Biodegradable PCL scaffolds with an interconnected spherical pore network for tissue engineering

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Abstract: A technique for producing controlled interconnected porous structures for application as a tissue engineering scaffold is presented in this article. The technique is based on the fabrication of a template of interconnected poly(ethyl methacrylate) (PEMA) microspheres, the introduction of a biodegradable polymer, poly-ε-caprolactone (PCL), and the elimination of the template by a selective solvent. A series of PCL scaffolds with a porosity of 70% and pore sizes up to 200 μm were produced and characterized (both thermally and mechanically). Human chondrocytes were cultured in monolayer on bulk PCL disks or seeded into porous PCL scaffolds. Cell adhesion, viability, proliferation, and proteoglycan (PG) synthesis were tested and compared with monolayer cultures on tissue-treated polystyrene or pellet cultures as reference controls. Cells cultured on PCL disks showed an adhesion similar to that of the polystyrene control (which allowed high levels of proliferation). Stained scaffold sections showed round-shaped chondrocyte aggregates embedded into porous PCL. PG production was similar to that of the pellet cultures and higher than that obtained with monolayer postconfluence cultures. This shows that the cells are capable of attaching themselves to PCL. Furthermore, in porous PCL, cells maintain the same phenotype as the chondrocytes within the native cartilage. These results suggest that PCL scaffolds may be a suitable candidate for chondrocyte culture. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 85A: 25–35, 2008

Key words: scaffold; porosity; cartilage tissue engineering; polycaprolactone; chondrocyte

INTRODUCTION

Tissue engineering is a promising field that aims at fabricating biological alternatives referring to harvested tissues and organs for transplantation.[1–10] In one approach, cells are seeded and cultured to develop into tissues on a biodegradable scaffold, which eventually disappears over time.

In this technique, the scaffolding should perform a number of critical functions: they must be as similar as possible to the *in vivo* environment[5,8,11] and they have to show a high surface/volume ratio[3,4]; in other words, they should have a highly porous structure,[3,4,12,13] with uniform pore size, distribution, and interconnectivity.[3,10] This allows for a cell distribution throughout the whole material with a high population density,[4,13] as well as facilitating the arrival of nutrients to, and the disposal of metabolic waste from, cells. Above all, they must have an appropriate surface chemistry to allow cell adhesion and growth[3,12,14–16] and, thus, compatibility with the culture medium.

Because of these requirements, biodegradable polymers (via hydrolytic or enzymatic degradation) are good candidates to produce tissue engineering scaffolds and a number of methods of obtaining macroporous polymeric materials have been reported.[1–4,10,13,17–22]

The purpose of this work is to use one of these polymeric materials, more specifically, polycaprolactone, a biodegradable material[23]; in the development of an appropriate three-dimensional porous substrate capable of supporting cellular culture.

In this work, a method to produce polycaprolactone (PCL) macroporous scaffolds is described. The three-dimensional pore structure is obtained by melt processing with porogen leaching. Morphological, thermal, and mechanical characterizations of the scaffolds are also determined. Here, we will describe the preparation process and the resulting porous structure, in addition to characterizing it according to its thermal and mechanical behavior.

Finally, the cell adhesion, viability, and proliferation of human chondrocytes on PCL scaffolds will be characterized, being these parameters essential in evaluating the performance of a biomaterial in tissue engineering applications.

Aggrecan is a core protein of large aggregating proteoglycans (PGs) and the main component of cartilage matrix; the detection of its presence can thus be used as a marker of chondrocyte differentiation. Therefore, the second step in the biological characterization was to examine the adhesion capacity, distribution, morphology, and aggrecan production of cells seeded in these new scaffolds. This study of the biological response of chondrocytes seeded on PCL is intended to be a preliminary study in order to elucidate the ability of the biomaterial to support chondrocyte activities and to regenerate the native cartilage.

MATERIALS AND METHODS

Polymeric materials

Poly(ε-caprolactone) (PCL) (M_W: 10,000–20,000, $\rho_{PCL} = 1.145$ g/cm^3; Polysciences) was employed to develop the porous materials. Poly(ethyl methacrylate) (PEMA) beads (Elvacite 2043; DuPont), having a size of around 200 $\pm$ 25 μm, were used as the porogen material.

Besides, ethanol (EtOH) (+99.5%; Scharlau) was used to leach out the filler material from the PCL. Each of these materials was employed as received.

Fabrication of scaffolds

Glass tubes with diameters ranging between 5 and 7 mm were sealed with porous stoppers and filled with PEMA beads. A sintering treatment was then applied, at 130°C for 60 min. Once the beads have been sintered, PCL was placed over them and fused at 80°C for 45 min. After that, the bottom of the tube was connected to a vacuum pump (Fig. 1), while keeping the whole system at 80°C until the end of the infiltration.

Once this operation finished, the sample was taken out of the glass tube and the filler removed by placing it in EtOH (which was changed daily) for 96 h, the result being porous polymeric scaffolds.
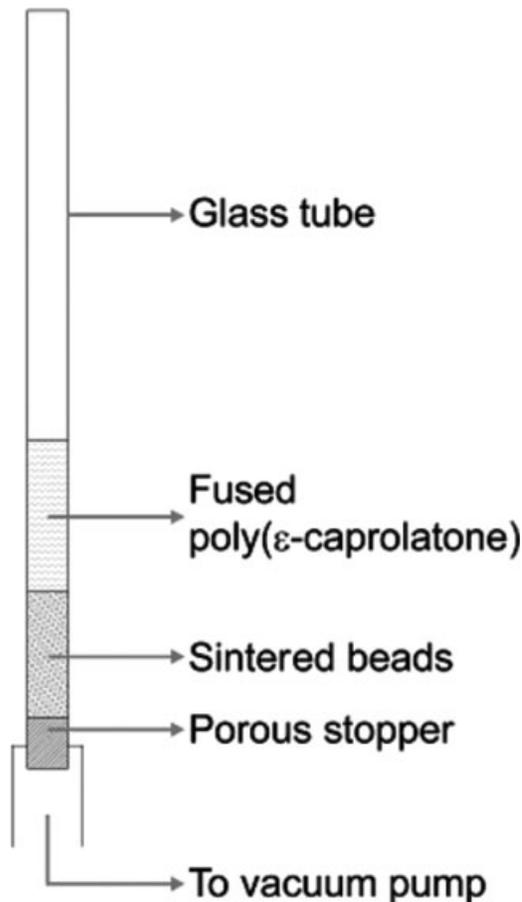

Figure 1. Porous scaffold manufacturing system. PEMA template is placed inside a glass tube and PCL is infiltrated because of the depression created with a vacuum pump.

Bulk (i.e. nonporous) PCL samples, were also prepared, by placing the polymer into a sealed tube and following the same process (PCL melting, cooling, and washing) as followed in the preparation of porous PCL scaffolds.

Cylinders of about 6 mm diameter and 4 mm height were prepared, both for porous and nonporous samples.

Scanning electron microscopy analysis

Scanning electron microscopy (SEM) (Jeol JSM 6300) was used to examine the morphology of the scaffolds. To observe their inner structure, the porous PCL samples were cryogenically fractured with liquid nitrogen, and then analyzed. Views of the (circular) top surface were also obtained. In this case, the material was cut with a hot blade and planed with a microtome (Microm HM 350 S rotation microtome).

In both cases, the surface to be observed was finally prepared by deposition of a gold coating (using a Baltec SCD 005 sputtering machine).

Porosity determination analysis

Porosity was characterized in two ways. First, the pore size was measured from different SEM images by means

of an image analysis process. Second, the overall porosity was determined from apparent density measurements of the scaffolds (immersion approach). In that way, samples were weighed in air and into distilled water, and their apparent density is calculated by means of the following expression:

$$\rho_{app} = \left[\frac{w_A}{(w_A - w_L)} (\rho_L - \rho_A) \right] + \rho_A \qquad (1)$$

being w_A the weight of the samples in air, w_L their weight when immersed in distilled water, ρ_L the density of distilled water (at the temperature conditions of measurement), and ρ_A the density of air (considered as a constant value of 0.0012 g/cm^3).

Then, porosity of scaffolds is determined from their apparent density and the density of bulk PCL, using the following expression:

$$\varepsilon\,(\%) = \left(1 - \frac{\rho_{app}}{\rho_{PCL}} \right) \times 100 \qquad (2)$$

These results were also compared with those that can be obtained from apparent density measurements (dimensional approach), and from the difference in weight between equal-dimensioned samples, porous and nonporous. All the samples used were cylinders of about 6 mm diameter (d) and 4 mm height (h).

In the dimensional approach, an apparent density is calculated from the exact dimensional values for each measured sample and their weight in air:

$$\rho_{app} = \frac{4\,w_A}{\pi d^2 h} \qquad (3)$$

and porosity is calculated using those values of apparent density and the density of bulk PCL, with the same expression that has been presented before.

Finally, the difference in weight between porous (w_P) and nonporous (w_{np}) samples (mean values), all of them having the same dimensions, permits obtaining directly a value of porosity:

$$\varepsilon\,(\%) = \left(\frac{w_{np} - w_p}{w_{np}} \right) \times 100 \qquad (4)$$

Thermal characterization

Differential scanning calorimetry (DSC) (Perkin–Elmer DSC 7) was carried out on three types of samples: commercial PCL (as received), porous PCL scaffolds, and bulk PCL samples. Runs were performed from −100 to 100°C at a rate of 10°C/min in an inert atmosphere of nitrogen with an inflow of 20 cm^3/min.

Mechanical testing

Both porous PCL scaffolds and bulk PCL samples were tested mechanically. Cylinder-shaped samples of about 6 mm diameter and 4 mm height were subjected to a dynamic-mechanical thermal analysis (Seiko DMS 210). The samples were submitted to cyclic loadings at a frequency of 1 Hz in compression mode, within the range from −100 to 50°C at a heating rate of 10°C/min.

Chondrocyte isolation

Human articular cartilage was obtained from osteoarthritic knee joints after prosthesis replacement. The study was conducted in accordance with the 1975 Declaration of Helsinki, as revised in 1983, and approved by our local Ethics Committee. All patients submitted written informed consent before their inclusion in the study.

Dulbecco's modified Eagle's medium (DMEM) (Life Technologies), hyaluronidase, collagenase-IA, and dimethyl sulphoxide (DMSO) (Sigma-Aldrich), pronase (Merck, VWR International SL), fetal bovine serum (FBS) (Invitrogen SA) were used for chondrocyte isolation and preservation.

The cartilage was dissected from subchondral bone, diced finely, and washed with supplemented DMEM. For chondrocyte isolation, the diced cartilage was incubated for 30 min with 0.5 mg/mL hyaluronidase in a shaking water bath at 37°C. The hyaluronidase was subsequently removed and 1 mg/mL pronase was added. After 60 min incubation in a shaking water bath at 37°C, the cartilage pieces were washed with supplemented DMEM. After removal of the medium, digestion was continued by addition of 0.5 mg/mL of collagenase-IA in a shaking water bath kept at 37°C overnight. The resulting cell suspension was filtered through a 70-μm pore size nylon filter (BD Biosciences) to remove tissue debris. Cells were centrifuged and washed with DMEM supplemented with 10% FBS. Finally, the cells were counted and cryopreserved in liquid nitrogen with DMEM containing 20% FBS and 10% DMSO, or plated in tissue culture flasks for chondrocyte culture.

Cell culture on the materials

After isolating or thawing, cells were plated in culture flasks at a high density in DMEM supplemented with 10% FBS and 50 μg/mL ascorbic acid (Sigma-Aldrich) at 37°C in a 5% CO$_2$ humidified atmosphere. The medium was changed every 3 days. After 7–14 days, adherent cells were harvested by incubation with trypsin/EDTA (Biological Industries, Israel) and seeded onto the bulk PCL disks previously moistened with Hank's balanced salt solution (Sigma-Aldrich) for 24 h in the incubator. In the case of porous PCL scaffolds, the cells were seeded into the premoistened material using an insulin syringe.

All the PCL samples (both bulk and porous ones) were placed on a 96-well polystyrene culture plate (Nunc A/S, Denmark). In every experiment, cells cultured onto the polystyrene of a 96-well plate (without biomaterial) were used as a control (CPS; tissue culture polystyrene). These monolayer cultures (CPS) were used to normalize the values from aggrecan quantification both in PCL bulk cultures and in PCL scaffold and pellet cultures. Monolayer cultures (both on bulk PCL disks and on polystyrene) were initiated at a density of 10,000 cells per disk and porous scaffolds were injected with 500,000 cells in 50 μL

of culture medium. After 2–3 days, the biomaterials were changed to a new well to test only the cells attached onto the sample material. First or second passage cultures were used in our experiments.

The chondrocytic phenotype was assessed in terms of the synthesis of aggrecan by means of immunoassay (Human aggrecan ELISA Kit). Data were normalized to CPS values within the experiment and means were calculated using these normalized values.

Pellet preparation

After cell harvesting from culture flasks, resuspended cells were transferred to a 30-mL polystyrene centrifuge tube (1,500,000 cells per tube) and culture medium was added up to 1 mL. Cell suspension was centrifuged for 4 min at 1200 rpm. The resulting pellet was still cultured with DMEM supplemented with 10% FBS and 50 μg/mL ascorbic acid at 37°C in a 5% CO_2 humidified atmosphere. The medium was changed every 3 days.

Cell viability and proliferation assay on bulk PCL disks

Human aggrecan ELISA Kit (Biosource), Mayer's haematoxylin (Sigma-Aldrich), MTT assay (Roche Diagnostics GmbH), colorimetric BrdU immunoassay (Roche Diagnostics GmbH), and bovine serum albumin (BSA) (Sigma-Aldrich) were used for the study of chondrocyte integrity, viability, and proliferation in the cultures.

Cell viability and proliferation were monitored at 7 and 14 days from the seeding. The cell viability was evaluated by MTT assay, based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. The resulting colored solution was quantified using a ELISA reader (A550).

Proliferation was determined using a colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis. In this case, cells were synchronized by incubation in serum-free medium with 0.1% BSA for 2 days. Serum-containing medium was added 24 h before the proliferation assay.

Cell adhesion was evaluated by cell staining using Mayer's haematoxylin and analyzed with optical microscopy: 2 days after seeding, the disks were changed to a new culture well, to ensure not taking into account nonattached cells. Moreover, the disk staining and mounting was done only over the material and only the cells adhered on disk were microscopically observed.

Cell viability and proliferation were monitored by cell staining, the MTT test and BrdU assay at 7 and 14 days from the seeding: cell staining was performed with Mayer's haematoxylin and analyzed with optical microscopy. Cell viability was evaluated by MTT assay and proliferation was determined using a colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis. In this case, cells were synchronized by incubation in serum-free medium with 0.1% BSA for 2 days. Serum-containing medium was added 24 h before the proliferation assay.

Data from each independent experiment were normalized to CPS values within the experiment. Each experiment was performed in triplicate and repeated at least three times using different chondrocyte populations.

PCL scaffold histology

The capacity for chondrocyte adhesion in the porous PCL scaffold was monitored by cell staining at 7, 14, and 28 days' culture using Mayer's haematoxylin and analyzed by optical microscopy. Briefly, scaffolds were embedded in optimum cutting temperature (OCT) compound, cryosectioned (8 μm thick), and stored at −20°C until use. Cryosections were air-dried and fixed in acetone for 10 min before haematoxylin staining. Alcian blue was used to stain the glycosaminoglycan.

RESULTS AND DISCUSSION

A variety of tissue engineering approaches are motivated by the clinical need for cartilage tissue repair. Among them, growing isolated chondrocytes on polymeric scaffolds for making a three-dimensional cartilage tissue suitable for implantation is a primary approach in tissue engineering involving the regeneration of tissue. It is considered that the concept of tissue engineering is widely based on a cell–polymer system in which a biodegradable polymer functions for a certain period of time as a substrate or scaffold to promote tissue formation.[24]

The process described in this study produced a three-dimensional porous PCL structure, which ensured connectivity of the pores throughout the foam structure after leaching out the filler material, as shown in Figure 2. As all filler beads are bonded together in the early stages of the scaffold processing, an open-pore structure is left after their removal.

The well-connected porous structure obtained consisted in spherical cavities, which retained the shapes of the original filler beads, linked to each other with circular communicating troughs with a mean size of 130 ± 25 μm. This macroporous network was the negative of the template formed by the sintered PEMA beads.

The SEM microphotographs of the scaffolds obtained for their surface and cross sections were similar, thus indicating that the entire polymer skeleton of the foam had a uniform morphology.

Table I shows the results from overall porosity determination of the scaffolds, both by means of apparent density measurements and from differences in weight between porous and nonporous samples. According to these data, an overall porosity of around 70% can be stated.
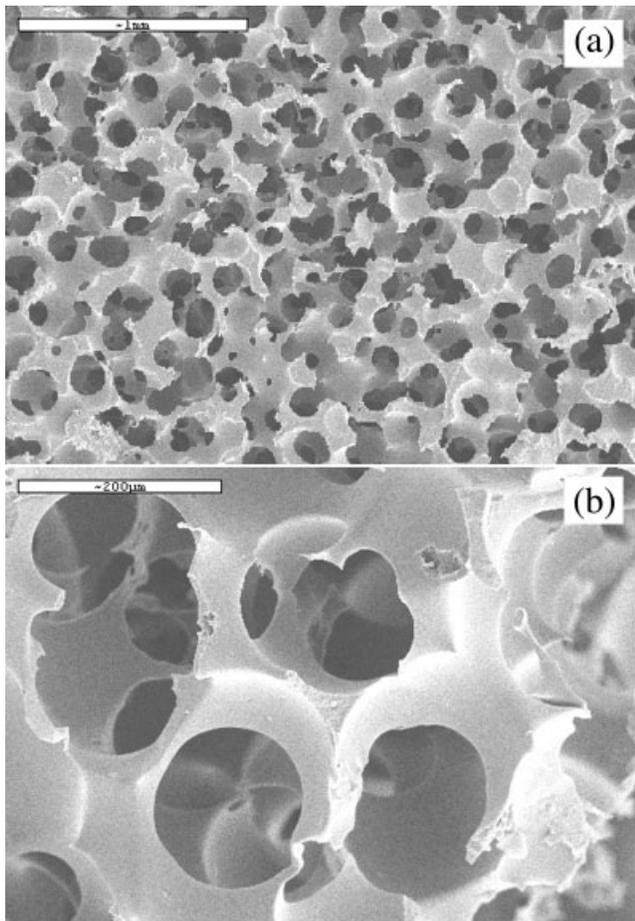
Figure 2. SEM micrographs of porous PCL scaffolds: (a) general view at 45×; (b) local view at 200×.

One approach to improve cartilage formation lies in the design of the substrate, as this has the potential to affect the quality of the formed tissue. In fact, the initial success of a bioengineered cartilage–substrate construct depends, in part, on the attachment of chondrocytes to the substrate, and their adhesion is going to influence the evolution and the quality of the *in vitro*-formed tissue; and porosity is a characteristic of the scaffold that may affect chondrocytes behavior.[25,26]

From that point of view, the pore size obtained in our scaffolds can be suitable for an appropriate growth of chondrocytes culture, as previous studies stated that pore sizes ranging from 100 to 300 µm are favorable size ranges for chondrocytes growth,[27] being a pore size from 80 to 200 µm suitable for chondrocyte attachment, proliferation, aggregation, and extracellular matrix production[28] but preferably around (or over) 100 µm, which is a pore space that may provide enough room for growth of chondrocytes.[29]

A series of DSC thermograms were obtained from the bulk and porous materials in the range of temperatures from −100 to 100°C at a heating rate of 10°C/min. The transition temperature values (−60°C) of both types of samples remains constant, which is an evidence that the manufacturing procedure does not have a significant effect on the amorphous structure of PCL. As PCL is a semicrystalline polymer, there is a second variation in the thermograms, a peak in the heat flow between 50 and 70°C which corresponds to the fusion of polymer crystallites. The fusion peak for all types of samples can be seen in Figure 3. The fusion enthalpy (ΔH_f) of a 100% crystalline PCL is 139.5 J/g,[30–32] and calculating this enthalpy for the sampled materials (commercial, bulk, and porous) the percentage of crystallinity of each type can be also obtained (Table II), using the equation:

$$\chi_c(\%) = \frac{\Delta H_f}{\Delta H_{f,100}} \cdot 100 \tag{5}$$

being χ_c the amount of crystallinity in the material, ΔH_f the fusion enthalpy of the samples, measured from DSC thermograms; and $\Delta H_{f,100}$ the fusion enthalpy of the 100% crystalline material, previously referred. It can be seen how both porous and bulk PCL samples have lower values of crystallinity and temperature values of the peaks than the ones corresponding to the commercial material. These variations can be attributed to thermal treatments (temperature increasing above the fusion point, followed

TABLE I
Values of Porosity of PCL Scaffolds Determined from Apparent Density Measurements, Immersion, and Dimensional Approaches (Mean Values of Apparent Densities Given), and from Difference in Weight between Equal-Dimensioned Porous and Nonporous PCL Samples (Mean Values of Weight of Each Type of Sample Given)

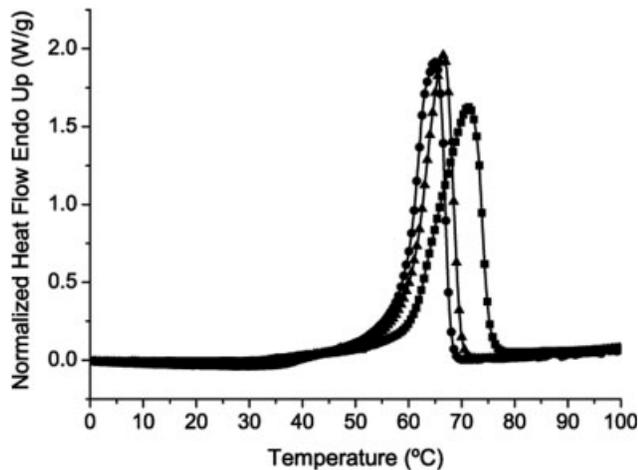| Measurement Technique	Apparent Density (g/cm³)		Porosity (%)
Immersion in water	0.348 ± 0.015		69.59 ± 1.33
Dimensional approach	0.347 ± 0.015		69.69 ± 1.30
	$W_{nonporous}$ (mg)	W_{porous} (mg)	Porosity (%)
Difference in weight between porous and nonporous samples	114.13 ± 2.74	35.69 ± 3.19	68.73 ± 4.01

Figure 3. DSC heat flow thermograms of commercial PCL (■), bulk PCL disks (▲), and porous PCL scaffolds (●) showing the melting peak between 50 and 70°C.

by a cooling stage back to room temperature) that can change the proportion between the amorphous and crystalline parts of the material, as well as the size of the crystalline microdomains.

Figure 4 shows curves for the loss tangent (tan δ) and the real part of the complex compressive modulus (E'), of the dynamic-mechanical test performed for both the bulk and the porous materials. The main dynamic-mechanical (or α) relaxation of amorphous or semicrystalline polymers is triggered by the glass transition of the system. As can be seen, the temperature of the maximum of the relaxation thermogram (T_α) displayed no shift towards higher or lower temperatures when compared with the bulk material. It can be said that there must not be any noticeable change in the amorphous part of the polymer due to the scaffold manufacturing procedure. It can be clearly seen that in the glassy state (in temperatures below the main mechanical relaxation) the modulus differences are of one magnitude order, and these differences are bigger after the relaxation. More specifically, at 37°C (body temperature) the modulus of the porous material drops by a factor of 25 with respect to the modulus of the bulk PCL.

However, in spite of that decrease of modulus values, the suitability of these porous scaffolds for tissue engineering has to be studied not only referring to proper cell culture and differentiation processes (that will be discussed later), but also dealing with mechanical similarities to cartilage tissues.[33]

Therefore, the mechanical response, in terms of compressive moduli, of different cartilage tissues, both from animal and human sources, are presented in Table III. Compressive modulus values for porous PCL scaffolds are also presented there.

In view of these values, the mechanical response of porous PCL scaffolds could be suitable for cartilage development, as they behave in a similar way than many cartilage types, both from animal and, above all, human sources.

It has to be noted, however, that compressive modulus values have been determined from different ways (confined and unconfined compression, indentation, uniaxial compression, dynamic compression, etc.), so they could vary slightly from one type of measurement to another, but, in general terms, they can be used to have an overview of the mechanical range of response of these types of cartilage tissues, being the properties of the porous PCL scaffolds within that range.

Besides, the cells cultured on the bulk PCL disks were stained with Mayer's haematoxylin to assess their adhesive capacity. Observations of the monolayer culture by optical microscopy showed the attachment of cells to the bulk disks. These cells had a fibroblast-like shape, similar to that shown on the CPS controls (Fig. 5). It is well known that cell proliferation depends on the capacity of adhesion onto the biomaterial. In terms of cell adhesion and proliferation, the results obtained at 14 days after seeding were similar to the results obtained on polystyrene [Fig. 5(b,d)].

The BrdU assay, the MTT test, and the ELISA assay were used to assess the proliferative activity, cellular viability, and PG production, respectively, on bulk PCL disks at 7 and 14 days after seeding. The results are shown in Figure 6. The data were normalized in each experiment with the value obtained with CPS. Normalization allowed comparisons to be made between experiments performed on different days and with different chondrocyte lines. The results were expressed as percentages (base one) with respect to the control (horizontal line corresponding to value 1).

TABLE II
Values of Fusion Enthalpy (ΔH_f), Onset Temperature of Fusion Peak (T_{onset}), Temperature of the Maximum of the Fusion Peak (T_p), and Percentage of Crystallinity (X_c) of Bulk and Porous PCL

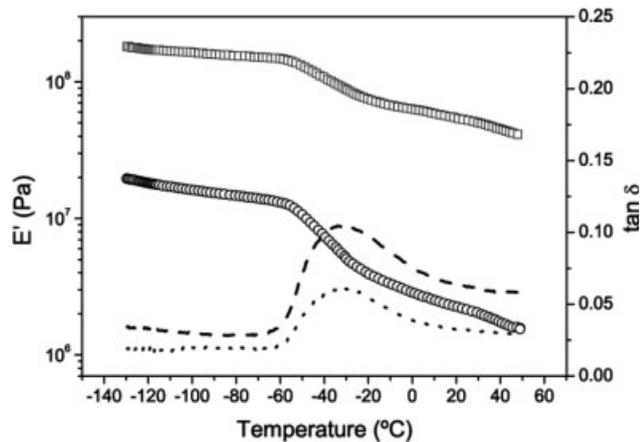|	Commercial PCL (as received)	Bulk PCL Samples	Porous PCL Scaffolds
ΔH_f (J/g)	93.84 ± 0.75	88.81 ± 1.85	88.88 ± 1.82
T_{onset} (°C)	59.18 ± 2.77	59.02 ± 1.36	58.06 ± 2.05
T_p (°C)	71.30 ± 0.44	67.50 ± 0.87	64.90 ± 0.26
X_c (%)	67.3 ± 0.5	63.7 ± 1.3	63.7 ± 1.3

Figure 4. Temperature dependence of the dynamic-mechanical loss tangent (tan δ) (..., bulk PCL; ---, porous PCL) and compressive storage modulus (*E'*) (□, bulk PCL; ○, porous PCL).

The different assays (MTT, BrdU, and PG ELISA) showed complementary results, which are consistent with cellular adhesion on the material and the cell number (Fig. 5). In all cases, PCL results at 14 days after seeding were comparable to the results on CPS, which is coherent with the microphotography results [Fig. 5(b,d)]. At 7 days' culture, the proliferation results were better in the control because there was a slight reduction in the number of cells on PCL [Fig. 5(a,c)]. One explanation could be that adhesion might be faster in the CPS culture. Another possibility would be that the material did not fit perfectly into the well and some cells could have fallen into the surrounding area and would therefore not be attached to the sample. However, the results on PCL were similar to CPS at 14 days after seeding because both cultures reached confluence.

Similar results in terms of chondrocyte proliferation were found in a study performed by Tsai et al.[37] In this case, chondrocytes from pig were seeded on PCL-coated glass dishes and the adherent

TABLE III
Mechanical Responses (Compressive Moduli)
of Different Cartilage Tissues, Both from Animal
and Human Sources, and Compressive Modulus
Values for Porous PCL Scaffolds

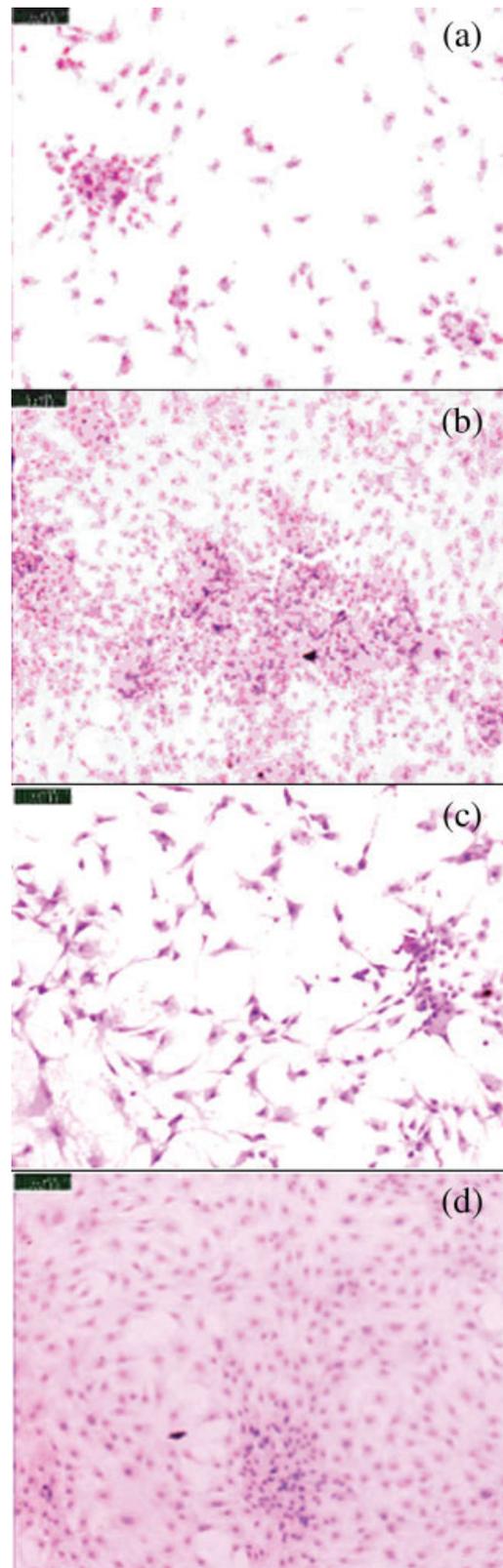| Cartilage Tissue	Compressive Modulus (MPa)
Meniscal tissue	Bovine knee: 0.41–0.79[34,35]
	Femoropatellar calf neocartilage: 0.15–0.20[36]
	Human: 0.22[34]
Temporomandibular joint disc	Canine: 16–31[34]
	Porcine: 0.02–0.03[34]
	Human: 2–30[34]
Articular cartilage	Porcine neonatal: 0.78–0.81[35]
	Human: 0.51–15.3[34]
Porous PCL scaffolds	At 37°C: 6.85 ± 1.83

Figure 5. Optical microscope pictures of Mayer's haematoxylin-stained monolayer chondrocytes seeded onto: (a) bulk PCL after 7 days' culture, (b) bulk PCL after 14 days' culture, (c) CPS control after 7 days' culture, and (d) CPS control after 14 days' culture. The length of the black bar represents 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
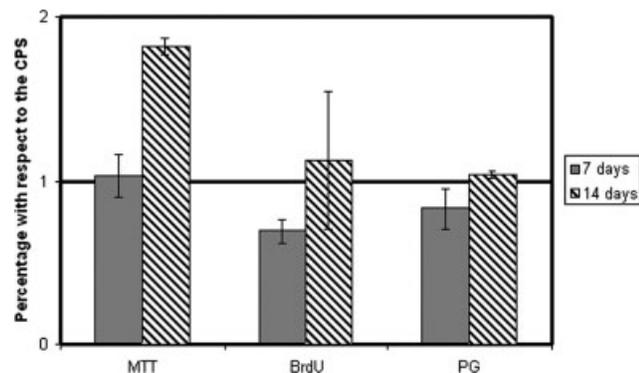
Figure 6. Viability (MTT), proliferation (BrdU), and proteoglycan (PG) synthesis measurements at 7 and 14 days after seeding of human chondrocytes cultured on PCL disks. The optical density (OD) of the MTT assay was determined at 550 nm, the BrdU test at 450 nm, and PG quantification at 450 nm. Data represent the mean ± standard deviation, and are expressed as a percentage (base one) with respect to the CPS values (threshold 1).

cells were quantified. At day 7, the adhered cells number onto CPS was higher than that onto PCL, but this difference was reduced at day 14.

Expansion of chondrocytes in two-dimensional culture systems results in their dedifferentiation.[38,39] Accordingly, expression of specific hyaline cartilage markers such as aggrecan decreases. In the present study, aggrecan synthesis was quantified at 7 and 14 days after seeding. A first subculture was used to seed bulk PCL disks and aggrecan production was tested to control the chondrocyte phenotype. The ELISA results revealed the presence of aggrecan in the medium supernatants and synthesis levels were similar to those achieved on CPS (Fig. 6). The differences in aggrecan values between the 2 days of measurement are consistent with the differences in the amount of cells with respect to the control and the results are not a proof of differences in cell differentiation. Thus, the amount of aggrecan expression would be equivalent to the values obtained in the MTT and BrdU assays.

Once the human chondrocyte cytocompatibility of the bulk PCL had been established, a second step was to evaluate the applicability of a porous PCL scaffold in a three-dimensional culture. Architectural and surface properties of the scaffold can affect adhesion, proliferation, and differentiation of cells.[9,25,40,41]

Chondrocytes were injected into the porous PCL scaffolds to test their adhesion and PG synthesis. The scaffold sections were stained with Mayer's haematoxylin to allow analysis of the cell arrangement within the material. Chondrocyte aggregates embedded in porous PCL are shown in Figures 7 and 8(a). Chondrocytes were able to attach to the PCL scaffold and acquired a round shape, very similar to that observed in native cartilage or in a pellet culture
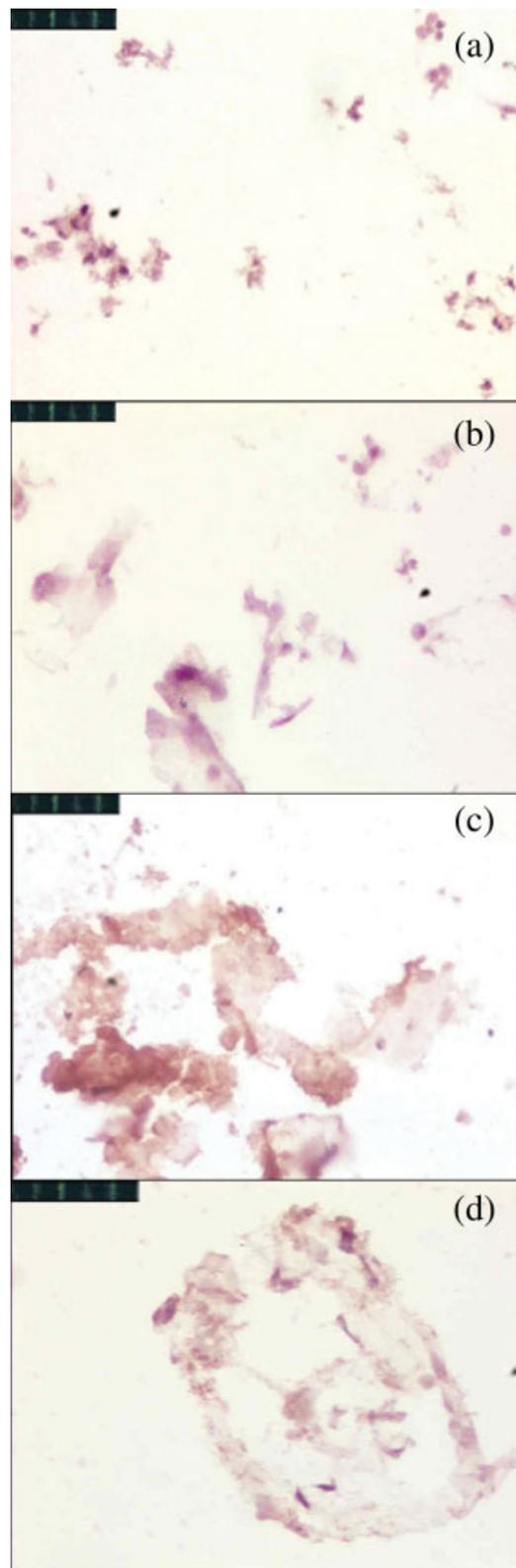

Figure 7. Histological sections of human chondrocytes seeded on porous PCL scaffolds at: (a) 7 days, (b) 14 days, (c) 21 days, and (d) 28 days of culture with Mayer's haematoxylin staining. The scale bars indicate 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
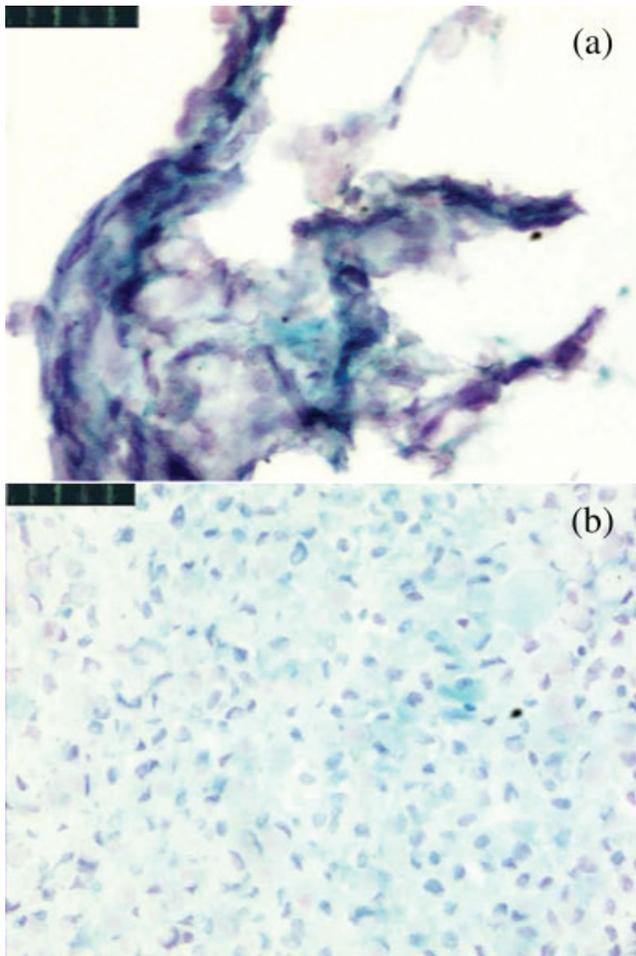
Figure 8. Histological sections of human chondrocytes seeded on (a) porous PCL scaffold and (b) high density pellet culture at 28 days' culture. PG was detected by Alcian blue staining. The scale bars indicate 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Figs. 7 and 8). This suggests that the cells keep the same phenotype as the chondrocytes within native cartilage, as a round morphology is known to be indicative of characteristics of differentiated chondrocytes.[27,42]

Similar results have been obtained throughout different studies, where mesenchymal stem cells are able to express a chondrogenic phenotype, during chondrogenic induction, when cultured within three-dimensional biomaterials,[43] or chondrocytes isolated from rabbit articular cartilage grow acquiring a predominantly spherical shape when cultured into polyhydroxyalkanoate scaffolds.[24]

No obvious increase in cell number could be appreciated and we could only see differences in the long term (from 21 days after seeding). The proliferative capacity was decreased, but they were capable of synthesizing new extracellular matrix (Fig. 7).

The results from the aggrecan ELISA assay showed that the porous PCL scaffolds enhance PG

production compared to monolayer cultures (CPS) for 14 days (Fig. 9). These monolayer cultures (CPS) were used to normalize experiments performed at different days and with different primary chondrocyte lines (value 1 in Fig. 9). In addition, the CPS allowed comparing the cell differentiation status in PCL and pellet cultures respect to monolayer cultures.

This confirms the possibility of being these porous structures a good substrate for developing chondrocytes in a differentiated state, as aggrecan (large PG) is one of the major components of articular cartilage, and the production of these macromolecules is used as a marker to indicate maintenance of the articular chondrocyte phenotype, as chondrocytes shape influence the synthesis of these extracellular matrix components.[25,44]

PCL results were also compared to pellet cultures as a positive control of differentiation. The pellet cultures are formed by centrifugation, which allows the cells to achieve a high density environment to promote cell–cell interactions.

The chondrocyte pellets cultured *in vitro* are capable of forming aggregates with round-shaped cells like in native cartilage. These cells are functional and express specific markers of the chondrocyte phenotype. Other authors[45,46] have proved that high-density culture pellets maintain the differentiated phenotype and favor the formation of extracellular cartilage matrix.

In addition, previous studies have reported that mesenchymal stem cells exhibited chondrogenic properties when maintained as cell aggregates or pellets.[47,48] However, the pellet culture system has several inherent disadvantages (small size and weak mechanical properties), whereas the biomaterial
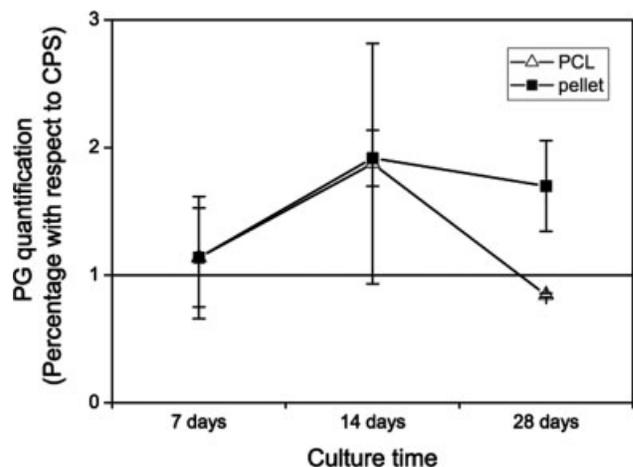

Figure 9. Comparisons of proteoglycan (PG) synthesis between PCL scaffolds and pellet three-dimensional cultures at 7, 14, and 28 days after seeding. Data represent the mean ± standard deviation, from normalized values with CPS. Threshold 1 corresponds to the monolayer culture (CPS) value.

scaffolds are able to provide three-dimensional environments and predesigned mechanical properties.

The behavior of chondrocytes seeded on a porous PCL scaffold was similar to the case of pellet culture. In both 28 days' culture, the presence of aggrecan in the supernatant was diminished with respect to monolayer culture. One hypothesis would be the inhibition of PG production due to accumulation within the porous scaffold. Another possibility would be the formation of an extracellular matrix that could retain the aggrecan and hence there would be no flow into the medium. This can be proved when analyzing histological slices, where both PCL and pellet cultures showed a new matrix synthesized around the chondrocytes with the presence of PG (Fig. 8).

Similar results were obtained in others studies using PCL scaffolds.[41,45,49,50] Li et al. fabricated fibrous three-dimensional scaffolds polymer using PCL polymer. They demonstrated that the scaffolds were biocompatible and supported cell differentiation, showing the normal rounded phenotype of chondrocytes, as well as good proliferation patterns, although they found reduced proliferation on PCL compared to CPS culture. These results agree with the reduced proliferation detected in our bulk PCL samples at 7 days' culture and, in particular, into PCL scaffolds.

Anyway, this is a logical response, as fast expansion of chondrocytes is usually carried out on monolayer cultures, but cells tend to dedifferentiate during that fast multiplication stage. After, if chondrocytes are to redifferentiate (or are already differentiated), they show a drastic drop in multiplication speed.[43,51]

Therefore, cell attachment, proliferation, and/or differentiation on a material are the indication of cellular compatibility of that material and its suitability for tissue engineering applications.[27] It has also been proved that scaffolds where the cultured chondrocytes remain spherical and produce extracellular matrix components could be potential candidates for cartilage tissue-engineering applications.[35,51]

Then, the viability of the culture of chondrocytes in a new type of PCL scaffold has been demonstrated in this study. Moreover, our results show that chondrocytes are able to maintain their differentiated phenotype, in terms of cell shape and glycosaminoglycan synthesis; although, more studies are needed to evaluate whether they are able to regenerate cartilage *in vitro* and, finally, *in vivo*.

CONCLUSIONS

In this study, new polycaprolactone scaffolds with interconnected spherical pores and 70% porosity have been produced successfully. The morphological study shows that there is an open pore structure and a good interconnectivity between pores. The porous structure, and the pore size that has been obtained, fulfills the morphological criteria for chondrocytes development.

Furthermore, compressive modulus of scaffolds is in the range of cartilage tissue responses.

Finally, the viability of the culture of chondrocytes in the PCL scaffold has been demonstrated (obtaining similar results as in Refs. 40, 41), although, more studies are needed to evaluate whether they are able to regenerate cartilage *in vitro* and, finally, *in vivo*. Human primary chondrocytes attached and proliferated on PCL disks similarly to CPS control. Three-dimensional culture of chondrocytes within PCL scaffolds showed similar results as pellet culture in terms of cellular shape and aggrecan synthesis.

Therefore, this scaffold is suitable to perform further studies in order to evaluate its capacity to cartilage regeneration.

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