# Synthesis and characterization of poly(L-lactic acid) membranes: Studies *in vivo* and *in vitro*

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The use of biodegradable polyesters as temporary structural supports in the recuperation of damaged live tissue is a promising area of research. Poly(L-lactic acid) (PLLA) membranes can act as a support for cell fixation and growth or as a barrier against soft tissues invasion in recuperating bone tissues. In this work, five different types of PLLA membranes, which varied in their polymer–solvent ratio and their content of plasticizer were studied. For the study *in vivo*, 6 mm diameter disks were inserted subcutaneously in the dorsal region of 15 Wistar rats, and the reactions on rats were studied 15 days later. In another series of experiments the samples were immersed in phosphate buffer, pH 7.4 at 37 °C, for 30 days. Membranes without plasticizer were morphologically dense and did not allow cell invasion nor tissue adherence, in contrast to membranes with plasticizer. While porosity enhanced cell fixation and growth, it made the membrane more fragile mechanically when compared to membranes without pores.


## Introduction

Biodegradable polyesters, such as polylactides, are promising materials in tissue engineering. This material is used to form dense membranes that function as barriers against soft tissue invasion during bone recuperation (a technique known as ''guided tissue regeneration'') or as porous membranes that provide a support for cell growth, fixation and tissue transplants [1, 5].

Dense poly(L-lactic acid) (PLLA) membranes of different thicknesses can be synthesized by the casting technique or by thermoplastic processing through injection molding or extrusion [6]. Porous membranes have also been synthesized by different methods. A common procedure involves the preparation of a polymer solution by adding salt granules of known size. After solvent evaporation and several washes to remove the salt, membranes with granulations determined by the size of the grains are obtained [7]. However, the membranes produced by this technique do not have regular-sized pores nor guaranteed interconnections, which is an important factor in tissue formation.

Macro-porous membranes can also be produced using high pressure $CO_2$ gas at room temperature. This technique has the advantage of not requiring the addition of organic solvent, but, as with the technique using salt, the membranes have poor mechanical properties [8].

The technique of phase inversion, which has been widely used to produce porous membranes for micro and ultra-filtration, consists of immersing of a polymeric solution in a vessel containing a non-solvent, to stimulate the separation of the different phases in solution, thereby affecting the porosity of the membrane during solvent evaporation [9–13].

The gel casting technique consists of polymer dissolution in a poor solvent. For PLLA, either acetone or ethyl acetate can be used as a solvent for gel phase formation. The porosity can be obtained by immersing the gel in a bath with a non-solvent to extract the solvent and induce polymer phase separation. The problem with this technique is that it creates very small pores (about 3 μm) [14, 15].

Another technique involves the agglomeration of polymer spheres of specific diameters to create a membrane. These spheres are obtained by mixing a gel solution and a polymer solution followed by agitation and cooling. Adhesion among the spheres is obtained by adding poly(vinyl acetate) and triethylcitrate [16].

Silva *et al*. [17] used the dry spray technique to obtain tubes for the regeneration of nerves. This technique can also be used for membrane synthesis.

All of these techniques present problems related to the size and interconnection of the pores as well as poor

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mechanical properties. In this report, we describe an alternative way to produce biodegradable membranes of PLLA with controled homogeneity, pore size and interconnectivity, as well as adequate mechanical properties. The membranes produced using this technique showed a good implant response and may be used as matrices for cell growth.

## Materials and methods

PLLA with a molar mass (Mw 300 000 daltons) in the form of pellets was obtained from Medsorb. The polymer was dissolved in methylene chloride ($CH_2Cl_2$, Merck) in a closed recipient in room temperature and triethylcitrate (Aldrich) was added as plasticizer. The concentrations used of polymer, the plasticizer added and the membrane identification are described below:

- Membrane 5% – 100 mL of $CH_2Cl_2$ + 5 g of PLLA
- Membrane 10% – 100 mL of $CH_2Cl_2$ + 10 g of PLLA
- Membrane 10%P – 100 mL of $CH_2Cl_2$ + 10 g of PLLA + 10 mL triethylcitrate
- Membrane 15%P – 100 mL of $CH_2Cl_2$ + 15 g of PLLA + 10 mL triethylcitrate
- Membrane 10%/10%P – Membrane type 10% + membrane type 10%P

After preparation, the solutions were spread on glass plates (100 $cm^2$) and then placed in a recipient at room temperature and dried (dry air outflow of 1 L $min^{-1}$) to remove solvent. After 15 h, the membranes were removed from the glass plates, vacuum dried for 24 h, and then submitted to *in vitro* and *in vivo* test.

### *In vitro* study

Samples of each type of membrane were cut and immersed in phosphate buffer solution, pH 7.4, at 37 °C, up to 30 days, as described for the ASTM D-1708 tension test. After 10, 20 and 30 days, the samples were removed from the bath, washed with distilled water and vacuum dried for 24 h. After drying, the samples were submitted to a tension test, differential scanning calorimetry (DSC), scanning electron microscopy (SEM) and gel permeation chromatography (GPC). The tension tests were done in an MTS 910-Materials Test System equipment, according to the procedure ASTM 1708/96, with constant a velocity of 2.2 mm $min^{-1}$ between spacing claws. The DSC measurements (DU PONT) were obtained from 0 to 250 °C at 10 °C $min^{-1}$. GPC measurements (Waters) were obtained using three Ultrastyragel columns (500, 1000 and 10 000 Å), coupled to a refraction index detector. The samples were dissolved in chloroform (MERK), and 200 µL were injected at an outflow of 1 mL $min^{-1}$. The Mw, Mn and polydiversity were calculated using polystyrene in tetrafluoroethylene standard (MERK). For SEM analysis (JEOL-300), the samples were fractured in liquid nitrogen and sputtered with gold.

### *In vivo* study

Membrane disks 6 mm in diameter obtained by casting were washed in distilled water for 30 min and then in ethanol 70% for 2 h. The disks were then dried at room temperature and stored in a vacuum.

The samples to be implanted were immersed in physiological solution, for a few minutes and subjected to a low vacuum to remove the air in the membrane pores and substitute this with physiological solution. This procedure prevented air from functioning as a possible site of infection [7].

Fifteen male Wistar rats approximately 4 months old were used. The rats were divided into five groups of three rats each (one group for each type of membrane studied). The rats were handled in accordance with established guidelines for the care and maintenance of laboratory animals. The rats had access to water and commercial animal feed *ad libitum*.

For use, the rats were anesthetized with ethyl ether and membrane samples were implanted subcutaneously by two parallel 1 cm incisions providing a ''pocket'' for the membranes. The rats were observed on a daily basis up to the 30th day after the implant, at which time the animals were killed and the tissue samples removed and fixed in Bouin solution for 24 h, at room temperature. The fixed material was dehydrated and embbeded in paraffin. Sections 5-µm thick were stained with hematoxylin-eosin and then observed and photographed with a photomicroscope (Zeiss).

## Results and discussion

### *In vitro* study

#### *Mechanical properties*

A knowledge of the maximum tensile strength that the membrane can present without harming its structure or disturbing its usefulness as a support for cells is extremely important during membrane synthesis and subsequent evolution of the final product. When the membrane is fixed in the host tissue or in cultured cells, the polymer starts degrading and its mechanical properties are adversely affected. Continuous monitoring of the changes in these properties over time is essential when assessing biodegradable membrane applications. Based on this knowledge it is possible to choose the type of membrane with mechanical properties compatible with the velocity of matrix growth that it will support.

During the first 10 days of degradation, all of the membranes that contained plasticizer (10%P, 15%P and 10%/10%P types) became very fragile, making it impossible to manipulate them for the tension tests at 10, 20 and 30 days. The analysis could be done with the 5% and 10% membranes up to 30th day. As shown in Fig. 1, membranes that contained plasticizer had lower tension values (Fig. 1). In addition, the tension limit varied significantly ($p < 0.05$, Student $t$-test) among these membranes, but not among those without plasticizer.

Fig. 2 shows an increase in the elongation values for membranes with plasticizer compared to membranes with no plasticizer. This characteristic is very important since membranes should be flexible enough to accommodate the movement and natural flexibility of soft

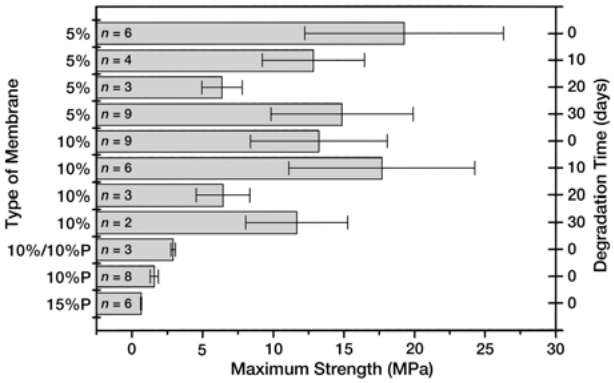


*Figure 1* The average tension values obtained in the yield limit for types of membranes at different degradation times ($t = 0$, 10, 20, 30 days); $n$ is the number of tests. Only 5% and 10% membranes could be tested up to the 30th day.

tissues. Statistical analysis showed that the values obtained for the 5% and 10% membranes were not significantly different among themselves during all four periods of degradation. Membranes with and without plasticizer gave significantly different results ($p > 0.05$).

Membranes containing 10 mL of plasticizer showed an extremely elastic mechanical behavior, but with little mechanical resistance, which limits their application. Varying the quantity of plasticizer added can therefore allow the production of membranes with the desirable characteristics of elongation and strength.

Few authors have analyzed their fraction test results statistically, so a comparison with other results is difficult. Another important factor relates to the criteria used by different authors to select the points from the curve stress vs. strain curve for subsequent calculation of the elongation, strength and elastic modulus. Thus some authors use stress of rupture, and others, strength at maximal limit of yield. Determination of the elastic modulus is a problem because there is not always a linear initial behavior in the curve, making it difficult to draw the tangent which identifies the modulus. For this reason the use of the elastic modulus to compare polymer properties has been contested with the dynamic elastic modulus being preferred.

Tsuji and Ikada [18] obtained pure PLLA membranes and analyzed variations in their mechanical properties at several temperatures. These properties were related to

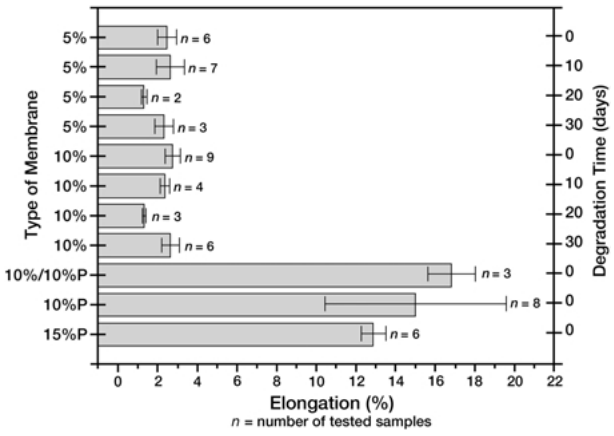


*Figure 2* The average elongation values obtained in the yield limit for different types of membranes at different degradation times ($t = 0$, 10, 20 and 30 days).

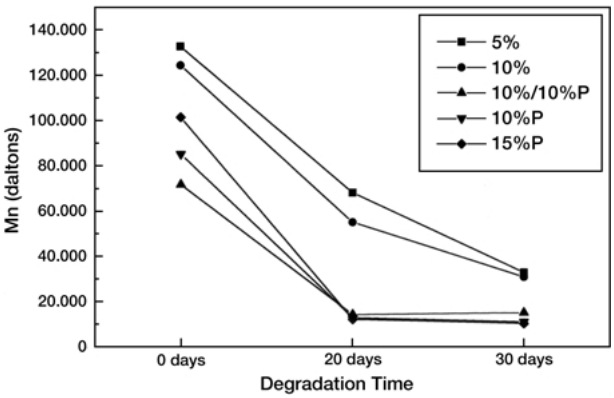


*Figure 3* Variation in Mn versus the degradation time for all membranes.

the degrees of crystallization. These authors concluded that the tension values, elastic modulus and elongation were closely related to the crystallization values. The increase in crystallization augmented the stress and modulus, but diminished the elongation.

Cordewener *et al*. [19], studied the mechanical, physical and histological properties of poly(96L/4D lactic acid) films containing 96% PLLA and 4% PDLA. The mechanical properties decreased steadily in the first 7 weeks with a 50% fall from an initial value of 51.3 MPa to 27.5 MPa, after 3 weeks. The elastic modulus gradually decreased and varied from 3.4 to 1.8 GPa after 7 weeks.

## Gel permeation chromatography

Variations in molar mass are indicative of degradation of the polymers which, when expressed as a function of time, can provide information on the rate of the process.

Molar mass measurements were done in PLLA membranes 0, 20 and 30 days after immersion in buffer solution. The average molar mass in number (Mn) are shown in Fig. 3.

The average molar mass in weight (Mw) values decreased with time for all membranes, with a sharper decrease between the periods 0 and 20 days than between 20 and 30 days. The loss of molar mass was greater in the membranes with plasticizer.

GPC data thus revealed a significant difference in the degradation of membranes with and without plasticizer, which agreed with the results obtained by DSC, see below, and for the mechanical properties.

## Differential scanning calorimetry

DSC is useful for determining variation in crystallinity during the degradation. These data are obtained by comparing the fusion enthalpy of a sample of the polymer with its theoretical fusion enthalpy, assuming that the polymer is 100% crystalline. For PLLA this value is 93.7 J g$^{-1}$ [20]. The degree of crystallinity is calculated using the equation:

$$\%\chi = \frac{\Delta H_f - \Delta H_c}{\Delta H_{f100\%}} \times 100$$

where $\chi$ is the degree of crystallinity (%); $\Delta H_f$ is the experimental fusion enthalpy (J g$^{-1}$); $\Delta H_c$ is the

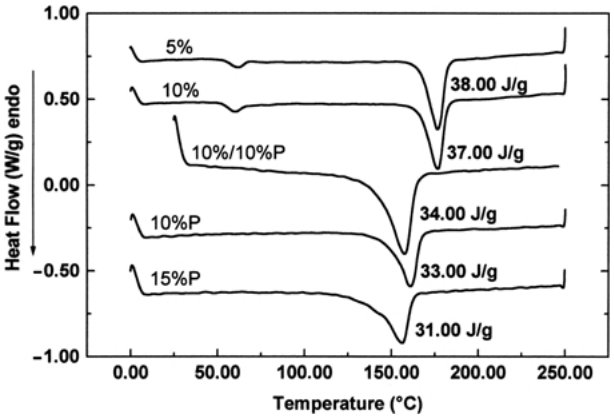


*Figure 4* Differential scanning calorimetry of the five types of membranes at zero days.

experimental crystallization enthalpy ($J\,g^{-1}$) and $\Delta H_{f100\%}$ is the theoretical fusion enthalpy for the 100% crystalline polymer.

Fig. 4 shows the thermogram for the five PLLA membranes studied. The samples had an endothermic fusion peak (Tm) between 165 and 176 °C and a glass transition temperature between 50 and 57 °C. The degree of crystallinity increased for all membranes during degradation, although this increase was sharper for membranes which contained plasticizer and in the first 10 days of immersion in phosphate buffer (Fig. 5).

The explanations for the increase in degree of crystallinity during degradation are controversial. Most authors claim that there is a rearrangement of the minor chains generated by the degradation process itself with the consequent formation of new crystals, while others believe that the amorphous parts of polymer degrade and a greater percentage of the crystalline structure remains.

The degradation of a polyester, such as poly(lactic acid) results from hydrolysis of the ester backbone, which occurs in amorphous regions of the polymer. This could explain the increase of crystallinity observed by several authors [22, 23].

The process of degradation is more complex than the relationship between amorphous and crystalline parts. Using different amorphous PDLA plates, Li *et al*. [22] observed that the degradation process is heterogeneous and faster in the center than on the surface in an aqueous environment. These authors summarized this processes

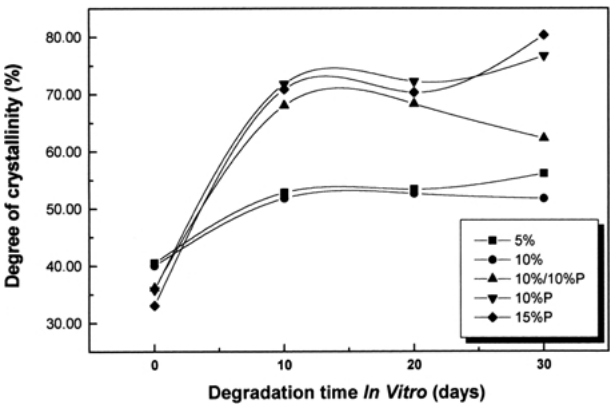


*Figure 5* Changes in the degree of crystallinity for the five types of membranes versus the degradation time, *in vitro*. The increase was much greater for the three types of membranes with plasticizer.

as follows. Initially, the sample is homogeneous, and hydrolysis begins in an aqueous environment as confirmed by the decrease in molar mass. At the beginning of the process, it is possible that degradation occurs on the surface as well in the interior of the molecule. However, regions near the surface can dissolve more easily in the medium than those located within the molecule. The latter have to diffuse through the bulk of the polymer mass at a very slow rate because of their size. Hence, the concentration of carboxylic end groups increases much more in the center than at the surface, resulting in a catalytic effect. This self-catalyzing behavior seems to be generic for the degradation of aliphatic poly(α-hydroxy acids), but depends on the chemical structure and configuration of the polymeric chains and the morphology of the device [22].

Lam *et al*. [6] confirmed this hypothesis when they showed that non-porous PLLA membranes degraded faster than porous membranes because in non-porous membranes the auto-catalyzing effect is favored while in porous membranes the products are easily dissolved.

In the membranes studied here, the plasticizer provides more mobility to the polymeric chains and the glass transition temperature diminishes. The ability of the plasticizer to diminish the chain interactions can explain the decrease in the initial degree of crystallinity in 10%P, 15%P, and 10%/10%P membranes when compared with the 5% and 10% membranes (Fig. 5). On the other hand, there was an increase in the degree of crystallinity of membranes with plasticizer after 10 days in phosphate buffer, when compared to the values for 5% and 10% membranes.

The plasticizer decreased the Tg to lower temperatures below 0 °C. Thus, at 37 °C in phosphate buffer, the membranes are found above the Tg and the chains show mobility, which facilitates their rearrangement during degradation.

For the PLLA membranes studied, as two morphologies were distinguished in SEM, the 5% and 10% membranes had a dense morphology while the membranes containing plasticizer had a globular and porous morphology.

Comparing our results with those obtained by Lam *et al*. [7], the membranes without plasticizer would be expected to show a greater increase in the degree of crystallinity, but the opposite was observed. These results indicate that the plasticizer has a greater effect on degradation than do simple morphological differences among the membranes, i.e. the mobility effect of the chains is more influential stronger than the morphology.

The increase in crystallinity is seen in amorphous membranes as well. Thus DSC analysis of PLA37.5/PGA25 copolymers, which are completely amorphous, showed that the Tg is in the range of 50–55 °C. After 12 weeks in a vessel filled with phosphate, a fusion peak at 95–100 °C also appeared, indicating the formation of crystalline regions as confirmed by X-ray analysis. The Tg can also decrease through an interaction of the plasticizer with water. In the case of the copolymer, this was influenced by the composition, the greater percentage of glycolic acid present compared to lactic acid, and the faster degradation process [24].

For porous membranes containing plasticizer, its

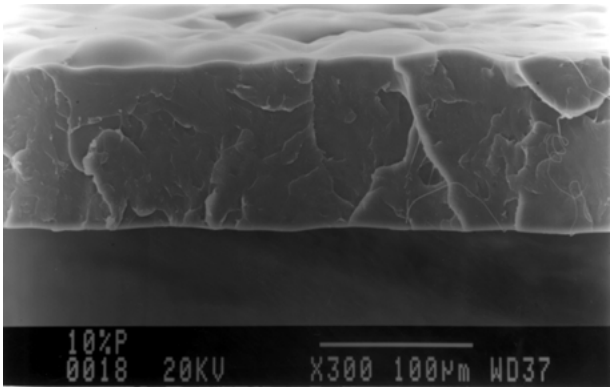


*Figure 6* Micrograph of the fractured surface of a 10% membrane without degradation, showing the dense and smooth surface.

action in decreasing the Tg and in providing more mobility to the chain is much more remarkable than the auto-catalitic effect, and its degradation was much faster, as seen in results of the tension and GPC tests. The porous and dense morphologies in the membranes can be visualized by electron micrographs.

## *Scanning electron microscopy*

The morphology of membranes, particularly the porous size and distribution, is extremely important for cell adhesion. A porous membrane is likely to be more fragile to mechanical actions. *In vitro* models do not fully evaluate the loss of mechanical properties of the membranes, because cellular invasion and the production of extracellular matrix are not assessed.

The electron micrographs of the membranes allowed a distinction between the morphology of the membranes and the changes caused by degradation. While membranes without plasticizer had a basically dense, smooth morphology, with rare globule formations, membranes containing plasticizer had a completely porous structure, formed by globules 60–100 µm in diameter with a rough surface. These globules was verified by polarized light microscopy, representing spherulites.

Fig. 6 shows the fractured surface of membranes of the 10%, before degradation and Fig. 7 shows the surface of this membrane after 30 days of degradation. With increasing time points of erosion appear on the surface of the membranes without plasticizer and spread uniformly over the whole membrane.

In the membranes with plasticizer, degradation was seen as appearance of scratches that originated in the center of the globules and propagated towards their roots. This morphology has observed by Shalaby *et al*. [25], who accelerated the degradation process by ''etching''. The greatest morphological changes *in vitro* occurred from the tenth day of degradation.

The micrographs in Figs. 8–14 show the appearance

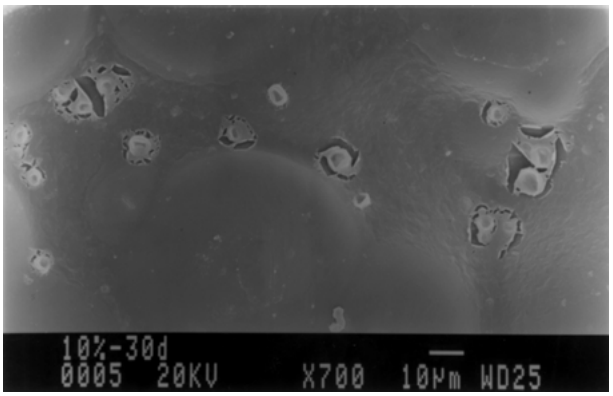


*Figure 7* Micrograph of the surface of a dense 10% membrane after a 30 days of degradation showing the globules. Note the points of erosion due to degradation, where the membrane is starting to break up.

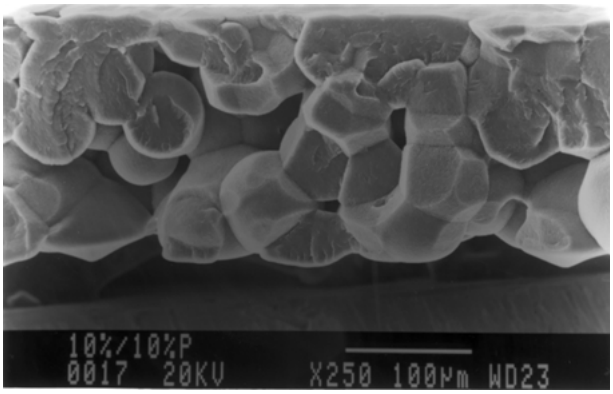


*Figure 8* Micrograph of the fractured surface of a 10%/10%P membrane, without degradation, showing that the membrane is formed from the agglomeration of globules. This results in a porous membrane that is denser at the surface.

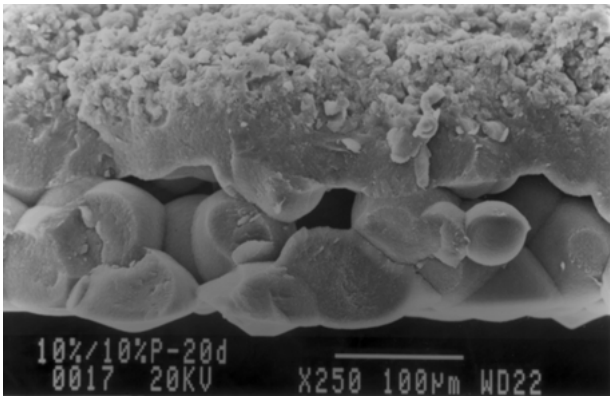


*Figure 9* Micrograph of a fractured surface of a 10%10%P membrane after 20 days of degradation *in vitro*. The upper surface shows many erosion points due degradation.

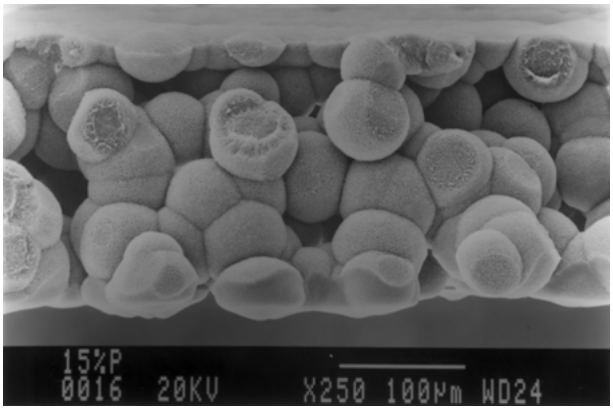


*Figure 10* Micrograph of a fractured surface of 15%P membrane, without degradation. The membranes with plasticizer always had the same type of structure, rough globules.

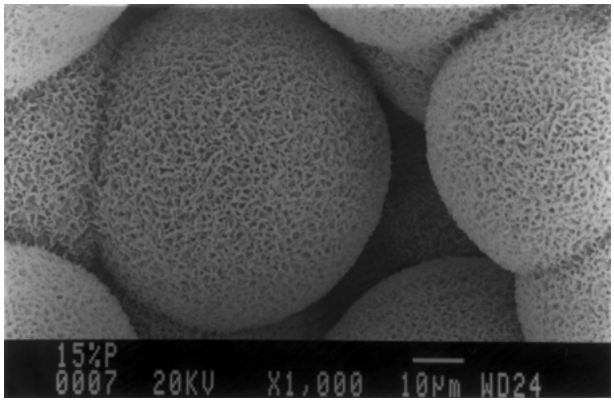


*Figure 11* Micrograph of a 15%P membrane, without degradation, showing the surface of the globules (similar to other membranes with plasticizer).

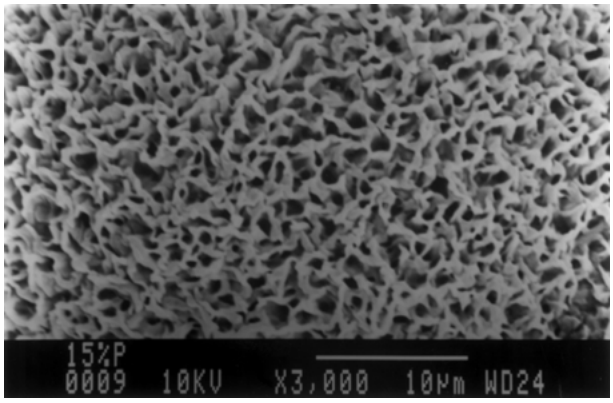


*Figure 12* Micrograph showing a detail of the surface of globules in a 15%P membrane without degradation. The rough, porous surface was present in all membranes containing plasticizer.

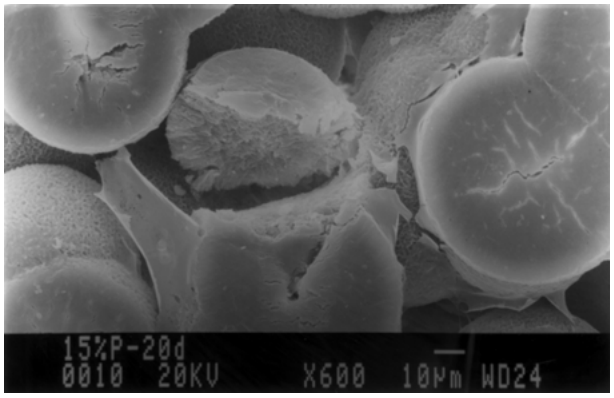


*Figure 13* Micrograph showing the surface of a 15% membrane after 20 days of degradation. The globules are starting to break up in the center because of degradation, and eventually divide (center of the photo).

five types of membranes during the 30 days of degradation *in vitro*. Clearly, as the porosity of the material increased, its resistance and complacency changed. Resistance usually decreased with the porosity increased, not just because of a decrease in the amount of material, but also because of tension on the membranes. The rate of loss must therefore be compatible with cell invasion and the deposition of extracellular matrix that will substitute the membrane.

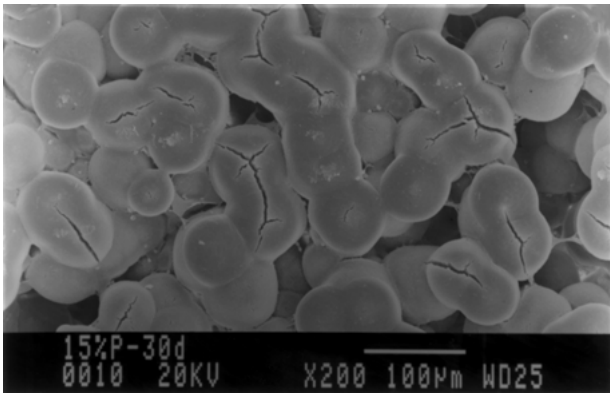


*Figure 14* Micrograph showing the broken aspect at the center of globules in a 15%P membrane, after 30 days of degradation.

## *In vivo* study

### *Macroscopic analysis*

The inflammatory response to biomaterials is determined by the composition and purity of the material, the shape and surface properties of the implant, the implantation site, positional stability at the implantation site, porosity, chemical stability, and so on [26–30].

Macroscopic observation of the samples without plasticizer (types 5% and 10%) showed that there was no adhesion of host tissue to the membranes during the 30 day after the implant. The membranes containing plasticizer (type 10%P, 10%/10%P and 15%P) were incorporated into the host tissue such that it was often difficult to locate the samples macroscopically in the skin in which they were implanted.

### *Microscopic analysis*

Light microscopy of membranes without plasticizer (types 5% and 10%) showed that there was no adhesion of cells to the membranes, thus confirming the macroscopic findings. Due to the absence of cell adhesion, it was difficult to demonstrate the presence of membranes in histological incisions because during microtomy the membrane became loose (there was no paraffin penetration in the membrane interstices), with only an empty site or some fragments surrounded by a fibrous capsule with a light inflammatory reaction being seen (Figs. 15 and 16).

Microscopic examination of samples from membranes with plasticizer (types 10%P, 10%/10%P and 15%) demonstrated that the plasticizer totally modified the tissue reaction. Since these membranes were porous, the host cell invaded the membrane interstices to cause subdivision of the membrane into globular units (Fig. 17). Blood vessels were surrounded by the membrane (Fig. 18) and by giant cells of foreign bodies in close contact with the spherulites, which composed the membrane (Fig. 19). There was a thick fibrous capsule of conjunctive tissue surrounding the membrane. There was no indication of an inflammatory reaction in the implants of membranes with plasticizer. The tissue reaction to the polylactides used in this study was manifested by the presence of fibroblasts, fibrocytes, macrophages, foreign body giant cells and polymorphonuclear cells.

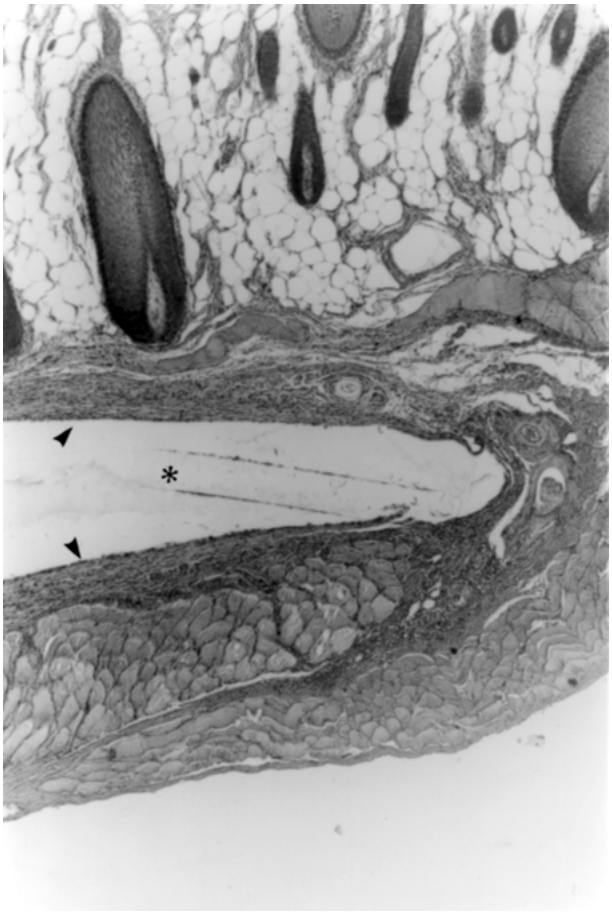

*Figure 15* Histological section of membrane without plasticizer 30 days after implantation. Note the membrane fragments (*) surrounded by a thick fibrous capsule. HE, ×50.

*Figure 16* Detail of the previous figure showing membrane without plasticizer (*) and a fibrous capsule (arrowheads) HE, ×90.

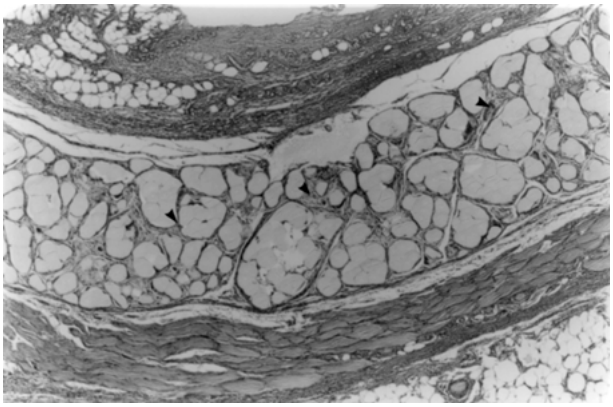

*Figure 17* Histological section of a membrane containing 10% plasticizer 30 days after implantation. Note the tissue invasion (arrowheads) where the membrane is fragmented. HE, ×200.

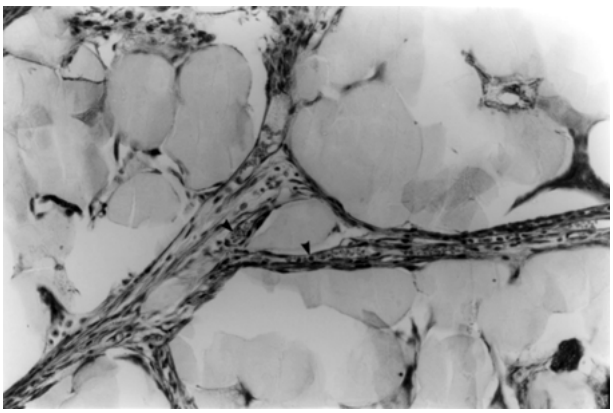

*Figure 18* Histological section of a membrane containing 15% plasticizer 30 days after implantation. Note the host tissue containing blood vessels (arrowheads), HE, ×400.

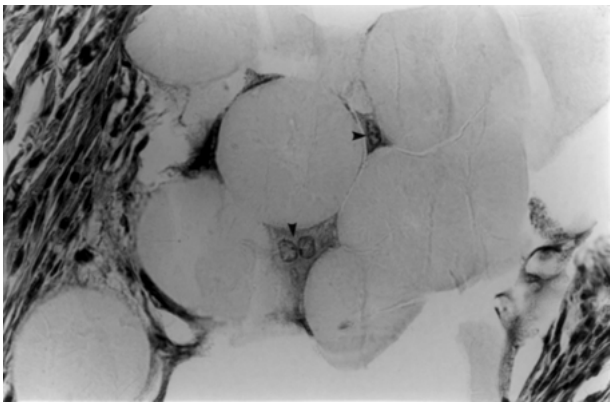

*Figure 19* Histological section of a membrane containing 10% plasticizer. Note the giant cells (arrowheads) of a foreign body adhering to the globules of the membrane. HE, ×700.

## Conclusions

The tissue analysis showed that the addition of plasticizer greatly improved the tissue-membrane interaction, but that the membrane quickly lost its mechanical properties. Another important factor is that the plasticizer used by Silva [17] and Schugens [16] in the tubes for guided neural cell regeneration and in this work, did not adversely affect cell growth in the 30 day after implantation.

Membranes with plasticizer degraded much faster than those without it. This finding limits their application to techniques including recovering from lesions or cell culture in which there is a fast cell migration and invasion by extracellular matrix to substitute for the degraded membrane. The amount of plasticizer used (10% v/v) reduced the tension values in the limit of yield, but increased the lengthening values. On the other hand, membranes without plasticizer retained their mechanical properties for much longer periods.

The plasticizer reduced the Tg and thus accelerated degradation by providing more mobility of the chains, particularly since the implanted membranes will be at a higher temperature those the Tg, i.e. 37 °C. An increase in the degree of crystallinity was observed and this increase was greater for membranes with plasticizer. The increase in the degree of crystallinity was enhanced by the scissions in the chains which rearranging themselves more easily in the presence of plasticizer.

SEM showed significant differences membranes morphology. Membranes without plasticizer were dense and those with plasticizer had a structure composed of a mass of spherulites about 100 μ in diameter arranged in a interconnected porous net. This type of morphology has been described by Coombes [15] as being ideal for invasion and growth. These spherulites had scissions that propagated towards the root from the 10th day onwards, indicating that degradation occurs within the spherulites as well.

Based on these results, two types of membranes were identified: (1) Dense membranes (5% and 10%) which, because their characteristics, are more indicated for applications such as guided tissue regeneration or any other recover technique that requires isolation from tissues through another biocompatible material with a long life time. (2) Porous membranes (10%P, 10%/10%P and 15%) which are indicated for situations that require support for cell growth, as in tissue reconstruction.

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