Innovative tissue engineering structures through advanced manufacturing technologies

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A wide range of rapid prototyping (RP) techniques for the construction of three-dimensional (3-D) scaffolds for tissue engineering has been recently developed. In this study, we report and compare two methods for the fabrication of poly-(ε-caprolactone) and poly-(ε-caprolactone)–poly-(oxyethylene)–poly-(ε-caprolactone) copolymer scaffolds. The first technique is based on the use of a microsyringe and a computer-controlled three-axis micropositioner, which regulates motor speed and position. Polymer solutions are extruded through the needle of the microsyringe by the application of a constant pressure of 10–300 mm Hg, resulting in controlled polymer deposition of 5–600 μm lateral dimensions. The second method utilises the heating energy of a laser beam to sinter polymer microparticles according to computer-guided geometries. Materials may be fed either as dry powder or slurry of microparticles. Both powder granulometry and laser working parameters influence resolution (generally 300 μm × 700 μm), accuracy of sintering and surface and bulk properties of the final structures.

The two RP methods allow the fabrication of 3-D scaffolds with a controlled architecture, providing a powerful means to study cell response to an environment similar to that found *in vivo*.

1. Introduction

Tissue engineering and guided tissue repair are rapidly developing new areas of science, which generally require the use of special biodegradable and biocompatible scaffolds, as three-dimensional (3-D) supports for initial cell attachment and subsequent tissue organisation and formation. Conventional techniques for scaffold fabrication include textile technologies, solvent casting, emulsion freeze drying, particulate leaching, membrane lamination, gas foaming and melt molding [1–7]. The drawbacks of these techniques include long fabrication time, incomplete removal of residual particulates, poor repeatability, irregular and poorly interconnected pores and thin structures.

Complex organs and tissues, such as liver, heart and neural tissue, have a specific 3-D cell distribution and their engineering requires biomaterial scaffolds with a known and well-defined topography. Advanced manufacturing technologies, known as rapid prototyping (RP) technologies, are now developing to fabricate scaffolds with controlled architecture [8–22]. RP methods combine computer-assisted design (CAD) with computer-assisted manufacturing (CAM): 3-D computer models are sliced into two-dimensional (2-D) layers to fabricate complex 3-D structures layer-by-layer [23]. As the scaffold design is based on a computer software model, the desired 3-D structure can be easily controlled and eventually repeated. In this paper, we present and test two new developing RP methods by applying them to the fabrication of poly-(ε-caprolactone) (PCL) and poly-(ε-caprolactone)–poly-(oxyethylene)–poly-(ε-caprolactone) (PCL–POE–PCL) scaffolds. The first technique, pressure assisted microsyringe (PAM), is an automated system using a microsyringe and a stage controller, developed at the Interdepartmental Research ''E. Piaggio'' at the University of Pisa. The second method, selective laser sintering (SLS) is a well-known RP technology through which 3-D structures are created layer-by-layer, by heating and sintering powdered materials by means of the heat generated from a CO_2 laser [24–32]. SLS has recently been proposed [17] and here tested for the first time as a promising rapid prototyping fabrication method of scaffolds for tissue engineering.

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In this work we also compare the advantages and limitations of each technique with regard to resolution and process variables.

2. Materials and methods

2.1. Materials

Polymers used were a commercial PCL supplied by Polysciences, Inc. with an average molecular weight (M_w) of 45 000 and a tri-block (PCL–POE–PCL) copolymer, with 85 wt % ε-caprolactone and 15 wt % poly(ethylene glycol), synthetised by Cerrai *et al.* through a simple synthetic procedure, without using potentially toxic initiators [33–35]. Results of cytotoxicity and haemocompatibility tests demonstrated that biocompatibility of PCL–POE–PCL copolymer was good [36].

Poly-(ε-caprolactone) microspheres for SLS were prepared by a solvent evaporation procedure based on a single oil-in-water emulsion [37, 38]. Briefly, 60 mL of a 5% w/v polymer solution in chloroform (reagent grade purity; Carlo Erba, Italy) were dipped into 1000 mL of demineralised water under moderate magnetic stirring. The resulting emulsion was homogenised by a high-speed homogeniser (Art. Miccra-D8, Falc Instruments) at 23 500 rpm for 10 min in a beaker cooled in an ice bath. The mixture was kept under moderate stirring at ambient temperature for 24 h, to allow complete removal of solvent. The resultant microspheres were collected by centrifugation at 4000 rpm for 5 min and dried in an oven at 35 °C for a week. The microspheres were evaluated for surface morphology by scanning electron microscopy (SEM, Jeol JSM-5600 LV).

Poly-(ε-caprolactone)–poly-(oxyethylene)–poly-(ε-caprolactone) and PCL solutions for PAM were prepared by dissolving the polymers in chloroform at a concentration of 20% w/v.

Poly-(ε-caprolactone) and PCL–POE–PCL films were obtained from a 4% w/v solution in chloroform, both by casting a volume of 20 mL of solution onto a 25 mm diameter glass dish and spin casting some drops of solution onto a glass slide, at 1000 rpm for 30 s. Films were left at room temperature for 48 h and then kept in an oven at 35 °C and under reduced pressure for a week to allow complete solvent evaporation.

2.2. Pressure assisted microsyringe (PAM)

The PAM technology is a RP system for the fabrication of 2-D and 3-D scaffolds of biodegradable polymers [11–13]. The method is based on the deposition of polymeric layers by means of a stainless-steel syringe with a 10–20 μm glass capillary needle and a capacity of about 10 mL. A small amount of the polymeric solution is placed inside the syringe and then it is extruded from the tip by the application of filtered compressed air at a pressure of 10–300 mm Hg. The syringe, which is mounted vertically on z-axis, is part of a three-axis micropositioning system with a resolution of 0.1 μm. Two stepper motors move the deposition substrate (usually a 3 × 3 cm glass slide) along the x–y plane.

The entire system is interfaced and controlled by a personal computer through an IRIS (Eclypse, Pisa, Italy) card. The software driving the system was developed in C with a user-friendly graphic interface that allows design and deposition of a wide range of patterns with a well-defined geometry. Deposited structures may have a lateral dimension ranging between 5 and 600 μm, depending on the applied pressure, the viscosity of the solution, the motor speed (0.5–2.5 mm/s) and the dimensions of the syringe tip [12].

In this study 2-D structures (in the shape of square-meshed grids of 1 mm sides) were realised as they are useful for initial studies on cellular adhesion to polymeric patterns. 3-D structures, which are required for the regeneration of implantable tissues, were also obtained through the deposition of stacked layers (square-meshed grids), by moving the syringe up along the z-axis by an amount corresponding to the height of each layer. Each layer was shifted laterally, with respect to the previous one, by an amount corresponding to half mesh side. We realised structures composed of three polymeric layers.

The surface characteristics of scaffolds deposited by PAM were examined by a Jeol JSM-5600 LV SEM and an OLYMPUS AX 70 optical microscopy (OM).

2.3. Selective laser sintering (SLS)

The SLS machine of this study is a prototype designed and built at the Department of Mechanical, Nuclear and Production Engineering of the University of Pisa, with the aim to explore SLS technology as a new method for the fabrication of scaffolds. The machine consists of a work chamber equipped with a platform able to shift along the z-axis and a laser block, including the laser beam and a system of galvanometric mirrors, which are positioned to precisely address the beam. Just before reaching the work plane, the laser beam goes through a focusing lens, which warrants the constancy of the laser operating parameters. The CO_2 laser used (SYNRAD model J48-5S) has a nominal power of 50 W. When the laser beam is off, its direction is indicated to by a red light emission diode (LED) laser with a low power (about 2 mW). The machine is PC-driven by means of a software for laser guidance (CADMARK, Quantasystem s.r.l). The materials to be processed may be either in powder or slurry form. Slurries are obtainable by dispersing the polymer in a small amount of a volatile, non-inflammable, non-toxic non-solvent.

Preliminary experiments in the fabrication of scaffolds by the SLS machine were performed using PCL, a well-known biocompatible material [39–42]. We used a slurry of PCL particles, obtained by dispersing the polymer in a small amount of demineralised water, in order to achieve a better sintering of the particles with respect to that attainable by dry powder. First, a very thin layer of PCL slurry (0.3 mm) was delivered and levelled on a glass slide fixed on the supporting cylinder, placed on the platform able to move along z-axis. Then the laser sintered specific areas of the layer according to the instructions of a CAD file, as to obtain 2-D scaffolds in the shape of square (2 mm × 2 mm)-meshed grids. Three-dimensional objects were fabricated by stacking more layers along the z-axis, according to the following procedure. After a layer is completed the platform lowers

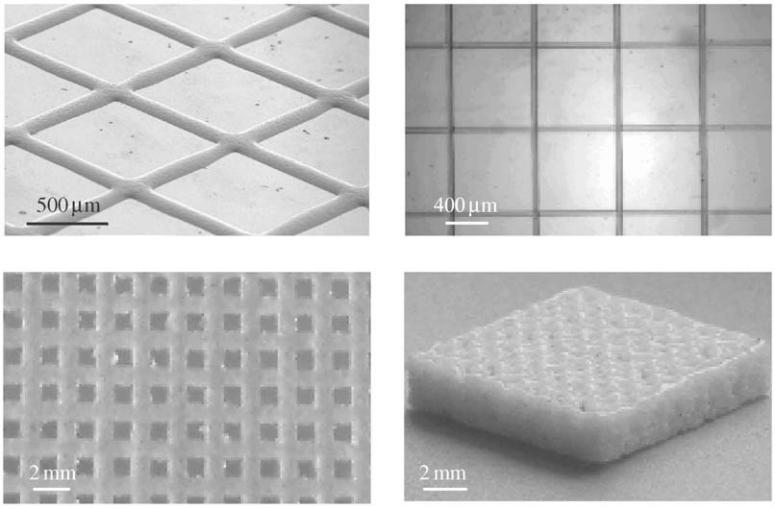

Figure 1 SEM picture of PCL–POE–PCL grids deposited by PAM (top left); OM images of PCL grids deposited by PAM (top right); photographs of laser sintered 2-D (bottom left) and 3-D (bottom right) PCL structures.

slightly by an amount fixed by the operator, another thin layer of polymer slurry is delivered and the laser scans selected areas of the current layer providing enough energy to fuse the powder within the layer and to bond it to the previous one. The process is repeated until a 3-D object is built from the bottom up. We fabricated square prisms, measuring 15 mm (length) by 15 mm (breadth) by 3.3 mm (height), through a sequence of 11 layers consisting of square (1.3 mm × 1.3 mm)-meshed grids. Our final structures were dried at ambient temperature for a week. The unsintered powder particles were brushed away. Selective laser sintered objects were characterised by means of OM.

2.4. Fibroblasts culture and characterisation

Two-dimensional scaffolds (grids and films) were prepared for cell culture according to the following procedure. The scaffolds realised were placed under vacuum for a week to ensure that all traces of solvent had evaporated. Dry samples were washed with a 70% ethanol solution in sterile water and UV-sterilised for 15 min on each side. Polymer structures were coated with gelatin (Sigma, Italy) (here selected as an adhesion protein), by leaving them in a bath of 1% gelatin aqueous solution for 1 h.

NIH-3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Cambrex, Italy) with high glucose, 10% foetal calf serum (Cambrex, Italy), 1% glutamine (Cambrex, Italy), penicillin (200 U/ml) (Cambrex, Italy) and streptomycin (200 µg/mL) (Cambrex, Italy). Culture was maintained in an incubator equilibrated with 5% CO_2 at 37 °C. The polymer structures and films were seeded with a 100 000 cells/mL suspension. After a culture time of 2, 4 and 24 h respectively, they were fixed and stained. Samples were analysed under an OM (Olympus AX 70) and the ratio between the number of cells on the polymeric structures and the total area of the polymer substrate was calculated as an index of cell density [13].

Both cells of a reference sample (a petri dish coated with gelatin) and cells seeded on scaffolds with different geometries (spin-cast films and square-meshed grids) were examined for comparison to evaluate the effect of scaffold topography on cell growth.

3. Results and discussion

3.1. Characteristics of scaffolds fabricated by PAM

Poly-(ε-caprolactone)–poly-(oxyethylene)–poly-(ε-caprolactone) and PCL 2-D grids deposited by PAM are reported in Fig. 1(a) and (b), respectively. Grid lines are about 80 µm wide for PCL and 150 µm for the copolymer. Fig. 2 shows the dependence of line width on the applied pressure for PCL–POE–PCL grids. It is evident that as applied pressure increases between 30 and 100 mm Hg, line width takes values close to those predicted by the model previously reported [12], increasing almost linearly as a function of pressure.

3.2. Characteristics of scaffolds fabricated by SLS

Scanning electron microscopy analysis of PCL particles confirmed their spherical shape, smooth surface and size uniformity (about 1 µm). Powder granulometry is a fundamental parameter for the precision degree of the

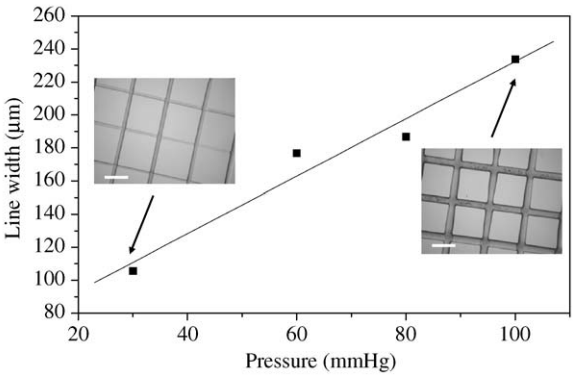

Figure 2 Effect of the applied pressure on line width for PCL–POE–PCL structures deposited by PAM and OM images relative to grids fabricated at 30 and 100 mm Hg. Bars indicate 500 µm.

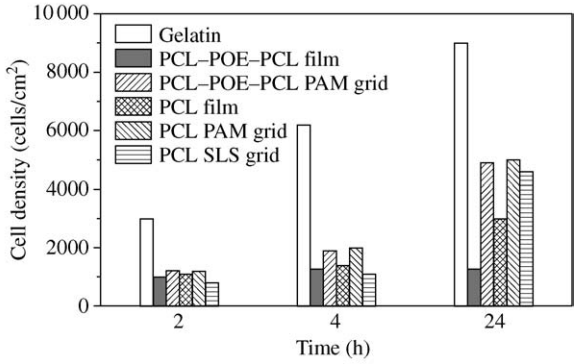

Figure 3 Cell density data for copolymer and PCL scaffolds (films and grids) as compared to control layer (gelatin) at different culture times (2, 4, 24 h). Column heights correspond to the mean values.

sintered structures. With small-sized particles with respect to laser spot ($< 50\,\mu m$), an increased vertical resolution of structures and lateral accuracy of sintering may be achieved, whereas a narrow polydispersity index assures an even thermal transfer and influences layer bonding and width uniformity of sintered lines. The first experiments with PCL were aimed at finding the optimal values of the fill laser power (P) and the beam speed (BS) to sinter the slurry. The fill laser power is a percentage of the duty cycle, which determines the power available from the laser beam at the bed surface. PCL laser sintered structures were produced under a fill laser power of 2 W and a beam speed of 20 mm/s. The energy density (ED), regarded as the applied laser energy per unit area, is directly proportional to the ratio between the fill laser power and the beam speed. Its value is a fundamental parameter as it affects properties such as surface roughness, density, tensile strength, dimensional accuracy, occurrence of curling and cracking [27]. Photos of 2-D and 3-D scaffolds fabricated by SLS are reported in Fig. 1(c) and (d), respectively.

3.3. Cell attachment results

Fig. 3 shows cell density on scaffolds (spin-cast films and grids) and on a gelatin layer, as a function of culture time. The ratio between cell density on structures and on the gelatin layer at any time may be taken as an index of cell adhesion efficiency.

For PCL–POE–PCL copolymer, mean values of efficiency were found to be 41%, and 33% after 2 h, 31% and 21% after 4 h and 55% and 14% after 24 h, respectively for scaffolds deposited by PAM and spin-cast films. Cells displayed a better adhesion to structures realised by PAM than to film-shaped scaffolds, both in terms of cell density and distribution (Fig. 4). In particular, for scaffolds in the shape of square-meshed grids, cell distribution becomes even with increasing culture time, until, after 24 h, the entire scaffold is covered with cells. Cell density on spin-cast films does not change with time. For PCL, mean value of efficiency was found to be 27%, 40% and 37% after 2 h, 18%, 32% and 23% after 4 h and 51%, 56% and 33% after 24 h culture time for grid-shaped scaffolds fabricated by SLS and PAM and for spin-cast films, respectively. Cell density on laser sintered grids is the lowest at low culture times (2–4 h), whereas, after 24 h, it increases greatly and becomes intermediate between that on films and that on scaffolds deposited by PAM. This finding suggests that cell adhesion is affected both by scaffold topography (geometry and surface roughness) and a greater affinity of cells for PCL. Adhesion is cell type specific and also depends on scaffold surface characteristics as well as material. Flexible RP techniques such as PAM and SLS can help in identifying the optimum architecture for a particular tissue.

3.4. Comparison of techniques

The two RP methods analysed differ in many ways: resolution, nature of processed material, 3-D assembling and surface characteristics of shaped objects. In the PAM microfabrication system, operating parameters can be adjusted in such a way to achieve a high resolution of about 10 μm [12]. Lateral resolution of SLS method is of the order of the laser spot (about 700 μm in our case), whereas vertical resolution is mainly limited by granulometry and it is about 300 μm for our structures. As for the type of feed material, PAM technique requires a small volume (a few millilitres) of a concentrated polymer solution, whereas materials processed by SLS are in the form of a dense aqueous slurry, thus avoiding the use of potentially toxic solvents. Since for SLS the unsintered material is used for the stacking of layers, no external supports are needed to build 3-D structures. On the contrary, fabrication of 3-D scaffolds by PAM technology implies the use of a support, generally a water-soluble polymer, between each layer. The main advantage of PAM and SLS is their simplicity, as they do not require special skills: once the geometry has been decided and input to the PC, the syringe is filled with a few millilitres of solution (PAM) or some slurry is delivered on a substrate (SLS), and then many scaffolds may be rapidly fabricated. Another advantage lies in their versatility: PAM offers the possibility to modulate line width and height, by varying operating parameters,

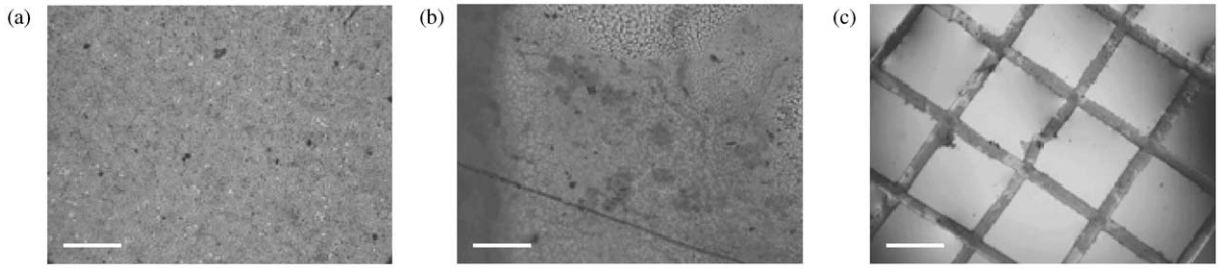

Figure 4 Photos of NIH 3T3 fibroblasts cultured over a gelatin layer (a), a copolymer spin-cast film (b), a copolymer grid (c) for 24 h. Bar indicates 500 μm.

such as applied pressure, motor speed and polymer concentration, whereas, for SLS, granulometry of particles and laser working parameters affect surface roughness and densification degree [27] and allow tailoring of superficial and bulk properties according to the type of application. As a consequence, PAM and SLS technologies are suitable methods for the study of cell motility, organisation and reaction to different topographies. The main limitations of PAM arise from the infrastructure investment, the narrow range of viscosities which can be employed to obtain high-resolution structures [12], the inability to incorporate particulates for leaching due to the risk of plugging the syringe tip. One of the disadvantages of SLS is the possibility that polymer degradation phenomena occur due to the laser energy. Degradation can be avoided by processing polymer under nitrogen atmosphere. Limitations may also arise from the difficulty to incorporate sensitive biomolecules as they can be easily degraded by laser energy.

4. Conclusions

In this paper two RP technologies, PAM and SLS, were investigated and successfully applied to produce simple 2-D and 3-D scaffolds.

Poly-(ε-caprolactone), already used for PAM microfabrications [12], has been here selected as a modelling material for the fabrication of laser sintered scaffolds by a new custom made prototype machine. The purpose was that to evaluate the actual performance of the machine in the scaffold production field and to eventually improve it through technical modifications. As PCL bioerosion can be increased by copolymerizing it with a hydrophilic polymer, a PCL–POE–PCL copolymer was tested for the construction of 2-D and 3-D scaffolds by PAM technology.

The degree of resolution of the two technologies was substantially different, as PAM allows the fabrication of fine-featured microstructures (down to 5–10 μm wide and 5 μm high), whereas SLS structures have a resolution of about 300 μm (height) × 700 μm (width). General advantages and disadvantages of the two methods as RP technologies for tissue engineering were investigated.

Cell attachment to scaffolds was higher than to membrane samples, indicating that surface topography and roughness have a positive influence on adhesion.

In future, detailed studies on the effect of scaffold architecture on cellular proliferation, differentiation and motility will be carry out, as the flexibility and simplicity of the two techniques allow to fabricate structures with different geometries in a short time. Furthermore, both techniques will be utilised to construct 3-D scaffolds that will allow the study of cell response to complex microenvironments which mimic those found *in vivo*.

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