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Evaluation of 3D hybrid microfiber/nanofiber scaffolds for bone tissue engineering

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Abstract. Fabrication of scaffolds for tissue engineering (TE) applications becomes a very important research topic in present days. The aim of the study was to create and evaluate a hybrid polymeric 3D scaffold consisted of nano and microfibers, which could be used for bone tissue engineering. Hybrid structures were fabricated using rapid prototyping (RP) and electrospinning (ES) methods. Electrospun nanofibrous mats were incorporated between the microfibrous layers produced by RP technology. The nanofibers were made of poly(L-lactid) and polycaprolactone was used to fabricate microfibers. The micro- and nanostructures of the hybrid scaffolds were examined using scanning electron microscopy (SEM). X-ray microtomographical (μCT) analysis and the mechanical testing of the porous hybrid structures were performed using SkyScan 1172 machine, equipped with a material testing stage. The scanning electron microscopy and micro-tomography analyses showed that obtained scaffolds are hybrid nanofibers/microfibers structures with high porosity and interconnected pores ranging from 10 to 500um. Although, connection between microfibrous layers and electrospun mats remained consistent under compression tests, addition of the nanofibrous mats affected the mechanical properties of the scaffold, particularly its elastic modulus. The results of the biocompatibility tests didn't show any cytotoxic effects and no fibroblast after contact with the scaffold showed any damage of the cell body, the cells had proper morphologies and showed good proliferation. Summarizing, using RP technology and electrospinning method it is possible to fabricate biocompatible scaffolds with controllable geometrical parameters and good mechanical properties.

Key words: rapid prototyping (RP), Electrospinning (ESP), Hybrid scaffolds.

1. Introduction

Most tissue engineering strategies used for replacement and regeneration of functional tissues or organs relay on the application of three-dimensional (3D) scaffolds, which can guide spreading and proliferation of seeded cells both in vitro and in vivo. An ideal scaffold should mimic natural Extracellular Matrix (ECM), it should have a proper porosity and pore size as well as good mechanical properties, which enable to provide a biologically functional implant site [1].

Currently there are several fabrication methods used for creation of 3D scaffolds with high porosity and interconnected pores. A rapid prototyping is one of the most interesting one and it allows for fabrication of scaffolds with predesigned external geometry and internal architecture, as well as desirable mechanical properties [2–4]. Using RP technology such as Solid Freeform Fabrications (SFF) it is possible to manufacture not only simple shapes but also patient-specific geometries [5–7]. Since scaffolds architectures has to fit the needs of individual patients. RP technologies meet these requirements by layer-by-layer construction, which allows for creation a well define shape, therefore making these technique so attractive for the fabrication of 3D-scaffolds [8]. Electrospinning is another method applied to form 3D nanostructures for regenerative medicine. ES structures are built from nanofibres, which makes them more similar to natural ECM [9, 10]. Electrospinning is a simple and versatile technique for fabrication of fibers from polymer solutions by application of high voltage [11–13]. However, both methods have their weaknesses. The scaffolds made by SFF methods consist mostly of strands that are too smooth and have large pores that form quite regular internal structure, which presents unfavourable conditions for initial cells attachment [14]. The electrospun scaffolds exhibit no problem with cell proliferation because of disordered porous/fibers system. This structure could be used in hybrid scaffold to improve cells attachement in first implant-medium contact [12, 14–16]. Combining RP and ES might allow for fabrication of a hybrid scaffold better mimicking structure and mechanical properties of natural tissue. There are several studies that indicate the high potential of utilising of such hybrid scaffolds for bone and cartilage regeneration [1, 4, 14, 17, 18]. Although, they present preliminary results for cell culture in vitro, the extensive characterization of the scaffolds structure and properties are omitted.

Among different kinds of biomaterials, polymeric materials such as polyesters have high potential to be used for

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scaffolds fabrication due to their good biocompatibility, controllable degradation, mechanical strength and good process ability. Polylactide (PLA), polyglycolide (PGA), polycaprolactone (PCL) and their copolymers have been extensively studied as materials for tissue engineering scaffolds [19]. They differ in physicochemical and mechanical properties as well as time of degradation. The PCL is hydrophobic polymer and degrades at a significantly slower rate than PGA and PLA. This makes the PCL a good candidate for long-term tissue engineering implants applications [14]. The degradation time of PCL is over two years, similarly for PLLA. Whereas PGA and PLDLA are completely absorbed after 6–12 and 12–16 months, respectively [20]. Thus, in long-term implants applications, the best solution is to combine of two biodegradable materials like PCL and PLLA.

The aim of the study was to characterise the properties of the 3D hybrid scaffolds consisted of alternating layers of micro-size strands and nanofibrous mats in order, to answer the question: what is the influence of combination of micro- and nanofibers on the structure and mechanical strength of the hybrid constructs. The properties of 3D scaffolds with and without nanofibers were compared. Additionally, the cytotoxicity test was performed to evaluate the influence of samples manufacturing processes on material biocompatibility. A hybrid technology combining a rapid prototyping method with electrospinning process was used to fabricate 3D scaffolds.

2. Experimental part

2.1. Materials. To fabricate microfibers the ε- polycaprolactone (PCL) from Sigma Aldrich (average Mn ca. 80 000) was used, whereas, poly(L-lactide) – Resomer®L207S (Beringer Ingelheim, average Mn ca. 66 000) was used to fabricate nanofibers.

2.2. Fabrication of microfibrous scaffolds. Cuboid scaffold ($10 times$ 10 mm, 5 mm height) with three-dimensional orthogonal periodic porous architectures were designed using Solid Works 3D CAD Design Software. The 3D geometrical scaffold model was exported to a Bioscaffolder®machine (SYS&ENG, Germany) as a STL file and then scaffolds fabrication was conducted using (SFF) rapid prototyping method [21].

The Bioscaffolder works like a high precision computer-controlled single screw extruder (Fig. 1a). The PCL granules were placed in a stainless steel reservoir and heated at $T = 90$°C through a heated cartridge unit. When the granules reached a molten phase a supply pressure of 6 bar was applied to the reservoir through a pressurized cap. Transferring viscous-liquid polymer to a turning single screw with spindle speed of 150 rpm. The fibers were plotted with deposition speed of 95 mm/min on a base plate by dispensing needle. The layer by layer printed microfiber scaffold was characterized by varying the fiber diameter (330 μm), the spacing between fibers in the same layer (420 μm), the layer thickness (240 μm) and the configuration of the deposited fibers within the whole structure (0°/90°/180°), (Fig. 1a).

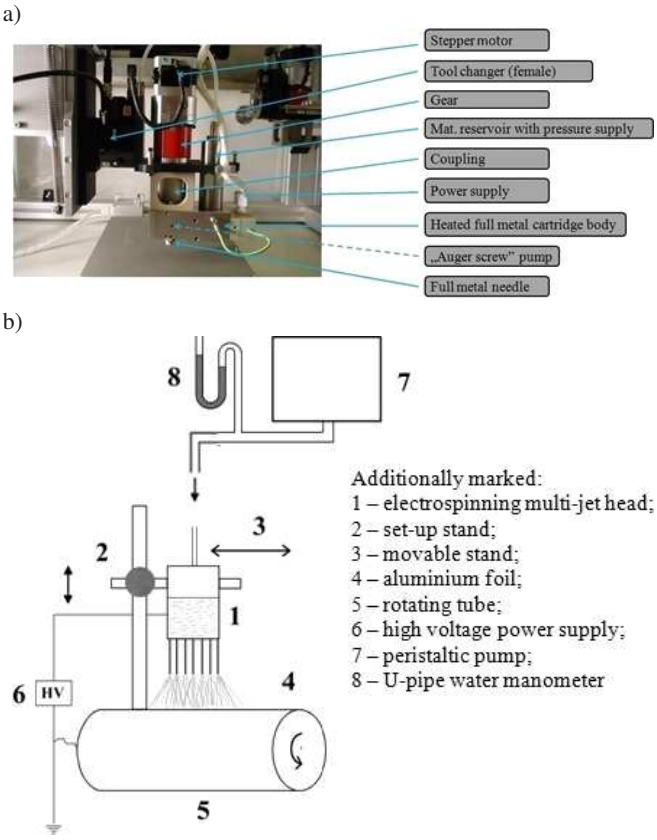

Fig. 1. 3D geometrical models of the hybrid scaffolds (a), hybrid scaffold consist of PCL microfibers (grey) and PLLA nanofibers (red) (b), example of hybrid scaffold (c)

2.3. Fabrication of nanofibrous mats. Nanofibers were fabricated using modified electrospinning method [16]. The electrospun solution was prepared by dissolving Resomer®L207S (Poly(L-lactide), PLLA, Beringer Ingelheim, average Mn ca. 66 000) in solvent mixture composed of chloroform and Dimethyl Sulfoxide (DMSO). The solvents were used in ratios of 90/10 wt.-% (chloroform/DMSO). The concentration of PLLA was 7 wt.-% in the solvent. The custom-made electrospinning system was used to fabricate nanofibrous mats (Fig. 1b), which was previously described by Tomaszewski et al. in [16]. The setup consisted of the grounded rotating collector and multijet spinneret (16 needles). The collector was made in the form of aluminium tube, which was covered with a sheet of thin aluminium foil. The spinneret was connected with 20 kV of applied voltage from a high voltage power supply ES50P-20W (Ormond Beach, USA). The collector rotated with velocity of about 10 rpm and simultaneously was moving forward and backward with typical velocity of 2 cm min^{-1}. The multijet spinneret was attached fixed at a distance of 15 cm from the collector. Fibers were electrospun at room temperature and further dried under vacuum (45 mb, 25°C) for 48 h.

2.4. Fabrication of hybrid scaffolds. During hybrid scaffold manufacturing, after every two layers of PCL microfibers

fabricated according to method described above, Bioscaffolder machine has been stopped for a while and pre-prepared nanofibrous PLLA mats with size of 8x8mm were manually incorporated. This procedure was repeated ten times during one sample fabrication (Fig. 2) [1, 17]. Finally, the hybrid scaffolds consisted of twenty two microfibers layers made of PCL and ten nanofibrous mats made of PLLA. It was assumed that the incorporation of mats every second layer allows for better cells seeding and growth.

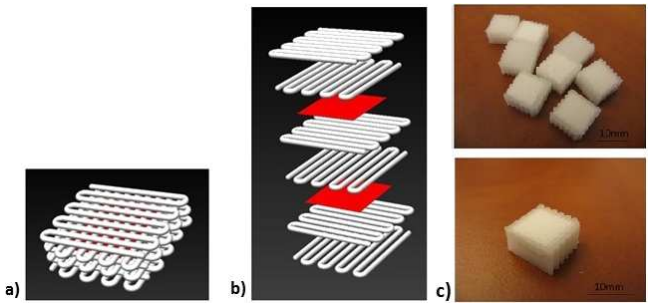

Fig. 2. Rapid prototyping machine (a), electrospinning set-up (b)

2.5. Scaffold characterization. The aim of the analysis was to visualize and evaluate the physical integrity of the PCL filaments and PLLA mats, as well as to understand if the previously defined pore geometry and size were maintained constant after fabrication process. Morphological analysis of the 3D scaffolds was carried out using two scanning electron microscopes, Hitachi SU8000 and Hitachi TM1000. To determine different fibers diameters, different resolutions was necessary so two different scanning electron microscopes were used. The microfibrous constructs were analyzed using SEM Hitachi TM1000, whereas Hitachi SU8000 was used to investigate connections between micro- and nanofibers in hybrid scaffolds.

X-ray microtomographical analysis and mechanical testing of the hybrid structures were performed using microcomputed tomography using SkySkan 1172 scanner. The scanner was set to a voltage of 59 kV and current of 167 μA. In order to mechanically test the samples in compression, a Material Testing Stage (MTS) was used in combination with the μ-CT scanner. The MTS was mounted in the chamber of μ-CT scanner between the source and camera. In the MTS, the lower clamp was moving downward, thereto compress the sample. The load-displacement characteristic was recorded at 10 samples per kind. In order to observe how samples behave at the different stages of compression, a step-wise loading mode was used and an intermediate μ-CT imaging of the samples was performed. The microfiber and hybrid scaffolds were scanned before and during compression loading. Resulting stress-strain curves were used to determine the Young Modulus (E) and the stress at 20% of compressive strain ($\sigma_{20\%}$). A scanning time of 14.5 minutes was used for each sample. An isotropic voxel size of 10μm was achieved in the reconstructed slices. Based on μCT scans 3D models of the scaffolds were generated. Scaffolds porosities were determined by image contrast of samples and the contrasting differences were converted in to the samples porosity using μCT software.

2.6. Cytotoxicity of the scaffolds. To determine whether biomaterials after processing can affect cells, the biomaterials were assayed for in vitro cytotoxic activities. The aim of this tests was to investigate the influence of manufacturing process – thermal extrusion and usage of solvent on scaffold cytotoxicity. Before cytotoxicity tests the samples have been sterilized by gamma radiation (25 kGy) in the air atmosphere, in a constant temperature of 30°C. Cell growth, cell morphology and cell viability were used as parameters to determine the cytotoxic effect of the materials. Investigation was realized in three times point: after 24 h, 48 h and 72 h of the cells contact with the samples. Cytotoxicity of the scaffolds was determined according to International Standard - ISO 10993-5:2009 (E) using the mouse cell line L929 (American Type Culture Collection Certified Cell Line-ATCC CCL1). This mouse fibroblast-like cell line was maintained in Eagle's medium (EMEM-minimum essential medium Eagle'a) supplemented with 10% c.s., antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and 2mM L-glutamine. For cytotoxicity test, the cells were seeded in the 24-well plates (Costar); 1 ml of 1x10^5 cells/ml in the culture medium Eagle'a with 2% calf serum, penicillin and streptomycin. Samples of the hybrid scaffolds (PCL microfibers + PLLA nanofibers) were added to prepared cells, which were then incubated for 24 h, 48 h and 72 h at 37°C in the atmosphere of 5% CO_2. Cell viability was determined on the basis of exclusion of Trypan Blue Staining. The cytotoxicity was defined as test of samples that causes 50% or greater destruction of cells. The cells in the in Eagle's medium served as control.

3. Results

The sizes of fabricated samples were approximately $9.8 \times 9.8 \times 5.4$ mm^3 for hybrid scaffolds and $9.8 \times 9.8 \times 5$ mm^3 for scaffolds without nanomats (Fig. 2). The SEM observations of the microfibrous RP scaffolds showed a well-defined internal geometry with square interconnected pores of dimensions in range of 300–500 μm, as well as uniform distribution of the pores. The extruded struts were mostly circular fibres with diameter of 300 μm, according to the nozzle tip used (330 μm) (Fig. 3a). They are slightly flattened when connected to each other.
The SEM analysis of the nanofibrous structure showed randomly oriented nanofibers with porous surface. The fibrous diameter was around 1μm and pore size ranged from 5 μm to 30 μm (Fig. 3b). The thickness of mats was about 200 μm.

The SEM images of the hybrid scaffold revealed that the nanofibrous mats were properly incorporated between microfibers layers and without delamination (Fig. 3c,d). It was also observed that some of the nanofibers were on the top of microfibers what was probably the result of transporting some of the ES fibers while struts depositing (Fig. 3c,d). This could improve cell attachment to the microfibers.

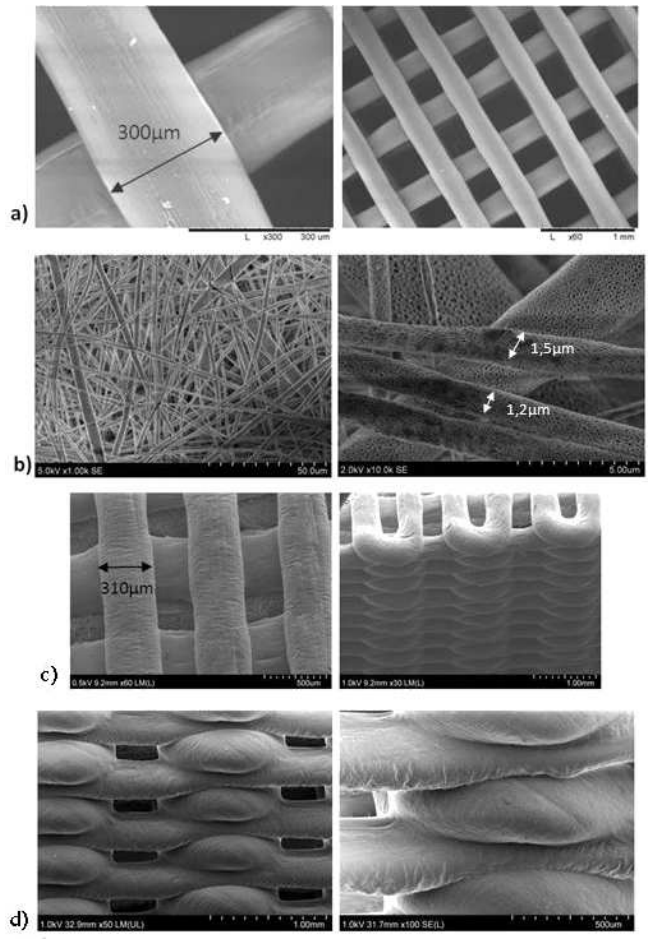

Fig. 3. SEM images, a) microfiber structure, b) nanofiber structure, c) hybrid scaffold top view, d) hybrid scaffold side view

3D computer model obtained based on μCT examination (Fig. 4) shows connection between micro and nanofibers, although spaces between two layers have been noticed (Fig. 4b). The compression test results have been analyzed to show the influence of presence of nanofibrous mats on mechanical properties of the hybrid scaffold (Table 1). The results revealed that the hybrid scaffolds had been less stiff than the scaffold without nanofibrous mats (Fig. 4a). For the scaffolds without mats, Young's Modulus was 25.2 MPa but for scaffolds with mats it was 5.4 MPa (Table 1). Comparing the obtained Young's Modulus to natural bone [22–24] more similar values were obtained for the scaffolds without mats. To obtain 20% of compressive strain the hybrid scaffold was compressed with 2.37 MPa stress, however the scaffold without nanofibers mats needed only 1.2 MPa of compressive stresses (Fig. 4a).

Table 1
Mechanical properties

Type of samples	E [MPa]	$\sigma_{20\%}$ [MPa]
Scaffolds	25.2±2	1.2
Scaffolds + Mats	5.4±0.4	2.37
Cortical bone	12.4–22 GPa	–
Cancellous bone	0.01–2 GPa	–

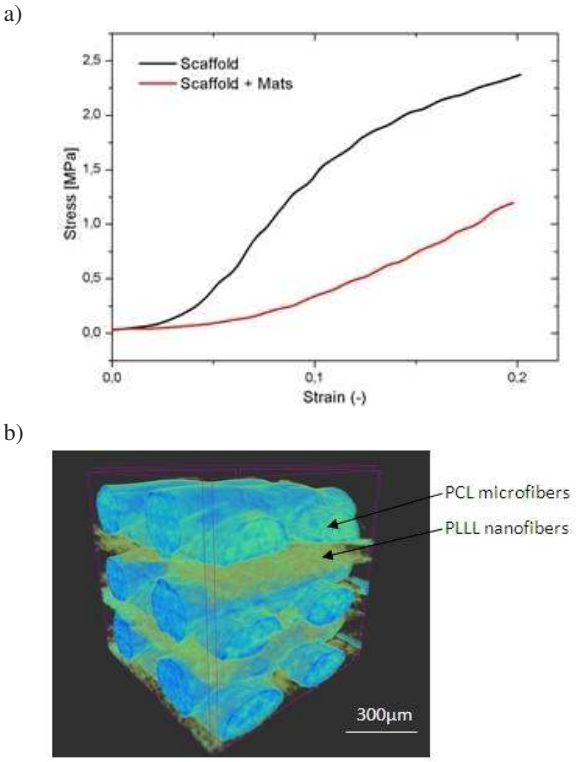

Fig. 4. Stress-strain curve obtained for a continuously loaded scaffold with and without mats (a), 3D computer model of the part of uncompressed hybrid scaffold (b)

The μCT images of the loaded scaffolds without nanofiber mats revealed that the deformation of the samples is accompanied by the change in their porosities from 58±1% to 54±1% (Fig. 5a,b). Applying load resulted in unfavourable relative sliding of the scaffold fibres and scaffold buckling. Pores diameters after compression tests were also slightly changed (Fig. 5a,b).

In case of the samples containing mats, no buckling after compression test was observed (Fig. 5c,d). However, adding nanomats resulted witch struts flattening. The shape of the struts cross-sections were changed from round to elliptical due placement of mats (Fig. 5c). The applied compressive load didn't cause more significant changes in the shape of individual fibres or whole structure (Fig. 5c,d). However, the porosity of microfibrous scaffold decreased from 60% to 55% (Fig. 5c,d). Connection between microfibrous layers interleaved with electrospinning mats remained consisted. Delimitation was not noticed.

The result of cytotoxicity tests using fibroblast cultures L929 didn't show any cytotoxic effects of the fabrication processes. A few dead cells were found after 24, 48 and 72 h, either in the control or in the contact with materials. No agglutination, vacuolization, separation from the medium or cell membrane lyses were observed. In cultures which had contact with the investigated material samples, single rounded cells were noted at all observation time points (Fig. 6). Proliferation of the cells in the control and the test cultures was normal, and the cells formed colonies on the whole surface of the plates. These results confirmed no cytotoxic effect of mate-

rial after heat treatment of PCL and after usage of solvent during PLLA nanofibers production. For the all investigated time points toxicity degree were still 0.

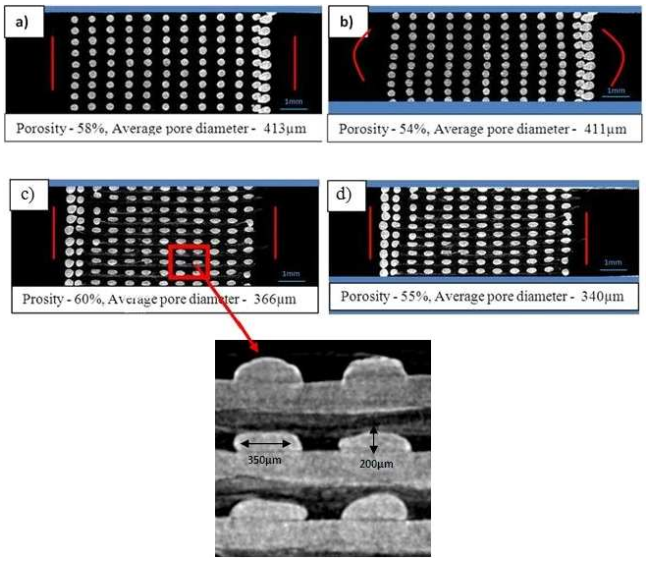

Fig. 5. CT images of the scaffolds; Without mats before (a) and after (b) compression tests; Hybrid scaffolds before (c) and after (d) compression tests

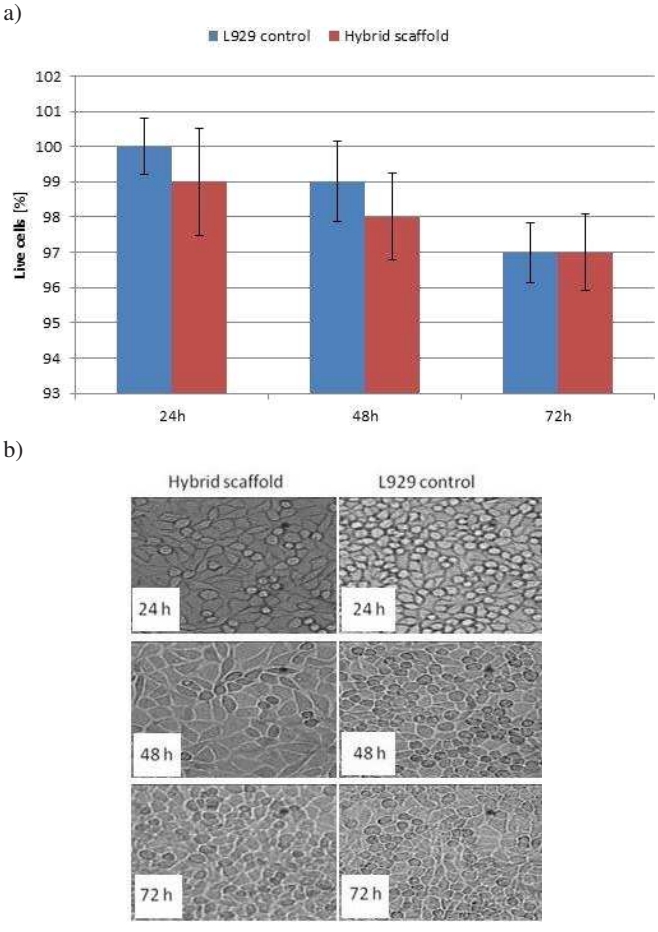

Fig. 6. Living cells changes (a) and morphology of the cells (b) after 24 h, 48 h and 72 h of incubation

4. Discussion

The mechanical properties of implants for bone tissue regeneration are very important. Application of polymeric scaffold as an implant requires designing of appropriate mechanical properties of the constructs similar to these exhibited by natural bone. The initial goal of hybrid micro/nanofibrous scaffolds was to add nanofibrous mats to the scaffolds, which would make them more similar to natural ECM. Authors hypothesis that application of mats with smaller pores (5 μm to 30 μm) than pores in microlayers of the 3D microfibrous scaffolds (300-500μm) can result in better cell adhesion and consequently better cell proliferation. The main goal of this work was to evaluate mechanical behaviour of samples after incorporation of nanofibrous meshes. Nanofibrous mats were added to support tissue in growth as well as improve cell attachment and proliferation to increase scaffold's functionality related to surface affinity and an additional surface area [13]. Our studies was focused mainly on the characterization of the samples manufacturing and investigation of mechanical properties of obtained structures. In a literature we can find the confirmation of the influence of fiber's diameter on improvement of cells proliferation and differentiation [13, 25–27]. Therefore, for the implementation of this work, we focused on mechanical behaviour of the scaffolds with two diameters of the fibers. The results of cytotoxicity studies demonstrated that after hybrid scaffolds manufacturing biomaterials didn't cause any damage in cell culture and the cells had proper morphologies and the level of proliferation was stable similar to positive control, which confirmed no cytotoxic effect of the material. On the other hand, mechanical properties have to be more extensively investigated to precisely evaluate the influence of incorporation of nanofibrous mats within 3D constructs. This study showed that the addition of the nanomats to the microlayers scaffolds significantly decreased compression strength of the scaffolds. Moreover, the Young's Modulus of the scaffolds with nanomats was significantly lower than for the scaffolds without nanomats. The μCT images of the hybrid scaffolds had shown spaces between nanomats and microlayers, which might result in decrease of Young's Modulus. However, all of the investigated structures had similar Young's Modulus to the natural bone [22, 23]. Moreover, the mechanical test demonstrated that addition of nanofibrous mats decreased mechanical properties of the scaffolds.

Additionally, flattened microfibers have been observed as a consequence of addition of nanomats on a top of warm and still viscous microfiber layers.

An automation manufacturing process with the use of the additional heating chamber may yield with more promising results in terms of obtaining a hybrid 3D micro/nanostructures. Follow up studies are undergoing aiming at examination of layers connection and the effect of addition of nanomats.

5. Conclusions

Combination of solid freeform fabrication and electrospinning process was utilised in this study in order to obtain

nanofiber/microfiber hybrid scaffolds. The hybrid scaffolds had a complex internal architecture with high porosity and interconnected pores ranging from 5 to 500 μm. It was observed that addition of the nanofibrous mats to microfibrous scaffolds reduced their stiffness and strength. The connection between the microfiber layers interleaved by electrospun mats remained consistent under compression. This combined hybrid process seems to be feasible technique for fabricating high quality 3D hybrid scaffolds with an open porous network and controllable geometry. However, the further studies on increase of the mechanical strength of the scaffolds, an increase of pore size to 10 μm within nanofibrous mats should be performed.

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