Virtual and Physical Prototyping, Vol. 5, No. 2, June 2010, 65–73

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Evaluation of *in vitro* degradation of PCL scaffolds fabricated via BioExtrusion. Part 1: Influence of the degradation environment

M. Domingos[1,2]*, F. Chiellini[2], S. Cometa[2], E. De Giglio[3], E. Grillo-Fernandes[2], P. Bártolo[1] and E. Chiellini[2]

[1]Centre for Rapid and Sustainable Product Development, Polytechnic Institute of Leiria (IPL), Leiria Portugal
[2]Laboratory of Bioactive Polymeric Materials for Biomedical & Environmental Applications (BIOlab) and Department of Chemistry & Industrial Chemistry, University of Pisa, Pisa, Italy
[3]Department of Chemistry, University of Bari, Bari, Italy

One of the most promising approaches in tissue engineering (TE) comprises the development of 3D porous scaffolds which are able to promote tissue regeneration. Biocompatible and biodegradable poly(ε-caprolactone) (PCL) structures are increasingly used as temporary extra-cellular matrices for bone tissue engineering. To ensure an appropriate bone restoration over the long term, the selected material must have a degradation rate that match the in-growth of new bone. The *in vivo* process, by which the scaffold degrades and is resorbed transferring the load and function back to the host tissue, is complex. Consequently, an appropriate preliminary *in vitro* study is required. A novel extrusion-based technology called BioExtruder was used to produce PCL porous scaffolds made with layers of directionally aligned microfilaments. The *in vitro* degradation behaviour in both simulated body fluid (SBF) and phosphate buffer solution (PBS) were investigated over 6 months. The characterization of the degradation behaviour of the structures was performed at specific times by evaluating changes in the average molecular weight, the weight loss and its thermal properties. Morphological and surface chemical analyses were also performed using a Scanning Electron Microscopy (SEM) and an X-ray Photoelectron Spectroscopy (XPS), respectively.

Keywords: in vitro degradation; PCL scaffolds; bioextrusion; degradation environment

1. Introduction

Resorbable polyesters were extensively studied for their potential application in different biomedical fields, such as the drug delivery and the tissue engineering fields (Middleton 2003, Domingos 2008, Lam 2008, Rath 2008, Yildirim 2008). Poly(ε-caprolactone) (PCL) is a biodegradable, biocompatible and semi-crystalline polymer not so fully explored in drug delivery applications as other members of the aliphatic polyester family such as poly(glycolic acid) (PGA) and poly(lactic acid) (PLA), due to its slow degradation rate. On contrary, PCL represents one of the most proposed materials in the tissue engineering field, taking advantage of its long degradation time and mechanical characteristics, enabling the production of porous 3D matrices adequate for cell adhesion, proliferation and further differentiation.

PCL has a low melting point at around 60°C and a high thermal stability over 350°C (Perrin 1998), so it presents an unusual thermal and structural stability along with a good processability. These PCL characteristics enable its melt

*Corresponding author. Email: domingos.marco@ns.dcci.unipi.it; marco.domingos@ipleiria.pt

Virtual and Physical Prototyping
ISSN 1745-2759 print/ISSN 1745-2767 online
http://www.tandf.co.uk/journals
DOI: 10.1080/17452751003769440

processing to obtain 3D porous scaffolds with a fully interconnected pore network using fused deposition modelling (FDM) (Hutmacher 2000, 2001, Zein 2002).

The design of a 3D scaffold takes in consideration not only the adequate selection of the material but also its behaviour once implanted, especially in terms of degradation mechanisms in the body fluids over time.

Polymer degradability in the body fluids is a phenomenon influenced by different and often conflicting variables, such as the ones related with the polymer structure (i.e. chemical composition, molecular weight and molecular weight distribution, crystallinity, morphology, etc.), its macroscopic features (i.e. implant shape or sizes, porosity, etc.) and environmental conditions (i.e. temperature, pH of the medium, presence of enzymes or cells and tissues). Consequently, a large number of studies were proposed about PCL long-term degradation in the body fluids, in the form of films and drug delivery devices (microspheres and rods) (Ye 1997, Yavuz 2002, Penã 2006). However, there is too little information about PCL-based 3D scaffolds degradation in body fluids to evaluate the influence of the material's macro and microstructure on its degradation kinetics and mechanism (Salgado 2008).

In this work, highly porous PCL 3D-scaffolds were produced employing a novel extrusion-based system called BioExtruder (Mota 2010). The ability to produce fully interconnected and reproducible 3D scaffolds without inducing relevant chemical and biocompatibility alterations of the polymeric material was already demonstrated by the authors in previous works (Domingos 2009a,b). Therefore, the present study is focused on the *in vitro* degradation in a phosphate buffer solution (PBS) and simulated body fluid (SBF) in a 6-month period. Weight loss measurements were followed at specific time intervals (1 month) and molecular weight changes were monitored by size exclusion chromatography (SEC). The evaluation of possible variations in chemical composition, oxidation state and electronic state of elements present on the surface of the material, before and after extrusion, as well as before and after degradation, was performed by XPS analysis. Differential scanning calorimetry (DSC) was carried out to study the relationship between scaffold's degradation and its thermal properties like glass transition (T_g) and melting (T_m) temperatures and crystallinity degree (X_c). The internal/external architecture of the scaffolds, namely pore size and topography, was assessed by using SEM. Initial inorganic salts deposition on the scaffold's surface, was analyzed by SEM/EDX. This study represents an important contribution for both the understanding of the degradation behaviour and kinetics of 3D PCL scaffolds and ultimately to determine their suitability for specific tissue engineering applications.

2. Materials and methods

2.1 *Materials*

CAPA® 6500 poly(ε -caprolactone) pellets with molecular weight around 50,000 were supplied by Perstorp UK Ltd (Cheshire, UK). SBF was prepared by dissolving reagent-grade NaCl, $NaHCO_3$, KCl, $K_2HPO_4{\cdot}3H_2O$, $MgCl_2{\cdot}6H_2O$, $CaCl_2$, and Na_2SO_4 in ion-exchanged and distilled water. Their ionic concentrations were Na^+, 142; K^+, 5.0; Mg^{2+},1.5; Ca^{2+}, 2.5; Cl^-, 147.8; HCO_3^-, 4.2; HPO_4^{2-}, 1.0; SO_4^{2-},0.5 (in mM). The solution was buffered at pH 7.4 with tris-hydroxymethyl aminomethane ($(CH_2OH)_3CNH_2$) and 1 M hydrochloric acid (HCl) at 36.5 ± 1.5 °C.

KCl, KH_2PO_4, NaCl and $Na_2HPO_4{\cdot}H_2O$ and ion-exchanged and distilled water were employed to prepare PBS 0.1M.

Chloroform, HPLC grade, and all the salts reported above were purchased from Sigma Aldrich.

2.2 *Scaffolds design and fabrication*

A BioExtruder device was employed for the fabrication of the PCL scaffolds (Figure 1). A detailed description of the equipment, along with the information flowchart required for the design and fabrication of scaffolds for tissue engineering, has been reported elsewhere (Domingos 2009).

Briefly, rectangular prisms measuring 40 (length) × 40 (width) × 8 mm (height), were initially designed in a CAD software named SolidWorks from Dassault Systèmes. The STL file format was then transferred to the BioExtruder slice generator software where it was automatically sliced. This routine consists of a slicing algorithm that slices the STL model into a number of 2D layers of pre-defined thickness to generate the contours of the model (SLI file). The deposition strategy (contour, raster and contour and raster deposition strategies), scanning velocity and filament distance for each layer were directly programmed through the BioExtruder scanning deposition generator routine, which was developed based on the ISO programming language for CNC machines. A 0/90° lay-down pattern and triple raster fill strategy (triple layers with filaments oriented in the same direction) was adopted to obtain highly porous scaffolds (76% porosity) with fully interconnected network of square channels.

2.3 *Degradation assay*

For the degradation tests, the extruded scaffolds were cut into smaller specimens with 4 (length) × 4 (width) × 3 mm (height), sterilized during 30 min with UV irradiation, and afterwards immersed for degradation in two different media: PBS (0.1 M) and SBF, prepared according to Kokubo (2006).

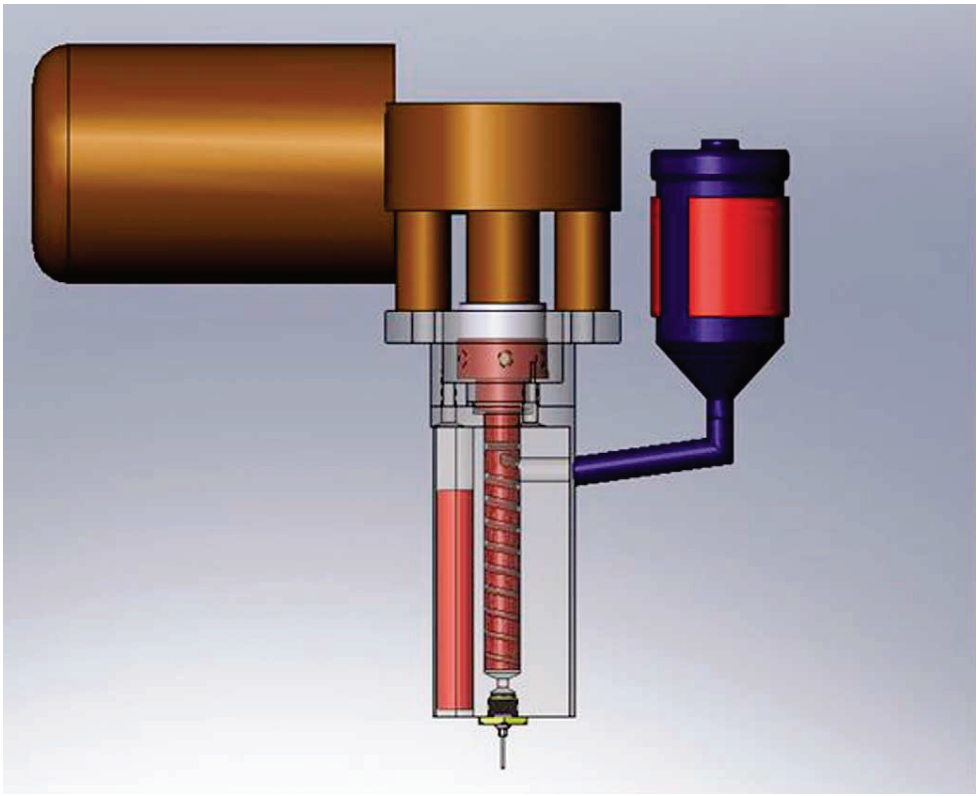

Figure 1. BioExtruder: Single-material extrusion system (Domingos 2009a).

SBF is an acellular, aqueous solution with an ionic composition closely resemblimg that of human plasma, and buffered to physiological pH 7.25–7.4. Microorganism contamination of samples was avoided by filtering the SBF with a 0.2-μm Millipore® system.

Sample weights before (W_0) and after degradation (W_t) were recorded for each specimen. After UV sterilization, three samples weighing 40 mg ($\pm$2 mg) were immersed in 2 mL of SBF in three separate sterile polyethylene containers, or in 2 mL of PBS, mimicking the physiological environment (37°C, pH 7.4). The volume of SBF (Vs in mL) required for this experiment was calculated based on the following equation proposed by Kokubo (2006):

$$V_s = S_a/10 \tag{1}$$

where S_a is the apparent surface area of the sample (mm^2).

Scaffold's sampling was carried out on intervals of 1 month up to complete 6 months. After the withdrawal from the degradation media, the samples were rinsed and immersed for some days in Millipore water to remove the excess of salts, carefully wiped, then dried under vacuum for 12 h at room temperature and weighed (W_t). The weight loss percentage (WL%) was determined by the following equation:

$$WL\% = \frac{(W_0 - W_t)}{W_0} \times 100 \tag{2}$$

2.4 *Statistical data treatment*

Experiments were carried out with at least three specimens of the samples, which were identically treated and processed. Relative changes of the assessed properties were statistically analysed with a $P < 0.05$ significance level using a one-way analysis of variance (ANOVA), using Tukey's test and the error of the mean was considered as the standard deviation.

2.5 *Characterization*

2.5.1 Size exclusion chromatography (SEC)

The weight and number average molecular weights (M_w and M_n, respectively), and the polydispersity index (PDI) of the scaffolds before and after degradation, were obtained using SEC. Analyses were performed at a flow rate of 1.0 ml/min by using a Jasco PU-1580 HPLC liquid chromatograph pump connected to a Jasco 830-RI detector, and equipped with two Mixed-D PLgel columns

(300 × 7.5 mm). Chloroform was used as the eluent and the calibration curve was established by using mono-dispersed polystyrene (PS) standards (Perkin-Elmer). The PS calibration was converted into one for PCL using universal calibration, in which the Mark–Houwink–Sakurada constants k and α were respectively $1.298 \cdot 10^{-4}$ and 0.828 for PCL (Sun 2006), and $0.49 \cdot 10^{-4}$ and 0.794 for PS (Ito 1996), at 30°C in chloroform.

2.5.2 *Differential scanning calorimety (DSC)*

Changes in the thermal properties of the scaffolds as a function of the degradation medium variables were evaluated by differential scanning calorimetry (DSC). Measurements were performed using a Mettler DSC 822^C module with FRS5 sensor and operated through the STAR software. Samples of 10–15 mg were scanned from −100°C to 100°C comprising first and second heating and first cooling under nitrogen atmosphere, at a rate of 10°C/min.

2.5.3 *Scanning electron microscopy (SEM)*

The morphology of the scaffolds, namely the pore size and surface topography of the filaments, were assessed through the SEM, using a JEOL (JSM5600LV, Tokyo, Japan) scanning electronmicroscope. Samples were air dried and gold sputtered under high vacuum. Microanalysis with an element mapping distribution was obtained from scaffolds not metalized using energy dispersive X-ray microanalysis spectroscopy (EDX – Oxford Instrument) accessory.

2.5.4 *X-ray photoelectron spectroscopy (XPS)*

X-Ray photoelectron spectroscopy (XPS) was used to assess the surface chemical composition of PCL-based materials before and after extrusion, and for both non-degraded and degraded scaffolds. Spectra were obtained using a Thermo VG Theta probe spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA), equipped with a microspot mono-chromatized AlKα source and a flood gun combined with an argon gun for compensation of electrostatic charging of samples. The AlKα line (1486.6 eV) was used throughout the work, and the base pressure of the instrument was 10^{-9} mbar. The survey and high-resolution spectra were acquired in fixed analyzer transmission mode with pass energies of 150 and 50 eV, respectively.

Data analysis was performed using the *Avantage* software package, which consists of a non-linear least-square fitting program. The values of binding energies (BE–eV) were taken relatively to the binding energy of C1s-electrons of hydrocarbon contaminants on the sample surface (from an adventitious carbon), which is accepted to be equal to 285.0 eV.

3. Results and discussion

The design and fabrication of biodegradable and biocompatible scaffolds, with adequate structural and material properties for bone tissue replacement, requires an extensive knowledge of the degradation kinetics mechanism of polymeric materials in the body fluids. Ideally, the scaffold should degrade at a rate matching the regeneration rate of the new tissue guaranteeing simultaneously structural support and the physical space for continuous growth of new bone.

The PCL is a polyester, so it is susceptible to hydrolysis (Figure 2) (Li 1999). The hydrolytic degradation mechanism is complex and involves several phenomena, namely water absorption, ester bond cleavage, diffusion and solubilization of the formed small molecules (Pitt 1990). In the case of scaffolds, the hydrolytic attack by the aqueous medium might occur on the internal part of the filaments, called

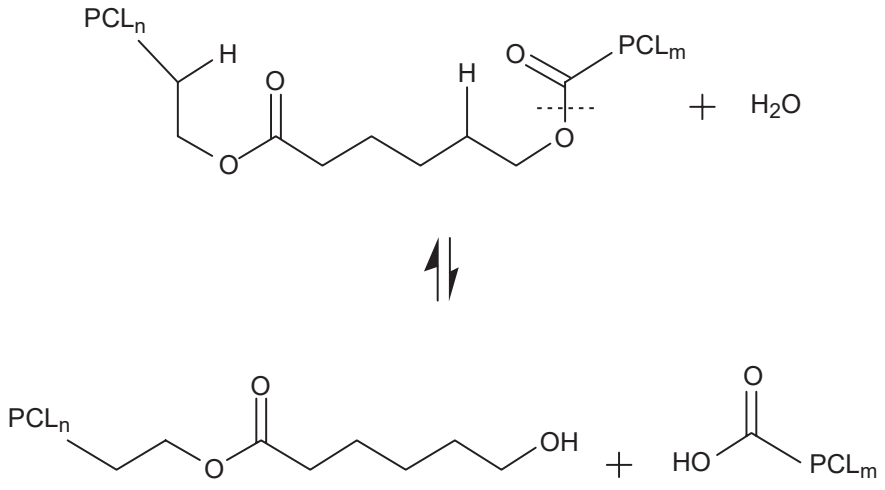

Figure 2. Typical polyester hydrolysis reaction.

bulk erosion, and/or externally through a process called surface erosion. The prevalence of one phenomenon upon the other depends on the diffusion/reaction mechanisms. If the rate of water diffusion into the polymer is relatively faster than the rate of hydrolysis reaction, the internal degradation will be faster and therefore bulk erosion will prevail. It is not possible to avoid the fact that the two degradation mechanisms may occur simultaneously.

In this study, the *in vitro* degradation properties of PCL scaffolds immersed in aqueous PBS and SBF were investigated. Degradation controls such as weight loss, variations in molecular weight, thermal properties and surface chemical composition were investigated. Bioactivity evaluation was also reported focusing on changes in scaffold surface morphology after immersion in SBF, as well as mineral content measurements.

3.1 *BioExtruded scaffolds*

The scaffolds produced with the BioExtruder present a well-defined internal geometry with square interconnected pores of regular dimensions ($\sim 600 \times 600$ μm) and uniform distribution, when viewed from the ZZ direction (Figure 3a). Micrograph images of the scaffolds cross section (Figure 3b) show that the triple raster fill strategy resulted in pores with 900 μm height.

The porosity of the structures was evaluated using the following methodology: (1) measuring the weight and volume of each sample, (2) calculating the apparent density of the PCL scaffolds and (3) applying the following equation:

$$Porosity = (1 - \frac{\rho^*}{\rho_{sub}}) \times 100 \quad (3)$$

where ρ^* is the apparent density of the cellular structure (scaffold) and ρ_{sub} is the density of the original substance ($\rho_{sub} = 1145$ g/cm^3). The overall porosity of the structures was found to be around 76%.

The extruded filaments present a regular circular geometry with 300 μm of diameter, and appear to have a good adhesion with the adjacent ones.

3.2 In vitro *degradation*

The morphologies of non-degraded and degraded PCL scaffolds non-degraded and degraded were evaluated by SEM photomicrographs from secondary electrons (SE) (Figure 4a) and backscattered electrons (BSC) (Figure 4b). Although the kind of electrons involved in both SEM techniques have different intensity, it can be observed that the scaffolds' architecture was almost retained during the 6-month period of degradation (Figure 4b). This observation suggests that the degradation process was still in its early stage. Similar observations were documented in the literature for the degradation of composite scaffolds with polymeric matrix containing ceramics (Maquet 1997, Kim 2004). However, brighter spots in the BSE image (Figure 4b) indicate the presence of heavier atoms than the one present in the PCL chains.

These heavy atoms result from the inorganic salts deposits on PCL scaffold surface after soaking in the highly rich SBF ionic solution. SEM/EDX analysis was used to confirm this statement.

The BSE image with higher magnification (Figure 5a) shows in detail the bright particles. EDX spectrum (Figure 5b) revealed the presence of Ca and P on the surface of PCL scaffolds, with Ca/P ratio of 1.65. This value is very close to that of bonelike apatite, where Ca/P ratio is equal to 1.67). As stated before, 6 months can be considered the first stage of the PCL degradation, so Ca/P deposits at this time cannot allow any claim about the bone mineralization process using PCL scaffolds, as well its microstructural features. Up to now, little is known about the nature of the CaP deposits on PCL-based scaffolds' surface subjected to degradation in body fluids.

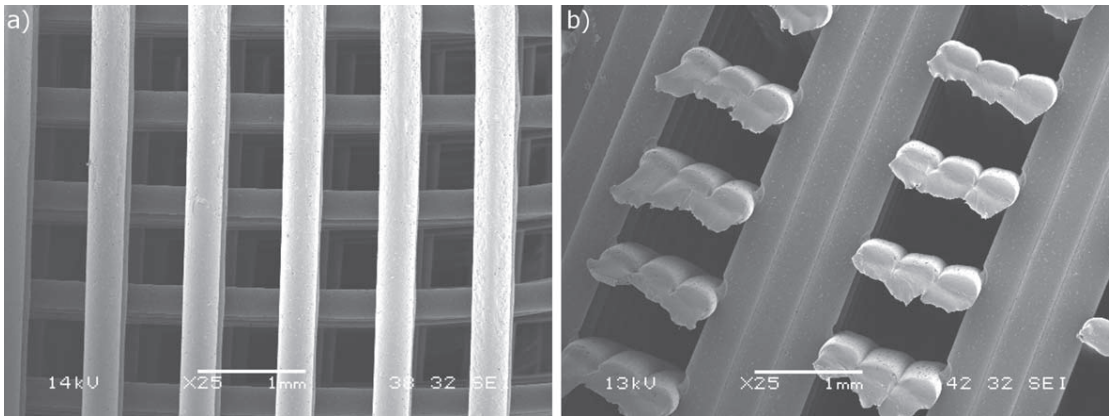

Figure 3. SEM micrograph of PCL scaffolds. (a) Top view; (b) cross-section view ($\times 25$ magnification).

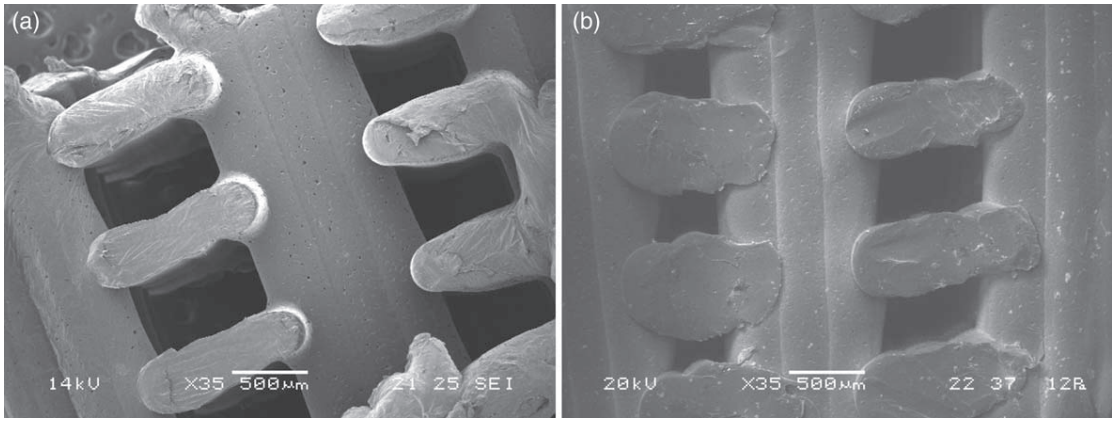

Figure 4. SEM micrographs of PCL scaffolds before (a) and after (b) immersion for 6 months in SBF ($\times 35$ magnification).

The observed salt deposit on the PCL scaffold surface can influence its erosion mechanism in the body fluid. One of the controls used to follow the erosion process is that of weight loss (Equation (2)). WL of the samples in aqueous medium reflects the release of soluble small molecules formed during the polymer hydrolysis reaction.

The average WL as a function of degradation time is depicted in Figure 6. The measurement errors from three specimens were calculated as the standard deviation. WL changes with degradation time were evaluated using one way ANOVA.

In the present study of PCL scaffolds, the very low WL% verified in the first 6 months of the experiment can be attributed to the polymer characteristics such as its crystallinity and hydrophobicity. ANOVA evaluation showed that the WL values of samples degraded in SBF, after 3, 4 and 6 months, are significantly different from the ones obtained after 1 month. However, as observed in Figure 5, there is a significant mean dispersion, principally for the WL values measured at 3 and 4 months. This can be attributed to the salt deposits, as previously verified by the SEM/BSE analysis, although the sample washing was thorough, as described in the experimental section.

It can be said that, for up to 3 months, there is a WL of about 1% due to erosion of PCL and that the salt deposit begin to influence WL measurements for degradation times higher than 2 months. PCL scaffold studied in PBS as degradation medium suggests an initial WL, from 0.2 to 0.4%, which was kept constant up to the end of the experiment. The invariable and very low WL of PCL in the PBS solution was confirmed by the ANOVA, showing no significant differences between the mean values obtained at each time interval.

During the PCL scaffold hydrolysis, the size of its chains decreased the forming of new chains. This process continued until a molecule of small size that is soluble in the acqueous medium has been reached. At this point, PCL scaffold erosion with WL began to be observed. Monitoring its molecular weight properties during and/or at the end of the degradation process will give additional information. Table 1 reports the changes in the number average molecular weight (M_n) and polydispersity index (PDI) of

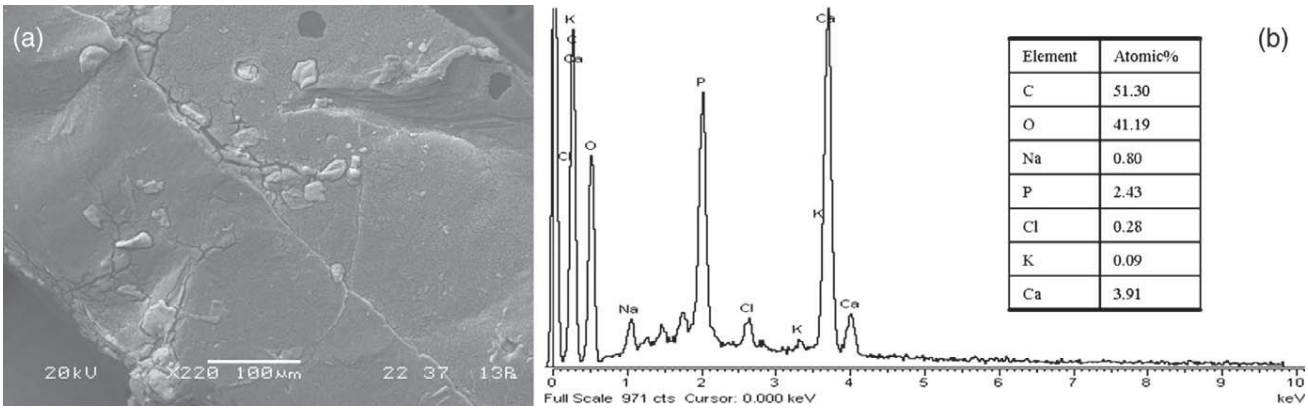

Figure 5. SEM/BSE photo-micrograph (a), and EDX spectrum (b) of PCL scaffold after 6 months in SBF.

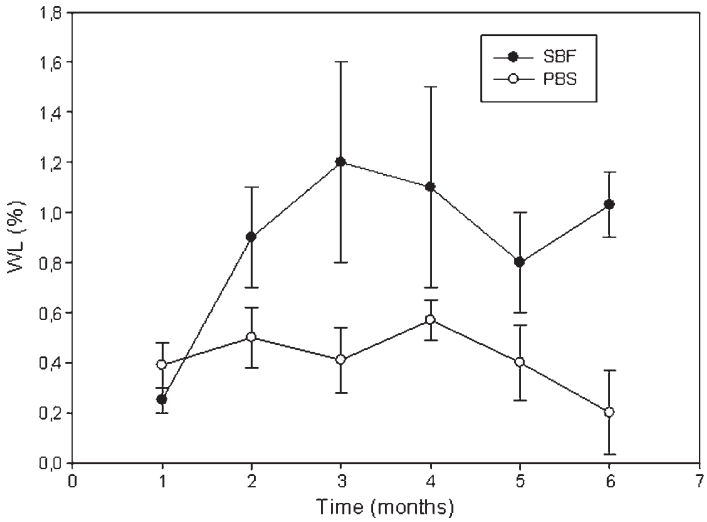

Figure 6. Weight loss percentages as a function of time of PCL scaffolds incubated in SBF and PBS.

pristine PCL scaffold after immersing in PBS and SBF for 6 months. The M_n of scaffolds decreased slightly after 6 months, in the aqueous PBS and SBF medium corresponding to 4 and 2%, respectively, and basically no change in the PDI was detected. These results corroborate the ones obtained with WL.

Although no significant hydrolysis was observed up to six months of degradation, it was sufficient to change in the same way the thermal properties as detected using DSC (Table 1). In both degradation media, the values of glass transition (T_g) and melting (T_m) temperatures decreased and the value of the degree of crystallinity (X_c) of PCL scaffold slightly increased in relation to that of the pristine material. After 6 months of immersion in SBF the values of T_g, T_m and X_c were -62°C, 53°C and 62%, corresponding to changes of 3, 7 and 5%, respectively. These results are in accordance with the changes in molecular weight, which means that the formation of small fraction of lower polymeric chains was sufficient to plasticize the bulk, decreasing the transition temperatures and increasing its crystallinity. A good agreement with literature was observed, where an increase of crystallinity from 61 to 77% was observed for PCL immersed in PBS for an 18-month period (Penã 2006).

The cleavage of the polymeric chains due to degradation reactions, promoted either by processing temperature or hydrolysis may change the proportions of the functional groups present on the scaffold's surface. XPS analysis then can help to identify environmental changes of the polymer chains. Figure 7 represents the high resolution C1s spectra of both pristine and scaffold PCL with curve fitting and peaks deconvolution performed on three specimens.

Looking at the survey scan spectra, an analogous surface chemical composition was determined for pristine and scaffold PCL (data not shown). Regarding the C1s curve fitting (Figure 7), four components relevant to different carbon chemical environments were employed which is in agreement with literature data (Beamson 2000). Attributions and the atomic percentages of the peaks are illustrated in Figure 7 and reported in Table 2. This comparison suggests that the scaffold manufacturing technique adopted does not change at all the surface chemistry of PCL.

As mentioned before, XPS was also employed on the detection of possible surface chemical changes in scaffolds

Table 1. SEC and DSC data of PCL scaffolds as a function of the aqueous medium and time of degradation.[a]

		SEC		DSC			
Medium	Time (months)	M_n	PDI	T_g (°C)	T_m (°C)	ΔH_m (J/g)	X_c (%)
No	0	33500	1.61	−64	57	82	59
PBS	6	32100	1.60	−63	53	85	61
SBF	6	32700	1.64	−62	53	86	62

[a] M_n is the number average molecular weight; PDI is the polydispersity index; T_g and T_m are the glass transition and melting temperatures, respectively; ΔH_m is the melting enthalpy and X_c is the crystallinity degree.

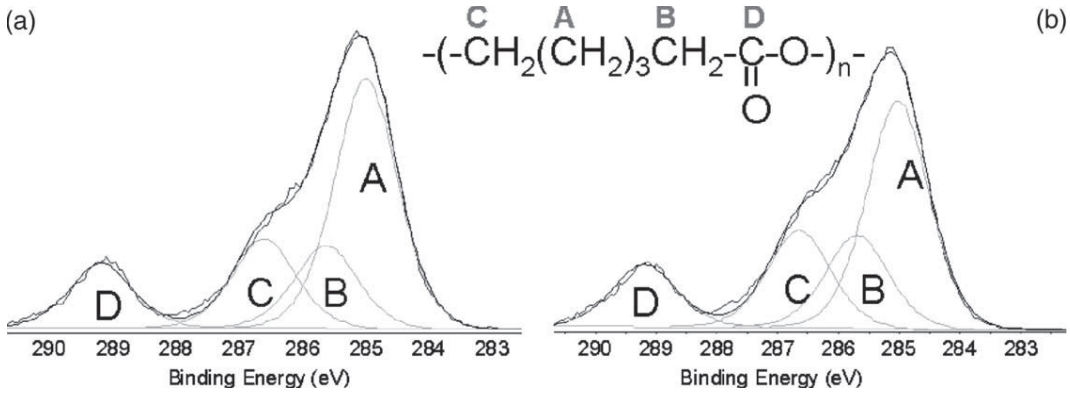

Figure 7. C1s high resolution spectra for pristine PCL (a) and PCL 3D-scaffold (b).

incubated for 6 months, either in PBS or in SBF. Figure 8 represents O1s curve fitting of PCL scaffold, before and after degradation in PBS. An almost similar amount of both C–O and C=O groups was present at the surface of the undegraded scaffold (Figure 8a). After 6 months of incubation in PBS, and despite minimal weight loss occurred, O1s shape was clearly modified (Figure 8b).

An increment of C=O/C–O ratio was detected, so it seems that there is surface erosion based on a random scission of polyester chains, in agreement with a previous work relevant to the hydrolysis of PCL films in basic solution (Sun 2009). This scission seems to be consistent with an increase of free –COOH groups (falling at the same B.E. of C=O present in un-hydrolyzed COOR groups) and a decrease of C–O groups on the surface of the polymer.

4. Conclusions

In vitro degradation behaviour of porous 3D PCL scaffolds, fabricated by the BioExtruder system, was carried out for 6 months, in two different media (PBS and SBF). It was observed that the degradation of the scaffolds after a 6-month period was still in its early stage, as clearly indicated by insignificant weight losses and molecular weight decreasing, independently of the aqueous medium. DSC results showed a slight increase of the degree of crystallinity. Finally, no significant changes were detected on the internal/external morphology of the scaffolds, as observed by SEM. Regarding the surface composition, an unambiguous change in C=O/C–O ratio was detected by the XPS analysis, indicating that a random chain scission has occurred as a result of being promoted by the hydrolysis. In addition, an heterogeneous and not conspicuous enough deposition of Ca/P salt illustrated that a possible precipitation of an inorganic phase occurred when the PCL scaffolds were incubated in a SBF solution. This observation may be correlated with the weight increase in opposition to the weight loss expected by the

Table 2. Binding energies (BE) and atomic percentages of PCL before and after scaffold manufacturing.

Carbon	BE (eV)±σ	Atomic%±σ		
		Literature*	Pristine	Scaffold
A	285.0±0.1	52	51±3	48±2
B	285.5±0.1	17	17±2	19±3
C	286.6±0.1	17	18±2	19±2
D	289.2±0.1	14	13±1	14±1

*Atomic % of PCL referred to in the literature (Beamson 2000).

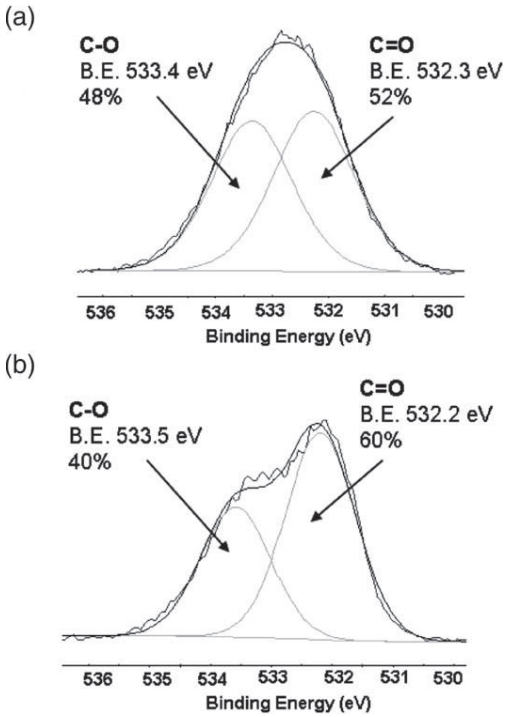

Figure 8. Curve-fitting of O1s high-resolution spectra of PCL scaffolds before (a) and after (b) immersion for 6 months in PBS.

degradation process. Further studies must be performed to understand the nature of these deposits.

The results of the *in vitro* degradation study allowed us to consider that PCL scaffolds, fabricated via BioExtrusion, have a great potential in bone tissue engineering, especially in the cases where the scaffold is required to maintain its structure and mechanical properties almost intact for a period of at least 6 months.

These preliminary *in vitro* screening tests show that the degradation kinetics of PCL scaffolds, especially in the SBF aqueous medium, appear to be governed by different and complex aspects.

To produce successful bone tissue engineering constructs, it is essential to correctly predict the biomechanical behaviour of the scaffolds after implantation, where they are subjected to dynamic mechanical loads and constant fluid flow. These factors may cause an excessive degradation rate, thus resulting in a premature decrease in the mechanical integrity of the scaffold, leading to the loss of tissue support and failure. Further experiments need to be carried out in order to understand the effect of the dynamic fluid flow on scaffold degradation, under conditions that closely mimic the host tissue environment. A perfusion bioreactor developed by our group is already being considered to perform this work. Different variables, such as the fluid flow rate, the scaffold porosity, the pore size and geometry are likely to affect the degradation kinetics, and will be studied using this system.

5. Acknowledgements

This work was performed within the framework of the European Network of Excellence "EXPERTISSUES" (Project NMP3-CT-2004-500283), with the partial financial support by Project PRIN – 2006 – prot. 2006038548. The authors wish to thank Mr Piero Narducci for recording SEM images of scaffolds.

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