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Influence of Enzymatic Degradation on Physical Properties of Poly(ε-caprolactone) Films and Sponges

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Summary: The effect of enzymatic degradation on poly(ε-caprolactone) (PCL) films and sponges was investigated at 37 °C using Pseudomonas lipase. Film samples were prepared by the solution casting method, while sponges were obtained by the freeze extraction method. The porosity was 17 and 60% respectively. Weight loss, morphology, crystallinity and mechanical properties were studied. The kinetic study on the enzymatic degradation of PCL porous samples depends on porosity, suggesting that degradation took place on the surface, not suffering bulk degradation. The non-dependence on crystallinity indicates that degradation occurred in both phases, amorphous and crystalline, at the same time.

Keywords: bioresorbable materials; differential scanning calorimetry (DSC); enzymatic degradation; mechanical properties; poly(ε-caprolactone)

Introduction

Biodegradable polymers are finding increasing number of applications as biomedical devices. The safe and predictable disappearance of these materials plays an important role in evaluating their performance. Among the chemical mechanisms of degradation, hydrolysis is the most common path taken by degradable polymers such as polyesters, polyamides, polyanhydrides and polyacetals. Theoretical models[1–3] predict that the mechanism of degradation, bulk or surface erosion, depends on the diffusivity of water inside the matrix, the degradation rate of the polymer and the matrix dimensions. Once the polymer hydrolyzes, there is a decrease in the molecular weight, followed by a loss of mechanical properties, and finally a loss of mass caused by the dissolution of small polymer fragments.

Poly(ε-caprolactone) (PCL) is a biocompatible, biodegradable and semicrystalline polyester proposed for a wide variety of biomedical applications, including resorbable sutures, drug delivery systems,[4,5] gene carriers,[6] and as scaffolds for tissue engineering.[7–10] The melting temperature is $T_m \sim 60\,^{\circ}C$ and the glass transition temperature, $T_g \sim -60\,^{\circ}C$. Its hydrolytic degradation is very slow due to its hydrophobicity and crystallinity,[11,12] which makes this polymer useful for long term applications. In order to improve PCL degradability and tailor polymer properties to specific applications, PCL copolymers or blends with a variety of other polymers have been proposed.[13–18]

Since Potts et al. reported the biodegradation of PCL[19] many studies have been carried out on PCL biodegradation, both non-enzymatic[20,21] and enzymatic.[22–26] Among them, it was Tokiwa and Suzuki[22] who first demonstrated that synthetic polyesters could be attacked by lipases. Some others[26,27] have shown that while pseudomonas (PS) lipase is very effective in the degradation of PCL, other lipases do not perform such a function. Different factors as the temperature[28] or the enzyme concentration influence the PCL degradation rate.[29,30] It has also been indicated that enzymatic biodegradation happens mainly on the surface because it is

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difficult for a hydrophilic enzyme to diffuse into a hydrophobic polymer.[31] The molecular weight of PS lipase is around 32000, and the water content of PCL is less than 10%. As a consequence, a linear relationship between the degradation rate and the surface area for film, PCL microparticles and fibers has been found.[18,30,32]

In a previous work[33] porous PCL samples with a very different morphology and porosity were obtained by the solvent casting and freeze drying techniques. In this work, sponges with a high porosity and surface area were obtained by freeze extraction.[34] This method presents advantages respect to freeze drying: the samples morphology is more regular with more interconnected pores and besides it is easier to manage. The aim of this work was to characterize the morphology and enzymatic degradation of porous PCL sponges. The results are compared with these obtained for samples with a lower porosity (film samples) and the corresponding to non-porous (bulk) samples. Enzymatic degradation was carried out in the presence of PS lipase at a temperature of 37 °C. The kinetics of degradation was examined by measuring weight loss, calorimetric and mechanical properties, as well as by visual observation using scanning electron microscopy (SEM).

Materials and Methods

Materials

Poly(caprolactone) (PCL) [Polysciences (Mw 43,000–50,000)] in the form of powder was used without further purification. Lipase from *Pseudomonas (PS) fluorescens*, in powder form, (EC 3.1.1.3, 40 units/mg) was purchased from Fluka. The solvent, tetrahydrofuran (THF) and sodium azide (NaN_3) 99% from Aldrich, and the buffer solution (disodium hydrogen phosphate) pH 7.00 (20 °C) $D = 1.01\ g \cdot cm^{-3}$ from Scharlau, were used as received.

Preparation of PCL Samples

Films and sponges were prepared from a solution of PCL in tetrahydrofuran (THF). The films were produced by evaporation of THF at room temperature while the sponges were obtained by freeze extraction.[34] In both types of samples, the PCL concentration was 30% and it was necessary to heat the solution to 40 °C for two hours to get the polymer to completely dissolve.

To obtain film samples the solution was poured into a glass Petri dish which was covered with a lid and placed in a fume hood at room temperature for slow evaporation. The solid samples were dried in vacuum to constant weight. The film samples obtained presented a flat, white surface. The thickness of the resulting films ranged from 0.8 mm to 1.4 mm.

In order to obtain more porous structures (sponges), solution was poured into a glass Petri dish and immersed in liquid nitrogen. The frozen polymer solution was immersed in an ethanol solution which was pre-cooled to −20 °C. Then, the THF was extracted out and replaced with ethanol, a non-solvent for PCL. After extraction, samples were dried under vacuum at room temperature. The thickness of the resulting sponges ranged from 0.7 mm to 2.9 mm (the immersion in liquid nitrogen did not allow a more uniform thickness, as would have been desirable).

For the sake of comparison, non-porous (bulk) samples were obtained by melting the PCL powder on glass plates to get a film of approximately 0.8 mm thick (ranging from 0.6 to 1.1 mm).

All samples were stored at room temperature for at least one month before physical measurements and enzymatic degradation.

Enzymatic Degradation

The degradation assay was carried out by immersing PCL samples, of known dry weight, (dimensions around 5×5 mm) in 5 ml of a phosphate buffer saline solution (pH 7) at 37.0 ± 0.5 °C in the presence of 0.5 mg PS lipase and 0.5 mg sodium azide to avoid proliferation of bacteria. The buffered enzyme solution was changed every 24 h to maintain the enzymatic activity. Three replicates were taken out of the

solution at predetermined time intervals, then washed with distilled water and dried under vacuum at room temperature to constant weight. The degradation rate was determined by the weight loss which is defined as:

$$\text{Weight Loss } (\%) = [(W_0 - W_d)/W_0] \times 100,$$

where W_0 is the initial weight and W_d the weight after degradation at different time intervals.

Analysis and Characterization

A Sartorius BP211D balance with a sensitivity of 0.01 mg was used to weigh the samples before and after degradation.

To investigate the surface and cross section morphology of dried samples, SEM micrographs of both the degraded and non-degraded samples were taken using a JEOL JSM-5410 scanning electron microscope.

The porosity of samples, ϕ, was estimated from the apparent volume, V_T, and the PCL volume V_M, defined from the actual amount of polymer, m, and its mean density, ρ ($\rho = 1.14$ g cm^{-3})

$$\phi = \frac{V_T - V_M}{V_T} = \frac{V_T - \frac{m}{\rho}}{V_T} \quad (1)$$

At different degradation times, the calorimetric properties of the samples were measured by using a Mettler Toledo differential scanning calorimeter, DSC, calibrated with indium. The measurements were carried out at a scan rate of 10 °C/min between −10 °C and 100 °C. The weight of the samples was around 15 mg, except for the most degraded samples, which were inferior. The crystallinity was calculated assuming proportionality to the experimental heat of fusion, using the reported heat of fusion of 139.5 J/g for the 100% crystalline PCL.[11] After this heating scan, the sample was cooled down to −10 °C at a rate of −10 °C/min and reheated again to 100 °C at a rate of 10 °C/min. The data from all three scans were collected for subsequent analysis. The reason why three consecutive scans were performed was because it was desired to identify the differences in the degradation of the amorphous and the crystalline phases by studying the first melting peak, or by differences in the molecular weight distribution that would affect the melting peak of the second heating scan.

Mechanical properties were evaluated using a Seiko TMA/SS6000 by applying a compression ramp from 0.5 g until 100.5 g at 50 g/min; the deformation of the sample was collected every 0.5 s. The section of the apparatus probe was 7 mm^2, being 0.15 MPa the maximum value of the stress applied on samples. Two samples of each type and degradation time, including non-degraded samples, were measured.

Results and Discussion

The porosity of the PCL samples, measured before degradation, was $17 \pm 4\%$ for the films and $60 \pm 5\%$ for the sponges. The error was taken as the standard deviation of the measured porosity. In a previous work,[33] sponges were obtained by freeze drying, in which solvent was extracted at a pressure of 10^{-4} bar and at a temperature of −30 °C. The obtained porosity and crystallinity were similar, but the main difference was that the microspheres obtained by freeze extraction seemed more interconnected. As an advantage, the freeze extraction method is simpler and requires less laboratory equipment.

Figure 1 shows SEM micrographs of the PCL sponge. It exhibits a cauliflower structure formed by microspheres of diameter 20–30 μm that leave interconnected pores ranging between 10 and 50 μm. The microspheres present a porous rough surface with a honeycomb structure whose cells have around 5 μm in size (see Figure 1b). As a result, the sponges present a very high surface area.

As far as the films were concerned, opposite faces of the same polymer film exhibited morphological differences in terms of the aggregates formed during phase separation and the microporosity of the surface. The surfaces of the PCL films appeared to be made up of fused aggregates

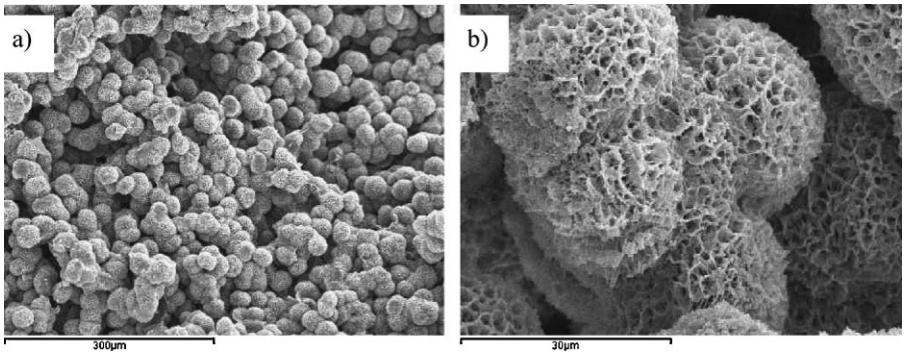

Figure 1.
SEM micrographs of the PCL sponge obtained by freeze drying: a) cauliflower structure bar = 300 µm, b) at a higher magnification the structure of the microspheres surface can be observed, bar = 30 µm.

showing rough surface morphologies on both sides. The surface in contact with glass was rough with visible pores and with spherule-like aggregations which were elongated and partially fused together, resulting in irregular voids on the surface. In contrast, the surface exposed to air was less porous but uneven, with visible cracks in places. Figure 2 shows the cross section and surfaces of PCL film where a transversal porosity gradient can be observed as well as the different morphology on both faces. Such single but heterogeneous porosity may have the potential to facilitate the growth of different tissues within a scaffold. It was described that different cells may have a preference for different pore sizes and optimizing these for the right application is necessary.[35]

Biodegradation of PCL samples was carried out at pH 7 and $37.0 \pm 0.5\,^{\circ}C$ with PS lipase. Results of weight loss showed in Figure 3 prove the influence of porosity (superficial area) on degradation. After 100 hours, the weight loss of bulk, film and sponge samples was 23%, 47%, and 92%, respectively. Depending on the thickness of the sample, some thin sponges degraded more (or even disappeared) than the thicker ones. In Figure 3 the average of three samples for every degradation time

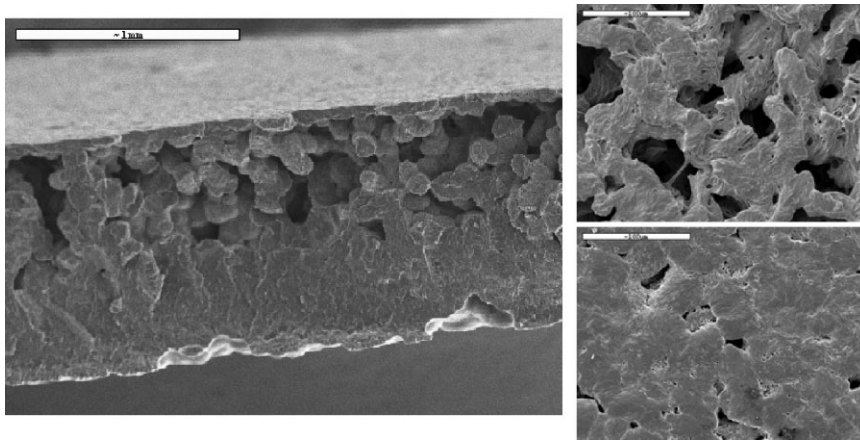

Figure 2.
Cross section of the PCL films. The upper part corresponds to that in contact with the Petri dish while the lower part was in contact with air, during THF evaporation. Bar = 1 mm.

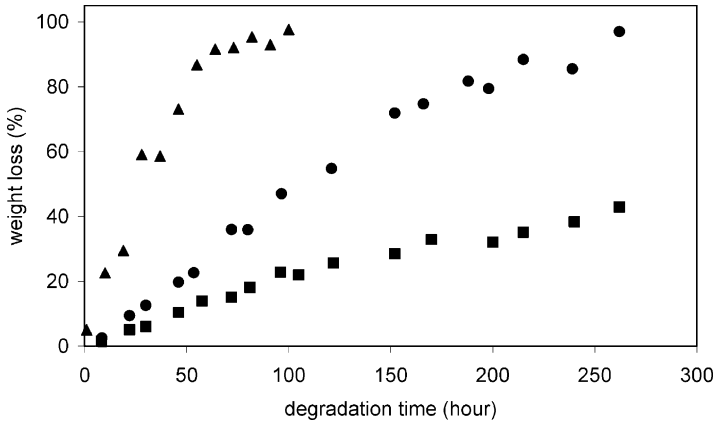

Figure 3.
Weight loss of PCL samples after degradation in Pseudomonas lipase. ■: bulk PCL, ●: film samples, and ▲: sponge samples.

are presented. Standard deviations, not shown in the figure are of the order of 10% or less for the bulk and film samples, but much higher for the sponges due to the variation of the thickness observed with this type of sample.

Weight loss gives a general trend about the rate of enzymatic hydrolysis, but it is not possible to find out how this hydrolysis proceeds. For this reason morphological examinations are necessary. SEM micrographs of the bulk, film and sponge samples taken before and after enzymatic hydrolysis are presented in Figure 4. Morphology changes indicate a superficial degradation that affects the roughness of the surface. Small holes and cracks, as well as surface irregularities appeared. As the degradation progressed the erosion became remarkable, the porosity was higher. The extent of hydrolysis was very high, even in the first 48 hours; and mass was removed from the samples, especially from the porous ones. In the case of porous samples (films and sponges) the degradation affected also the inner parts of the samples as can be observed in the micrographs of the cross section (Figure 5).

Figure 6 shows the crystallinity of the degraded samples as a function of time corresponding to the first and second heating scan for each sample. Looking at the results measured in the first heating scan (solid symbols in Figure 6) it was observed that bulk PCL samples had a crystallinity around 55% that remained constant during the degradation duration. The same happened with PCL films; their crystallinity value of around 66% does not change, although there has been a much greater loss of mass. A gradual increase of crystallinity can be noticed in the PCL sponges. It, however, becomes significant only after 2 days of degradation, when the loss of mass is more than 50% (see Figure 3) and, then, samples of smaller mass had to be measured with the corresponding increase in uncertainty. On the other hand, it can be observed that the crystallinity measured in the second heating scan is the same at any time independent of the type of sample. This is because the melting process removes the influence of sample preparation. Nevertheless, this value ($45 \pm 3\%$) is different than that measured for block samples in the first heating scan due to the elapsed time between sample preparation and the measurements (more than a month at room temperature, a temperature much higher than the glass transition temperature and not far from the melting temperature, which allows the progression of the crystallization process). According to this, we have considered the standard deviation of

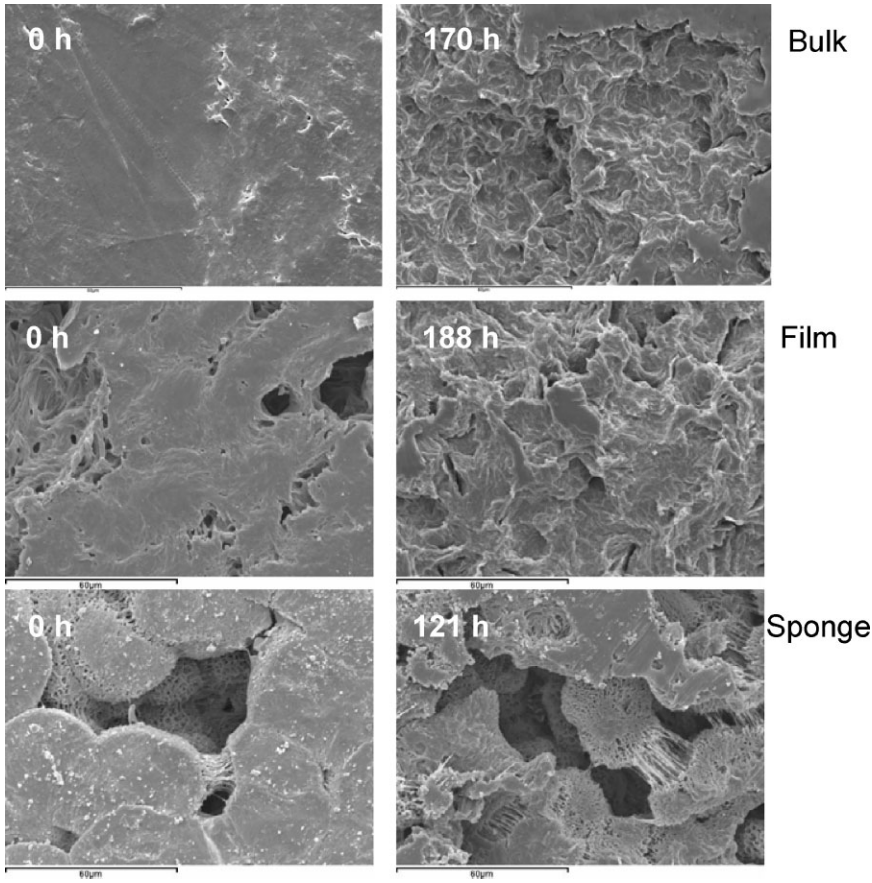

Figure 4.
Micrographs of the surface of non-degraded and degraded samples in PS lipase. The labels indicate the degradation duration. Bar = 60 μm.

the crystallinity measured in the second heating scan as a measure of the uncertainty of the crystallinity.

Comparison of weight loss and crystallinity indicates that PS lipase can degrade both, the amorphous and the crystalline phases, and is in agreement with literature data.[27] These results indicate that the degradation is influenced by porosity, in agreement with a surface erosion phenomenon characteristic of enzymatic degradation.

In the second heating scan, all the samples have the same value of crystallinity, 46% (see Figure 6), independent of the degradation duration and the initial morphology. However, the thermograms show significant differences. Figure 7 shows, as an example, the thermograms corresponding to the bulk samples. Those that correspond to the first heating scan (labeled 1 and 2 in Figure 7) show that the melting peak of the degraded sample is narrower and slightly displaced to lower temperatures with respect to the non-degraded sample, which could be explained by the existence of crystals with a more uniform size in the degraded samples. A shoulder can also be observed around 50 °C, and that could be due to crystallization while heating or to the melting of small crystals formed during degradation.

It could also be observed in the second scans (labeled 3 and 4 in Figure 7) that the

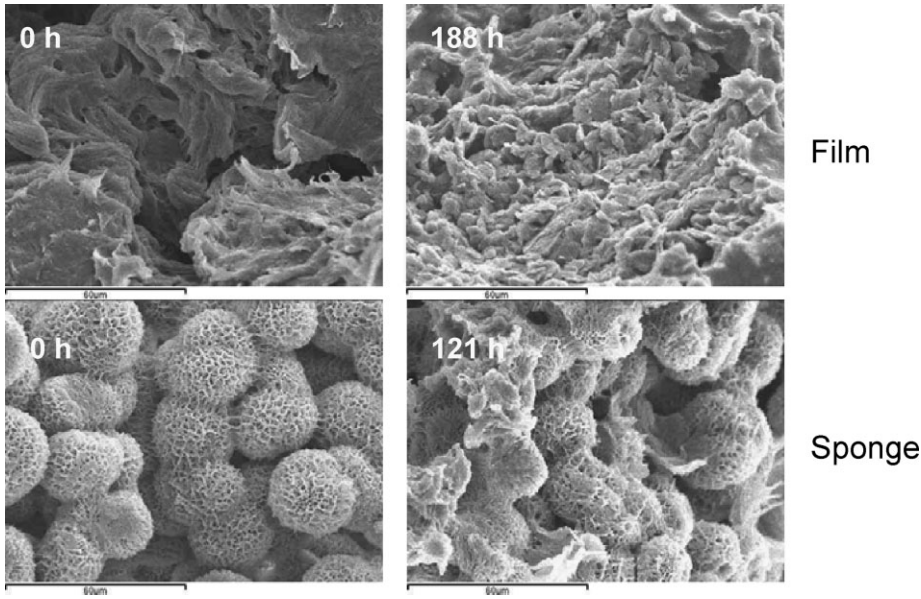

Figure 5.
Micrographs of the cross section of non-degraded and degraded samples in PS lipase. The labels indicate the degradation duration. Bar = 60 μm.

width of the melting peak of the degraded sample is lower than that of the non-degraded one. As this second scan is obtained after melting the sample and after a further crystallization, under the same conditions for all the samples, the observed narrowing of the melting peak could only be explained by a modification in the molecular weight of the samples as a consequence of the degradation process.

Figure 8 shows the results of the mechanical test of the film samples. The

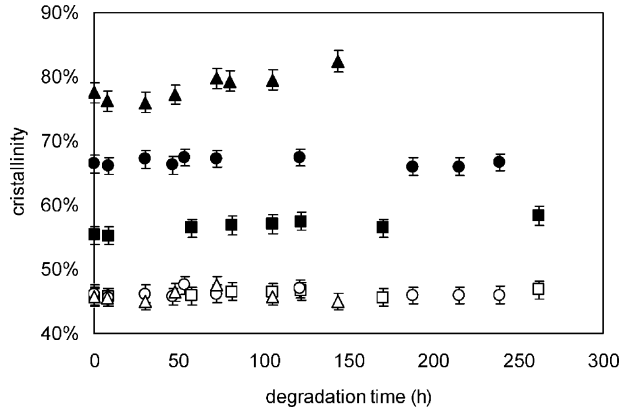

Figure 6.
Crystallinity of PCL samples measured by DSC after degradation. Solid symbols correspond to the crystallinity measured in the first scan. Open symbols correspond to the crystallinity measured in the second heating scan; square symbols: bulk PCL, circle: PCL films, and triangle: PCL sponges. Error bars equal to the standard deviation of the crystallinity obtained in the second heating scan for all the samples.

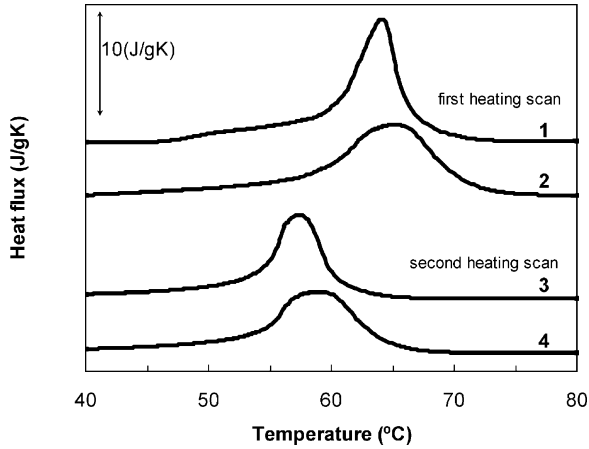

Figure 7.
Heat flux from the first and second heating scans measured on the bulk sample. Scans 1 and 3 correspond to a degradation time of 262 h, and scans 2 and 4 to a non-degraded sample. The scans have been shifted for better visualization.

maximum force available on the experimental device did not allow us to obtain significant conclusions. According to the obtained results in previous work by this group[33] at 0.15 MPa, the probe of the apparatus was contacting the sample, and showed the irregularities of the surface. At this stage, the linear region was not yet reached, and the stress-strain curve shows the initial contact between the probe and the surface of the sample. It is noticeable that the initial deformation of the samples increases with degradation duration. This result has also been observed with two other types of samples (not shown). The increasing initial deformation could be a consequence of the increasing roughness of the sample surface, and it is related to the fact that enzymatic degradation produces superficial erosion. To obtain more overall features, greater forces should be applied.

Conclusion

Porous PCL samples with interconnected pore network were obtained by freeze extraction from a PCL/THF solution. The

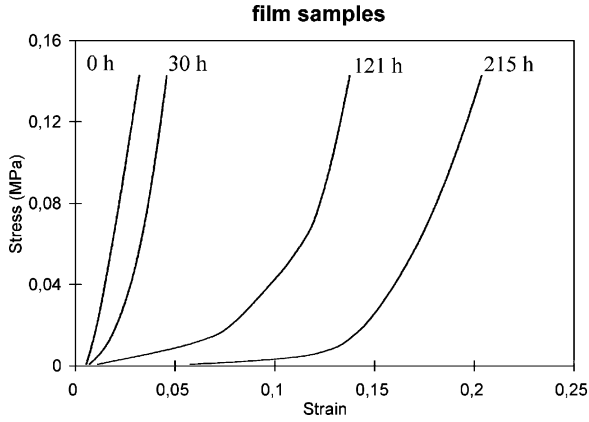

Figure 8.
Stress-strain curve obtained for the non-degraded and degraded film samples.

samples presented a cauliflower structure formed by microspheres that show a rough honeycomb morphology. As a result samples with a very high surface area were obtained. A gradient pore structure was created within the film samples. The degradation, carried out at pH 7 and 37 °C with PS lipase, produced erosion and roughness of the samples surface. In the case of porous samples the degradation also affected the inner part. The weight loss depended more on porosity and surface area than on the degree of crystallinity of the samples. It was also found that the degradation process did not change the crystallinity and, therefore, it seemed to affect both the amorphous and the crystalline phases. However, it modified the structure of the crystalline phase, which is a consequence of the chain fragmentation caused by the decrease in the molecular weight of the polymer chains.

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