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The membrane with polylactide and hyaluronic fibers for skin substitute

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Purpose: Skin substitutes are heterogeneous group of scaffolds (natural or synthetic) and cells. We hypothesize that nanofibers with layer composition made of polylactide (PLA) and sodium hyaluronate (HA) obtained using electrospinning method are a good matrix for cell adhesion and proliferation. *Methods*: Optimal conditions of electrospinning of PLA and HA nanofibers to create layered compositions (PLA membrane covered with HA nonwovens) were determined by modifying parameters such as the appropriate amount of solvents, polymer concentration, mixing temperature and electrospinning process conditions. By changing the parameters, it was possible to control the diameter and properties of both polymer fibers. The spinning solution were characterized by surface tension and rheology. A scanning electron microscope (SEM) was used to determine the morphology and fiber diameters: PLA and HA. Structure of the PLA/HA nonwoven was analyzed using spectroscopy (FTIR/ATR). Biocompatibility of the nonwoven with fibroblasts (ECM producers) was assessed in the *in vitro* conditions. *Results*: The results showed that stable conditions for the formation of submicron PLA fibers were obtained using a 13% wt. solution of the polymer, dissolved in a 3:1 mixture of DCM:DMF at 45 °C. The hyaluronic fibers were prepared from a 12% wt. solution of the polymer dissolved in a 2:1 mixture of ammonia water and ethyl alcohol. All materials were biocompatible but to a different degree. *Conclusions*: The proposed laminate scaffold was characterized by a hydrophobic-hydrophilic domain surface with a maintained fiber size of both layers. The material positively underwent biocompatibility testing in contact with fibroblasts.

Key words: *nanofibrous scaffold, electrospinning, skin substitute, hyaluronic acid, polylactide*

1. Introduction

Skin substitutes are defined as a heterogeneous group of materials that are used to close the wound and temporarily or permanently take over skin functions [10], [20]. They consist of natural or synthetic scaffold (a substitute for dermis) and fibroblasts that produce the extracellular matrix (ECM) compounds themselves and grow into a three-dimensional matrix [1], [5]. Due to their complexity, substitutes can be divided into three classes. The first class includes temporary dressings, the second class – one-layer skin substitutes, and the third class – complex skin substitutes. Substitutes can be used as protection of damaged tissue, a dressing that supports regeneration before transplantation or as a supplement to the resulting tissue defect. Each substitute class needs porous scaffolds that can work together with the patient's fibroblast.

Nanofibres obtained by electrospinning of resorbable materials are a good matrix for cell adhesion and proliferation, and the fibrous phase of the scaffold material is a biomimetic form of the collagen fibers present in the extracellular matrix (ECM) [6], [8], [13]. The fi-

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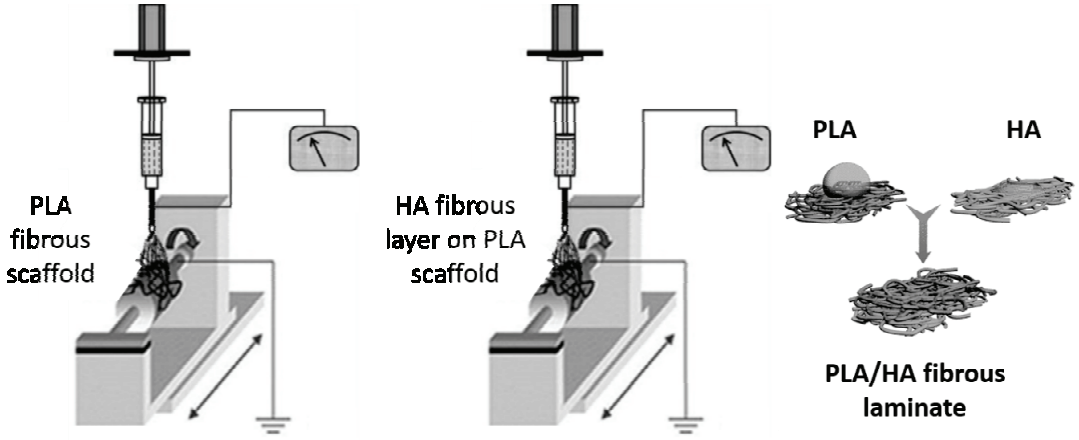

Fig. 1. Schematics of preparation of fibrous materials: PLA (support layer), HA (top layer) and PLA/HA laminate materials with modified surface properties (wettability, chemistry)

bers belong to the group of nanomaterials if the diameter of a single fiber ranges within 50–500 nm or when the cross-section is about 100 times smaller than the length. From the material point of view, the nanofibres guarantee a larger specific surface area of the nonwoven fabric, the possibility of obtaining a porosity (e.g., pores between fibers or pores in the fiber). This translates into high permeability to gases and liquids while maintaining a simultaneous barrier function (against external factors). The fibrous microstructure gives the surface unique properties that improve protein adsorption and cell adhesion. Such fibers can be additionally modified both by volume and by surface with bioactive agents (HAp, TCP, BG or RGD, fibronectin) [18]. These phenomena are used in both tissue engineering and regenerative medicine, but also in cosmetics and dermatology [3], [18], [20], [21].

An example of the use of fibrous scaffolds is the combination of known polymers (e.g., PLA, gelatin) with plant extracts that accelerate skin regeneration, e.g., in the rat model [11]. Other studies show that metabolic activity of chondrocytes and osteoblasts deposited on fibrous substrates is higher, which encourages the authors to carry out further studies with animals [12], [19]. Increasingly, fibers are used in widely understood dermatology, and an example of such applications are special revitalizing masks [7]. An electrospun nonwoven (fiber membrane) composed of collagen fibers and polyvinyl alcohol fibers (PVA) was enriched with gold nanoparticles and retinoic acid. Antioxidants in the hydrated form of the Col/PVA mask have been shown to be more stable and more durable than in cotton masks, and a high development of the fiber surface ensured deep penetration of the ingredients inside the skin [22].

Bearing the multifunctional use of fibrous substrates in mind, an interesting combination may be modification of the fibers with other fibers (fibrous form of another polymer). Their presence may affect the release rate of bioactive particles, activation of particular groups of cells, etc., and hyaluronic acid (HA) appears to be a good candidate for these purposes.

Hyaluronic acid is a typical hydrogel, responsible for the elasticity and hydration of the skin as well as for the transport of ions and nutrients. It is a component of tissues and body fluids, e.g., synovial fluid providing a proper lubrication to the joints. It is used in eye surgery, where it protects tissues from damage, but also in dermatological treatment as a matrix for autologous skin transplants. In addition, it helps to heal wounds, ulcers, scars, and reduces the effects of joint diseases, thus it can also be helpful in the application of nanofibers [2], [4], [23].

The aim of this work was to develop the conditions of the manufacturing process of fibrous substrates composed of polylactide fibers (PLA) and hyaluronic fibers (HA) and their combinations (PLA/HA), as well as their initial characterization, also biological, for the use on skin substitutes (Fig. 1). The layered arrangement of this type of material could significantly modify their surface (wettability, surface energy, functional groups), which is crucial for cell adhesion and proliferation of fibroblasts and keratinocytes.

2. Materials and methods

Commercial polymers: polylactide (PLA) (3251D, NatureWorks) and sodium hyaluronate (HA) (Contipro) were used in the experiment. A mixture of dichloromethane (DCM) and N, N-dimethyloforamid (DMF) in a 3:1 ratio was used as a solvent in the PLA spinning solution, and a 2:1 mixture of ammonia water

(WA) and ethanol (EA) was used as a solvent in the HA spinning solution (all reagents manufactured by Avator). In the first stage, PLA solutions were prepared at various concentrations in a mixture of DCM and DMF at various volume ratios. In the second one, HA solutions of various concentrations using various solvent systems of ammonia water with ethyl alcohol (EA), N-methylpyrrolidone (NMP) or DMF were prepared. In both stages, nanofibers were obtained by electrospinning. The stability of spinning solutions (rheology of polymer-solvent systems) was determined using the Brookfield RST-CPS V3.1.1 rheometer. The measurements were made using a 20 ml SC4-21 spindle and 20 and 50 RPM spindle rotation rates. The tests were carried out after 24 hours of stirring (dissolving the polymer in a solvent) at room temperature and after one hour of stirring at a temperature of 45 °C (at which PLA fibers were spun).

Evaluation of the morphology of nonwoven PLA, HA and PLA/HA was made by means of microscopic examination using scanning electron microscopy (SEM) (Nova Nano SEM 200, FEI Company). The structural characteristics of PLA/HA was performed using FTIR-ATR (BIO-RAD FTS60V). The tests were carried out on a diamond crystal doped with zinc selenide (ZnSe) in the wavenumber range of 600–4000 cm^{-1}. The physicochemical properties of the fibrous membrane were evaluated based on wettability and surface free energy (DSA 10 Kruss) measurements. The free surface energy was calculated using the Owens–Wendt method. The viability and activity of the cells were evaluated in the biocompatibility studies.

Cell culture of fibroblasts Hs68.Tr

The Hs680.Tr fibroblast cell line (ATCC) was used in the studies. Cells were cultured in 25-ml plastic bottles (Nunc, Denmark) in DMEM medium supplemented with high glucose, L-glutamine (Lonza, USA), 10% foetal bovine serum (Biowest, USA) and 5% antibiotic solution containing penicillin 10 UI/ml and streptomycin 10 mg/ml (Sigma, USA). The cells were cultured in an incubator (Nuaire, USA) at 37 °C and 5% CO_2. Every 2–3 days, when cells formed monolayers with high confluency, cell cultures were passaged by trypsinization (0.25% trypsin solution, Sigma-Aldrich, Germany).

Cell-biomaterial studies in vitro

Biomaterials were subjected to UV sterilization for cell culture studies (20 minutes on each side) and placed on the bottom of 24-well plates (Nunc, Denmark). Cells harvested after 4–5 passages were counted in a Bürker hemacytometer, diluted to 3×10^4 cells/ml and placed in wells of 24-well culture dishes containing discs of test biomaterials. Thus prepared cell cultures grown for 1 or 7 days, and then, using an inverted microscope, the morphology of cells was observed (Jenamed, Germany) and verified by the test staining with crystal violet (CV). In the test, the cells adhered to the film of biomaterials or CTR were fixed with 2% paraformaldehyde for 1 hour and then stained with crystal violet (CV 0.5% in 20% methanol, for 5 minutes). After this time, the wells were washed with water and their contents transferred to a 24-well culture plate.

A PLA polymer was used as a control material for PLA/HA. However, the control Nunclon tissue-culture polystyrene (CTR) (Nunc, Denmark) was also used as a control for the biocompatibility of PLA.

3. Results

Obtaining reproducible fibrous substrates using electrostatics requires optimization of the basic parameters of the solution (i.e., concentration, solvent system, the viscosity, surface tension), ambient parameters (i.e., humidity, temperature) and the parameters of device (i.e., voltage, distance nozzle-collector). For each polymer-solvent system (solvent system), preliminary tests are necessary to pre-select a polymer solution that would allow submicron filaments to be obtained. In the case of polylactide, the concentration, proportions of solvents and nozzle diameters as well as voltage and temperature of the electrospinning process were selected. The basic process parameters for individual materials are summarized in Table 1.

The results of rheological tests indicated that the polymer-solvent solutions were unstable over time. Macroscopically, this was manifested by the solidification of the polymer on the nozzle, which hindered the electrospinning process. The test results of rheology and surface tension of a spinning solution are shown in Table 2.

The developed process parameters significantly influenced the polymer solution (viscosity – η, surface tension – γ). The determined viscosity and surface tension for PLA and HA indicated that the electrospinning process at elevated temperature (45 °C) and reduced humidity improved the stability of the solution. The PLA solution was stable for about 2 h while the HA solution did not change for 1 h (Table 2).

The rheological tests proved that the proposed polymer:solvent systems, both of PLA and HA, were stable during the electrospinning process under given

Table 1. Parameters of the PLA spinning process

Samples name	Concentration [%]	Solvents ratio DCM : DMF for PLA WAM : EA for HA	Voltage [kV]	Nozzle diameter [mm]	Temperature [°C]	Humidity [%]
PLA 1	10	1 : 2.3	12.5	0.9	25	22–25
PLA 2				1.1		
PLA 3		1 : 1	13.2	0.45		
PLA 4			12.5	0.9		
PLA 5		2.3 : 1	12.5	0.8		
PLA 6				0.9		
PLA 7		2.5 : 1	12.5	0.9		
PLA 8	13	1 : 1.5	12.7	0.8		
PLA 9		1 : 1	12.5	0.8		
PLA 10		1.5 : 1	12.8	0.8		
PLA 11	13	3 : 1	12.5	0.8	36	12
PLA 12	15	2.5 : 1	12.5	0.8	45	
HA 1	12	2 : 1	14.5	0.6	25	30
HA 2	12		14.3	0.7		
HA 3	12		14.2	0.9	45	15

Table 2. Rheology and surface tension of a spinning solution under various conditions

	Viscosity η after 24 h stirring in 20 °C, [cP]	Viscosity of spinning solution with extra 2 h in 45 °C, [cP]	Viscosity of spinning solution with extra 1 h in 45 °C, [cP]	Surface tension γ [mN/m]
PLA 11 (DCM : DMF ratio 2.5 : 1)	687.8	645.5	662.7	75.5
PLA 12 (DCM : DMF ratio 3 : 1)	68.4	67.8	68.4	51.8
HA 3 (WAM : EA, ratio 2 : 1)	212.6	184.7	182.5	58.9

conditions (temperature, humidity) for 60 min (PLA) and 120 min (HA), respectively. The morphology of selected PLA fibrous membranes obtained under various experimental conditions is shown in Fig. 2.

The obtained HA fiber layer was characterized by a larger spread of the fiber diameter, which was caused by the presence of a non-conductive support (PLA) on the collector (Fig. 3).

Finally, a two-layer system with comparable fiber sizes (around 800 nm) was obtained in both the PLA layer and the HA layer (Fig. 4).

In order to confirm the composition of the complex two-layer and bi-component laminate (PLA/HA) FTIR-ATR spectroscopic studies were performed, and FTIR-ATR spectra of fibrous materials are presented in Fig. 5. The spectra presented in the figure show that the presence of the characteristic bands for the polymers used in the experiment strongly depend on the thickness of the top polymer layer (HA) applied onto the support (PLA). If a thin HA layer is present in the fiber laminate, both PLA and HA bands are visible in the spectrum (Fig. 5c). If the HA layer above 4 μm (FTIR-ATR penetration depth with diamond crystal doped with zinc selenide) tightly covers the PLA support, the characteristic bands of PLA are absent in the spectrum (Fig. 5a). The reference material is the spectrum of pure, fibrous PLA support (Fig. 5b).

The physicochemical properties of the material were examined, which may also be critical for the cell's interaction with a given material, and results of surface wettability as well as surface energy are shown in Fig. 6. The smaller the diameter of the fibers forming the nonwoven fabric, the more hydrophobic PLA nonwoven. When the nonwoven fabric consisted of fibers with an average size of 560–720 nm, its contact angle was 117° (PLA11). When the nonwoven fabric was made of 780 nm fibers and larger (as is the case of PLA12), the wettability dropped to 93°.

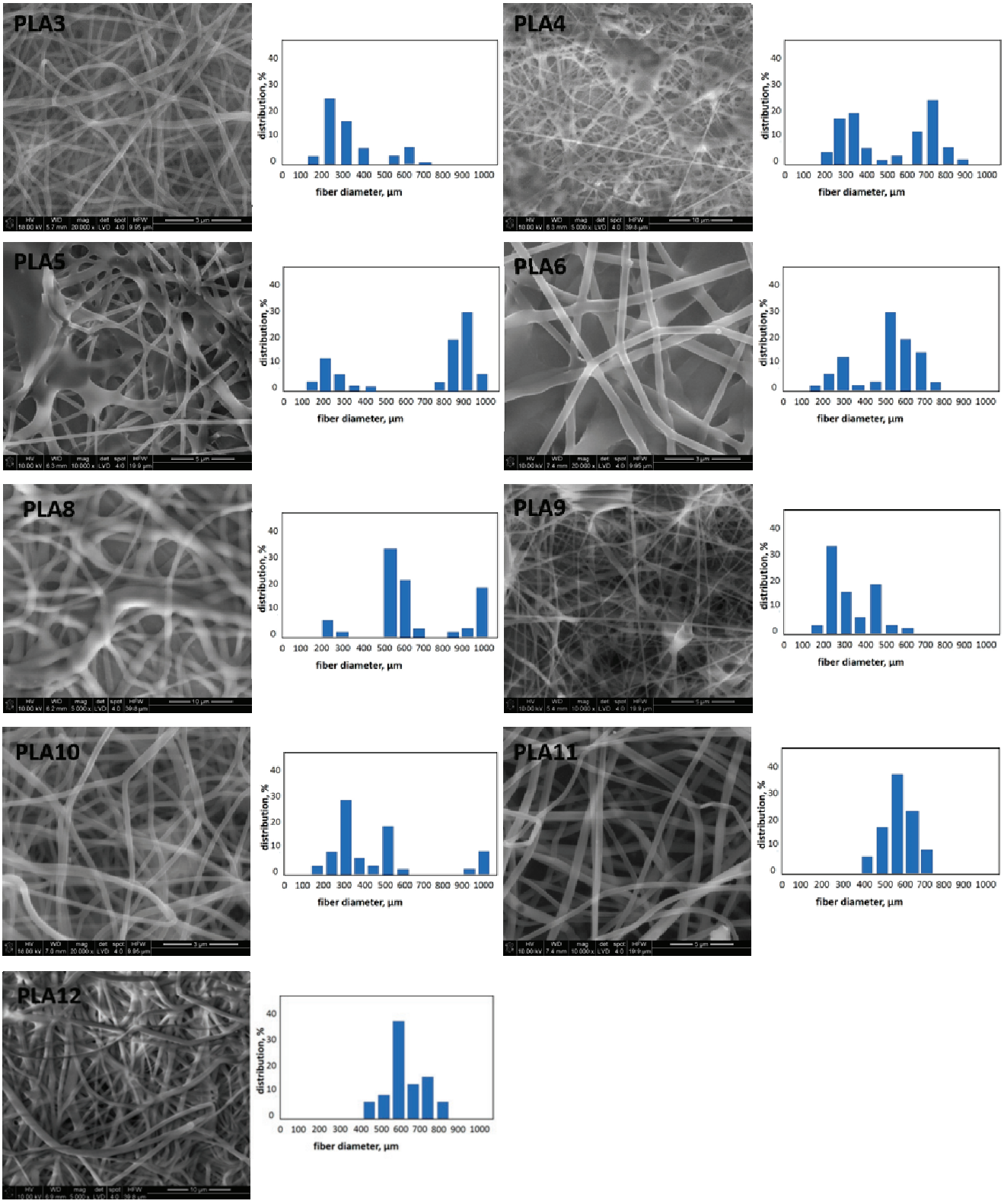

Fig. 2. Morphology of selected fibrous membrane with PLA
obtained under various experimental condition with fibers size distribution

Similar values of the contact angle were also noted for the nonwoven made of HA. The domain character of the surface layer of the PLA/HA fibrous laminate significantly affected the wettability of material, whose average value was 83°, while maintaining the high component value of polar surface energy.

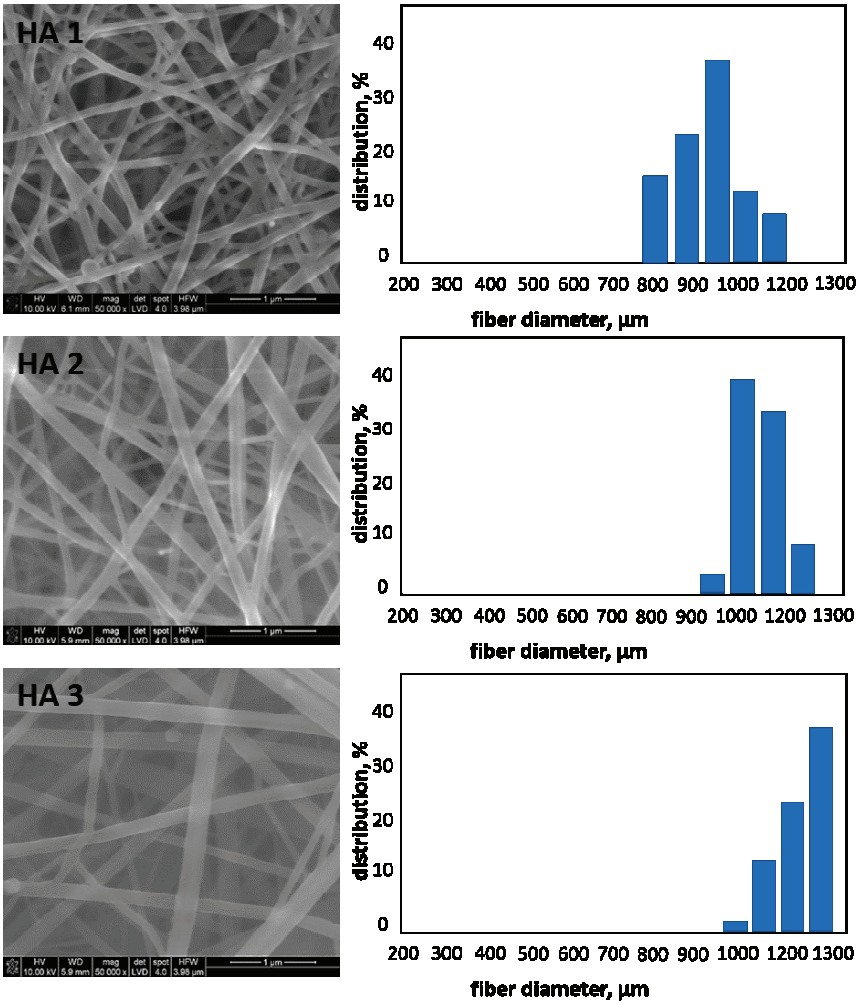

Fig. 3. Morphology of selected fibrous membrane with HA
obtained under various experimental condition with fibers size distribution

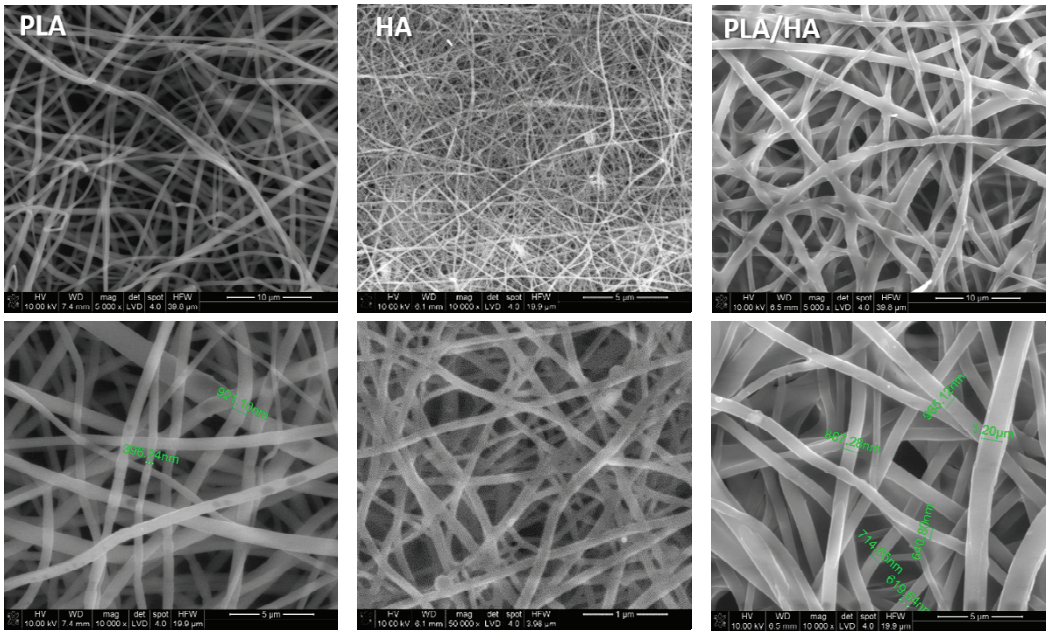

Fig. 4. Morphology of fibrous materials: support fibrous layer of PLA,
modification layer of HA, fibrous laminate of PLA/HA

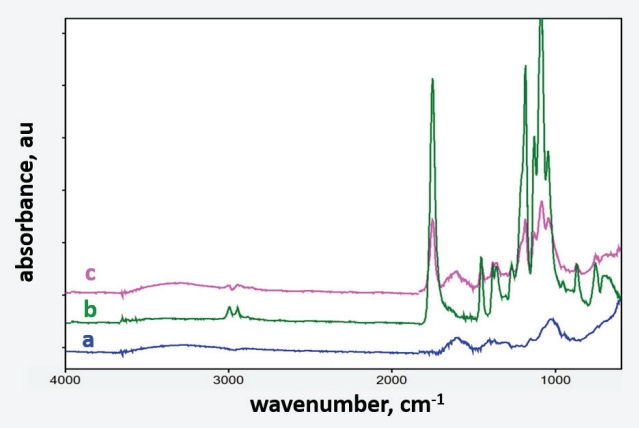

Fig. 5. FTIR-ATR spectra of fibrous materials:
PLA support with HA surface layer (thinner HA layer (a),
PLA support (b), PLA support with thicker HA layer (c)

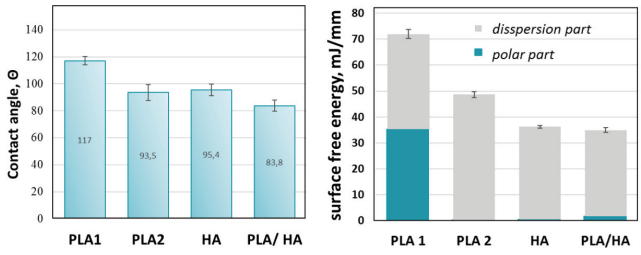

Fig. 6. Wettability and free surface energy of fibrous materials:
PLA, HA and fibrous laminate PLA/HA.

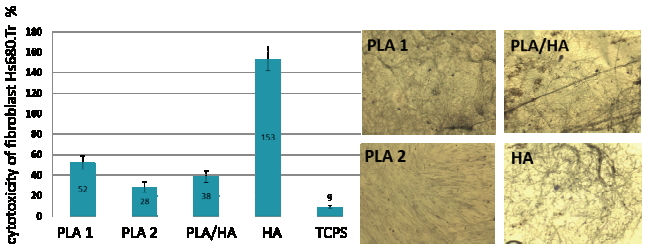

Fig. 7. Morphology of fibrous materials
and their cytotoxicity against fibroblasts for:
PLA, HA and PLA/HA laminate

The survival of human Hs680.Tr fibroblasts contacted with the surface of the fibrous materials was also tested to evaluate material biocompatibility with the living tissue and the results are presented in Fig. 7. The survival of human Hs680.Tr fibroblasts contacting the surface of the fibrous materials tested was at the control level (TC PS) after 24 hours of culture. Both after 1 and 7 days of cultivation, a larger number of cells were observed on the PLA/HA laminate compared to the control controls – PLA11 and PLA12. On the first day of cultivation, cells contacted with all fibrous substrates were characterized by a circular shape deviating from normal morphology. Observations after 7 days of culture indicated that the correct morphology of fibroblasts was obtained the fastest for cells adhering to the PLA/HA composite material.

4. Discussion

Analyzing the parameters of the solution (Table 1), apparatus and environment, it was found that for systems containing a mixture of solvents in which the amount of DMF predominates (i.e., ratio of DCM: DMF 1 : 2,33 – PLA1 and PLA2) it is impossible to achieve a fibrous form, since secondary dissolution occurs and polymer foil is formed. A significant reduction in the amount of DMF relative to DCM (i.e., in PLA8) allowed to obtain heterogeneous fibers with a bimodal distribution of diameters (first max at 560 nm, second max at 1250 nm). In the solvent system with an equal ratio of DMF to DCM (PLA3, PLA4 and PLA9), the resulting fibers contained defects in the form of "beads". The same problem occurred for fibers produced in systems with a much higher proportion of DCM (PLA5, PLA6, PLA7, PLA10), and the fiber size distribution was more uniform, but still bimodal (e.g., for PLA5 first max at 200 nm, second max at 960 nm). In SEM, "beads" or stains, due to insufficient evaporation rate of the solvent, are observed (Fig. 2). The effect of the polymer concentration on fiber morphology is also visible in the pictures: increasing the concentration from 10 to 13%, while maintaining the ratio of solvents, resulted in obtaining fibers of a larger diameter (two fiber populations: at 260 nm and 450 nm for PLA 10%, compared to PLA 13% when the diameter of the fiber was characterized by the first maximum of 210 nm and the second maximum of 370 nm. In turn, increasing the concentration of the spinning solution to 15% of PLA resulted in the production of larger, more homogeneous and flat fibers with an average diameter of about 720 nm. It is known from the literature that PLA is a polymer that can be processed into a fibrous form using alloying methods (melt spinning), so the expected effect of raising the temperature in the chamber of the apparatus (36–45 °C) while reducing its humidity (up to 12%) was obtaining homogeneous fibers. Nonwovens obtained under such conditions (PLA11 and PLA12) did not have "beads" and were homogenous (about 560 nm for PLA11 and 780 nm for PLA12), and therefore for further studies on the persistence of PLA/DCM:DMF systems, these process and solution parameters as well as environmental conditions were selected, that allowed to obtain non-woven PLA11 and PLA12. The fiber size distribution in these two substrates was unimodal, while the

other nonwovens obtained by electrospinning were characterized by a bimodal fiber size distribution. The choice of the substrate with unimodal fiber distribution was aimed at limiting the influence of topography on the cellular response.

The concentration and solvent system for hyaluronic acid was developed in earlier works [4], [9], [16], and the proposed (three) HA-solvent systems guaranteed obtaining a fibrous microstructure. Analyzing the parameters of the solution and their effect on the morphologies of the obtained fiber substrates, it was found that the system allowing to obtain a fibrous homogeneous microstructure (unimodal fiber size distribution) is a mixture of solvents ammonia water (WAM) and ethyl alcohol (EA) in the 2:1 ratio. As in the case of PLA, also more stable process parameters in terms of polymer rheology were obtained when the ambient temperature was at the level of 45 °C and the humidity did not exceed 40%.

The predicted electrospinning conditions of PLA (PLA11 and PLA12) enabled us to obtain a support medium for the HA fibrous layer. Due to the greater stability of the solution and repeatable fiber diameters (780 nm), PLA12 was selected for further work. PLA 11 was still a type of reference, especially that the effect of fiber size on cell-material interaction was expected. The HA layer was applied onto the fibrous PLA layer (after changing the nozzle), and the electrospinning process of the HA layer was carried out for 15 minutes maintaining the pre-determined conditions (Figs. 3 and 4).

The carrier layer contains PLA typical bands (Fig. 5): a strong band originating from tensile vibrations of the carbonyl group (1730 cm^{-1}), a band of COC ester vibrations (1050–1300 cm^{-1}) and vibrations of methylene groups (2850 cm^{-1}, 2930 cm^{-1}). The surface layer is not homogeneous, having domain character that is visible on the spectrum set (Fig. 5), and in places where the HA layer is thicker there are only bands belonging to hyaluronan visible: 1° order amide band (1610 cm^{-1}), vibrating bands stretching OH (3500 cm^{-1}) and NH (3200–3500 cm^{-1}) [9], [24]. Confirmation of the domain character of the surface layer is the spectrum being a PLA/HA composition in which there are bands typical for both polymers. This surface condition can have a significant impact on the interaction of material with the biological environment [17].

The heterogeneous surface layer affects the physicochemical properties of the material, which are also an important parameter considered from the point of view of the cell's interaction with the material, since both the surface wettability and surface energy induce proliferation of fibroblasts and keratinocytes [15], [25]. The smaller the fiber diameters (topography on the nanometer scale), the more hydrophobic nature of the top layer. This is particularly evident in the case of PLA fibers building the support: the fiber size in the range of 500–600 nm has influenced the increase in the wetting angle. The increasing fiber diameter reduced the wettability to 93° for PLA and 83° for HA fibers, respectively. As can be seen from the data obtained, the secondary role for wettability values is played by the nature of the polymer, whereas the dominant parameter is the topography of the fibrous substrate. The synergistic effect of surface properties is, however, certainly important for cell-material interaction. The strongly hydrogel character of the HA layer is visible in the first days of incubation of the material with fibroblasts. The cells get stuck in the hydrogel, hence their low survival and difficulty in capturing their morphology on the material (Fig. 7). The number of cells is greater after 7 days, however, the hydrogel effect is still visible, the cells are trapped in a gelatinous suspension. From the literature data on cultures on hydrogel substrates as well as from our earlier works, it should be assumed that the biopolymer (HA) layer should be as thin as possible so that the concentration of hyaluronate on the support surface does not exceed 0.025 μg/ml [14]. While chemical mimicry is an important parameter in the first days of contact of cells with the medium, in long-term studies it is difficult to fully interpret the behavior of cells on the surface of the biomaterial.

5. Conclusions

The research on the selection and optimization of environmental conditions as well as equipment settings and physicochemical parameters allowed to receive non-woven PLA and HA. The obtained substrates were pre-characterized in terms of surface morphology and physicochemistry. It has been shown that depending on the conditions of electrospinning, the size of fibers and their distribution can be controlled. In the second part of the experiment, a material that combined the previously obtained material was prepared, where PLA was the support layer and HA the top layer. The described laminate was characterized by a hydrophobic-hydrophilic domain surface with a maintained fiber size of both layers (about 750 nm). The material positively underwent biocompatibility testing in contact with fibroblasts. The preliminary results obtained are a good starting point for

further research on PLA/HA material for skin regeneration (skin substitute).

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