

# Effect of in vitro enzymatic degradation on 3D printed poly( $\epsilon$ -caprolactone) scaffolds: morphological, chemical and mechanical properties

Joana Ferreira<sup>1</sup>, Antonio Gloria<sup>2</sup>, Stefania Cometa<sup>3</sup>, Jorge F.J. Coelho<sup>4</sup>, Marco Domingos<sup>5</sup>

<sup>1</sup>Centre for Rapid and Sustainable Product Development, Polytechnic Institute of Leiria, Leiria - Portugal

<sup>2</sup>Institute of Polymers, Composites and Biomaterials, National Research Council of Italy, Naples - Italy

<sup>3</sup>Jaber Innovation s.r.l., Rome - Italy

<sup>4</sup>CEMUC-Chemical Engineering Department, University of Coimbra, Coimbra - Portugal

<sup>5</sup>School of Mechanical, Aerospace and Civil Engineering, University of Manchester, Manchester - UK

J. Ferreira, A. Gloria and S. Cometa contributed equally to this work.

## ABSTRACT

**Background:** In recent years, the tissue engineering (TE) field has significantly benefited from advanced techniques such as additive manufacturing (AM), for the design of customized 3D scaffolds with the aim of guided tissue repair. Among the wide range of materials available to biomanufacture 3D scaffolds, poly( $\epsilon$ -caprolactone) (PCL) clearly arises as the synthetic polymer with the greatest potential, due to its unique properties – namely, biocompatibility, biodegradability, thermal and chemical stability and processability. This study aimed for the first time to investigate the effect of pore geometry on the in vitro enzymatic chain cleavage mechanism of PCL scaffolds manufactured by the AM extrusion process.

**Methods:** Morphological properties of 3D printed PCL scaffolds before and after degradation were evaluated using Scanning Electron Microscopy (SEM) and micro-computed tomography ( $\mu$ -CT). Differential Scanning Calorimetry (DSC) was employed to determine possible variations in the crystallinity of the scaffolds during the degradation period. The molecular weight was assessed using Size Exclusion Chromatography (SEC) while the mechanical properties were investigated under static compression conditions.

**Results:** Morphological results suggested a uniform reduction of filament diameter, while increasing the scaffolds' porosity. DSC analysis revealed an increment in the crystallinity degree while the molecular weight, evaluated through SEC, remained almost constant during the incubation period (25 days). Mechanical analysis highlighted a decrease in the compressive modulus and maximum stress over time, probably related to the significant weight loss of the scaffolds.

**Conclusions:** All of these results suggest that PCL scaffolds undergo enzymatic degradation through a surface erosion mechanism, which leads to significant variations in mechanical, physical and chemical properties, but which has little influence on pore geometry.

**Keywords:** Biomanufacturing, Enzymatic degradation, Polycaprolactone, Scaffolds, Tissue engineering

## Introduction

Tissue engineering (TE) products are generally based on the combination of artificial scaffolds – characterized by sat-

isfying porosity, biocompatibility and biodegradability properties – with cells. This approach arises as one of the most promising strategies for the successful reconstruction of damaged tissues and organs (1). Scaffolds are engineered to provide an immediate biomechanical support at the site of implantation, to stimulate the new tissue ingrowth and to be gradually eliminated through a degradation mechanism, characterized by a precise balance between the implant degradation duration and the tissue regeneration rate (2). The inability to synchronize these rates may generate instability in the neo tissue, leading to premature mechanical failure of the entire system (3).

The most common materials used to design and construct artificial tissues (i.e., synthetic polymers) must possess a wide

Accepted: May 4, 2017

Published online: June 15, 2017

### Corresponding author:

Prof. Marco Domingos  
School of Mechanical, Aerospace and Civil Engineering  
The University of Manchester  
Sackville Street, M13 9PL Manchester, UK  
marco.domingos@manchester.ac.uk

range of properties that span the range from mechanical to biochemical properties, including biodegradability and its mutability with time (4, 5). Furthermore, the degradation of polymeric matrices is a phenomenon that can be influenced by different variables. Among these, we can distinguish between the inherent chemical-physical properties of the polymer (i.e., chemical structure, degree of polymerization, degree of crystallinity, etc.) and properties related to the scaffold architecture (dimension, geometry and porosity of the implant), as well as many external factors (including pH of degradation medium, temperature or presence of enzymes, cells or tissues, etc.). Despite its importance in the overall performance of TE scaffolds, the breakdown process of a synthetic polymer is defined by a terminology that appears to be quite inconsistent. Hence, to avoid the misleading use of the term *biodegradable* and to provide some clarification, Vert et al (6) proposed 4 terms – namely, biodegradable, bioresorbable, bioabsorbable and bioerodible – to accurately describe the breakdown mechanism (Tab. I).

Poly( $\epsilon$ -caprolactone) (PCL) is one of the most important polymers used in the production of long-term degradable TE implants, thanks to its high stability, ease of processability under mild conditions, excellent biocompatibility and consistent degradation rates (7-9). PCL is generally categorized as a biodegradable aliphatic polyester; however, the most precise definition for PCL should be “bioresorbable,” since its chain breakdown pathway leads to the formation of degradation products which can be rapidly eliminated by the body without cytotoxic effects (10). However, as Pitt et al suggested (11), in studying the PCL degradation behavior both in vitro and in vivo, the term *bioerodible* should also be employed to describe the polymer erosion mechanism occurring at the surface. Different studies have well documented the degradation behavior of PCL-based scaffolds, which seems to be mainly driven by hydrolytic and enzymatic processes (10-14). Hydrolysis is responsible for the bulk degradation, whereas the enzymatic mechanism occurs mainly at the surface of the

polymer, causing its erosion (15, 16). Bulk degradation takes place when the medium penetrates inside the polymer matrix promoting a random hydrolytic chain scission in a uniform manner and with consequent molecular weight decrease.

On the other hand, surface erosion occurs when non-equilibrium is reached in the reaction-diffusion mechanism: in this case, penetration of the degradation medium into the polymer matrix is significantly slower than hydrolysis, thus enabling the surface degradation by-products to rapidly migrate in the aqueous medium, while preventing the medium itself from moving inside the matrix interior. Consequently, the degradation mainly occurs at the more exposed polymer layers, causing the thinning of the constructs without changing polymer molecular weight.

The enzymatic degradation process can be driven by an ester bond cleavage caused by enzymes like lipase. This cleavage occurs mainly due to similarity of polyesters to lipids where, normally, an ester bond is formed between carboxylic acid functionalities present in fatty acids and alcoholic ones present in molecules such as glycerol (17). Based on the literature, there are 3 types of lipase capable of accelerating the degradation of PCL: *Rhizopus delemar* lipase (18), *Rhizopus arrhizus* lipase and *Pseudomonas* lipase (19, 20).

The degradation time of PCL-containing structures, evaluated by simulating the in vivo conditions, is relatively high and can last up to 4 years (21). As reported by Zhang et al (22), the degradation process, evaluated in vitro of 3-dimensional (3D) PCL scaffolds fabricated by combining a salt leaching process and thermally induced phase separation, is strongly influenced by the porosity of the structures. PCL scaffolds with higher porosity, when immersed in phosphate-buffered saline (PBS) solution for 72 weeks, presented a higher degree of degradation compared with scaffolds with smaller porosity.

Duarte and coworkers (13) investigated the effect of  $\alpha$ -amylase and/or lipase on the in vitro degradation rate of starch-based PCL scaffolds produced using supercritical fluid technology. The results showed that, in contrast to  $\alpha$ -amylase, lipase strongly affected the degradation of PCL that underwent a bulk degradation process, mainly based on hydrolysis of polymer chains.

Castilla-Cortazar et al (23) investigated the PCL degradation process under hydrolytic and enzymatic conditions, evaluating the weight loss, swelling ratio and mechanical property changes. The results obtained showed that the enzymatic degradation mechanism was different from that which occurred by hydrolysis. Moreover, the degradation rate was found to be faster in the presence than in the absence of enzymes. In agreement with other works, the enzymatic degradation followed a surface erosion route, while the hydrolytic degradation involved the whole sample through a bulk degradation mechanism.

Domingos et al (24, 25) evaluated the influence of the degradation media (i.e., PBS and simulated body fluid [SBF]) and scaffold internal architecture on the degradation mechanisms of PCL scaffolds obtained using a highly reproducible and low-cost BioExtruder system (26), which had been used to produce 3D scaffolds with a more controlled micro-architecture in comparison with conventional manufacturing techniques (27). Results of degradation in the chosen

**TABLE I** - Terminology and definitions applied for the degradation of synthetic polymers (6)

Terminology	Definition
Biodegradable	Solid polymeric devices which break down to macromolecule degradation with dispersion in an animal body but no proof for elimination from the body.
Bioresorbable	Solid materials which can degrade and further re-sorb in vivo – i.e., which are eliminated through natural pathways either because of simple filtration of degradation by-products or after their metabolization.
Bioabsorbable	Solid polymeric materials or devices which can dissolve in body fluids without any polymer chain cleavage or molecular mass decrease.
Bioerodible	Solid polymeric materials or devices which undergo surface degradation via an erosion mechanism when in contact with living elements such as tissues, cells or fluids.

simulating fluids highlighted a strong influence of both the degradation media and scaffold pore size on the degradation kinetics of PCL. These 3D PCL scaffolds have been successively studied in terms of the (i) effect of process parameters on the morphological and mechanical properties of the scaffolds (28) and (ii) effect of pore size and geometry on compressive mechanical behavior and human mesenchymal stem cell viability (29).

Despite the relevant amount of work completed as well as the numerous data available in the literature, it is interesting to note that it is still not clear how some architectural parameters such as pore geometry can actually influence the in vitro enzymatic degradation process of PCL scaffolds. Hence, this present work aimed to analyze the influence of pore geometry on the enzymatic degradation in vitro of 3D PCL scaffolds, with a special focus on morphological, chemical-physical and mechanical properties. The novelty of this work consists in its use of 3D printing technology to produce highly accurate and reproducible scaffolds to systematically study the influence of pore geometry on the in vitro enzymatic degradation kinetics of PCL scaffolds. This is something that is not achievable with any other manufacturing technique (i.e., solvent casting, freeze drying, etc.) due to the high variability between the samples produced by those techniques. The results from this work can then be combined with biological and mechanical information, to generate a design database for the production of scaffolds with optimized architectural features (i.e., dimensions and distribution of the pores) for the regeneration of different tissues.

## Materials and methods

### Materials

Linear thermoplastic PCL with a molecular weight (Mw) of 50,000 Da (Capa® 6500; Perstorp Caprolactones, Cheshire, UK) in the form of pellets was employed as received, for the production of 3D scaffolds. Lipase enzyme (Amano Lipase PS, from *Burkholderia cepacia* [was *Pseudomonas cepacia*]) was obtained from Sigma Aldrich.

### Scaffold design and fabrication

3D structures based on PCL were fabricated using an extrusion-based additive manufacturing (AM) device called the BioExtruder, which was equipped with a nozzle with a 300- $\mu$ m inner diameter (26). Block samples were initially designed (30  $\times$  30  $\times$  8 mm<sup>3</sup>) using computer-aided design (CAD) software (Solid Works, Dassault Systemes, SA). Three different lay-down patterns were adopted with a filament distance (FD) fixed at 1,000  $\mu$ m and a slice thickness (ST) at 280  $\mu$ m: square (0/90°), triangular (0/60/120°) and complex polygonal (0/45/90/135°) internal pore geometries were obtained, as shown elsewhere (29).

PCL in the form of pellets was directly melted in the material chamber of the BioExtruder and then transferred to the screw-extrusion chamber via compressed air. Based on previous results, the structures were produced considering the optimized processing conditions indicated in Table II (28). After fabrication, the scaffolds destined to further

**TABLE II** - Process and instrument parameters employed to fabricate 3D bioextruded poly( $\epsilon$ -caprolactone) scaffolds

Process and instrument parameters			
DV (mm/s)	LT (°C)	EP (bar)	SRV (rpm)
10	80	4	30

DV = deposition velocity; LT = liquefier temperature; EP = extrusion pressure; SRV = screw rotation velocity.

analysis were cut into smaller pieces, taking care to avoid or minimize damage to their architecture.

### Degradation assay

Seven specimens of each scaffold topology, after being sterilized with UV radiation for 30 minutes, were cut into smaller samples, measuring 4  $\times$  4  $\times$  8 mm, accurately weighed ( $w_0$ ), immersed in PBS solution containing 0.5 mg/mL of lipase (Amano Lipase PS, from *Burkholderia cepacia* [was *Pseudomonas Cepacia*]) and incubated (37°C, pH 7.4) for 25 days. The enzyme concentration was established based on previous studies reported in the literature (30). At predetermined intervals of 5 days, scaffolds were withdrawn from the degradation medium. The removal of salt excess was carried out by immersion of the scaffolds in ultrapure water (with a Millipore purification system) for 24 hours. Finally, the scaffolds were carefully wiped, stored in a vacuum desiccator for 12 hours at room temperature and quickly weighed ( $w_T$ ). The weight loss percentage (WL%) was calculated as follows:

$$WL\% = \frac{W_0 - W_T}{W_0} \times 100 \quad \text{Eq. [1]}$$

### Scaffold characterization

#### Morphological analysis

Morphological features of nondegraded and degraded scaffolds, including the architecture, pore shape and filament size, were assessed through scanning electron microscopy (SEM) analysis using a FEI (FEI Quanta 600F) apparatus. Micro-computed tomography ( $\mu$ -CT) analysis was carried out using a SkyScan 1072 system (Aartselaar, Belgium). Rotational steps of 0.9° and rotation angle of 180° were set up. In this manner, information on the internal structure of degraded and nondegraded scaffolds (i.e., porosity, pore shape, size and interconnectivity, as well as surface area to volume) was obtained. Cross-sections and 3D models were reconstructed at different degradation times using the software package SkyScan and Image J software.

#### Differential scanning calorimetry analysis

Differential scanning calorimetry (DSC) was employed to monitor changes in the thermal properties of the scaffolds linked to the degradation time, using a DSC Q2000 (TA Instruments) apparatus. Nondegraded and degraded scaffolds were

subjected to heating and cooling cycles at 2°C/min between 20°C and 100°C. The crystallinity degree ( $X_c$ ) was determined as follows:

$$X_c = \Delta H_f / \Delta H_{f0} \quad \text{Eq. [2]}$$

where  $\Delta H_f$  is the enthalpy of fusion of the sample and  $\Delta H_{f0}$  is the enthalpy of fusion of 100% crystalline PCL (equal to 139.5 J/g, as reported in the literature).

#### Size exclusion chromatography analysis

Weight average molecular weight (Mw), number average molecular weight (Mn) and polydispersity index (PDI), of the scaffolds having different pore geometries were determined through high-performance liquid chromatography (HPLC) using a WellChrom Maxi-Star k-1000 pump (Knauer) connected to Light Scattering (LS) (PL-EMD 960), before (time T0) and after degradation (time T25). The instrument was equipped with 2 Mixed-C columns (Mixed C 5  $\mu$ m; Polymer Laboratories). Chloroform was used as eluent, while monodisperse polystyrene (PS) standards (Perkin-Elmer) were used to build up the calibration curve. The chromatography parameters were determined using universal calibration, as described elsewhere (24).

#### Compressive mechanical tests

Compression tests were performed at a rate of 1 mm/min up to a strain value of 0.5 mm/mm using an INSTRON 5566 testing system. Both nondegraded and degraded block-shaped specimens were cut to the following dimensions: length (l) = 4.0 mm, width (w) = 4.0 mm and height (h0) = 8.0 mm. Engineering stress  $\sigma$  was calculated as the ratio between the force  $F$  measured by the load cell divided by the total area of the apparent cross-section of the scaffold ( $A = l w$ ):

$$\sigma = \frac{F}{A} \quad \text{Eq. [3]}$$

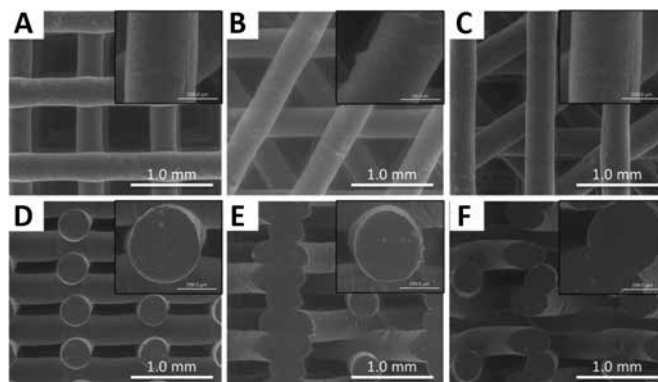
while strain  $\varepsilon$  was defined as the ratio between the scaffold height variation ( $\Delta h$ ) and its initial height ( $h_0$ ):

$$\varepsilon = \frac{\Delta h}{h_0} \quad \text{Eq. [4]}$$

Five specimens per time point were analyzed to determine the influence of the degradation process on the PCL scaffolds' mechanical behavior.

#### Statistical analysis

The data from the measurements were reported as means and standard deviations and analyzed using 1-way analysis of variance (ANOVA) and Tukey post hoc test (using SigmaPlot 13 software). The significance level was set at a p value of <0.05.



**Fig. 1** - Results from SEM analysis: images of 3D poly( $\epsilon$ -caprolactone) (PCL) scaffolds with 0/90° (A, D), 0/60/120° (B, E) and 0/45/90/135° (C, F) lay-down patterns, before the degradation process (i.e., day 0): top view (A-C) and cross-section (D-F).

**TABLE III** - Results from SEM analysis performed before the degradation process

Lay-down pattern	RW ( $\mu$ m)	FG ( $\mu$ m)	LG ( $\mu$ m)
0/90°	360 $\pm$ 13	600 $\pm$ 18	159 $\pm$ 14
0/60/120°	377 $\pm$ 8	651 $\pm$ 18	154 $\pm$ 8
0/45/90/135°	383 $\pm$ 18	610 $\pm$ 18	160 $\pm$ 23

Values are means  $\pm$  standard deviation.

RW = road width; FG = filament gap; LG = layer gap.

## Results and discussion

### Morphological analysis of nondegraded scaffolds

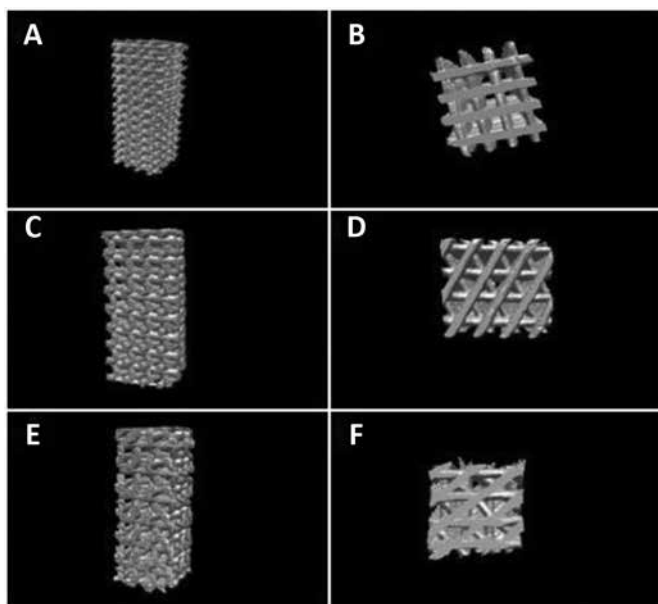
SEM micrographs were used to assess the scaffolds' architectural features – namely, road width (RW), filament gap (FG) and layer gap (LG) (Fig. 1; Tab. III).

$\mu$ -CT results depicted in Figure 2 show that the PCL scaffolds produced presented a repeatable microstructure with precise pore size and shape and interconnectivity. The results obtained supported the SEM observations, and the experimental values sufficiently substantiated the theoretical ones, defined during the scaffold fabrication by the process and instrument parameters. As shown in Figure 2, all scaffolds were characterized by a mean fiber diameter of 360–380  $\mu$ m, and a center-to-center fiber distance of 610–650  $\mu$ m between 2 fibers in a common layer was evaluated, independently of the pore geometry. Scaffold characteristics obtained by  $\mu$ -CT are summarized in Table IV.

### Degradation studies

Degraded PCL scaffolds were investigated in terms of WL%, SEM and  $\mu$ -CT, focusing attention on the possible influence of architectural parameters on the in vitro enzyme-mediated degradation process.

The influence of these parameters has already been analyzed in earlier studies on 3D PCL scaffolds subjected to in vitro hydrolytic degradation in PBS or SBF (24, 25). The results evidenced that PCL scaffolds, incubated in both the media for



**Fig. 2** - Three-dimensional reconstructions obtained from micro-computed tomography ( $\mu$ -CT) analysis of poly( $\epsilon$ -caprolactone) (PCL) scaffolds with 0/90° (A, B), 0/60/120° (C, D) and 0/45/90/135° (E, F) lay-down patterns, before degradation: lateral view (A, C, E) and top view (B, D, F).

**TABLE IV** - Results from micro-CT analysis: typical values of porosity, surface area to volume ratio and interconnectivity

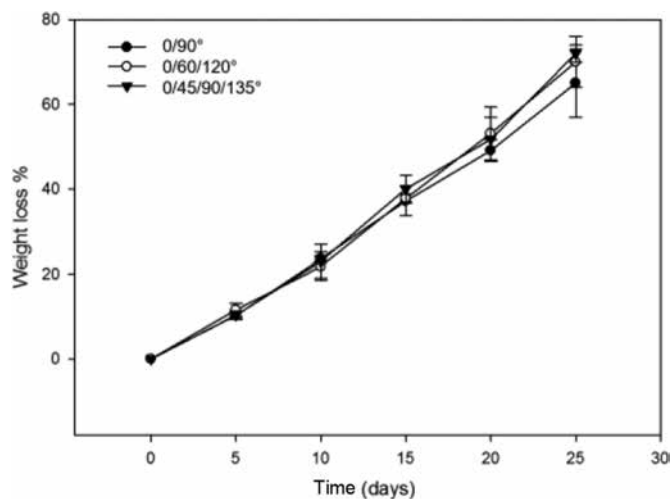
Lay-down pattern	Porosity (%)	Surface area to volume ratio ( $\text{mm}^{-1}$ )	Interconnectivity (%)
0/90°	72.0	18.4	100
0/60/120°	69.7	14.7	100
0/45/90/135°	68.9	13.6	100

6 months, showed a WL% in the range of 0.2%-1%, independently from the geometry of the pores. Similar results (i.e., WL% in the range of 0.2%-1.2%) were achieved by Idaszek et al (31), who investigated the degradation kinetics in SBF for 3 months of composite PCL-based scaffolds obtained by a solvent casting technique.

In the present study, results for the enzymatic degradation of PCL 3D scaffolds revealed that, regardless of the scaffold pore geometry, the weight loss determined 5 days after the beginning of the degradation experiment maintained a linear and uniform trend throughout the entire period, reaching a maximum WL% of 72.2% at day 25 (Fig. 3).

Brugmans et al (32) studied the enzymatic degradation of PCL scaffolds obtained by a conventional electrospinning method. They found a WL% equal to 44% by lipase treatment up to 56 hours. Murray et al (33) investigated the enzymatic degradation of graphene/PCL composites in lipase solution at 37°C for 4 days. The results revealed that both for thin films made of PCL alone and for the composites, a 50%-60% weight loss over the first 24 hours was achieved.

When comparing our data with those from preceding studies, a slower degradation kinetic was found. This observa-

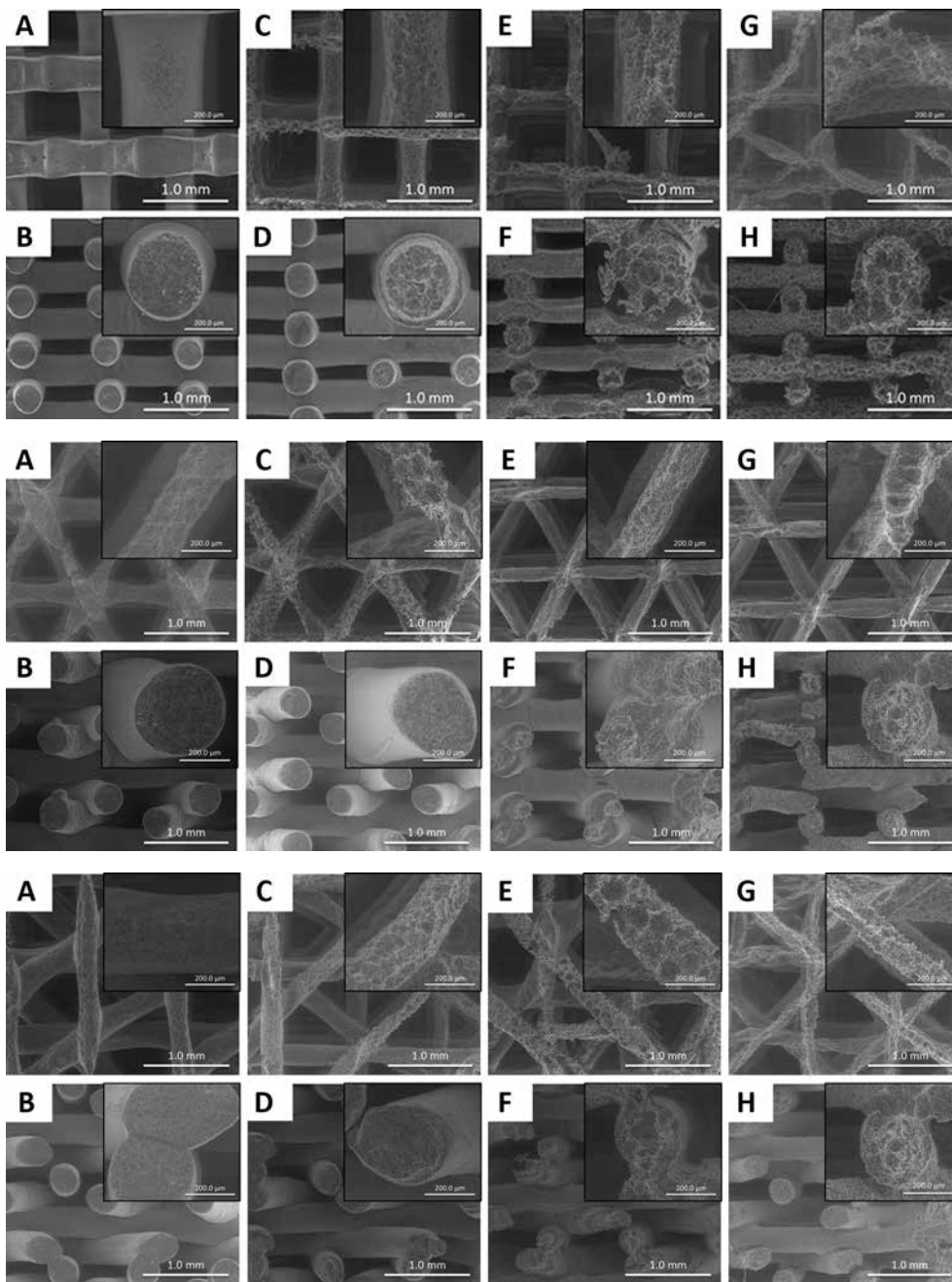


**Fig. 3** - Percentage of weight loss (WL%) as a function of degradation time and pore geometry.

tion can be explained considering that PCL systems were subjected to a superficial erosion mechanism with an initial loss of the amorphous zones located on the surface of the scaffold. In particular, Bölgen et al (34) demonstrated that the surface area to volume ratio (SA/Vol) of a material affects the rate of degradation: samples with higher SA/Vol and porosity are expected to degrade faster, due to the greater water penetration into their structures. In contrast, Athanasiou et al (35) showed that scaffolds based on poly(lactide-co-glycolide) with a higher porosity degraded at a slower rate than those with 0% porosity. A possible explanation was that a greater surface area allowed easier removal of acidic breakdown products, thus reducing autocatalytic degradation phenomena. Bosworth and Downes (36) carried out a degradation study investigating the hydrolysis of PCL scaffolds obtained by different techniques (i.e., 3D bundles, 2D fibrous mats and solvent cast films, with SA/Vol of 11.48, 13.23 and 10.26, respectively) in PBS at 37°C, over a 3-month period. Their work confirmed the hypothesis of Bölgen et al (34).

In our case, as shown in Figure 3, no particular trend was observed for WL% as a function of pore geometry. Based on the hypothesis of Bölgen et al, a degradation kinetic 0/90° > 0/60/120° > 0/45/90/135° should be expected. A possible explanation for these results may rely on the geometry of the pores. As the number of deposition angles increases from 0/90° to 0/45/90/135° the diffusion of acidic breakdown products is reduced, thereby promoting a higher autocatalytic degradation process. This phenomena appears to counterbalance the effect of SA/Vol ratio (which is smaller in the 0/45/90/135° scaffolds) thus promoting a similar weight loss trend among all scaffold geometries.

SEM observations and  $\mu$ -CT analysis revealed that the physical features strongly changed over time as an effect of the degradation process. Despite the diameter reduction of the fibers, adhesion with the neighboring fibers was still maintained. On the other hand, as the fiber diameter decreased, FD consequently increased. This means that the increasing of



**Fig. 4** - Results from SEM analysis: images of 3D poly( $\epsilon$ -caprolactone) (PCL) scaffolds with 0/90° (A), 0/60/120° (B) and 0/45/90/135° (C) lay-down patterns, at different degradation times: day 5 – T5 (A, B), day 10 – T10 (C, D), day 15 – T15 (E, F) and day 20 – T20 (G, H).

FD led to an increased pore size and porosity (Fig. 4). Furthermore, it was also possible to note the appearance of numerous cavities in the filaments (T10, T15 and T20), as a result of the surface erosion mechanism.

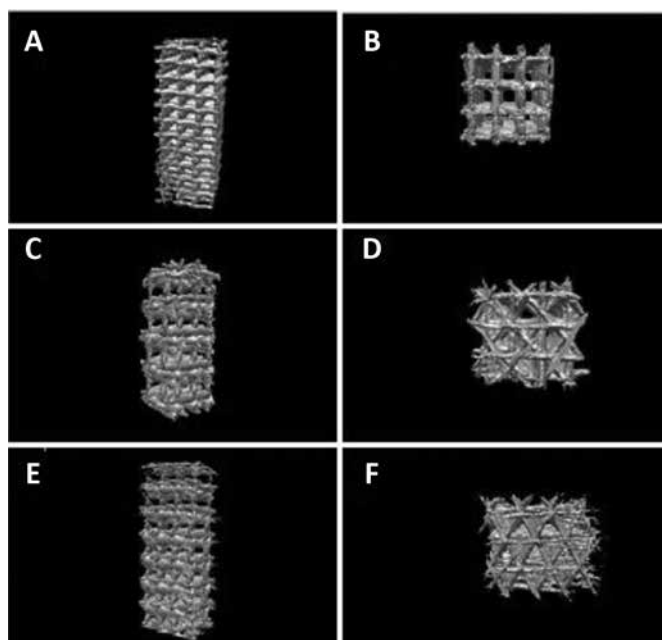
Results obtained from  $\mu$ -CT analysis were consistent with SEM observations, showing an increase of pore size and porosity as a consequence of the filament thinning (Fig. 5).

#### DSC analysis

DSC results are reported in Table V and suggested that, during the degradation period, there were no significant differences among the architectures investigated, in terms of

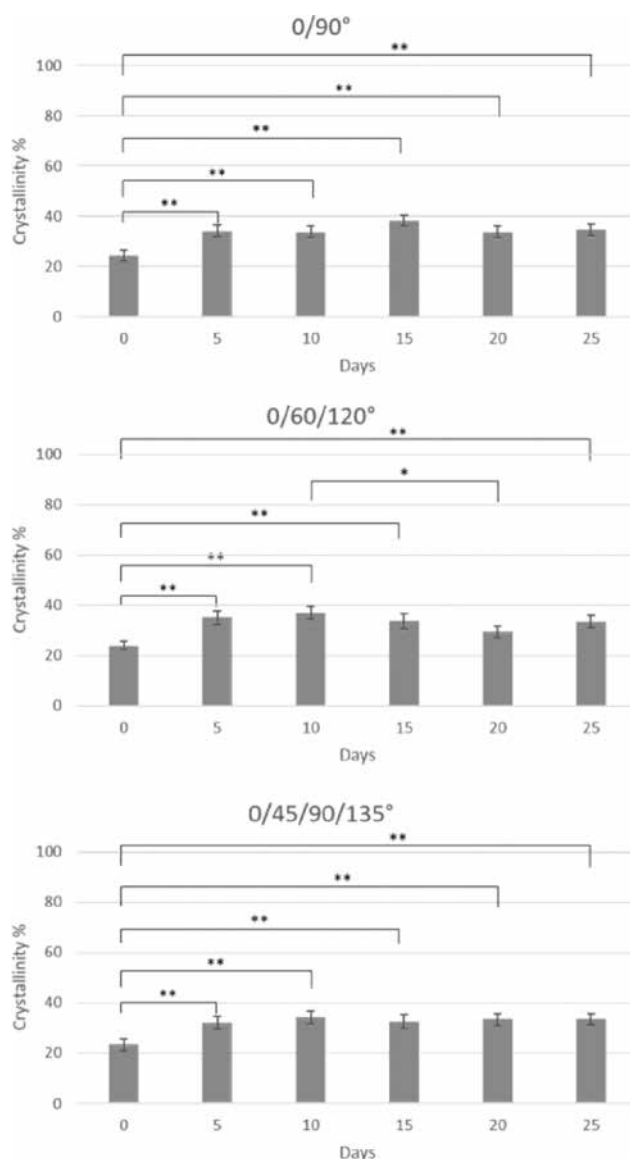
melting temperature, melting enthalpy and crystallinity degree.

The enzymatic degradation process did not have a significant impact in the melting temperature of PCL, which remained in the range 61°C–64°C over 25 days. The same observations were reported by Murray et al (33). Furthermore, for all of the analyzed architectures, the crystallinity degree generally increased over time, with statistically relevant differences between day 0 and the successive days, except for the 0/60/120° architecture, where no significant differences were found between T0 and T20 (see Fig. 6). However, during the period analyzed, all of the scaffolds showed an increase of the crystallinity degree, which should



**Fig. 5** - Three-dimensional reconstructions obtained from micro-computed tomography ( $\mu$ -CT) analysis on 3D poly( $\epsilon$ -caprolactone) (PCL) degraded scaffolds with 0/90° (A, B), 0/45/90/135° (C, D) and 0/60/120° (E, F) lay-down patterns, at day 15: lateral view (A, C, E) and top view (B, D, F).

probably be ascribed to the random hydrolytic bond cleavage of the amorphous regions present in PCL, since it is well recognized that the degradation medium is facilitated more to penetrate into a disordered network than into an ordered one (37). After 5 days, the crystallinity degree seemed to become constant over time (Tab. V; Fig. 6). This observation suggested that the degradation of amorphous and crystalline regions might occur at the same time but at different rates, with crystalline regions presenting a lower degradation profile when compared with amorphous regions. The results also suggested that the overall degradation process, being related to both crystalline and amorphous PCL regions, might lead to alternate increases and decreases in crystallinity percentage throughout the incubation period investigated.



**Fig. 6** - Variation of crystallinity percentage as function of time for the 3 pore geometries analyzed. Results are reported as means  $\pm$  standard deviation; \* $p < 0.01$ , \*\* $p < 0.05$  (Tukey post hoc test).

**TABLE V** - Data obtained from DSC analysis, as a function of degradation time

Degradation time (days)	0/90°			0/60/120°			0/45/90/135°		
	$T_m$ (°C)	$\Delta H_m$ (J/g)	$X_c$ (%)	$T_m$ (°C)	$\Delta H_m$ (J/g)	$X_c$ (%)	$T_m$ (°C)	$\Delta H_m$ (J/g)	$X_c$ (%)
0	62.8 $\pm$ 2.1	33.9 $\pm$ 2.8	24.3 $\pm$ 2.1	62.8 $\pm$ 2.0	33.4 $\pm$ 2.9	24.0 $\pm$ 1.7	62.8 $\pm$ 2.3	32.6 $\pm$ 2.5	23.4 $\pm$ 2.2
5	63.9 $\pm$ 2.4	47.6 $\pm$ 3.1	34.1 $\pm$ 2.4	63.1 $\pm$ 2.2	49.1 $\pm$ 3.0	35.2 $\pm$ 2.6	63.9 $\pm$ 2.1	44.8 $\pm$ 3.3	32.1 $\pm$ 2.5
10	64.4 $\pm$ 2.0	47.0 $\pm$ 3.5	33.7 $\pm$ 2.3	64.0 $\pm$ 2.3	51.5 $\pm$ 3.7	36.9 $\pm$ 2.5	63.8 $\pm$ 2.3	47.8 $\pm$ 3.2	34.3 $\pm$ 2.6
15	64.8 $\pm$ 2.1	53.5 $\pm$ 3.9	38.4 $\pm$ 2.1	63.1 $\pm$ 2.0	47.0 $\pm$ 3.3	33.7 $\pm$ 3.0	63.1 $\pm$ 2.1	45.4 $\pm$ 3.4	32.5 $\pm$ 2.7
20	64.0 $\pm$ 2.5	47.1 $\pm$ 3.2	33.8 $\pm$ 2.2	63.8 $\pm$ 2.2	41.0 $\pm$ 3.1	29.4 $\pm$ 2.2	63.8 $\pm$ 2.3	46.7 $\pm$ 3.0	33.5 $\pm$ 2.3
25	61.3 $\pm$ 2.2	48.2 $\pm$ 3.0	34.6 $\pm$ 2.4	62.1 $\pm$ 2.1	46.6 $\pm$ 2.8	33.5 $\pm$ 2.5	62.1 $\pm$ 2.2	54.6 $\pm$ 3.1	33.6 $\pm$ 2.2

Results are reported as means  $\pm$  standard deviation.

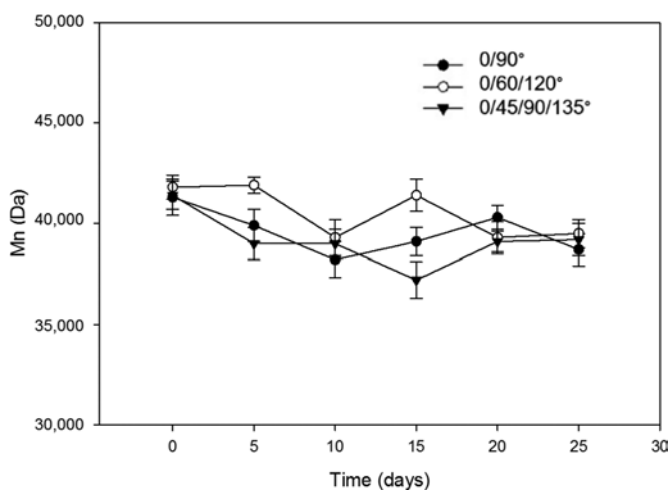
DSC = differential scanning calorimetry;  $T_m$  = melting temperature;  $\Delta H_m$  = melting enthalpy;  $X_c$  = crystallinity degree.

**TABLE VI** - Mn and PDI values as function of degradation time, for each specimen typology

Degradation time (days)	0/90°		0/60/120°		0/45/90/135°	
	Mn	PDI	Mn	PDI	Mn	PDI
0	41,300 ± 900	1.45	41,400 ± 700	1.42	41,800 ± 600	1.41
5	39,900 ± 800	1.49	39,000 ± 800	1.49	41,900 ± 400	1.44
10	38,200 ± 900	1.50	39,000 ± 700	1.46	39,300 ± 900	1.51
15	39,100 ± 700	1.45	37,200 ± 900	1.56	41,400 ± 800	1.46
20	40,300 ± 600	1.43	39,100 ± 500	1.47	39,300 ± 800	1.45
25	38,700 ± 800	1.45	39,200 ± 800	1.47	39,500 ± 700	1.43

Results are reported as means ± standard deviation.

Mn = number average molecular weight; PDI = polydispersity index.



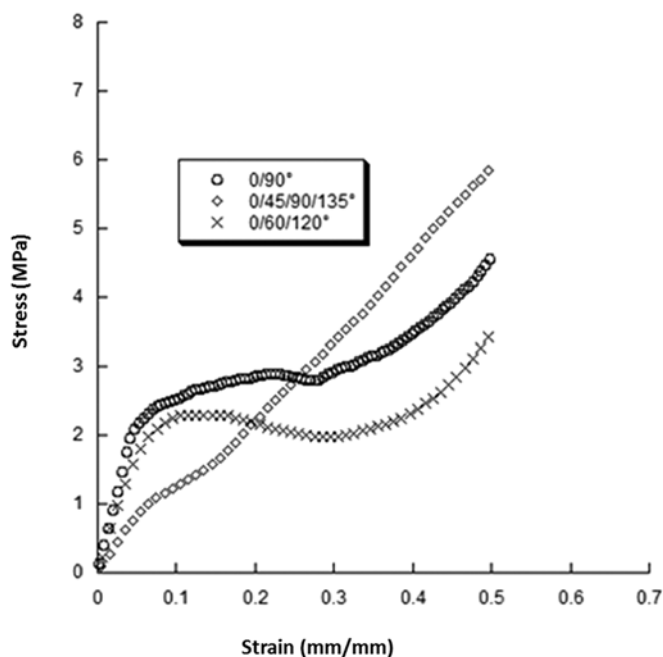
**Fig. 7** - Variation of the number average molecular weight (Mn) as function of degradation time.

### SEC analysis

Table VI and Figure 7 report the changes in Mn and PDI of PCL scaffolds with different pore geometries, which were degraded for 25 days in enzymatic solution. Before degradation, all scaffolds presented a similar Mn of approximately 41,000 Da, with a PDI between 1.41 and 1.45.

Bosworth and Downes (36) found a decrease in Mn for PCL 3D bundles (6.2%), followed by 2D fibrous mats (5.6%) and then solvent cast films (5.0%). Løvdaal et al (38) investigated the mechanical properties of an electrospun PCL exposed to in vitro physiological fluids or to accelerated in vitro degradation conditions (i.e., at pH 12 for 29 days), at 37°C. Mw evaluations after 29 days showed a Mw reduction of 48% compared with that of pristine PCL, with an increased PDI (from 1.9 at day 0 to 4.5 at day 29). On the other hand, enzymatic degradation of PCL scaffolds obtained by a conventional electrospinning method by Brugmans et al (32) resulted in a significant mass loss by lipase treatment up to 56 hours, while Mw remained constant over time.

Our results showed that PCL scaffolds produced with different lay-down patterns and comparable porosities pre-



**Fig. 8** - Typical stress-strain curves obtained for nondegraded scaffolds with 0/90°, 0/60/120° and 0/45/90/135° lay-down patterns, compressed at a rate of 1 mm/min up to a strain value of 0.5 mm/mm.

sented an almost similar degradation rate, resulting in a very slight, almost negligible Mn decrease from day 0 to day 25. Moreover, the differences observed were within the range of error associated with the SEC measurements. These observations suggested that PCL scaffolds were degraded mainly by surface erosion mechanisms with significant weight loss but almost invariable molecular weight values throughout the degradation period.

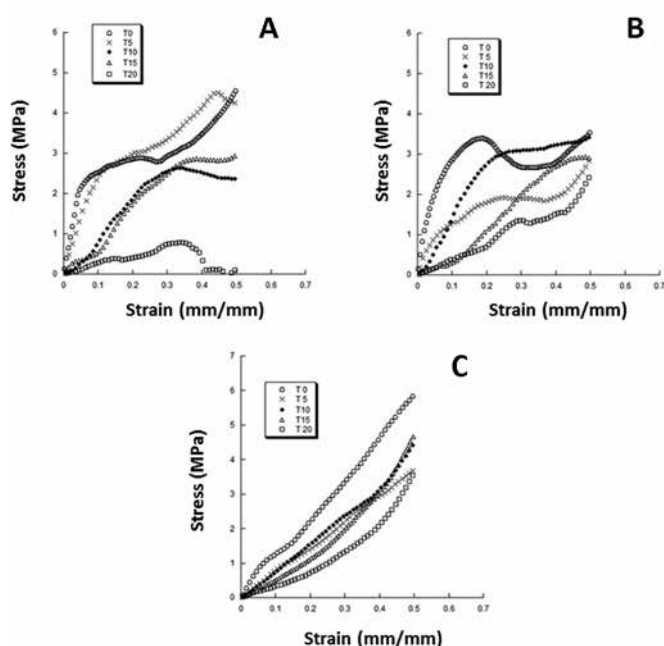
### Compressive mechanical tests

Figure 8 reports typical stress-strain curves relevant to the 3D bioextruded scaffolds before the degradation, evidencing the effect of lay-down pattern on the mechanical features of the structures.

**TABLE VII** - Effect of lay-down pattern and in vitro enzymatic degradation on the mechanical properties of 3D bioextruded poly( $\epsilon$ -caprolactone) scaffolds

Degradation time (days)	0/90°		0/60/120°		0/45/90/135°	
	E (MPa)	$\sigma_{\max}$ (MPa)	E (MPa)	$\sigma_{\max}$ (MPa)	E (MPa)	$\sigma_{\max}$ (MPa)
0	52.1 ± 2.8	4.3 ± 0.4	44.0 ± 6.2	3.3 ± 0.4	16.2 ± 2.1	5.8 ± 0.7
5	29.9 ± 5.1	3.5 ± 0.8	40.0 ± 5.5	2.9 ± 0.9	8.6 ± 0.8	4.3 ± 0.9
10	15.1 ± 1.3	3.0 ± 1.4	15.1 ± 2.6	3.6 ± 0.3	6.6 ± 0.4	4.5 ± 0.6
15	9.2 ± 0.7	3.2 ± 0.9	8.5 ± 1.8	2.5 ± 0.7	5.2 ± 1.6	3.6 ± 0.6
20	3.3 ± 0.5	0.8 ± 0.1	5.0 ± 2.5	2.1 ± 0.7	8.2 ± 0.2	2.8 ± 1.1

Scaffolds were characterized by a filament distance of 1,000  $\mu\text{m}$  and different lay-down patterns (0/90°, 0/60/120° and 0/45/90/135°). Compressive modulus (E) and maximum stress ( $\sigma_{\max}$ ) are reported as means  $\pm$  standard deviation at different degradation times.



**Fig. 9** - Typical stress-strain curves obtained for degraded scaffolds with 0/90° (A), 0/60/120° (B) and 0/45/90/135° (C) lay-down patterns, compressed at a rate of 1 mm/min up to a strain value of 0.5 mm/mm, at each time point (day 0: T0, day 5: T5, day 10: T10, day 15: T15 and day 20: T20).

The results confirmed that, at a selected FD, the lay-down pattern had a significant effect on the mechanical behavior of the 3D scaffolds. Table VII shows that scaffolds with 0/90° lay-down pattern exhibited a greater compressive modulus (52.1  $\pm$  2.8 MPa at T0) than those obtained for 0/60/120° (44.0  $\pm$  6.2 MPa at T0) and 0/45/90/135° (16.2  $\pm$  2.1 MPa at T0) patterns. When the amplitude of the deposition angle between the filaments of adjacent layers decreased – i.e., passing from a 0/90° to a 0/45/90/135° lay-down – a larger contact area occurred. This in turn should lead to a reduction of the local stress endured by the 3D scaffold, with consequent reduction of the overall mechanical performance.

Figure 9 and Table VII summarize the influence of the in vitro enzymatic degradation on the mechanical performances

of PCL scaffolds with the proposed lay-down patterns over time. The data present an overall deterioration of mechanical properties as a function of degradation time, as was also observed by other researchers (38).

In the results obtained, an evident reduction in the overall mechanical properties of the samples, independently of the pore geometry, was observed. This may be related to the significant mass loss caused by the enzymatic degradation mechanism. As observed in the morphological analysis, the surface erosion process led to the thinning of the filaments thus increasing the FD and porosity of the structures. In general, for all of the materials, the variation in mechanical properties as a function of FD and porosity was ascribable to the fact that the filament junctions, when subjected to compression, acted as columns. A scaffold having a higher FD, for a defined area presented a smaller number of columns. This in turn led to a decrease in the scaffold compressive modulus and maximum stress (39).

## Conclusions

This study investigated the effect of pore geometry on the enzymatic degradation process carried out in vitro in 3D PCL scaffolds. The results suggest that PCL scaffolds, when immersed in PBS solution containing lipase enzyme, degrade mainly at the polymer surface, with a remarkable mass loss. Independently of the pore geometry, DSC analyses revealed an increment in the crystalline percentage, probably linked to the major susceptibility to degradation of amorphous regions of the polymer. The molecular weight remained almost constant during the entire incubation period, which confirmed the absence of bulk degradation. Furthermore, as indicated by the morphological analysis, surface erosion caused the thinning of the filaments with consequent increase in porosity and decrease of mechanical properties. As opposed to pore dimension (porosity), the internal architecture and geometry did not seem to represent a crucial parameter in the mechanism involved in the in vitro degradation of the proposed scaffolds.

## Disclosures

Financial support: This work was partially performed within the framework of the SKELGEN project: Establishment of a Cross Con-

tinant Consortium for Enhancing Regenerative Medicine in Skeletal Tissues (Marie Curie Action, International Research Staff Exchange Scheme [IRSES]; Project reference: 318553).

Conflict of interest: None of the authors has any financial interest related to this study to disclose.

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