Elastic three-dimensional poly (ϵ -caprolactone) nanofibre scaffold enhances migration, proliferation and osteogenic differentiation of mesenchymal stem cells

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Abstract

Objectives: We prepared 3D poly (ϵ -caprolactone) (PCL) nanofibre scaffolds and tested their use for seeding, proliferation, differentiation and migration of mesenchymal stem cell (MSCs).

Materials and methods: 3D nanofibres were prepared using a special collector for common electrospinning; simultaneously, a 2D PCL nanofibre layer was prepared using a classic plain collector. Both scaffolds were seeded with MSCs and biologically tested. MSC adhesion, migration, proliferation and osteogenic differentiation were investigated.

Results: The 3D PCL scaffold was characterized by having better biomechanical properties, namely greater elasticity and resistance against stress and strain, thus this scaffold will be able to find broad applications in tissue engineering. Clearly, while nanofibre layers of the 2D scaffold prevented MSCs from migrating through the conformation, cells infiltrated freely through the 3D scaffold. MSC adhesion to the 3D nanofibre PCL layer was also statistically more common than to the 2D scaffold ($P < 0.05$), and proliferation and viability of MSCs 2 or 3 weeks post-seeding, were also greater

on the 3D scaffold. In addition, the 3D PCL scaffold was also characterized by displaying enhanced MSC osteogenic differentiation.

Conclusions: We draw the conclusion that all positive effects observed using the 3D PCL nanofibre scaffold are related to the larger fibre surface area available to the cells. Thus, the proposed 3D structure of the nanofibre layer will find a wide array of applications in tissue engineering and regenerative medicine.

Introduction

The goal of regenerative medicine is to replace damaged functional tissues or an organ with engineered material (1, 2) that will ultimately support, then become, a living surrogate. Scaffolds that support cell adhesion, proliferation and migration are needed, and hence, substances have been developed and tested for this purpose. Novel materials characterized by superb cell biocompatibility and suitable biomechanical parameters are in high demand in tissue engineering. In addition, the surrogate must allow ample supply of nutrition and adequate waste removal – this is vital for cell accommodation, proliferation, and/or differentiation. Consequently, appropriate scaffold structure with sufficient pore size for inherent circulation is essential for preparation of artificial tissues. Notably, optimal pore size requirement differs significantly between different size and type of cell. Pore sizes $>300\ \mu\text{m}$ have been recommended for vascularization in bone tissue

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engineering (3), whereas fibroblasts have been demonstrated to prefer a pore size of 6–20 μm (4).

Several approaches have been reported for preparation of scaffolds of definitive pore size. Systems combining insoluble fibres and sacrificial (temporary) co-fibres have been reported (5, 6). Pores can be introduced into a solid support by salt particles that can subsequently be leached out (7), ice crystals (8), or photopatterning (9). After removal of sacrificial components, voids are introduced, and the appropriate scaffold pore size is obtained. Scaffolds based on fibrous structures are typified by favourable structural properties by several aspects, electrospun nanofibres, for example, being characterized by high porosity and an abundance of interconnected pores. Diameter of nanofibres is similar to that of natural fibres of the extracellular matrix (10, 11). In addition, nanofibre scaffolds exhibit high surface-to-volume ratio, facilitating cell adhesion and proliferation (12–15); due to this unique property, nanofibre scaffolds offer numerous contact points for cells.

Importantly, pore size of electrospun fibre mesh strongly correlates with diameter and orientation of fibres (16, 17); pore size usually decreases as diameter of fibres is reduced. Thus, preparation of finest fibre scaffolds is difficult and also unquestionably cell-specific. Ideal electrospun scaffolds should have nano- or microscale fibres with macroscale pores (10–500 μm) (18). These parameters permit population growth and differentiation of human mesenchymal stem cells (MSCs) towards osteoblasts (19, 20), amongst other things. Major limitations of a broader application of traditional electrospun scaffolds (for example, 2D fibrous scaffolds) remain their tightly packed layers, clearly, this hinders stem cell motion. Although we have recently demonstrated unaffected diffusion of molecules of up to 10 kDa in nanofibre meshes (21), structure of 2D fibrous mesh enables only superficial penetration of cultured cells (19, 22–24). Cell penetration into 3D scaffolds has been expected to be significantly enhanced.

Vaquette and Cooper-White prepared 3D structured nanofibre layers using several patterned collectors (25). Pore size enlargement and heterogeneous 3D distribution of pores in the structure enabled deeper cell infiltration into the scaffold compared to that observed in conventional electrospun layers. Milleret *et al.* prepared porous scaffolds from poly(lactic-co-glycolic acid) and the polyurethane Degrapol®, using a co-fibre method (26). Increased porosity enhanced infiltration of seeded 3T3 fibroblasts, but no difference in cell proliferation was observed. 3D nanofibre structure in the form of a cotton ball-like scaffold has been prepared using ground spherical dishes and arrays of needle-like probes (18). The structure produced consisted of accumulated low-density nanofibres in an

uncompressed manner, which increased space for cell ingrowth, and resulted in higher rate of cell proliferation.

In our present study, we attempted to prepare 3D fibre scaffolds using a novel patterned cylindrical collector. We examined scaffold biomechanical and stereological properties and focused on effects of 3D fibre structure on adhesion, proliferation and differentiation of MSCs in the presence of osteogenic medium, and compared the findings to those observed using 2D scaffolds. Poly (ϵ -caprolactone) (PCL) was chosen as the model polymer for this study. PCL is a biocompatible and Food and Drug Administration-approved material for biomedical applications. In addition, nanofibres from PCL have commonly been used to facilitate proliferation of MSCs (27–29).

Materials and methods

Scaffold preparation

2D PCL fibre materials (PCL 2D) were prepared using an electrospinning method. Molecular weight of PCL produced by Sigma Aldrich is 45 000 Da. electrospinning was performed using 14wt% PCL solution dissolved in chloroform:ethanol at a ratio of 9:1. A high-voltage source, Spellman SL 150, generates up to 50 kV, and polymer solution on the spinning electrode was connected to the positive pole of the high-voltage source. Electrospun fibres were deposited on a ground collecting electrode (rotating drum collector, approximately 60 rpm). Nanofibres were stored in a desiccator until used.

3D PCL fibre materials (PCL 3D) were prepared from PCL with the same molecular weight as those of PCL 2D. Polymer solution concentration was 14wt%, chloroform:ethanol at a ratio of 9:1 employed as solvent. The electrospinning setup is depicted in Fig. 1.

Two high-voltage sources were used to generate positive and negative potentials. The positive source was connected to a syringe needle, whereas the negative source was connected to a specific collector (described in the Results section, below); polymer pump 'KDS-100-CE' was used to feed the polymer solution. Voltage applied to the syringe needle was +46.5 kV, whereas voltage of –5 kV was applied to the collector. Distance between syringe needle and collector was 120 mm. Environmental conditions during the experiments were temperature 19.8 °C and relative humidity of 20%; processing time was 2 h for preparing compact 3D layers.

Scaffold characterization

Fibre layers prepared were visualized using an optical microscope (Olympus IX51, Tokyo, Japan) and also by

Figure 1. Electrospinning setup for the production of 3D nanofibres and macroscopic visualization of the prepared layers. Electrospinning setup for the laboratory production of PCL 3D (a) 1, high-voltage source; 2, high-voltage source; 3, feeding pump; 4, syringe with polymer; 5, nanofibres; 6, special collector; 7, frame for adjustment distances; and 8, electromotor. (b) The special collector is shown. The nanofibrous PCL 3D layer created by a special collector has a knitted-like structure (c and d)

scanning electron microscopy (SEM, Phenome G2; FEI, Eindhoven, The Netherlands). To quantify and describe fibre structures in detail, SEM photomicrographs were used and analysed using NIS Elements 3.0 software for image analysis. This software enables measurements of pore size, surface area and fibre diameter.

X-ray micro-tomography

X-ray micro-tomography is a non-destructive technique based on properties of absorption of X-rays by the scanned material. X-rays are absorbed by nuclei of atoms, and higher the atomic number, greater the X-ray absorption. The sample is rotating during scanning, while beams of transmitted X-rays are collected by the detector; so-called transmission images are generated at different angles of rotation. From these images, 3D structure of the scanned object is finally computed. Our micro-tomograph is capable of scanning with voxel size as small as 200 nm, depending on sample size and material. The general rule for obtaining best resolution is for the sample to be less than 500 times the resolution. Presented images were scanned on a Xradia XCT 400 at very low energy, 20 kV.

Profilometry

Profiles of 2D and 3D nanofibre layers was determined using an Alpha-step IQ mechanical profilometer (KLA Tencor, USA) with a diamond tip (Stylus, radius 10 μm , full angle 45°). Pressure force was 80 μN , sampling frequency 10 Hz and scan speed was 20 $\mu\text{m/s}$. Scanning scale was 550 μm (accuracy at the z-axis was

0.04 μm); average values were determined from 5 independent measurements.

Differential scanning calorimetry

Calorimetric measurements were performed using a DSC8500 apparatus (Perkin–Elmer). Purge gas (nitrogen, Waltham, MA, USA) passed through the DSC cell at flow rate of 20 ml/min and temperature of the equipment was calibrated using water, gallium and indium – the melting point of indium was used for calibrating heat flow. Samples with mass of approximately 6 mg were placed in pressure-tight aluminium pans and subjected to heating scan from 20 °C to 150 °C at 10 °C/min.

Melting temperatures (T_m) were determined from positions of the melting peaks. Degree of crystallinity (X_c) of the PCL nanofibres was calculated from melting enthalpy of PCL (ΔH_m), determined from area below the melting peak in the DSC thermogram, as

$$X_c = \Delta H_m / \Delta H_{\text{PCL}},$$

where ΔH_{PCL} is the melting enthalpy of fully crystalline PCL ($\Delta H_{\text{PCL}} = 139.5 \text{ J/g}$) (30).

Biomechanical testing

Twelve specimens of PCL 2D and 9 specimens of PCL 3D were loaded for tensile testing using Zwick/Roel traction apparatus equipped with a 1kN load cell, to obtain mechanical parameters, namely Young's moduli of elasticity, ultimate stresses and ultimate strains. Specimens

mens were thin strips of composite with 5 mm paper grips, prepared according to previous studies (31, 32). Initial length of all specimens was 10 mm of width also 10 mm. Thickness of individual specimens was measured using a digital caliper, and the values ranged from 90 μm up to 220 μm . Thickness of 3D PCL was measured at the thinnest region of specimens. Composites were loaded at loading velocity of 10 mm/min until the sheet of composite ruptured, according to earlier studies (31, 32). Young's moduli of elasticity were determined using linear regression analysis of stress-strain curves at strain of approximately 1–6% (depending on shape of the curve). Ultimate stresses and ultimate strains were determined at initiation of the rupture. Stress was defined as force divided by initial area, and strain was defined as elongation of the specimen divided by its initial length. Our own software written in Python, an Open Source object-oriented programming language, was used for the evaluation.

Isolation and culture of MSCs

Bone marrow aspirates were obtained from os ilia of anaesthetized miniature pigs (age 6–12 months, Institute of Animal Physiology and Genetics of the ASCR, Libechov, CZ). Animal care was in compliance with the Act of the Czech National Convention for Protection of Vertebrate Animals. Bone marrow was aspirated into 10-ml syringes with 5 ml Dulbecco's phosphate-buffered saline (PBS) with 2% foetal bovine serum (FBS, StemCell Technologies) and 25 IU heparin/ml connected to a bioptic needle (15G/70mm). Under sterile conditions, bone marrow (approximately 20 ml) was introduced into 50-ml centrifuge tubes, and 5 ml of Gelofusine (B. Braun Melsungen, Germany) was added. After 30 min incubation at room temperature, samples were centrifuged at 400 g for 15 min. Thus, the layer of mononuclear cells was removed and seeded into culture flasks and cultured at 37 °C in humidified atmosphere of 5% CO₂. α -minimum essential medium (MEM) with Earle's salt and L-glutamine supplemented with 10% FBS and penicillin/streptomycin (100 IU/ml and 100 $\mu\text{g}/\text{ml}$, respectively) was used as culture medium.

MSC seeding on scaffolds

Scaffolds 6 mm diameter and 2 mm thickness, were sterilized using low-temperature hydrogen peroxide gas plasma (Sterrad 100 S sterilizer, ASP, USA). Cells were seeded on scaffolds at $90 \times 10^3/\text{cm}^2$ density, in a 96-well plate, corresponding approximately to 25×10^3 cells/scaffold. Scaffolds with seeded MSCs were cultured in 250 μl differentiation medium per well

(α -MEM supplemented with 10% FBS and penicillin/streptomycin (100 IU/ml and 100 $\mu\text{g}/\text{ml}$, respectively), 100 nM dexamethasone, 40 $\mu\text{g}/\text{ml}$ ascorbic acid 2-phosphate, and 10 nM glycerol 2-phosphate disodium salt hydrate). Medium was changed every 3 days.

MSC penetration through nanofibre layers

Penetration of MSCs through 2D and 3D nanofibre layers was investigated. Sheets of nanofibres were cut and fixed in 24-well-plate inserts (Scaffdex, Finland). Bottoms of 24-well plates were covered by microscope glass, and inserts with nanofibres were placed in the wells. Cells at density of $90 \times 10^3/\text{cm}^2$ were seeded on nanofibre layers inside the inserts to prevent leakage of cells into surrounding areas. On days 1, 4, and 7, MSCs were visualized using fluorescence staining. Inserts with fibres were removed, and fibre sheets were excised. Samples and glass from bottoms of 24-well plates were rinsed in PBS (pH 7.4), fixed in frozen methyl alcohol (–20 °C) for 10 min, and rinsed in PBS. Subsequently, fluorescent probe 3,3'-diethyloxacarbocyanine iodide (DiOC6; 0.1–1 $\mu\text{g}/\text{ml}$ in PBS; pH 7.4) was added and incubated with the samples for 45 min at room temperature. Samples were rinsed in PBS, and propidium iodide (PI; 5 $\mu\text{g}/\text{ml}$ in PBS) was added for 10 min, followed by rinsing in PBS and visualization using a ZEISS LSM 5 DUO confocal microscope (PI: λ_{exc} = 561 nm, λ_{em} = 630–700 nm; DiOC6: λ_{exc} = 488 nm, λ_{em} = 505–550 nm).

Cell adhesion on scaffolds

DiOC6 staining was used to detect cell adhesion to scaffolds. Samples were fixed in frozen methyl alcohol (–20 °C) for 10 min and stained with DiOC6 and PI as described above. Specimens were visualized using the ZEISS LSM 5 DUO confocal microscope. Areas of adherent cells were counted using Ellipse software.

Cell viability analysis

Metabolic activity of cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Fifty microlitres of MTT (1 mg/ml in PBS, pH 7.4) was added to 150 μl of sample medium and incubated for 4 h at 37 °C. Then, 100 μl of 50% N, N-dimethylformamide in 20% sodium dodecyl sulphate (pH 4.7) was added. Results were examined using spectrophotometry in an ELISA reader at 570 nm (reference wavelength, 690 nm).

For detecting cell viability, live/dead staining (2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluoresceinacetoxymeth-

yl ester (BCECF-AM)/propidium iodide) and visualization using confocal microscopy were performed. BCECF-AM (diluted 1:100 in medium) was added, and cells were incubated for 45 min at 37 °C in atmosphere of 5% CO₂ for live cell detection. Cells were then rinsed in PBS (pH 7.4), and PI (5 µg/ml in PBS) was added for 10 min, after which cells were rinsed in PBS and visualized using the Zeiss LSM 5 DUO confocal microscope (PI: λ_{exc} = 561 nm, λ_{em} = 630–700 nm; BCECF-AM: λ_{exc} = 488 nm, λ_{em} = 505–550 nm).

Cell proliferation analysis using PicoGreen

The PicoGreen assay was performed using the PicoGreen assay kit (Invitrogen Ltd., Paisley, UK) and proliferation of MSCs on scaffolds was tested on days 1, 7, 14, and 21. To process material for analysis of DNA content, 250 µl of cell lysis solution [0.2% v/v Triton X-100, 10 mM Tris (pH 7.0), and 1 mM EDTA] was added to each well containing a scaffold sample. To prepare cell lysate, samples were processed through 3 freeze/thaw cycles; the scaffold sample was first frozen at –70 °C and thawed at room temperature. Between each freeze/thaw cycle, scaffolds were roughly vortexed. Prepared samples were stored at –70 °C until analysis. To quantify cell number on scaffolds, a cell-based standard curve was prepared using samples with known cell numbers (range, 1×10^2 – 1×10^6 cells). DNA content was determined by mixing 100 µl of PicoGreen reagent with 100 µl DNA sample. Specimens were loaded in triplicate, and fluorescence intensity was measured on a multiplate fluorescence reader (Synergy HT, λ_{ex} = 480–500 nm, λ_{em} = 520–540 nm).

Quantitative real-time polymerase chain reaction (PCR) analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufac-

turer's protocol. This kit is based on technology that combines selective binding properties of silica gel-based membranes with the speed of microspin technology. At the end of the procedure, total RNA was stored at –20 °C.

cDNA from 1 µg total RNA was used as template. Synthesis of cDNA was performed using a standard procedure described in our previous work (33). Osteocalcin (OC) and bone sialoprotein (BS) mRNA expression levels were quantified by means of LightCycler 480 (Roche Diagnostics, Mannheim, Germany) using doublestrand-specific dye, SYBR Green I (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. The complete list of primer sets, including their sequences and annealing temperatures, is presented in Table 1.

PCR conditions were initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 54 °C for 10 s, and extension at 72 °C for 20 s. Expression levels of OC and BS mRNA were adjusted, using level of beta-actin mRNA as housekeeping gene, and were expressed as ratio to beta-actin. Evaluation of mRNA expression of OC and BS was performed by quantitative real-time PCR analysis ($P < 0.05$, 2-sided t -test).

Detection of osteogenic markers using indirect immunofluorescence staining

Presence of OC, as marker of osteogenic differentiation, was confirmed using indirect immunofluorescence staining as described previously (34). In brief, samples were fixed in 10% formaldehyde/PBS for 10 min and incubated with the primary antibody against OC (mouse anti-OC, diluted 1:20, Abcam, US) for 1 h at room temperature. Then, secondary antibody (Alexa Fluor 635-conjugated Goat Anti-Mouse IgG (H+L), Invitrogen) was diluted 1:300 and added for 45 min at room temperature. OC staining was visualized using the ZEISS LSM 5 DUO confocal microscope.

Table 1. List of primers used for real-time PCR analysis

Gene	Primer	Sequence (5' to 3')	T _a (°C)	PCR product (in bp)
Beta-actin	Sense	AGG CCA ACC GCG AGA AGA TGA CC	53	332
Beta-actin	Antisense	GAA GTC CAG GGC GAC GTA GCA C	53	332
OC	Sense	TCA ACC CCG ACT GCG ACG AG	67	204
OC	Antisense	TTG GAG CAG CTG GGA TGA TGG	67	204
BS	Sense	CGA CCA AGA GAG TGT CAC	57	498
BS	Antisense	GCC CAT TTC TTG TAG AAG C	57	498

Each primer was chosen to span introns. The specific annealing temperature (T_a) of each primer and the size of the expected PCR products are listed below.

Figure 2. Visualization of nanofibrous PCL 2D and 3D layers. Optical microscopy (a,b,c) and SEM (d,e,f) were used for visualization of PCL 3D (a,b,d,e) and 2D (c,f) layers. 3D structure of PCL 3D layer is good visible (a,d) as well as bigger porosity in thin areas of PCL 3D layer (b,e).

Statistical analysis

Quantitative data are presented as mean $\pm$ standard deviation (SD). For *in vitro* tests, average values were determined from at least 3 independently prepared samples. Results were evaluated statistically using 1-way analysis of variance and Student-Newman-Keuls test. Normality of Young's moduli of elasticity, ultimate stresses and ultimate strains was tested using the Shapiro-Wilk W test. Differences in mechanical parameters between PCL 2D and PCL 3D specimens were determined using the Mann-Whitney U test. For both tests, Statistica base 9.1 (Statsoft, Tulsa, OK, USA) was used.

Results

Novel collector and 3D scaffold characterization

The 3D nanofibre scaffold was prepared using a classic electrospinning setup (Fig. 1a) but with a novel special collector. The characteristic feature of the collector was that it was fitted with a rotating drum with a structured surface; a metal chain was employed for surface structure modification (Fig. 1b). The fibre scaffold was formed on the conductive metal strips of the rotating cylinder, and the pattern of the scaffold produced corre-

sponded to the structure of the collector surface (Fig. 1c). Thus, the newly formed scaffold was characterized with spots of different mass per unit area (weight of polymer per scaffold area), resembling a knitted structure (Fig. 1d). Clearly, the metal chain ensured formation of a 3D fibre scaffold characterized by unique properties and parameters.

To compare the novel 3D nanofibre scaffold with the classic 2D nanofibre scaffold, structures were examined using optical microscopy (Fig. 2a–c) and SEM (Fig. 2d–f). Optical microscopy identified that the 3D fibrous mesh consisted of more dense and less dense areas; morphology of 2D PCL was more homogenous. Ultrastructure of the fibres was visualized using SEM. Clearly, pore size differed between 2D and 3D samples. Visual analysis revealed that pores of 3D PCL were larger than those of 2D PCL. To quantify observed fibre arrangements, SEM photomicrographs were analysed using NIS Elements 3.0 software. Image analysis data of these PCL samples revealed a significant increase in average pore size area from $5 \mu\text{m}^2$ for 2D PCL to $10 \mu\text{m}^2$ for 3D PCL. However, within these average values were included significant contributions of very tiny pores of no importance for cell migration. Interestingly, maximum pore size increased from $20\text{--}30 \mu\text{m}^2$ for 2D scaffolds to more than $60 \mu\text{m}^2$ for 3D scaffolds. Nota-

bly, scaffold mass per unit area was identical for both types of sample.

Fibre diameters were similar in both 2D and 3D samples; average fibre diameter was $3.53 \pm 1.48 \mu\text{m}$ for 2D scaffolds and $2.91 \pm 0.91 \mu\text{m}$ for 3D fibres. For detailed analysis, fibres were separated into microfibrils ($\leq 1 \mu\text{m}$) and nanofibrils ($>1 \mu\text{m}$). Mean diameter of microfibrils in 2D mesh was $5.15 \pm 2.55 \mu\text{m}$, whereas nanofibrils had average diameter of $0.24 \pm 0.12 \mu\text{m}$. 3D nanofibrils had a mean diameter less than of respective 2D microfibrils ($2.7 \pm 1.2 \mu\text{m}$) and nanofibrils ($0.14 \pm 0.21 \mu\text{m}$). Interestingly, when ratios between microfibrils and nanofibrils were estimated, 3D meshes had significantly more microfibrils ($66.5\% \pm 8.8\%$). In contrast, 2D scaffolds contained more nanofibrils, and proportion of microfibrils was just $36.9\% \pm 8.3\%$. From the data presented, it is apparent that our novel technique results in a more homogenous fibre mesh, despite being composed of more microfibrils.

Contact profilometry was used to measure samples' surfaces. Surfaces of 2D and 3D PCLs were scanned, and z-axis profiles were obtained. While z-axis profile of 2D PCL nanofibrils peaked at $70 \mu\text{m}$ (Fig. 3a), z-axis profile of 3D PCL revealed two distinct peaks with heights

around $240 \mu\text{m}$ (Fig. 3b). The peaks corresponded to dense components of the knitted-type structure. Results confirmed 3D nature of the prepared patterned scaffolds.

Additionally, three dimensional morphology of 3D PCL was also confirmed by X-ray micro-tomography. While in the case of 2D PCL, samples were predominantly planar (Fig. 4a), tomography of 3D PCL indicated elevation of structure in the dense areas (Fig 4b). Additionally, larger pore size of the 3D PCL mesh was clearly visible from the tomograph. Results support the observation that 3D PCL nanofibrils consist of less dense zones with higher porosity and more dense areas with tightly condensed nanofibrils.

Thermal properties of the 2D and 3D scaffolds were analysed using differential scanning calorimetry. Figure 5 (d) presents DSC thermograms of 2D PCL and 3D PCL nanofibrils. Melting point (T_m) of 2D PCL was 59.89°C , melting enthalpy (ΔH_m) being 88.66 J/g and degree of crystallinity (X_c) at 62.12% . Thermal properties of 3D PCL were almost identical to those obtained for 2D PCL: $T_m = 59.89^\circ\text{C}$, $\Delta H_m = 86.66 \text{ J/g}$ and $X_c = 62.12\%$. Results indicated similar thermal properties and identical physicochemical properties of 2D and 3D samples to each other.

Figure 3. Surface topography of PCL 2D and 3D layers measured by contact profilometry. The heights of profilometer peaks of PCL 2D were not higher than $70 \mu\text{m}$ (b). On PCL 3D reached peaks almost $240 \mu\text{m}$ (a).

Figure 4. X-ray micro-tomography of PCL 2D and 3D layers. Micro-CT was used to visualize the structure of PCL 2D (a) and PCL 3D (b) samples. Difference in porosity on PCL 3D sample is clearly visible. The object in the bottom of image (a) is a part of sample fixation.

Figure 5. The mechanical and thermal properties of PCL 2D and 3D layers. No difference was shown in thermal properties of both samples (a). Significant differences between groups were found in the Young's moduli of elasticity (b) and ultimate stresses (c) (determined by Mann–Whitney *U*-test, $P < 0.001$). No significant difference was found in the ultimate strains (d) ($P = 0.24$).

To examine samples' mechanical properties, dynamic mechanical testing was performed. Results of these measurements demonstrated that in the case of 2D PCL, ruptures originated at a single point, typically in the middle of a specimen, and a single rupture line propagated perpendicularly to loading, while in the case of 3D PCL, ruptures originated in multiple locations; these rupture points occurred in thinner parts of the specimens. Dense locations held specimens together while thinner zones ruptured; with increasing load, dense parts also tore. Moreover, it was obvious that specimens with higher amounts of dense structures were stiffer than those with lower quantities of such structures.

Resultant Young's moduli of elasticity, ultimate stresses and ultimate strains are shown in Fig. 5. Mann–Whitney *U* testing showed that Young's modulus of elasticity of 2D PCL was significantly lower than that of 3D PCL ($P < 0.001$) (Fig. 5b). Ultimate stress was also significantly lower in the case of 2D PCL when compared to 3D PCL ($P < 0.001$) (Fig. 5c). No significant differences were observed for ultimate strain between

2D PCL and 3D PCL specimens ($P = 0.24$) (Fig. 5d). Obtained data clearly identified that 3D samples were more elastic and were firmer, despite having identical chemical composition to 2D samples.

3D PCL nanofibre scaffold facilitated MSC penetration through nanofibre layers

Undamaged 2D PCL scaffolds have been demonstrated (in our laboratory and others) to be tightly sealed and protective against cell incursion on obverse sides of scaffolds. However, higher average pore diameters of 3D scaffolds, as well as their unique stereology, were compared to 2D scaffolds, and questions concerning possible cell migration throughout 3D scaffolds arose. To address these notions MSC penetration through the 3D nanofibre layers was investigated.

MSCs were seeded on both types of nanofibre layer in a manner that prevented leakage of cells out to surrounding areas (for details, see Materials and methods section). MSCs were fluorescently stained and detected

Figure 6. Confocal microscopic observation of penetrated cells. Confocal microscopic observation of cells penetrating through the nanofibrous layers of the 2D (a) and 3D (b) scaffolds on day 7; cells were stained using DiOC6 (green colour) and propidium iodide (red colour). Colour-coded projection of cells adhered to the 2D (c) and 3D (d) scaffolds on day 7.

Figure 7. Penetration of MSCs through the nanofibrous layers. The number of MSCs penetrating through the nanofibrous layers was counted on 1, 4, and 7 days using the confocal microscopy images. The number of cells under the PCL 3D scaffold was increased over the 7 days. No cells penetrated through the PCL 2D scaffold.

using confocal microscopy, on days 1, 4 and 7 of the investigation. Cells attached to nanofibre scaffolds, as well as those attached to well bottoms, were visualized.

Visualization included information concerning cell diffusion through the scaffolds.

In accordance with our previous results, nanofibre layers of 2D scaffolds with average pore sizes of $5 \mu\text{m}^2$ prevented MSC migration through scaffold structures. This was clearly documented by absence of any fluorescence signal from well bottoms, even after 7 days seeding, whereas MSCs seeded on 3D samples exhibited strong fluorescence signals from these locations (Fig. 6a, c). A significantly different picture was observed for 3D scaffolds, here, cells were visible at bottoms of wells 24 h after seeding (Fig. 6b,d). Numbers of cells penetrating increased gradually during incubation periods (up to 7 days), proving our hypothesis that MSCs were able to work their way through 3D scaffolds (Fig. 7).

Metabolic activity of cells significantly increased on 3D PCL scaffolds

Both 2D and 3D PCL scaffolds have different potential applications. Thus, both were compared and tested for

Figure 8. Cell adhesion. Cell adhesion was measured as the spreading area of cells 24 h after seeding. Cells were stained using DiOC6 and visualized using confocal microscopy. Images were analysed using the Ellipse software, and the spreading areas of cells were calculated (mean $\pm$ standard deviation; $P = 0.45$).

MSC biocompatibility. MSC proliferation and viability were followed and tested for 21 days.

First, cell adhesion was measured 24 h after seeding. MSCs were stained using DiOC6, visualized using confocal microscopy, and areas of cell spreading measured. Interestingly, spreading area of cells cultured on 3D nanofibre PCL layers were statistically larger ($P = 0.045$) than those on 2D scaffolds (Fig. 8). We

attribute this observation to higher values between surface-to-volume ratio of 3D scaffolds compared to 2D scaffolds.

Cell seeding efficiency and proliferation were estimated from DNA values measured using PicoGreen assay (Fig. 9a). Cell seeding efficiency was counted as ratio of number of seeded cells (25×10^3 cells/scaffold) and number of cells on scaffold determined by PicoGreen assay, 24 h after seeding. Seeding efficiency was $59.4 \pm 4.61\%$ for 2D PCL and $44.3 \pm 8.64\%$ for 3D PCL. Results of PicoGreen assay on days 7, 14 and 21 clearly indicated substantial cell proliferation on both types of scaffold. Nevertheless, there were still significant differences observed between scaffolds. Cell number on 2D scaffolds increased gradually from 1 to 21 days; conversely, cells seeded on 3D nanofibres exhibited lower levels of proliferation between 1 and 14 days, but then cell number rapidly increased between 14 and 21 days ($P < 0.001$).

A similar pattern was observed with MSC viability as assayed by MTT testing (Fig. 9b). Cells grew well on both scaffolds for the first 14 days; however, viability considerably differed between 14 and 21 days. On the other hand, viability of MSCs on 2D scaffolds decreased during this period ($P = 0.019$), but a significant increase in metabolic activity was observed for MSCs seeded on 3D scaffolds ($P = 0.027$).

Figure 9. MSCs viability, proliferation, and differentiation. Cell viability was determined using MTT assay (a). PicoGreen assay was used for measuring of MSCs proliferation (b). Osteogenic differentiation was determined by quantification of osteogenic markers, bone sialoprotein and osteocalcin, using real-time PCR (c,d).

Figure 10. Cell viability and expression of OC visualized by confocal microscopy. Live cells and dead cells on PCL 2D (a and c) and PCL 3D (b and d) scaffolds were stained using BCECF-AM (green colour) and propidium iodide (red colour), respectively, on 7 (a and b) and 21 days (c and d). Immunofluorescence staining of OC on PCL 2D (e) and PCL 3D (f) was assessed on day 21

Data from confocal microscopy supported findings of the MTT assay (Fig. 10). Cell viability was determined as a ratio of live to dead cells, and was significantly higher on 3D PCL scaffolds than on 2D scaffolds on day 7. Viability of cells on 2D scaffolds decreased from 87% on day 14 to 53% on day 21, while on 3D scaffolds, it did not decrease below 86% over the entire experiment (Table 2).

Adherence to 3D PCL nanofibre scaffolds accelerated MSC differentiation

Different types of interactions between MSCs and both 2D and 3D PCL scaffolds, reflected by differences in MSC proliferation, could possibly influence differentiation potential of the cells. Thus, MSCs seeded on each type of scaffold were tested for osteogenic differentia-

Table 2. Cell viability [%]

	d 1	d 7	d 14	d 21
PCL 2D	71 ± 15	85 ± 22	87 ± 7	53 ± 14*
PCL 3D	89 ± 13	86 ± 11	93 ± 4	89 ± 7

Cell viability was measured as the ratio of live to dead cells detected using confocal microscopy (mean ± standard deviation; $P < 0.05$).

tion, determined by real-time PCR analysis and immunofluorescence staining. OC and BS were used as osteogenic markers. Interestingly, expression of both proteins was significantly higher on cells 3D scaffolds than on those of 2D scaffolds, during the entire observation period (Fig. 9c,d). In contrast to cell proliferation, MSC differentiation was significantly elevated 7 days after seeding on 3D meshes, which indicated early cell differentiation compared to those on 2D scaffolds (BS $P < 0.001$; OC $P = 0.003$).

OC expression was also confirmed using immunofluorescence staining. Samples were stained using anti-OC monoclonal antibody and visualized on days 7, 14 and 21. Increasing production of OC was clearly documented over the whole period (Fig. 10).

Discussion

Novel electrospinning setup for laboratory production of 3D nanofibre layers

Simple modification of our electrospinning apparatus – adding a special collector composed of a rotating drum with a metal chain – led to production of knitted-like nanofibrous material with 3D structure arrangement. Results of optical microscopy, SEM, profilometry and X-ray micro-tomography demonstrated that structure of 3D PCL consisted of thin (less dense) areas and elevated (dense) areas. Stereological measurements showed that less dense areas had significantly higher porosity and pore size than had classic planar 2D nanofibres; dense structures were made of tightly packed nanofibres. Composite scaffolds of this kind seem to be very promising for tissue engineering applications.

There are other methods of determining pore size of a mesh, using a variety of special collectors. Zhu *et al.* (35) developed a technique based on a rotating frame cylinder, to prepare parallel mesh fibres. Parallelization of fibres increased pore size between the meshes without altering fibre diameter. A further approach was introduced by Zussman *et al.* (36). This group used a rotating table placed on a rotating disc moving 90° between steps of deposition. The fibrous mesh produced had a 'square mesh' orientation of nanofibres, as any follow-

ing layer had a perpendicular orientation to the one before. Li *et al.* (37–39) developed patterned static collectors consisting of conductive and non-conductive voids; nanofibres were aligned across a non-conductive voids (36). They also developed various collectors enabling production of 3D multi-layered structures with various organizations of fibres (36).

Fibrous 3D scaffolds prepared using special collectors had a clear advantage over methods employing sacrificial (that is, temporary) co-fibres, as here it would not be necessary to remove the pore-forming agents (25). Advantages of our method, as presented in this study, are its simplicity and ability to prepare 3D nanofibres with pores of any desired diameter. This is a key condition for appropriate seeding of each particular cell type.

Surfaces of prepared fibrous 3D PCL meshes were characterized by dense strings (dense areas) elevated over planar fibre layers (thin areas). This phenomenon of non-uniform deposition of fibres could be explained by changes to the electric field due to the collector's structure, as described earlier (above). In our case, the metal chain served as a field concentrator inducing greater fibre deposition and resulting in formation of dense zones. Electric fields around the void gaps between the metal chains was non-homogenous and resulted in less fibre deposition and greater stretching. This arrangement was explained by Li *et al.* (37) as occurring due to changes in Coulombic interactions when fibres approach the vicinity of wires. Coulombic interactions were responsible for fibres' alignment along edges of holes or along metal strips. However, Vaquette *et al.* (25) demonstrated that this theory had limitations, as observed patterns were lost when electrospinning was performed for several hours.

Morphological analysis of our knitted-type mesh revealed the 3D nature of prepared scaffolds. The z-axis height profile of 2D and 3D PCL revealed that while z-axis profile of 2D PCL mesh demonstrated only a slightly rough surface, with fluctuations in the order of 70 µm, the z-axis profile of 3D meshes had much higher peaks, corresponding to a rough surface. Interestingly, both dense and thin areas with less rough surface, were observed in 3D PCL scaffolds with significantly higher average pore sizes in less dense areas. Mean area of pores in less dense areas of scaffolds exceeded 60 µm², approximately 2-fold higher than of 2D PCL. We assume that 60-µm² pores were suitable for cell migration as concluded from previous cell experiments. On the other hand, 30-µm² pores of 2D PCL nanofibres were too small and hindered cell penetration. Thus, 3D scaffolds would be more suitable for cell culture in tissue engineering, where 3D systems are predominantly required.

Results of biomechanical testing showed higher Young's modulus of elasticity and strain/stress resistance of 3D PCL mesh compared to 2D mesh; 3D PCL scaffolds were more elastic and more stress-resistant. Importantly, this is not a consequence of any phase change of the polymers as thermal analysis performed by DSC did not show any difference between the two types of scaffold. Thus, the more favourable biomechanical properties were a consequence of physical scaffold structure and were caused by nano- and micro-stereology of the scaffold. The improved biomechanical properties of PCL 3D mesh were caused by presence of dense areas that reinforced the entire structure. We can easily imagine that elevated structures could be brought down when strain stress is applied, with no damage to side fibres. Consequently, this could provide a simple explanation for higher elasticity of our 3D scaffold. Thus, the proposed scaffold combined advantages of improved cell infiltration in the thin zones and greater mechanical stability in dense parts.

3D PCL nanofibre scaffold improved MSC proliferation and facilitated their differentiation

Properties of the 3D nanofibre PCL scaffold open widespread application of opportunities for it in tissue engineering and regenerative medicine. Cell migration through nanofibre layers is undoubtedly among these attractive features. Our data have clearly proven that the 3D scaffold was characterized by larger pores, and this was obvious, at least for certain locations. Clearly, identical surface mass of 3D samples, characterized with plentiful spikes, must naturally result in larger pore size compared to that of 2D scaffolds with the same surface mass. Larger pores can be expected at ends of spikes, thus facilitating MSC migration through the nanofibre layers. In addition, the 3D scaffold has different structural and stereological features that should result in different local mechanical properties at the nanofibrous spikes. We suppose that these properties alone can influence cell penetration through the layers. On the other hand, larger pores negatively influenced cell seeding efficiency on 3D PCL while small pores of 2D PCL hampered passive penetration of cells and caused their attachment to the surface, resulting in higher seeding efficiency.

Our study has indicated that different structural properties of the 3D nanofibre scaffold also resulted in differences in biocompatibility of MSCs. Biocompatibility of MSCs to PCL nanofibres has been widely reported (40–42). In accordance with the results here, we observed good cell adhesion and proliferation on both 2D and 3D PCL scaffolds. However, MSC adhesion was notably better to 3D samples. We suppose that enhanced MSC adhe-

sion reflects the more extensive surface of 3D scaffolds with more contact sites available for cell adhesion.

The novel 3D scaffold described here was characterized by better proliferation and viability of the cells; probably contact inhibition of cells, which naturally occurs earlier on 2D surfaces than on 3D scaffolds, offers more space for cell proliferation. Interestingly, cell proliferation significantly increased at the later times (day 21), but not in the early stages of this experiment. The explanation of this may be related to cell differentiation. Relationships between proliferation and differentiation of cells has been described in many works (43,44). Coordination of proliferation and differentiation is a fundamental process in normal tissue formation and regeneration. At the cellular level, proliferation and differentiation seem to correspond to independent cell processes that can sometimes be seen separated by a proliferation/differentiation switch (45). Gene and protein expression and activity profiles between proliferation and differentiation significantly differ and the processes are often resolved in time. Due to the multi-factor nature of this coordination process, the exact mechanism of regulation is not completely understood; however, key aspects are associated with signaling networks associated with G₁/G₀ cell cycle arrest and expression of differentiation-associated transcription factors (45,46).

This corresponds to our observation that 3D PCL scaffolds were also characterized by better osteogenic differentiation. This is in good accordance with osteogenic differentiation detected on 3D scaffolds with larger surface areas (47–49). In addition, the relationship between cell spreading and differentiation was reported. It was demonstrated that MSCs that were allowed to adhere, flatten and spread, underwent osteogenesis, whereas unspread, round cells became adipocytes (50).

Thus, we hypothesized that a larger surface available resulted in expression of both OC and BS markers, in our study. However, higher expression of both bone markers was also accompanied by lower cell proliferation between days 7 and 14. In addition, increase in cell proliferation with maintained osteogenic differentiation followed. Meanwhile, MSCs on 2D scaffolds proliferated steadily over the entire 21 day period of the experiment, with reduced cell viability by day 21. Cell differentiation on the 2D scaffold was detected on day 21; this indicated that MSCs differentiated in the earlier stage, which resulted in temporarily slower proliferation.

Consequently, we can conclude that 3D structure of the nanofibre layer can support proliferation and viability of MSCs, and is suitable for application in tissue engineering and regenerative medicine.

Conclusion

A special, novel collector for classic electrospinning has been developed. This collector served as a simple system for production of 3D nanofibres with easily modulated patterns. The 3D PCL scaffold was characterized by more favourable biomechanical properties, particularly greater elasticity and resistance against stress and strain. Such 3D nanofibres can be used for seeding cells of different origin and type, with different requirements for cell lacunae. We prepared 3D PCL nanofibre scaffolds with pore sizes exceeding $60\ \mu\text{m}^2$ and tested them for seeding, proliferation, differentiation and migration of MSCs. We demonstrated that nanofibre layers of 2D scaffolds prevented MSCs from migrating through the scaffold, while these cells infiltrated easily through 3D scaffolds. Adhesion of MSCs to 3D nanofibre PCL layers was also statistically more common than to 2D scaffolds, and proliferation and viability of MSCs 2 or 3 weeks after seeding were greater on 3D PCL scaffolds. In addition, the 3D PCL scaffolds were characterized by better osteogenic differentiation. We suppose that all these positive effects observed on 3D PCL nanofibre scaffolds are related to the larger surface-to-volume ratio of 3D structures. This novel system for producing 3D nanofibre scaffolds with modulated surface patterns can be effectively used in tissue engineering and regenerative medicine.

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