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Mesenchymal stem cells cultivated on scaffolds formed by 3D printed PCL matrices, coated with PLGA electrospun nanofibers for use in tissue engineering

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Abstract
Materials, such as biopolymers, can be applied to produce scaffolds as mechanical support for cell growth in regenerative medicine. Two examples are polycaprolactone (PCL) and poly(lactic-co-glycolic acid) (PLGA), both used in this study to evaluate the behavior of umbilical cord-derived mesenchymal stem cells. The scaffolds were produced by the 3D printing technique using PCL as a polymer covered with PLGA fibers obtained by electrospinning. The cells were seeded in three concentrations: \(8.5 \times 10^3\); \(25.5 \times 10^3\) and \(51.0 \times 10^3\) on the two surfaces of the scaffolds. With scanning electron microscopy (SEM), it was observed that the electrospun fibers were integrated into the 3D printed matrices. Confocal laser scanning microscopy and SEM confirmed the presence of attached cells and the lactate dehydrogenase release test showed the scaffolds were not cytotoxic. The cells were able to differentiate into osteogenic and chondrogenic lineages on the scaffolds. Mechanical test showed that the cells seeded on the 3D printed PCL matrices coated with PLGA electrospun nanofibers (3D + ES + SC) did not show significant difference in tensile modulus than the pure PCL matrix (3D) or PCL matrices coated with PLGA electrospun nanofibers (3D + ES). The combination of the two polymers facilitated the production of a support with greater mechanical stability due to the presence of the 3D printed PCL matrices fabricated by melted filaments and greater cell adhesion due to the PLGA fibers. The scaffolds are suitable for use in cell therapy and also for tissue regeneration purposes.

1. Introduction

Tissue engineering (TE) has attracted considerable attention in recent years as a promising area of medicine for reconstructing and replacing damaged organs and tissue. Regenerative medicine combines the principles of engineering and biology for the development of biomaterials, using scaffolds and cells for tissue regeneration (Langer and Vacanti 1993, Chan and Leong 2008).

 Ideally, scaffolds used in TE have several requirements that characterize a successful approach, including particular biological, mechanical, physical and chemical ones (Dhandayuthapani et al 2011). These are:

(a) biocompatibility, supporting cell growth and maintenance, with a negligible immune reaction (Teo et al 2006, Dhandayuthapani et al 2011, O’Brien 2011, Rana et al 2015, Skardal and Atala 2015, Hussein et al 2016);

(b) provision of appropriate structural support, shape and mechanical stability for the cells, including stiffness and swelling consistent with the anatomical site into which they are to be implanted and
with sufficient strength to allow surgical handling during implantation \( (\text{Hollister \, 2005, \, Teo \, et\, al \, 2006, \, Chan \, and \, Leong \, 2008, \, Dhandayuthapani \, et\, al \, 2011, \, O’Brien \, 2011, \, Giannitelli \, et\, al \, 2015}) \); architecture with interconnected pore structure and porosity to ensure cellular adhesion, penetration, engraftment and migration, adequate diffusion of nutrients to cells, gas exchange (i.e., \( O_2 \) and \( CO_2 \)), metabolic waste removal, and signal transduction because the microenvironment can create additional sites for cell anchorage and may provide distinct biochemical cues to guide cell behavior \( (\text{Hollister \, 2005, \, Dhandayuthapani \, et\, al \, 2011, \, O’Brien \, 2011, \, Giannitelli \, et\, al \, 2015, \, Rana \, et\, al \, 2015}) \);

(d) surface properties, e.g., ability to conduct signals, surface energy, chemistry, charge, surface area and suitable surface finishing;

(e) biodegradability, with a degradation rate in synchrony with defect healing rate \( (\text{Minuth \, et\, al \, 2005, \, Dhandayuthapani \, et\, al \, 2011, \, O’Brien \, 2011, \, Rana \, et\, al \, 2015}) \);

(f) sterilization facility because scaffolds are implanted into medical devices and as such are required to meet stringent sterility regulations to ensure patient safety \( (\text{Baume \, et\, al \, 2016}) \);

(g) in addition to physical and spatial cues, the scaffold itself can be the carrier of signaling biomolecules, thus emphasizing the need for scaffold-based tissue engineering \( (\text{Rana \, et\, al \, 2015}) \).

The requirements to be taken into account when leading with a scaffold can be seen in figure 1.

Modern technologies exist which may be used to produce scaffolds based on biomaterials processed by additive manufacturing (AM). There is a wide range of areas where AM is applied and medicine (health) is one of the fastest growing areas. Furthermore, there is substantial research focused on obtaining future autologous implantable tissue and organs through 3D printing \( (\text{Skardal \, and\, Atala \, 2015}) \).

The consolidated technique called electrospinning facilitates the manufacturing of nanostructured fibers with dimensions similar to components in the extracellular matrix (ECM), mimicking its fibrillar structure and providing essential cues for cellular organization, survival and function \( (\text{Dhandayuthapani \, et\, al \, 2011}) \). A typical electrospinning set up is shown in figure 2(a). Electrospinning and AM are technologies that converge toward the same goal and can complement each other. They are independently applied sciences for manufacturing scaffolds for a variety of tissue engineering applications \( (\text{Dalton \, et\, al \, 2013}) \).

Many types of biomaterials can be used to produce scaffolds for tissue engineering \( (\text{Teo \, et\, al \, 2006, \, Dhandayuthapani \, et\, al \, 2011, \, Ulery \, et\, al \, 2011}) \). Aliphatic polyesters, such as polycaprolactone (PCL) and poly(lactide-co-glycolide) (PLGA), have excellent tissue compatibility and degradability, which are critical aspects for their successful use in the human body. Moreover, these polymers have been approved by the United States Food and Drug Administration and European Medicine Agency for biomedical applications \( (\text{Ulery \, et\, al \, 2011}) \).

Mesenchymal stem or stromal cells (MSC) serve as a fundamental part of tissue engineering. These cells are often used in combination with biomaterials with the aim of repairing or reconstructing tissue and organs \( (\text{Langer \, and\, Vacanti \, 1993}) \). MSC are multipotent cells found in a variety of human tissue and organs \( (\text{Kang \, et\, al \, 2012, \, Ullah \, et\, al \, 2015}) \). They contribute to the regeneration of many types of tissue and organs, such as bone, cartilage, muscle, ligament, tendon, adipose tissue, stroma and fibrous tissue \( (\text{Kang \, et\, al \, 2012}) \). Besides their aptitude for tissue replacement via multipotent differentiation, MSC have been recognized for their therapeutic immunomodulatory and anti-inflammatory effects and also for the secretion of molecules or bioactive factors that instigate or assist in tissue repair \( (\text{Lavoie \, and\, Rosu-Myles \, 2013, \, Paul \, and\, Anisimov \, 2013}) \). Among the non-invasive sources to obtain MSC, human umbilical cord is an interesting source as the umbilical cord is normally discarded after birth. Favorable characteristics of human umbilical cord mesenchymal stem cells (UCMSC) include good differentiation and proliferation capabilities \( (\text{Kern \, et\, al \, 2006, \, Baksh \, et\, al \, 2007}) \).

The present study has investigated the interaction between biomaterials and MSC. The scaffolds were produced through 3D printing to manufacture 3D PCL matrices with suitable mechanical properties covered by electrospun PLGA to improve cell attachment (figure 2).

2. Materials and methods

2.1. Materials

The products used were heat-inactivated Fetal Bovine Serum (FBS) (Cultilab, Campinas/SP), collagenase type I and penicillin/streptomycin (Gibco), LDH Liquiform Ref.: 86-1/100 (Labtest Diagnóstica SA), optimum cutting temperature (OCT) compound (Sakura). The surface markers used were: CD14, CD29, CD34, CD45, CD90, CD105 and HLA-DR (Becton Dickinson, San Diego, CA) and growth factor Rh-TGF-β-1 (Peprotech). The polymers used were poly(DL-lactide-co-glycolide) 75/25 (PLGA) (PURAC) and polycaprolactone (PCL) (Solvay Capa 6505). The following products were purchased from Sigma-Aldrich: Dulbecco’s Modified Eagle’s Medium (DMEM/HEPES)—low glucose, amphotericin B, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), trypsin-EDTA solution 10x, dimethyl
sulphoxide (DMSO), dexamethasone, L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (ASAP), ITS Liquid Media Supplement (100x), alcian blue 8GX, insulin from bovine pancreas, indomethacin minimum, rosiglitazone, oil red O, β-glycerophosphate disodium salt hydrate, alizarin red S and Triton X-100. TRIzol® Reagent and Power SYBR® Green qPCR Master Mix kit were purchased from Life Technologies and M-MLV Reverse Transcriptase kit, actin, osteocalcin and osteopontin were purchased from Invitrogen.

2.2. Hybrid fabrication: 3D printing plus electrospinning

The poly(lactic-co-glycolic acid) (PLGA) fibers were deposited by an electrospinning apparatus onto
printed PCL matrices to improve the biofouling of the MSC on the scaffolds.

2.2.1. 3D printed PCL matrices

The PCL matrices (porous woodpile-like structures, figure 2(b)) were fabricated by the 3D printing process with the 3D printer, Fab@CTI (figure 3(a)), which consisted of the extrusion of fused filaments (Lixandrão et al. 2009). The most common hydroplastic forming technique is extrusion, in which a stiff plastic ceramic mass is forced through a die orifice, having the desired cross-sectional geometry. A forming technique is applied whereby a material is forced by compression through a die orifice. The extrusion process is the molding of a viscous thermoplastic under pressure through an open-ended die. A mechanical screw or auger propels the pelletized material through a chamber, which is then compacted, melted, and formed into a continuous charge of viscous fluid. The technique is especially adapted for producing continuous lengths, having constant cross-sectional geometries—for example, rods, tubes, hose channels, sheets, and filaments (Callister 2007). Scaffolds can also be fabricated in the same way.

The scaffolds were printed with 2 layers, each of 0.4 mm, and cut in circular forms with diameters of approximately 6 mm. The PCL printed matrices were produced with a 1 mm air gap, which refers to the space found between the raster lines. The Fab@CTI was built and adapted at the Center for Information Technology Renato Archer (CTI) with the purpose of collaborating in research initiatives in the bioengineering and biomaterials fields through partnerships with many research institutions. Fab@CTI can use materials under different conditions, such as filament, powder and fluid state.

The printer head (figure 3(b)) used in this work has the potential of being adapted to different filament diameters and different melting temperatures (room temperature to 400 °C) (Inforçatti Neto et al. 2011).

2.2.2. Electrospun PLGA coating

PLGA was used for electrospinning, as previously described (Acasiguia et al. 2014). 12% PLGA (75/25) was dissolved in 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol. The polymer solutions were placed between electrodes connected to a high voltage. The voltages applied for the nanofiber production were 11 kV and 1 kV, corresponding to the positive and negative electrodes, respectively. The polymer solution was electrospun with a syringe equipped with a 21 G × 1’ steel needle (0.8 × 25 mm), at 15 cm distance from the stationary collector plate with a flow rate of 0.019 ml min$^{-1}$ (Acasiguia et al. 2014). Random fibers were electrospun on a collector containing PCL with 6 mm diameter on both sides of the 3D matrices (figure 2(a)). 0.45 ml of the PLGA polymer solution was used on each side of the 3D matrices, forming the 3D + ES scaffolds (figure 2(d)). Subsequently, the 3D + ES scaffolds were placed under ultraviolet light for 1.5 h in a vertical laminar flow hood (45 min for each side) for sterilization and then placed in 96 well culture plates.

2.3. UCMSC isolated from humans

2.3.1. Cell isolation

The samples of the umbilical cord were obtained in partnership with the Hospital Moinhos de Vento de Porto Alegre, Brazil. This work was approved by the Research Ethics Committees of the Pharmacy
School/Universidade Federal do Rio Grande do Sul (protocol number 27315) and by the Brazil Platform Committee for Ethics and Research, called Plataforma Brasil. This program is under the auspice of the Ministry of Health for the registration of studies involving humans, based on the opinion of the Certificate of Presentation for Ethical Consideration (CAAE—Certificado de Apresentação para Apreciação Ética) under number 36211214.3.0000.5347. Participants were informed regarding the objectives and procedures of the study and signed a consent form.

The umbilical cord was harvested after birth. Immediately after collection, the umbilical cord was immersed in MSC culture medium, consisting of DMEM/Hepes, pH 7.4 supplemented with 10% FBS, 100 U ml\(^{-1}\) penicillin, 100 \(\mu\)g ml\(^{-1}\) streptomycin and 0.25 \(\mu\)g ml\(^{-1}\) amphotericin. The umbilical cord was cut in a laminar flow, washed with phosphate buffered saline (PBS) and Wharton’s jelly from the umbilical cord was digested in a 0.1% solution of collagenase type I. The cells were seeded into a culture bottle and detached with trypsin/EDTA. After being washed, the cells were cut in a laminar flow, washed with phosphate buffered saline (PBS) and Wharton’s jelly from the umbilical cord was digested in a 0.1% solution of collagenase type I. The cells were seeded into a culture bottle (T75 cm\(^2\)). The medium was replaced after 24 h in order to isolate adherent cells and, thereafter, it was refreshed once every 3 d to allow further growth. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO\(_2\). After reaching confluence, the cells were detached with trypsin–EDTA 0.05% solution and re-seeded at a density of 5000 cells cm\(^{-2}\) until they reached the 6th passage for characterization and use in the experiments described below.

### 2.3.2. Cell characterization

The UCMSC were characterized by flow cytometry and by the ability to differentiate into osteoblasts, chondrocytes and adipocytes in vitro (Acasiguá et al 2014, Andrade et al 2016).

#### 2.3.2.1. Immunophenotypic profile by flow cytometry

The immunophenotypic profile of the MSC was performed after cell dissociation with Trypsin/EDTA and posterior incubation with specific monoclonal antibodies. The cells were counted, re-suspended in PBS buffer at 10\(^6\) cells ml\(^{-1}\) and incubated with the human antibodies. The positive markers were CD29, CD90 and CD105 and the negative markers were CD14, CD34, CD45 and HLA-Dr. The antibodies were conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC). After 30 min incubation in the dark, the cells were washed with PBS to remove unbound antibody excess. Appropriate isotype controls were used and exclusion of dead cells was performed by incubation with 7-aminoactinomycin D (7AAD). The analyses were performed by flow cytometer FACS东亚 III (Becton Dickinson) and analyzed by FACS DiVa software, version 6.0 (Acasiguá et al 2014, Andrade et al 2016).

#### 2.3.2.2. Cell differentiation in vitro

The cell cultures were evaluated for their capacity to differentiate into osteoblasts, chondrocytes and adipocytes through induction medium until acquiring morphological change for about one month, as detailed below. The cells were fixed with 4% paraformaldehyde. The photomicrographs were captured by an optical microscope, as previously described (Acasiguá et al 2014, Andrade et al 2016).

#### 2.3.2.3. Adipogenic differentiation

The cells were cultivated in MSC culture medium supplemented with dexamethasone (1 \(\mu\)M), insulin (10 \(\mu\)g ml\(^{-1}\)), indomethacin (50 \(\mu\)M), rosiglitazone (1 \(\mu\)M) and IBMX (0.5 mM). The deposits of lipid droplets were observed after oil red O staining.

#### 2.3.2.4. Chondrogenic differentiation

For chondrogenic differentiation, the cells were cultivated in differentiation medium for about 30 d. The inducing medium consisted of MSC culture medium supplemented with 10 ng ml\(^{-1}\) TGF-\(\beta\) 1, 0.1 \(\mu\)M dexamethasone, 50 nM solution of ascorbic acid 2-phosphate (ASAP) and 1x of ITS 100x. The chondrogenesis was made apparent by alcian blue staining.

#### 2.3.2.5. Osteogenic differentiation

The cells were cultivated for 3–4 weeks in a supplemented MSC culture medium containing dexamethasone (0.1 \(\mu\)M), ascorbic acid 2-phosphate (5 \(\mu\)M) and \(\beta\)-glycerophosphate (15 mM). The deposition of the mineralized matrix was observed by alizarin red S staining.

#### 2.3.2.6. Control

The cells were cultivated in MSC culture medium in the same plates for the same time as the cells treated with a differentiation induction medium. After change in the cell morphology in the differentiation cultures, the MSC (control) were fixed and stained with each of the three different dyes at the end of each differentiation, as described above.

#### 2.3.3. Cell cultivation in the scaffolds

The UCMSC were seeded in three concentrations: 8.5 × 10\(^3\), 25.5 × 10\(^3\) and 51.0 × 10\(^3\) on one of the surfaces of the 3D + ES scaffolds. The 96-well culture plates without scaffolds were used as control. After 48 h, the scaffolds were turned over and the same number of cells was seeded on the other surface of the scaffolds, totaling 1.7 × 10\(^4\), 5.1 × 10\(^4\) and 10.2 × 10\(^4\) UCMSC seeded for the treatment in scaffolds and in the controls wells. After a further 48 h, the tests were considered completed.

#### 2.4. Cytotoxicity assay

Cytotoxicity was assessed by measuring the culture supernatant using enzyme lactate dehydrogenase...
(LDH) delivery assay. It is an intracellular enzyme and its increased presence in the extracellular environment is indicative of problems in the cytoplasmic membrane integrity and, consequently, provoking cell damage. The higher the concentration of this enzyme, the higher the cell death. The test (Labtest kit) was performed 48 h after the second seeding. As negative control, cells cultivated directly on the wells were used and as positive control, cells cultivated directly on the wells treated with Triton X-100 (Sigma-Aldrich), 1% (v/v) for 20 min. Triton X-100 causes cell death, permitting maximal LDH release. Measurement was performed with the equipment 560 Labmax (Labtest Diagnóstica SA) (Andrade et al 2016). Data is mean ± SEM for three experiments (n = 12 for treatment).

2.5. Cell viability (MTT assay)
Cell viability was determined using the colorimetric MTT assay. Two days after seeding, the cells were incubated with 0.25 μg ml⁻¹ MTT; 4 h later, the supernatant was carefully removed and DMSO (200 μl) was added per well to dissolve the crystals formed. Absorbance was measured at 570 and 630 nm in the apparatus SpectraMax® 250 (Molecular Device, Sunnyvale, CA, USA), with the results being calculated by the absorbance label subtraction (570–630 nm) (Andrade et al 2016). Data is mean ± SEM for four experiments (n = 14 for treatment).

2.6. Image analysis
2.6.1. Optical microscopy
Both cell differentiation and the PCL matrices were analyzed by the images obtained in a Nikon microscope (Nikon Ti Eclipse microscope). The matrix diameter was determined using the ImageJ program (National Institutes of Health).

2.6.2. Scanning electron microscopy
The scaffold morphology and the PLGA fiber diameter, together with the cell presence were observed by scanning electron microscope Zeiss Evo 50 (Carl-Zeiss, Oberkochen, Germany). Fiber diameter was determined using the ImageJ program. The scaffolds were washed in phosphate buffer (pH 7.4), fixed in 3% glutaraldehyde buffer for 30 min, rinsed in buffer PBS and dehydrated in an ethanol series. Dried samples were metalized with a thin layer of platinum and were placed on stabs using adhesive carbon discs. The images were obtained using accelerating voltage of 10 kV with a magnification range of 1000–20 000x (Zanatta et al 2012).

2.6.3. Confocal laser scanning microscopy
The presence of cells was analyzed by confocal laser scanning microscopy. The samples were washed in phosphate buffer (pH 7.4), fixed in 4% paraformaldehyde buffer for 20 min and rinsed in buffer PBS. The stem cells were permeabilized with Triton X and the actin of the cytoplasms were stained red using 50 μg ml⁻¹ rhodamine conjugated phalloidin (40 min). They were washed with PBS and the cell nuclei were blue stained with 0.5 μg ml⁻¹ of 4, 6-diamidino-2-phenilindole, DAPI (1 min). Photographs were obtained by confocal-laser scanning fluorescence microscope with an objective of EC-Plan-Neofluar 10x/0.30 M27 (Zeiss Model LSM 700) (Zanatta et al 2012).

The quantitative analyses of the results were made by normalization: the cells attached on the scaffolds and stained with DAPI were counted (cell attachment number) and then normalized in relation to the seeded cell number (cell attachment number/seeded cell number). Three images were used for each cell concentration.

2.6.4. Cell differentiation on the scaffolds
Capacity of osteogenic and chondrogenic differentiation of stem cells was performed on the 3D + ES scaffolds (3D printed PCL matrices coated with PLGA electrospun nanofibers). The UCMSC (51.0 × 10⁵) were seeded on one of the surfaces of the scaffolds (3D + ES).

After the differentiation period on the scaffolds (about 2 weeks), the osteogenic differentiation of cells was analyzed by the following methodologies: PCR, alkaline phosphatase activity assay and histology. Total RNA from the constructs (3D + ES + UCMSC) was extracted using the TRizol® Reagent method, in accordance with the manufacturer’s directions. After 10 d each constructs was washed with PBS, vortexed in 2 ml TRizol® for 5 min and stored at −80 °C until further use. Proteins were removed with 0.4 ml chloroform extraction, and the RNA pellets were washed once with isopropyl alcohol and once with 75% ethanol. The total number of RNA pellets were reconstituted in diethyl pyrocarbonate-treated water (DEPC). The concentration and purity of the RNA was measured using a NanoDrop (ND-2000) Spectrophotometer. cDNA was synthesized using a moloney murine leukemia virus reverse transcriptase enzyme M-MLV Reverse Transcriptase kit. The single-strand cDNA synthesis occurred by incubating the complete reaction mixture for 5 min at 65 °C, followed by ice and 50 min at 37 °C and terminated by an incubation at 70 °C for 15 min. PCR reactions were prepared using Power SYBR® Green qPCR Master Mix kit. Each 20 μl sample was composed of 10 μl of qPCRSupermix, 1 μl of albumin, 0.5 μM of each primer (table 1) and 1 μl of cDNA, diluted in DEPC water. PCR cycle conditions were as follows: 95 °C for 2 min followed by 44 cycles at 95 °C for 15 s (denaturation), 30 s of annealing (temperature dependent on the gene, table 1) and 72 °C for 30 s (extension) were carried out in an Applied Biosystems® Thermo Cycler. PCR-amplified fragments were resolved by 1.5% agarose/ethidium bromide gel electrophoresis.
Table 1. PCR primers of osteogenic markers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5′–3′)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>Sense: AGCACAGAGGCTGCGCTT</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Antisense: CCGGGATATCATCATC</td>
<td></td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>Sense: GCAGAGTCCCAAGGCTGC</td>
<td>61.4</td>
</tr>
<tr>
<td></td>
<td>Antisense: TCACGCAACTGTGATAGTC</td>
<td></td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Sense: GCAAAGTGTCTTCATCTCC</td>
<td>58.4</td>
</tr>
<tr>
<td></td>
<td>Antisense: TGAGGTGATGGTCCCGT</td>
<td></td>
</tr>
</tbody>
</table>

2.7. Statistical analysis
The results were expressed as the mean ± standard error of the mean and evaluated using one-way ANOVA, followed by Bonferroni’s test. Significant differences were established at p < 0.05. Data analyses were performed with the program BioEstat 5.0 (Ayres et al 2007).

3. Results and discussion

3.1. Scaffolds 3D of PCL covered by PLGA electrospun (3D + ES)
Figure 2(d) shows the macroscopic aspect of the scaffolds produced. It is possible to see the electrospun fibers integrated with the 3D matrices. The aspect of the 3D matrices is shown in figure 4(a) (optical microscopy). The PCL 3D-printed matrices were constructed with two different layer diameters: at the bottom, the average diameter was 797.5 μm with standard error of 61.0 μm and at the top layer of the matrix it was 581.2 ± 13.9 μm, measured in the center of the rod. The 3D-printed matrices covered with electrospun PLGA nanofibers can be seen in figure 4(b). The SEM of the PLGA nanofibers is shown in figure 4(c). It was possible to observe that the fibers were distributed in a random manner over the entire scaffold structure and presented a large number of interconnected pores.

The average diameter of the electrospun PLGA fibers was 109.4 ± 42.7 nm and the histogram in figure 4(d) illustrates the fiber diameter distribution as a function of frequency (number of fibers that lie in the fiber diameter group). In this study, the produced scaffolds showed a favorable geometry for facilitating their manipulation. Moreover, the scaffolds exhibited fibers with different nanometric dimensions, interconnected pores and an elevated superficial area.

The fibers had diameters compatible with the fibers of the natural ECM. In the native tissue, the structural ECM proteins range in diameter from 50 to 500 nm (Barnes et al 2007). In this work, the diameter of the fibers varied from 34 to 391 nm, with the main concentration of between 75 and 150 nm. Conventional polymer processing techniques have difficulty in producing fibers smaller than 10 μm in diameter, which are several orders of magnitude larger than the native ECM. For this reason, there has been a concerted effort to develop methods for producing...
nanofibers to more closely simulate the ECM geometry, such as electrospinning (Barnes et al 2007). The influence of the ECM on cellular activities occurs via the binding of specific factors to specific ECM molecules and the binding of ECM molecules to cell surface receptors, known as integrins. The participation of integrin-β1 in MSC adherence on PLGA nanofibers produced by electrospinning has been previously demonstrated (Zanatta et al 2012). Integrin-mediated cell adhesion is of substantial importance as it links ECM with cytoskeleton and signal transduction cascade to regulate cell shape, differentiation, adhesion, migration and proliferation in many systems (Schwartz and Assoian 2001).

Moreover, the PLGA electrospun nanofibers produced in previous studies of the group were able to give support to cell differentiation of MSC (Zanatta et al 2012, Acasiguca et al 2014). This behavior is desirable because this system gives enough mechanical support for cellular adherence and spreading, providing adequate material to support differentiation for chondrocyte and ECM production. This system can be used as carrier material in transplantation procedures, allowing MSC to be firmly adhered to the material during the transport to the injured site (Acasiguca et al 2014).

### 3.2. Mesenchymal stem cell characterization

The MSC from human umbilical cord were isolated, cultivated and characterized successfully from three different umbilical cords. The cells showed typical MSC morphology, characteristics of plastic adherence, colony forming unit and immunophenotyping showed positivity for the surface markers CD29/PE (figure 5(a)), CD90/PE (figure 5(b)), CD105/PE (figure 5(c)) (>95%) with low expression for CD11b/FITC (figure 5(d)), CD34/PE (figure 5(e)), CD45/FITC and HLA-DR/FITC (figure 5(f)) (<2%).

The cells were able to differentiate into the three analyzed mesodermal cell lineages (osteogenic, adipogenic and chondrogenic). Photomicrographs of a representative sample of the UC-MSC from differentiated umbilical cord cultures are shown in figure 5. Adipogenic differentiation was demonstrated by staining with oil red O, without evidence of lipid vacuoles in the control (figure 5(h)), but showed droplets of fat, highlighted by oil red O (figure 5(k)). Chondrogenic differentiation was demonstrated by staining with alcian blue, indicated by the blue staining of glycosaminoglycans deposits (figure 5(i)), but not in the control (figure 5(i)). Osteogenic differentiation was demonstrated by staining with alizarin red S, indicated by the red staining of the calcium deposits (figure 5(m)), but without evidence of the calcified matrix stained with alizarin red S in the control (figure 5(j)).

In this study, mesenchymal stem cells were characterized following the International Society for Cellular Therapy standard criteria: the cells must be plastic-adherent when maintained in standard culture conditions; MSC must express positivity (≥95%) to CD73 (ecto-5′-nucleotidase), CD90 (Thy1) and CD105 (endoglin), while negatively expressing (≤2%) CD11b or CD14, CD34, CD45 and HLA-DR surface...
molecules and the MSC must differentiate into osteoblasts, adipocytes and chondroblasts in vitro (Dominici et al. 2006).

Human umbilical cord derived MSC can be isolated and expanded easily in vitro without ethical concerns because the cord is discarded after birth (Moretti et al. 2010, Cui et al. 2015). This postnatal organ has been found to be rich in primitive stromal cells, showing typical characteristics of bone-marrow (BM) MSC (BMSC) (Moretti et al. 2010). Compared to BM, umbilical cord tissue carries a higher frequency of stromal cells with a higher in vitro expansion potential. Therefore, MSC from umbilical cord have attracted interest as a promising candidate for several potential clinical applications due to the immune-privileged and immune-modulatory properties (Moretti et al. 2010). Thus, MSC from umbilical cord are a powerful cellular alternative for regenerative medicine and tissue engineering applications and open highly interesting perspectives for clinical applications (Moretti et al. 2010).

3.3. Cytotoxicity assay
The cytotoxicity of the scaffolds of PLGA fiber-coated PCL was evaluated by LDH concentrations measured on the supernatant of the cell culture. The LDH delivery assay showed that the scaffolds were not cytotoxic (figure 6). In the test, with a $17 \times 10^3$ MSC...
seeded density, the average LDH (U/L) released and standard deviation of the control well was $103.1 \pm 22.5$ and the scaffold was $125.1 \pm 36.2$. In the death control (Triton) it was $300.2 \pm 177.3$. At the concentration of $51 \times 10^3$ cells, the average was $144.6 \pm 36.7, 181.2 \pm 79.0$ and $512.4 \pm 369.3$, in the control, scaffold and death control groups, respectively. When $10^2 \times 10^3$ MSC were seeded in the wells, the average LDH (U/L) released was $327.0 \pm 313.7$. When seeded on the scaffolds, it was $291.0 \pm 168.9$ and when seeded in the Triton-treated wells it was $950.9 \pm 558.1$. No significant statistical difference occurred between the LDH leakage (U/L) in the control (cells cultivated on the plates) and in the 3D + ES scaffolds ($p > 0.05$) with each cell density used. Cells treated with Triton were used as the positive control for cell death and, statistically, they delivered more LDH than the cells cultivated on the plates ($p < 0.05$) and in the scaffolds ($p < 0.05$).

For clinical success, an ideal scaffold must not release cytotoxic substances but should support growth, adherence and proliferation of the seeded cells (Açıl et al 2014). In order to evaluate the chemical characteristics of the biomaterials, the cytotoxicity of eluates from the biomaterials was investigated by using the LDH test. The LDH assay is an effective means of measuring the membrane integrity (cytotoxicity) as a function of the amount of leaked LDH, a cytoplasmic enzyme released from injured cells into the medium (Açıl et al 2014).

The organic chemical solvents used in electrospinning are a source of cytotoxic compounds. For example, most of the polymers used in electrospinning are not soluble in an aqueous media, as, for example, PLGA. Therefore, the use of organic solvents possessing high toxicity is necessary. However, during the electrospinning procedure, the solvent will evaporate as the fluid jet accelerates toward the collector. In this path, if most of the solvent evaporates, individual fibers are formed. If not, the fibers may not be formed at all, or a thin film of polymer solution is deposited on the collector (Ramakrishna et al 2005). It was shown that, despite the fact that HFIP residue was present in the PLGA electrospun fibers the amount of retained HFIP was too low to impart any in vitro or in vivo toxicity (Weldon et al 2012). The possible presence of solvent residuals; however, or other interference cannot be excluded and cytotoxicity assay is necessary. The LDH test demonstrated that the tested scaffolds formed by 3D + ES, did not cause an increase of LDH release, which indicates that the scaffolds were not cytotoxic to the MSC.

### 3.4. Cell viability

MTT is reduced to formazan in viable cells, thus the level of reducing MTT into formazan can reflect the level of cellular metabolism and cell proliferation. After the second seeding, cell viability assay showed that there were no statistically significant differences between the wells and the scaffolds in terms of MSC concentration. In the test with a $17 \times 10^3$ MSC seeded density, the average absorbance and standard deviation of the control well was $0.31 \pm 0.19$ and the scaffold was $0.26 \pm 0.10$. At the concentration of $51 \times 10^3$ cells, the absorbance was $0.29 \pm 0.10$ and $0.35 \pm 0.13$ in the control and scaffold groups, respectively. When $10^2 \times 10^3$ MSC were seeded in the wells (control), the absorbance was $0.35 \pm 0.24$ and when seeded on the scaffolds, it was $0.41 \pm 0.16$. It is therefore possible to conclude that the scaffolds behaved similarly to the wells (gold standard).

In high doses of seeded cells ($51 \times 10^3$ and $10^2 \times 10^3$ MSC), there was a trend of better viability in the scaffolds. This is possibly due to the approach used in this study to seed cells on both sides of the scaffolds. Thus, the cells on both sides of the scaffolds had more space for growth than in the wells.

Previous studies of the group have demonstrated that MSC from dental pulp of deciduous teeth proliferated in 12% PLGA fibers obtained by electrospinning (Acasigua et al 2014). There was an increase in cell viability (by MTT assay) in both groups (PLGA fiber scaffold and plate control) on the 7th day of cultivation.
The presence of the cells attached in the scaffolds was investigated by confocal laser scanning microscopy and scanning electron microscopy. Figure 7 shows the cells stained with DAPI (blue nuclei) and phalloidin (red cytoskeleton) and the merge of DAPI and phalloidin images. Scanning electron microscopy of the cells and the PLGA fibers stained with DAPI is shown by scanning electron microscopy, with 8.5 $\times 10^3$ (figure 7(j)), 25.5 $\times 10^3$ (figure 7(k)) and 51 $\times 10^3$ (figure 7(l)).

Representative micrographs of three individual experiments. (a)–(e): 100x magnification.

when compared to the first day of the experiments. The same occurred on the 14th day when cell viability increased in both groups. However, on the 21st day of cultivation, cell viability decreased in both groups in comparison to the 14th day.

Cell viability remained similar in the test and control groups in the different experiment time periods, with no statistical difference between them (Acasigua et al 2014). In addition, the study demonstrated that the association of MSC seeded into biodegradable PLGA scaffolds has the ability of promoting bone regeneration in rats, which is a promising alternative for application in regenerative medicine (Acasigua et al 2014).

3.5. Interaction of cells with scaffolds

The presence of the cells attached in the scaffolds was confirmed by confocal laser scanning microscopy and scanning electron microscopy. Figure 7 shows the cells stained with DAPI (blue nuclei) and phalloidin (red cytoskeleton) and the merge of DAPI and phalloidin images. Scanning electron microscopy of the cells and the PLGA fibers stained with DAPI is shown by scanning electron microscopy, with 8.5 $\times 10^3$ (figure 7(j)), 25.5 $\times 10^3$ (figure 7(k)) and 51 $\times 10^3$ (figure 7(l)).

Cell attachment on the scaffolds was investigated by staining the cell nucleus using DAPI. The cytoskeletal characteristics were investigated by using phalloidin by fluorescent microscopic and scanning electron microscopy images of the cells on the scaffolds.

The quantitative analyses using normalization of data (number of cells attachment after 4 d they were seeded on the scaffolds in relation to the number of cells seeded at day 0) resulted the rates of 1.78, 1.77 and 1.88 of MSC with 8500, 25 500 and 51 000 of cells seeded, respectively. The similar ratio in the different number of cells seeded on the scaffolds (1.78, 1.77 and 1.88) indicates that regardless of the number of cells used to start the experiment (8500, 25 500 and 51 000), the presented scaffold approach allows for similar cell proliferation. Besides this, the rate greater than 1 suggests a higher cell attachment number in relation to the seeded cell number and indicates cell proliferation on the scaffolds after 4 d in cultivation. This result suggests that the UCMSC can proliferate in the proposed scaffolds used in this work. It was confirmed that the seeding of 51 000 cells resulted in a higher attachment than the 25 500 and 8500 cells seeded on each side of the scaffold, with similar proliferation rates. Consistent with the cell attachment results by
fluorescent microscopic, the SEM on the 51 000 cell seeding manifested the largest number of cells.

In general, the experiments use $2 \times 10^4$ to $3 \times 10^5$ plated cells per cm$^2$ and allow proliferation and performing analyses at different points in the growth curve. This approach has been used in previous experiments of the group in scaffolds with 12% PLGA and MSC with analysis at 7, 14 and 21 d (Acasigu et al 2014). In the present study, the lower cell density used ($8.5 \times 10^3$ cells per well in 96 well plates) corresponds to this interval ($2.6 \times 10^4$ cells per cm$^2$).

At this density, it is possible to observe spaces without cells in the scaffolds (figures 7(a), (d) and (g)). The cells use these spaces to proliferate in experiments of longer duration. The higher density MSC seeded approach in this work was used to reduce the time of the experiment.

After four days of experiments, it can be concluded that the 51 000 seeded cells are suitable for transplant to the scaffold because the cells have a confluent layer on the scaffold (figures 7(c), (f) and (i)).

A different approach of seeding cells on both sides of the scaffolds was used with the aim of increasing cell density. Thus, it is possible to have stem cells on both sides of the scaffold when transplanting this material to injured tissue, presumably accelerating the regeneration time.

The architecture of scaffolds used for tissue engineering is of critical importance to cell adhesion, attachment, growth, proliferation and metabolic activity. Scaffolds should have an interconnected pore structure and high porosity to ensure cellular penetration and adequate diffusion of nutrients to cells within the construction and to the extra-cellular matrix formed by these cells. Furthermore, a porous interconnected structure is required to allow diffusion of waste products out of the scaffolds and the products of scaffold degradation should be able to leave the body without interference with other organs and surrounding tissue (O’Brien 2011). This study has shown that electrospun PLGA fibers were able to provide these properties.

3.6. Differentiation of cells on the scaffolds

Scaffolds in regenerative medicine for cartilage and bone engineering strategies have been shown in this study. The capacity of osteogenic and chondrogenic differentiation of stem cells was performed on the scaffolds (3D printed PCL matrices coated with PLGA electrospun nanofibers). The cells were able to perform osteogenic and chondrogenic differentiation on the scaffolds.

UCMSC on the scaffolds which underwent the osteogenic differentiation protocol expressed the osteogenic genes osteocalcin and osteopontin (figure 8(a)). The osteogenic differentiated cells on the scaffolds showed positive activity for alkaline phosphatase (figure 8(b)). Figure 8(c) shows the macroscopic aspect of undifferentiated control (left) and osteogenic differentiation (right) on the scaffolds produced. Photomicrographs by an optical microscope of control cells on the scaffolds without evidence of the calcified matrix stained with alizarin red S (figure 8(d)) and cryosectioned (figure 8(e)) and osteogenic differentiated of UCMSC staining by alizarin red S indicated by the red staining of deposited calcium (figure 8(f)) and cryosectioned (figure 8(g)).

The absence of chondrogenic differentiation in the controls was demonstrated by staining with alcian blue without blue staining of glycosaminoglycans deposits at figure 8(h) (left), figures 8(i) and (j) (cryosectioned) and the chondrogenic differentiation was demonstrated by blue staining of glycosaminoglycans deposits with alcian blue macroscopically at figure 8(h) (right), microscopically (figure 8(k)) and cryosectioned (figure 8(l)).

3.7. Mechanical test of cells on the scaffolds

The results of mechanical properties of the scaffolds composed of PCL 3D printed matrix (3D), scaffolds 3D covered with electrospun PLGA nanofibers (3D + ES) and 3D + ES with seeded stem cells from umbilical cord (3D + ES + SC) are shown in table 2 and figure 9.

The results indicate typical stress $\times$ strain curves of scaffolds. The tensile properties indicate that the strength and modulus were not significantly affected by the electrospinning process or the cell cultivation in comparison with neat PCL 3D matrices. Nevertheless, for the scaffolds submitted to cell cultivation, a tendency of depletion of tensile at break and Young modulus was observed (blue line), indicating that the culture media might affect the mechanical properties. Although the cells used in the experiment could promote bone regeneration, no differences were observed in the mechanical properties. In order to investigate the scaffold reinforcement promoted by bone tissue formation, more extensive experiments are required.

However, the fibrous scaffolds produced by electrospinning are thin. The thickness of the PLGA fiber scaffolds produced by this group is about 37 $\mu$m (Acasigu et al 2014). Moreover, ideally, the scaffold should have mechanical properties consistent with the anatomical site into which it is to be implanted and, from a practical perspective, it must be strong enough to allow surgical handling during implantation (O’Brien 2011). The PCL matrices produced in this work were able to provide mechanical stability to the scaffolds.

Recently, several articles have focused on the fabrication of hybrid scaffolds for potential tissue engineering applications (Hoque et al 2014). Electrospinning may be combined with other scaffold fabrication technologies to compensate for its potential limitations, such as inadequate mechanical strength for load-bearing applications (O’Brien 2011). This was the strategy used in
Figure 8. Osteogenic and chondrogenic differentiation of UCMSC on scaffolds (3D printed PCL matrices coated with PLGA electrospun nanofibers). (a)–(f): osteogenic differentiation. (a) PCR; (b) alkaline phosphatase positive activity; (c)–(g): alizarin red S staining on 3D + ES scaffolds. (c) macroscopic aspect of 3D + ES with UCMSC undifferentiated (left) and differentiated (right); (d) scaffolds with undifferentiated cells (osteogenic control); (e) cryosections of undifferentiated tissue constructs (osteogenic control); (f) osteogenic differentiation in scaffolds; (g) cryosection of osteogenic differentiation. 3D: 3D printed matrix; ES: electrospun PLGA nanofibres. OsteoC: osteocalcin; OsteoP: osteopontin. (h)–(l): 100x magnification.
this work by combining support with greater mechanical stability due to the presence of 3D printed PCL matrices deposited in the filament and the electrospun PLGA fibers, which allows for better cell adhesion. The scaffolds are suitable for use in cell therapy and also for tissue regeneration purposes.

4. Conclusions

The combined polymeric structure used in this study made it possible to produce a support of PCL with controlled design and three-dimensional print. Moreover, it increased cell adhesion due to the PLGA nanostructured fibers which mimicked the ECM, and which did not present cytotoxicity. The cells were shown to be viable and they adhered to the 3D + ES scaffolds. This hybrid scaffold produced by electrospun PLGA fibers coated with cells on both sides of the 3D structure can be used in cell therapy and regeneration of hard tissue.

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Table 2. Mechanical properties (average values and standard deviations) of 3D printed matrices (3D), 3D printed PCL matrices coated with PLGA electrospun nanofibers (3D + ES); and cells on the scaffolds (3D + ES + SC).

<table>
<thead>
<tr>
<th>Sample</th>
<th>3D</th>
<th>3D + ES</th>
<th>3D + ES + SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young modulus (MPa)</td>
<td>20.72 ± 6.54</td>
<td>19.57 ± 2.43</td>
<td>18.53 ± 0.80</td>
</tr>
<tr>
<td>Tensile at break (MPa)</td>
<td>2.59 ± 0.45</td>
<td>2.60 ± 0.03</td>
<td>2.53 ± 0.21</td>
</tr>
<tr>
<td>Elongation at break (%)</td>
<td>73 ± 17</td>
<td>65 ± 9</td>
<td>74 ± 20</td>
</tr>
</tbody>
</table>

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