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Development, Characterization and Cell Cultural Response of 3D Biocompatible Micro-Patterned Poly- ϵ -Caprolactone Scaffolds Designed and Fabricated Integrating Lithography and Micromolding Fabrication Techniques

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Abstract

Scaffold design and fabrication are very important subjects for biomaterial, tissue engineering and regenerative medicine research playing a unique role in tissue regeneration and repair. Among synthetic biomaterials Poly- ϵ -Caprolactone (PCL) is very attractive bioresorbable polyester due to its high permeability, biodegradability and capacity to be blended with other biopolymers. Thanks to its ability to naturally degrade in tissues, PCL has a great potential as a new material for implantable biomedical micro devices. This work focuses on the establishment of a micro fabrication process, by integrating lithography and micromolding fabrication techniques, for the realization of 3D microstructure PCL devices. Scaffold surface exhibits a combination in the patterned length scale; cylindrical pillars of 10 μ m height and 10 μ m diameter are arranged in a hexagonal lattice with periodicity of 30 μ m and their sidewalls are nano-sculptured, with a regular pattern of grooves leading to a spatial modulation in the z direction. In order to demonstrate that these biocompatible pillared PCL substrates are suitable for a proper cell growth, NIH/3T3 mouse embryonic fibroblasts were seeded on them and cells key adhesion parameters were evaluated. Scanning Electron Microscopy and immunofluorescence analysis were carried out to check cell survival, proliferation and adhesion; cells growing on the PCL substrates appeared healthy and formed a well-developed network in close contact with the micro and nano features of the pillared surface. Those 3D scaffolds could be a promising solution for a wide range of applications within tissue engineering and regenerative medicine applications.

Keywords: Microfabrication; Micromolding; Tissue engineering; Biomaterials; 3D scaffold; Poly-epsilon-caprolactone; Fibroblasts

Abbreviations

PCL: Poly- ϵ -caprolactone; ECM: Extracellular Matrix; PLGA: poly-L-(glycolic acid); PLLA: poly-L-(lactic acid); PLA: poly (lactic acid); PEG: polyethylene glycol; Si: Silicon; DRIE: Deep Reactive Ion Etching; ATCC: American Type Culture Collection; DMEM: Dulbecco's Modified Eagle's Medium; BSA: Bovine Serum Albumin; PBS: Phosphate Buffered Saline; SEM: Scanning Electron Microscopy

Introduction

The latest improvements in biomaterials design and characterization, along with the impressive progress achieved in nanotechnologies and microfabrication techniques, have led to the production of highly performant micro- and nano-structured materials and devices designed for a variety of biomedical tissue engineering applications [1-5]. In recent years, micro- and nanotechnologies are giving extraordinary answers to the specific

needs of regenerative medicine, by allowing the fabrication of new three-dimensional (3D) biocompatible cell culture systems. Despite the 3D *in vivo cells and tissue* growth, cell culture studies are mainly daily performed on two-dimensional (2D) glass or plastic surfaces, thus resulting in flat monolayers, which limit the biological relevance of assays in cellular and clinical research. To overcome such limitations due to the use of 2D cell culture systems use, recently 3D scaffolds have recently been fabricated by offering the possibility to regulate cell/tissue morphogenesis through the mimicking of the extracellular matrix (ECM) architecture. ECM, with its complex 3D interlacement of elastin, collagen, glycosaminoglycans, glycoproteins and proteoglycans, regulates the regeneration and maintenance of tissues and organs, thus playing a key role in successful tissue engineering applications, such as wound healing [6,7], skin, cartilage, bone and neural regeneration [8,9]. Several tissue engineering methods involve cell seeding onto 3D scaffolds for *in vitro* [10] or *in vivo* neotissue growth [11]. Microfabrication is one such technology with the higher potential to improve scaffold fabrication. Indeed thanks to its control over micro- and nano design, it allows the realization of devices with superficial features ranging from several nanometers to hundreds of microns, to be used for *in vitro* and *in vivo*

medicine regeneration and drug delivery use. Within tissue engineering, photolithography is one of the most applied microfabrication technique, it transfers a specific pattern from a mask to a thin film by light localization. By combining steps of masked exposure and thin film application, resists can be processed with high aspect ratios. In most cases, photolithography is incorporated in a multi-technique micromachining process characterized by steps such as chemical etching, which can be either wet, with acids and bases, or dry, commonly with plasmas, chemical vapor deposition or oxide formation.

Micromachining and photolithography can also be used in the process of micromolding fabrication for the transfer of a master with features to a polymer through techniques like injection molding, hot embossing, and plastics casting. Natural polymers, such as collagens, fibrin, gelatin, albumin, elastin, fibrinogen, silk fibroin, glycosaminoglycan, starch, cellulose and chitosan, have been used to repair bone, nerves, cartilage and skin [12-15]. Whilst naturally derived biomaterials may closely simulate the *native cellular* environment, they are frequently characterized by poor mechanical performances. Each biomimetic scaffold should have the mechanical strength and elasticity needed for the fabrication of a macro-microporous scaffold able to keep its structure also after implantation. In order to overcome the aforementioned problems associated with natural polymers, many synthetic polymeric materials, such as poly-L-(glycolic acid) (PLGA), poly-L-(lactic acid) (PLLA), poly(lactic acid) (PLA), polyethylene glycol (PEG), have been developed. Such synthetic biomaterials can be fabricated and characterized, in order to tune many more physical and chemical properties. Within the biomedical field, PCL, bioresorbable hydrophobic polyester, as synthetic biomaterial, has been extensively investigated [16-18]; it is, in fact, a semi-crystalline polymer with low melting point (59–64°C) and a low glass-transition temperature (–62°C). Compared to PLA, PGA and many other resorbable polymers, the degradation of PCL is relatively slow [19], thus resulting as a much more suitable material for long-term implants and drugs delivery [19-22]. As fibroblasts could be readily cultured in the laboratory and have a key role in ECM deposition and epithelial–mesenchymal interactions, this paper will describe a fabrication process employing a microfabricated silicon (Si) master, which allows the rapid realization of economical and versatile biocompatible and bioresorbable 3D PCL scaffolds for NIH/3T3 cell cultures.

Materials and Methods

Microfabrication

Silicon negative master fabrication. Pillars textured superhydrophobic surface was fabricated by using Poly- ϵ -caprolactone (PCL) in a micromolding technique approach by means of a negative silicon master as mold. For what concern master fabrication, optical lithography and Deep Reactive Ion Etching (DRIE) techniques were employed. P-type silicon wafers (100) with resistivity 5-10 Ω /cm were cleaned with a standard procedure: wet cleaning with acetone (Sigma-Aldrich), isopropanol (Sigma-Aldrich) and O₂-Plasma cleaning (TUCANO GABETTI, V_{bias}= -320 V, P=100 W), then etched in a 4% (w/v) Hydrofluoric Acid solution, rinsed several times in deionized water and finally blown with N₂. Optical lithography masks were fabricated using direct write lithography equipment (DWL66fs, HEIDELBERG Instruments). Standard microlithography was employed: SPR220 positive tone resist (thickness 2 μ m SPR220) was

spin coated obtaining, after lithography, a periodic texture of void disks to be used as mask for DRIE of silicon (ICP-RIE, SI 500, SENTECH Instruments GmbH). The DRIE process operated a pulsed time-multiplexed etching, with repeated cycle of (i) C₄F₈ - chemically inert passivation layer deposition and (ii) SF₆ - based anisotropic silicon etching. Each cycle was followed by a refractory period of reactor cleaning and gas evacuation. In this manner bulk silicon wafers were transformed obtaining a periodic hexagonal lattice of cylindrical holes, with nano-patterned vertical sidewalls. As obtained, silicon molds were subjected to standard wet cleaning, followed by a piranha solution cleaning (H₂SO₄:H₂O₂, 3:1 volume) and, finally, to an oxygen plasma cleaning to remove residual resist and contaminant. In order to reduce the adhesion of polymer during the nano-imprinting process, an anti-sticking layer was deposited by phase vapor chemical deposition. Few drops of silanizing agent (Trichloro(1H,1H,2H,2H-perfluorooctyl) silane 97%, Sigma) were placed into a glass vial and let evaporate in vacuum condition by exposing the silicon master surface to be functionalized. Silicon masters were then heated (T=110 °C) to stabilize the silane layer and then washed several times with acetone, isopropanol and deionized water.

Micromolding

Microstructured nanopatterned PCL surface was obtained by micromolding as follows. Poly- ϵ -caprolactone (PCL) (Sigma Aldrich, M_n = 70000-90000, T_{melt} = 60°C) was melted on a glass slide previously cleaned (Acetone, 2-propanol and several rinses with H₂O) and placed on a hot plate (T=130°C). Negative master was put in contact with the melted polymer avoiding the formation of air bubbles at the polymer-silicon interface. Polymer molding was performed with a press (Nanoimprinter, Obducat) in a two steps process: first, molding at T=68 °C and P= 5 bar; second, cooling and polymer solidification at constant pressure (P=5 bar) until reaching Tamb. The peel-off (silicon master detachment) was performed in water by applying a slight pull. Positive polymer structures, with aspect ratio more than 2, were obtained. Textures of a periodic hexagonal lattice of cylindrical biopolymer pillars were obtained. Microstructured nanopatterned PCL scaffolds were washed in deionized water overnight.

Cell culture

The NIH/3T3 mouse embryonic fibroblast cells were obtained from the American Type Culture Collection (ATCC). The NIH/3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, supplemented with 10% (v/v) fetal calf serum, penicillin G (100 U/ml) and streptomycin sulfate (100 μ g/ml). The cells were grown at 37°C in a humidified 5% CO₂ atmosphere.

Immunofluorescence

Single substrates were sterilized by immersion in 99.9% ethanol (34963, Sigma-Aldrich), washed twice with sterile deionized water and further sterilized by UV irradiation for 1.5 h. Then the substrates were treated with Poly-D-lysine hydrobromide (P6407, Sigma-Aldrich) and DMEM medium complete in a 1:1 (v/v) ratio respectively for 48 hours in a cell culture incubator (37°C, 5% CO₂, 95% humidity). After soaking, substrates were washed twice with sterile deionized water and, once dried, 4 x 10⁴ cells were plated on each substrate and grown for 24 hours. Afterwards the cells were fixed with 4% (w/v) paraformaldehyde for 1 hour at room temperature, permeabilized with 0.5% (v/v) Triton X-100 for 10 min and blocked with 1% (w/v) of

Bovine Serum Albumin (BSA) in phosphate buffered saline (PBS). At the end the samples were stained for 1 hour with antibody against Vinculin (Abcam ab18058) and detected with a secondary antibody (chicken anti-mouse Alexa Fluor 488, Invitrogen). Further for F-Actin staining, the cells were incubated with Alexa Fluor 546 phalloidin for 30 min at 4°C. Images were acquired at an upright Leica TCS SP5 AOBs TANDEM confocal microscope equipped with a 40X/0.80 APO L W UVI objective. Images were visualized and processed by using the Leica LAS AF, ImageJ and Adobe Photoshop CS3 softwares.

Scanning Electron Microscopy

Fibroblasts were grown for 24 hours and then fixed for 1 h in a solution of 1.2% (v/v) glutaraldehyde (G5882, Sigma) and 0.1 M sodium cacodylate (C0250, Sigma) at 4°C. After fixation, cells were extensively washed in the 0.1 M pH 7.4 sodium cacodylate buffer, and postfixed for 1 h in a solution of 1% (v/v) osmium tetroxide (CAS #20816-12-0, 19110, Electron Microscopy Sciences) and 0.1 M sodium cacodylate. After several washes in Milli-Q water, fixed samples were rinsed for 5 min in increasing concentrations of filtered (0.2 μm, Millex-LG Millipore) ice-cold ethanol (30%, 50%, 70%, 80%, 90% and 96% v/v), followed by 2 x 15 min rinses with filtered ice-cold 100% (v/v) ethanol. Graded dehydration with ethanol was followed by gradual replacement with hexamethyl disilazane (379212, Sigma) that was allowed to evaporate overnight in a fume hood. Samples were then glued with silver paint (Pelco) to Scanning Electron Microscopy (SEM) stubs, coated with 10 nm gold/palladium in a sputter coater (108 auto/SE Cressington), and observed with a JEOL JSM-6490LA variable pressure SEM.

Results

The fabrication approach of these biocompatible substrates consisted in obtaining a positive structure by polymer micromolding in a negative silicon master, fabricated by the means of standard optical lithography and Deep Reactive Ion Etching techniques as schematically summarized in Figure 1.

The optical resist layer was deposited onto cleaned Si wafer by spin coating and a void disk pattern (disk diameter and periodicity was set to be 10 μm and 20 μm, respectively) was defined by optical lithography (Figure 1Ai). Time multiplexed Deep Reactive Ion Etching process was employed to determine 10 μm depth cylindrical wells by digging the void disks pattern (Figure 1Aii). Si molds were imaged with a scanning electron microscope (Figure 1Aiii). The mold's surface was primed by means of anti-sticking layer deposition (Figure 1Bi). A direct nanoimprint process was performed by placing PCL pellets onto a silanized glass support, pre-melting at 130°C and placing the silicon mold in contact with the polymer to imprint (Figure 1Bii). After the nanoimprinting process, mold and support were peeled-off and a 500 μm freestanding PCL pillared surface was obtained (Figure 1Biii).

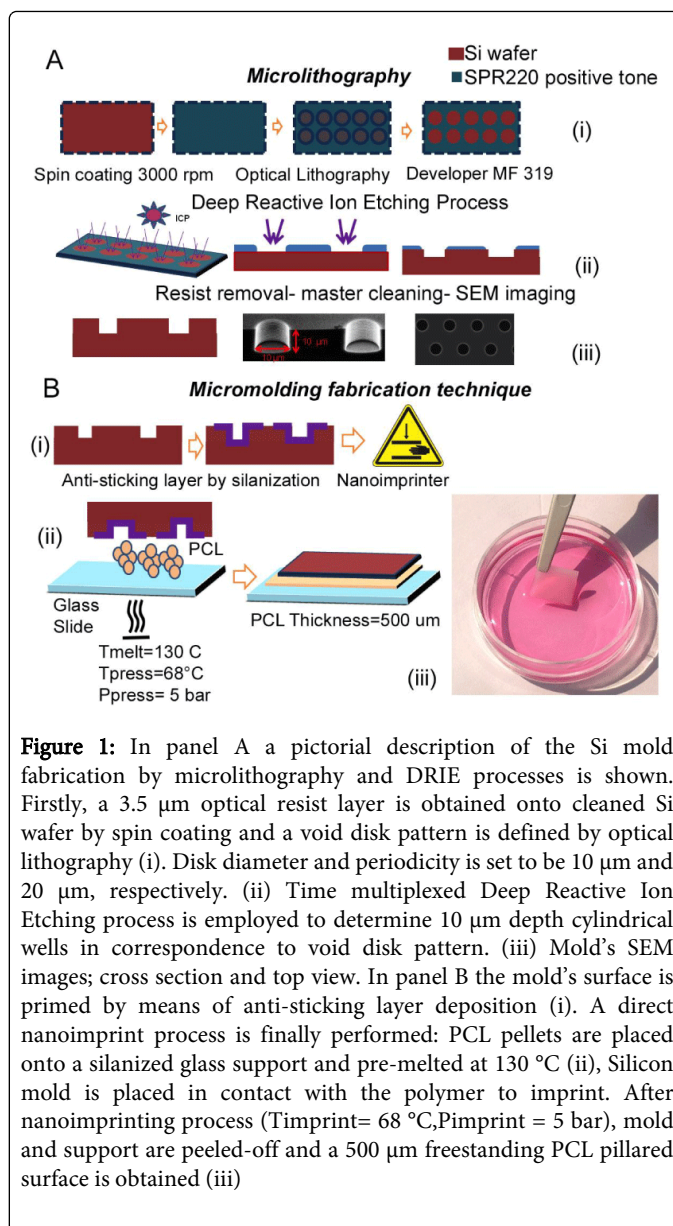


Figure 1: In panel A a pictorial description of the Si mold fabrication by microlithography and DRIE processes is shown.

Firstly, a 3.5 μm optical resist layer is obtained onto cleaned Si wafer by spin coating and a void disk pattern is defined by optical lithography (i). Disk diameter and periodicity is set to be 10 μm and 20 μm, respectively. (ii) Time multiplexed Deep Reactive Ion Etching process is employed to determine 10 μm depth cylindrical wells in correspondence to void disk pattern. (iii) Mold's SEM images; cross section and top view. In panel B the mold's surface is primed by means of anti-sticking layer deposition (i). A direct nanoimprint process is finally performed: PCL pellets are placed onto a silanized glass support and pre-melted at 130 °C (ii), Silicon mold is placed in contact with the polymer to imprint. After nanoimprinting process (T_{imprint}= 68 °C, P_{imprint} = 5 bar), mold and support are peeled-off and a 500 μm freestanding PCL pillared surface is obtained (iii)

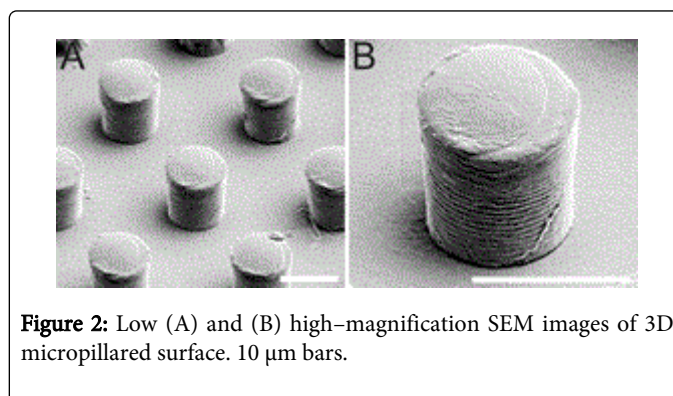


Figure 2: Low (A) and (B) high-magnification SEM images of 3D micropillared surface. 10 μm bars.

Cylindrical pillars of 10 μm in height and 10 μm in diameter were arranged in a hexagonal lattice with periodicity of 30 μm (Figure 2A); the sidewall of the pillars was nano-sculptured with a regular pattern

of grooves (Figure 2B). The pillared surface was characterized by an interconnected structure with controlled space size and shape. Pillars interconnection facilitated cell ingrowth providing higher surface area for cells adhesion and expansion. The proposed architecture of the scaffold resulted as crucial for an efficient nutrient, gas and waste exchange system.

Small areas of confluence, with tightly packed cells, were observed on the microfabricated surface (Figure 3A) although few patches of grouped cells were observed (Figure 3B); however, there were still large number of isolated cells (Figure 3C-3F) that could be seen to strongly interact with the pillars. Cells were healthy and formed a well-developed suspended network in close contact with the micro and nano features of the PCL substrate. In Figures 3B and 3E, it is evident how the particular pillars arrangement promoted along the z-axis a double order of healthy growing cells. Fibroblasts within 24 hours produced filopodia (white arrows) sensing the microstructured biopolymer, and thicker pseudopodia-like (white asterisks) processes appeared to use pillars as stepping-stones (Figures 3D and 3G).

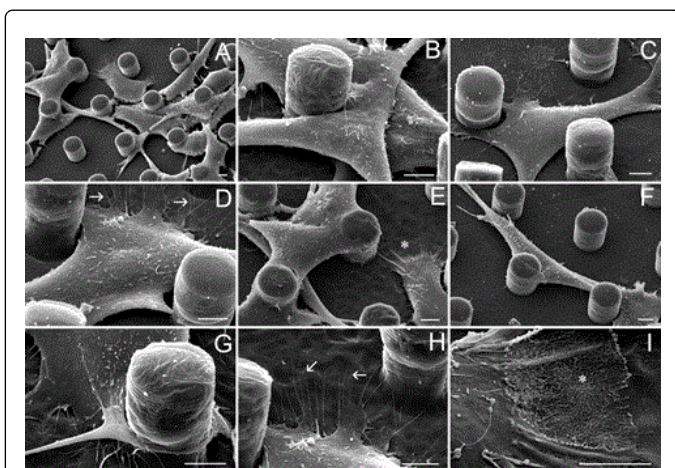


Figure 3: SEM images of NIH/3T3 cells cultured on micropillared surface. 5 μ m bars.

PCL substrates were able to spatially direct cells growth and promote the formation of extending bridges between adjacent pillars (Figure. 3 E, F). Cells moved by means of actin filaments, extension of sheet-like and rod-like protrusions at the cell-surface front; many filopodia (Figure. 3 D, H) and large lamellipodia (Figure. 3 A, E, I) were formed.

In Figure 4, an optimal fibroblasts proliferation on the surface is reported, showing as cells grow mostly suspended in the inter-pillar spaces. F-actin and vinculin proteins were stained, thus making visible the formation of solid cellular adhesion on the microfabricated substrate. The optimal expression and localization of these proteins allowed the visualization of lamellipodial focal adhesions structures, which are essential for cellular spreading and polarisation. Lamellipodia were associated with filopodia that actively probed the external environment, by collecting topographical and biochemical information from the Extracellular Matrix (ECM) and material surface. The presence of lamellipodial and filopodia focal adhesions structures evidenced the strong adhesion and spreading of cells, which indicates the high cytocompatibility of the substrates.

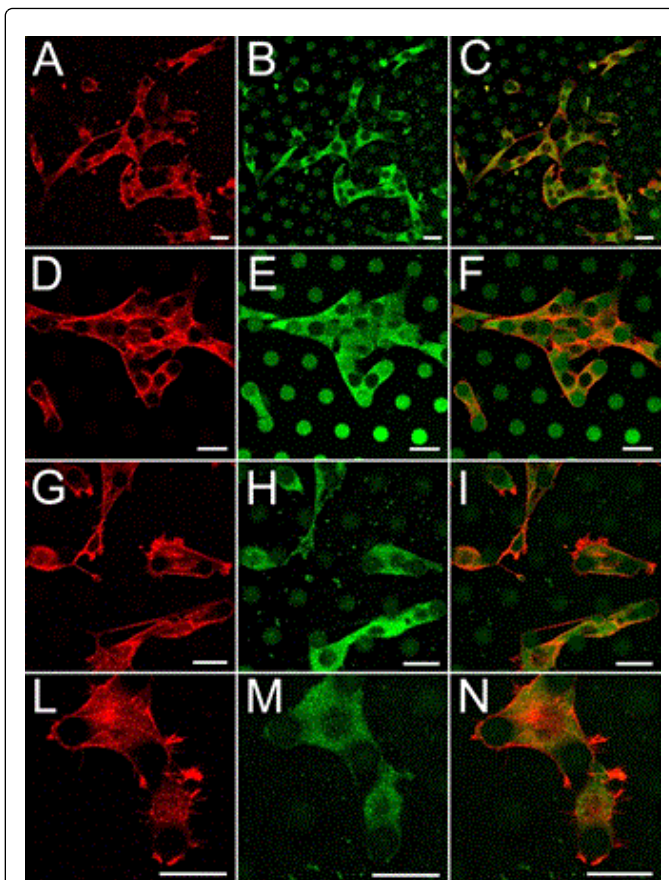


Figure 4: Confocal analysis of NIH/3T3 cells cultured on pillared PCL substrates; F-actin (red) and vinculin (green) expression is reported. The overlay between F-actin and vinculin is shown in the right panel. 20 μ m bars.

Discussion

The use of 3D polymeric scaffolds for cell lines, primary cells or stem cells is an increasingly widespread practice in tissue engineering. The requirements of scaffolds for medicine regeneration are different and strictly related to the structure and function of the tissue of interest. For these simple reasons, fabrication techniques need to be further developed to allow the realization of scaffold with the desired shape, degradation rate, porosity and mechanical properties. New design and manufacture methodologies are required and, in this context, our 3D microfabricated PCL scaffold represents an effective support for the realization of in vitro healthy cell cultures. Cells that are daily cultured on 2D cell culture system, flat dishes made of glass or polystyrene, adhere and spread on this surfaces forming unnatural attachments and proliferation. In the proposed pillared 3D cell culture system, cells grow suspended to one another forming, with the aid of the pillars, cell-to-cell attachments and double or multi order of growing cells, needed to achieve tissue and organ regeneration. Using the proper cell type, for instance embryonic stem cells, these layers could grow by differentiating into different tissues and organs with structural and physiological functionality. In this 3D cell culture environment, cells can exert forces on one another and can easily move and migrate; an efficient fluid transport is guaranteed by the

particular arrangement of the pillars on the device allowing optimal nutrients, gas and waste exchanges by growing cells suspended between the pillars. It should be noted that, due to the successful application of the PCL in tissue engineering therapeutics [23-26], our 3D scaffold could be also used as simple and inexpensive drug-laden bioresorbable solution. Thus, we highlight the suitability of the material used and the techniques applied for future development of in vitro and in vivo applications in the field of regenerative medicine and tissue engineering improving patients' survival and quality of life.

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