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# Microfabrication of PDLLA scaffolds

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## Abstract

This study aimed to comprehend the potentialities of the microfabrication to produce tissue-engineering scaffolds. Structures presenting homogeneously distributed pores of size 100 and 200  $\mu\text{m}$  were fabricated through layer-by-layer deposition of filaments of poly(D,L-lactic acid) (PDLLA) prepared from dichloromethane/dimethylformamide solutions. Rheological tests on the solution and molecular weight distributions of PDLLA, solvent cast films and microfabricated scaffolds were performed to determine which material conditions are optimal for the microfabricated system and to identify any possible material modification induced by the process. *In vitro* qualitative preliminary cell culture studies were conducted using MG63 osteoblast cell lines after assuring the non-cytotoxicity of the scaffold material by the lactate dehydrogenase *in vitro* toxicology assay; biological evaluations were initially performed using scaffolds with the smaller (100  $\mu\text{m}$ ) pore size. Scanning electron microscopy imaging was used to determine cell morphology distribution. A second cell culture test was performed, using the scaffold with the higher (200  $\mu\text{m}$ ) porosity. Confocal laser microscopy (CLM) was utilized to examine cell morphology and growth behaviour. Cellular metabolic activity and viability were also examined using Alamar Blue assay and further verifications were performed using CLM. Cell culture studies indicated homogeneous distribution, high viability and metabolic activity. Pore dimension affects cell distribution: pores <100  $\mu\text{m}$  acted as barrier structures for the MG63 osteoblast cell line; penetration inside the matrix was hindered and cells grew on the outer part. Increasing pore size resulted in a more homogeneous cell distribution and penetration of cells inside the structure was achieved. Copyright © 2010 John Wiley & Sons, Ltd.

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**Keywords** microfabrication; scaffold; tissue engineering; PDLLA; pore size; biocompatibility

## 1. Introduction

In a typical tissue-engineering application, a scaffold is used to provide a three-dimensional (3D) support where cells can adhere, proliferate and produce extracellular matrix (ECM). The scaffold materials should be easily

processable in the desired shapes and sizes, exhibit a degradation rate compatible with effective repair of the tissue and produce non-toxic degradation products (Langer and Vacanti, 1993; Salgado *et al.*, 2004; Huttmacher, 2000; Chen *et al.*, 2001; Mikos and Temenoff, 2000).

There are many synthetic and natural polymers that have been used as scaffold materials in different tissue-engineering applications. Among them, poly ( $\alpha$ -hydroxyl) acids such as polyglycolic acid (PGA), polylactic acid (PLA) and their copolymers or blends are widely used

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(Vozzi *et al.*, 2002, 2003a; Hong *et al.*, 2008; Ghosh *et al.*, 2008; Yang *et al.*, 2006; Athanasiou *et al.*, 1996).

For a given material, besides biocompatibility and degradability requirements, the scaffold should possess a specific spatial architecture enabling cell penetration and guidance of the early assembly of the produced extracellular matrix. In particular, the ideal scaffold should have interconnected pores (Aydin *et al.*, 2009) and a proper surface area:volume ratio to maximize cell adhesion, transport of nutrients and removal of metabolic waste. Moreover, depending on the specific application, it should possess adequate mechanical stiffness and strength to maintain its structural integrity during cell culture *in vitro* and support loads when *in vivo*.

In recent years, considerable attention has been paid to fabrication methods for producing porous scaffolds with certain predetermined designs. The design of the scaffold can, in fact, significantly alter cell proliferation and differentiation (Ma and Langer, 1998; Bhatia and Chen, 1999; Bhatia *et al.*, 1997) and the geometry defines the adhesion sites of the cells. The architecture can influence cell and cytoskeleton organization and conformation. It has been stated that cellular gene expression is controlled by the shape and anchorage of the cells (Yilgor *et al.*, 2008; Benya and Schaffer, 1982).

There are several conventional processing techniques to produce scaffolds with pores having different sizes and organizations, such as solvent casting (Mikos *et al.*, 1994; Kose *et al.*, 2003), freeze drying (Silva *et al.*, 2008; Ulubayram *et al.*, 2002), phase inversion (Holly *et al.*, 2000), fibre bonding (Kim and Mooney, 1998; Tuzlakoglu *et al.*, 2005), melt-based technologies (Thomson *et al.*, 1995; Costa-Pinto *et al.*, 2008), high pressure-based methods (Mooney *et al.*, 1996) and computer-assisted design and manufacture (CAD/CAM) techniques (Vozzi *et al.*, 2003a, 2003b; Lam *et al.*, 2002).

The most common microfabrication systems are based on the use of a micro-extruder, which deposits successive layers of polymer solution or melt on a support substrate that a computer-controlled mechanism moves in the *x*–*y*–*z* directions (Chen *et al.*, 2005; Yamada *et al.*, 2007; Vozzi *et al.*, 2007). The geometry and properties of the prepared scaffolds depend on the orifice of the extrusion die, the viscosity of the polymer melt or solution, the pressure applied to extruder and the speed at which the base support moves in each direction.

In this study, starting from polymer solutions, ordered geometry PDLLA scaffolds were microfabricated. The study shows the potential of the microfabrication system to produce scaffolds with ordered and predetermined structure at a micro-scale level. The proposed apparatus was designed and developed to produce scaffolds for tissue-engineering applications having repeated and regular pores. The system is versatile, adaptable and cost-effective. Different polymeric solutions can be used. Furthermore, since the final geometry is defined and described by a software platform, different shapes, pore sizes and pore distributions can be obtained.

## 2. Materials and methods

### 2.1. Materials

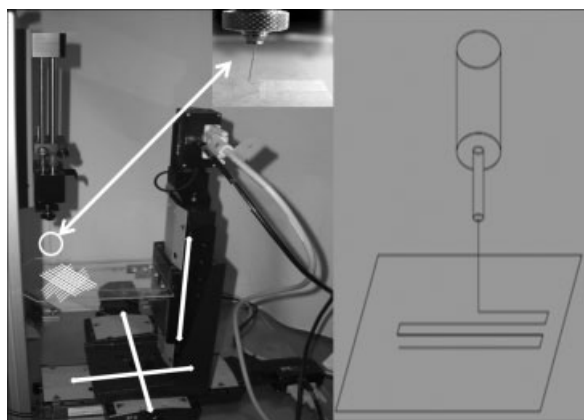
Poly(D-lactic acid) (PDLLA, type RESOMER<sup>®</sup> 207, MW = 252 kDa) was purchased from Boehringer Ingelheim (Germany). The polymer was used without further purification. Dichloromethane (DCM) and dimethylformamide (DMF) were obtained from BDH Chemicals (UK) and J. T. Baker (The Netherlands), respectively.

### 2.2. The microfabrication apparatus

Scaffolds were produced using a home-made microfabrication apparatus. It consists of three independent slides (supplied by National Instruments, Austin, TX, USA) that can move independently with 1  $\mu$ m resolution under the control of a computer through a labVIEW software platform. The slides, having a travel range up to 100 mm, can displace a support in the three directions of space. A glass syringe connected to an automatic pumping system (11 Plus, Harvard Apparatus, MA, USA), is used to extrude a filament of the polymer solution at set flow rates through a metal micro-needle (34 gauge; 60  $\mu$ m i.d., 1 cm length, 90° point style). By adjusting the solution injection rate and the speed of the collecting plate, polymer solution filaments were deposited layer-by-layer, forming filament constructs with different predetermined architectures and sizes which were fabricated on poly(ethylene terephthalate) (PET) sheet fixed to the support. In particular, scaffolds composed of orthogonal layers were produced by moving the plate alternatively in the *x* and *y* directions with two separate slides. After the completion of each layer, the third slide moved the plate down in the *z* direction by an amount corresponding to the thickness of the forming polymer fibre, i.e. the distance between the needle and the deposition plane was kept constant. The number of iterations of this sequence determined the number of layers composing the scaffold, while fibre dimension depended on the solution concentration and flow rate. The microfabrication apparatus and scaffold preparation are shown schematically in Figure 1.

### 2.3. Scaffold microfabrication

PDLLA solution (20% w/v) was prepared in dichloromethane:dimethylformamide (70:30 v/v). These solvents and concentrations were selected as being optimal after performing multiple microfabrication tests and viscosity measurements of the polymer solutions (data not shown). Before starting the microfabrication process, the polymer solution was magnetically stirred at 40 °C for 18 h and then filtered through Millipore nylon net filters with a 20  $\mu$ m pore size. Scaffolds were prepared by injection of polymer solution through the syringe needle, in equidistant rows. For the selected polymeric solution, the speed of stage movement was set at 15 cm/min and flow



**Figure 1.** Schematic presentation of the microfabrication system and scaffold preparation

rate at 1 m/min. The distance between layers was fixed at 25  $\mu$ m. The microfabrication process was performed at room temperature. The dimensions of the scaffolds were 1  $\times$  1 cm with ca. 350  $\mu$ m thickness and 25 layers. Scaffolds having 100  $\mu$ m (s-100) and 200  $\mu$ m (s-200) pore size were produced. Solid PDLLA films were also prepared from the same polymer solutions by the solvent casting technique as controls.

## 2.4. Rheological tests

The flow behaviour of the polymer solution was evaluated by measuring its complex viscosity over shear rate. In particular, the solution determined to be the optimal one in the microfabrication system was analysed to determine the solution working parameters. A rotational rheometer (ARES TA Instrument) with a cone-plate configuration (50 mm plate diameter, 0.04 rad cone angle, 0.050 mm initial gap between cone and plate) and a dynamic frequency sweep test mode (strain control, 3% strain, 0.1 rad/s initial frequency, 22  $^{\circ}$ C temperature) were used. A humidity chamber was prepared by enclosing the cone-plate apparatus and lined with a solvent-soaked sponge to prevent solvent evaporation during the experiments.

## 2.5. Gel permeation chromatography (GPC)

The molecular weights of the polymers before and after scaffold fabrication, and of the cast film polymers, were determined by gel permeation chromatography (GPC; Spectra System P1500) by using a Shodex K-804 column (Shodex, Tokyo, Japan) and a KG pre-column with 1 ml/min eluent constant flux. Chloroform was the solvent for both polymers and the instrument was calibrated by using polystyrene standards.

## 2.6. Cytotoxicity assays

The cytotoxicity of the scaffolds was examined by enzyme lactate dehydrogenase (LDH; Sigma Aldrich)

assay. LDH found in the cytoplasm is a marker for membrane integrity of cells; it can be correlated to cell viability and proliferation. It is released when damage to the cytoplasmic membrane occurs. To perform the LDH toxicology assay, human osteosarcoma-derived osteoblasts (MG63;  $10^5$  cells/cm<sup>2</sup>) were seeded using a culture medium with reduced serum. Microfabricated scaffolds were left for 72 h in a phosphate-buffered saline (PBS) solution at 37  $^{\circ}$ C with a concentration of 10 mg/ml (polymer/solution). After incubation, PBS was collected and added to the culture medium (1/3 v/v). The enzymatic analysis was assessed following the manufacturer's instructions. TCP seeded with the same concentration of cells and standard culture medium was used as control. The absorbance of the assay mixture was measured at 490 nm using a microplate reader (Thermo Labsystems, Finland). For each sample four readings were performed; average value and standard deviation (SD) are reported.

## 2.7. Cell culture tests

Microfabricated scaffolds (0.5  $\times$  0.5 cm, thickness 350  $\mu$ m) were used for preliminary qualitative biological evaluations. For this purpose, all samples were washed in water several times to eliminate possible traces of solvents, then sterilized by immersion in 70% ethanol followed by washing with distilled water, then placed in 48-well plates. Human osteosarcoma-derived osteoblasts (MG63) were seeded by using 0.5 ml cell suspension for each well at a cell concentration of  $5 \times 10^5$  cells/ml. Minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin, 1% glutamax, 1% vitamins, 1% non-essential amino acids was used as culture medium. The cells were incubated at 37  $^{\circ}$ C in a 5% CO<sub>2</sub> atmosphere incubator, changing the medium every 2 days.

Two different cell culture tests were carried out. At first microfabricated scaffolds having pore size of 100  $\mu$ m (s-100) were seeded and two different time points were considered (7 and 14 days). SEM evaluation of the scaffold before and after cell culture was used to show cell attachment and growth over the structure.

After this preliminary test, a second cell culture was performed on scaffolds having 200  $\mu$ m (s-200) pore size to determine whether the cells would penetrate this structure better. Cell culture was stopped at 2, 9 and 16 days; the morphology of the cells and their distribution on the scaffolds were observed by confocal laser microscopy (CLM) imaging after phalloidin–Rhodamine and DAPI staining; viability and proliferation analyses were also performed.

## 2.8. Sample imaging: SEM imaging

Morphological observations of scaffolds before and after cell culture were performed by scanning electron microscopy (SEM; Cambridge Stereoscan 200; operating



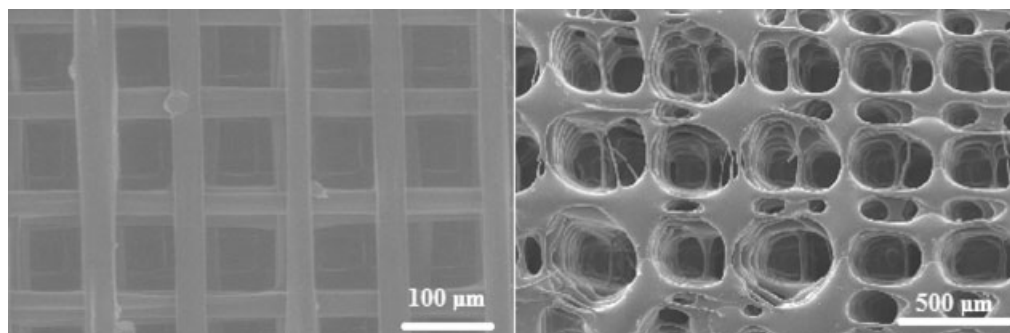


Figure 2. SEM images of PDLLA scaffolds with 100  $\mu\text{m}$  (left) and 200  $\mu\text{m}$  (right) pore size

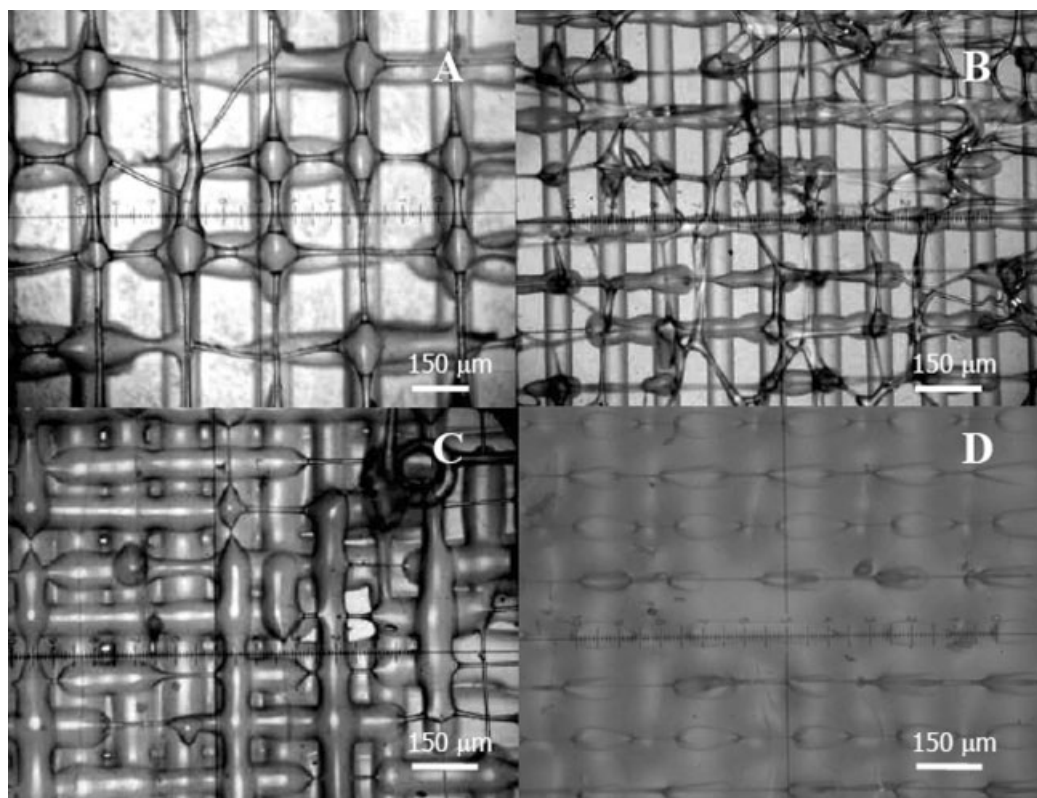


Figure 3. Optical images of microfabricated PDLLA scaffolds with defects

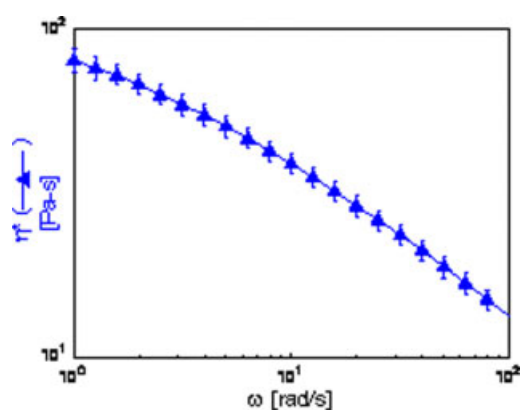


Figure 4. Rheological test results for PDLLA solution

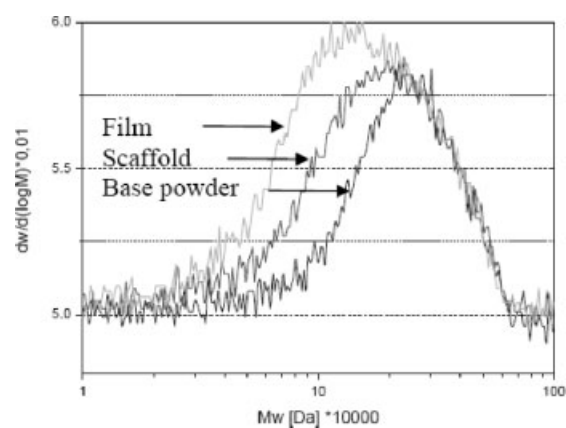
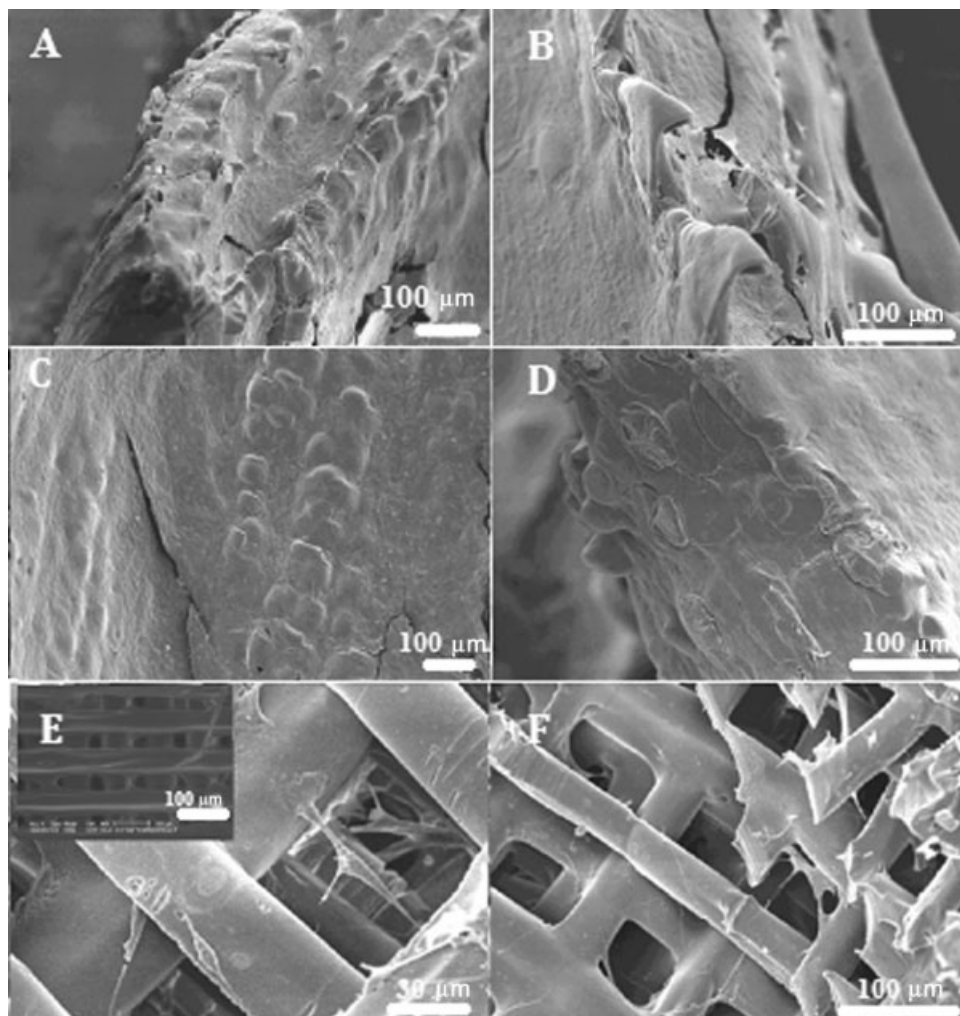


Figure 5. Molecular weight distribution of PDLLA-based materials: base powder, cast film and microfabricated scaffold



**Figure 6.** SEM images of s-100 scaffolds after 7 days (A, B) and 14 days (C, D) of MG63 cell seeding; internal parts of the scaffolds after 7 days (E, F)

mode, high vacuum, secondary electron SE detector). Cell cultured samples, prior to imaging, were fixed with glutaraldehyde solution (25% glutaraldehyde in cacodylic buffer solution 0.1 M, pH 7.2) and then dehydrated by dipping in aqueous ethanol solutions at increasing alcohol concentrations. For SEM imaging, dried samples were sputter-coated (SEM Coating Unit PS3, Assing S.p.A., Rome, Italy) with a thin layer of gold in an argon atmosphere (20 mA at  $5 \times 10^{-7}$  Pa for 30 s).

## 2.9. Sample imaging: CLM imaging

Evaluation of cell attachment and growth on the seeded scaffolds was performed by confocal laser microscopy (CLM; Nikon Eclipse, Ti-E, Japan) after rhodamine phalloidin and DAPI staining according to the manufacturer's protocol (Molecular Probes Inc., OR, USA). Fixation with a formaldehyde solution (4% formaldehyde in PBS solution) and permeabilization with Triton X (0.2% Triton X in PBS solution) was performed before staining.

## 2.10. Cellular metabolic activity and viability assays

The metabolic activity and viability of the cells were examined by Alamar Blue (Biosource International Inc., USA) assay. The active constituent of Alamar Blue is resazurin and it is a non-toxic, cell-permeable compound, blue and non-fluorescent. Once entered in the cells, resazurin is reduced to resorufin, which produces very bright red fluorescence. Viable cells constantly convert resazurin to resorufin, thereby generating a quantitative measure of viability. The assay was performed according to the manufacturer's instructions. Samples, placed in 48-well plate, were seeded with  $1 \times 10^4$  MG63 cells and incubated.

To measure cell metabolic activity at the desired time points (2, 9, 16 and 18 days), in the Alamar Blue assay an extra time point was added to better analyse the metabolic activity over time, since a rapid increase of absorbance was registered after day 16. The seeded scaffolds were incubated for 4 h at 37 °C with fresh culture medium (RPMI without phenol red) supplemented with Alamar Blue solution diluted according to the manufacturer's

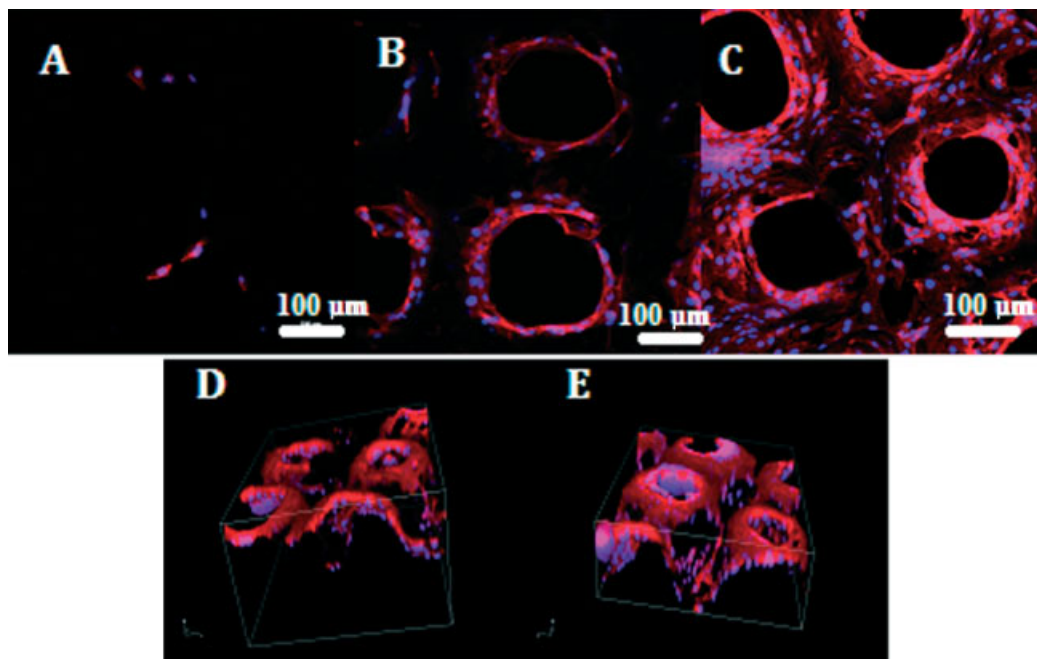


Figure 7. CLSM images of MG63 cells adhering to PDLLA scaffolds after (A) 2 days, (B) 9 days and (C) 16 days of cell culture, and 3D CLSM images of scaffolds after 9 days of cell culture (D); height is 318  $\mu\text{m}$  and after 16 days (E) height is 188  $\mu\text{m}$

data sheet (simply adding the Alamar Blue reagent as 10% of the sample volume). A total of three replicates was used for each sample and each replicate was split into four other samples in the final reading. The blank reference was taken from wells without cells incubated with the Alamar Blue solution. Absorbance was measured at 570 and 620 using a microplate reader.

Cell viability was further assessed with confocal laser microscopy (CLM) after staining with fluorescein diacetate–propidium iodide (FDA–PI; Molecular Probes). FDA stains viable cells green, while PI stains necrotic and secondary apoptotic cells red. The assay was performed according to previously published methods (Chim *et al.*, 2000; Martina *et al.*, 2005).

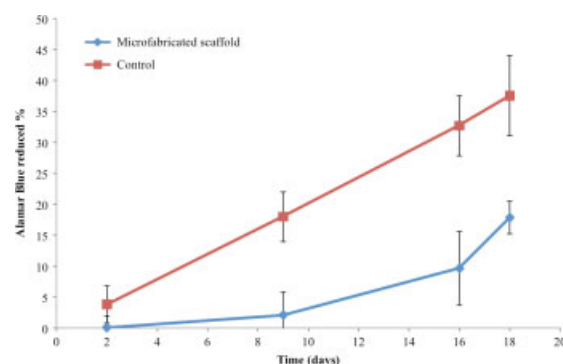


Figure 8. Alamar Blue assay for MG63 cell culture on PDLLA scaffolds

### 3. Results and discussion

PDLLA scaffolds with predetermined pore size (100 and 200  $\mu\text{m}$ ) and shape were prepared by a home-made microfabrication apparatus by adjusting process parameters. SEM images of these scaffolds are given in Figure 2.

In the preparation of the well-ordered scaffold structures, setting process parameters is very important. Wrong setting of the machine combined with improper polymer solution viscosity resulted in defects in the final scaffolds. The choice of the solvent (or combination of solvents) and polymer solution concentration are supposed to guarantee a continuous flow of the material out of the needle with a proper solvent evaporation rate. Low boiling temperature solvents such as chloroform or dichloromethane evaporated too fast, even on the needle tip, causing discontinuous flow of the polymer solution

and accumulation of polymer at the tip of the needle. Similar results could also be observed when the solution concentration was too high. For the preparation of PDLLA scaffolds, non-continuous flow created defects such as big droplets and broken fibres in different layers (Figure 3A, B). High boiling temperature solvents such as dimethyl formamide, on the contrary, are difficult to remove from the material, so that the polymer would spread on the collecting plate and the following layers would collapse over the others (Figure 3C, D). The same effect could be observed when the polymer concentration was too low.

Needle dimensions together with micrometric resolution of the slides are fundamental process parameters to obtain a proper microporous scaffold. The final matrix should be designed to prevent physical distortions due to cell culture conditions. PDLLA scaffolds, prepared from the proper combination of process and material parameters, showed repetitive and ordered structures (Figure 2).



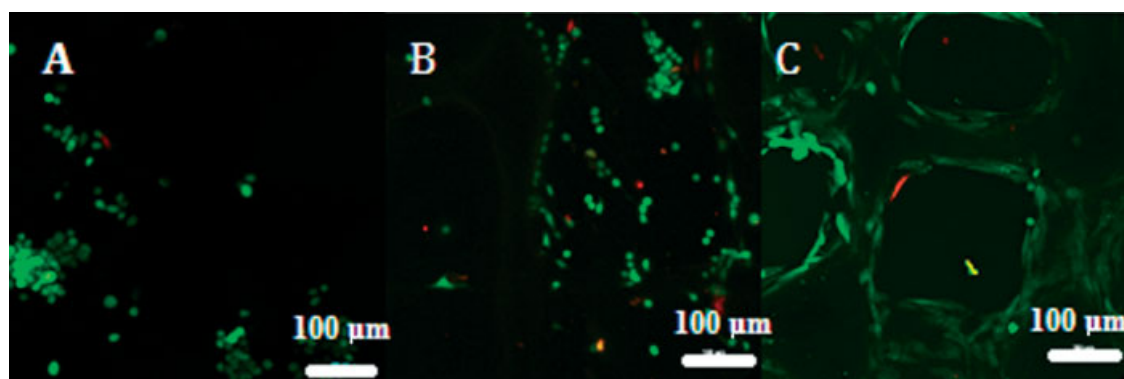


Figure 9. Cell viability test after (A) 2 days, (B) 9 days and (C) 16 days of cell culture

Some defects were still present in the 200  $\mu\text{m}$  structure; however, the pore sizes and their distribution were controlled and appropriate comparison with the 100  $\mu\text{m}$  structure can be made.

For this scaffold microfabrication production process, the viscosity of the solution is the most important parameter. The results obtained by the rheological tests performed on the solutions are related to the flow viscosity (shear stresses) under dynamic conditions, i.e. the complex viscosity,  $\eta^*$ , at varying frequencies. According to the Cox–Merz rule (Gupta, 2005), the complex viscosity as a function of the frequency corresponds to the shear viscosity as a function of the shear rate.  $\eta^*$  for the PDLLA solution showed a significantly decreasing trend over the chosen frequency range (1–100 rad/s) (Figure 4).

Molecular weight (MW) distribution of the polymer was examined by GPC tests. The average molecular weights of the starting PDLLA, microfabricated PDLLA scaffold and PDLLA film were found to be 252 kDa (pdi = 2.03), 206 kDa (pdi = 1.83) and 177 kDa (pdi = 1.88), respectively. The MW distributions of the starting powder, the cast film and the microfabricated scaffold are given in Figure 5. Differences in the MWs of PDLLA before and after microfabrication and in PDLLA cast films may be caused by DMF solvent. Most probably, the MW reduction could therefore be attributed to hydrolytic degradation caused by the presence of water in the hygroscopic DMF.

Since the presence of traces of solvent may affect the biocompatibility of the material, LDH-based *in vitro* toxicology assay was used to estimate the cytotoxicity of the produced scaffold. The average values of the measured absorbance and the standard deviations (SDs) for the microfabricated scaffold and the control (PBS and medium) are  $0.052 \pm 0.004$  a.u. and  $0.0470 \pm 0.003$  a.u., respectively. The absorbance values in the case of microfabricated scaffolds fall within the range of the SD of the control sample. Therefore the test showed that the material, after microfabrication, is not cytotoxic.

Preliminary biological evaluations were performed by seeding human osteosarcoma-derived osteoblasts (MG63) on microfabricated scaffolds with scaffolds having 100  $\mu\text{m}$

pore size. SEM images refer to both external and internal areas of seeded scaffolds (Figure 6). The inner regions were made visible after removing the outer layers and following glutaraldehyde fixation for SEM imaging. The images show that cells completely covered the surfaces of the scaffolds and penetrated to the inside by 7 days of cell culture. Osteoblasts could invade the inner parts of the scaffolds, with cells adhering to single filaments or bridging between different filaments making the pore walls. However, the amount of cells in the bulk of the scaffold appears to be much lower compared to the surface. Cell migration was thus hindered by the small size of the pores.

Several studies have reported on the effect of porosity and pore size on osteoblast growth (Ishaug-Riley *et al.*, 1998; Tsuruga *et al.*, 1997). Pore sizes in the range 100–400  $\mu\text{m}$  are usually recommended in order to have proper cell growth and tissue regeneration, to enable waste removal from metabolic activity and for vascularization (Karageorgiou *et al.*, 2005; Hollister 2005). A porous scaffold can be infiltrated by cells or not, depending on its morphology and cell-seeding conditions. In order to evaluate cell behaviour in terms of cell attachment, growth and migration, we used the same cell-seeding conditions (i.e. cell seeding density and seeding procedure) for comparison. Under these conditions, cell adhesion was observed on scaffolds with 100  $\mu\text{m}$  pore size and displayed a uniform superficial cell layer, arguably due to the spread of cells by a bridging method, whereas scaffolds with larger pores enhanced cell infiltration, hindering the bridging mechanism and disrupting the formation of a cell layer. As a result, cell penetration becomes evident.

It was therefore decided to increase the size of the pores from 100 to 200  $\mu\text{m}$ ; a new set of scaffolds was prepared and cell culture experiments were done using the same cell line. The cells were fixed and stained with rhodamine phalloidin/DAPI to examine using confocal laser microscopy on days 2, 9 and 16. Confocal microscopy images showed that the cells started invading the pores, adhering to the matrix, and with increased culture time a deeper growth of cells inside the pores was observed (Figure 7). As the incubation time was increased, an increase in cell number was observed.



Cellular metabolic activity and viability were examined using Alamar Blue. By comparing the absorbance values of control and microfabricated scaffolds (Figure 8), it is possible to notice that there is a considerable increase in the absorbance of control samples, which represent high metabolic activity of the cells to reduce the Alamar Blue. The increase is slower for microfabricated scaffolds until day 16, then a rapid increase is shown. This trend was expected according to the seeding procedure. After seeding, the scaffolds were moved from the original well plate to a new one. Following this practice, most cells were lost at the first time point, being attached to the well plate and lowering the kinetic. Alamar Blue results indicate that the cell number continuously increased between 2 and 16 days, in accordance with the confocal images (Figure 7).

For cell viability, further verification was performed using CLM after FDA/PI staining. FDA stained the viable cells and resulted in green fluorescence, while PI stained apoptotic cells red (Figure 9). During the observed period, viable cells were predominant and they increased with time. After 16 days the cells were still viable, fully spread and well distributed on the scaffold surface.

## 4. Conclusion

Many studies have reported that the design of scaffolds for tissue engineering is important for directing cell proliferation and differentiation. With most of the scaffolding techniques there is a lack of well-ordered structure formation and reproducibility. Our microfabrication technique provides a good alternative for producing scaffolds with higher resolution, controlled geometry and porosity at  $\mu\text{m}$  size. In this study, a microfabrication technique was applied to fabricate PDLLA scaffolds with optimized parameters, and the adhesion and migration of osteoblast cells into the scaffolds were investigated. The 200  $\mu\text{m}$  pore size appeared to be optimal for permitting osteoblast cells to migrate inside the scaffolds and also to bridge the pore walls. It can be concluded that the scaffolds prepared in the desired geometry produced by our microfabrication technique could be good candidates for tissue-engineering applications.

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