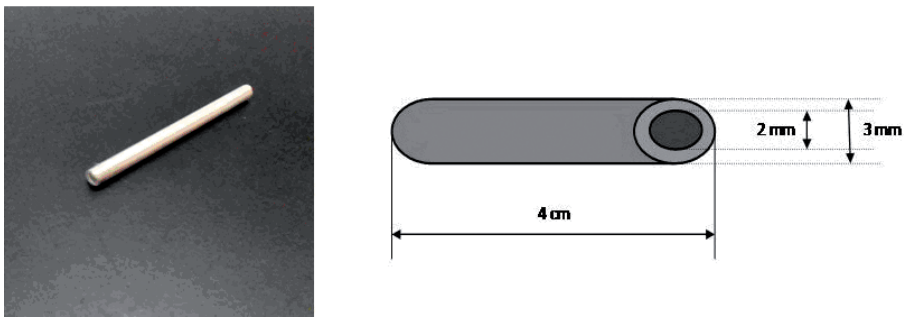


Figure 1. Metal tube collector (mandrel) presented as photo images (left) and as schematic diagram (right).

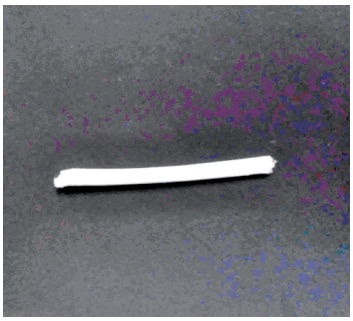


Figure 2. Tubular scaffold (one representative specimen).

cular cells produce extracellular matrix (ECM) and form functional vessels through a tissue remodeling process in vivo (6, 7). The vascular scaffold should be composed of a durable biomaterial capable of withstanding physiological hemodynamic forces while maintaining structural integrity until mature tissue forms in vivo (6). Electrospinning technology has been widely used for this purpose because this technique permits fabrication of nano- to microscale fibrous matrices and allows for control of the composition, structure, and biomechanical properties of scaffolds (8-10).

The aim of this study was to prepare scaffolds by means of electrospinning as well as to test morphological and biomechanical properties of the scaffolds aiming to create small diameter bilayered blood vessel grafts.

MATERIAL AND METOD

Scaffold fabrication

In order to prepare bioengineered circumferential bilayered scaffolds, biodegradable materials were used. For the first (inner) layer polycaprolactone (PCL, Sigma-Aldrich, U.K., Mn 80000, pellets (~3 mm)) and polyethyleneglycol (PEG, Sigma-Aldrich, Germany, Mn 4000, platelets) were dissolved in chloroform solution (Chloroform, Sigma-Aldrich, France, ACS, reagent, ≥ 99.8%) in the period of 24 hours. Different ratios of PCL and PEG were used (1:1.1 ratio and 1:1.25 ratio) dissolved in chloroform solution as 16.5% (w/v), 22% (w/v), and 30% (w/v), in order to obtain proper concentrations which would in the end have gratifying amount of pores (important for coating with cells). A metal tube collector with the diameter of 3-5 mm (mandrel) was used for manufacturing the inner layer of circumferential scaffolds (Figure 1). After 24 h drying scaffolds were immersed into the water for another 24h (in order to dissolve some amount of PEG to obtain pores). After drying, scaffolds were ready for creating outer layer (Figure 2).

Electrospinning method

Outer layer was obtained using electrospinning method and apparatus. The electrospinning system consists of syringe pump, high voltage supply and rotating rod (outer diameter, 2.1 mm). The Acopian Power Supply Model P030HP1 was used (in collaboration with Faculty of Engineering and BioIRC, University of Kragujevac). The positive electrode was connected to the cusp of the syringe needle and negative electrode was attached to the rotating rod. One-layered scaffolds were put onto the rod collector. A polymeric solution for electrospinning was prepared as 20% (w/v) PCL using an organic solvent mixture composed of chloroform and N,N-dimethylformamide (DMF, Sigma-Aldrich, USA, anhidrous, 99.8%) (7:3 ratio) (11). For the electrospinning process the polymeric solution was placed in a 10 ml plastic syringe fitted with a metal needle with a tip diameter of 0.8 mm. The syringe pump provided constant flow rate of polymer of 1 ml/h through syringe needle. The polymer solution was converted to nanofiber in presence of electric field. The voltage supply between 15 kV and 18 kV was applied to polymer solution. These nanofibers were collected to the already prepared one-layered scaffold which was put onto 436 stainless steel rod with outer diameter 2.7 mm. Different numbers of rotations were applied (50 revolutions per minute and 60 revolutions per minute) for solvent cast PCL layer. Electrospinning was performed in 1 and 2 min. The distance between the syringe tip and the collection rod was 20 cm that allows fibers to be wider and better spread on collector in order to achieve better ar-

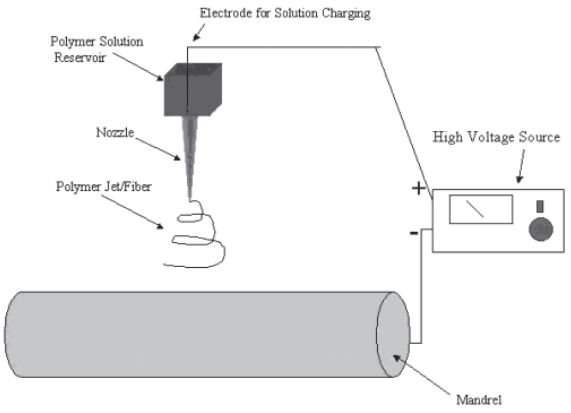
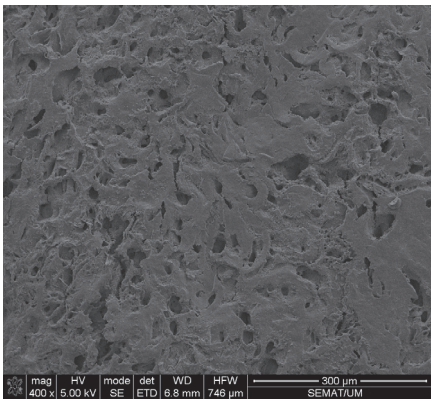


Figure 3. Electrospinning apparatus setting



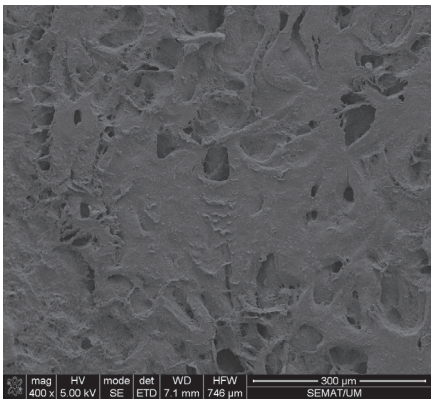


Figure 4. Photomicrographs obtained by SEM represent: a) inner layer of scaffold 16.5% (w/v) chloroform solution, PCL:PEG=1:1.1, b) inner layer of scaffold 16.5% (w/v) chloroform solution, PCL:PEG=1:1.25.

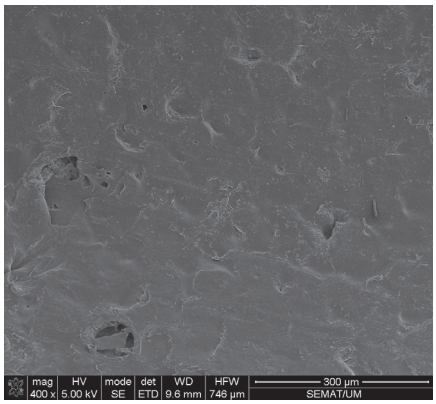
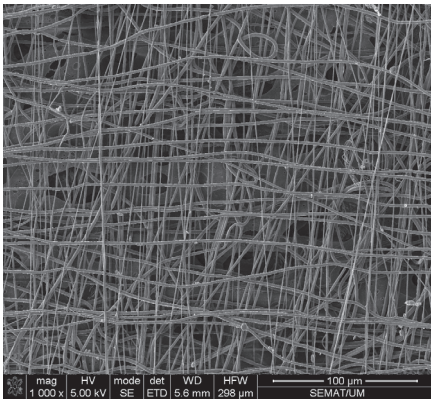


Figure 5. Photomicrograph obtained by SEM represents an inner layer of scaffold which consists chloroform solution 30% (w/v) and PCL:PEG= 1:1.1.



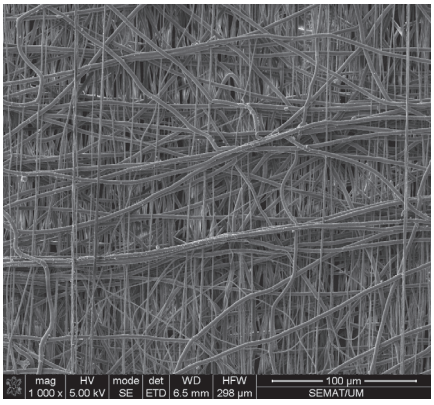


Figure 6. Photomicrographs obtained by SEM represent: a) outer layer of scaffold 60 rpm, 1 min, b) outer layer of scaffold 60 rpm, 2 min.

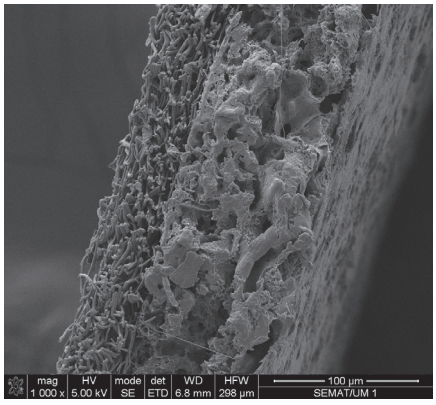


Figure 7. Photomicrograph of bilayered scaffold (cut at the edge, one representative specimen).

rangement of fibers. Solvent evaporation was performed at room temperature for 2 days. Many conditions were changed in order to obtain gratifying scaffold that will be used for cell seeding (Figure 3).

Mechanical characterization of tubular scaffolds

Electrospun bilayered tubular specimens were used for mechanical testing. Different ratios of PCL and PEG, as well as the percentage of chloroform solutions (w/v), of scaffolds' were used in order to obtain optimal biomechanical characteristics of specimens (Young's tensile modulus, Max stress and Strain at break). Tensile properties were measured with load test machine (Bose ElectroForce® 3200, TA Instruments®, USA) (in collaboration with Faculty of Engineering and BioIRC, University of Kragujevac) equipped with a maximum 225 N load cell.

Morphological characterization of tubular scaffolds

Bilayered parts of membranes were observed using scanning electron microscopy (SEM) (Pegasus X4M). The membranes were cut into square specimens (1 cm^2), glued with carbon tape to copper supports and sputter coated with gold to a thickness between 10 to 15 nm. Images were acquired using SEM operating at accelerating voltage of 5 kV.

Statistical analysis

Statistical analyses of data obtained after mechanical tests were performed using independent t-test with significance threshold $p<0.05$. All statistical calculations were done with the computer program SPSS, version 19.0. Data are presented as mean ± standard deviation (SD).

RESULTS

In order to adjust experimental conditions for preparing bioengineered scaffolds that would have desired properties for creating vascular grafts, several adjustments were made. Scaffolds were made using different values and combinations of materials and conditions, as we already described in the Material and Methods section and presented in the Table 1.

All specimens then subjected to scaffolds' analysis consisted of:

1. Morphological characterization (Figures 4-7),
2. Biomechanical characterization (Young's tensile modulus, Max stress and Strain at break) (Table 2, Figures 8-11)

Table 1. The values and combinations of used materials and conditions in order to create bioengineered scaffolds.

The inner layer of scaffold		The outer layer of scaffold		
Ratio PCL:PEG	w/v %	PCL	Rotations	Time of rotations (electrospinning)
1:1.25	16.5	7:3	50	1 min and 2 min
	22			
	30		60	1 min and 2 min
1:1.1	16.5	7:3	50	1 min and 2 min
	22		60	1 min and 2 min
	30			

PCL is polycaprolactone, PEG is polyethyleneglycol, PCL is dissolved in chloroform and N,N-dimethylformamide (ratio 7:3)

Table 2. Results of biomechanical characterization of scaffolds (PCL:PEG=1:1.1 and PCL:PEG=1:1.25, made under 60 rpm, duration 1 minute), performed by estimation of Young's tensile modulus, Max stress and Strain at break.

Scaffold ratio PCL : PEG	Young's tensile modulus (Mpa)[1]	Max stress (Mpa)	Strain at break (%)
1 : 1.1	50.2 ± 5.2	5.3 ± 0.6	320 ± 128.8 **
1 : 1.25	49.2 ± 14.5	4.3 ± 0.9	179 ± 39.2 **

* results are presented as Mean ± SD
** stat. significant difference, p<0.05
[1]- Tensile modulus calculated as difference between two points in linear part of graph
[2]- Tensile modulus calculated using function LINEXP, and all the spots between two chosen in linear part of the graph

1. Morphological characterization - by scanning electron microscopy (SEM)

All created scaffolds were subjected to analysis by scanning electron microscopy (SEM). Analysis of the inner layer of the specimens showed that scaffolds made as 16.5% chloroform solution with both ratios PCL:PEG=1:1.1 and PCL:PEG=1:1.25 had the most appropriate characteristics in respect to desirable size and number of pores (Figures 4a) and 4b)). As an example, we presented in the fig. 5, how one of the scaffolds looks like (30% chloroform solution, PCL:PEG=1:1.1), which showed no suitable morphological characteristics.

Analysis of the outer layer of the specimens (by SEM) showed that scaffolds made using wire collector under 60 revolutions per minute (with the duration of electrospinning of 1 minute) had the most appropriate characteristics in respect to arrangement and amount of fibers and pores (Figure 6a), according to Nam and coauthors (12). As an example, we presented in the Figure 6b, how one of the scaffolds looks like (30% chloroform solution, PCL:PEG=1:1.1), which showed no suitable morphological characteristics.

In the Figure 7, we presented photomicrograph of one representative specimen of bilayered scaffold (cut at the edge).

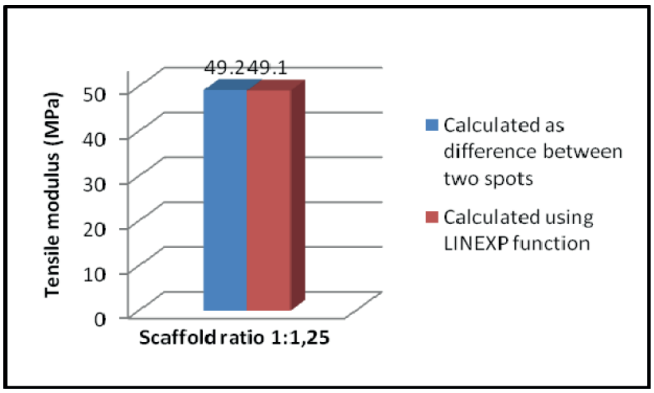


Figure 8. Calculated Tensile modulus; scaffold ratio (PCL:PEG) 1:1.25.

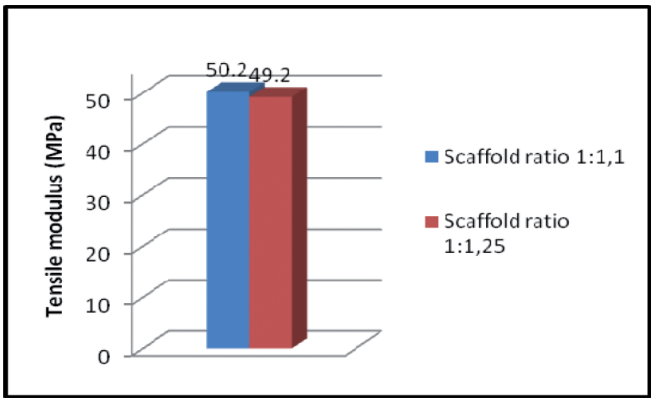


Figure 9. Comparison of Tensile modulus between scaffold ratios (PCL:PEG), calculated as difference between two spots.

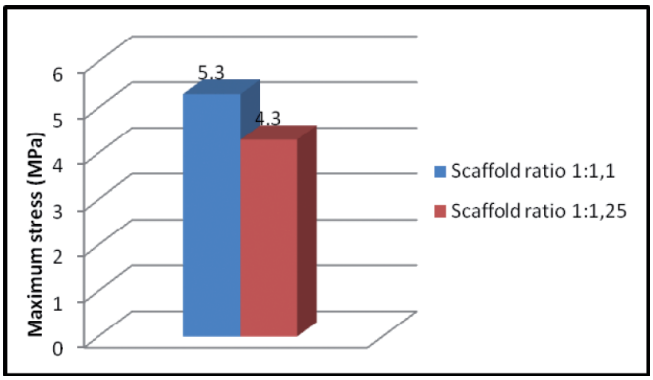


Figure 10. Maximum stress; scaffold ratios PCL:PEG.

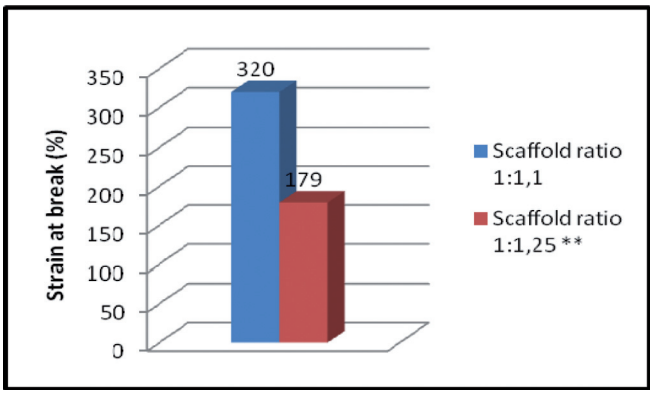


Figure 11. Strain at break; scaffold ratios PCL:PEG. (** stat. significant difference, p<0.05)

2. Biomechanical characterization

Electrospun bilayered tubular scaffolds which showed the most suitable morphological characteristics (PCL:PEG=1:1.1 and PCL:PEG=1:1.25, made under 60 revolutions per minute (rpm), 1 minute duration) were used for mechanical testing in order to evaluate biomechanical characteristics of specimens. Biomechanical characterization was performed by estimation of Young's tensile modulus, Max stress and Strain at break. Obtained results are presented in Table 2 and Figures 8-11.

DISCUSSION

Having in mind that each year over a million patients worldwide need arterial prostheses which cost more than US$25 billion (13), the need for vascular grafts replacement is growing. It is also known that pathologies (mainly caused by atherosclerosis) affecting small and medium-sized blood vessels are the primary cause of death (14, 15). In these situations cardiac and peripheral bypass surgeries are necessary, requiring the replacement of a segment of blood vessels. The currently available options for these transplants are autologous grafts, allografts, xenografts and synthetic vascular grafts (16). The use of autografts and allografts is limited due to the lack of tissue donors, previous harvesting or anatomical variability (17). Xenografts suffer from their relatively shorter life span (18). Synthetic prosthetic grafts become rejected by the immune system of the body if the diameter of the vessel is smaller than 6 mm (reocclusion, thrombosis and aneurysm) due to mismatch of compliance (17-20). So far it has been shown that tissue engineering could be an alternative approach for creating new vascular grafts. However, synthetic vascular grafts have rarely been proved successful in small blood vessel replacements (inner diameter < 6 mm) (21). The principle of designing tissue engineered scaffolds is simple: the scaffold should mimic the structure and biological function of native extracellular matrix (ECM) as much as possible, and in addition not to be toxic for tissues. In tissue engineering, the basic idea is that cells seeded onto scaffold produce ECM while the polymer is degraded, gradually creating the intended tissue. Moreover, vascular grafts should have further characteristics: enough strength to resist rupture or excessive dilation when subjected to pulsatile pressure *in vivo*; also to have stable mechanical properties during their expected lifetime. Many previous studies have used PCL as biodegradable polymer for vascular tissue engineering (22) or to make *in vitro*, scaffolds coated with neuronal cells (23) which show its nontoxic effects. That was one of the reasons why it was used for this research.

Poly(ε)-caprolactone (PCL) is wide used biopolymer in many studies (24). PCL is semicrystalline, aliphatic polyester synthesized by the ring-opening polymerization of ε-caprolactone (25-27). It shows good mechanical properties, specifically high elongation and strength, and good biocompatibility (27, 28). Furthermore, PCL degrades very slowly *in vivo* by enzymatic action and by hydrolysis to caproic acid and its oligomers (25, 27). It takes more than one year to completely degrade in vivo (27, 28). PCL is a FDA approved polymer (25). The degradation products are ultimately removed by giant macrophage cells (29, 30). PCL elasticity closely matches native values (31) and has a high extension rate before breakage, but tensile strength of PCL is less appreciable (31). PCL based small diameter vascular constructs have been reported to have good suture retention value and compliance to withstand physiological conditions of blood vessels (32). Electrospun PCL with differential porosity in two layers (inner low porosity than outer), when implanted in a rat showed complete endothelization and perfect patency with no thrombosis (33, 34). In another study, an electrospun PCL tubular scaffold implanted into rat proximal native artery showed excellent structural integrity throughout the study, with no aneurysmal dilation, and perfect patency with no thrombosis and limited intimal hyperplasia (34).

In the first phase of our research we wanted to create scaffolds with the most suitable characteristics, which could be used for seeding cells, in the second phase of our research.

Regarding the morphological characteristics, all the adjustments performed in this research (Figures 4-7) were made in order to obtain effective pore diameters for cell ingrowths. Pores in a tissue-engineered scaffold make up the space in which cells reside. In this study, the majority of pore diameters are limited to the range of 25 to 100 μm (Figures 4-7) According to literature data of vascular grafts, the effective pore diameters for cell ingrowths are between 20 and 60 μm while for bone ingrowths the pore-diameters between 75 and 150 μm are required (35). The pore size that would permit adequate cellular infiltration has been suggested to be greater than 10 μm (9). For the engineering of blood vessels, small pore size does not present a problem with respect to coating of the lumen using endothelial cells (EC). However, this pore size would limit the ability of smooth muscle cells (SMC) to colonize the outer portion of neo-vessel and facilitate remodeling of ECM (12). As already described in the Material and Methods section, all manufactured specimens were subjected to evaluation of morphological properties by scanning electron microscopy (SEM). Results of these evaluations showed that the most appropriate properties in respect to desirable size and number of pores for the inner layer showed scaffolds made as 16.5 % chloroform solution with both ratios PCL:PEG = 1:1.1 and PCL:PEG = 1:1.25 (Figures 4a) and 4b)). Regarding to outer layer using electrospinning method the most gratifying properties showed scaffolds made using thicker wire collector under 60 revolutions per minute (duration of electrospinning was also one minute) (Figure 6a) because arrangement and amount of fibers and pores would be satisfactory for smooth muscle cells' growth (12).

As far as biomechanical tests are concerned (Figures 8-11), our results showed that no statistical significance were obtained between scaffolds' Young's modulus values.

Young's modulus defines the relationship between stress (force per unit area) and strain (proportional deformation) in a material, it predicts how much a material sample extends under tension or shortens under compression. This value of the Young's modulus provides strong and compact tissue formation. More flexible tissue formation can be achieved using thinner layer of scaffold material (36). Maximal stress was no significantly different regarding the scaffold's ratio, which is desirable from mechanical point of view. However, the material with scaffold PCL:PEG ratio of 1:1.1 has approximately 20% greater maximal stress value and due to this fact can be a better choice in elastic tissue formation (Figure 10). Maximal stress value is in fact maximal force value per area of material for which material reserves elastic behavior what is crucial at blood vessels application due to presence of the cyclic mechanical stress. Further, statistically significant difference was obtained for value of Strain at break (%) ($p=0.021$, group of scaffolds made as ratio PCL:PEG=1:1.1 shows higher level for Strain at break). Strain at break represents ratio between changed length and initial length after breakage of the test specimen. It expresses the capability of a material to resist changes of shape without crack formation. Before break material loses the elastic behavior. The mixture of the scaffold ratio PCL:PEG=1:1.1 has approximately twice larger Strain at break value than mixture with ratio of 1:1.25. In the normal condition this value has never been achieved (37) and both materials are satisfied toward potential crack in application for tissue growth.

CONCLUSIONS

The morphological analysis of results in this study has showed that scaffolds with inner layer made as 16.5 % (w/v) PCL:PEG ratio 1:1.1 and PCL:PEG ratio 1:1.25 have more desirable size and number of pores than scaffolds made as 22% and 30%. Further, biomechanical characterization showed that both specimens with PCL:PEG ratios 1:1.1 and 1:1.25, respectively, have very similar elasticity characteristic. However, scaffolds with PCL:PEG ratio 1:1.1 are stronger and more resistive to the straining and mechanical stress. This fact point, that considered scaffold material, represents a better choice in application for tissue growth. In the same time this material has slightly better elastic characteristics and in the same time can be used for more different types of tissues which is an additional reason for its application.

We believe that described experimental procedures and obtained results extend our knowledge in the technologies for vascular grafts, and represent a good basis for further research that would include and the cells seeded onto scaffold in order to develop artificial blood vessels as vascular prostheses.

Disclosures

Conflict of Interest: None.

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