TISSUE ENGINEERING: Part A
Volume 14, Number 10, 2008

DOI: 10.1089/ten.tea.2007.0307

Assessment of the Suitability of Chitosan/PolyButylene Succinate Scaffolds Seeded with Mouse Mesenchymal Progenitor Cells for a Cartilage Tissue Engineering Approach

JOÃO T. OLIVEIRA, B.Sc.,[1,2] VITOR M. CORRELO, B.Eng.,[1,2] PAULA C. SOL, B.Eng.,[1,2] ANA R. COSTA-PINTO, B.Sc.,[1,2] PATRÍCIA B. MALAFAYA, Ph.D.,[1,2] ANTONIO J. SALGADO, Ph.D.,[3] MRINAL BHATTACHARYA, Ph.D.,[4] PIERRE CHARBORD, Ph.D.,[5] NUNO M. NEVES, Ph.D.,[1,2] and RUI L. REIS, Ph.D.[1,2]

ABSTRACT

In this work, scaffolds derived from a new biomaterial originated from the combination of a natural material and a synthetic material were tested for assessing their suitability for cartilage tissue engineering applications. In order to obtain a better outcome result in terms of scaffolds' overall properties, different blends of natural and synthetic materials were created. Chitosan and polybutylene succinate (C-PBS) 50/50 (wt%) were melt blended using a twin-screw extruder and processed into 5×5×5 mm scaffolds by compression moulding with salt leaching. Micro-computed tomography analysis calculated an average of 66.29% porosity and 92.78% interconnectivity degree for the presented scaffolds. The salt particles used ranged in size between 63 and 125 μm, retrieving an average pore size of 251.28 μm. Regarding the mechanical properties, the compressive modulus was of 1.73 ± 0.4 MPa (E_{sec} 1%). Cytotoxicity evaluation revealed that the leachables released by the developed porous structures were not harmful to the cells and hence were noncytotoxic. Direct contact assays were carried out using a mouse bone marrow–derived mesenchymal progenitor cell line (BMC9). Cells were seeded at a density of 5×10^5 cells/scaffold and allowed to grow for periods up to 3 weeks under chondrogenic differentiating conditions. Scanning electron microscopy analysis revealed that the cells were able to proliferate and colonize the scaffold structure, and MTS test demonstrated cell viability during the time of the experiment. Finally, Western blot performed for collagen type II, a natural cartilage extracellular matrix component, showed that this protein was being expressed by the end of 3 weeks, which seems to indicate that the BMC9 cells were being differentiated toward the chondrogenic pathway. These results indicate the adequacy of these newly developed C-PBS scaffolds for supporting cell growth and differentiation toward the chondrogenic pathway, suggesting that they should be considered for further studies in the cartilage tissue engineering field.

[1]3B's Research Group—Biomaterials, Biodegradables, and Biomimetics, Department of Polymer Engineering, University of Minho, Braga, Portugal.
[2]IBB—Institute for Biotechnology and Bioengineering, PT Associated Laboratory, Braga, Portugal.
[3]Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, Braga, Portugal.
[4]Department of Biosystems Engineering, University of Minnesota, St. Paul, Minnesota.
[5]INSERM, Equipe ESPRI/EA-3855, Faculty of Medicine, University François Rabelais, Tours, France.

INTRODUCTION

Trauma and disease of bone and joints, frequently involving structural damage to both the articular cartilage surface and the subchondral bone, result in severe pain and disability for millions of people worldwide. Such problems were initially addressed by performing different surgical procedures, which included debridement, drilling, abrasion arthroplasty, and microfracture.[1,2] Some of these clinical experiments turned out to be successful in some cases, and are still being performed in hospitals and clinics throughout the world, but most of their outcomes are variable and dependent on a wide range of factors that can limit its wider application.[3,4]

Tissue engineering was brought up as a new way to address these problems and grew as a new field of knowledge not only in cartilage regeneration but also in several other types of tissue.[5–7] The fundamental goal of tissue engineering is to develop biological substitutes that restore, maintain, or improve tissue function and to apply these to clinical scenarios where tissue is lost through trauma or disease.[8] The cell support structure—a scaffold—should serve as a three-dimensional template for initial cell attachment and subsequent tissue formation, both *in vitro* and *in vivo*. It must not trigger strong immunological responses or cause severe cytotoxicity effects, and should present mechanical properties similar to the tissue of interest.[5]

Scaffolds are made of materials that can be broadly divided into synthetic and natural.[9,10] Synthetic polymers include the polylactides, such as polylactic acid (PLA)[9] and polyglycolic acid (PGA),[11] although a wide range of others such as polyethylene oxide (PEO),[12] poly(lactic-co-glycolic) PLGA,[13] and poly-2-hydroxyethyl methacrylate (PHEMA)[14] exist. Even though they possess some controllable and advantageous features, such as reproducible manufacturing at a large scale and controlled degradation time, they also have several disadvantages, like the lack of cell-recognition signals. Further, specific features of some polymers, like the acidic by-products released by PLA, for example, pose additional difficulties to their use.[15]

Natural-origin materials seem to overcome some of these limitations.[16,17] Most of them are normal components of the tissue to be regenerated, possess specific sites for cell recognition, and share some similarities with the native tissue components.[18–20] Collagen,[19] hyaluronic acid,[18] chitosan,[10,20] and starch[21] are examples of those materials. The conjugation of natural and synthetic materials in the fabrication of a scaffold taking advantage of the individual features of each one is a strategy that has been tried by different research groups. Chen *et al.* produced a hybrid mesh of PLGA and collagen that enabled the aggregation of mesenchymal stem cells and provided them with a microenvironment that allowed chondrogenic differentiation to occur.[22] In another study, Wang *et al.* fabricated a hybrid matrix based on polyglycolide and chitosan that allowed fibroblast proliferation and revealed promising further tissue engineering applications.[23]

In this work, scaffolds of a blend of chitosan (a natural polymer derived from the deacetylation of chitin) and polybutylene succinate (a synthetic polymer) were produced and put through preliminary *in vitro* tests. The rationale is to combine the biological properties of chitosan with the mechanical support conferred by polybutylene succinate, expecting this will render a better performance to the tissue-engineered construct once implanted. Chitosan is a partially deacetylated derivative of chitin, which is the primary structural polymer in arthropod exoskeletons, shells of crustaceans, and the cuticles of insects.[24] Chitosan is a semicrystalline polysaccharide that is normally insoluble in aqueous solutions above pH 7. However, in dilute acids (pH 6), the free amine groups are protonated and the molecule becomes soluble. This pH-dependent solubility provides a convenient mechanism for processing under mild conditions.[25] Chitosan is reported to be nontoxic, biodegradable, and biocompatible,[26] and has structural similarities to glycosaminoglycans, which are structural components of the cartilage extracellular matrix.[27] It serves different applications, and its use ranges from the food industry to the biomedical and pharmaceutical fields.[28,29] Polybutylene succinate is one of the most accessible biodegradable polymers, and has been extensively studied for its potential use as a future conventional plastic, serving also as a support for different approaches in the medical field.[30,31] It is an aliphatic polyester presenting good processability and flexibility, and having degradation products that are nontoxic and can enter the metabolic cycles of bioorganisms. Its conjugation with chitosan aims at providing mechanical support to the scaffold, which should be advantageous considering the clinical scenario of constant load-bearing efforts in articular cartilage. Little research has been conducted in the melt blending of synthetic polyesters and chitosan,[26] and the preliminary results described herein for their use as potential scaffolds for cartilage regeneration are important. The herein developed chitosan and polybutylene succinate (C-PBS) scaffolds were seeded with cells originated from a mouse mesenchymal stem cell line (BMC9)[32] and cultured under chondrogenic inductive conditions, in order to assess their suitability for cartilage tissue engineering approaches.

MATERIALS AND METHODS

Scaffold production and processing

The chitosan/polybutylene succinate 50/50 (%wt) blend was compounded in a twin screw extruder. The details of the processing conditions are summarized elsewhere.[26] The methodology used for the scaffold production was based on compression moulding followed by salt leaching. Before using it in the scaffold processing, the salt was ground and sieved to obtain particles with size between $63\,\mu m < d < 125\,\mu m$. The compounded polymeric blend was ground, mixed with salt, and compression molded into discs. The salt

content was 80% by weight. The discs were cut into 5×5×5 mm cubes. These cubes were then immersed in distilled water to leach out the salt, dried, and used for cell culture and proliferation studies after sterilization by ethylene oxide.

Scaffold characterization

The C-PBS scaffold structure was analyzed by scanning electron microscopy (SEM) using a Leica Cambridge S360 (Leica Cambridge, Cambridge, UK). Micro-computed tomography (μ-CT) equipment (SkyScan, Kortich, Belgium) was used for more detailed analysis of the morphology of the developed scaffolds, and CT Analyser and CT Vol Realistic 3D Visualization were used as image-processing softwares, both from SkyScan. Uniaxial compression tests were performed to assess the mechanical properties of the scaffolds (dry state) using a Universal tensile testing machine (Instron 4505 Universal Machine, Instron, Norwood, MA). The details regarding these methods are presented elsewhere.[26]

In vitro *cytotoxicity tests*

To assess the short-term cytotoxicity of the developed C-PBS scaffolds, minimum essential medium (MEM) extraction and 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium (MTS) tests, both within a 24 h extraction period, according to ISO/EN 10993 part 5 guidelines,[33] were used in order to establish the possible toxic effects of leachables released from the scaffolds during extraction. Latex rubber was used as positive control for cell death due to its high cytotoxicity to cells, and culture medium was used as a negative control representing the ideal situation for cell proliferation. The results are presented after normalization with the negative control. The objectives of the MEM extraction test are to evaluate changes in cell morphology and growth inhibition, whereas the MTS test determines whether cells are metabolically active.

Cell culture. A rat lung fibroblast cell line, L929, acquired from the European Collection of Cell Cultures (ECACC), was used. The cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany) and 1% of an antibiotic–antimycotic mixture (Sigma). Trypsin (Sigma) was used to detach the cells from the culture flasks before the experiments were conducted.

MEM extraction test. The ratio of material weight to extract fluid was constant and equal to 0.2 g/mL for porous samples. For the positive control the ratio of material outer surface to extraction fluid was 2.5 cm^2/mL. Test material ($n = 6$) and positive control were extracted for 24 h at 37°C, using complete culture medium as extraction fluid. Before the tests, culture medium was removed from the wells and an identical volume (2 mL) of extraction fluid was added. For the MEM extraction test, the cells were seeded in 24-well plates ($n = 3$) at a density of 1.25×10^5 cells/well. Then they were incubated for 24 h at 37°C in a humidified atmosphere with 5% CO_2. Cell response was evaluated after 24, 48, and 72 h of incubation. Confluence of the monolayer, degree of floating cells, and changes in morphology were analyzed by visual observation. After 72 h, the percentage of growth inhibition was determined by cell counting with a hemocytometer and trypan blue exclusion method. Final measurements were corrected for the negative control.

MTS test. For the MTS test, cells were seeded in 96-well plates ($n = 6$) at a density of 1.8×10^4 cells/well. Then they were incubated for 24 h at 37°C in a humidified atmosphere with 5% CO_2. As referred, the MTS was performed to evaluate the cytotoxic effects of the developed scaffolds. A CellTiter 96 One solution Cell Proliferation Assay kit (Promega, Madison, WI) was used. It is based on the bioreduction of the substrate MTS into a brown formazan product by dehydrogenase enzymes in metabolically active cells, and is commonly used for cell viability evaluation. Briefly, the procedure was conducted as follows. The extraction procedure was the same as described previously for the MEM extraction test, using 200 μL of extraction fluid per well. After 72 h, the extraction fluid was removed and 200 μL of a mixture containing serum-free culture medium without phenol red, and MTS was added to each well. Cells were then incubated for 3 h at 37°C in a humidified atmosphere containing 5% CO_2. After this, optical density (OD) was measured with a plate reader (Molecular Devices, Sunnyvale, CA) at 490 nm. The mean OD value obtained was standardized taking into account the values for the negative control. Statistical analyses were conducted using a two-sample *t*-test assuming unequal variances for $n = 3$ and $\alpha = 0.05$.

Direct contact assays

Cell seeding and culturing. For the direct contact assays, a mouse mesenchymal progenitor cell line (BMC9) was used. The BMC9 cell line has been shown to exhibit four mesenchymal cell phenotypes: chondrocytic, adipocytic, stromal (supports osteoclast formation), and osteoblastic.[32] The cells were grown as monolayer cultures in a culture medium consisting of α-MEM (Sigma), 10% FBS, and 1% A/B (penicillin G sodium 10,000 U/mL, amphotericine B Fungizone® (Invitrogen, Carlsbad, CA) 25 μg/mL, and streptomycin sulfate 10,000 μg/mL, in 0.85% saline). When the adequate cell number was obtained, cells at passage 9 (P9) were trypsinized, centrifuged, and resuspended in cell culture medium. Cells were seeded at a density of 5×10^5 cells/scaffold ($5 \times 5 \times 5$ mm^3) under static conditions, using for this purpose aliquots of 15 μL loaded on top of the scaffolds that had been previously placed in 24-well culture plates. Two hours after seeding, 1 mL of culture medium

was added to each well and the cell-seeded scaffolds were allowed to develop for periods up to 3 weeks, in a humidified atmosphere at 37°C, containing 5% CO_2, under chondrogenic differentiation inducing medium. This culturing medium consisted of DMEM (Sigma), dexamethasone (Sigma) 1.0×10^{-4} M, sodium pyruvate (Sigma) 1.0×10^{-3} M, ascorbate-2-phosphate (Sigma) 0.17 mM, proline (Sigma) 0.35 mM, ITS 1× (Sigma), and hBMP-2 (R&D BioSystems, Minneapolis, MN) 100 ng/mL. The culture medium was changed every 3 to 4 days until the end of the experiment.

Cellular viability by MTS test. The principle of the MTS test has already been previously herein described for the *in vitro* cytotoxicity tests. The MTS test was performed for the cell-seeded scaffolds for different time periods, specifically 1, 2, and 3 weeks. Briefly, the procedure is as follows. The cell-seeded C-PBS scaffolds ($n=3$) were rinsed in 0.15 M phosphate-buffered saline (Sigma) and immersed in a mixture consisting of serum-free cell culture medium and MTS reagent at 5:1 ratio. Incubation for 3 h at 37°C in a humidified atmosphere containing 5% CO_2 followed. After this, 100 µL were transferred to 96-well plates and the OD determined at 490 nm. Controls consisting of scaffolds without any cells seeded were also used. Statistical analyses were conducted using a two-sample *t*-test assuming unequal variances for $n=3$ and $\alpha=0.05$.

Cell adhesion and morphology by SEM. Cell adhesion, morphology, and average distribution were observed by SEM analysis. The cell–scaffold constructs were washed in 0.15 M phosphate-buffered saline and fixed in 2.5% glutaraldehyde (in phosphate-buffered saline). The constructs were then rinsed three times in phosphate-buffered saline, and subjected to a series of ethanol increasing conditions (30%, 50%, 70%, 90%, and 100% ethanol), 10–15 min each, to allow dehydration of the samples. The samples were let to air dry afterward, and then sputter coated with gold (JEOL JFC-1100) and analyzed with a Leica Cambridge S360 scanning electron microscope.

Western blot: collagen type II. For the protein extraction, the cell–scaffold constructs ($n=3$) were washed in 0.15 M phosphate-buffered saline, lysed in 750 µL of lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, and Triton X-100), and sonicated three times at 40 kV (15 s). After sonication the C-PBS scaffolds were removed and the resulting suspension was centrifuged for 10 min at 13,000 rpm and 4°C, at the end of which the formed pellet was discarded. The supernatants containing the protein fraction were stored for quantification. Western blot was performed using the protein extracts collected at 3 weeks. A 5% stacking polyacrylamide gel (30% acrylamide mix, 1.0 M Tris (pH 6.8), 10% SDS, 10% ammonium persulfate, and TEMED) and an 8% resolving gel (30% acrylamide mix, 1.5 M Tris (pH 8.8), 10% SDS, 10% ammonium persulfate, and TEMED) were prepared. The collected supernatant was heated at 100°C for 5 min in the water bath, and then an aliquot (15.64 µL) was loaded in the gels and subjected to electrophoresis (30 mA, 3 h) and electrotransferred to a Hybond P membrane (Amersham Biosciences, Piscataway, NJ). Afterward, the membrane was washed in phosphate-buffered saline-Tris, submerged in Coomassie blue (isopropylic alcohol 0.25% (v/v), acetic acid 0.1% (v/v), and Coomassie Brilliant Blue R250 2 g/L), and left overnight. The membrane was washed in Ponceau solution, and the transfer from the gel to the membrane was visually confirmed. Membranes were then incubated with a blocking solution of 5% (wt/v) powdered milk in TBS (Tris base 2.42 g/L, NaCl 8 g/L, and HCl 3.8 g/L) with Triton X-100 0.002% (v/v), for 1 h under constant stirring at room temperature. Incubation with an equal solution followed, but altering the powdered milk concentration to 2.5% (wt/v). This solution also included the primary antibody against collagen type II (University of Iowa, Iowa City, IA) at a 1:500 dilution. The membrane was left overnight with constant stirring at 4°C. The membrane was then washed three times in phosphate-buffered saline-Tris under stirring, and incubated with the secondary antibody (1/1000) for 1 h at room temperature, under stirring. The secondary antibody was diluted in the same solution as the one described above for the primary antibody. The membranes were washed three times in phosphate-buffered saline-Tris and passed to a phosphate-buffered saline solution. The immune complex was detected by incubation of the membrane as described in SuperSignal® Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL).

RESULTS

Scaffold characterization

As seen in the SEM and µ-CT images (Figs. 1 and 2, respectively), the structure of the processed C-PBS scaffolds

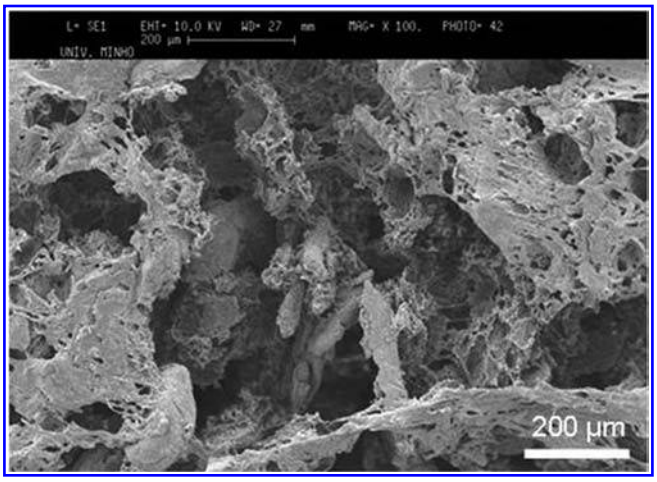

FIG. 1. SEM micrograph showing the surface of a chitosan–polybutylene succinate scaffold 50:50 (wt%), with 80% porosity.

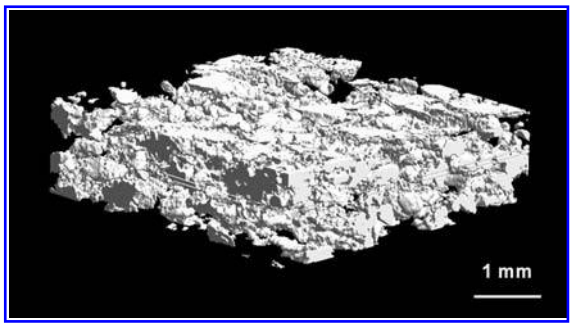

FIG. 2. μ-CT image evidencing the structure and interconnectivity of the processed chitosan–polybutylene succinate scaffold 50:50 (wt%), with 80% porosity scaffolds.

appears to be quite interconnected (Fig. 2) and with suitable pore size to provide support for cell growth and development. μ-CT analysis calculated an average of 66.29% (± 2.55%) porosity and 92.78% (± 1.69%) interconnectivity degree for these C-PBS scaffolds. The salt particles used ranged in size between 63 and 125 μm, retrieving an average pore size of 251.28 μm (± 61. 9 μm). In terms of mechanical properties, the scaffolds exhibited a compressive modulus of 1.73 ± 0.4 MPa (E_{sec} 1%).

In vitro *cytotoxicity tests (MEM extraction and MTS tests)*

Regarding the MEM extraction test, the results showed that the materials did not cause any morphological changes or induce any deleterious alteration to the metabolic activity of L929 cells and thus can be considered noncytotoxic. There was no growth inhibition detected after the 72 h time period (0.0 ± 0.0%) when using the trypan blue exclusion method. The negative control did not affect cell proliferation and morphology, and a monolayer of spread cells was observed.

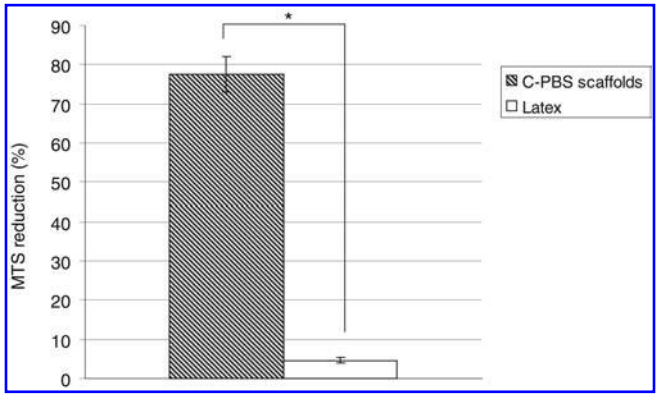

FIG. 3. Graphical representation of the results obtained after cytotoxicity evaluation of the processed scaffolds (MTS test) using L929 cells, derived from a rat lung fibroblast cell line. Statistical analyses were conducted using a two-sample *t*-test assuming unequal variances for $n = 3$ and $\alpha = 0.05$.

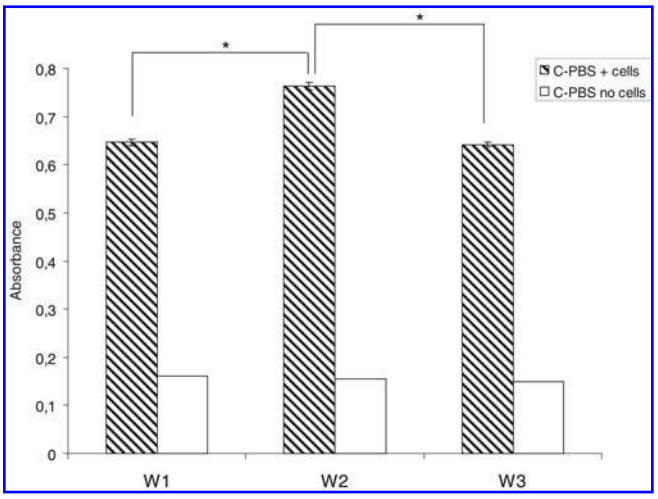

FIG. 4. Graphical representation of the results obtained after performing MTS test with samples taken at 1, 2, and 3 weeks of culturing with BMC9 cells. Statistical analyses were conducted using a two-sample *t*-test assuming unequal variances for $n = 3$ and $\alpha = 0.05$.

The toxic effect of the positive control (latex) was evident, given the severe changes on morphology and the inability of cells to proliferate. The extracts from the C-PBS scaffolds did not exert any deleterious effect on L929 cells morphology, presenting morphological and proliferative features similar to those encountered for the negative controls (data not shown). Concerning the MTS test, L929 cells were able to metabolize the MTS into a brown formazan product after a 72 h incubation period with the collected extracts and the values obtained were similar to the negative control (Fig. 3).

Direct contact assays

Direct contact assays were performed to evaluate BMC9 cell response to the C-PBS scaffolds. MTS was performed to assess cell viability at defined time periods, specifically 1, 2, and 3 weeks. Figure 4 represents the obtained results, where it can be observed that the cells were able to remain viable within the C-PBS scaffolds during the whole time of the experiments. The values were higher than the control for all time points and had a significant increase ($p < 0.05$) from 1 week to 2 weeks, which is a good indicator of cell viability. At 3 weeks, a significant decrease ($p < 0.05$) is observed in the OD values.

SEM analysis (Figs. 5–7) evidenced that BMC9 cells were well adhered onto the C-PBS scaffold surface, and appeared to be morphologically normal throughout the whole time of the experiments. Further, a morphological transition in these cells was observed from an initial fibroblastic-like shape to a round-shaped phenotype, which is a feature present in articular cartilage chondrocytes that underwent culturing under 2D conditions.[34] From the SEM picture at 1 week, it is clear that cells adhered to the scaffolds and spread along its structure (Fig. 5A), forming multilayers (Fig. 5B). Cell

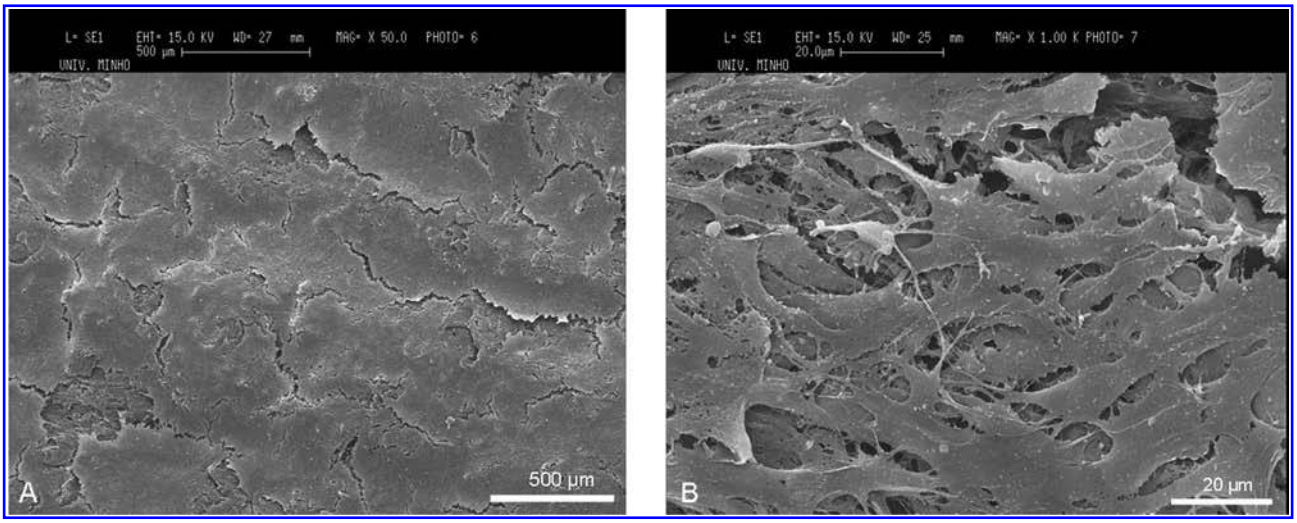

FIG. 5. SEM micrographs of 1 week of culturing (**A**, **B**), showing that BMC9 cells were able to adhere and appear to remain viable within the scaffold structure.

morphology is still clearly fibroblastic-like, with extensive cell-to-cell interactions.

After 2 weeks of culture, cell morphology passed from the initial fibroblastic-like shape with some evident cytoplasmic membrane extensions to a round-shaped phenotype, as shown in Figure 6A and B. SEM analysis also revealed that the chondrocyte-like cells were widely present in the pores of the scaffolds, as shown by a representative example in Figure 6E.

After 3 weeks, almost all the cells exhibited a round-shaped phenotype and were widely distributed throughout the scaffold (Fig. 7). Further, on the cell surface, some structures previously described as collagen fibrils[35] were observed (Fig. 7D, arrows). Even though speculative, one may correlate this observation with the results shown afterward for collagen type II expression, performed by Western blot immunological analysis.

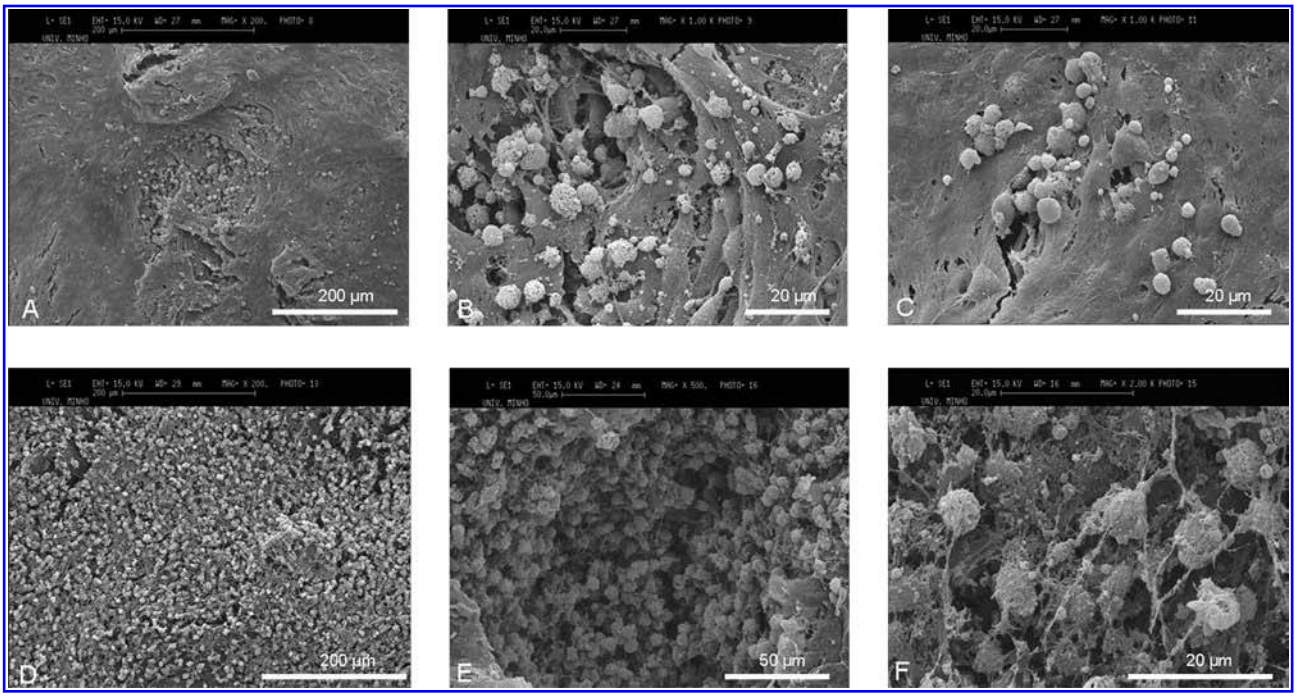

FIG. 6. SEM micrographs of 2 weeks of culturing (**A**–**F**). We can observe the cell morphological transition, passing from the initial fibroblastic-like shape with some evident cytoplasmic membrane extensions to a round-shaped phenotype, as evidenced in (**A**) and (**B**).

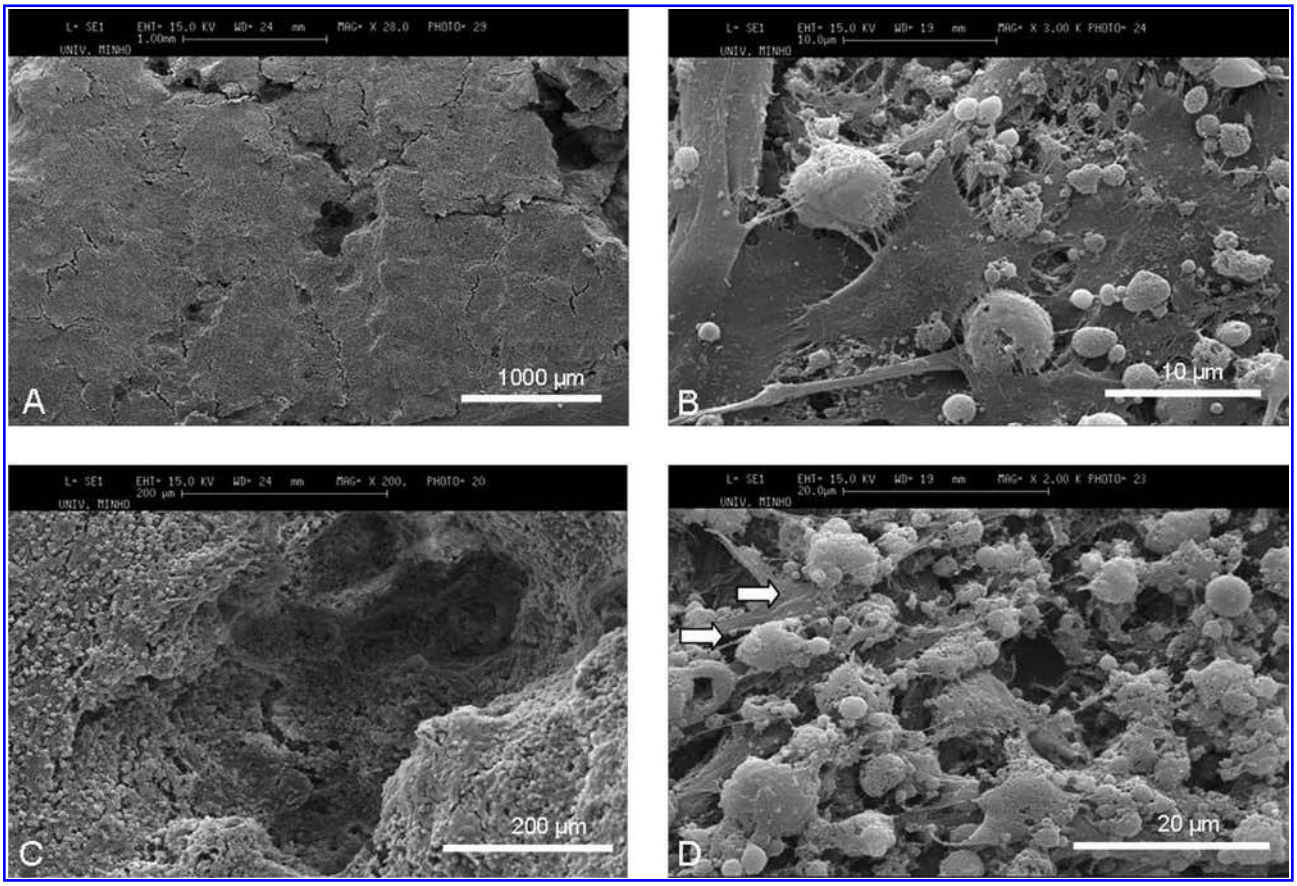

FIG. 7. SEM micrographs showing BMC9 cells onto the developed scaffolds at 3 weeks (**A**–**D**). Most cells exhibited a round-shaped phenotype and were widely distributed throughout the scaffold (**C**). Structures previously described as collagen fibrils are indicated by white arrows.

Western blot analysis at 3 weeks (Fig. 8) demonstrated that collagen II was being expressed, which is a good indicator for the successful differentiation of the BMC9 cells toward the chondrogenic pathway, and suggests at the same time that cartilage-like extracellular matrix was being produced as suggested from the SEM micrograph observations (Fig. 7). The band obtained correlates with data presented in the literature for collagen type II protein.[36,37]

DISCUSSION

In this work, scaffolds produced from a blend of chitosan and polybutylene succinate were tested for assessing their suitability for cartilage tissue engineering applications. The scaffolds were characterized to evaluate their potential for future uses in the cartilage regeneration field by employing techniques such as µ-CT and performing their mechanical behavior evaluation. Their cytotoxicity was determined, and in a final stage the direct contact with BMC9 cells under chondrogenic differentiating conditions was conducted, as well as analysis of their differentiation status. Considering the salt particle sizes used (63–125 µm), one would expect that the created pores after the salt leaching step will be within this range. Nevertheless, some disperse particle agglomerations take place during processing, creating therefore bigger pore sizes, which may arise as an advantage regarding interconnectivity. In fact, results show that the scaffolds present a high range of pore sizes exhibiting a 251.28 µm average pore size with a $\pm$61.9 µm standard deviation. These measurements show that pores of less than 100 µm and more than 300 µm are also present in the scaffolds, granting them greater versatility in terms of pore size profiles for cells to distribute. The average porosity of 66.29% ($\pm$2.55%) is also adequate and quite interesting considering the processing technology used, while the 92.78% ($\pm$1.69%) interconnectivity degree renders these scaffolds with a quite interconnected structure, thereby enabling seeded cells to proliferate and establish communication paths throughout the 3D support, as well as allowing nutrients and metabolic waste flow to be conducted. Such data prompt these C-PBS scaffolds to go through further screenings in order to evaluate their potential in the generation of a functional cartilage tissue-engineered construct. The mechanical properties of the scaffolds disclosed values

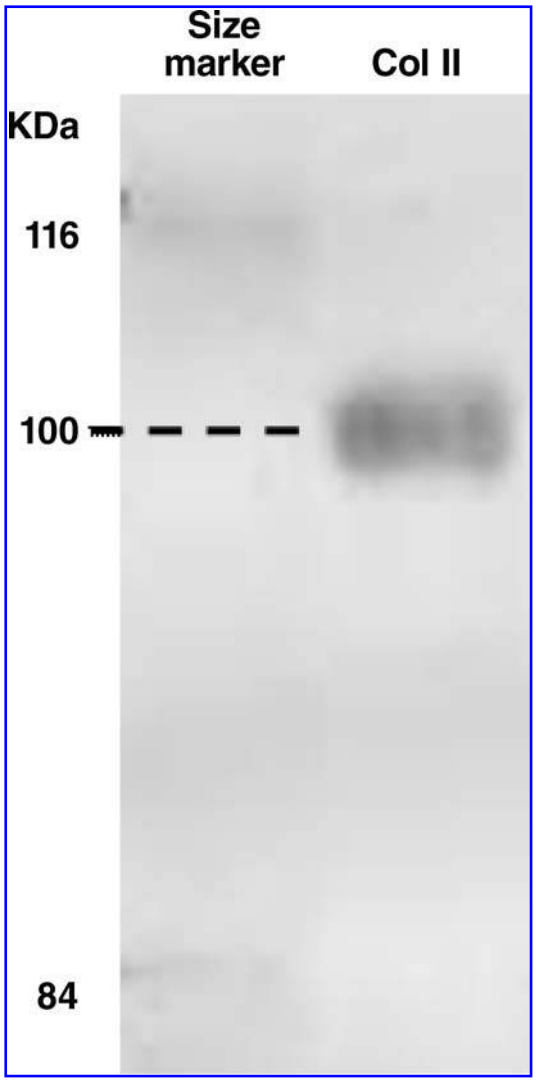

FIG. 8. Western blot analysis performed for collagen type II. The band obtained correlates with data presented in the literature for collagen type II protein (approximately 100 kDa). Collagen type II expression is a good indicator for the successful differentiation of the BMC9 cells toward the chondrogenic lineage.

that are in the range of those shown in the literature for human articular cartilage[38], which is an indication of the suitability of the C-PBS scaffolds in terms of mechanical performance for cartilage regeneration strategies. In an *in vivo* scenario, the authors expect a balance between chitosan absorption by the organism, and cells proliferation and tissue ingrowth, in such a way that the mechanical support and biological role of the structures are constantly maintained. Cytotoxicity evaluation performed using MEM and MTS tests showed that the C-PBS were noncytotoxic. The leachables released from the scaffolds did not cause any severe alteration to the L929 cell metabolism, thereby reinforcing their adequacy for moving through further studies in the regeneration of cartilaginous tissues. The following step involved the direct contact of cells with the fabricated C-PBS scaffolds. The cells used were derived from a mouse mesenchymal stem cell line (BMC9), and these were cultured under chondrogenic inductive conditions onto the C-PBS scaffolds, assessing in this way the suitability of these systems in future cartilage regeneration studies. Bone morphogenetic protein type 2 (BMP-2) was used as the promoting agent for chondrogenic differentiation. BMP-2 is a protein belonging to the TGF-β superfamily that can stimulate the chondrogenic lineage development of cells from mesenchymal origin, and its inclusion was a key factor in cell differentiation. The cell seeding was performed using the cell drop seeding method, which is currently used for seeding cells into scaffolds. Although it is disadvantageous in terms of cell seeding homogeneity throughout the support structure, it is a good way to perform a first evaluation of how the cells react once in contact with the proposed scaffolds. In the case of the C-PBS, although this method gives rise to an inhomogeneous cell distribution, it was observed that the scaffold surface was highly colonized throughout the time of the experiments. When analyzing the MTS results, it is possible to observe that the values were higher than the control for all time points and had a significant increase ($p < 0.05$) from 1 week to 2 weeks, which is a good indicator of cell viability. This may be the consequence of two factors: either the cells are proliferating and thereby more MTS is metabolized, justifying therefore the higher OD values, or the proliferation rates are not that high, but on the other hand the mitochondrial machinery is highly active, converting therefore higher amounts of MTS. Any combination of these two situations is possible, but either mechanism is indicative of cell viability within the scaffold structure. This fact is highly relevant for the following steps because although the leachables released from the scaffolds did not present any *in vitro* cytotoxicity, the direct cell contact with the structures could be affected by factors such as surface chemistry, topography, and wettability, which were shown not to occur. At 3 weeks, a significant decrease ($p < 0.05$) is observed in the OD values, which may be directly related to normal changes in cell metabolism, given that the active protein synthesis normally associated with this stage usually implies a decrease in cellular proliferation. As the mitochondria have a prominent role in cell growth, the reduction in their activity leads to a diminished metabolization of the MTS. The C-PBS scaffolds appear to be able to support BMC9 cell proliferation and differentiation toward the chondrogenic lineage, once subjected to the specific medium used which contained BMP-2. This occurrence was observed by SEM after 2 weeks of culture, since the cells passed from an initial fibroblast to a more round chondrocyte-like morphology. In fact, this situation is similar to the one obtained when growing primary culture chondrocytes under a 2D environment for extended periods of time. Initially, the chon-

drocytes dedifferentiate and adopt a more fibroblastic-like phenotype, with alteration in protein expression, decreasing, for example, collagen type II expression and increasing collagen type I.[34,39] Nevertheless, once confluence is reached under a 2D environment, cells start to pack themselves into multilayers, reflecting a consequence of high cell density culturing, and start to regain their globular morphology. In fact, this multilayered arrangement somehow mimics a 3D environment such as the one created under pellet or 3D support cultures. Once this change takes place, protein expression patterns also modify, and, for example, collagen type II levels increase again. In this work, this change in morphology is an indication for a possible BMC9 cell chondrogenic lineage differentiation. This was an expected result due to the culturing of the cells in a 3D environment supplied with BMP-2. Further, on the cell surface, some structures previously described as collagen fibrils[35] were observed (Fig. 7D, arrows). Even though speculative, one may correlate this observation with the results shown afterward for collagen type II expression, performed by Western blot immunological analysis. Finally, Western blot analysis after 3 weeks (Fig. 8) demonstrated that collagen type II was being expressed, which is a good indicator for the successful differentiation of the BMC9 cells toward the chondrogenic pathway, and suggests at the same time that cartilage-like extracellular matrix was being produced as suggested from the SEM micrograph observations (Fig. 7). The band obtained correlates with data presented in the literature for collagen type II protein.[36,37] Given the mesenchymal progenitor origin of BMC9 cells, which have not been shown to normally express collagen type II, this result leads us to believe that the cells were actually being directed toward the chondrogenic lineage. As a preliminary screening, such suggestions are indeed important for the future application of these systems in cartilage regeneration approaches. The results obtained so far show that these C-PBS scaffolds have fulfilled the basic requirements to be put through sequential testing. This work revealed that they can support the growth and differentiation of undifferentiated cells and create an environment suitable for their chondrogenic differentiation. These results can be considered as a base for following experiments that can combine undifferentiated cells from other sources, direct them toward the chondrogenic lineage, and culture them *in vitro* for adequate periods so that a functional tissue-engineered construct is formed. This possesses the mechanical stability provided by polybutylene succinate and the biological similarity properties conferred by chitosan.

CONCLUSIONS

In the present work, it was observed that scaffolds made of a blend of chitosan and polybutylene succinate 50:50 (%wt) are adequate to be used in cartilage tissue engineering approaches. These scaffolds were shown to present mechanical properties and morphological features suitable for cell development and to be noncytotoxic and cytocompatible. Direct contact assays evidenced that cells from a mouse mesenchymal progenitor cell line (BMC9) were able to adhere to the scaffold surface and penetrate its pores, as well as remaining viable for at least 3 weeks of culturing. SEM analysis indicated that the cells were directed toward the chondrogenic lineage due to observed morphological transitions occurring around 2 weeks of culture. This was one of the expected outcomes, due to the 3D environment onto which the cells were cultured, as well as the specific medium used, which contained BMP-2. BMC9 cell chondrogenic differentiation was further corroborated by the collagen type II expression obtained after 3 weeks of culturing. The obtained data so far present good perspectives for the use of chitosan and polybutylene succinate scaffolds in cartilage regeneration approaches.

ACKNOWLEDGMENTS

J.T. Oliveira would like to acknowledge the grant (SFRH/BD17135/2004) from Portuguese Foundation for Science and Technology (FCT). The authors would like to thank Fernanda Marques, at the Institute for Health and Life Sciences (ICVS), University of Minho, Braga, Portugal, for her help with the Western blot analysis, as well as the staff at ICVS for allowing to use their facilities. The monoclonal antibody for collagen type II was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa (Iowa City, IA). This work was carried out under the scope of the European NoE EXPERTISSUES (NMP3-CT-2004-500283), and partially supported by the European Project GENOSTEM (LSHB-CT-2003-503161) and the FCT Project CartiScaff (POCTI/SAU-BMA/58991/2004).

REFERENCES

1. Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O., and Peterson, L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med **331**(14), 889–895, 1994.
2. Gross, A.E. Cartilage resurfacing: filling defects. J Arthroplasty **18**(3 Suppl 1), 14–17, 2003.
3. Duda, G.N., Maldonado, Z.M., Klein, P., Heller, M.O., Burns, J., and Bail, H. On the influence of mechanical conditions in osteochondral defect healing. J Biomech **38**(4), 843–851, 2005.
4. Richardson, J.B., Caterson, B., Evans, E.H., Ashton, B.A., and Roberts, S. Repair of human articular cartilage after

implantation of autologous chondrocytes. J Bone Joint Surg Br **81**(6), 1064–1068, 1999.
5. Salgado, A.J., Gomes, M.E., Chou, A., Coutinho, O.P., Reis, R.L., and Hutmacher, D.W. Preliminary study on the adhesion and proliferation of human osteoblasts on starch-based scaffolds. Mater Sci Eng C Biomimetic Supramol Syst **20**(1–2), 27–33, 2002.
6. Chen, G., Sato, T., Ohgushi, H., Ushida, T., Tateishi, T., and Tanaka, J. Culturing of skin fibroblasts in a thin PLGA-collagen hybrid mesh. Biomaterials **26**(15), 2559–2566, 2005.
7. Holmes, T.C., de Lacalle, S., Su, X., Liu, G., Rich, A., and Zhang, S. Extensive neurite outgrowth and active synapse formation on self-assembling peptide scaffolds. Proc Natl Acad Sci USA **97**(12), 6728–6733, 2000.
8. Langer, R., and Vacanti, J.P. Tissue engineering. Science **260**(5110), 920–926, 1993.
9. Jung, Y., Kim, S.S., Kim, Y.H., Kim, S.H., Kim, B.S., Kim, S., and Choi, C.Y. A poly(lactic acid)/calcium metaphosphate composite for bone tissue engineering. Biomaterials **26**(32), 6314–6322, 2005.
10. Malafaya, P.B., and Reis, R.L. Porous bioactive composites from marine origin based in chitosan and hydroxylapatite particles. Key engineering materials vol. **240–242**, 39–42, 2003.
11. Zammaretti, P., and Jaconi, M. Cardiac tissue engineering: regeneration of the wounded heart. Curr Opin Biotechnol **15**(5), 430–434, 2004.
12. Koegler, W.S., and Griffith, L.G. Osteoblast response to PLGA tissue engineering scaffolds with PEO modified surface chemistries and demonstration of patterned cell response. Biomaterials **25**(14), 2819–2830, 2004.
13. Yang, Y., Basu, S., Tomasko, D.L., Lee, L.J., and Yang, S.T. Fabrication of well-defined PLGA scaffolds using novel microembossing and carbon dioxide bonding. Biomaterials **26**(15), 2585–2594, 2005.
14. Hoffman, A.S. Hydrogels for biomedical applications. Adv Drug Deliv Rev **54**(1), 3–12, 2002.
15. Kim, B.S., and Mooney, D.J. Development of biocompatible synthetic extracellular matrices for tissue engineering. Trends Biotechnol **16**(5), 224–230, 1998.
16. Gomes, M.E., Ribeiro, A.S., Malafaya, P.B., Reis, R.L., and Cunha, A.M. A new approach based on injection moulding to produce biodegradable starch-based polymeric scaffolds: morphology, mechanical and degradation behaviour. Biomaterials **22**(9), 883–889, 2001.
17. Solchaga, L.A., Dennis, J.E., Goldberg, V.M., and Caplan, A.I. Hyaluronic acid-based polymers as cell carriers for tissue-engineered repair of bone and cartilage. J Orthop Res **17**(2), 205–213, 1999.
18. Solchaga, L.A., Temenoff, J.S., Gao, J., Mikos, A.G., Caplan, A.I., and Goldberg, V.M. Repair of osteochondral defects with hyaluronan- and polyester-based scaffolds. Osteoarthritis Cartilage **13**(4), 297–309, 2005.
19. Boccafoschi, F., Habermehl, J., Vesentini, S., and Mantovani, D. Biological performances of collagen-based scaffolds for vascular tissue engineering. Biomaterials 2005. Vol 26, Issue 35. December 2005, Pages 7410–7417.
20. Suh, J.K., and Matthew, H.W. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomaterials **21**(24), 2589–2598, 2000.
21. Reis, R.L., Cunha, A.M., Allan, P.S., and Bevis, M.J. Mechanical behavior of injection-molded starch-based polymers. Polym Adv Technol **7**(10), 784–790, 1996.
22. Chen, G., Liu, D., Tadokoro, M., Hirochika, R., Ohgushi, H., Tanaka, J., and Tateishi, T. Chondrogenic differentiation of human mesenchymal stem cells cultured in a cobweb-like biodegradable scaffold. Biochem Biophys Res Commun **322**(1), 50, 2004.
23. Wang, Y.-C., Lin, M.-C., Wang, D.-M., and Hsieh, H.-J. Fabrication of a novel porous PGA-chitosan hybrid matrix for tissue engineering. Biomaterials **24**(6), 1047–1057, 2003.
24. Freier, T., Koh, H.S., Kazazian, K., and Shoichet, M.S. Controlling cell adhesion and degradation of chitosan films by N-acetylation. Biomaterials **26**(29), 5872–5878, 2005.
25. Madihally, S.V., and Matthew, H.W.T. Porous chitosan scaffolds for tissue engineering. Biomaterials **20**(12), 1133–1142, 1999.
26. Correlo, V.M., Boesel, L.F., Bhattacharya, M., Mano, J.F., Neves, N.M., and Reis, R.L. Properties of melt processed chitosan and aliphatic polyester blends. Mater Sci Eng A **403**(1–2), 57–68, 2005.
27. Mahmoudifar, N., and Doran, P.M. Tissue engineering of human cartilage and osteochondral composites using recirculation bioreactors. Biomaterials **26**(34), 7012–7024, 2005.
28. Devlieghere, F., Vermeulen, A., and Debevere, J. Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. Food Microbiol **21**(6), 703–714, 2004.
29. Silva, R.M., Malafaya, P.B., Mano, J.F., and Reis, R.L. Bioactive composite chitosan membranes to be used in bone regeneration applications. Key engineering materials vol. **240–242**, 423–425, 2003.
30. Lyoo, W.S., Kim, J.H., Yoon, W.S., Ji, B.C., Choi, J.H., Cho, J., Lee, J., Yang, S.B., and Yoo, Y. Effects of polymer concentration and zone drawing on the structure and properties of biodegradable poly(butylene succinate) film. Polymer **41**(26), 9055–9062, 2000.
31. Henry, E.W., Chiu, T.-H., Nyilas, E., Brushart, T.M., Dikkes, P., and Sidman, R.L. Nerve regeneration through biodegradable polyester tubes. Exp Neurol **90**(3), 652–676, 1985.
32. Dennis, J.E., Merriam, A., Awadallah, A., Yoo, J.U., Johnstone, B., and Caplan, A.I. A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse. J Bone Miner Res **14**(5), 700–709, 1999.
33. Biological evaluation of medical devices as on ISO 10993–1:2003 http://www.iso.org/iso/.
34. Cancedda, R., Dozin, B., Giannoni, P., and Quarto, R. Tissue engineering and cell therapy of cartilage and bone. Matrix Biol **22**(1), 81–91, 2003.
35. Fuss, M., Ehlers, E.M., Russlies, M., Rohwedel, J., and Behrens, P. Characteristics of human chondrocytes, osteoblasts and fibroblasts seeded onto a type I/III collagen sponge under different culture conditions: a light, scanning and transmission electron microscopy study. Ann Anat **182**(4), 303–310, 2000.
36. Gartland, A., Mechler, J., Mason-Savas, A., MacKay, C.A., Mailhot, G., Marks, S.C., and Odgren, P.R. *In vitro* chondrocyte differentiation using costochondral chondrocytes as a

source of primary rat chondrocyte cultures: an improved isolation and cryopreservation method. Bone **37**(4), 530–544, 2005.

37. Estrada, L.E., Dodge, G.R., Richardson, D.W., Farole, A., and Jimenez, S.A. Characterization of a biomaterial with cartilage-like properties expressing type X collagen generated *in vitro* using neonatal porcine articular and growth plate chondrocytes. Osteoarthritis Cartilage **9**(2), 169–177, 2001.
38. Woodfield, T.B.F., Malda, J., de Wijn, J., Peters, F., Riesle, J., and van Blitterswijk, C.A. Design of porous scaffolds for cartilage tissue engineering using a three-dimensional fiber-deposition technique. Biomaterials **25**(18), 4149–4161, 2004.
39. Cancedda, R., Descalzi Cancedda, F., and Castagnola, P. Chondrocyte differentiation. Int Rev Cytol **159,** 265–358, 1995.

Address reprint requests to:
João Teixeira de Oliveira, B.Sc.
3B's Research Group—Biomaterials, Biodegradables, and Biomimetics
Department of Polymer Engineering
University of Minho
Campus de Gualtar
4710-057 Braga
Portugal

E-mail: Joao.oliveira@dep.uminho.pt

Received: September 19, 2007
Accepted: February 21, 2008

This article has been cited by:

1. Cornelia Theresa Brunner, Erkan Türker Baran, Elisabete Duarte Pinho, Rui Luís Reis, Nuno Meleiro Neves. 2011. Performance of biodegradable microcapsules of poly(butylene succinate), poly(butylene succinate-co-adipate) and poly(butylene terephthalate-co-adipate) as drug encapsulation systems. *Colloids and Surfaces B: Biointerfaces* . [CrossRef]
2. Albino Martins , Elisabete D. Pinho , Vítor M. Correlo , Susana Faria , Alexandra P. Marques , Rui L. Reis , Nuno M. Neves . 2010. Biodegradable Nanofibers-Reinforced Microfibrous Composite Scaffolds for Bone Tissue EngineeringBiodegradable Nanofibers-Reinforced Microfibrous Composite Scaffolds for Bone Tissue Engineering. *Tissue Engineering Part A* **16**:12, 3599-3609. [Abstract] [Full Text] [PDF] [PDF Plus]
3. Vitor M. Correlo, Ana R. Costa-Pinto, Paula Sol, José A. Covas, Mrinal Bhattacharya, Nuno M. Neves, Rui L. Reis. 2010. Melt Processing of Chitosan-Based Fibers and Fiber-Mesh Scaffolds for the Engineering of Connective Tissues. *Macromolecular Bioscience* n/a-n/a. [CrossRef]
4. Pierre Charbord, Erella Livne, Gerhard Gross, Thomas Häupl, Nuno M. Neves, Pierre Marie, Paolo Bianco, Christian Jorgensen. 2010. Human Bone Marrow Mesenchymal Stem Cells: A Systematic Reappraisal Via the Genostem Experience. *Stem Cell Reviews and Reports* . [CrossRef]
5. M.L. Alves da Silva, A. Crawford, J.M. Mundy, V.M. Correlo, P. Sol, M. Bhattacharya, P.V. Hatton, R.L. Reis, N.M. Neves. 2010. Chitosan/polyester-based scaffolds for cartilage tissue engineering: Assessment of extracellular matrix formation. *Acta Biomaterialia* **6**:3, 1149-1157. [CrossRef]
6. Albino Martins, Wu Gang, Elisabete D. Pinho, Esther Rebollar, Stefano Chiussi, Rui L. Reis, Betty León, Nuno M. Neves. 2010. Surface modification of a biodegradable composite by UV laser ablation: in vitro biological performance. *Journal of Tissue Engineering and Regenerative Medicine* n/a-n/a. [CrossRef]
7. Ana M. Martins, Catarina M. Alves, F. Kurtis Kasper, Antonios G. Mikos, Rui L. Reis. 2010. Responsive and in situ-forming chitosan scaffolds for bone tissue engineering applications: an overview of the last decade. *Journal of Materials Chemistry* **20**:9, 1638. [CrossRef]
8. M. L. Alves da Silva, A. Martins, A.R. Costa-Pinto, V.M. Correlo, P. Sol, M. Bhattacharya, S. Faria, R. L. Reis, N. M. Neves. 2010. Chondrogenic differentiation of human bone marrow mesenchymal stem cells in chitosan-based scaffolds using a flow-perfusion bioreactor. *Journal of Tissue Engineering and Regenerative Medicine* n/a-n/a. [CrossRef]
9. V. M. Correlo, L. F. Boesel, E. Pinho, A. R. Costa-Pinto, M. L. Alves da Silva, M. Bhattacharya, J. F. Mano, N. M. Neves, R. L. Reis. 2009. Melt-based compression-molded scaffolds from chitosan-polyester blends and composites: Morphology and mechanical properties. *Journal of Biomedical Materials Research Part A* **91A**:2, 489-504. [CrossRef]
10. J. T. Oliveira, T. C. Santos, L. Martins, M. A. Silva, A. P. Marques, A. G. Castro, N. M. Neves, R. L. Reis. 2009. Performance of new gellan gum hydrogels combined with human articular chondrocytes for cartilage regeneration when subcutaneously implanted in nude mice. *Journal of Tissue Engineering and Regenerative Medicine* **3**:7, 493-500. [CrossRef]
11. M. K. El Tamer, R. L. Reis. 2009. Progenitor and stem cells for bone and cartilage regeneration. *Journal of Tissue Engineering and Regenerative Medicine* **3**:5, 327-337. [CrossRef]