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The influence of pore size on colonization of poly(L-lactide-glycolide) scaffolds with human osteoblast-like MG 63 cells in vitro

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Abstract A degradable copolymer of L-lactide and glycolide (PLG) was synthesized by ring opening polymerization using zirconium acetylacetonate [$Zr(acac)_4$] as a biocompatible initiator. The structure of the copolymer was studied by nuclear magnetic resonance spectroscopy (NMR) and gel permeation chromatography (GPC). Porous scaffolds of defined microstructure were prepared by solvent casting/salt particulate leaching, which resulted in the creation of three types of scaffolds with the same porosity (87% ± 1%) but with different diameters of the pores (600, 200 and 40 μm) and degree of interconnectivity. The potential of the scaffolds for cell colonization was tested in a conventional static cell culture system using human osteoblast-like MG 63 cells. As revealed by conventional fluorescence and confocal microscopy on days 5 and 7 after seeding, the cells on the scaffolds of large or medium pore size infiltrated the inside part of the material, whereas on the scaffolds of small pore size, the cells were retained on the material surface. On day 7 after seeding, the highest number of cells was found on the scaffolds of the largest pore size (more than 120,000 cells per sample of the diameter 15 mm and thickness 2 mm), whereas on the scaffolds with medium and smallest pore diameter, the number of cells was almost three times lower and similar for both pore sizes. These results corresponded well with the incorporation of bromodeoxyuridine into newly synthesized DNA, which was significantly higher in cells on scaffolds of the largest pore size than on the material with medium and smallest pore diameter. As indicated by the MTT test, the mitochondrial activity in cells on scaffolds with medium pore size was similar to that on the material with the highest pore size, and significantly higher than on scaffolds of the smallest pore diameter. These results suggest that PLG scaffolds with the largest pore diameter (600 μm) and better pore interconnectivity are the most suitable for colonization with osteogenic cells.

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Introduction

Copolymers of L-lactide and glycolide (PLG) have been widely used in clinical and experimental medicine as resorbable materials for surgical sutures, various tissue replacements and implants for internal fixation of bone fractures; the Food and Drug Administration of the United States of America (FDA) has approved them for many medical applications [1]. Thanks to their good biocompatibility and resorbability, these copolymers have been regarded as the most promising materials for tissue engineering.

Tissue engineering is an advanced interdisciplinary scientific field which aims at constructing so-called bioartificial tissues or organs, i.e. structures containing a synthetic component which mimics natural extracellular matrix and is colonized with regenerated well-functioning

autologous cells [2, 3]. Cell colonization can be facilitated by an appropriate architectural design of the artificial material, especially by its three-dimensional scaffold-like structure [4, 5]. Another important prerequisite for a biomaterial is absence of cytotoxicity. It was shown recently that PLG materials can be synthesized using an initiator (zirconium acetylacetonate) of lower toxicity than that of commercially-used but more toxic tin compounds [6–8].

The influence of the macro- and microstructure of scaffold materials on interaction with cells has been addressed in many papers, but the findings published in the literature are very heterogeneous. Most researchers believe that a pore size of around 200 μm is appropriate or even optimal for ingrowth of osteoblasts and the creation of bone tissue [9–12]. In contrast, in other works it has been shown that the formation of newly regenerated tissue inside porous materials increases proportionally to pore size. For example, the rate of movement of osteoblasts into polymer-based matrices (pore diameters from 40 μm to 500 μm) and their proliferation was highest for the largest pore size [13, 14]. Similar results have also been obtained in human osteosarcoma cells cultured on ceramic scaffolds with channel diameters ranging from 170 to 421 μm [4]. On poly(DL-lactic-*co*-glycolic acid) scaffolds with pore sizes from 150 μm to 710 μm, the mineralized osseous tissue newly formed in vitro reached a maximum penetration depth into samples with an intermediate pore diameter of 300–500 μm [10, 11].

Therefore, the aim of the present study is to find out what microstructure of PLG scaffolds, regarding pore diameter and interconnectivity, is most appropriate to enable the scaffold to be colonized with osteoblast-like cells in vitro. The scaffolds were manufactured from a copolymer of glycolide and L-lactide, synthesized with a low-toxic initiator, zirconium acetylacetonate.

Materials and methods

Synthesis of the polymeric material

Glycolide and L-lactide (both from Purac, the Netherlands) were purified by re-crystallization from dry ethyl acetate and dried in a vacuum oven at room temperature. Zirconium (IV) acetylacetonate $Zr(acac)_4$ was purchased from Aldrich Corp., Germany and was used without purification. Copolymerization was performed in bulk at 100 °C with an initiator/monomer molar ratio of 1.25×10^{-3} by a conventional method using a vacuum line for degassing and sealing the ampoules [6]. In order to remove non-reacted monomers, the obtained copolymer was dissolved in chloroform and precipitated with cold methanol and finally dried in a vacuum at 50 °C to a constant weight.

Scaffold manufacturing procedure

The scaffolds were produced by a solvent casting/particulate leaching technique. Sieved sodium citrate particles (POCh, Gliwice, Poland) of defined sizes: I (600 ± 100 μm), II (200 ± 40 μm) and III (40 ± 10 μm), were mixed with 10% (w/v) copolymer solution in methylene chloride (POCh, Gliwice, Poland) in such proportions that a salt volume fraction of 85% was obtained. The size of the sodium citrate particles was measured by optical microscopy (Lanametr, Poland). The average diameter and confidence interval (at a confidence level $\alpha = 95\%$) were calculated from 100 measurements of individual salt grains originating from each experimental group: I, II and III. The mixture was cast on glass Petri dishes (diameter 5 cm) and dried overnight in the air, followed by vacuum treatment at a reduced pressure for 24 h. In the next step, the salt was leached out in distilled water until the conductivity of the water was close to that of distilled water (about 5 μS/cm), which took about 5 days. The samples were then dried in a vacuum oven at 35 °C for at least 24 h and stored in a desiccator prior to use.

Evaluation of the physical and chemical properties of the material

The chemical composition of the copolymer was determined by ^{1}H NMR (Varian Unity Inowa spectrometer) at 300 MHz using a 5-mm sample tube. Dried dimethyl sulfoxide-d_6 was used as a solvent. The spectra were obtained at 100 °C with 32 scans, acquisition time 3.74 s and pulse width 7 μs.

The molecular weight and polydispersity were determined by gel permeation chromatography (GPC) using the Spectra Physics SP 8800 chromatograph (chloroform as the eluent, flow rate 1 mL/min, Styragel columns 104, 103 and 500 A, Shoedex SE detector).

The porosity and water uptake of the scaffolds were calculated from the weight of the dry and water-soaked samples. After salt leaching, the foams were wiped with a wet cotton tissue in order to remove water from their surfaces, and then weighed (m_{wet}). Next, the samples were dried in a vacuum oven at 35 °C for at least 24 h and weighed once again (m_{dry}). The water uptake (W_u) was calculated using formula (1).

$$W_u(\%) = 100\ (m_{wet} - m_{dry})/m_{dry} \quad (1)$$

The percent porosity was calculated using formula (2),

$$P(\%) = 100\ (1 - \rho_a/\rho_c) \quad (2)$$

where ρ_a is the apparent density of the scaffold and ρ_c is the density of the solid copolymer (1.29 g/cm^3). The value of ρ_a was determined using Eq. 3,

$$\rho_a = m_{dry}/[m_{dry}/\rho_c + (m_{wet} - m_{dry})/\rho_w \quad (3)$$

where ρ_w is the density of water at 20 °C (0.9982 g/cm^3). The results were presented as the average and standard deviation calculated for three individual scaffolds (5 cm in diameter).

The scaffold permeability was measured using a method that applies Darcy's law according to a procedure described previously [15, 16]. It involved measuring the flow rate of water through the scaffold under a known hydrostatic pressure. The permeability, κ, was calculated from Eq. 4,

$$\kappa = \Delta Q L \mu / \Delta P A \quad (4)$$

where ΔQ is the induced flow (m^3/s), L is the sample length (m), μ is the kinematic viscosity of water (Pa· s), ΔP is the pressure drop across the sample (Pa) and A is the cross-sectional area of the sample (m^2). The permeability results were presented as the average and standard deviation for six individual scaffolds (13 mm in diameter). The parameter of permeability, κ having dimensions of area (μm^2), may be thought of as representing the cross sectional area of an effective channel for fluid flow through the pore space [17], and it can be used to compare the pore interconnectivity of different scaffolds, provided that their porosities are similar [16].

The microstructure of the scaffolds was studied with the use of a scanning electron microscope (JSM 5400, JEOL, Japan; accelerating voltage 15 kV, magnification 50×). Before the analysis, the samples were sputter-coated by a thin carbon layer in order to make them conductive.

Cell source and culture conditions

The scaffolds in the form of discs (diameter 15 mm, thickness 2 mm) were sterilized by the H_2O_2–plasma method (Sterrad 120, ASP, Johnson & Johnson) and inserted into 24-well polystyrene Nunclon Multidishes (Nunc, Denmark, diameter 15 mm). The scaffolds, very prone to floating in the culture media, were fixed to the well bottom by polyethylene rings (inner and outer diameter of 7 and 14 mm, respectively) and placed on top of the discs. By analogy, polyethylene rings were put on PLG foils and the bottom of the Nunclon tissue culture polystyrene (TCPS) wells that served as control samples. Other control samples were PLG foils and TCPS without the rings.

The scaffolds were then pre-wetted by two-step immersion in ethanol and phosphate-buffered saline (PBS) in order to remove air from the pores [18, 19], soaked overnight in the complete culture medium (see below), and seeded with human osteoblast-like cells of the line MG 63 (European Collection of Cell Cultures, Salisbury, UK). The cells were suspended in the Dulbecco-modified Eagle Minimum Essential Medium (DMEM; Sigma, USA, Cat. N° D5648) supplemented with 10% fetal bovine serum (FBS; Sebak GmbH, Aidenbach, Germany) and gentamicin (40 μg/mL, LEK, Ljubljana, Slovenia) to the concentration of 30,000 cells/mL and poured on top of the samples. Each well contained 45,000 cells (i.e., 25,000 cells/cm^2) and 1.5 mL of the culture medium. The cells were cultured for 5 or 7 days at 37 °C in a humidified air atmosphere containing 5% of CO_2. For each experimental group and time interval, three samples were used, and the experiments were performed twice.

Morphology and distribution of cells colonizing the scaffolds

On days 5 and 7 after seeding, the scaffolds were rinsed in PBS and the cells were fixed with pre-cooled (–20 °C) 70% ethanol for 5 min. Afterwards, the cells were stained with propidium iodide (5 μg/mL of PBS, Sigma, USA, Cat. N° P4170). Their morphology and distribution on the samples were observed in an IX 50 inverse fluorescence microscope (Olympus, Japan). The pictures were taken after focusing either on the material surface or into deeper parts of the scaffold in order to visualize the ingrowth of the cells into the pores. The penetration of cells into the pores was also evaluated in a confocal microscope (Leica TCS SP2, Germany), using horizontal and vertical optical sections through the scaffolds every 20 μm. The series of horizontal sections, parallel to the upper surface of the material disc, started from the pore entrance up to a depth of 640 μm. For the vertical sections, the discs were cut perpendicularly with a razor blade along their axis into two halves, and then blocks about 2 mm in width were cut out of the discs towards their periphery. The scanning then started from the central cutting surface of the blocks towards their peripheral face up to a distance of 640 μm. The area of the optical vertical sections involved the entire thickness of the scaffold discs, i.e. 2 mm.

Evaluation of cell number on the scaffolds

On day 7 after seeding, the cells were detached from the scaffolds, the underlying bottom of the culture wells, the control TCPS as well as the polyethylene rings by treatment with a trypsin-EDTA solution (Sigma, USA, cat N° T4174) in phosphate buffered saline (PBS) for 10 min at 37 °C. The cells were washed out of the scaffold by repeatedly rinsing the samples with the trypsin solution using a needle and a syringe, including perfusion of the scaffolds from the rear side through the pores towards the surface of the samples. In addition, the release of cells was

facilitated by mechanical disintegration of the scaffolds, relatively fragile and prone to degradation in aqueous media, into tiny fragments (less than 0.5 mm^3). The released cells were then counted in the Bürker haemocytometer.

Evaluation of DNA synthesis

Small discs of the scaffolds (diameter 6 mm; used in quadruplicates) were tightly inserted into gamma-sterilized 96-well polystyrene test plates (Nunc, Denmark, well diameter 6 mm) in order to prevent them floating in the culture media. The material was seeded with MG 63 cells in the density of 20,000 cells/well and incubated for 5 days in 200 μl of DMEM with 10% of FBS and gentamycin (see above). The medium was changed once (day 1–2) or twice (day 3–5) per day. On day 5 after seeding, DNA synthesis in cells colonizing the scaffolds was evaluated by a commercially available Cell Proliferation ELISA BrdU (Colorimetric) kit (Roche s.r.o., Diagnostic Division, Prague, CR; Cat. No. 11647229001) in accordance with the manufacturer's protocol. Briefly, the cells were incubated with 5-bromo-2-deoxy-uridine (BrdU) labeling reagent (4 h, 37 °C, atmosphere of 5% of CO_2 in air), FixDenat solution (30 min, room temperature), Anti-BrdU POD solution (90 min), washing solution (3 × 2 min), substrate solution (10 min, dark place), and finally STOP solution (1 M H_2SO_4) was added. The resulting solution was then moved into fresh plates and the absorbance was measured using a Multilabel Counter Wallac Victor 1420 (Perkin Elmer Life and Analytical Sciences, Inc., Wellesley, MA, USA) at a wavelength of 450 nm (the reference value was 690 nm). Two types of control samples were used: (1) cells in scaffolds in medium without BrdU (background) and (2) scaffolds in medium + BrdU without cells (blank). For each control group, three samples were used.

MTT assay

The growth and viability of cells colonizing the scaffolds was also evaluated by measuring the mitochondrial dehydrogenase activity using a modified MTT (3-(4,5-dimetyl-2-tiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) reduction assay [20]. Briefly, the cells on day 7 after seeding on the scaffolds (see paragraph 2.6.) were incubated with 150 μL DMEM and 50 μL of 0.2% MTT (Sigma, Cat. No. M5655) in PBS for 4 h at 37 °C. Then, 90 μL of 0.69 M sodium dodecyl sulphate in 50% *N,N*-dimethylformamide (pH 4.7) was added and incubated for 3 h at 37 °C. Then, after stirring 8 times, the resulting solution was moved into fresh plates and the absorbance was measured using TECAN Spectra A5082 (TECAN, Austria) at a wavelength of 570 nm (the reference value was 690 nm). Two types of control samples were used: (1) scaffolds with cells in medium without MTT (background) and (2) scaffolds without cells in medium with MTT (blank). For each control group, three samples were used.

Statistics

The quantitative data obtained in the cells was presented as averages ± SEM (Standard Error of the Mean). The statistical significance of the differences was evaluated by a one-way analysis of variance (ANOVA, Student-Newman-Keuls method), using SigmaStat software (Jandel Corp. USA). The *P* values equal to or less than 0.05 were considered significant.

Results

Properties of poly(glycolide-L-lactide) and scaffolds

As determined by ^{1}H NMR, the molar ratio of L-lactide to glycolide in the PLG copolymer was 82:18. Gel permeation chromatography showed that the number-average molecular mass (Mn) of the copolymer was 85 kD and the polydispersity index (d = Mw/Mn) was 2.5.

Figure 1 shows the gross morphology of the scaffolds on pictures taken with a digital camera. The initial samples of the scaffolds (i.e., before cutting into smaller discs) had a diameter of 5 cm and a thickness of 2 mm. It is apparent that in addition to pore entrances, the surface of all three materials (i.e., with large, medium and small pore size) also contains other irregularities, i.e. protuberances and depressions.

Figure 2 demonstrates representative microstructures of the scaffolds obtained with the use of a scanning electron microscope (SEM). The scaffolds consist of interconnected, tortuous pores with circular, oval or irregularly-shaped pore entrances on the surface. The size of the pores in all scaffolds was close to the size of the salt particles used as porogens. The pores usually ended blindly inside the material, their depth being similar to their diameter. However, some pores were deeper and even perforated the whole thickness of the discs. The porosity of all scaffolds was about 88% (Table 1), and was slightly higher than had been assumed in the preliminary theoretical design of the scaffolds (salt volume fraction 85%). The permeability of scaffolds depended on the size of the pores, and it was the highest for the scaffolds with the biggest pores (~600 μm; Table 1). The obtained data could be compared with the results of permeability of cancellous bone (1.5–140 μm^2) [15] or PEGT/PBT scaffolds produced by other researchers (60–180 μm^2) [16].

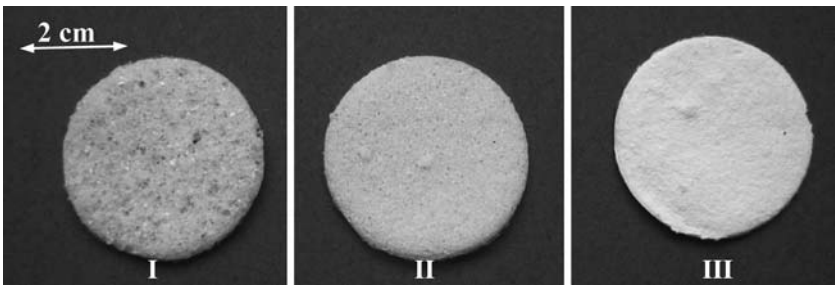

Fig. 1 Morphological appearance of scaffolds with defined diameter of pores: I (600 μm), II (200 μm) and III (40 μm) made of poly(L-lactide-*co*-glycolide) by solvent casting/salt particle leaching; the original scaffold discs were 5 cm in diameter

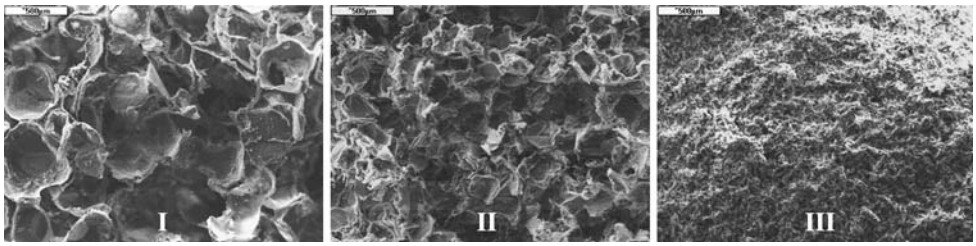

Fig. 2 Microphotographs of scaffolds of defined pore size: I (600 μm), II (200 μm) and III (40 μm) made of poly(L-lactide-*co*-glycolide) by solvent casting and salt particulate leaching. Scanning electron microscope, magnification 50×

Cell morphology and distribution

Figure 3 presents the morphology and distribution of the cells in 5-day-old cultures on scaffolds of decreasing pore size (I, II and III, i.e. average pore size of 600, 200 and 40 μm, respectively) as well as on the control TCPS. Propidium iodide, a dye for nucleic acids, stained the nuclei preferentially but the cytoplasmic part of the cells was also stained, though only faintly. Therefore, at higher magnification it was apparent that the cells were relatively well spread on the material surface, being polygonal or spindle-shaped (data not presented). On scaffolds I and II, the cells were well visible not only on the material surface but also inside the pores, especially on the pore bottoms or on horizontally-oriented parts of the tortuous pore walls. It should be noted that pictures 3A–D were taken after focusing the cells either on the material surface or inside the pores; and for this reason some parts of the pictures are blurred. On scaffold III, i.e., on the material with the smallest pore size, the cells did not grow inside the pores. Some of them spanned the pore entrances, so that the cultures on scaffolds III tended to form a continuous cell monolayer resembling that obtained on the flat control TCPS. These results were verified by confocal microscopy on day 7 after seeding. The horizontal optical sections revealed that the cells were able to penetrate inside the pores to a distance of 640 μm for scaffolds I and 580 μm for scaffolds II (Fig. 4). By contrast, in scaffolds with a pore size of 40 μm, no cells were found below a depth of 60 μm. These findings were confirmed by the perpendicular sections. In scaffolds of both large and medium pore size, the cells were distributed through the entire thickness of the sample, i.e. up to 2 mm from the surface (Fig. 5A–D and E–H), although the majority of them were located up to a depth of about 600 μm. On the other hand, in scaffolds with the pore size of 40 μm, the cells were concentrated almost exclusively on the upper or lower surface of the scaffold disc and rarely inside the material (Fig. 5I–L).

On the control TCPS, it was clearly visible that the cells adhered preferentially to the central region of the material not covered with polyethylene rings. Below these rings, few cells were observed, and their spindle-shaped morphology and orientation (data not presented) suggested that most of these cells were migrating from the central region of the dish not covered by the ring. Similar cell behavior was observed in the scaffolds, where the cell concentration on the surface as well as inside the material was higher in the central than in the peripheral part covered by the ring.

Table 1 Properties of porogens and scaffolds

Sample	D [μm]	P [%]	W_u [%]	κ [μm^2]
I	600 ± 100	88.3 (0.2)	580 (20)	85 (32)
II	200 ± 40	87.7 (0.7)	550 (40)	51 (27)
III	40 ± 10	87.0 (1.3)	540 (60)	17 (8)

D–diameter of salt particles; P–porosity; W_u–water uptake; κ–permeability. Results are presented as means ± confidence interval at $\alpha = 0.05$, $n = 100$ (D) and means and standard deviation in parentheses, $n = 3$ (P and W_u) and $n = 6$ (κ)

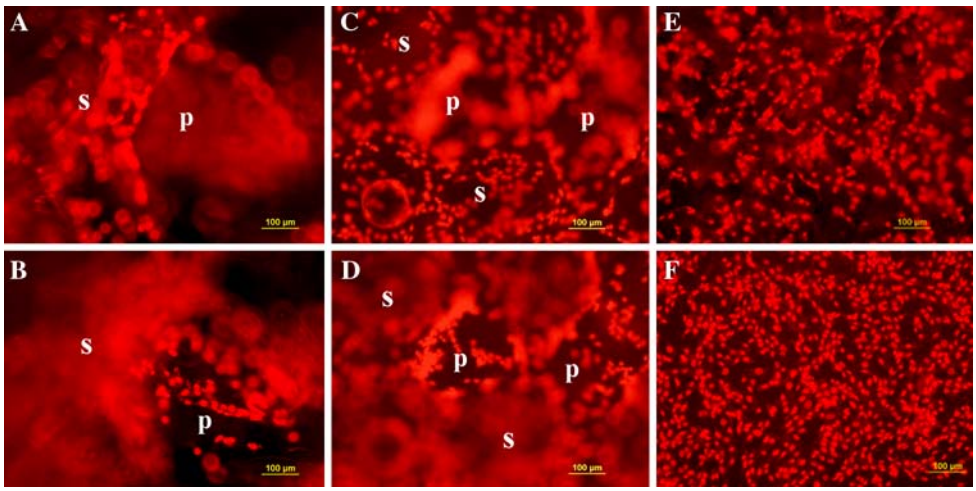

Fig. 3 Morphology of MG 63 cells on day 5 after seeding on poly(L-lactide-*co*-glycolide) scaffolds of large (**A**, **B**), medium (**C**, **D**) and small (**E**) pore size (about 600 μm, 200 μm and 40 μm, respectively) or the bottom of a control polystyrene well (**F**). The pictures were taken after focusing on the material surface (**A**, **C**, **E**; labeled as "s") or the pore interior (**B**, **D**; labeled as "p"). Propidium iodide staining, inverted epifluorescence microscope Olympus IX 51 with digital camera DP 70, obj. 20×

Cell number, DNA synthesis and mitochondrial activity

The number of cells released by trypsinization from scaffold I on day 7 after seeding, was significantly higher than the numbers obtained from scaffolds II and III. It was also higher than the number found on the bottom of the control PLG foil or TCPS wells partially covered by polyethylene rings, and comparable to the values obtained in the control foils or wells without rings (Fig. 6A). In accordance with these findings, the DNA synthesis, measured by BrdU incorporation, was significantly higher in cells colonizing scaffolds I than in cells on scaffolds of a lower pore size, i.e. II and III, and similar as in cells on the control TCPS. Moreover, the DNA synthesis was significantly higher than in cells on PLG foil (Fig. 6B). The activity of mitochondrial enzymes, measured by MTT assay, was lower in cells on all types of the scaffolds as well as PLG foil than in cells on TCPS. In cells on scaffolds II (pore size of 200 μm), it was similar as on scaffolds with the highest pore diameter of 600 μm and significantly higher than on scaffolds III with the smallest pore size of 40 μm (Fig. 6C).

Discussion

In the present study, the microstructure of poly(L-lactide-*co*-glycolide) scaffolds developed for bone tissue engineering was substantially modified by the size of the sodium citrate particles acting as porogens. Relatively low-toxic zirconium acetylacetonate was used as an initiator of polymerization; low toxicity is an important prerequisite for materials evaluated for tissue engineering [7, 8].

As revealed by conventional fluorescence and confocal microscopy, the osteoblast-like MG 63 cells were able to penetrate inside scaffolds I and II, i.e. the material with the large and medium pore diameter of 600 and 200 μm, respectively. On vertical optical sections, the cells in both types of scaffolds were detected throughout the entire thickness of the scaffold discs, i.e. up to 2 mm from the pore entrances, although the cells were concentrated preferentially on the material surface and in the underlying layer up to a depth of about 600–800 μm. For comparison, in other studies performed on a similar material, i.e. poly(DL-lactic-*co*-glycolic acid) foams of pore size from 150 μm to 300 μm, the maximum penetration depth of rat osseous tissue was smaller, being 220 ± 40 μm after 56 days of cultivation. At the same time, it was only 190 ± 40 μm for constructs of pore size from 500 μm to 710 μm [11]. This could be explained by the use of bone cells in primary cultures, whereas in our study, a quickly growing cell line well adapted to the in vitro conditions was applied. On the other hand, on polyHIPE polymer with a pore diameter of 100 μm, rat osteoblasts in primary cultures migrated to a maximum depth of 1.4 mm inside the scaffold after 35 days of cultivation [13], which is closer to our findings. On the titanium implants with drill channels, the ingrowth of human osteoblasts within 20 days amounted on average to 504, 573, 644, 838 and 266 μm into channels 300, 400, 500, 600 and 1000 μm in diameter, respectively [21].

Scaffolds III with the smallest pore size of 40 μm in our study did not allow considerable ingrowth of cells inside the material. This could be due to cell spreading over the pore entrances (i.e., bridging of the pore entrances by cells) and the creation of a cell monolayer, which functioned as a barrier preventing the migration of cells into deeper parts of the scaffolds. Similar behavior was observed in microvascular epithelial cells cultured on poly(lactic acid) scaffolds with pore diameter less than 38 μm. These cells formed a multilayered lining on the surface of the scaffolds without any ingrowth inside the pores [22]. Similarly,

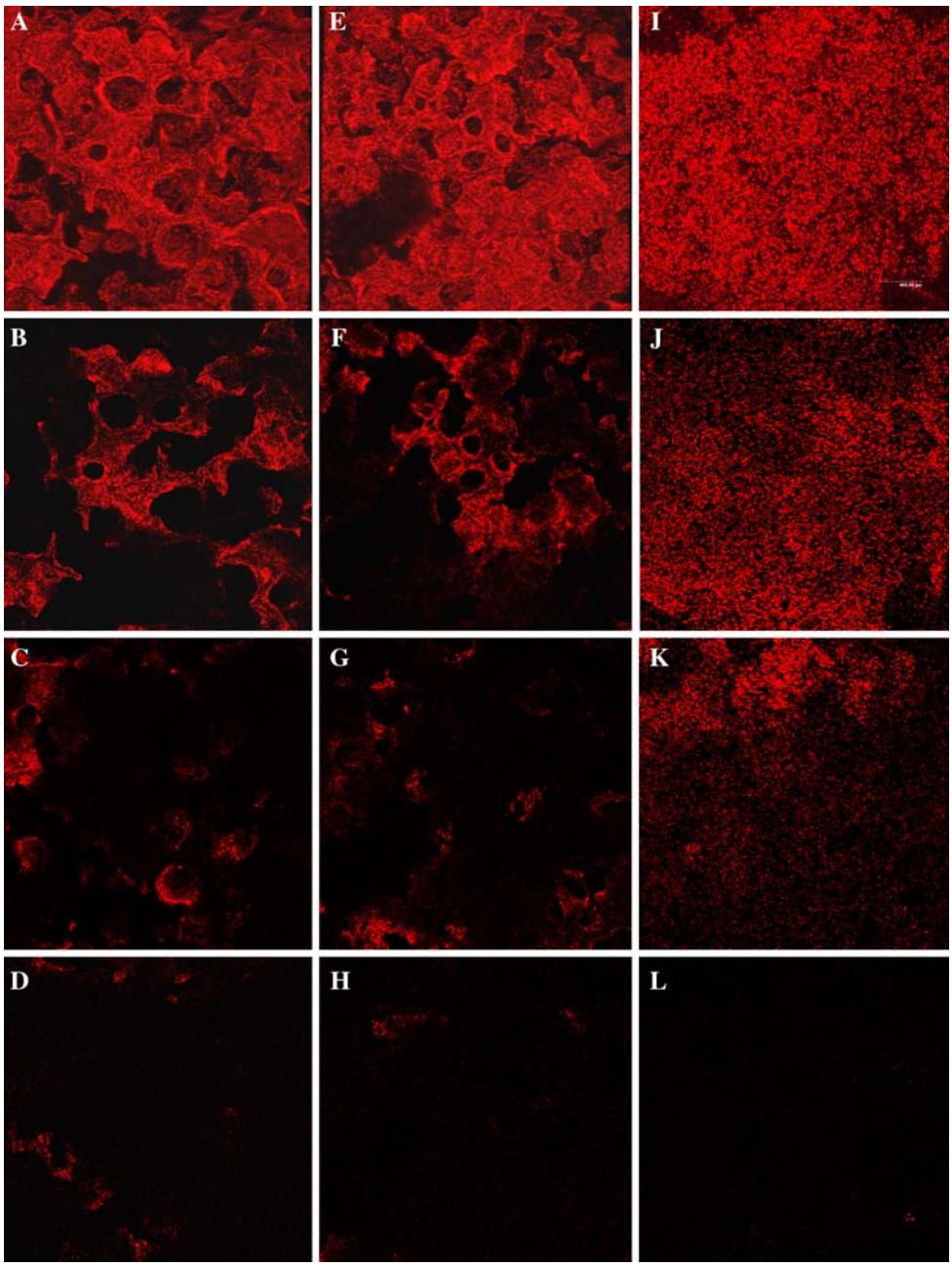

Fig. 4 Horizontal optical sections (2974 × 2974 mm) through the central region of poly(L-lactide-*co*-glycolide) scaffolds on day 7 after seeding with human osteoblast-like MG 63 cells. (**A**–**D**) average pore diameter 600 μm; (**E**–**H**) average pore diameter 200 μm; (**I**–**L**) average pore diameter 40 μm. (**A**, **E**, **I**) summarizing pictures of all optical sections; (**B**, **F**, **J**) surface layer; (**C**, **G**, **K**) depth of 340 μm, 300 μm and 40 μm, respectively; (**D**, **H**, **L**) depth of 640 μm, 580 μm, and 60 μm, respectively. Stained with propidium iodide, confocal microscope Leica TCS SP2, obj. 5×

osteoblasts on polyHIPE polymer of pore sizes of 40 μm formed cell multilayers on the polymer surface, although some limited migration inside the pores was observed [13]. Also porous Ti6Al4V discs (pore diameters from 68 μm to 13 μm), seeded with bovine chondrocytes, were covered by a thicker sheet of cartilaginous tissue when the pore size was smaller [23]. It can be recapitulated that if the pores are too small, with low interconectivity, rapid formation of tissue preferentially on the outer edge of the scaffold is observed, probably due to limitations of cell penetration as well as nutrient and waste exchange. For human osteosarcoma cells of the line HOS TE85, i.e. a similar cell type as used in our present study, the minimum channel or pore diameter required for cell penetration into hydroxyapatite scaffolds was estimated to be approximately 80 μm [4].

The number of cells released by trypsinization from scaffold I, on day 7 after seeding, was significantly higher than the numbers obtained from scaffolds II and III. It was also higher than that found on the PLG foils or the bottom of the control TCPS wells partially covered by polyethylene rings, and comparable to the values obtained in the control foils or wells without rings (Fig. 5). However, it can be supposed that the cell numbers obtained by trypsinization from the scaffolds were not complete. During the

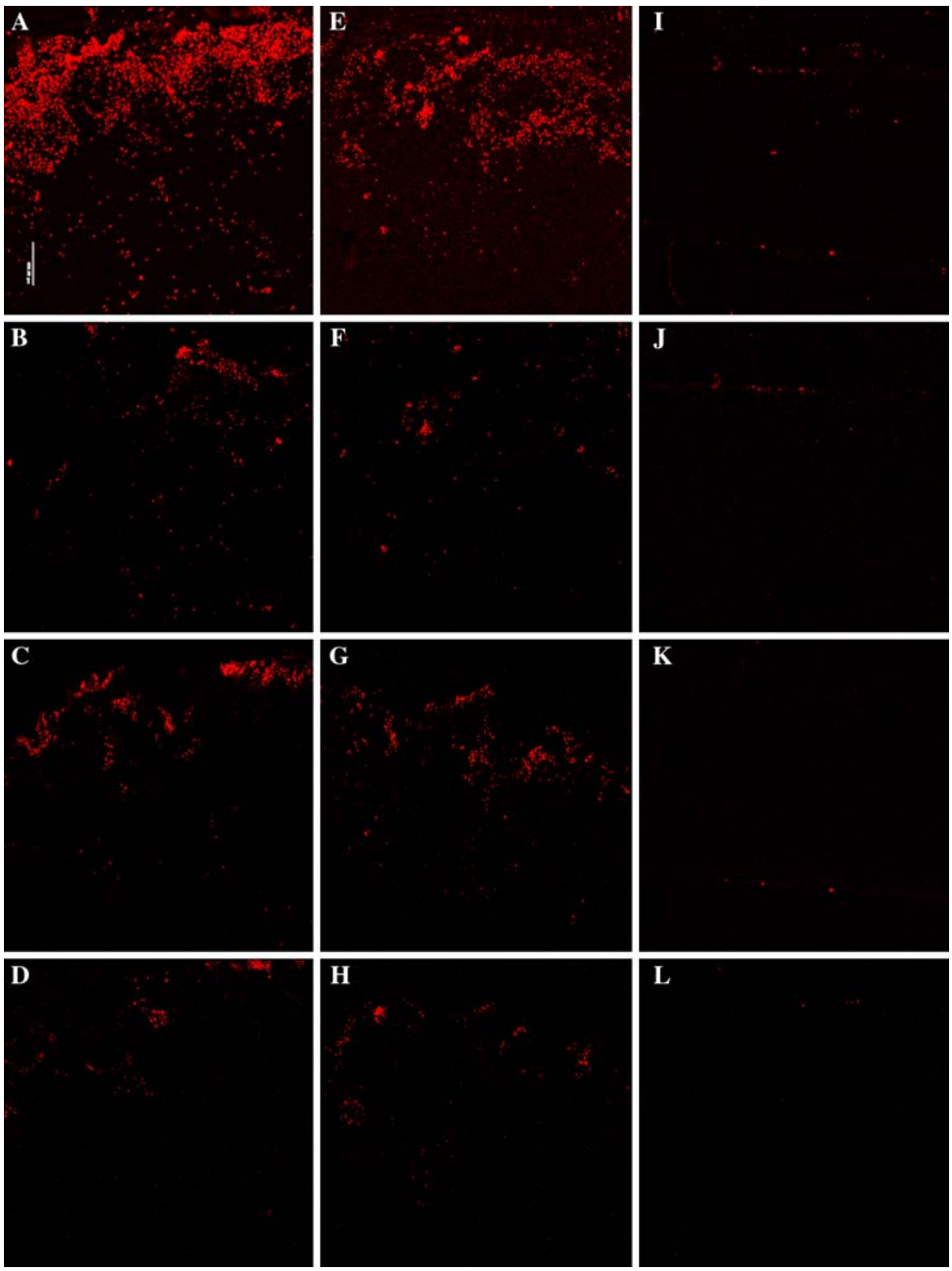

Fig. 5 Vertical optical sections (2974 × 2974 mm) through the central region of poly(L-lactide-*co*-glycolide) scaffolds on day 7 after seeding with human osteoblast-like MG 63 cells. (**A**–**D**) average pore diameter 600 µm; (**E**–**H**) average pore diameter 200 µm; (**I**–**L**) average pore diameter 40 µm. (**A**, **E**, **I**) summarizing pictures of all optical sections; (**B**, **F**, **J**) cutting surface of the blocks; (**C**, **G**, **K**) distance of 340, 220 and 280 µm, respectively; (**D**, **H**, **L**) distance of 640, 520, and 560 µm, respectively. Stained with propidium iodide, confocal microscope Leica TCS SP2, obj. 5×

cell harvesting procedure, some cells may have been be retained in the relatively complicated pore system with tortuous and interconnected parts and not washed out from the material, especially in scaffolds of smaller pore sizes. It has been reported that human osteoblasts were able to penetrate interconnections of at least 20 µm in diameter even in hard, non-dilatable materials, such as hydroxyapatite and beta-tricalcium phosphate ceramics [24]. On the other hand, our findings on the cell number were confirmed by the results on DNA synthesis in cells growing on the tested materials. The BrdU incorporation into cells was significantly higher on scaffolds I than on scaffolds II or III, and was similar or even higher in comparison with PLG foil or TCPS. The mitochondrial activity, which is considered as a marker of cell growth and viability [14, 25], was similar in cells on scaffolds I and II and significantly higher on scaffolds II than on scaffolds III.

In addition, in our experiments some of the cells adhered to the well bottom underlying the scaffolds. On day 7 after seeding, the number of well-attached cells below all scaffold discs was relatively constant and very close to 40,000 cells/well. Provided that these cells proliferated at a similar rate during the 7 days of culture, it may be assumed that the number of cells initially attached to all scaffold types

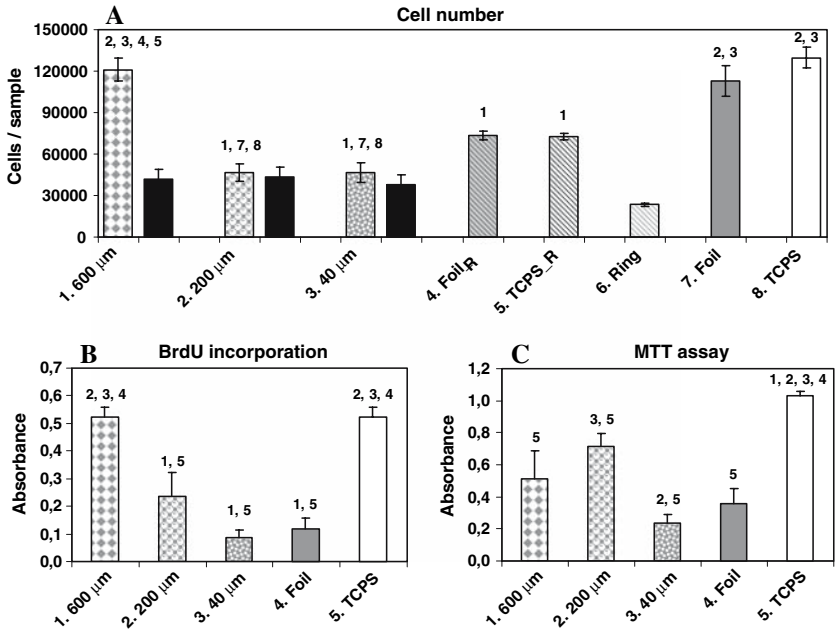

Fig. 6 Number (**A**), incorporation of bromodeoxyuridine (BrdU) into newly synthesized DNA (**B**) and mitochondrial activity measured by MTT assay (**C**) in MG 63 cells on day 7 after seeding on poly(L-lactide-*co*-glycolide) (PLG) scaffolds with average pore sizes of 600, 200 and 40 µm, PLG foil with or without a polyethylene ring (Foil_R and Foil, respectively), bottom of tissue culture polystyrene dishes with or without a polyethylene ring (TCPS_R and TCPS, respectively) and on the polyethylene ring (Ring). Black columns: cells on the dish below the scaffolds. Averages ± SEM from 16–18 measurements made on two independent samples for each group (**A**) or from four independent samples (**B**, **C**). One-way ANOVA, Student-Newman-Keuls method; the statistical significance ($P \leq 0.05$) in comparison with a certain experimental group is indicated by the number of that group above the column

was similar. Thus, the highest final cell number found on scaffold I was probably reached by the most intensive cell division on this material, which was also suggested by a higher incorporation of BrdU in these cells. The presence of pores is known to enlarge the specific surface area, i.e. the surface area per volume unit [5, 26]. Therefore, in comparison with flat 2D materials, the cells on porous materials can use more space for their expansion, which may stimulate their proliferation. Although the scaffolds with the largest pore size had the smallest specific surface area among all tested scaffolds, the accessibility of this area for cell colonization was the greatest, because the large pore diameter facilitated the penetration of cells inside the material and the pores also had the highest degree of interconnectivity. On the other hand, the activity of mitochondrial enzymes was found to be significantly lower in cells on all PLG scaffolds than on flat TCPS surfaces. The cells inside the pores could suffer from hypoxia, lower supply of nutrients, slower removal of the waste products of the metabolism as well as acidic pH resulting from the degradation of PLG [4, 27], which could limit their proliferation and viability.

Since some cells adhered to the well bottom underlying the scaffolds, and also to the polyethylene rings fixing the material to the well bottom (ca. 23,000 cells per ring), all cells seeded on the scaffolds were not utilized for colonizing this material. Therefore, the actual capacity of scaffolds for cell colonization could be higher than the values found in this study, especially in materials with medium and largest pore diameters. In addition, the capacity of these scaffolds for cell colonization could be better exploited if a larger number of cells were seeded and cultured for a longer time. In comparison with our study, the seeding densities used by other authors have usually been several times higher, ranging from hundreds of thousands to millions of cells per ml or cm^2 [4, 5, 10, 11]. Also the cultivation periods have been much longer, lasting up to 4–8 weeks [10, 11, 21]. On the other hand, the tests of low seeding densities and short culture periods, provided by our study, are equally important—clinical applications of scaffolds for bone tissue reconstruction could be associated with lower availability of a patient's autologous cells grown from a small biopsy [25], and also with the need for quick colonization of the material with cells. At low cell densities, the seeding efficiency, the homogeneity of cell distribution within the scaffolds, as well as cell expansion, could be markedly enhanced by dynamic methods of cell seeding, especially those using centrifugation [25].

Scaffolds with pore sizes of 600 and 200 µm allowed colonization with human osteoblast-like MG 63 cells on the material surface as well as inside the pores. The number of adhered cells was significantly higher on scaffolds with

the largest pore size and having the highest water permeability when compared to samples with medium and small pore diameters. Similar results were obtained on titanium implants containing drill channels of diameters ranging from 300 μm to 1000 μm seeded with osteoblasts isolated from human patients. In 20-day-old cultures, the deepest ingrowth and the highest osteogenic differentiation of cells were found in channels with a diameter of 600 μm [21]. A recent study performed on hydroxyapatite scaffolds revealed that the penetration of human osteosarcoma cells into the material, as well as the cell coverage of the channel walls and bottoms, was greater in samples with larger channels of 420 μm than in matrices with smaller channels of 170, 200 or 300 μm [4]. Even highly macroporous scaffolds of pore diameter from 0.8 mm to 1.8 mm, i.e. much larger than that in our study, recently constructed for bone tissue engineering from poly(lactide-*co*-glycolide) combined with calcium phosphate, are expected to enhance ingrowth of osteogenic cells inside the material both in vitro and in vivo [28].

Scaffolds with a pore diameter of 200 μm in our study also promoted ingrowth of MG 63 cells into the pores, but the cell number on this material was found to be significantly lower than on samples with a pore size of 600 μm. This result is relatively inconsistent with some literature data, which refers to a pore size of about 200 μm as optimal for osteoblast culturing [9–12]. For example, on biodegradable poly(DL-lactic-*co*-glycolic acid) foams with porosity of 90%, i.e. on a material very similar to our scaffolds, seeded with a similar density of rat osteoblasts (i.e., 22.1×10^5 cells per cm^2), the maximum penetration depth of the osseous tissue was obtained in foams of 150–300 μm pore size [10, 11]. In accordance with these findings, the cells on the scaffolds with a pore size of 200 μm in our study displayed relatively high mitochondrial activity, which tended to be on an average higher than on scaffolds with a pore diameter of 600 μm.

Certain disproportions between our results and the findings of other authors on the optimum pore size for bone tissue regeneration can be explained, at least partly, by different material composition and structure, cell source and type, the parameter chosen for evaluating tissue regeneration (e.g., cell number, DNA synthesis, activity of various enzymes, production of extracellular matrix molecules, etc.) and many other experimental conditions mentioned above (e.g., cell seeding density, duration of cultivation, static or dynamic cell culture condition, composition of cell culture media, etc.)

Thus, it can be concluded that, in our experiments, PLG scaffolds with a pore size of about 600 μm appeared to be more appropriate for colonization with human osteoblast-like cells than those with a smaller pore size of 200 or 40 μm. In further experiments, the ingrowth of bone cells into the scaffolds, followed by formation of mineralized bone tissue, could be enhanced by the nanoarchitecture of the pore walls, e.g. by introducing nanophase ceramics or nanofibres into the polymer material or by preparing PLG in a semicrystalline form. Nanostructured materials are known to improve the adsorption of cell adhesion-mediating extracellular matrix proteins, namely vitronectin, which selectively enhances the adhesion of osteoblasts [29–32]. At the same time, the manufacturing procedure could be modified in order to improve the pore interconnectivity.

Conclusion

Poly(L-lactide-*co*-glycolide) scaffolds of three different pore diameters (600, 200 and 40 μm) and degree of interconnectivity were constructed for potential application in bone tissue engineering, and used for cultivating human osteoblast-like MG 63 cells in a conventional static cell culture system. The samples with the highest and medium pore sizes allowed cell colonization on the material surface as well as inside the pores, whereas scaffolds with the smallest pore size allowed cell colonization only on the material surface. On day 7 after seeding, the highest number of cells and the highest DNA synthesis was obtained on the samples with the largest pores. The mitochondrial activity was similar in cells on the scaffolds with the largest and medium pores and significantly lower in cells on the material with the smallest pore size.

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References

1. B. L. SEAL, T. C. OTERO and A. PANITCH, *Mater. Sci. Eng. R* **34** (2001) 147
2. S. YANG, K.-F. LEONG, Z. DU and C.-K. CHUA, *Tissue Eng.* **7** (2001) 679
3. Y. TABATA, *Drug Discovery Today* **6** (2001) 483
4. F. R. ROSE, L. A. CYSTER, D. M. GRANT, C. A. SCOTCHFORD, S. M. HOWDLE and K. M. SHAKESHEFF, *Biomaterials* **25** (2004) 5507
5. F. J. O'BRIEN, B. A. HARLEY, I. V. YANNAS and L. J. GIBSON, *Biomaterials* **26** (2005) 433
6. P. DOBRZYNSKI, J. KASPERCZYK, H. JANECZEK and M. BERO, *Macromolecules* **34** (2001) 5090
7. B. CZAJKOWSKA, P. DOBRZYŃSKI and M. BERO, *J. Biomed. Mater. Res.* **74A** (2005) 591

8. E. PAMULA, M. BLAZEWICZ, B. CZAJKOWSKA, P. DOBRZYNSKI, M. BERO and J. KASPERCZYK, *Ann. Transplant.* **9(1A)** (2004) 64
9. S. I. JEONG, S. H. KIM, Y. H. KIM, Y. JUNG, J. H. KWON, B. S. KIM and Y. M. LEE, *J. Biomater. Sci. Polym. Ed.* **15** (2004) 645
10. S. L. ISHAUG-RILEY, G. M. CRANE, A. GURLEK, M. J. MILLER, A. W. YASKO, M. J. YASZEMSKI and A. G. MIKOS, *J. Biomed. Mater. Res.* **36** (1997) 17
11. S. L. ISHAUG-RILEY, G. M. CRANE-KRUGER, M. J. YASZEMSKI and A. G. MIKOS, *Biomaterials* **19** (1998) 1405
12. M. YAMAMOTO, Y. TABATA, H. KAWASAKI and Y. IKADA, *J. Mater. Sci. Mater. Med.* **11** (2000) 213
13. G. AKAY, M. A. BIRCH and M. A. BOKHARI, *Biomaterials* **25** (2004) 3991
14. G. TORUN KOSE, S. BER, F. KORKUSUZ and V. HASIRCI, *J. Mater. Sci. Mater. Med.* **14** (2003) 121
15. P. W. HUI, P. C. LEUMG and A. SHER, *J. Biomechanics* **29** (1996) 123
16. J. SOHIER, R. E. HAAN, K. DE GROOT and J. M. BEZEMER, *J. Contr. Release* **87** (2003) 57
17. M. A. KNACKSTEDT, C. H. ARNS, T. J. SENDEN and K. GROSS, *Biomaterials* **27** (2006) 2776
18. S. H. OH, S. G. KANG, E. S. KIM, S. H. CHO and J. H. LEE, *Biomaterials* **24** (2003) 4011
19. A. G. MIKOS, M. D. LYMAN, L. E. FREED and R. LANGER, *Biomaterials* **15** (1994) 55
20. L. BACAKOVA, K. WALACHOVA, V. SVORCIK and V. HNATOWICZ, *J. Biomater. Sci.–Polym. Ed.* **12** (2001) 817
21. K. H. FROSCH, F. BARVENCIK, V. VIERECK, C. H. LOHMANN, K. DRESING, J. BREME, E. BRUNNER and K. M. STURMER, *J. Biomed. Mater. Res.* **68A** (2004) 325
22. J. ZELTINGER, J. K. SHERWOOD, D. A. GRAHAM, R. MUELLER and L. G. GRIFFITH, *Tissue Eng.* **7** (2001) 557
23. T. BHARDWAJ, R. M. PILLIAR, M. D. GRYNPAS and R. A. KANDEL, *J. Biomed. Mater. Res.* **57** (2001) 190
24. J. X. LU, B. FLAUTRE, K. ANSELME, P. HARDOUIN, A. GALLUR, M. DESCAMPS and B. THIERRY, *J. Mater. Sci. Mater. Med.* **10** (1999) 111
25. W. T. GODBEY, S. B. HINDY, M. E. SHERMAN and A. ATALA, *Biomaterials* **25** (2004) 2799
26. K. WHANG, K. E. HEALY, D. R. ELENZ, E. K. NAM, D. C. TSAI, C. H. THOMAS, G. W. NUBER, F. H. GLORIEUX, R. TRAVERS and S. M. SPREGUE, *Tissue Eng.* **5** (1999) 35
27. C. SCHILLER and M. APPLE, *Biomaterials* **24** (2003) 2037
28. L. GUAN and J. E. DAVIES, *J. Biomed. Mater. Res.* **71A** (2004) 480
29. T. J. WEBSTER, C. ERGUN, R. H. DOREMUS, R. W. SIEGEL and R. BIZIOS, *J. Biomed. Mater. Res.* **51** (2000) 475
30. R. L. PRICE, M. C. WAID, K. M. HABERSTROH and T. J. WEBSTER, *Biomaterials* **24** (2003) 1877
31. K. M. WOO, V. J. CHEN and P. X. MA, *J. Biomed. Mater. Res.* **67A** (2003) 531
32. G. WIE and P. X. MA, *Biomaterials* **25** (2004) 4749